IDENTIFICATION AND CHARACTERIZATION OF NOVEL KINETOCHORE COMPONENTS IN THE YEAST *SACCHAROMYCES CEREVISIAE*

by

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ABSTRACT

To maintain a high fidelity of chromosome transmission during mitosis, the genetic material must be segregated accurately to daughter cells. Failures in this process lead to aneuploidy and may contribute to the development of cancer. The multiprotein kinetochore complex contributes to faithful chromosome segregation by mediating the attachment of a specialized chromosomal region, the centromere, to the mitotic spindle. The goal of this project was to identify and characterize novel components of the budding yeast kinetochore.

A subset of kinetochore proteins, referred to as the central kinetochore, provides a link between centromere DNA-binding proteins of the inner kinetochore and microtubule-binding proteins of the outer kinetochore. A combination of chromatin immunoprecipitation, *in vivo* localization and protein co-immunoprecipitation was used here to establish that yeast Chl4p and Iml3p are central kinetochore proteins, by demonstrating that they localize to the kinetochore and interact with known central kinetochore proteins; kinetochore components needed for these proteins to interact with centromere DNA were also identified. Chl4p is proposed to be an important structural component of the central kinetochore, as it is required for protein-protein interactions of several members of the Ctf19 central kinetochore complex. These physical interaction dependencies provide insights into the molecular architecture and centromere DNA loading requirements of the kinetochore complex.

Proper chromosome segregation is monitored by the spindle assembly checkpoint that delays anaphase if defects in kinetochore-microtubule attachments occur. Ame1p is an essential component of the Ctf19 complex. Here conditional *ame1* mutants were used to probe the molecular localization of Ame1p and investigate its function. *ame1* mutants are defective in chromosome segregation, and Ame1p likely participates in kinetochore-microtubule attachment through its interaction with Ctf19p. *ame1* mutants are also sensitive to overexpression of a microtubule-associated protein, Bik1p. In addition, *ame1* mutants break through an initial spindle assembly checkpoint-dependent delay in cell cycle progression; this defect is restored by overexpression of another essential component of the Ctf19 complex, Okp1p. Finally, *ame1* mutants seem to exhibit premature spindle elongation, even in the presence of a microtubule-depolymerizing drug, which frequently occurs in the

mother cell and appears to be uncoupled from sister chromatid separation. Together these results indicate that Ame1p functions in kinetochore-microtubule attachment and may contribute to the maintenance of the spindle checkpoint signal.

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LIST OF ABBREVIATIONS

3-AT:	3-AminoTriazole
APC/C:	Anaphase-Promoting Complex/Cyclosome
Cdk:	Cyclin-Dependent Kinase
CDE:	Centromere DNA Element
CEN DNA:	CENtromere DNA
CF:	Chromosome Fragment
ChIP:	CHromatin ImmunoPrecipitation
CIN:	Chromosomal INstability
Ddk:	Dbf4p-Dependent Kinase
FACS:	Fluorescence-Activated Cell Sorting
G1:	Gap 1 (Growth before DNA replication phase of cell cycle)
G2:	Gap 2 (Growth after DNA replication phase of cell cycle)
GAP:	GTPase Activating Protein
GEF:	Guanine nucleotide Exchange Factor
HU:	HydroxyUrea
IP:	ImmunoPrecipitation
MAP:	Microtubule-Associated Protein
M:	Mitosis (nuclear division phase of cell cycle)
MEN:	Mitotic Exit Network
MIN:	Microsatellite INstability
NZ:	NocodaZole
Plk:	Polo-Like Kinase
RSC:	Remodels the Structure of Chromatin
S:	Synapsis (DNA replication phase of cell cycle)
SPB:	Spindle Pole Body
YFP:	Yellow Fluorescent Protein
VFP:	"Venus" yellow Fluorescent Protein
CFP:	Cyan Fluorescent Protein
GFP:	Green Fluorescent Protein
ts:	Temperature Sensitive

A NOTE FROM THE AUTHOR ABOUT YEAST "JARGON"

Throughout this thesis, I have tried to be consistent with the nomenclature used to designate genes and proteins in accordance with accepted standards as follows:

For "your favorite gene" in *S. cerevisiae*:

YFG1 denotes the wild type gene

yfg1 denotes a recessive mutant allele of *YFG1*

Yfg1p denotes the wild type protein encoded by *YFG1*

yfg1p denotes the mutant protein encoded by *yfg1*

(in cases where a protein has two commonly used names, both names might be mentioned, separated by a / , e.g. "Your Favorite Gene" might also be named "Someone Else's Baby" and thus might appear as Yfg1p/Seb1p)

(For a corresponding mammalian gene or protein I have adopted the notation of YFG1, and included the mammalian name followed by / and the yeast name, e.g. YFG1/Yfg1p; in specific cases with other organisms I have tried to follow the relevant literature standards as closely as possible)

Moreover, for *S. cerevisiae*:

- Gene replacements (where a section of DNA is replaced with a selectable marker, for example for the deletion of a gene) are marked with the symbol Δ and appear as *yfg1Δ::[SELECTABLE MARKER]* (usually abbreviated *yfg1Δ* in the text)
  e.g. *yfg1Δ::HIS3*

- Conditional mutant alleles of a gene appear with a number that allows differentiation of the various alleles, e.g. *yfg1-1*, *yfg1-3*

- Tagged versions of genes appear as [*YFG1 or yfg1*]-[*tag*]-[*SELECTABLE MARKER*], and the corresponding proteins as [Yfg1p or yfg1p]-[tag]
  e.g. *YFG1-13Myc-HIS3*, Yfg1p-13Myc

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CHAPTER I

INTRODUCTION:

CHROMOSOME SEGREGATION IN EUKARYOTES

I.1. Mitosis and the chromosome cycle

Cell division, which involves the division of the nucleus (mitosis) and of the cytoplasm (cytokinesis) of a cell, is a fundamental biological process. The goal of cell division is to give rise to two daughter cells that are identical to the parent, and one of the main challenges during this process is to ensure proper distribution of the genetic material. Errors in chromosome transmission lead to aneuploidy (an abnormal number of chromosomes), often with dire consequences and even cell death. Thus, it is crucial to identify and understand the mechanisms that ensure proper chromosome transmission and promote genome stability. Moreover, because cell division is such a basic and fundamental cellular event, there is significant conservation of the proteins involved among eukaryotes (Kitagawa and Hieter, 2001). The baker's yeast *Saccharomyces cerevisiae* can thus serve as a good model organism for studying this process.

The partitioning of genetic material is coordinated with other events that occur during cell division, and chromosomes undergo a cycle of replication and segregation that is synchronized with the cell cycle (Figure I.1.A) (Harper et al., 2002; Hartwell and Weinert, 1989). The mitotic cell cycle is divided into four phases, named G1, S (DNA replication), G2 and M (cell division). Two substages of M phase, metaphase and anaphase, are particularly important to the study of chromosome segregation: in metaphase, duplicated chromosomes (sister chromatids) achieve attachment to the mitotic spindle (see section I.3); in anaphase, the sister chromatids are pulled apart from each other and segregate into each daughter cell (Figure I.1.A). Progression from metaphase to anaphase is a major step within mitosis and can be imagined as the passage through a gate; in many cases, the mutation of a gene involved in proper chromosome segregation results in a cell cycle pause at the metaphase-to-anaphase transition – like being held at the gate because not all requirements for passage are fulfilled.

Yeast cells reproduce by budding, and cellular morphology (the presence and size of a bud) allows the assessment of cell cycle stage (Figure I.1.A). For example, cells that are undergoing mitosis have a bud that is practically as large as the mother cell, while newly formed daughter cells that have not started undergoing replication of their DNA are unbudded. In higher eukaryotes, the chromosome cycle can be followed microscopically. In yeast however, cytological examination of chromosomes is hindered by limitations in the

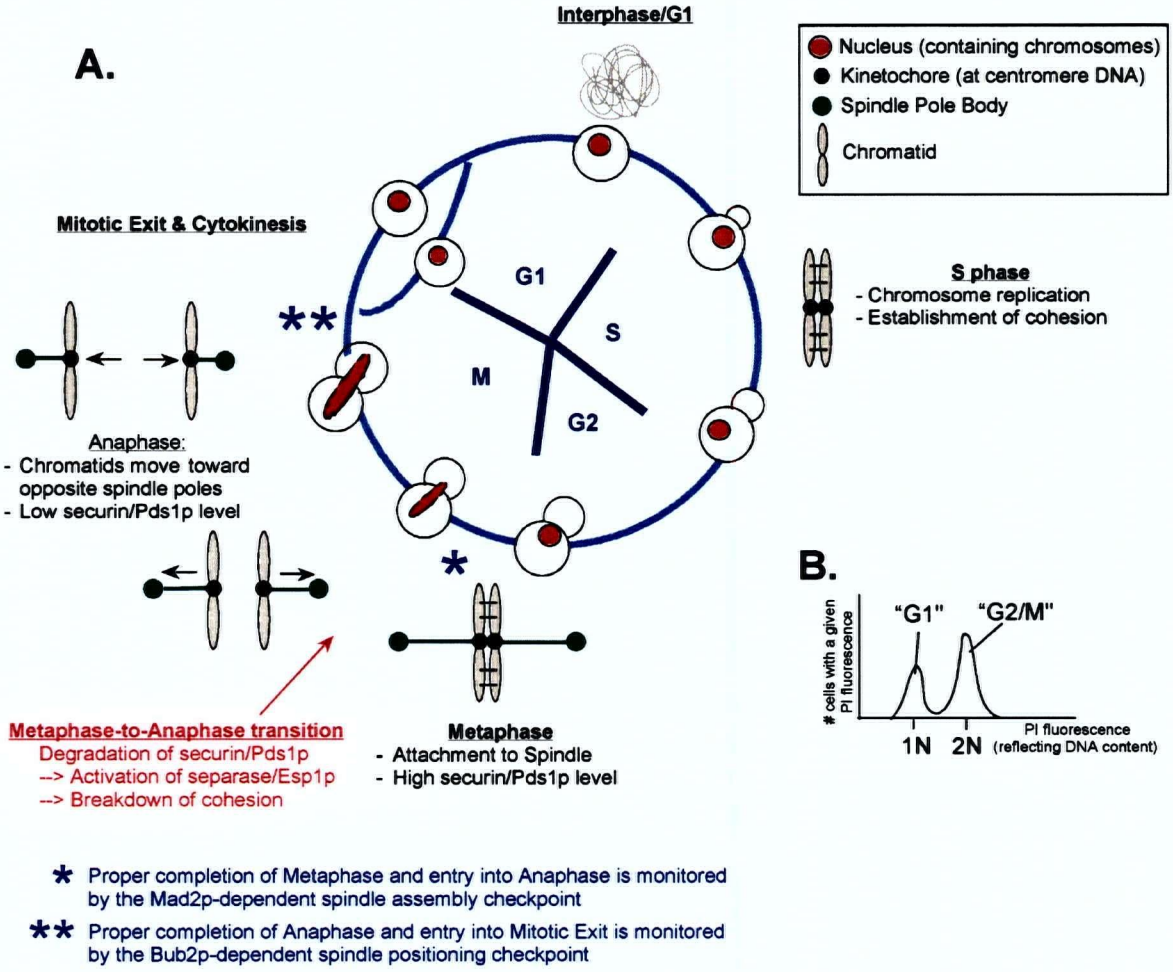

Figure I.1. The budding yeast cell cycle and the eukaryotic chromosome cycle

A. Yeast cells reproduce by budding. The cell cycle is divided into four stages [G1, S (DNA replication), G2 and M (mitosis)]. The size of the bud gives an approximate indication of cell cycle stage. The nucleus is shown in red; in yeast, the nuclear membrane does not break down during mitosis. During cell division, chromosomes undergo a replication and segregation cycle that is synchronized with the cell cycle. A specialized region of DNA, the centromere, onto which kinetochore proteins assemble (black dot), is the site of attachment of chromosomes to microtubules of the mitotic spindle, emanating from the spindle pole body (green lines, green dot is the SPB). At metaphase, sister chromatids (grey), held together by cohesins (small black lines), attach to microtubules of the spindle. The metaphase-to-anaphase transition, which happens once all chromosomes have achieved bipolar attachment, is characterized by the breakdown of cohesion that occurs when the Mad2p-dependent spindle checkpoint is silenced, resulting in degradation of the separin protease inhibitor securin; this allows sister chromatids to separate and segregate to opposite poles of the spindle, which constitutes anaphase. Proper spindle elongation, orientation and migration are monitored by the Bub2p-dependent spindle positioning checkpoint, which regulates entry into the mitotic exit and cytokinesis stages. B. To follow the chromosome cycle in yeast, DNA content can be analyzed by flow cytometry of cells in which DNA has been stained with a fluorescent dye such as propidium iodide. A typical histogram showing the fluorescence distribution of a population of cycling cells is shown. Haploid cells in G1 phase have a 1N DNA content, while cells that have replicated their DNA and are undergoing mitosis (G2/M) have a 2N DNA content.

resolution of microscopy, so progression of the chromosome cycle is followed by flow cytometry (FACS analysis), which gives an indication of cellular DNA content and allows one to distinguish cells with one or two (i.e. replicated) copies of the genetic material (Figure I.1.B). Uncoupling of the chromosome cycle from the cell cycle, such as completion of mitosis without proper chromosome replication or segregation, can be a source of severe genomic instability.

Regulation of cell cycle progression is achieved mainly through phosphorylation and proteolysis of key substrates (reviewed in McGowan, 2003). Defects in cell cycle regulation lead to uncontrolled proliferation, which is observed in most cancer cells (Malumbres and Carnero, 2003). Specific kinases, such as cyclin-dependent kinases (Cdks), are involved in the phosphorylation of these cell cycle regulators. Polo-like kinase (Plk)/Cdc5p and the Aurora/Ipl1p family of kinases are also emerging as key regulators of mitotic progression (reviewed in Donaldson et al., 2001; Katayama et al., 2003; Nigg, 2001). To ensure that cell cycle events occur in the proper order (e.g. that DNA does not re-replicate before chromosome segregation and cytokinesis have occurred), Cdk activity is controlled by a number of variant regulatory subunits called cyclins; additional regulation of Cdk activity is achieved by Cdk inhibitors such as Sic1p (Harper et al., 2002; Zachariae and Nasmyth, 1999). Specific cyclins, as well as other key substrates whose destruction is instrumental to mitotic cell cycle progression, are targeted for ubiquitin-mediated degradation by the 26S proteasome (Ciechanover, 1994; Jentsch, 1992) via a large E3 ubiquitin ligase complex called the anaphase-promoting complex/cyclosome (APC/C) (reviewed in Harper et al., 2002; Irniger, 2002; Page and Hieter, 1999; Zachariae and Nasmyth, 1999).

The APC/C is composed of between 11 and 13 core components, most of them conserved among eukaryotes, and its activity is modulated through phosphorylation of some of the subunits by mitotic kinases such as Cdk, Plk and protein kinase A (PKA), and dephosphorylation by PP1- or PP2A-type phosphatases (Harper et al., 2002; Kotani et al., 1999; Page and Hieter, 1999). The APC/C also transiently interacts with specificity factors (also called activators) such as Fizzy/Cdc20p and Fizzy-related/Cdh1p (Visintin et al., 1997), and the meiosis-specific Ama1p (Cooper et al., 2000), that activate APC/C by targeting the appropriate substrates for degradation at each stage of the cell cycle. The specificity factors

are themselves regulated: in the case of Cdc20p, regulation occurs at the transcription level and by phosphorylation, protein degradation, and inhibition due to checkpoint protein binding (see below and section I.2.b.ii); in the case of Cdh1p, regulation occurs mainly by inhibitory Cdk phosphorylation, which is reversed by the Cdc14p phosphatase (see section I.2.b.iii) (Harper et al., 2002; Kotani et al., 1999). Typically, substrates of APC/C^{Cdc20} contain a motif called the destruction box, whereas those of APC/C^{Cdh1} have a KEN box motif (reviewed in Harper et al., 2002). Sequential binding of the specificity factors to APC/C ensures a timely degradation of substrates that allows orderly mitotic progression. APC/C^{Cdc20} is active at the metaphase-to-anaphase transition for destruction of securin (see below) and mitotic cyclins, whereas APC/C^{Cdh1} is active in telophase and G1 and mediates degradation of mitotic cyclins and other proteins (Irniger, 2002). Thus, both specificity factors contribute to the degradation of mitotic (B-type) cyclins that is required for cells to exit mitosis, perhaps as a mechanism to ensure that this critical set of regulators will be completely destroyed to allow for the start of a new cell cycle (Yeong et al., 2000). Whether the specificity factors actually bind APC/C substrates is still under debate, and exactly how the specificity factors bind the APC/C is unknown (see Harper et al., 2002; Peters, 2002). Beside a critical role in anaphase initiation involving securin destruction (see below), APC/C activity has also been proposed to be required for the regulation of other events that occur during the metaphase-to-anaphase transition. These include sensing the tension created by bipolar chromosome attachment to the mitotic spindle and its loss at anaphase; moving chromosomes toward poles of the mitotic spindle; and gradually separating poles, which results in spindle elongation. Proteins regulated by APC/C that impinge on these processes include specific kinesins (such as Cin8p and Kip1p), and proteins such as Aurora/Ipl1p that localize to the spindle midzone at anaphase, similar to the known APC/C substrate Ase1p (Gordon and Roof, 2001; reviewed in Harper et al., 2002; Hildebrandt and Hoyt, 2001; Juang et al., 1997; Thornton and Toczyski, 2003).

Prior to mitosis, during S phase, the genetic material is duplicated and the two newly synthesized sister chromatids are paired. Sister chromatids are then held together until anaphase, a delay that distinguishes the eukaryotic and bacterial cell cycles (Nasmyth et al., 2000). The "molecular glue" that holds chromatids together is an essential multiprotein

complex called cohesin, which is made up of RAD21/Mcd1p (Scc1p), SA1, SA2/Irr1p (Scc3p), SMC1/Smc1p and SMC3/Smc3p (Guacci et al., 1997; Michaelis et al., 1997; reviewed in Nasmyth, 2001). The concatenation of DNA (intertwining of strands) is also thought to contribute to overall cohesion between chromatids. Cohesion occurs along the whole length of chromosomes but is particularly concentrated at the centromere (the chromosomal region involved in microtubule attachments, see section I.4), and depends on an active centromere, although specific cohesin-associated regions have not yet been defined with certainty (Guacci et al., 1994; reviewed in Meluh and Strunnikov, 2002; Tanaka et al., 1999). The mechanism of cohesion establishment is unclear, but it is thought to occur at the time of DNA replication (S-phase); in yeast, it involves Eco1p (Ctf7p) (Ivanov et al., 2002; Skibbens et al., 1999; Toth et al., 1999), a variant of replication factor C (RFC^{Ctf18}) involved in the loading of the proliferating cell nuclear antigen (PCNA) DNA polymerase clamp that would facilitate a polymerase switch (Mayer et al., 2001), the chromatin-remodelling complex RSC (Baetz et al., 2004), the helicase Chl1p, and a number of other proteins (reviewed in Meluh and Strunnikov, 2002; Nasmyth, 2001). Cohesin loading and stable association with chromosomes is also facilitated in yeast by the Scc2p/Scc4p complex (Ciosk et al., 2000). Additionally, yeast Pds5p associates with cohesin and participates in the maintenance of cohesion (Hartman et al., 2000). Some of the cohesin subunits contain a long coiled-coil tail and globular ends with dimerization and ABC-type ATPase domains. Cohesin has been proposed to form a ring structure that wraps around the two sister chromatids, holding them together; opening of the ring would require ATP hydrolysis, and would be hindered by the presence of Scc1p (Arumugam et al., 2003; Gruber et al., 2003; Haering et al., 2002; Weitzer et al., 2003). An additional role of cohesin is to sterically force a back-to-back orientation of sister centromeres that promotes biorientation as opposed to attachment of both centromeres to the same spindle pole (Hagstrom and Meyer, 2003; Meluh and Strunnikov, 2002; Nasmyth, 2001). Cohesin holds sister chromatids together until the onset of anaphase, resisting the force exerted on sister centromeres during metaphase by the attachment of chromosomes to spindle microtubules emanating from opposite spindle poles. Thus, cohesion provides a mechanism to ensure that the two chromatids of a sister pair will be segregated to opposite poles of the spindle – that is, because of the time delay between replication and segregation, cohesion provides a way by which cells can "remember" which

chromatids are sister pairs. Ultimately, this mechanism greatly contributes to faithful chromosome transmission (Nasmyth et al., 2000; Tanaka et al., 2000). In addition, the force pulling on centromeres due to microtubule attachment generates tension and provides a way to recognize when a pair of sister chromatid has achieved bipolar attachment, a process that is monitored by the Aurora/Ipl1p kinase; Aurora promotes microtubule detachment until this tension is sensed (see Chapter IV) (Tanaka et al., 2002). Tension at centromeres due to opposing forces of microtubule-binding and cohesion is so great, that sister centromeres split before anaphase onset and chromatin in this region is stretched; chromatid pairing is maintained through cohesion at centromere-surrounding regions (Pearson et al., 2001; Tanaka, 2002).

In budding yeast, cohesin remains associated with whole chromosomes until anaphase (Tanaka et al., 1999); in contrast, mammalian cohesin dissociates from chromosome arms in prophase and pro-metaphase in a Plk-dependent way, while a small amount persists, mostly at centromeres, suggesting the existence of two independent mechanisms for removal of mammalian cohesin (reviewed in Peters, 2002; Waizenegger et al., 2000). It has been suggested that condensation of chromosomes (which doesn't occur to a great extent in budding yeast) accounts for the loss of cohesion prior to anaphase in mammalian cells (Kitagawa and Hieter, 2001; Peters, 2002). Once all chromosomes are properly attached to the spindle, anaphase can proceed. Cohesion between sister chromatids is dissolved after cleavage of the Scc1p cohesin subunit by the separase/Esp1p protease (Uhlmann et al., 1999; Uhlmann et al., 2000); the activation of this protease requires targeting of its inhibitor securin/Pds1p for degradation through the APC/C^{Cdc20} (Cohen-Fix et al., 1996; Shirayama et al., 1999). Cdc20p is the downstream target of a signaling mechanism (the spindle assembly checkpoint) that oversees proper attachment of all chromosomes to the spindle before anaphase onset (see section I.2.b.ii and Figure I.3) (Hwang et al., 1998). At anaphase, sister chromatids move toward opposite poles of the spindle, which is followed by cytokinesis and the formation two daughter cells with the proper complement of chromosomes (Figure I.1.A). Timely release of active separase from securin is critical, as early separase activity leads to precocious sister chromatid separation; separated sister chromatids then run the risk of attaching to the same spindle pole, leading to chromosome segregation errors. Interestingly, while securin inhibits separase activity, it also promotes separase's proper function, possibly

by enhancing its nuclear localization and other means such as protecting it from degradation; thus, cells use a complex interplay between these cell cycle progression regulators to fine-tune the timing of anaphase initiation (Agarwal and Cohen-Fix, 2002). However, since securin is non-essential, other mechanisms are postulated to regulate cohesion independently; for example, Plk phosphorylation of Scc1p enhances the ability of separase/Esp1p to cleave it (Alexandru et al., 2001; reviewed in Meluh and Strunnikov, 2002; Nasmyth, 2001).

Modifications in the mechanism of cohesion are required during meiosis (reviewed in Page and Hawley, 2003; Petronczki et al., 2003). In that case, paired sister chromatids segregate to the same pole during meiosis I, and it is the paired homologous chromosomes, held together at chiasmata formed during recombination events, that segregate to opposite poles (Buonomo et al., 2000). Biorientation of sister chromatids is suppressed by monopolin/Mam1p-Csm1p-Lrs4p (Toth et al., 2000), and cohesion between sister chromatids, which is mediated by a modified form of cohesin containing Rec8p instead of Scc1p (Klein et al., 1999), is protected at centromeres during meiosis I through the action of Sgo1p, a homolog of *Drosophila* MEI-S332 (Kitajima et al., 2004; Marston et al., 2004; Rabitsch et al., 2004). Meiosis II resembles mitosis with loss cohesion at centromeres and sister chromatids segregating to opposite poles.

Cohesin components, as well as subunits of another complex called condensin that functions in chromosome condensation, show wide evolutionary conservation among eukaryotes. Beside the obvious fact that cohesin has a major role in ensuring the correct segregation of chromosomes at anaphase, this conservation of subunits emphasizes the overall critical importance of promoting efficient and accurate structural reconfiguration, packaging and sorting, and movement of chromosomes in the maintenance of genomic stability (Meluh and Strunnikov, 2002). Cohesin and condensin have also been proposed to participate in many other events that require chromosomal rearrangements, such as gene expression, gene silencing, and DNA repair (Hagstrom and Meyer, 2003).

I.2. Chromosome transmission fidelity

I.2.a. Consequences of errors in chromosome transmission

A hallmark of tumor cells is their inherent genomic instability, and an abnormal karyotype, which includes chromosome gain or loss as well as gross chromosomal

aberrations such as translocations, duplications and deletions; other cytological abnormalities commonly occur, such as multipolar spindles, defective microtubule-organizing centres (centrosomes, see section I.3), an abnormal number of centrosomes, and lagging chromosomes (Gisselsson, 2003; Jallepalli and Lengauer, 2001). That variations in chromosome numbers are at the origin of tumors was already recognized almost a century ago by Theodor Boveri, who thought that individual chromosomes must have different qualities and that it was the proper complement of chromosomes that would lead to normal cell development. Boveri hypothesized that errors occurring during mitosis, including problems in separating chromosomes, were at the origin of tumorigenesis, because malignant cells frequently showed abnormalities in chromosome combinations (reviewed in Manchester, 1995).

Tumor cells are believed to arise as a clonal population from a cell that has acquired a threshold amount of mutations in critical pathways, such as those that control cell growth, mortality, and apoptosis, which gives that cell a selective growth advantage (Nowell, 1976). An alteration of the karyotype leading to aneuploidy has also been proposed to create the initial environment necessary for the development of clonal neoplastic populations, including advantageous gene dosage imbalances (Li et al., 2000). Tumor cells generally show an increase in genomic instability; however, although in many cases this instability makes the cell more susceptible to becoming cancerous, and allows faster progression of tumorigenesis, it is still debated whether genomic instability is actually a driving force for tumor development that is required for cellular transformation (reviewed in Marx, 2002; Sieber et al., 2003). The causes of this genomic instability are also not clearly defined. Genomic instability could be the consequence of a specific mutation, for instance as in tumors that have the 'MIN' phenotype (microsatellite instability) (Lengauer et al., 1998), and predominantly show small changes at the DNA sequence level. This type of instability is likely to result from mutations in genes involved in DNA repair or replication (reviewed in Charames and Bapat, 2003), and fits a hypothesis that proposes that a cell must first acquire a "mutator phenotype" (a mutation that will lead to a higher rate of mutation than in normal cells) (Loeb, 1991; Nowell, 1986). However, not all cancers show mutations in such genes, and this type of instability seems most prevalent in hereditary tumor-predisposing syndromes and does not occur as frequently in sporadic tumors (Gisselsson, 2003); therefore, other

sources of genomic instability must exist. Some tumors, such as colorectal tumors that don't have MIN, exhibit a 'CIN' phenotype (chromosomal instability) (Lengauer et al., 1997; Lengauer et al., 1998), suggesting that genomic instability could arise from mutations that lead to errors in chromosome segregation, resulting in aneuploidy (Duesberg et al., 1998). Interestingly, aneuploidy is not well tolerated in normal cells, and the positive effect (i.e. growth advantage) of an altered karyotype is expected to occur only in a subset of cells; also, tumor cells may be able to survive aneuploidy better because they concomitantly acquire anti-apoptotic qualities (Dobles and Sorger, 2000; Li et al., 2000). In fact, not only do tumor cells have to gain genomic instability and bypass the mechanisms that would normally protect cells from this instability, but they also have to amplify some protecting factors in order to ensure their own survival; for example, an increased expression of telomerase, to counteract the inevitable shortening of telomeres that occurs through multiple rounds of division, is frequently observed in cancers. Thus, a certain balance needs to be achieved to ensure successful growth of a cancer cell population; it is that population that will be naturally selected for its ability to survive (Gisselsson, 2003).

The various steps of the chromosome cycle provide many opportunities for errors and inaccuracies to occur, that can lead to unfaithful chromosome transmission (Kolodner et al., 2002). Errors in chromosome segregation can result in loss or gain of genetic material in daughter cells. Possible consequences of aneuploidy during mitosis include an altered dosage of specific gene products, or the uncovering of recessive mutations (Figure I.2). Since the accumulation of mutations is thought to be one contributing factor in tumorigenesis, loss of heterozygosity in a diploid cell has a significant impact on the likelihood of cancer cell development, as it decreases the number of steps required to inactivate a gene, such as a tumor suppressor: only one copy now needs to be mutated to obtain the phenotype (Charames and Bapat, 2003). Increased copies of oncogenes or their positive regulators due to chromosome gain, which contributes to their increased expression, also affects cellular proliferation ability. Increased expression of several proteins involved in the process of chromosome segregation has been observed in tumor tissue. For example, the Aurora family of kinases and their interacting partners are overexpressed in several cancers (reviewed in Katayama et al., 2003); deregulated Plk also results in tumorigenesis (Dai and

Cogswell, 2003); and securin shows increased expression in cancer cells (Zou et al., 1999). Since these proteins are involved in orchestrating cellular events during G2/M, including monitoring of proper bipolar attachment of chromosomes and regulating sister chromatid cohesion, they must be tightly regulated temporally. It is thus likely that their overexpression contributes to an increase in aneuploidy due to deregulated chromosome segregation, which can be viewed almost as a vicious circle, since increases in aneuploidy will lead to ever increasing amounts of mutations and increased genome destabilization, which in turn might result in enhanced expression of these proteins. The strength of the aneuploidy hypothesis as the initial engine for tumorigenesis, as opposed to the single gene mutation hypothesis, is the fact that an event that creates aneuploidy affects thousands of structural and regulatory genes at once, making it more likely that the cell will reach and surpass the threshold of misregulation necessary for uncontrolled proliferation and invasion (Li et al., 2000).

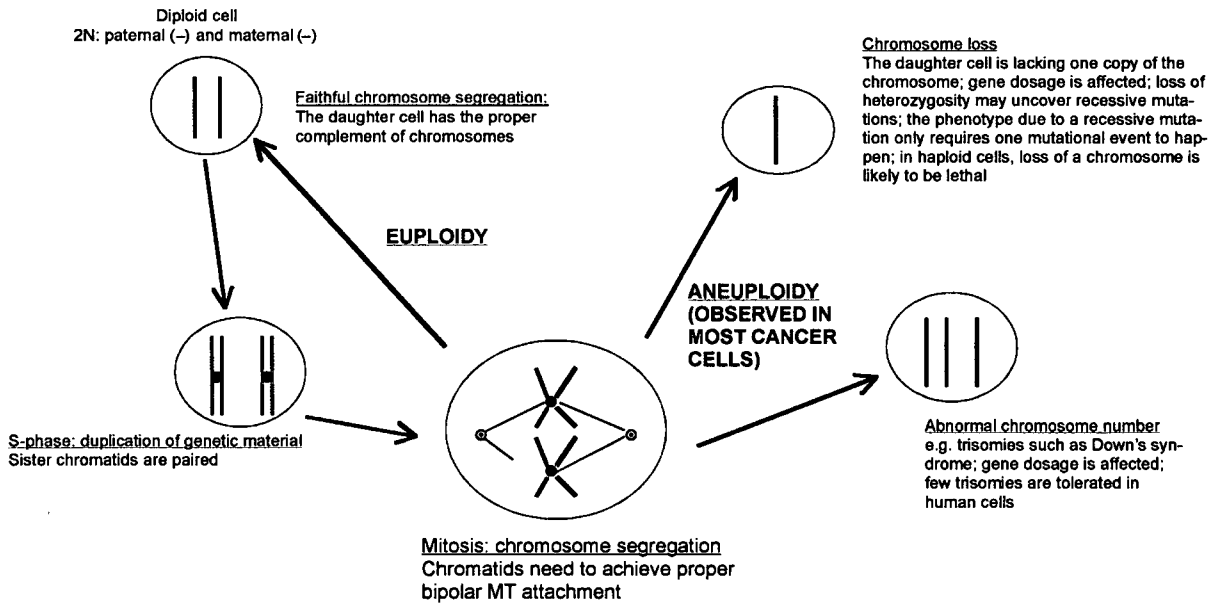

Figure I.2. Consequences of chromosome missegregation on genome integrity.
A diploid eukaryotic cell, which has two copies of each chromosome (blue and red lines), can become aneuploid during mitosis due to errors in chromosome segregation, for example due to improper attachment of the centromere/kinetochore (black dot) to microtubules emanating from the centrosome (green dot). If chromosome loss occurs, existing recessive mutations are uncovered, and fewer new mutations are needed to inactivate a gene. Gene dosage could be affected. If the two chromatids segregate to the same pole, chromosome gain ensues, and gene dosage is also affected. Such chromosome segregation errors occurring during meiosis in humans lead to trisomies such as Down's syndrome.

Chromosome transmission errors during mitosis and meiosis also lead to other defects. In haploid organisms, and in human gametes, loss of an entire chromosome is likely to be lethal. Many miscarriages in humans are due to errors in chromosome segregation, and aneuploidy can also cause infertility (McFadden and Friedman, 1997). While most aneuploidies resulting from chromosome segregation errors in meiosis lead to embryonic death, a small number of trisomies and rarely, monosomies, survive albeit with severe consequences (McFadden and Friedman, 1997). Aneuploidies are the leading cause of mental retardation, combined with poor growth and malformations; the most commonly cited example of a viable trisomy is Down's syndrome (an extra copy of chromosome 21) (Hassold and Hunt, 2001; McFadden and Friedman, 1997).

I.2.b. Surveillance mechanisms that ensure proper chromosome transmission

Since over their life time, most cells have to undergo many cell division cycles, there are mechanisms in place (called checkpoints) to ensure that the chromosome cycle and other cell cycle events proceed properly; underlying the concept of checkpoints is the dependence of each step of the cell cycle upon the successful completion of the previous one (Elledge, 1996; Hartwell and Weinert, 1989; Kolodner et al., 2002). The purpose of checkpoint mechanisms is to contribute to the maintenance of euploidy, and checkpoint effectors have the ability to slow down or stop progression of the cell cycle when an error is detected, so that the cell has time to repair it before proceeding further. Three checkpoints impinge on the chromosome cycle (summarized in Figure I.3): 1) the DNA damage and DNA replication checkpoints mediate repair of damaged DNA and monitor the duplication of DNA, which is coupled with the establishment of cohesion between sister chromatids (reviewed in Kelly and Brown, 2000; Longhese et al., 2003; Murakami and Nurse, 2000; Nyberg et al., 2002); 2) prior to chromosome segregation, the mitotic (or spindle assembly) checkpoint ensures that the mitotic spindle has been properly assembled and that all chromosomes have reached bipolar attachment to the spindle before cohesion is removed and chromatids are pulled apart (reviewed in Amon, 1999; Lew and Burke, 2003; Musacchio and Hardwick, 2002; Skibbens and Hieter, 1998); 3) during anaphase, the mitotic exit (or spindle positioning) checkpoint monitors proper spindle position, orientation and migration prior to cytokinesis and

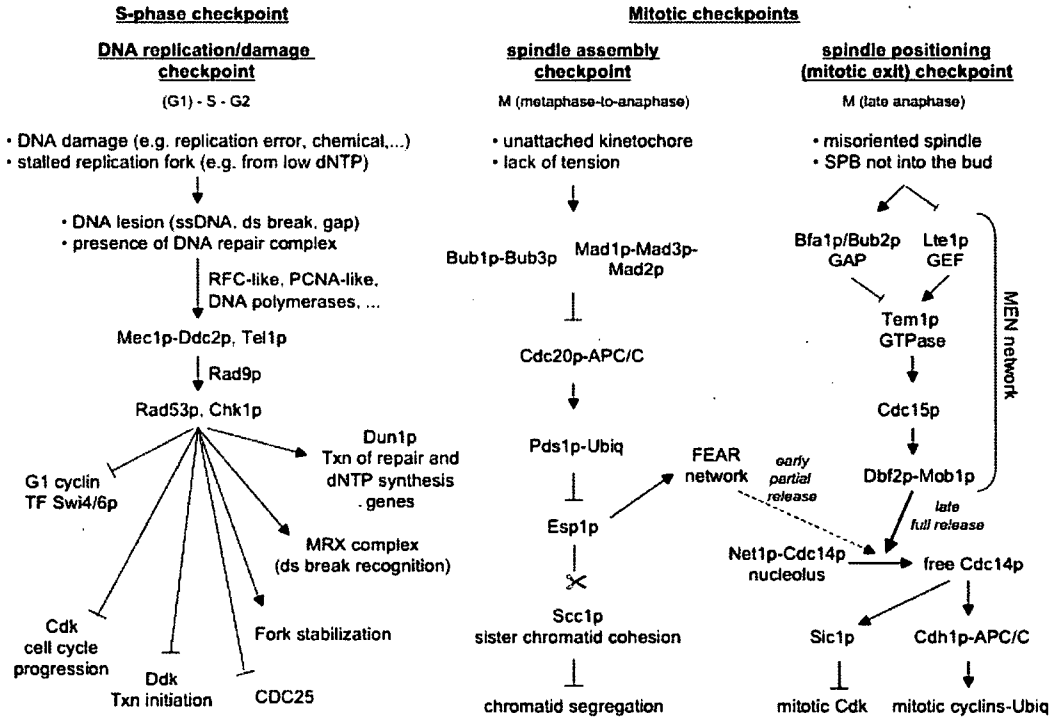

Figure I.3. Summary of genome stability surveillance mechanisms
Overview of the three checkpoints that impinge upon the maintenance of genomic stability. The proteins shown are mainly from budding yeast, except CDC25 which is a mammalian phosphatase. For reasons of simplicity not all relationships within and between pathways are shown. Txn, transcription; TF, transcription factor; ss, single stranded; ds, double stranded; Ubiq, ubiquitin. See text for more details.

completion of mitosis, to ensure that one of the spindle poles is segregated to the daughter cell (reviewed in Hoyt, 2000; Lew and Burke, 2003).

I.2.b.i. The DNA replication/DNA damage checkpoint

During S-phase of the cell cycle, the genetic material is replicated with the aim of producing two identical copies of DNA. Preparation for this enormous task starts at the end of M-phase and continues during G1, and involves the formation of pre-replicative complexes at origins of replication, as well as sensing of environmental and internal states such as nutrient availability and completion of previous cellular processes (sporulation, differentiation, etc.) (Sidorova and Breeden, 2003). DNA replication requires a large number of events to be coordinated spatially and temporally: timely and efficient firing of origins of

replication, production and assembly of the basic building blocks of chromatin (dNTPs, histones, and other chromatin proteins), and DNA damage surveillance and repair. These processes require the activity of many enzymes and leave ample room for errors to occur; moreover, a number of environmental and chemical agents can cause DNA damage. The DNA replication/damage checkpoint triggers a delay in the cell cycle in S-phase to allow for repair of errors, preventing chromosome segregation when DNA replication hasn't been completed or if DNA is damaged, and stabilizing the partially replicated chromosomes; it thereby contributes to the maintenance of genome integrity. A number of inherited diseases and cancer susceptibility syndromes have now been shown to be linked to mutations in genes involved in the DNA replication/damage checkpoint pathway, demonstrating the importance of this control mechanism in proper cellular function (Laiho and Latonen, 2003).

The major components of the checkpoint are: 1) Sensors, proteins that recognize the defect – for example by detecting a stalled replication fork due to low availability of dNTPs, or detecting a lesion in DNA (gap, double-stranded break), single-stranded DNA, or the presence of a DNA repair complex – and then trigger the response; 2) Transducers of the signal, which consist of phosphatidylinositol-3-kinase (PI3K)-like kinases (ATR/Mec1p and ATM/Tel1p) bound to ATRIP/Ddc2p, and their downstream effector kinases CHK2/Rad53p and CHK1/Chk1p; these two kinases may be linked to the PI3K-like kinases by adaptor proteins such as BRCA1/Rad9p; 3) Target effector proteins (Nyberg et al., 2002). Some of the proteins that initially detect defects are part of the replication machinery (e.g. DNA polymerases, helicases) or they are checkpoint-specific proteins, some of which have homology to proteins of the replication machinery (e.g. RFC-like proteins, PCNA-like proteins). It is still unclear whether the signal that triggers the checkpoint is the actual DNA lesion or the distortion in DNA structure that the lesion causes, or if the lesion first has to be processed to a common structure such as a single-stranded DNA or a double-stranded break, to be recognized by the checkpoint machinery (Foiani et al., 2000; Muzi-Falconi et al., 2003). Downstream targets of the checkpoint include cell cycle regulators such as Cdks, whose activity is inhibited to halt cell cycle progression. Checkpoint activation leads to inhibition of assembly of the replication complex and inhibition of DNA replication initiation through inhibition of Ddks (Dbf4p-dependent Cdc7p kinase; Duncker and Brown, 2003). Activation of the checkpoint also results in fork stabilization (thereby preventing collapsing of the

stalled fork), suppression of late origin firing, double-stranded break recognition (by the Mre11p-Rad50p-Xrs2p MRX complex), the transcription of genes needed for repair (involving activation of the Dun1p kinase), and regulation of dNTP pools. The S-phase checkpoint may also serve as a mechanism to prevent uncontrolled replication of telomeres, as an excess of unprotected single-stranded DNA overhangs would be detected as DNA damage and lead to checkpoint activation (Longhese et al., 2003).

It should be noted that while in mammalian cells, there exists a G1 checkpoint that delays entry into S-phase in the presence of damaged DNA, this checkpoint is very weak in budding yeast and is effected by Rad53p-dependent phosphorylation of the G1 cyclin transcription factors Swi4/6p (Nyberg et al., 2002). Similarly, DNA damage in mammalian cells triggers a G2/M arrest that is conveyed by inhibitory phosphorylation of the CDC25 mitosis-promoting phosphatase (Hoffmann, 2000). Both the G1 and G2/M mammalian responses to DNA damage involve the tumor suppressor p53, one of the most frequently mutated genes in cancer, which underlines the fact that genome instability is likely to contribute to tumorigenesis (Laiho and Latonen, 2003). As mentioned above, sister chromatid cohesion ensures that the replicated DNA strands stay together until the appropriate time to segregate, thus contributing to genomic stability. Since cohesion is established during S-phase (coupled with DNA replication), the budding yeast G2/M arrest triggered by DNA damage, in parallel to triggering transcription of DNA repair genes, is mainly conveyed by promoting activity of anaphase inhibitors rather than inhibiting Cdks (Clarke, 2003). Budding yeast S-phase checkpoint kinases communicate with mitotic regulators to ensure metaphase arrest and maintenance of cohesion: Pds1p is phosphorylated in a Mec1p-dependent manner and yeast Plk (Cdc5p) is phosphorylated by Rad53p; Cdc5p is actually thought to function in checkpoint adaptation and recovery from the arrest (Nyberg et al., 2002). Since budding yeast assembles its spindle during S-phase (in other eukaryotes spindle assembly occurs during mitosis), a downstream effector of the DNA replication/damage checkpoint is also expected to inhibit premature spindle elongation (Clarke, 2003; Smith et al., 2002). Lastly, mammalian cells are thought to have a topoisomerase II-dependent checkpoint active in G2 to ensure the decatenation of newly replicated DNA molecules, which is necessary for proper chromosome condensation and faithful segregation. This checkpoint seems absent from yeast (Smith et al., 2002).

I.2.b.ii. The spindle assembly checkpoint

For the purpose of this work, the most critical checkpoint is the mitotic/spindle assembly checkpoint that monitors the metaphase-to-anaphase transition. This checkpoint system is composed of a set of proteins that were first described in yeast but are largely conserved among eukaryotes (Amon, 1999; Smith et al., 2002). Five non-essential genes, *BUB1*, *BUB3* (budding uninhibited by benzimidazole), *MAD1*, *MAD2* and *MAD3* (mitotic arrest-deficient) are part of a signaling cascade that receives and processes information about unattached chromosomes and lack of bipolar attachment (Hoyt et al., 1991; Li and Murray, 1991; reviewed in Wells, 1996). Deletion of these genes leads to increased sensitivity to microtubule-depolymerizing drugs because cells progress through mitosis despite disruption of the mitotic spindle; these strains also show increased chromosome loss rate, even in the absence of the microtubule poison, reflecting a defect in monitoring occasional failures in chromosome attachment (Li and Murray, 1991; Warren et al., 2002).

Similar to the DNA replication/damage checkpoint, the spindle checkpoint is expected to have three components: sensors, transducers and effectors. The checkpoint is triggered upon defects in microtubule depolymerization or centrosome duplication, or in the presence of malfunctioning microtubule motors or defective kinetochore components (proteins that link chromosomal centromeres to the spindle, see section I.5) (Hardwick et al., 1999). Several signals have been proposed to be sensed by the checkpoint, including the level of free tubulin subunits, centrosome function, or even the distance between the centrosome and an attached chromosome. But the two main signals are believed to be kinetochore attachment to microtubules (i.e. unbound "free" kinetochores would generate a "wait-anaphase" signal), and bipolarity of attachment leading to tension (i.e. a pair of sister chromatids not attached to opposite spindle poles is not under tension and would generate a signal) (Amon, 1999; Wells, 1996). It is known that even a single unattached kinetochore is sufficient to activate the checkpoint (Rieder et al., 1994). While there is still much debate about tension versus attachment being the checkpoint-triggering signal, it is likely that both contribute to full checkpoint activation (reviewed in McIntosh et al., 2002). Moreover, exactly how a mechanical signal (tension) is translated into a biochemical signal is not known, but it has been proposed to involve tension-regulated phosphorylation; for example, tension could cause a conformational change in a kinase, modifying its activity (Nicklas, 1997). There is

some evidence that phosphorylation of a kinetochore-associated component is part of the signal, as the 3F3/2 antibody, which recognizes an as yet unidentified phosphorylated epitope at the mammalian kinetochore, stains more strongly in the presence of unattached kinetochores (Gorbsky and Ricketts, 1993). There are several kinases in the checkpoint machinery, and many of the checkpoint components themselves are phosphorylated; however the exact role of these phosphorylations remains unclear to date (Lew and Burke, 2003). Interestingly, Bub1p activity seems to be modulated by Cdk phosphorylation (and possibly other kinases) (Yamaguchi et al., 2003), which illustrates a possible way for the cell to ensure that full activation of the checkpoint is coordinated with the cell cycle.

The signal transduction cascade of the spindle assembly checkpoint involves interactions between the known checkpoint components. Bub1p is a kinase that is known to phosphorylate Bub3p, which in turn binds to and activates Bub1p; Mad1p is a coiled-coil protein that is hyperphosphorylated in checkpoint-activated cells, and might function in the recruitment of Mad2p to kinetochores; Mad2p is a putative calcium binding protein that plays a central role in checkpoint function; and Mad3p has some homology to Bub1p (reviewed in Lew and Burke, 2003; Li and Murray, 1991; Musacchio and Hardwick, 2002). The three Mad proteins are thought to form a complex that binds Cdc20p (Figure I.3. and see below). Mammalian spindle checkpoint proteins were shown to localize to unattached kinetochores (reviewed in Cleveland et al., 2003) and recently the yeast checkpoint components were determined to localize to the kinetochore and interact with centromere DNA (Gillett et al., 2004). In mammalian cells, different localization patterns for different checkpoint components suggest that the checkpoint is able to differentiate between unattached kinetochores (where most checkpoint component can be observed to localize) and attached kinetochores that are not under tension (where BUB1 and BUBR1 - a putative Mad3p homolog - strongly localize, but little MAD2 is found) (Skoufias et al., 2001; Waters et al., 1998). The localization of checkpoint components to the kinetochore is interdependent; some of the components might act as a scaffold onto which other components can assemble (Musacchio and Hardwick, 2002). One model of checkpoint activation proposes that Mad2p interaction with unattached kinetochores, which is highly dynamic, renders it competent to interact with Cdc20p and transmit the checkpoint signal. This activated form of Mad2p could be an oligomerized form, and the kinetochore might act

as the catalytic site of polymerization of Mad2p subunits; alternatively, the activated form of Mad2p could consist of a Mad-Bub complex (summarized in Shah and Cleveland, 2000). In any case, complex formation between the different checkpoint components, conformational changes induced by differential binding of components and effectors, and localization of these complexes all seem to be important in checkpoint activity (reviewed in Musacchio and Hardwick, 2002). Additionally, the kinase Mps1p (monopolar spindle), which functions in centrosome duplication and is crucial to ensure the formation of a bipolar spindle, also has a role in the spindle checkpoint (reviewed in Nigg, 2001; Winey and Huneycutt, 2002). Mps1p phosphorylates Mad1p, and might function to recruit other checkpoint components (Abrieu et al., 2001; Hardwick et al., 1996; Winey and Huneycutt, 2002). *MPS1* overexpression constitutively activates the checkpoint, and this activation requires the Mad and Bub components; interestingly, it does not depend on an intact kinetochore (Fraschini et al., 2001; Hardwick et al., 1996). Experiments with the mammalian MPS1 indicate that its activity is necessary for establishment and maintenance of the checkpoint and that it acts upstream of MAD2 (Abrieu et al., 2001). Mps1p might also participate in other processes, such as mitotic exit (see section I.2.b.iii) (Luca and Winey, 1998; Winey and Huneycutt, 2002).

While checkpoint activation ultimately leads to decreased Cdk activity through APC/C-mediated destruction of Cdk-associated mitotic cyclins (Hoyt et al., 1991; Li and Murray, 1991; Li et al., 1997b), the direct target of checkpoint proteins is the APC/C specificity factor Cdc20p (described in section I.1). Mad2p binds to Cdc20p, leading to inhibition of APC/C^{Cdc20} activity, and thus stabilization of Cdc20p-dependent APC/C substrates (reviewed in Amon, 1999). It is not entirely clear whether Cdc20p is prevented from binding APC/C when bound to Mad2p, but substrate binding by Cdc20p does not seem to be prevented by the Cdc20p-Mad2p interaction; rather the inhibition of APC/C activity might occur through lack of ubiquitination of the substrates (reviewed in Harper et al., 2002; Hwang et al., 1998; Zachariae and Nasmyth, 1999). Interestingly, Cdc20p and Mad1p possess a similar domain to interact with Mad2p, which suggests that an exchange of Mad1p for Cdc20p in the Mad2 complex might occur upon checkpoint activation (Lew and Burke, 2003). The major consequence of activating the Mad2p-dependent checkpoint is a delay in sister chromatid segregation and in anaphase: the securin Pds1p, which is normally targeted for degradation

by APC/C^{Cdc20}, inhibits cohesion breakdown by restricting the activity of the separase/Esp1p that cleaves cohesins and promotes release of Cdc14p (Figure I.3.; and see section I.2.b.iii).

Other proteins are thought to participate in the spindle assembly checkpoint. One putative component is the tension sensor Aurora/Ipl1p that promotes biorientation by destabilizing kinetochore-microtubule attachments until sisters are under tension due to their attachment to opposite poles (see Chapter IV) (Biggins and Murray, 2001; Tanaka et al., 2002); however, it is difficult to distinguish whether Ipl1p actually sends a signal to the checkpoint, or whether its destabilizing action produces transiently unattached kinetochores that will themselves be the signal (Lens and Medema, 2003; Lew and Burke, 2003). A number of microtubule motor proteins might also be involved in the checkpoint. For example, mammalian CENP-E is a kinesin-like protein that is required for efficient capture of microtubules at kinetochores; CENP-E interacts with BUBR1 and is proposed to increase the efficiency of checkpoint signaling (Abrieu et al., 2000; Weaver et al., 2003, and see Chapter III). The microtubule motor dynein has been implicated in the highly dynamic turnover of checkpoint components at the kinetochore, where they would be replenished by an ATP-dependent mechanism; dynein has been proposed to be involved in checkpoint silencing and inactivation by moving checkpoint proteins away from kinetochores (towards centrosomes). ZW10 and ROD are two proteins that were first identified in *Drosophila* and form a complex, and are required for the spindle assembly checkpoint, and possibly for kinetochore localization of dynein (Musacchio and Hardwick, 2002). Kinetochore components are also candidates for checkpoint signaling; however, since an intact kinetochore is required for the checkpoint to detect defects in kinetochore-microtubule attachments (e.g. some strains with mutations in core kinetochore components do no trigger the checkpoint even though they have defective spindle attachment), it might be difficult to distinguish a specific checkpoint role from an effect on kinetochore structure (Gardner and Burke, 2000). A good candidate for checkpoint signaling at the kinetochore is the conserved Ndc80 kinetochore complex (Ndc80p-Spc24p-Spc25p-Nuf2p) (reviewed in Lew and Burke, 2003).

One could expect that mitotic checkpoint genes functioning during chromosome segregation are good candidate tumor suppressor genes, since they contribute to the maintenance of genomic stability. In fact, most tumors with a CIN phenotype have a

defective checkpoint (Cahill et al., 1998), and loss of mitotic checkpoint activity was observed at the early stage of tumorigenesis in a mouse model (Gascoyne et al., 2003). However, so far, only some human cancer cells have been shown to have mutations in conserved checkpoint components (reviewed in Jallepalli and Lengauer, 2001). hBUB1 has been shown to be mutated in colorectal cancer (Cahill et al., 1998) and mutations in hBUB1 and hMAD3 were proposed to contribute to the pathogenesis of breast cancer (Lee et al., 1999). hMAD2 expression was shown to be reduced in breast cancer cell lines (Li and Benezra, 1996; Percy et al., 2000) and mice heterozygous MAD2 knock-outs have an increased rate of lung tumor development (Michel et al., 2001). These studies reveal that cellular genomic integrity is sensitive to a reduction in the level of functional checkpoint proteins. However, despite these observations, a large panel of aneuploid colorectal tumors that show defective checkpoint activity did not show mutations in components of the mitotic checkpoint (Cahill et al., 1999), indicating that other genes that function in chromosome segregation are likely mutated in these cells, causing high levels of aneuploidy. These genes could function in any of the processes that impinge on chromosome segregation: centrosome function, microtubule dynamics, chromosome condensation, kinetochore, or cohesion (Lengauer et al., 1998). Thus, a detailed characterization of the function of genes involved in chromosome segregation could ultimately provide clues about mechanisms of tumorigenesis. For example, the adenomatous polyposis coli gene APC, which normally participates in the regulation of the cytoskeleton by binding and stabilizing microtubules, is frequently mutated in familial colon cancer; these mutations cause chromosome missegregation. It was shown that the APC protein binds components of the mitotic checkpoint, suggesting that a mutated version of APC unable to bind microtubules would not be able to properly signal defects in chromosome-microtubule attachments (Kaplan et al., 2001). Checkpoint function is also important during meiosis, especially meiosis I. It was suggested that checkpoint deficiency may be responsible for abnormalities caused by non-disjunction of chromosomes at meiosis I, such as Down's syndrome (Shonn et al., 2000).

I.2.b.iii. The spindle positioning (mitotic exit) checkpoint

BUB2 was one of the genes found in the initial screen for mutants that failed to arrest their cell cycle in response to microtubule poisons; thus, it was originally thought that Bub2p also functioned in the spindle assembly checkpoint. However, a number of observations hinted that Bub2p was actually part of a second pathway that acted later in mitosis: first, *mad2* cells undergoing spindle damage are able to block cytokinesis and DNA re-replication, suggesting the existence of another monitoring system for cell cycle progression in late anaphase; second, in *bub2* cells Pds1p degradation is still inhibited in response to checkpoint activation, indicating that these cells are competent to regulate Cdc20p's activity (i.e. their Mad2p-dependent checkpoint is functional) (Alexandru et al., 1999). Thus, only *mad2 bub2* double mutants progress through the cell cycle without arresting in the presence of spindle damage. The mitotic checkpoint has now been divided into two branches: the spindle assembly checkpoint (described above), and the spindle positioning checkpoint that monitors mitotic events in late anaphase/telophase and restricts cell cycle progression into mitotic exit and cytokinesis. In budding yeast, faithful distribution of the segregating chromatids requires the mitotic spindle to properly orient in the mother-bud axis, which depends on the interaction of cytoplasmic microtubules emanating from the spindle pole body (SPB; the yeast equivalent of a centrosome) with proteins at the cell cortex and involves microtubule-associated proteins and motors (see section I.3.d). Defects in orientation and elongation of the spindle are detected by the spindle positioning checkpoint. Evidence for monitoring of this process was first obtained with the study of dynein mutants that delayed cell cycle progression because their spindle remained in the mother cell instead of elongating into the bud (Yeh et al., 1995); this arrest could be relieved by deletion of *BUB2* (Bloecher et al., 2000). There is now evidence that this checkpoint monitors entry of the SPB into the bud, regardless of its association with kinetochores, and that even a transient bud entry of the SPB is enough to deactivate the checkpoint (reviewed in Lew and Burke, 2003). Proteins that mediate the signaling response of the spindle positioning checkpoint, including Bub2p, form the mitotic exit network (MEN) (reviewed in Hoyt, 2000; Jensen and Johnston, 2002). Mutants in MEN genes have a characteristic cell cycle arrest in late anaphase/telophase (Jaspersen et al., 1998).

Unlike the checkpoint pathways described above, where checkpoint components are activated in the presence of damage, the signaling cascade of MEN is inactive (i.e. checkpoint active) as long as the spindle has not reached the bud. Thus, the checkpoint "signal" in this case is the absence of the activated cascade, and the consequence of checkpoint activation is failure to degrade or activate specific mitotic progression regulators (Figure I.3). Bub2p exists in a complex with Bfa1p, and together they act as the GTPase activating protein (GAP) for the GTPase Tem1p, thus inhibiting its activity by keeping it in the GDP-bound state; conversely, the Tem1p guanine nucleotide exchange factor (GEF) Lte1p promotes loading of GTP and thus activates Tem1p (Bardin et al., 2000; Pereira et al., 2000). The regulatory aspect of MEN is contained in the spatial distribution of its components: most MEN components are localized to the cytoplasmic side of the SPB until the end of anaphase where they relocate to the bud neck (Daum et al., 2000). The GAP Bfa1p/Bub2p preferentially associates with the SPB that is destined for the bud, which is dictated by differential interactions of the SPB with the mother and bud cortexes via cytoplasmic microtubules (Pereira et al., 2001, and see section I.3.d). In contrast, the GEF Lte1p localizes exclusively to the bud, where it concentrates at the cortex. This spatial separation provides a way in which Tem1p can only be activated when the SPB has entered the bud, when Tem1p and Lte1p can interact (Bardin et al., 2000; Pereira et al., 2000). Activation of Tem1p also seems to occur by an Lte1p-independent mechanism, which involves downregulating the Bfa1p/Bub2p GAP activity, through phosphorylation by Plk/Cdc5p, allowing for spontaneous GDP/GTP exchange on Tem1p to occur. Tem1p activation at anaphase leads to recruitment of other MEN component to the poles. Active Tem1p-GTP targets the Cdc15p kinase, and activates it to phosphorylate the Dbf2p kinase (or its homolog Dbf20p) (reviewed in Burke, 2000; Lew and Burke, 2003; Smith et al., 2002). Dbf2p exists in a complex with Mob1p, and Mob1p might promote the phosphorylation-dependent activation of Dbf2p. As mentioned above, Mps1p also appears to be involved in mitotic exit. Mps1p is thought to interact with Mob1p, suggesting that Mps1p plays a role in monitoring spindle position; alternatively Mob1p could have a role in centrosome duplication (Luca and Winey, 1998; Winey and Huneycutt, 2002).

The downstream target of MEN is the phosphatase Cdc14p (Figure I.3). Cdc14p mediates mitotic exit by several routes that converge on inhibition of the mitotic Cdk, the most

essential event required to reset origins of replication for the next cell cycle (Noton and Diffley, 2000). First, Cdc14p dephosphorylates the APC/C specificity factor Cdh1p, allowing full APC/C^{Cdh1} activity for targeting mitotic cyclins and other late anaphase substrates for ubiquitin-mediated degradation. Second, dephosphorylation of the Cdk inhibitor Sic1p stabilizes it; moreover dephosphorylation of the Sic1p transcription factor Swi5p allows it to enter the nucleus and induce expression of Sic1p, contributing to Sic1p accumulation (Jaspersen et al., 1999; Visintin et al., 1998). Cdk inactivation is also critical for cytokinesis, as components of the MEN must relocalize from the SPB to the bud neck before cytokinesis can occur; this relocalization depends on Cdk inactivation (Hwa Lim et al., 2003). Cdc14p is sequestered in the nucleolus during most of the cell cycle in an inhibitory complex with Net1p/Cfi1p (Visintin et al., 1999). In a still poorly understood mechanism, which could involve MEN proteins affecting the trafficking of Cdc14p in and out of the nucleus, and possibly Plk/Cdc5p phosphorylation of Net1p (Lew and Burke, 2003; Shou et al., 2002), a large part of Cdc14p gets released from the nucleolus through the activation of the MEN pathway in late mitosis. However, a small amount of Cdc14p is also released in early anaphase by a MEN-independent pathway called the FEAR network (Cdc14p early anaphase release), that consists of separase/Esp1p, Slk19p, Spo12p and Plk/Cdc5p (Stegmeier et al., 2002). The early-released Cdc14p interacts with MEN components at the SPB and - not necessarily by dephosphorylation - promotes inhibition of Bfa1p/Bub2p activity, which is required for activation of Tem1p (Pereira et al., 2002); it also dephosphorylates Cdc15p, which renders it more active. Perhaps the low dose of early-released Cdc14p forms a positive feedback loop through pre-activation of the MEN, which results in full activity and release of Cdc14p at the end of anaphase (Figure I.3) (reviewed in Jensen and Johnston, 2002; Visintin et al., 2003).

It is not known whether this checkpoint pathway exists in higher eukaryotes; some components of the pathway are conserved, at least by sequence homology (Smith et al., 2002), and it has been suggested that the principles of surveillance for proper spindle positioning and exit from mitosis are important in all eukaryotes (Seshan and Amon, 2004). While the orientation of the spindle towards the bud is specific to budding yeast, it may be that other eukaryotes use a similar pathway to monitor the position of the spindle within the cell, since that will determine the location of the cleavage plane, which is critical for cell fate

(see section I.3.d). Preliminary evidence indicates that mammalian cells do monitor spindle orientation and that this process depends on dynein and astral microtubules (O'Connell and Wang, 2000).

I.2.b.iv. Crosstalk between checkpoints

Many instances demonstrate that there is a crosstalk between checkpoints, especially the two spindle checkpoints (Lew and Burke, 2003). A good example is Pds1p. Beside its major function as an anaphase inhibitor, controlled by the spindle assembly checkpoint, Pds1p is also the target of the DNA damage checkpoint: phosphorylation by the Rad53p and Chk1p kinases leads to inhibition of Pds1p ubiquitination (Agarwal et al., 2003; Cohen-Fix and Koshland, 1997; Tinker-Kulberg and Morgan, 1999). Additionally, *cdc20* mutants arrest in mitosis with stable Pds1p levels, but this is suppressed by a *bub2* mutation, indicating that Pds1p can be degraded by a Cdc20p-independent pathway (Tavormina and Burke, 1998). Pds1p also affects the activity of APC/C^{Cdh1}, and thus mitotic cyclin degradation, by preventing dephosphorylation of Cdh1p (Cohen-Fix and Koshland, 1999; Tinker-Kulberg and Morgan, 1999). Other examples include the degradation of mitotic cyclins being carried out by both the metaphase-to-anaphase form of APC/C (bound to Cdc20p) and the anaphase-to-telophase form (bound to Cdh1p); the activity of separase/Esp1p in both cohesion breakdown and Cdc14p early release; Plk/Cdc5p having multiple roles during mitosis (e.g. as a target of the DNA replication/damage checkpoint; for Cdc14p early release through the FEAR network; and for activation of the MEN pathway); and the Bfa1p/Bub2p complex being the target of multiple checkpoints, including the DNA damage checkpoint, to ensure a mitotic arrest (Hu and Elledge, 2002; Hu et al., 2001). An obvious goal for this crosstalk between checkpoints is the temporal coordination of events to ensure proper progression of mitosis (reviewed in Seshan and Amon, 2004). However, it could also provide a backup safety mechanism to slow down mitosis when a defect is detected. For example, if in the presence of DNA damage the DNA damage checkpoint is not able to completely arrest progression through the cell cycle, crosstalk of this pathway with a downstream checkpoint will ensure arrest of the mitotic cycle, giving the cell more time to repair the damage (Lew and Burke, 2003).

I.3. Chromosomes segregate to the two daughter cells by moving along fibers of the mitotic spindle

I.3.a. Microtubules and the mitotic spindle

In order to segregate into daughter cells, sister chromatids are guided by a polarized network of fibers called the mitotic spindle. The spindle is made up of microtubules, which are one of the main components of the cellular cytoskeleton. Microtubules consist of a heterodimer of two types of tubulin, α and β, that are encoded in yeast by the *TUB1/TUB3* and *TUB2* genes, respectively. Microtubules are polar, with their "minus" end at the microtubule-organizing centre (MTOC, see below) and their "plus" end projecting away from the MTOC. Furthermore, microtubules are dynamically unstable and undergo constant growing and shrinking due to the addition and removal of tubulin subunits, typically at the plus end (Maddox et al., 2000). Tubulins are associated with a GTP molecule that hydrolyses upon a conformational change induced by polymerization. It is thought that microtubule ends with GTP-bound tubulin are flat sheets that close up into a tube concomitantly with GTP hydrolysis (Andersen, 2000; Schuyler and Pellman, 2001). While the addition of GTP-tubulin subunits to the microtubule end depends on the concentration of free subunits (at least *in vitro*), hydrolysis of GTP is only dependent on the conformational change. Thus, at high free subunit concentration there is a delay between subunit addition and GTP hydrolysis, which results in a "GTP cap" in which subunits are protected from depolymerization, allowing growth of the microtubule; conversely, at low subunit concentration shrinkage would be promoted due to the absence of the cap (Mitchison and Kirschner, 1984). Transitions between growth and shrinkage can happen rapidly, and are referred to as "catastrophe" (growth → shrinkage) and "rescue" (shrinkage → growth). "Pauses" are times at which no growth or shrinkage is occurring. The polymerization/depolymerization rate can be influenced by the interaction of microtubules with other cellular structures such as the cortex or the kinetochore (see below). Microtubule growth rate also varies during the cell cycle; for example, in yeast the growth rate is highest during G1, a time at which the SPB gets duplicated and positioned prior to mitosis (Segal and Bloom, 2001). The dynamic instability of microtubules is a critical property that allows for rapid reorganization of the cytoskeleton, for example during mitosis or cellular movement; it is thus expected to be a highly regulated process. Interestingly, the metastable nature of the

spindle has lead to a hypothesis for the existence of an underlying spindle matrix, composed of proteins such as yeast Fin1p or *Drosophila* Skeletor, that would provide an anchor for spindle motors or promote centromeric clustering (Bloom, 2002).

Microtubules originate at the MTOC (called centrosome in higher organisms and SPB in yeast). The centrosome is made up of two barrel-shaped centrioles surrounded by a matrix of pericentriolar material, while the SPB is a disk-shaped structure. Unlike in higher eukaryotes, the yeast nuclear membrane does not break down during mitosis, so the SPB remains embedded within the membrane during the whole cell cycle (Hoyt and Geiser, 1996; McIntosh et al., 2002). Thus, the SPB has two faces: nuclear and cytoplasmic. Some of the constituent SPB proteins occur on both faces, but other proteins are unique to one or the other (Francis and Davis, 2000). The SPB is made up of several layers, divided into three major plaques. The outer plaque (on the cytoplasmic face) and the inner plaque (on the nuclear face) are linked by a central plaque embedded in the nuclear envelope (Francis and Davis, 2000; O'Toole et al., 1999). The structural components of the SPB are well described and contain several coiled-coil proteins, some of which are phosphorylated. Six major components of the SPB are: Spc110p, which functions as a linker between the inner and central plaques, and interacts with calmodulin, leading to calcium-dependent interaction with other SPB components (Hoyt and Geiser, 1996); Spc29p, which interacts with Spc110p and Spc42p, in the central plaque; Spc42p (an internal scaffold for the SPB), Cnm67p and Nud1p in the intermediate layers between the central and outer plaques; and Spc72p in the outer plaque; both Spc110p and Spc72p also interact with the γ-tubulin complex (see below) (reviewed in Francis and Davis, 2000).

SPB duplication and segregation constitutes a cycle that follows the cell and chromosome cycles (Winey and O'Toole, 2001): duplication and separation occur during prophase and metaphase, while segregation occurs at anaphase. SPB duplication depends on the calcium-binding protein centrin/Cdc31p, Kar1p, and a number of other proteins, and is regulated by the kinase Mps1p (Francis and Davis, 2000). Separation of the duplicated SPBs involves the activity of Cdks and microtubule motors (Winey and O'Toole, 2001). Subsequent insertion of the new pole in the nuclear envelope involves Mps2p and Ndc1p (Hoyt and Geiser, 1996). SPB mitotic maturation also depends on phosphorylation of some of its components, possibly by Cdk (McIntosh and Hering, 1991). In addition to its major role as a microtubule

nucleation site, the MTOC also provides a platform for the localization of regulators of cell cycle progression, including kinases (such as Cdk) and phosphatases, which are often associated with scaffold proteins such as AKAP and pericentrin (Cassimeris and Skibbens, 2003). Examples of localized regulators include the tumor suppressor p53 and components of the MEN (described in section I.2.b.iii).

Nucleation of microtubules involves the γ-tubulin ring complex that resides at the MTOC, and contains the ubiquitously conserved microtubule minus-end binding protein γ-tubulin/Tub4p (Joshi et al., 1992; Schiebel, 2000; Stearns et al., 1991; Vogel and Snyder, 2000). Regulation of Tub4p by phosphorylation has been proposed to control the number of microtubules nucleated by the SPB (Vogel et al., 2001). In yeast Tub4p is found in the inner and outer plaques, from which microtubules are nucleated. Growth of microtubule arrays also requires motor enzymes that bridge microtubules together. There are three types of microtubules: 1) kinetochore microtubules, which link kinetochores to spindle poles; 2) interpolar microtubules, which keep poles apart and mediate spindle elongation after anaphase; these microtubules project towards the spindle midzone and either interact with chromosome arms or overlap with microtubules emanating from the other pole, through the interaction of microtubule-associated factors and motors; and 3) astral (or cytoplasmic) microtubules, which project from the cytoplasmic face of the SPB towards the cortex and are instrumental in spindle orientation and positioning (Wittmann et al., 2001). Since the three kinds of microtubules are composed of the same tubulin subunits, their identity must be specified by associated structural and regulatory factors (Hoyt and Geiser, 1996). Once the microtubules have been nucleated, their minus-end resides near the MTOC but is not directly attached to it; rather, tethering occurs through a number of structural and motor proteins (Cassimeris and Skibbens, 2003).

I.3.b. Attachment of chromosomes to spindle microtubules allows their movement toward mitotic poles

During mitosis, the attachment of sister kinetochores to kinetochore microtubules emanating from opposite poles is crucial for faithful chromosome segregation. In many organisms, centromeres reside close to the centrosome even before mitosis. In budding yeast, the SPB is so close to the kinetochore that proteins initially purified with the SPB have

now been shown to be components of the kinetochore (Wigge et al., 1998); in fact, yeast kinetochores may be attached to the SPB for most of the cell cycle (Winey and O'Toole, 2001). Microtubules growing from SPBs probe the interpolar space until they find a kinetochore, and then associate with outer kinetochore components, such as the Dam1 complex in budding yeast (Hofmann et al., 1998). Chromatin is thought to have a stabilizing effect on microtubules (see below). After one of the sisters has captured a microtubule emanating from one pole, the chromosome is mono-oriented and tends to move toward the SPB (with the help of a minus-end directed motor), but eventually also moves away from the pole; thus chromosomes oscillate due to alternating poleward and anti-poleward movements. This movement of chromosomes depends on microtubule dynamics and on minus-end and plus-end directed microtubule motors, such as dynein and CENP-E, respectively, as well as on SPB-associated motors that promote depolymerization at the minus end, such as Kar3p. Movement toward poles is also counteracted by polar ejection forces (or polar wind), a pressure exerted on chromosome arms by unattached or interpolar microtubules, which depends on non-kinetochore associated motors (Heald, 2000; McIntosh et al., 2002). In most organisms, the back-to-back orientation of sister kinetochores renders attachment of both sisters to the same pole unlikely for steric reasons. Once the sister kinetochore of a mono-oriented chromosome connects to a microtubule emanating from the opposite pole, tension ensues and is thought to stabilize the kinetochore-microtubule connections. Although microtubules are highly dynamic, the exchange rate of tubulin subunits in kinetochore-associated microtubules is much slower than in unattached microtubules (McIntosh et al., 2002). Kinetochores thus have a stabilizing effect on kinetochore microtubules and are sites of microtubule growth up to the onset of anaphase, after which depolymerization largely occurs; this suggests that there are factors at kinetochores (described below) that have the ability to influence microtubule stability, and that are regulated during the cell and chromosome cycles.

In a poorly understood mechanism, chromosomes then gradually move towards the spindle equator to position themselves at approximately equidistance from each pole (congression). In yeast, only centromeres adopt a metaphase-like conformation by moving to the metaphase plate (Pearson et al., 2001), whereas in higher eukaryotes, chromosome arms are also subject to positioning by microtubule motors (Heald, 2000; Hoyt and Geiser, 1996).

Segregation of the poles and their attached chromosomes ensues at anaphase, which consists of two steps: at anaphase A, the chromosomes move closer to the poles, while at anaphase B the poles and their attached chromosomes segregate in opposite directions. As mentioned above, yeast kinetochores are close to SPBs during most of the cell cycle, and thus anaphase A is very short (Hoyt and Geiser, 1996); most of anaphase consist of the pole segregation stage, which occurs in two steps: a first, rapid elongation of the spindle, followed by a pause, and a slower elongation to the final length (Francis and Davis, 2000). At the end of anaphase, kinetochores are held very close to SPBs by short microtubules; the remaining spindle consists of a few interpolar microtubules that interdigitate at the spindle midzone, where a number of proteins also reside, such as the yeast kinetochore protein Ndc10p and the Aurora kinase Ipl1p (Buvelot et al., 2003). One of these proteins, Ase1p, is degraded in an APC/C^{Cdh1}-dependent manner, thus tying in cell cycle progression control to the spindle cycle (Winey and O'Toole, 2001). Spindle disassembly, which is necessary for cytokinesis, is thought to occur by depolymerization of the interpolar microtubules from their plus end (Winey and O'Toole, 2001).

I.3.c. Microtubule-associated proteins contribute to proper chromosome-microtubule attachment and chromosome movement

Microtubules are not just bundles of tubulin (Barnes et al., 1992); they interact with microtubule-associated factors and motor proteins that can influence microtubule behavior, such as modifying growth and shrinkage rates (McIntosh and Hering, 1991; Mitchison and Kirschner, 1984) or enhancing or preventing tube closure at the microtubule ends (Andersen, 2000). These proteins also promote microtubule attachment to structures such as membranes or kinetochores (Schuyler and Pellman, 2001), and help to organize the microtubules into a bipolar array (Wittmann et al., 2001). As mentioned above, chromatin interaction with microtubules seems to be a stabilizing factor. One way this could be achieved is by chromatin suppressing the activity of microtubule polymerization inhibitors. One such factor is Op18/stathmin, which is thought to act by sequestering tubulin subunits, increasing catastrophe rate, or inducing GTP hydrolysis at the microtubule cap; Op18/stathmin is negatively regulated by phosphorylation, possibly when in proximity to chromatin (Andersen, 2000; Andersen et al., 1997; Walczak, 2000). Interestingly, Op18/stathmin is

highly upregulated in some tumors, suggesting that deregulated microtubule dynamics can affect cell proliferation (Curmi et al., 2000). A second catastrophe inducer is *Xenopus* XKCM1 (mammalian MCAK), a kinesin of the KinI family. Its effect on microtubule depolymerization is not dependent on its motor domain; rather it is though to bind microtubule ends, inducing a conformational change that promotes microtubule destabilization. While most motors use ATP-derived energy to move along microtubules, in XKCM1 ATP hydrolysis might be used to release the motor from a dimer with tubulin after microtubule depolymerization (Walczak, 2000). Counteracting these microtubule depolymerization-inducing proteins are the microtubule-associated proteins (MAPs; e.g. XMAP125/Stu2p), microtubule stabilizers that are negatively regulated by cell cycle-dependent phosphorylation. Although such proteins generally increase polymerization rate at the plus-end of microtubules, their main role is to reduce the activity of catastrophe inducers, probably by binding to and stabilizing microtubules (Wittmann et al., 2001). Proper activity of Stu2p in microtubule dynamics is required for microtubule-kinetochore attachment and chromosome alignment by affecting centromere motility, and for microtubule-cortex interaction and proper spindle orientation (Kosco et al., 2001; Pearson et al., 2003). The overall microtubule polymerization rate is therefore controlled by a balance between the activities of catastrophe inducers and MAPs, which is spatially and temporally regulated. For example, the increased activity of XKCM1 at mitosis could be due to loss of the stabilizing influence of MAPs, whose activity is modulated by Cdks (Walczak, 2000; Wittmann et al., 2001). Spindle length is also controlled by the interplay between these polymerization rate modulators (Karsenti and Vernos, 2001).

The GTPase Ran, which is involved in nucleocytoplasmic trafficking, also stimulates polymerization; it has been suggested that chromatin localization of its GEF Rcc1p would facilitate Ran activation near mitotic chromosomes to promote microtubule assembly (Arnaoutov and Dasso, 2003; Nemergut et al., 2001). Although possible downstream substrates of Ran-GTP have been identified, such as the structural proteins TPX2, NuMA and the kinesin Eg5 (see below), the exact mechanism for the action of Ran-GTP on microtubules is still unclear; it may involve the regulated import of these microtubule regulators into the nucleus (Cassimeris and Skibbens, 2003; Karsenti and Vernos, 2001; McIntosh et al., 2002;

Wittmann et al., 2001), and the interaction of Ran-GTP with centrosomal proteins including the γ-tubulin ring complex, as well as MAPs and motors (Schiebel, 2000).

Microtubules are stabilized at their minus-end at spindle poles, via crosslinking by SPB-associated components (e.g. NuMA, TPX2) (Cassimeris and Skibbens, 2003; Wittmann et al., 2001). Stu2p has also been proposed to have a stabilizing role in anchoring of microtubules at the SPB independent of their γ-tubulin nucleation, by binding microtubules laterally and interacting with the SPB component Spc72p (Francis and Davis, 2000).

Soluble and chromosome-bound motor proteins, such as kinesins and dynein, have an important role in spindle dynamics, by providing not only motility but also structural integrity (Sharp et al., 2000). Two types of motors are thought to be involved in pole separation: the plus-end directed bimC/KinN kinesin-related proteins, and the minus-end directed dynein. bimC/KinN motors (e.g. Eg5, Cin8p and Kip1p) are involved in spindle assembly and maintenance, and are thought to crosslink interpolar microtubules; subsequent sliding along two adjacent antiparallel microtubules toward the plus end simultaneously would then provide a pushing force to separate poles (Hoyt and Geiser, 1996). Interestingly, Cin8p and Kip1p may have differentiated roles in pole separation, by acting during the different steps of anaphase B (fast and slow, respectively) (Straight et al., 1998). The minus-end directed motor dynein and its associated activator complex dynactin promote the transit of the nucleus into the bud and spindle elongation and positioning. Dynein associates with the cell cortex and with SPBs in a microtubule-dependent manner (Yeh et al., 1995) and is thought to slide along microtubules while being anchored at the cortex, thus resulting in a pulling action on pole separation. Another type of kinesin-related protein, KinC (e.g. ncd, Kar3p), is a minus-end directed motor that has an antagonistic role to the bimC/KinN motors, and also possesses the ability to crosslink microtubules (Hoyt and Geiser, 1996). Additionally, Kar3p may have a role as a poleward kinetochore motor. Finally, higher eukaryotes have chromokinesins (e.g. XKid, Xklp1), which are KinN-type motors acting on chromosome arms that participate in the maintenance of spindle integrity as well as mediate polar ejection forces (Cassimeris and Skibbens, 2003; Heald, 2000; Hoyt and Geiser, 1996; Wittmann et al., 2001). Such chromokinesins may require cell cycle regulation, as exemplified by the APC/C-dependent degradation of XKid at anaphase required for proper chromosome movement (Funabiki and Murray, 2000). Motors have also been shown to be

localized to kinetochores and may not only contribute to microtubule attachment, but could also provide some of the force required to move chromosomes along microtubules. Dynein is thought to function in the initial capture of microtubules. The plus-end directed KinN kinesin, CENP-E, could provide kinetochore tethering to microtubules as they are depolymerizing. And the KinI-type microtubule-destabilizing kinesin (XKCM1/MCAK/Kip3p) has also been localized to kinetochores and is thought to bind microtubule ends and promote their depolymerization (Heald, 2000; Wittmann et al., 2001). Thus, overall spindle assembly and integrity and chromosome movement appear to depend on the coordinated action of several motors proteins.

Additionally, non-motor microtubule-associated proteins such as EB1/Bim1p and CLIP170/Bik1p, which are part of a class of proteins called "plus-end tracking proteins" or +TIPs that generally associate with the plus end of microtubules, have been shown to be associated with kinetochores and affect microtubule dynamics (Schuyler and Pellman, 2001; Tirnauer and Bierer, 2000; Tirnauer et al., 2002). The mechanisms by which +TIPs are loaded onto microtubules is unclear, but may involve binding to free tubulin dimers and subsequent assembly onto microtubule during polymerization. +TIPs specifically associate with the polymerizing end of microtubules, getting released from microtubules when they get behind the region of growth; release might be controlled by phosphorylation or by a conformational change in the protein induced by folding of the microtubule end into a tube (Schuyler and Pellman, 2001). CLIP170/Bik1p is thought to guide microtubules to their target destinations by locally controlling their dynamics and stabilizing them until they reach the target (Schuyler and Pellman, 2001). The role of Bik1p is discussed in more detail in Chapter III. CLIPs might be regulated by interacting CLASP/Stu1p proteins that have homology to XMAP125/Stu2p and also bind microtubules; CLASPs are thought to have a role specifically at the kinetochore (Maiato et al., 2003; Schuyler and Pellman, 2001). Additionally, interaction of CLIP170/Bik1p with dynein/dynactin might be mediated by an interacting partner, LIS1/Pac1p (Coquelle et al., 2002). EB1/Bim1p has a role in bridging the interaction of spindle positioning determinants (such as APC and Kar9p) with microtubules (see section I.3.d) (Tirnauer and Bierer, 2000).

I.3.d. Proper spindle positioning is essential for accurate partioning of the genetic material

The proper orientation of the mitotic spindle contributes to faithful chromosome segregation. For example, in yeast cells proper spindle orientation and elongation ensures the correct segregation of the dividing nucleus towards the bud cell, thus equally partitioning the genetic material. In a broader sense spindle orientation is important because in higher eukaryotes it determines the plane of cleavage, which is perpendicular to the spindle axis. The position of the spindle is crucial as it may result in the assymetric distibution of cell fate determinants that is necessary for differentiation and development. In yeast the plane of division depends on bud site selection, which is preprogrammed according to the ploidy of the cell; thus there is an interplay between factors that specify bud site selection, cell polarity and spindle orientation.

Spindle orientation and nuclear migration in yeast depend on astral microtubules and the motor proteins described above as well as additional proteins interacting with the SPB and the cell cortex (Segal and Bloom, 2001; Stearns, 1997). In *C. elegans* spindle positioning also appears to require activation of heterotrimeric G proteins that would "read out" spatial information provided by cortical factors (PAR proteins) and translate it into forces acting on microtubules, perhaps through dynein or actin, or by regulating microtubule stability (see Goldstein, 2003; Kusch et al., 2003). In fission yeast, the spindle aligns with the cell cortex, thus using cellular geometry as a determinant for spindle positioning (reviewed in Kusch et al., 2003). The actin cytoskeleton is involved in proper spindle orientation, as actin cables serve as tracks for the bud tip positioning of either astral microtubules or components required for their cortical anchoring (summarized in Bloom, 2001; Goode et al., 2000). For example, an actin-bound motor of the myosin family may interact with one of the microtubule-bound kinesins, linking the two filaments, and pulling the microtubule toward the bud tip where the actin cable is anchored. Alternatively myosin may bind a microtubule-associated non-motor protein such as CLIP170/Bik1p, or the critical nuclear positioning protein Kar9p, which binds microtubules via EB1/Bim1p (based on this interaction, Kar9p is thought to be the functional equivalent of APC; Kusch et al., 2003), to transport them to the bud via actin filaments (Hwang et al., 2003). An actin-associated complex containing Bni1p, Spa2p, Pea2p and Bud6p, which functions in cell polarity, bud site selection and spindle orientation, may anchor Kar9p and other proteins needed for microtubule attachment at the

bud tip. The cortical site of microtubule attachment also contains Num1p, which functions as an anchor for dynein (reviewed in Bloom, 2001; Heil-Chapdelaine et al., 2000).

In a model of spindle positioning, astral microtubules initially probe the cortex early during bud emergence, in a Kar9p-assisted search and capture mechanism (Adames and Cooper, 2000); the duplicated SPBs are then placed near the bud neck. The SPB destined to the bud makes contact preferentially with the bud cortex, and then also with the bud neck, while the mother-bound SPB interacts with the mother cortex, first near the bud neck and then progressively away from the neck towards the distal end, which orients the spindle. During these events, dynein is first associated with the daughter-bound SPB, and then redistributes to both SPBs. This assymetric distribution depends on Cdk-Clb5p activity, thus linking spindle positioning with cell cycle control. The nucleus then migrates to the bud neck, and spindle elongation through the neck is powered by dynein anchored at the cortex by Num1p. However, rather than being first recruited to the cortex and then capturing microtubules, dynein is thought to reside at the plus end of microtubules via a number of microtubule-associated proteins such as its associated dynactin or a CLIP170/Bik1p-LIS1/Pac1p complex (Coquelle et al., 2002); Num1p would then function not only as a cortical anchor for dynein but also as an enhancer of its motor activity or processivity (Sheeman et al., 2003). Microtubule capture at the cortex is thought to remain a dynamic process which, as described above for spindle pole separation, requires a balance between the antagonistic activity of several motors (dynein, Kip2p, Kar3p, and Kip3p). Once the spindle is properly positioned at the neck, interactions between astral microtubules and Bud6p localized at the neck are thought to prevent its further migration into the bud (Bloom, 2001; Segal and Bloom, 2001).

SPB duplication is known to be conservative. Interestingly, it was shown that the "old" SPB is destined for the bud in most instances, while the "new" SPB remains in the mother (Pereira et al., 2001). The significance of this pattern of inheritance is unknown. The differential behavior of the two SPBs depends on microtubule-cortex interactions mediated by Kar9p. Kar9p only associates with the old (daughter-bound) SPB, due to the inhibitory action of Cdk-Clb3p or Clb4p that assymetrically associates with the new (mother-bound) SPB and phosphorylates Kar9p, preventing its association with Bim1p at the new SPB. Kar9p at the old SPB (associated with Bim1p and therefore microtubules) is then transported

to the bud via interaction with myosin (Hwang et al., 2003). The differential activity of Cdk at the mother-bound pole was shown to be mediated by preferential association of the Clb3/4p with that pole, which might be established during spindle pole duplication (Liakopoulos et al., 2003).

I.4. Overview of the centromere in eukaryotes

The consequences of faulty chromosome transmission and the mechanisms that are in place to prevent errors, described in section I.2, highlight the importance of proper chromosome segregation in the maintenance of genomic stability. A central component in this process is a specialized region of the chromosome called the centromere, which functions as the site of attachment for microtubules of the mitotic spindle. In chromosomes of higher eukaryotes, the centromere is recognized in condensed metaphase chromosomes as a region with a distinct cytological structure, known as the primary constriction, that can be visualized microscopically; this structure may arise from the increased amount of cohesion at centromeres (Petronczki et al., 2003; Vig and Rattner, 1989).

A great variation in centromere size and base composition is observed among eukaryotes; hence, no universal consensus has been defined for centromeric DNA sequences. In higher eukaryotes, centromeres often (but not always) consist of a large number of repeated sequences that range from 12bp to 240bp, such as human 171bp α-satellites, and tend to have high A/T nucleotide content (reviewed in Craig et al., 1999; Sullivan et al., 2001). Human centromeres can be several Mb long. The fission yeast *S. pombe* centromere is 35-100kb long and contains inverted repeats that flank a core sequence (reviewed in Karpen and Allshire, 1997; Wiens and Sorger, 1998); while the core sequence is sufficient to confer mitotic stability, conserved elements within the flanking repeat are thought to stimulate establishment of centromere function (reviewed in Sharp and Kaufman, 2003). In contrast, the minimal DNA sequence required to specify the centromere of budding yeast (described in more detail in Chapter II) is a conserved 125bp-long stretch of DNA that is protected from nuclease digestion (Cottarel et al., 1989). To some extent the variation in centromere size may be due to differences in the complexity of microtubule attachment: in yeast, each kinetochore binds only one microtubule, whereas the kinetochores of higher eukaryotes bind 10-45 microtubules, which is likely to require a larger and more complex structure (whether

this structure consists of repeated subunits of single-microtubule attachment sites is unclear). However, the relationship between number of microtubules per centromere and size to explain centromere complexity is limited, as the fission yeast centromere is significantly larger than the budding yeast centromere, despite the fact that it binds 2-4 microtubules (Hoyt and Geiser, 1996; Maney et al., 2000; Rieder and Salmon, 1998).

Centromeres are not just naked DNA; similar to all genomic DNA, they exist as chromatin, a complex between DNA and proteins. Chromatin itself is further organized into fibrous structures that greatly influence its function, for example in gene regulation. At the most basic level, DNA strands are wrapped around nucleosomes, octamers of histone proteins (two of each of H2A, H2B, H3 and H4); 146bp of DNA (two turns) is wrapped around each nucleosome, and nucleosomes occur periodically along DNA, each being associated with about 160-200bp of DNA. The nucleosomal array is then packed into a chromatin fiber by association with stabilizing linker histones (histone H1) and other proteins, and chromatin is then condensed into higher order structures (reviewed in Hayes and Hansen, 2001; Wolffe and Guschin, 2000; Woodcock and Dimitrov, 2001). Histones are small basic proteins composed of a globular domain forming the core of the nucleosome and a charged amino-terminal tail that binds DNA and other proteins. The histone tail can be post-translationally modified (acetylation, phosphorylation, methylation, ribosylation, ubiquitination) to modulate the strength of the interaction with DNA, the compaction of chromatin, and the interaction with trans-acting factors (Wolffe and Guschin, 2000). Because of the large influence of different combinations of tail modifications on many processes, including inherited structural information and activity, they have come to be known as the "histone code" (Jenuwein and Allis, 2001). Nucleosome positioning, which is influenced by DNA sequence, determines access to DNA and is known to affect many processes, for instance transcription; some transcription factors access DNA by rapid and transient unwrapping of DNA from nucleosomes, while others are able to bind nucleosomal DNA directly (reviewed in Hayes and Hansen, 2001; Lewin, 1994).

Chromatin exists in two main forms: euchromatin, a transcriptionally active region, and heterochromatin, a transcriptionally inert, silenced form. Heterochromatin is maintained throughout mitosis, is more condensed and typically less accessible to enzymes, and often associates with the nuclear envelope. Heterochromatic properties are thought to arise from a

nucleosomal array that is more highly ordered, and in which histones have a specific post-translational modification patterns (e.g. hypoacetylation, hypermethylation). Specific chromatin-associated factors are enriched in heterochromatin, such as the HP1 and Su(var) proteins, which are conserved in most eukaryotes and contain conserved domains (e.g. bromo-, chromo- and SET domains); these domains are also conserved in antagonistic, euchromatin-promoting factors (Jenuwein and Allis, 2001). While budding yeast does not have a homolog of HP1, it contains a unique set of proteins (Sir) that mediate silencing and are considered to be the functional equivalent of HP1. Targeting of HP1/Sir to specific chromosomal sites may occur through the recognition of specific DNA sequences by DNA-binding proteins (e.g. Rap1p at telomeres) that will then interact with HP1/Sir proteins (reviewed in Kellum, 2003).

Histones are deposited onto DNA as nucleosomes during DNA replication, and this process involves histone chaperones, the conserved chromatin assembly factor I (CAF-I) complex, the CAF-I related protein Asf1, and the histone regulatory proteins (Hir) (reviewed in Sharp and Kaufman, 2003). CAF-I is recruited by association with PCNA at the replication fork, and is involved in the initial deposition of H3 and H4. CAF-I also function in silencing at heterochromatic loci (e.g. telomeres, mating-type loci, rDNA), possibly by recruiting silencing complexes. Recently, CAF-I and Hir proteins have been demonstrated to be concentrated at centromeres, where they might serve as a scaffold for recruitment of other chromatin and non-chromatin proteins that allow the formation of an active centromere, thus participating in proper centromere formation and function (Sharp et al., 2002). Interestingly, the yeast silencer Sir1p interacts with the CAF-I subunit Cac1p independently of other Sir proteins, which suggests that mammals and yeast use similar mechanisms (Sir or HP1 recruiting CAF-I) to recruit chromatin factors involved in centromere formation (Sharp et al., 2003).

Centromeres and pericentromeric flanking region are typically heterochromatic and don't generally contain gene-coding sequences, but they display transcriptional repression if genes are artificially placed within centromeric sequences (a phenomenon referred to as position effect variegation). Centromeric silencing has been shown to require HP1 and Su(var)3-9 (a histone methyltransferase), and seems to be critical for centromere function, as loss of silencing or mutation in HP1 or Su(var)3-9 leads to increased chromosome instability.

Su(var)3-9 may mediate its silencing role through methylation of histone H3, which would act as a signal to recruit HP1. The recruitment of Su(var)3-9 appears to be mediated by histone deacetylases (Jenuwein and Allis, 2001; Sharp and Kaufman, 2003).

The lack of a specific sequence defining centromeres in most organisms has prompted the search for other mechanisms that determine what gives certain regions of chromatin a centromeric character. For example, centromeric chromatin could have centromere-specific histone modification patterns or factors that promote a specialized chromatin structure (Karpen and Allshire, 1997). These could influence the spacing and arrangement of nucleosomes, and therefore the packing order of DNA, or they could modulate the interaction of specific proteins with centromeric chromatin. One possible centromere determinant is a specialized form of histone H3 that occurs in all eukaryotes (CENP-A/Cse4p) and is hypothesized to replace histone H3 in centromeric or pericentromeric nucleosomes (Smith, 2002; reviewed in Sullivan, 2001). Although the histone fold domain of CENP-A/Cse4p is conserved, the N-terminal tail diverges greatly among eukaryotes; this region might thus mediate interaction with organism-specific kinetochore proteins, perhaps through post-translational modifications (Chen et al., 2000). CENP-A/Cse4p was shown to be required for the proper recruitment of many kinetochore components, spindle checkpoint proteins, cohesins and centromere-associated kinases such as Plk. However, CENP-A/Cse4p is not sufficient in itself to direct formation of a centromere at an ectopic site (Meluh and Strunnikov, 2002; Sharp and Kaufman, 2003).

Centromere formation has also been proposed to be epigenetically (i.e. independent of DNA sequence) determined, through inheritable methylation of DNA, and acetylation, phosphorylation, or other modifications of histones and chromatin remodelling proteins (Choo, 2000; Karpen and Allshire, 1997). An epigenetic mechanism would explain the presence of fully functional mammalian centromeres among sequences that are not "centromere-like", or the fact that in a dicentric chromosome only one of the two centromeres is functional and assembles a kinetochore (Craig et al., 1999; Wiens and Sorger, 1998). It has been suggested that epigenetic mechanisms also influence *de novo* centromere formation in budding yeast (Mythreye and Bloom, 2003). A specialized chromatin structure, affecting for instance the level of DNA packing and compactness, is thought to exist at centromeres and might contribute to the specification of centromeres on DNA that has

greatly diverging primary sequence (Choo, 2000; Smith, 2002). Centromere identity could also be determined by spatial and temporal separation of centromeric heterochromatin formation; for example, regulation of the expression of centromeric determinants such as CENP-A/Cse4p, the timing and regulation of their deposition into chromatin, as well as the timing of centromere DNA replication, have all been proposed to contribute to the propagation of the specialized centromere DNA structure (Mellone and Allshire, 2003; Sullivan et al., 2001). Finally, Mellone & Allshire (2003) propose that the tension and/or microtubule attachment occurring during one mitotic cycle could imprint chromatin at the centromere so that it will be recognized as the site where centromere formation should occur in the next cycle; this could be mediated by chromatin factors including CENP-A, or by enzymes that reside at active centromeres during mitosis (Mellone and Allshire, 2003).

Beside a function in determining centromere identity, chromatin proteins have also been proposed to be important for centromere structure and function by contributing to the stability of kinetochore-microtubule attachments. Centromeric nucleosome assembly is monitored by the spindle assembly checkpoint, suggesting that compromised histone deposition affects proper microtubule attachment (Sharp et al., 2002). Pericentromeric regions contain phased nucleosome arrays, the integrity of which is essential for centromere integrity and proper chromosome segregation (Pinto and Winston, 2000; Saunders et al., 1990). It is thought that nucleosomes flanking the core centromere sequence serve to stabilize centromere DNA-kinetochore interaction as well as kinetochore structure itself. In addition, histone modifying enzymes are required for proper establishment of pericentric heterochromatin; defects in specific modifications of histone tails affect chromosome segregation. For example, mutation of the serine 10 phosphorylation site in histone H3 leads to extensive chromosome loss (Wei et al., 1999), and mutation of a component of histone deacetylase leads to aneuploidy and defective cytokinesis (David et al., 2003). The critical importance of pericentromeric heterochromatin in maintaining a functional centromere was also demonstrated when cells artificially inhibited for histone deacetylases displayed loss of proper centromere function and defective chromosome segregation (Ekwall et al., 1997; Taddei et al., 2001).

Recently, budding yeast chromatin remodelling complexes such as RSC have also been implicated in centromere function (Hsu et al., 2003; Smith, 2002). RSCs are

ATPase–containing complexes related to the chromatin remodeller SWI/SNF complex. They contain helicase-like domains and function by repositioning nucleosome arrays, thus influencing chromatin access (Flaus and Owen-Hughes, 2001). Mutants in some of the RSC subunits have a chromosome loss phenotype, activate the spindle assembly checkpoint, and enhance the phenotypes of a centromere mutant (Baetz et al., 2004). Additionally human RSC shows a concentrated localization at kinetochore (Xue et al., 2000). Chromatin remodellers are thought to have a general role in regulating higher order folding of chromatin by acting together with other chromatin or DNA modifications (Flaus and Owen-Hughes, 2001). Chromatin remodelling complexes may have a role in CENP-A deposition (which happens at G2) as it is uncoupled from replication (Sharp and Kaufman, 2003; Sullivan, 2001).

Chromatin proteins are also thought to have a role in the recruitment of cohesin. Both chromatin proteins and cohesin are loaded at the time of DNA replication. The fission yeast HP1 (Swi6) was shown to be specifically required in heterochromatin at centromeres to promote association of cohesin subunits, although it was not required for cohesion along chromosome arms (Bernard et al., 2001). Cohesion near centromeres might also be critical to establish stable bipolar attachments, and thus pericentromeric heterochromatin and cohesion may function as a "tensiometer" to detect proper microtubule attachment (He et al., 2000; Meluh and Strunnikov, 2002). The chromosome instability phenotypes observed in some of the chromatin remodeller mutants may be due to their inability to properly recruit cohesin (Baetz et al., 2004). Finally, cohesin may also participate in chromatin silencing (reviewed in Sharp and Kaufman, 2003).

I.5. The eukaryotic kinetochore

The kinetochore is defined as the set of proteins that bind centromeric DNA to provide a platform for interaction with microtubules of the mitotic spindle, and mediate chromosome movement and checkpoint signaling (Maney et al., 2000) (Figure I.4). The kinetochore of higher eukaryotes is organized in a number of substructures distinguishable by electron microscopy (reviewed in Rieder and Salmon, 1998). In a simplistic way the kinetochore can be viewed as two main layers: an outer layer that mediates interaction with microtubules and

microtubule-associated proteins and motors, and an inner layer that interacts with specialized centromeric chromatin (Sullivan, 2001).

In mammals, the first kinetochore proteins were identified when sera from patients with autoimmune disorders such as CREST were shown to contain antibodies that localized to the primary constriction of chromosomes (Earnshaw and Rothfield, 1985; reviewed in Maney et al., 2000). The antigenic proteins recognized by these antibodies were named centromere proteins (CENPs). Other CENPs were subsequently defined from sera and by other techniques and to date there are ten known CENP's (CENP-A, B, C etc.), some of which have been shown to specifically bind centromeres (Choo, 2000). The centromere-specific

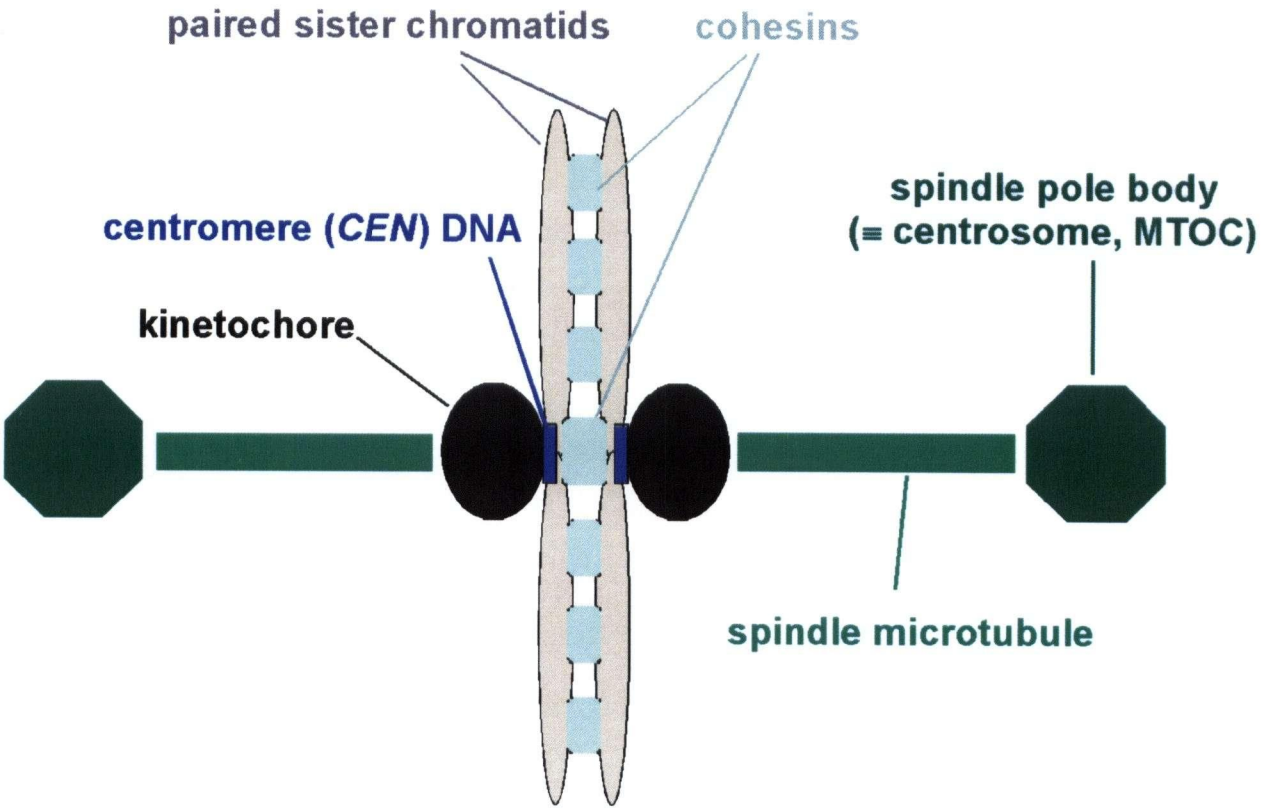

Figure I.4. The central role of the kinetochore complex in chromosome-microtubule attachment. The kinetochore mediates attachment of chromosomes to microtubules of the mitotic spindle. It is thus a central player in the process of chromosome segregation, and it is thought to act as a structural, regulatory, and signaling platform for many proteins that participate in the process: microtubule- and DNA-associated factors, motor proteins, checkpoint proteins, etc.

modified histone H3 CENP-A/Cse4p was described above (section I.4). The KinN-type kinesin CENP-E that was mentioned previously for its role in microtubule dynamics (section I.3), has a central role at the kinetochore. Beside promoting the motility of chromosomes along microtubules, it functions in the capture of microtubules by the kinetochore, in congression of chromosomes to the metaphase plate as well as the maintenance of a metaphase configuration, and in the spindle checkpoint pathway (reviewed in Wittmann et al., 2001). Some aspects of CENP-E's role will be discussed in more detail in Chapter III. CENP-F/Mitosin, which preferentially associates with unaligned chromosomes, has been proposed to work together with CENP-E in spindle checkpoint function (Yang et al., 2003). And CENP-I, a protein with homology to budding yeast Ctf3p and fission yeast Mis6, is important for the kinetochore localization of some checkpoint components including MAD2; thus CENP-I has been proposed to have a role in promoting the sustained "wait-anaphase" signal that results in mitotic arrest in cells where microtubule attachment is defective (Liu et al., 2003).

As mentioned in previous sections, other proteins were also shown to function at the kinetochore, including the Aurora/Ipl1p kinase (reviewed in Carmena and Earnshaw, 2003), chromosomal passenger proteins (INCENPs), microtubule-associated proteins and motors (dynein and kinesin-like proteins), and components of the mitotic checkpoint (reviewed in Maney et al., 2000). Some of these proteins are localizing to the centromere for only part of the cell cycle; it could be that they only have a transient function to perform at the kinetochore, or that they use the kinetochore as a site of assembly before moving on to their site of action; alternatively, proteins such as the chromosomal passengers may provide a link between chromosomal and cytoskeletal function (Adams et al., 2001; Craig et al., 1999). In budding yeast, the number of kinetochore proteins has literally exploded over the past 5 years, as illustrated in Figure I.5. The introduction to Chapter II gives a more detailed description of yeast kinetochore proteins. Several yeast kinetochore proteins have mammalian homologues; in addition, some of the homologous kinetochore components appear to be similarly post-translationally modified; finally, several kinetochore components in mammals and yeast have a similar behavior during anaphase, such as a relocalization from the centromere to the spindle midzone (Adams et al., 2001). These observations indicate that the chromosome segregation machinery may be more evolutionarily conserved than might

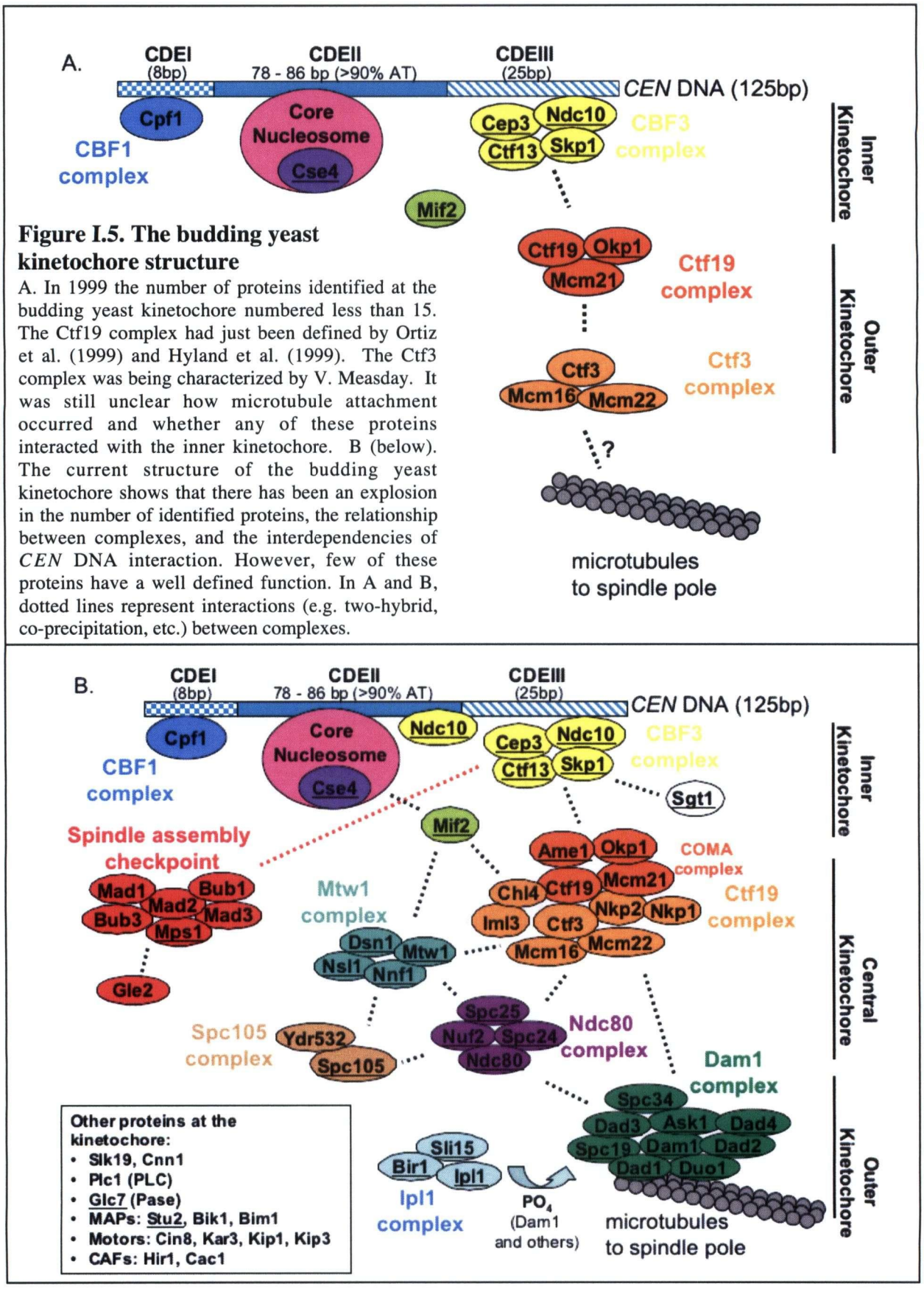

Figure I.5. The budding yeast kinetochore structure

A. In 1999 the number of proteins identified at the budding yeast kinetochore numbered less than 15. The Ctf19 complex had just been defined by Ortiz et al. (1999) and Hyland et al. (1999). The Ctf3 complex was being characterized by V. Measday. It was still unclear how microtubule attachment occurred and whether any of these proteins interacted with the inner kinetochore. B (below). The current structure of the budding yeast kinetochore shows that there has been an explosion in the number of identified proteins, the relationship between complexes, and the interdependencies of *CEN* DNA interaction. However, few of these proteins have a well defined function. In A and B, dotted lines represent interactions (e.g. two-hybrid, co-precipitation, etc.) between complexes.

have been previously thought (Kitagawa and Hieter, 2001), which validates the use of model organisms such as yeast in studies of this process.

I.6. Overview of thesis

The next two chapters describe the identification of three new proteins of the budding yeast kinetochore. In an era when the genome sequence of yeast has been completed for nearly ten years, the challenge has switched from the sole identification of genes to the characterization of their function. As such, the sequence of the proteins characterized here was already known when this project started, and some preliminary information about their involvement in a specific cellular pathway was available, mainly through the use of genetic assays. Several proteomic methods developed in the past five years have also allowed the identification of many of the proteins involved in kinetochore function. However, these large-scale studies rarely go beyond the identification step, except for a few chosen components. Chapters II and III provide not only definitive evidence that yeast Chl4p, Iml3p and Ame1p are kinetochore proteins by rigorous methods that test for recognized hallmarks of such proteins, but also provide insight into the molecular organization of the kinetochore complex by detailing dependency of interactions among kinetochore proteins and between these proteins and centromere DNA. Moreover, Chapter III presents data that suggest that Ame1p has additional roles at the kinetochore, such as a possible contribution to the maintenance of checkpoint function. Chapter IV draws conclusions about the possible function of these proteins, mentioning some additional data obtained by other groups during the course of this work, and discusses the overall recent advances in the understanding of kinetochore structure and function. Finally, Chapter IV also speculates on the meaning of the research in the treatment of diseases and on possible outcomes in the long term.

CHAPTER II

CHARACTERIZATION OF TWO NEW COMPONENTS OF THE BUDDING YEAST KINETOCHORE, CHL4P AND IML3P

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II.1. Introduction

The budding yeast kinetochore is a multiprotein complex that assembles at the yeast centromere and contributes to high fidelity of chromosome transmission. The 125bp segment of yeast DNA that is sufficient for centromere function contains three conserved DNA elements (CDEs) (see Figure I.5). CDEI and III are conserved sequences while CDEII is an intervening A/T-rich region of conserved length and base composition (Hyman and Sorger, 1995). One model proposes that CDEI and III serve as sites of interaction with kinetochore proteins, while CDEII wraps around a specialized centromeric nucleosome (reviewed in Cheeseman et al., 2002b). However, recent data (discussed further in Chapter IV) support a different model in which yeast centromere DNA is nucleosome-free (Espelin et al., 2003). CDEI is not essential for centromere function, whereas the lack of CDEII and CDEIII or point mutations in CDEIII result in an inactive centromere (Hegemann et al., 1988; McGrew et al., 1986).

Although a large number of proteins interact with centromere DNA (*CEN* DNA) specifically (see Figure I.5.B), only a subset of these (proteins of the inner kinetochore) has been shown to make direct contacts with DNA. Cbf1p binds to CDEI and *cbf1* mutants, while viable, display increased chromosome loss (Baker and Masison, 1990; Cai and Davis, 1990). Cbf1p is thought to influence chromatin structure and also functions to regulate transcription at other chromosomal sites (Cai and Davis, 1990; Mellor et al., 1990). The CBF3 complex, which is made up of the essential proteins Ndc10p/Cbf2p, Cep3p, Ctf13p and Skp1p, has been shown to bind CDEIII directly (Lechner and Carbon, 1991; Stemmann and Lechner, 1996). Interestingly, Skp1p functions not only at the kinetochore but also in the SCF E3 ubiquitin ligase complex that targets proteins for degradation (Bai et al., 1996; Connelly and Hieter, 1996); kinetochore assembly has been shown to involve regulatory ubiquitination (Kaplan et al., 1997; Russell et al., 1999). Sgt1p is an interacting partner of Skp1p and participates in its functions at the kinetochore and at the SCF (Kitagawa et al., 1999). While the binding of specific proteins has been mapped to CDEI and III, until recently no proteins had been shown to interact with CDEII, although a few candidates had been proposed. These include the modified histone H3 Cse4p (CENP-A homolog), which is thought to be part of a specialized centromeric nucleosome (Meluh et al., 1998), and Mif2p, a

homolog of human CENP-C that may bind centromeric A/T rich regions (Meluh and Koshland, 1997). However, it has now been demonstrated that Ndc10p is able to bind CDEII, extending the idea that Ndc10p is not only part of the CBF3 complex but also polymerizes along *CEN* DNA outside of CDEIII (see Chapter IV) (Espelin et al., 1997).

The CBF3 complex provides a platform for the assembly of the central and outer kinetochore, which is comprised of several protein complexes that associate with *CEN* DNA chromatin in a CBF3-dependent manner (reviewed in Cheeseman et al., 2002b; McAinsh et al., 2003; Ortiz and Lechner, 2000) and serve as probable links to microtubules, motor proteins or regulatory proteins. At the early stages of this project, there were four known central/outer kinetochore protein complexes: the Ctf19 complex, containing Ctf19p, Mcm21p and Okp1p (Ortiz et al., 1999); the Ctf3 complex, containing Ctf3p, Mcm16p and Mcm22p (Measday et al., 2002); the Ndc80 complex, containing Ndc80p, Nuf2p, Spc24p and Spc25p (Janke et al., 2001; Wigge and Kilmartin, 2001); and the Dam1 complex, containing Dam1p, Duo1p, Spc19p, Spc34p, Ask1p, Dad1p, Dad2p, Dad3p and Dad4p (Cheeseman et al., 2001; Janke et al., 2002; Li et al., 2002). These central/outer kinetochore complexes contain proteins that localize to the kinetochore, interact specifically with *CEN* DNA, and contribute to the fidelity of chromosome transmission. Some components of the Dam1 complex bind microtubules (Hofmann et al., 1998), placing this complex at the periphery of the outer kinetochore. However, the exact physical interactions and molecular architecture of complexes within the kinetochore and the pattern of assembly of individual proteins in this supra-molecular complex still remain to be elucidated.

Hallmarks for the identification of kinetochore proteins include their ability to crosslink to *CEN* DNA, as assayed by chromatin immunoprecipitation (ChIP), and to localize next to the nuclear side of the SPB. These criteria have been used to assess candidate genes that potentially encode kinetochore proteins (He et al., 2001), including genes required for faithful chromosome transmission identified by genetic screens. Several independent screens have led to the isolation of mutants that lose chromosomes at a higher rate than a wild type strain (the *chl*, *mcm*, *ctf* and *cin* mutants) (Hoyt et al., 1990; Kouprina et al., 1988; Maine et al., 1984; Spencer et al., 1990). *CHL4/CTF17/MCM17* was identified in three of these mutant collections (Kouprina et al., 1988; Kouprina et al., 1993b; Roy et al., 1997; Spencer et al., 1990). A strain deleted for *CHL4* is viable (Roy et al., 1997), and several secondary

phenotypes suggest that *chl4* mutants have a compromised kinetochore: strains with *chl4* mutations are able to maintain a dicentric plasmid with the same fidelity as a monocentric plasmid (Doheny et al., 1993; Kouprina et al., 1993a); a mutation in *chl4* weakens the transcription block that occurs when a *CEN* sequence is placed between a promoter and a reporter construct (Doheny et al., 1993); and two mutant alleles of *chl4* become inviable upon increased dosage of *CTF13* or *NDC10* (Kroll et al., 1996; Measday et al., 2002). Chl4p was also shown to have a two-hybrid interaction with Iml3p/Mcm19p (Ghosh et al., 2001). *iml3* mutants exhibit an increased rate of centromere plasmid loss (Entian et al., 1999; Roy et al., 1997) as well as a number of phenotypes suggesting that *IML3* encodes a previously uncharacterized kinetochore protein.

This chapter establishes Chl4p and Iml3p as *bona fide* kinetochore proteins, and their physical juxtaposition and *CEN* DNA loading requirements within the kinetochore complex are examined. Chl4p crosslinks to *CEN* DNA chromatin, localizes to the kinetochore and interacts with two known central kinetochore proteins, Ctf19p and Ctf3p. In addition, Chl4p interacts with a new kinetochore component, Iml3p, that displays a Chl4p-dependent *CEN* DNA interaction and a kinetochore localization pattern. A combination of biochemical and *in vivo* localization techniques are used to determine specific requirements and dependencies for Chl4p, Iml3p, Ctf19p and Ctf3p interactions and kinetochore localization, providing insights into the molecular architecture of the kinetochore complex.

Note: I performed all the work described in this chapter except for the following:

- Microscopy images and quantitation (Sections II.3.c and II.4.cegh; Figures II.3 and II.4) were done by Bryan Snysdman in Trisha Davis and Eric Muller's laboratories at the Yeast Resource Center (YRC) in Seattle, WA, using strains I had constructed.
- The genome-wide two-hybrid screen (Sections II.3.d and II.4.h) was performed by Gerard Cagney in Stanley Fields' laboratory at the YRC, using the bait Chl4p strain I had constructed. I subsequently reconfirmed the results manually using my bait strain and prey strains provided by the YRC (Section II.4.h; Figure II.7).

II.2. Materials and methods

II.2.a. Yeast strains and media

Strains used in this study are listed in Table II.1. Media for growth and sporulation were described previously (Rose et al., 1990). To visualize chromosome fragment loss, strains were first grown on SC medium lacking uracil (selecting for the chromosome fragment), and then streaked onto YPD medium. In strains with a high rate of chromosome loss, a colony will consist of cells containing the chromosome fragment (white), and cells that have lost it (red), resulting in a white and red sectored phenotype. For the microtubule-depolymerizing drug sensitivity assay, benomyl from DuPont (Wilmington, DE) was added at the indicated concentration to YPD media; DMSO was used in the control plate (0μg/ml benomyl). Epitope tagging and gene deletions were made directly at the endogenous loci according to Longtine et al. (1998), and functionality of tagged proteins was determined using the sectoring assay. Yeast transformations were done according to Gietz and Schiestl (1995).

II.2.b. ChIP assays and co-immunoprecipitations

ChIP assays and co-immunoprecipitations (co-IPs) from yeast lysates were performed as in Measday et al. (2002) with the following changes. For ChIP analysis, multiplex PCR was performed, with three sets of primers added to a single PCR reaction. The primer pairs used to amplify specific regions of DNA are described in Meluh and Koshland (1997). The expected sizes of PCR products are 302bp (*CEN1*), 288bp (*PGK1*) and 243bp (*CEN3*). To equilibrate the amount of PCR products obtained, 0.6 mM primer was added for each of the *CEN3* and *PGK1* pairs, whereas 0.8 mM primer was used for the *CEN1* pair. The amount of total chromatin added varied from 1/1500 to 1/600 of the available template, while that of immunoprecipitated chromatin template varied from 1/10 to 1/30 of the available template, depending on the linear range for PCR.

II.2.c. Fluorescence microscopy

Proteins were tagged at their C-terminus with YFP and CFP as described in Hailey et al. (2002). In all cases the tag was integrated into the genome to maintain gene expression from the endogenous promoter. To ensure that wild type and mutant strains expressed similar amounts of YFP or CFP fusion proteins, 50μg of yeast lysate was run on an SDS-PAGE gel

and Western blotted with anti-GFP antibody from Boehringer Mannheim (Indianapolis, IN) for all strains used in fluorescence imaging. Cells were imaged using a DeltaVision microscopy system from Applied Precision (Issaquah, WA). The system incorporates an Olympus IL-70 microscope, a u-plan-apo 100X oil objective (1.35NA), a CoolSnap HQ digital camera from Roper Scientific (Tucson, AZ) and optical filter sets from Omega Optical (Battleboro, VT). Live cells were imaged on a thin pad of media containing 1% agarose (Hailey et al., 2002). Analysis was carried out on single-plane, non-deconvolved pictures using SoftWoRx software. To quantify the image intensities at the kinetochore and in the nucleus, the image intensity values in a 5x5 pixel square centered either on the kinetochore or within the nucleus, respectively, were summed. A background value from a 5x5 pixel square either adjacent to the kinetochore (within the nucleus), or a 5x5 pixel square within the cytoplasm was subtracted from the summed values for the kinetochore or nucleus, respectively. For the conversion of the image files to the TIFF format, the output was 8-bit grayscale and all images of a particular protein in different mutant backgrounds were scaled with the same set minimum and maximum values.

II.2.d. Genome-wide two-hybrid assay

CHL4 was cloned into pOBD2 as described in Cagney et al. (2000). The Chl4p-DNA binding domain fusion was functional as judged by rescue of the *chl4Δ* sectoring phenotype described in Figure II.1. Two-hybrid screens were performed as described in Uetz et al. (2000). To confirm positive two-hybrid interactions, strains containing the DNA binding domain fusion plasmid (or pOBD2 vector alone) and the activation domain fusion plasmid (or pOAD vector alone) were mated. Diploid strains containing both plasmids were grown to log phase in media selecting for the plasmids, and five-fold dilutions of $5x10^6$ cells were plated on media selecting for the two-hybrid interaction as indicated on Figure II.7.

Table II.1. List of yeast strains used in experiments described in this chapter

Strain	Genotype	Reference
YCTF30	MATα *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1* CFIII (*CEN3.L.* YPH278) *URA3 SUP11 ctf13-30*	(Spencer et al., 1990)
YCTF42	MATα *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1* CFIII (*CEN3.L.* YPH278) *URA3 SUP11 ctf14-42*	(Spencer et al., 1990)
YPH499	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63*	P. Hieter
YPH500	MATα *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63*	P. Hieter
YPH1027	MATa *ura3-52 leu2-3,112 ndc10-2*	T. Huffaker
YPH1124	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63* CFIII (*CEN3.L.* YPH982) *URA3 SUP11*	P. Hieter
YPH1315	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ctf19Δ::HIS3*	(Hyland et al., 1999)
YPH1316	MATα *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ctf19Δ::TRP1*	(Hyland et al., 1999)
YPH1534	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 chl4Δ::His3MX6* CFIII (*CEN3.L.* YPH982) *URA3 SUP11*	This study
YPH1535	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 chl4-20* CFIII (*CEN3.L.* YPH982) *URA3 SUP11*	This study
YPH1536	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 chl4-61* CFIII (*CEN3.L.* YPH982) *URA3 SUP11*	This study
YPH1537	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 chl4Δ::His3MX6*	This study
YPH1538	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 chl4Δ::kanMX6*	This study
YPH1539	MATα *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 chl4Δ::kanMX6*	This study
YPH1540	MATα *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 chl4-20*	This study
YPH1541	MATα *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 chl4-61*	This study
YPH1542	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 CHL4-13Myc-TRP1*	This study
YPH1543	MATα *ura3-52 lys2-801 ade2-101 leu2 trp1Δ63 ndc10-2 CHL4-13Myc-TRP1*	This study
YPH1544	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ctf19Δ::HIS3 CHL4-13Myc-TRP1*	This study
YPH1545	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ctf3Δ::HIS3 CHL4-13Myc-TRP1*	This study
YPH1546	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 CHL4-13Myc-TRP1 CTF3-3HA-His3MX6*	This study
YPH1547	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 CHL4-13Myc-TRP1 CTF3-3HA-kanMX6*	This study
YPH1548	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ctf19Δ::HIS3 CHL4-13Myc-TRP1 CTF3-3HA-kanMX6*	This study
YPH1549	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 iml3Δ::kanMX6 CHL4-13Myc-TRP1*	This study
YPH1550	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 CTF19-13Myc-TRP1*	This study
YPH1551	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 chl4Δ::His3MX6 CTF19-13Myc-TRP1*	This study
YPH1552	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 chl4Δ::His3MX6 CTF3-13Myc-TRP1*	This study
YPH1553	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 CTF3-3HA-His3MX6*	This study
YPH1554	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ctf19Δ::HIS3 CTF3-3HA-kanMX6*	This study

(continues)

Table II.1 (continued)

Strain	Genotype	Reference
YPH1555	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 NCD10-13Myc-kanMX6*	This study
YPH1556	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 chl4Δ::His3MX6 NCD10-13Myc-kanMX6*	This study
YPH1557	MATα *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 IML3-3HA-kanMX6*	This study
YPH1558	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 chl4Δ::His3MX6 IML3-3HA-kanMX6*	This study
YPH1559	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ctf19Δ::HIS3 IML3-3HA-kanMX6*	This study
YPH1560	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 CHL4-13Myc-TRP1 IML3-3HA-kanMX6*	This study
YPH1561	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ctf19Δ::HIS3 CHL4-13Myc-TRP1 IML3-3HA-kanMX6*	This study
YPH1562	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 IML3-13Myc-His3MX6*	This study
YPH1563	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 chl4Δ::His3MX6 IML3-13Myc-kanMX6*	This study
YPH1564	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ctf19Δ::HIS3 IML3-13Myc-kanMX6*	This study
YPH1565	MATa/α *ura3-52/ura3-52 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 trp1-901/trp1-901 gal4Δ/gal4Δ gal80Δ/gal80Δ GAL2-ADE2/GAL2-ADE2 LYS2::GAL1-HIS3/LYS2::GAL1-HIS3 met2::GAL7-LacZ/met2::GAL7-LacZ pOBD2-CHL4 pOAD-MIF2*	This study
YPH1566	MATa/α *ura3-52/ura3-52 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 trp1-901/trp1-901 gal4Δ/gal4Δ gal80Δ/gal80Δ GAL2-ADE2/GAL2-ADE2 LYS2::GAL1-HIS3/LYS2::GAL1-HIS3 met2::GAL7-LacZ/met2::GAL7-LacZ pOBD2-CHL4 pOAD*	This study
YPH1567	MATa/α *ura3-52/ura3-52 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 trp1-901/trp1-901 gal4Δ/gal4Δ gal80Δ/gal80Δ GAL2-ADE2/GAL2-ADE2 LYS2::GAL1-HIS3/LYS2::GAL1-HIS3 met2::GAL7-LacZ/met2::GAL7-LacZ pOBD2 pOAD-MIF2*	This study
YPH1568	MATa/α *ura3-52/ura3-52 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112 trp1-901/trp1-901 gal4Δ/gal4Δ gal80Δ/gal80Δ GAL2-ADE2/GAL2-ADE2 LYS2::GAL1-HIS3/LYS2::GAL1-HIS3 met2::GAL7-LacZ/met2::GAL7-LacZ pOBD2 pOAD*	This study
YPH1569[a]	MATa/α *ura3-1/ura3-1 ade2-loc/ade2-loc his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 CHL4-YFP-His3MX6/CHL4-YFP-His3MX6 SPC29-CFP-kanMX6/SPC29-CFP-kanMX6*	This study
YPH1570[a]	MATa/α *ura3-1/ura3-1 ade2-loc/ade2-loc his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/TRP1 ctf19Δ::kanMX6/ctf19Δ::kanMX6 CHL4-YFP-His3MX6/CHL4-YFP-His3MX6 SPC29-CFP-kanMX6/SPC29-CFP-kanMX6*	This study
YPH1571[a]	MATa/α *ura3-1/ura3-1 ade2-loc/ade2-loc his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 chl4Δ::His3MX6/chl4Δ::kanMX6 CTF19-YFP-His3MX6/CTF19-YFP-His3MX6 SPC29-CFP-kanMX6/SPC29-CFP-kanMX6*	This study
YPH1572[a]	MATa/α *ura3-1/ura3-1 ade2-loc/ade2-loc his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ctf19Δ::kanMX6/ctf19Δ::kanMX6 NDC10-YFP-His3MX6/NDC10-YFP-His3MX6 SPC29-CFP-kanMX6/SPC29-CFP-kanMX6*	This study

(continues)

Table II.1 (continued)

Strain	Genotype	Reference
YPH1573[a]	MATa/α *ura3-1/ura3-1 ade2-loc/ade2-loc his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/TRP1 chl4Δ::kanMX6/chl4Δ::kanMX6 NDC10-YFP-His3MX6/NDC10-YFP-His3MX6 SPC29-CFP-kanMX6/SPC29-CFP-kanMX6*	This study
YPH1574[a]	MATa/α *ura3-1/ura3-1 ade2-loc/ade2-loc his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/TRP1 ctf19Δ::kanMX6/ctf19Δ::kanMX6 CTF3-YFP-His3MX6/CTF3-YFP-His3MX6 SPC29-CFP-kanMX6/SPC29-CFP-kanMX6*	This study
YPH1575[a]	MATa/α *ura3-1/ura3-1 ade2-loc/ade2-loc his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1//trp1-1 chl4Δ::His3MX6/chl4Δ::kanMX6 CTF3-YFP-His3MX6/CTF3-YFP-His3MX6 SPC29-CFP-kanMX6/SPC29-CFP-kanMX6*	This study
YPH1576[a]	MATa/α *ura3-1/ura3-1 ade2-loc/ade2-loc his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/TRP1 IML3-YFP-His3MX6/IML3-YFP-His3MX6 SPC29-CFP-kanMX6/SPC29-CFP-kanMX6*	This study
YPH1577[a]	MATa/α *ura3-1/ura3-1 ade2-loc/ade2-loc his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/TRP1 chl4Δ::His3MX6/chl4Δ::His3MX6 IML3-YFP-His3MX6/IML3-YFP-His3MX6 SPC29-CFP-kanMX6/SPC29-CFP-kanMX6*	This study
YPH1578[a]	MATa/α *ura3-1/ura3-1 ade2-loc/ade2-loc his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 ctf19Δ::kanMX6/ctf19Δ::kanMX6 IML3-YFP-His3MX6/IML3-YFP-His3MX6 SPC29-CFP-kanMX6/SPC29-CFP-kanMX6*	This study
YPH1579[a]	MATa/α *ura3-1/ura3-1 ade2-loc/ade2-loc his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 MIF2-YFP-His3MX6/MIF2-YFP-His3MX6 SPC29-CFP-kanMX6/SPC29-CFP-kanMX6*	This study
YPH1580[a]	MATa/α *ura3-1/ura3-1 ade2-loc/ade2-loc his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 CHL4-YFP-His3MX6/CHL4-YFP-His3MX6 MIF2-CFP-kanMX6/MIF2-CFP-kanMX6*	This study
YPH1582	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 iml3Δ::kanMX6 CTF19-13Myc-TRP1*	This study
YPH1583[a]	MATa/α *ura3-1/ura3-1 ade2-loc/ade2-loc his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/trp1-1 iml3Δ::kanMX6/iml3Δ::kanMX6 CTF19-YFP-His3MX6/CTF19-YFP-His3MX6 SPC29-CFP-kanMX6/SPC29-CFP-kanMX6*	This study
YPH1602	MATα *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 iml3Δ::kanMX6*	This study
YPH1603	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 iml3Δ::kanMX6 CHL4-13Myc-TRP1*	This study
YVM111	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ctf3Δ::HIS3*	V. Measday
YVM112	MATα *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ctf3Δ::HIS3*	V. Measday
YVM218	MATα *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 CTF3-13Myc-TRP1*	(Measday et al., 2002)
YVM1176[a]	MATa/α *ura3-1/ura3-1 ade2-loc/ade2-loc his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 trp1-1/TRP1 NDC10-YFP-His3MX6/NDC10-YFP-His3MX6 SPC29-CFP-kanMX6/SPC29-CFP-kanMX6*	(Measday et al., 2002)
DHY201[a]	MATa/α *ura3-1/ura3-1 ade2-loc/ade2-loc his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 CTF19-YFP-His3MX6/CTF19-YFP-His3MX6 SPC29-CFP-kanMX6/SPC29-CFP-kanMX6*	(Measday et al., 2002)
DHY202[a]	MATa/α *ura3-1/ura3-1 ade2-loc/ade2-loc his3-11,15/his3-11,15 leu2-3,112/leu2-3,112 CTF3-YFP-His3MX6/CTF3-YFP-His3MX6 SPC29-CFP-kanMX6/SPC29-CFP-kanMX6*	(Measday et al., 2002)
1cAS281	MATa *ura3 lys2 ade2 his3 leu2 trp1 cep3-1*	(Strunnikov et al., 1995)
2bAS282	MATa *ura3 lys2 ade2 his3 leu2 trp1 cep3-2*	(Strunnikov et al., 1995)
PMY1002-3A	MATα *ura3 leu2 trp1 mif2-3*	P. Meluh

[a] *His3MX6* in these strains is the his5 gene of *Schizosaccharomyces pombe*. In all other strains *His3MX6* is the *HIS3* gene from *Saccharomyces kluveri*. Both complement the *S. cerevisiae his3* mutation.

II.3. Results

II.3.a. chl4Δ displays phenotypes commonly observed in kinetochore mutants

In order to confirm the chromosome loss phenotype observed in *chl4* point mutants, *CHL4* was deleted from a strain containing a non-essential marked chromosome fragment, and chromosome missegregation in the deletion mutant was scored by a colony sectoring assay (Koshland and Hieter, 1987). *chl4Δ* sectored heavily, similar to two *chl4* alleles isolated in the *ctf* mutant collection (*chl4-20* and *chl4-61*), indicating a high rate of chromosome fragment loss (Figure II.1.A) (Spencer et al., 1990).

Many chromosome segregation and spindle integrity mutants have heightened sensitivity to microtubule-destabilizing drugs, perhaps due to the synergistic effect of a mutation affecting a microtubule-dependent process together with compromised microtubule networks (Poddar et al., 1999). Several central kinetochore mutants have been shown to be sensitive to sublethal doses of the microtubule-depolymerizing drug benomyl (Hyland et al., 1999; Poddar et al., 1999; Roy et al., 1997). The ability of *chl4Δ*, *chl4-20* and *chl4-61* to grow on benomyl-containing media at 25°C was tested (Figure II.1.B). All mutant alleles exhibited increased sensitivity to 15μg/ml benomyl and were inviable at 20μg/ml benomyl. The chromosome loss and benomyl sensitivity phenotypes are consistent with *chl4Δ* having a defect in kinetochore function.

II.3.b. chl4Δ genetically interacts with kinetochore mutants

To test for genetic interactions with kinetochore mutants, *chl4Δ* was mated to strains carrying individual mutations in three of the CBF3 subunits (*ndc10*, *cep3* and *ctf13*) (Kopski and Huffaker, 1997; Spencer et al., 1990; Strunnikov et al., 1995), or to a *mif2* mutant strain (Meluh and Koshland, 1995). Dissection of the heterozygous diploids showed that *chl4Δ* was synthetically lethal with *mif2-3* and *ndc10-42*, and that *chl4Δ* lowered the permissive temperature of *ctf13-30*, *cep3-1* and *cep3-2* mutants (Table II.2). The synthetic interaction observed with *ndc10-42* was allele-dependent, as *ndc10-2* was not synthetically lethal with *chl4Δ* (Table II.2). Genetic interaction between *chl4Δ* and two central kinetochore deletion mutations, *ctf19Δ* (Hyland et al., 1999) and *ctf3Δ* (Measday et al., 2002), was also tested, and no synthetic effect on growth was observed, even when the three deletion mutations were present in a single strain (Table II.2). Thus, a *chl4* deletion mutation genetically interacts

with mutations in genes of the inner kinetochore, but not with mutations in two central kinetochore genes.

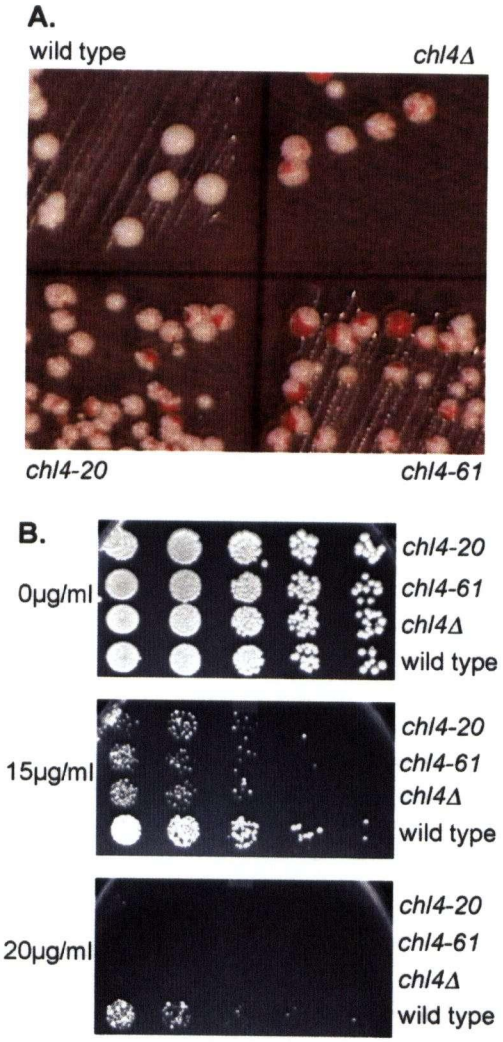


Figure II.1. Phenotypes of *chl4* mutants.
A. Chromosome loss phenotype visualized using the *SUP11* system. A non-essential chromosome fragment carrying *SUP11* can suppress the *ade2-1* mutation that leads to accumulation of a red pigment in the cells (Koshland and Hieter, 1987). Thus, cells containing the chromosome fragment are white, while those that have lost it are red. Wild type, *chl4Δ*, *chl4-20* and *chl4-61* strains containing a non-essential chromosome fragment marked with *URA3* were grown in SC medium lacking uracil and then plated on YPD plates. B. Benomyl sensitivity. Wild type, *chl4Δ*, *chl4-20* and *chl4-61* strains were spotted in five-fold dilutions on YPD plates containing the amount of benomyl indicated on the left.

Table II.2 Genetic interactions between *chl4Δ* and kinetochore mutants

Strain[a]	Genetic interaction[b]
chl4Δ ndc10-42	SL[c]
chl4Δ ndc10-2	viable
chl4Δ cep3-1	CSL[d]
chl4Δ cep3-2	CSL[d]
chl4Δ ctf13-30	CSL[d]
chl4Δ mif2-3	SL[c]
chl4Δ ctf19Δ	viable
chl4Δ ctf3Δ	viable
chl4Δ ctf19Δ ctf3Δ	viable
iml3Δ chl4Δ	viable
iml3Δ ctf19Δ	viable
iml3Δ ctf3Δ	viable
iml3Δ chl4Δ ctf3Δ	viable
iml3Δ ctf19Δ ctf3Δ	viable

[a] The strains used in the crosses (*chl4Δ*, *ndc10-42*, *ndc10-2*, *cep3-1*, *cep3-2*, *ctf13*-30, *mif2-3*, *ctf19Δ*, *ctf3Δ* and *iml3Δ*) are listed in Table I.1.

[b] Strains were incubated at 25°C.

[c] SL = synthetic lethality: individual mutations do not cause inviability on their own, but a strain containing both mutations is inviable.

[d] CSL = conditional synthetic lethality: spores are viable at 25°C but die at a lower temperature than the restrictive temperature of the single temperature-sensitive mutant. *chl4Δ ctf13-30* double mutants died at 32°C while the non-permissive temperature of the *ctf13-30* single mutant is 35°C; *chl4Δ cep3-1* and *chl4Δ cep3-2* double mutants were inviable at 31°C and 30°C, respectively, while the non-permissive temperature of the *cep3-1* and *cep3-2* single mutants is 34°C.

II.3.c. Chl4p associates with CEN DNA and localizes to the kinetochore

Genetic and phenotypic evidence strongly suggested that the *CHL4* gene product was a candidate kinetochore protein. In order to obtain biochemical evidence that Chl4p is located at the centromere, ChIP was used to assay whether Chl4p could interact with *CEN* DNA. Myc-tagged Chl4p was immunoprecipitated from formaldehyde-crosslinked extracts using anti-Myc conjugated beads. The co-precipitated DNA was analyzed by PCR using primer pairs specific to centromeric regions of chromosomes I and III (*CEN1* and *CEN3*), and to a non-centromeric region (*PGK1*) as a control for binding specificity. Chl4p interacted

specifically with *CEN* DNA but not with a non-*CEN* locus, similar to Ctf19p and Ndc10p, two known kinetochore proteins (Figure II.2.A, lanes 4, 6 and 8).

The localization of several kinetochore proteins tagged with the green fluorescent protein (GFP) or its yellow and cyan variants (YFP and CFP), has recently been determined in yeast cells by fluorescence microscopy. The resulting images have shown that kinetochore proteins reside next to the nuclear side of the SPB in cells with short spindles, and co-localize with the SPB in late anaphase cells (He et al., 2001; Measday et al., 2002; Pearson et al., 2001). To visualize the localization of Chl4p, it was tagged with YFP and Chl4p-YFP was imaged in a strain containing a tagged SPB protein, Spc29p-CFP (Figure II.3.A, wild type panel). Chl4p-YFP was found to have a pattern of localization similar to Ctf19p-YFP and Ctf3p-YFP (Figures II.3.B and II.3.D, wild type panels) and other kinetochore proteins in cells with short and long spindles. The specific interaction of Chl4p with *CEN* DNA chromatin and its cellular localization suggest that Chl4p is part of the budding yeast kinetochore complex.

II.3.d. Chl4p requires Ndc10p and Ctf19p, but not Ctf3p, to interact with CEN DNA

While components of the CBF3 complex bind directly to *CEN* DNA (Lechner and Carbon, 1991), central kinetochore complexes, such as the Ctf19 complex, interact with *CEN* DNA via CBF3 (Ortiz et al., 1999). In addition, the Ctf3 complex was shown to require both Ctf19p and Cse4p to interact efficiently with *CEN* DNA (Measday et al., 2002). The *CEN* DNA loading requirements of kinetochore proteins can thus be used to probe their molecular organization within the kinetochore complex. To determine whether the interaction of Chl4p with *CEN* DNA requires the CBF3 complex, ChIP analysis was performed with Chl4p-Myc in a strain containing a temperature-sensitive mutation in *NDC10*. In the *ndc10-2* background, Chl4p interacted with *CEN* DNA at permissive temperature (25°C) (Figure II.2.B, lane 10), but not at restrictive temperature (37°C) (Figure II.2.B, lane 12). Since Chl4p is able to co-immunoprecipitate *CEN* DNA in the wild type strain at 37°C (Figure II.2.B, lane 8), the lack of interaction in *ndc10-2* is not simply due to a failure of Chl4p to associate with *CEN* DNA at high temperature. Western blots also demonstrated that Chl4p-Myc was immunoprecipitated efficiently in wild type and mutant backgrounds at both temperatures (data not shown). Conversely, deletion of *CHL4* did not affect the ability of

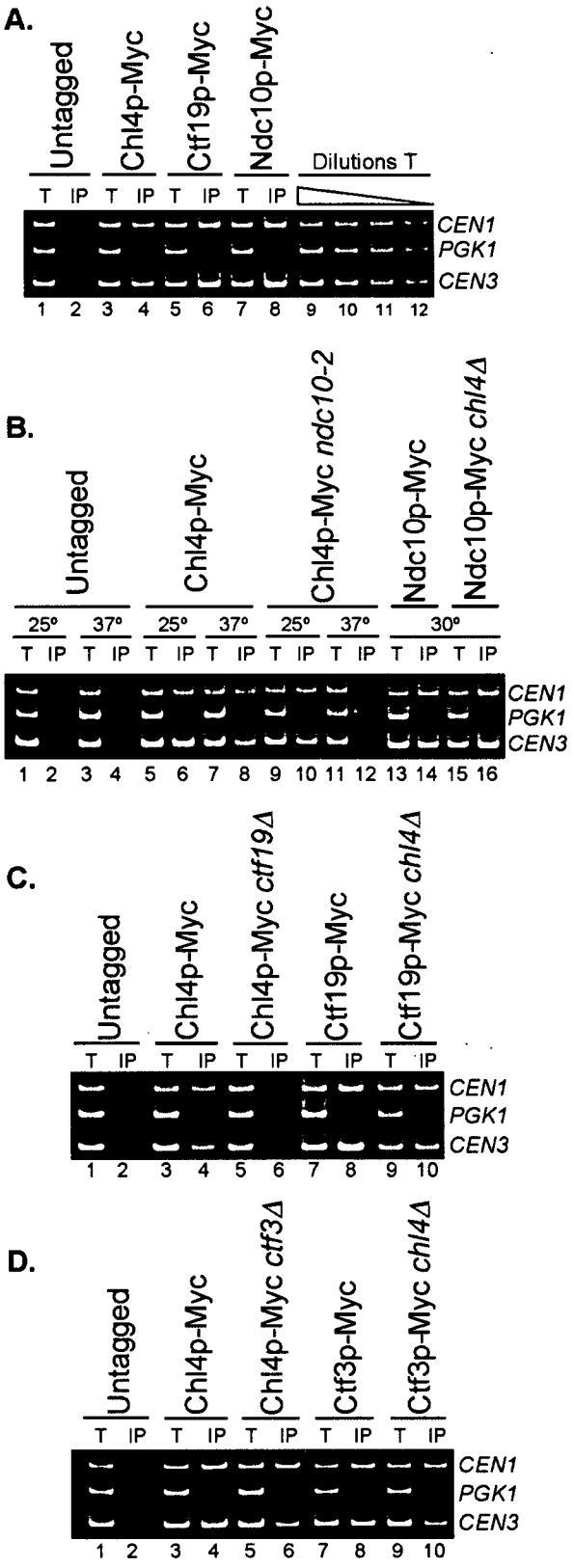


Figure II.2. Chl4p interacts with *CEN* DNA in an Ndc10p- and Ctf19p-dependent, but Ctf3p-independent manner.

A. Chl4p interacts with *CEN* DNA. ChIP assay performed by immunoprecipitation of Myc-tagged Chl4p, Ctf19p or Ndc10p followed by multiplex PCR analysis. Lanes 9 to 12: 2.5-fold dilutions of one of the total templates, showing that PCR is in the linear range. B. The interaction of Chl4p with *CEN* DNA depends on Ndc10p. ChIP assay performed by immunoprecipitation of Myc-tagged Chl4p or Ndc10p from wild type, *ndc10-2* or *chl4Δ* strains, followed by multiplex PCR analysis. For the temperature sensitive assay, wild type and *ndc10-2* strains were grown to log phase at 25°C and half of the culture was then shifted to 37°C for 3 hours. C. The interaction of Chl4p with *CEN* DNA depends on Ctf19p. ChIP assay performed by immunoprecipitation of Myc-tagged Chl4p or Ctf19p from wild type, *ctf19Δ*or *chl4Δ* strains, followed by multiplex PCR analysis. D. The interaction of Chl4p with *CEN* DNA is independent of Ctf3p. ChIP assay performed by immunoprecipitation of Myc-tagged Chl4p or Ctf3p from wild type, *ctf3Δ* or*chl4Δ* strains, followed by multiplex PCR analysis. In A-D, T = total lysate, IP = immunoprecipitated fraction.

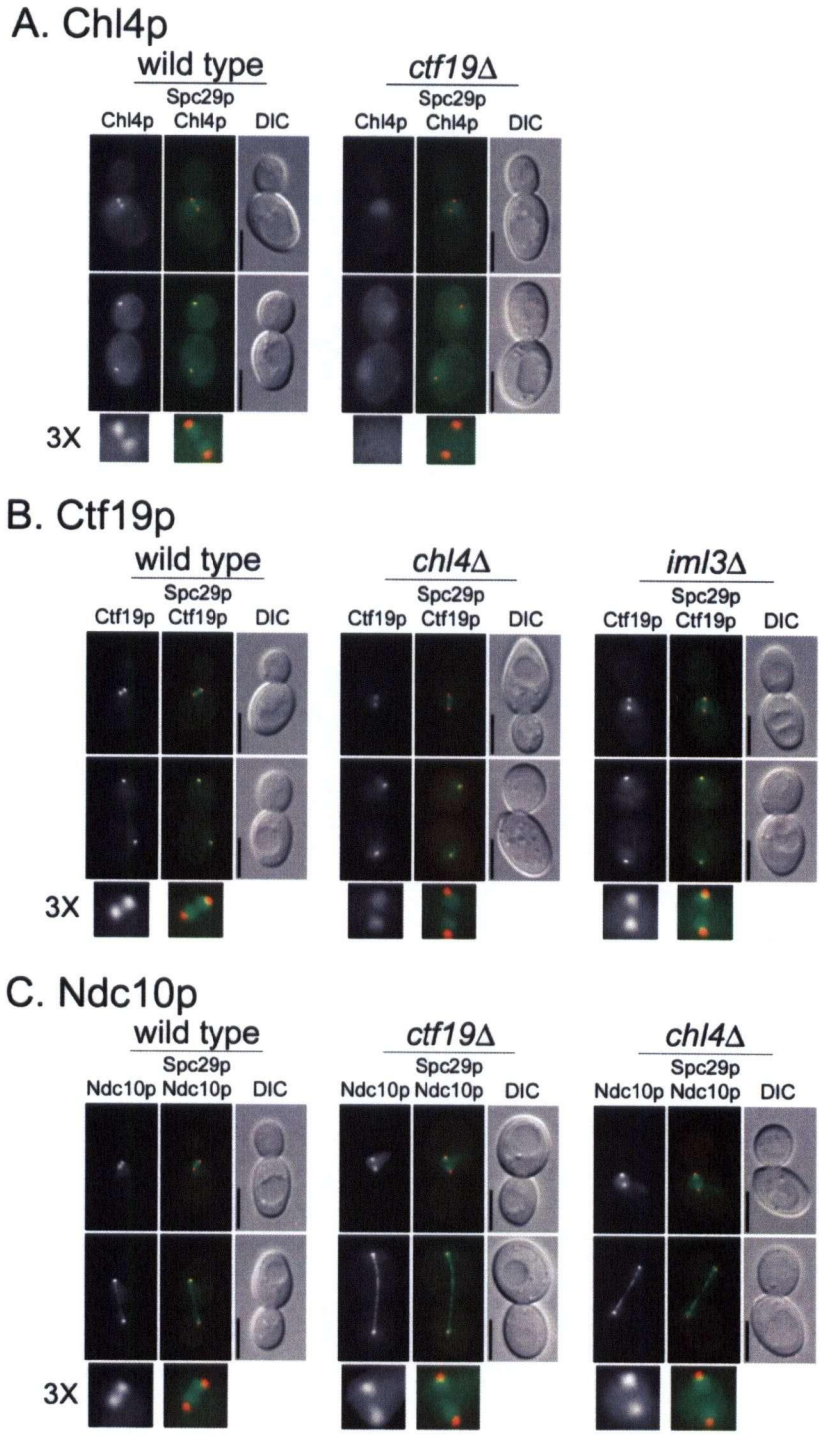


Figure II.3. Interdependence of kinetochore protein localization.

A-E. The indicated proteins were tagged with YFP and imaged as described in materials and methods. Spc29p-CFP was included in the genetic background as an SPB marker, which allowed determination of the relative location of the kinetochore. In all panels, upper images are cells with short spindles and lower images are cells with long spindles. The left frame has the YFP signal, the middle frame has merged YFP and CFP signals, and the right frame is the corresponding differential interference contrast (DIC) image. In the color images, YFP is pseudo-colored green and CFP is pseudo-colored red. A three-fold enlargement of the YFP and CFP signals in cells with short spindles, indicated by '3X', is shown at the bottom of each panel. The relevant genetic background (wild type or kinetochore mutant) is indicated on top of each panel. F. For co-localization, Chl4p was tagged with YFP and Mif2p was tagged with CFP in a wild type strain, and imaged as in A-E. All strains are homozygous diploids, and all contain Spc29p-CFP (except in F). wt, wild type. Bar, 5μm.

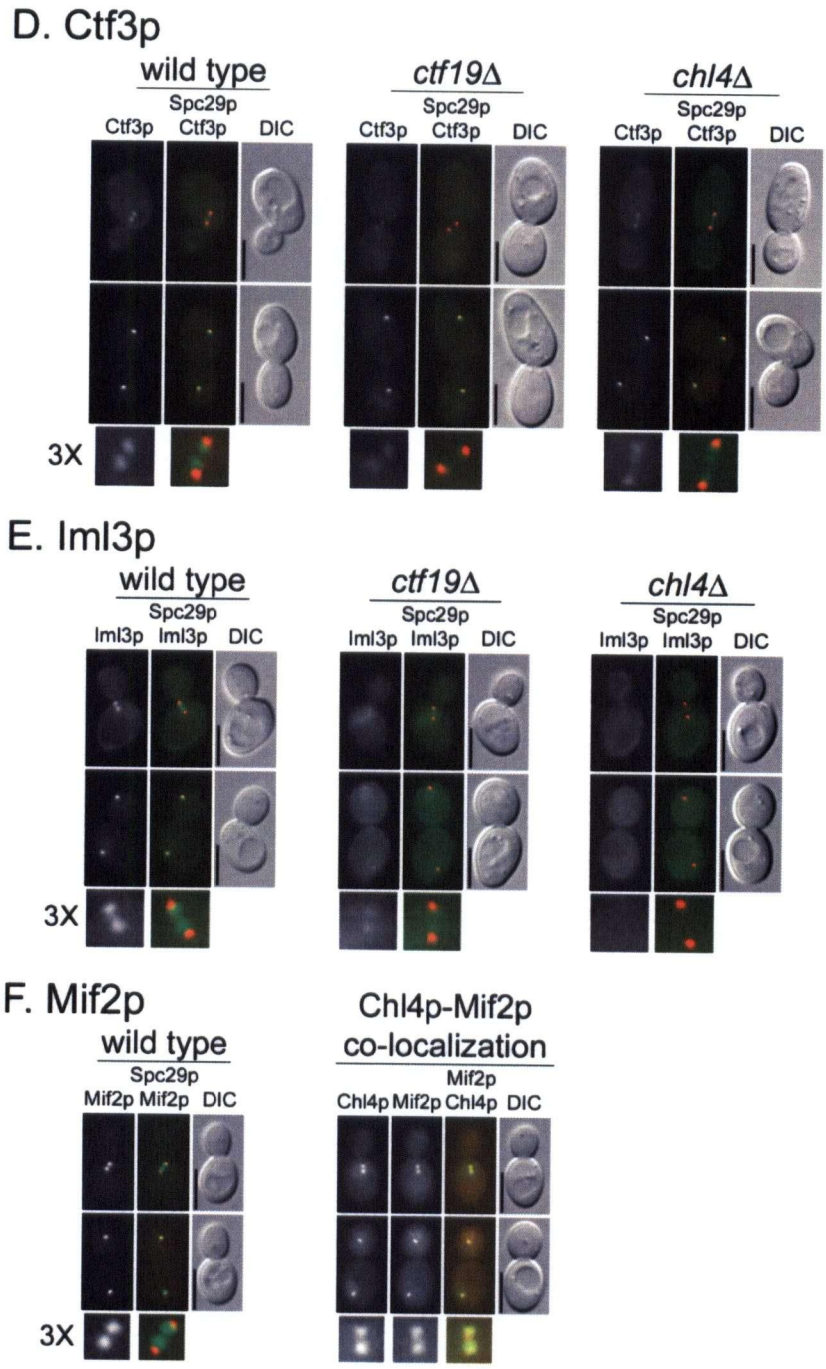


Figure II.3. Interdependence of kinetochore protein localization (continued).

Ndc10p to interact with *CEN* DNA (Figure II.2.B, lanes 14 and 16). Thus, functional Ndc10p is required for the Chl4p-*CEN* DNA interaction. In accordance with this data, Chl4p-GFP localization becomes diffuse in an *ndc10-2* strain incubated at the restrictive temperature (Mythreye and Bloom, 2003). Therefore, Chl4p requires an intact CBF3 complex to properly localize to and interact with the centromere.

To investigate whether Chl4p requires other kinetochore components, such as Ctf19p or Ctf3p, for its interaction with *CEN* DNA, ChIP was performed with Chl4p-Myc in strains lacking *CTF19* or *CTF3*. It was found that while Chl4p required Ctf19p to interact with the centromere (Figure II.2.C, lane 6), it did not require Ctf3p (Figure II.2.D, lane 6). Conversely, immunoprecipitation of Ctf19p-Myc in strains lacking *CHL4* revealed that Ctf19p co-immunoprecipitated *CEN* DNA in the absence of Chl4p (Figure II.2.C, lane 10). Similarly, Ctf3p was able to interact with *CEN* DNA in the absence of Chl4p (Figure II.2.D, lane 10), although in some instances the *CEN* DNA co-immunoprecipitation appeared to be less efficient (data not shown). Western blots demonstrated that in all cases, efficient immunoprecipitation of the Myc-tagged protein was not affected in the deletion strain compared to the wild type strain (data not shown, and see also Western blots in Figure II.5 showing immunoprecipitations in wild type and mutant strains). Thus, Chl4p, like the Ctf3 complex, requires Ctf19p to interact with *CEN* DNA, while neither Chl4p nor the Ctf3 complex are required for the Ctf19p-*CEN* DNA interaction (Figure II.2.C and D and Measday et al., 2002). Moreover, Chl4p and Ctf3p are able to interact with the centromere independently of each other.

II.3.e. Chl4p requires Ctf19p for proper kinetochore localization, and lack of Chl4p or Ctf19p affects the localization of Ctf3p in early anaphase

Since Ctf19p is required for Chl4p to interact with *CEN* DNA, the next question was whether lack of *CTF19* disturbed the localization of Chl4p-YFP. It was found that in a *ctf19Δ* strain, Chl4p-YFP did not localize to the kinetochore. Instead, the YFP signal was diffuse throughout the nucleus (Figures II.3.A and II.4.A). The intensity of the diffuse nuclear signal was four-fold greater in the *ctf19Δ* mutant than in the wild type strain (Figure II.4.A). Conversely, the localization of Ctf19p-YFP was only modestly impaired by the absence of Chl4p (Figures II.3.B and II.4.B). In agreement with the ChIP data (Figure II.2.B

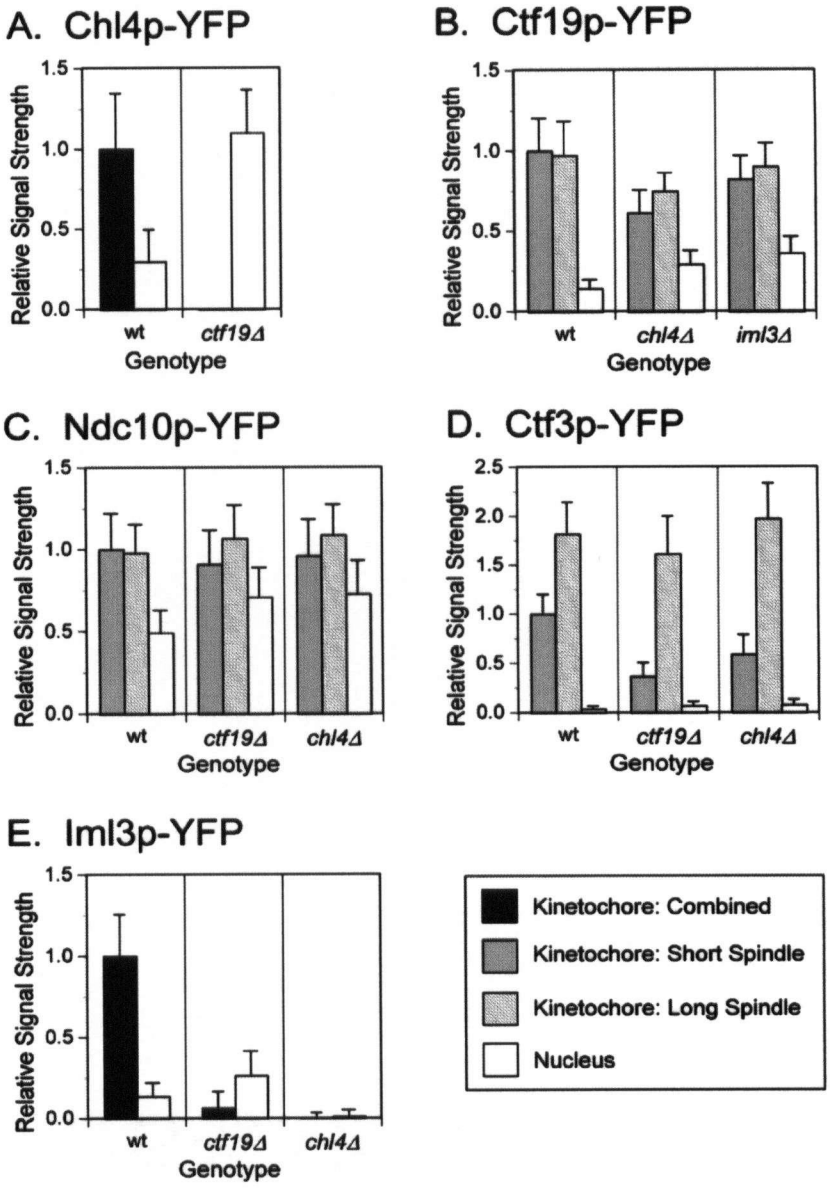


Figure II.4. Quantification of kinetochore localization signals.

A-E. Kinetochore protein signal strengths of the strains imaged in Figure II.3.A-E were determined as described in materials and methods. For each graph the values were normalized to the mean value of the wild type strain. In B-D the mean value of the kinetochore signal from cells with a short spindle was used for normalization. Bars represent the standard deviation. The mean value of the signal intensity in wild type strains and the number of kinetochores or nuclei examined for each panel are as follows, where n1 is the sample size for kinetochore signals in cells with either short or long spindles, n2 is the sample size for kinetochore signals in cells with short spindles, and n3 is the sample size for kinetochore signals in cells with long spindles (sample sizes for determining the nuclear signal strength are equal to either n1 or n2): A, mean value (wt) = 2297; for wt, n1 = 78; for *ctf19Δ*, n1 = 45. B, mean value (wt) = 12368; for wt, n2 = 33 and n3 = 26; for *chl4Δ*, n2 = 36 and n3 = 30; for *iml3Δ*, n2 = 22 and n3 = 14. C, mean value (wt) = 12587; for wt, n2 = 86 and n3 = 62; for *ctf19Δ*, n2 = 64 and n3 = 38; for *chl4Δ*, n2 = 82 and n3 = 33. D, mean value (wt) = 4035; for wt, n2 = 24 and n3 = 16; for *ctf19Δ*, n2 = 45 and n3 = 24; for *chl4Δ*, n2 =38 and n3 = 19. E, mean value (wt) = 5740; for wt, n1 =74; for *ctf19Δ*, n1 = 33; for *chl4Δ*, n1 = 63. wt, wild type.

and Measday et al., 2002), deletions of either *CTF19* or *CHL4* do not significantly disturb the inner kinetochore structure, as Ndc10p-YFP still localizes to the kinetochore in *ctf19Δ* and *chl4Δ* strains (Figures II.3.C and II.4.C). A slight increase in a diffuse nuclear signal in these strains will require further investigation. In all cases, the wild type and mutant strains expressed similar levels of fusion proteins (data not shown; see materials and methods). In summary, the ChIP and *in vivo* localization data indicate that Ctf19p, which interacts with *CEN* DNA via CBF3 (Ortiz et al., 1999), is essential not only for the Chl4p–*CEN* DNA interaction, but also for the proper localization of Chl4p to the kinetochore.

Because Ctf3p, like Chl4p, requires Ctf19p to interact with *CEN* DNA (Measday et al., 2002), the localization of Ctf3p in the absence of Ctf19p was examined. Interestingly, in the *ctf19Δ* strain, Ctf3p-YFP showed a three-fold decrease in kinetochore localization signal in cells with short spindles, whereas in cells with long spindles, Ctf3p-YFP showed normal co-localization with the SPB (Figures II.3.D and II.4.D). To investigate whether this behavior was specific to the lack of Ctf19p, Ctf3p-YFP was imaged in a *chl4Δ* strain. A 40% decrease in the intensity of the Ctf3p-YFP kinetochore signal was observed in *chl4Δ* cells with short spindles, while there was no decrease in Ctf3p-YFP signal intensity in *chl4Δ* cells with long spindles, similar to *ctf19Δ* (Figures II.3.D and II.4.D). These data suggest that Ctf19p, and possibly Chl4p, are important for Ctf3p localization to the kinetochore during early stages of mitosis, but not in later stages.

II.3.f. Chl4p interacts with known central kinetochore proteins

Given its interaction with *CEN* DNA and its localization to the kinetochore, the interaction of Chl4p with known central kinetochore proteins was investigated. Immunoprecipitation using anti-Myc conjugated beads was performed in a strain containing Myc-tagged Chl4p and both Chl4p and Ctf19p were detected in the immunoprecipitate (Figure II.5.A, lane 4). Interaction of Chl4p with Ctf3p was also tested using a strain that contained HA-tagged Ctf3p. Ctf3p-HA was detected in anti-Myc immunoprecipitates only when Chl4p-Myc was present (Figure II.5.A, lane 8). Similar results were obtained when the same strain was used in an anti-HA immunoprecipitation (data not shown). To determine if the interaction of Chl4p with Ctf3p depended on Ctf19p, immunoprecipitations were performed in a *ctf19Δ*

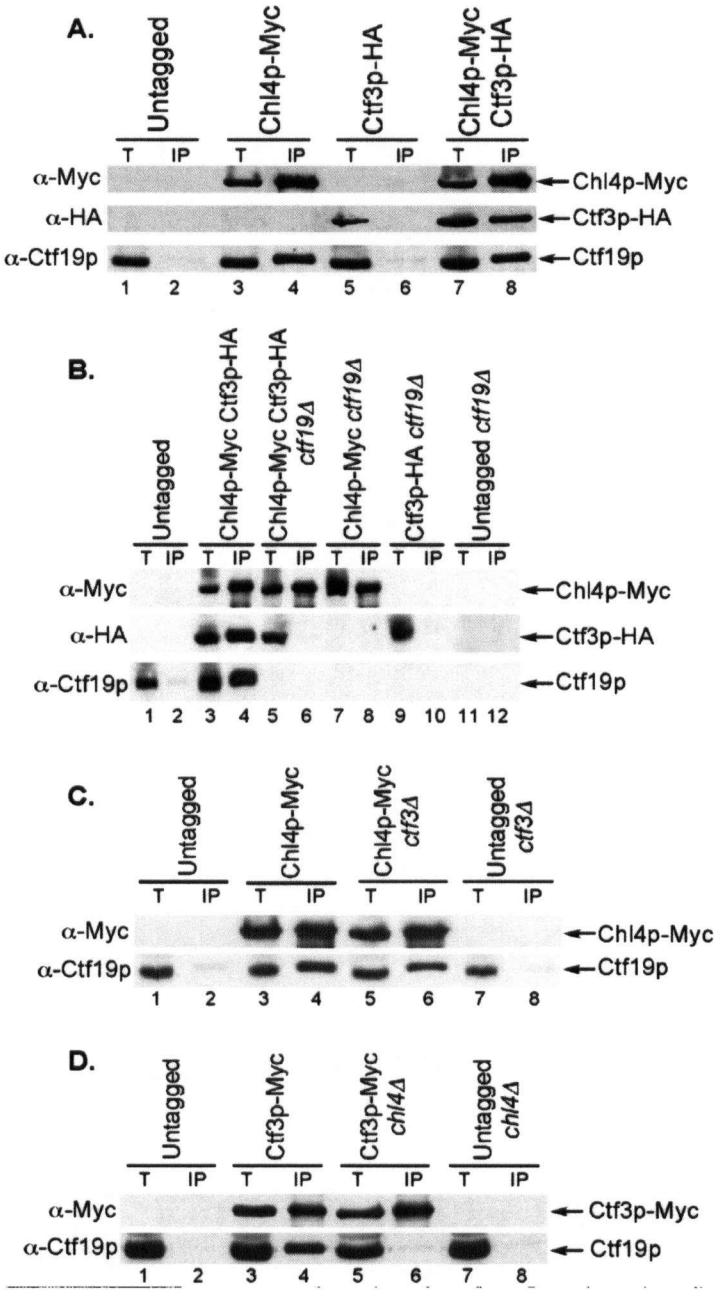


Figure II.5. Chl4p co-immunoprecipitates with central kinetochore proteins.

A. Chl4p co-immunoprecipitates with Ctf19p and Ctf3p. Anti-Myc immunoprecipitations were performed in a strain containing Myc-tagged Chl4p and HA-tagged Ctf3p and control strains containing one or no tagged proteins. 40μg of total lysate and 15% of the immunoprecipitated fraction were loaded on SDS-PAGE gels, and Western blots were used to detect Myc- and HA-tagged proteins, and Ctf19p, using the antibodies indicated on the left. B. Ctf19p is required for the co-immunoprecipitation between Chl4p and Ctf3p. Anti-Myc immunoprecipitations and Western blots were performed as in A in strains lacking Ctf19p. Lanes 7 to 12 are controls. C. Ctf3p is dispensable for the co-immunoprecipitation between Chl4p and Ctf19p. Anti-Myc immunoprecipitations and Western blots were performed as in A in strains lacking Ctf3p. Lanes 7 and 8 are controls. D. Chl4p is required for the interaction of Ctf3p with Ctf19p. Anti-Myc immunoprecipitations and Western blots were performed as in A in strains containing Myc-tagged Ctf3p in the presence or absence of Chl4p. Lanes 7 and 8 are controls. In A-D, T = total lysate, IP = immunoprecipitated fraction.

strain using both anti-Myc (Figure II.5.B) and anti-HA (data not shown) conjugated beads. It was found that Chl4p no longer interacted with Ctf3p in the absence of Ctf19p (Figure II.5.B, lane 6). Immunoprecipitation of Chl4p-Myc was also performed in a *ctf3Δ* strain, and revealed that Ctf19p was still present in the immunoprecipitate, indicating that the Chl4p-Ctf19p interaction is independent of Ctf3p (Figure II.5.C, lane 6). Conversely, immunoprecipitating Ctf3p-Myc in the absence of Chl4p disrupted the interaction between Ctf3p and Ctf19p (Figure II.5.D, lane 6). Thus, the immunoprecipitation data suggest that Chl4p is connected to the centromere and to the Ctf3 complex via Ctf19p. Moreover, since the Ctf3p-*CEN* DNA interaction depends on Ctf19p (Measday et al., 2002), and Chl4p is essential for the Ctf3p-Ctf19p interaction, Chl4p may contribute to the efficient association of the Ctf3 complex with the centromere.

II.3.g. The Chl4p two-hybrid interactor Iml3p is a new central kinetochore protein

Phenotypic analyses of *iml3/mcm19* mutants indicated that Iml3p was a putative kinetochore protein (Entian et al., 1999; Ghosh et al., 2001). Genetic interactions between an *iml3* deletion mutation and mutations in other central kinetochore genes were tested. Similar to *chl4Δ*, *iml3Δ* did not compromise growth when combined with *chl4Δ*, *ctf19Δ* or *ctf3Δ*, or various multiple combinations of these mutations (Table II.2). Iml3p was shown to interact with Chl4p by two-hybrid assay (Ghosh et al., 2001). This interaction was confirmed biochemically by performing an anti-HA immunoprecipitation in a strain containing Iml3p-HA and Chl4p-Myc. Anti-Myc and anti-Ctf19p Western blots showed that Iml3p was able to co-immunoprecipitate both Chl4p and Ctf19p (Figure II.6.A, lanes 4 and 8). The Iml3p-Chl4p interaction was also observed when anti-Myc beads were used for immunoprecipitation (data not shown). Since Ctf19p is required for Chl4p to interact with Ctf3p, the requirement of Ctf19p for the Iml3p-Chl4p interaction was tested. In contrast to Ctf3p, Iml3p still co-immunoprecipitated with Chl4p in a *ctf19Δ* strain (Figure II.6.A, lane 10). Thus Iml3p and Chl4p can form a complex independently of Ctf19p. The next question was whether lack of Chl4p would disrupt the Iml3p-Ctf19p interaction. When Iml3p-HA was immunoprecipitated from a *chl4Δ* strain, Ctf19p was no longer present in the immunoprecipitate (Figure II.6.A, lane 14), indicating that Chl4p is required for the Iml3p-Ctf19p interaction. Finally, to determine if Iml3p was required for the Chl4p-Ctf19p

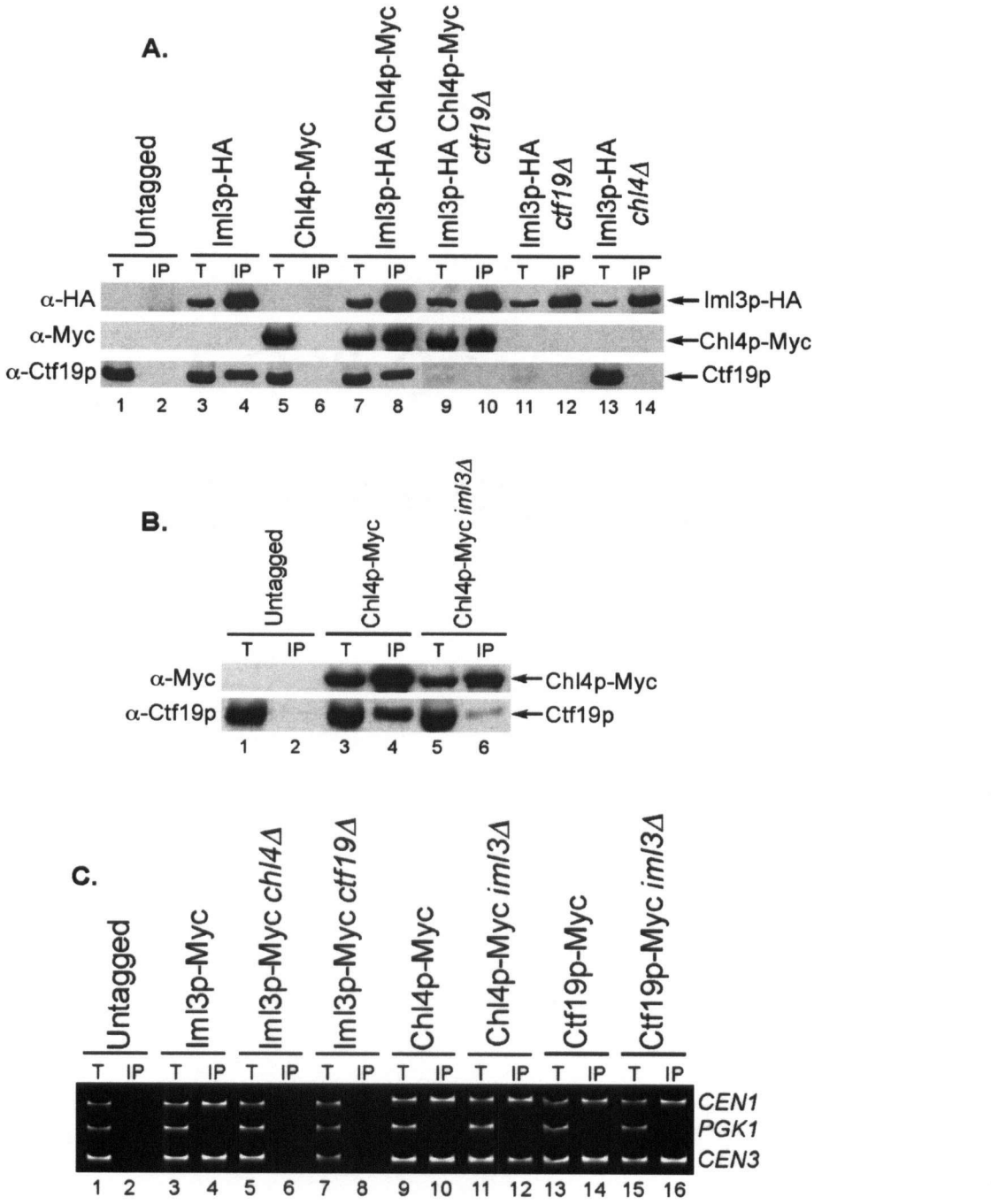


Figure II.6. Iml3p is a new central kinetochore protein.

A. Iml3p interacts with Chl4p and with Ctf19p in a Chl4p-dependent manner. Anti-HA immunoprecipitations and Western blots were performed as in Figure II.5 in wild type, *ctf19Δ* or *chl4Δ* strains containing Myc-tagged Chl4p and HA-tagged Iml3p or control strains containing one or no tagged proteins. B. The interaction of Ctf19p with Chl4p is reduced in the absence of Iml3p. Anti-Myc immunoprecipitations and Western blots were performed as in Figure II.5 in wild type or *iml3Δ* strains containing either Myc-tagged Chl4p or no tagged protein. C. Iml3p interacts with *CEN* DNA in a Chl4p- and Ctf19p-dependent manner. ChIP assay performed by immunoprecipitation of Myc-tagged Iml3p from wild type, *chl4Δ* or *ctf19Δ*strains, or by immunoprecipitation of Myc-tagged Chl4p or Ctf19p from wild type or *iml3Δ* strains, followed by multiplex PCR analysis. In A-C, T = total lysate, IP = immunoprecipitated fraction.

interaction, Chl4p-Myc was immunoprecipitated from an *iml3Δ* strain, and it was found that the amount of Ctf19p in the immunoprecipitate was reduced, albeit not completely absent (Figure II.6.B). Thus, Iml3p is part of the outer kinetochore complex and interacts with the Ctf19 complex in a Chl4p-dependent manner, and Iml3p contributes to the efficient interaction of Chl4p with Ctf19p.

The next question asked was whether Iml3p met the criteria that were previously used to define a kinetochore protein: association with *CEN* DNA and a kinetochore localization pattern. *CEN* DNA was specifically isolated from an Iml3p-Myc immunoprecipitate, similar to Chl4p (Figure II.6.C, lane 4). The Iml3p-*CEN* DNA interaction also depended on Chl4p and Ctf19p, as *CEN* DNA no longer co-precipitated with Iml3p-Myc in *chl4Δ* and *ctf19Δ* strains (Figure II.6.C, lanes 6 and 8). In contrast, Iml3p was not required for the Chl4p-*CEN* DNA or Ctf19p-*CEN* DNA interactions (Figure II.6.C, lanes 12 and 16), although in some instances the efficiency of *CEN* DNA co-immunoprecipitation appeared slightly reduced for Chl4p (data not shown), perhaps due to the reduced interaction of Chl4p with Ctf19p in the absence of Iml3p. These data suggest that Iml3p lies more distal to the centromere than Ctf19p and Chl4p. Iml3p-YFP was then imaged in a strain containing Spc29p-CFP and was observed to localize to the kinetochore (Figure II.3.E). In accordance with the ChIP data described above, Iml3p-YFP failed to localize to the kinetochore in a *ctf19Δ* or a *chl4Δ* strain (Figures II.3.E and II.4.E). Interestingly, a diffuse nuclear Iml3p-YFP signal was visible in *ctf19Δ*, as was observed for the Chl4p-YFP signal in *ctf19Δ*; no Iml3p-YFP signal was detected in *chl4Δ* (Figure II.4.E). In contrast, lack of Iml3p did not disturb Ctf19p-YFP localization to the kinetochore (Figures II.3.B and II.4.B). Thus, these results strongly suggest that Iml3p is a new central kinetochore protein that is connected to the centromere via Chl4p and Ctf19p.

II.3.h. A genome-wide two-hybrid screen reveals that Chl4p interacts with the kinetochore protein Mif2p

To uncover other potential protein partners of Chl4p, a Chl4p-DNA binding domain fusion was constructed and tested for two-hybrid interactions with a genome-wide array of activation domain fusions (Cagney et al., 2000; Uetz et al., 2000). Two independent screens showed that Mif2p interacted with Chl4p (Figure II.7). However, attempts to co-

immunoprecipitate the two proteins from yeast lysates using various combinations of epitope tags have so far been unsuccessful. Mif2p has been shown to localize to the kinetochore by ChIP assay (Meluh and Koshland, 1997). Here, fluorescence microscopy imaging determined that the localization of YFP-tagged Mif2p in relation to Spc29p-CFP was similar to that of other kinetochore proteins (Figure II.3.F). YFP-tagged Chl4p was also observed to co-localize with Mif2p-CFP in cells with both short and long spindles (Figure II.3.F). Thus, Chl4p shows not only a genetic interaction (Table II.2) but also a two-hybrid interaction and co-localization with Mif2p, an established kinetochore protein.

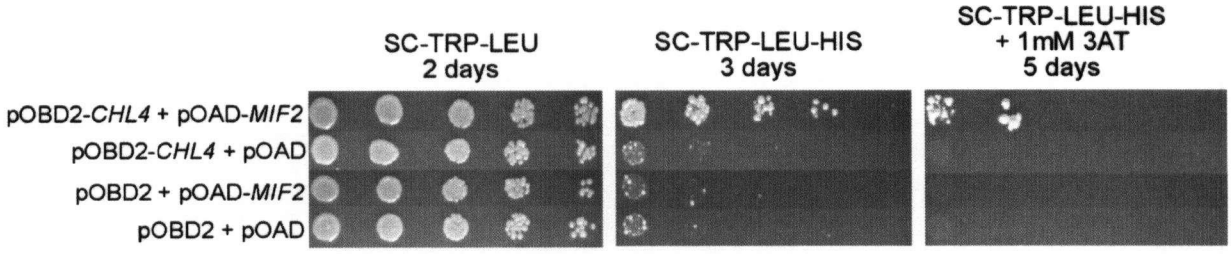


Figure II.7. Chl4p interacts with the kinetochore protein Mif2p.
Growth of PJ694a/α strains containing the Chl4p–DNA-binding domain fusion in plasmid pOBD2 (or vector alone as control) and the Mif2p–activation domain fusion in plasmid pOAD (or vector alone as control) on selective media. SC medium lacking tryptophan and leucine only selects for the vectors, while medium lacking tryptophan, leucine and histidine selects for the vectors and the two-hybrid interaction. 3–aminotriazole (3-AT) is added to prevent growth due to leaky expression from the *HIS3* promoter.

II.4. Discussion

The co-immunoprecipitation, ChIP and fluorescence imaging data presented here contribute to the mapping of protein complexes within the kinetochore, by determining requirements for protein-protein interactions, *CEN* DNA loading and proper kinetochore localization of four central kinetochore proteins. The data first demonstrate that Chl4p is a protein of the central kinetochore, confirming earlier suggestions from genetic data (Kouprina et al., 1993a), and then investigate the location of Chl4p within the kinetochore complex. Chl4p specifically co-immunoprecipitates with *CEN* DNA and localizes to the kinetochore in an Ndc10p- and Ctf19p-dependent manner, suggesting that Chl4p is located more distal to the DNA than the CBF3 and Ctf19 complexes. Moreover, since the Ctf19 complex itself requires CBF3 for interacting with *CEN* DNA (Ortiz et al., 1999), a plausible hypothesis is that Chl4p interacts with CBF3 and *CEN* DNA via the Ctf19 complex. Other kinetochore proteins, such as Mtw1p, have also been shown to depend on Ndc10p for interaction with the centromere (Goshima and Yanagida, 2000), suggesting that disruption of this inner kinetochore component will affect the localization and *CEN* DNA interaction of many kinetochore proteins. However, the data presented here demonstrate further specificity in dependency relationships by showing that association of Chl4p with *CEN* DNA requires one central kinetochore protein (Ctf19p) but not another (Ctf3p).

These studies with Chl4p have led to the identification of another component the kinetochore complex, Iml3p. Previous genetic data suggested that the *IML3* gene product might function at the kinetochore (Ghosh et al., 2001). The mutant phenotypes of *iml3*, which include a high rate of chromosome and plasmid loss, benomyl sensitivity, relaxation of a transcription block, and stable maintenance of a dicentric plasmid (Entian et al., 1999; Ghosh et al., 2001), are very similar to those of *chl4*. Iml3p also interacts with Chl4p by two-hybrid assay (Ghosh et al., 2001). This two-hybrid interaction was confirmed by co-immunoprecipitation of Chl4p with Iml3p from yeast lysates (Figure II.6.A). Interestingly, it has now been shown that Chl4p is present in a tandem-affinity purification of Iml3p (Gavin et al., 2002, and see Chapter III). Here Iml3p is shown to both interact with *CEN* DNA and localize to the kinetochore in a Ctf19p- and Chl4p-dependent manner, which establishes Iml3p as a component of the central kinetochore. Unlike the Ctf3p-Chl4p interaction, the Iml3p-Chl4p interaction does occur in the absence of Ctf19p, indicating that Chl4p and

Iml3p could form a separate complex from the Ctf19 and Ctf3 complexes. It should be noted that the interactions of Chl4p and Iml3p with each other and with other kinetochore proteins may not be direct, and that it is not known whether they require the presence of centromere DNA or an intact inner kinetochore.

The co-immunoprecipitation data suggest that Chl4p connects the Ctf3 complex to the Ctf19 complex. Whereas Chl4p interacts with Ctf19p and *CEN* DNA in the absence of Ctf3p (Figures II.2.D and II.5.C), Ctf3p does not interact with Ctf19p in the absence of Chl4p (Figure II.5.D), and since the interaction of Ctf3p with *CEN* DNA requires Ctf19p (Measday et al., 2002), a simple interpretation of this result is that Chl4p is more proximal to *CEN* DNA than the Ctf3 complex. However, deletion of *CHL4* does not abolish the Ctf3p-*CEN* DNA interaction and only reduces the localization signal of Ctf3p to the kinetochore by 40% (Figures II.3.C and II.4.C). Therefore, it is likely that a more complex network of protein interactions occurs. For example, other members of the Ctf3p complex may allow for interaction with the Ctf19 complex in the absence of Chl4p, as is schematically depicted in Figure II.8.B. Alternatively, the lack of Ctf3p-Ctf19p co-immunoprecipitation in the absence of Chl4p could reflect a change in the overall conformation of the central kinetochore that may result in reduced *CEN* DNA binding efficiency by the Ctf3 complex. Moreover, it was observed here that proper *in vivo* localization of Ctf3p requires Ctf19p, and to some extent Chl4p, only in cells with short spindles; in cells with long spindles, Ctf3p localizes to the kinetochore even in the absence of Ctf19p and Chl4p. Since kinetochores overlap with the SPB near the end of anaphase, the SPB may provide an additional anchor for kinetochore proteins at this stage of the cell cycle.

In addition to the interactions of Chl4p with proteins of the central kinetochore, the data presented here show that Chl4p interacts with the kinetochore protein Mif2p by two-hybrid assay. The fact that the Chl4p-Mif2p interaction was not detected by co-immunoprecipitation suggests that the conditions in which the experiment was performed may not have been ideal to preserve the interaction. For instance, since Mif2p has been proposed to interact with DNA at CDEII or CDEIII, owing to the presence of potential A–T hooks in its structure, it is possible that a certain DNA conformation is required to position Mif2p so that it can interact with other kinetochore proteins. This particular conformation may be lost during the immunoprecipitation procedure. In fact, up to recently, there had been

no report of proteins interacting with Mif2p by standard immunoprecipitation from yeast lysates, and thus the exact positioning of Mif2p within the kinetochore complex had not been determined yet. Interestingly, Mif2p was also found to interact with the CBF3 component Cep3p in the two-hybrid array (data not shown), suggesting that Mif2p lies close to the inner kinetochore. More recent data highlighting the position of Mif2p within the kinetochore will be discussed in Chapter III. The studies presented here show that Mif2p has a kinetochore localization pattern and that it co-localizes with Chl4p by fluorescence microscopy (Figure II.3.F).

One major difficulty in understanding the function of kinetochore proteins is their genetic and phenotypic redundancy. Several central kinetochore proteins, including Ctf19p, Mcm21p, Ctf3p, Mcm16p, Mcm22p, Chl4p and Iml3p, are not essential for cell viability. Simultaneous deletion of several of these components does not seem to compromise cell viability or have a synergistic effect on the chromosome loss phenotype (Table II.2 and Ghosh et al., 2001; Measday et al., 2002), which may be indicative of the presence of a "mega-complex" within the outer kinetochore, encompassing the Ctf19 and Ctf3 complexes, Chl4p, Iml3p and additional proteins found in affinity purifications (Gavin et al., 2002, and see Chapter III). Whole genome synthetic lethal screens of *chl4Δ* and *iml3Δ* are currently underway with the aim of uncovering genetic interactions and possibly other proteins performing a similar function, and may provide some insights into Chl4p and Iml3p function.

Chl4p is a 453 amino acid protein that has no recognizable domains as assessed by standard conserved domain homology searches. It has been suggested that part of Chl4p is similar to a small region of *E.coli recA*, and some Chl4p residues have been predicted to fold into a helix-turn-helix motif that could be part of a putative DNA-binding domain (Kouprina et al., 1993a). A BLASTP analysis (Altschul et al., 1997) of the Chl4p sequence against the nr database revealed that two predicted proteins have significant similarity to Chl4p, the *Neurospora crassa* protein B2A19.050 "related to trfA protein" (Genbank accession CAB98235) and the *Schizosaccharomyces pombe* pi022/SPBP22H7.09c gene product (Genbank accession CAC37377). Additionally, a *Candida albicans* protein, orf6.7000 (CandidaDB CA4453) shows 27% identity to Chl4p. Although these putative homologues of Chl4p have yet to be functionally characterized, conservation of this protein in distant fungal

species indicates that it probably plays an important role in the maintenance of genomic integrity.

The data presented here demonstrate that Chl4p is required for interactions between known central kinetochore complexes, and that it may affect their *CEN* DNA loading efficiency. Thus, Chl4p could be an important structural component of the outer kinetochore. It has been proposed that Chl4p is involved in the initial step of kinetochore formation rather than maintenance of pre-existing ones, as *de novo* kinetochores were more affected by deletion of *CHL4* than established ones (Mythreye and Bloom, 2003). Thus Chl4p could be instrumental in establishing the structure of nascent kinetochores by holding together the building blocks in the proper conformation. Additionally, the data show that strains with *chl4* mutations are sensitive to a drug that disrupts microtubule networks. Thus, a kinetochore lacking *CHL4* may not attain the optimal structural conformation required for efficient interaction with spindle microtubules. Whether dynamic rearrangements of the kinetochore occur during the progression of the cell cycle is unknown. The data obtained in this study with Ctf3p localization in *chl4* and *ctf19* mutants suggest that changes do occur, as the localization of Ctf3p is disturbed only in early anaphase in the absence of Ctf19p and Chl4p. Future studies will determine if Chl4p and other proteins of the outer kinetochore actively participate in kinetochore dynamics during mitosis.

Combining the fluorescence imaging, ChIP and immunoprecipitation data presented here (summarized in Table II.3) then allows one to propose an extension of a previous model (Measday et al., 2002) of kinetochore structure, which includes Chl4p and Iml3p as central components of the kinetochore (Figure II.8.A). Although the budding yeast centromere is much simpler than the centromere of other eukaryotes, the number of proteins comprising the budding yeast kinetochore is quite large and still growing (Cheeseman et al., 2002b, and see Figure I.5). The studies described in this chapter not only establish two new protein components in the kinetochore complex, but also give a deeper understanding of the spatial relationships existing among several of its components.

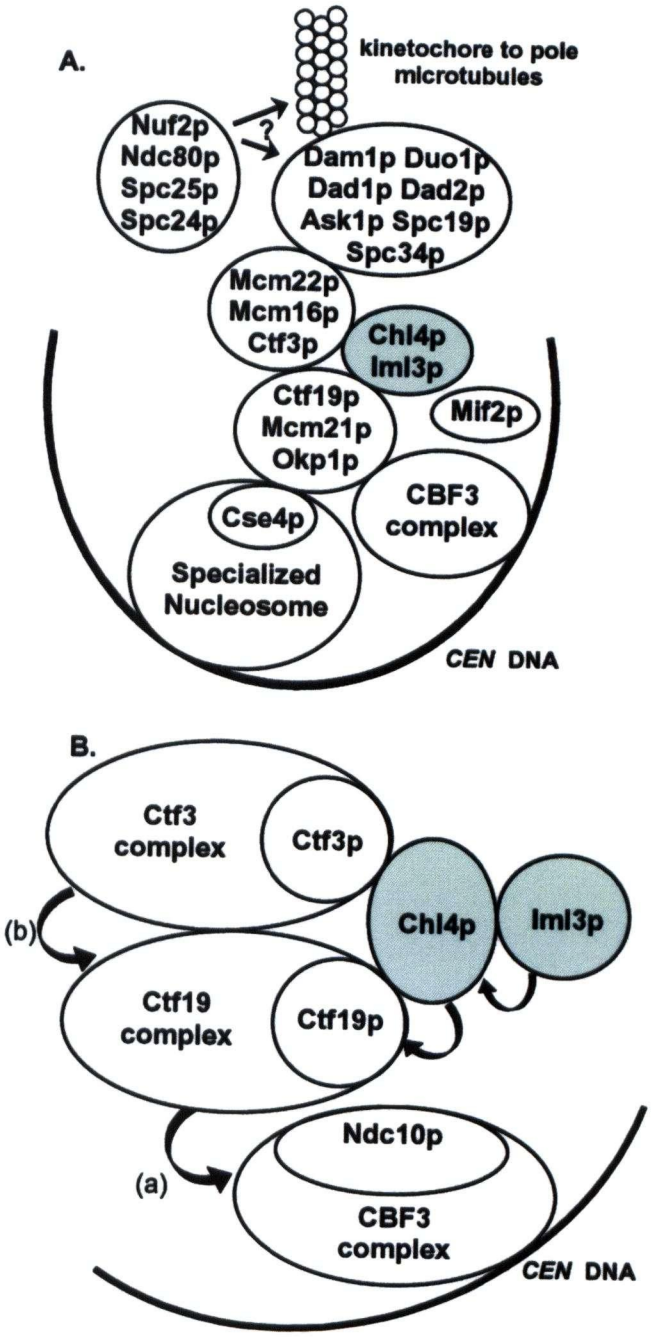
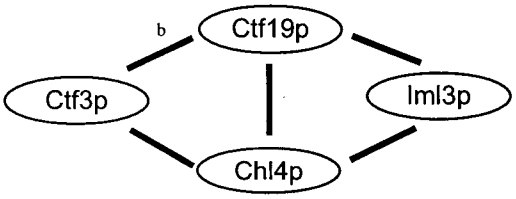
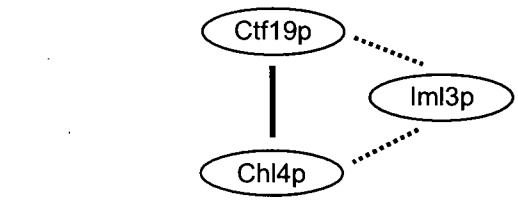
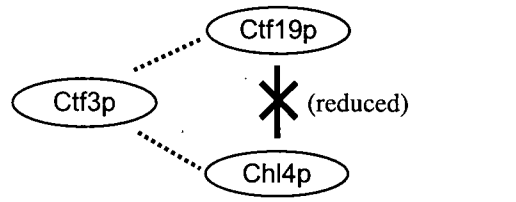


Figure II.8. Model of the budding yeast kinetochore.

A. Revision of the kinetochore model presented in Measday et al. (2002), which includes Chl4p and Iml3p as part of the outer kinetochore. Mif2p has also been added to the diagram because of its two-hybrid interaction with Chl4p. B. Proposed molecular architecture of the proteins that have been examined in this paper, deduced from requirements for *CEN* DNA interaction, localization and protein-protein interactions. Arrows represent requirements for interaction with *CEN* DNA, beginning with the complex/protein interacting with *CEN* DNA, and pointed towards the complex/protein required for the interaction. (a) From Ortiz et al. (1999). (b) From Measday et al. (2002).

Table II.3. Summary of dependency relationships among kinetochore proteins studied here

Strain Background	*CEN* ChIP (Myc-tagged protein)	Kinetochore Localization (YFP-tagged protein)	Immunoprecipitation (━ observed; ···· n/d [a]; ✕ disrupted)
wild type	Ndc10p + [b] Ctf19p + [b] Ctf3p + [b] Chl4p + Iml3p +	Ndc10p + [b] Ctf19p + [b] Ctf3p + [b] Chl4p + Iml3p +	
ndc10-2 (non-permissive temperature)	Ctf19p – [c] Chl4p –	n/d [a] Chl4p – [d]	n/d [a]
ctf19Δ	Ndc10p + [b, e] Ctf3p – [b, e] Chl4p – Iml3p –	Ndc10p + Ctf3p – (ss [f]), + (ls [f]) Chl4p – Iml3p –	
ctf3Δ	Ctf19p + [b, c, g] Chl4p +	n/d [a]	
chl4Δ	Ndc10p + Ctf19p + Ctf3p + [h] Iml3p –	Ndc10p + Ctf19p + Ctf3p – (ss [f]), + (ls [f]) Iml3p –	
iml3Δ	Ctf19p + Chl4p + [h]	Ctf19p + n/d [a]	

[a] n/d = non-determined
[b] From Measday et al. (2002).
[c] From Ortiz et al. (1999).
[d] From Mythreye and Bloom (2003).
[e] ChIP assay was done in nocodazole-arrested cells.
[f] ss = in cells with short spindles; ls = in cells with long spindles.
[g] Ctf19p was tagged with HA instead of Myc for this particular assay.
[h] *CEN* ChIP may be less efficient in the mutant strain than in the wild type strain.

CHAPTER III

THE KINETOCHORE PROTEIN AME1P FUNCTIONS IN PROPER MICROTUBULE ATTACHMENT AND MAY HAVE A ROLE IN SPINDLE CHECKPOINT MAINTENANCE

The work presented in this chapter is part of a collaboration with Dr. Jackie Vogel (McGill University). A large part of the data presented here is included in a manuscript that is being prepared for publication by Isabelle Pot, Victoria Aneliunas, James Knockleby, Sonia Ah-Kye, Gregory Liszt, Nancy Burns, Michael Snyder, Philip Hieter and Jackie Vogel.

III.1. Introduction

High-fidelity chromosome segregation relies on a dynamic interaction between the plus ends of spindle microtubules and the kinetochore, and on the presence of a checkpoint system that delays anaphase if defects in kinetochore-microtubule interactions occur. As mentioned in Chapter I, while only one of the kinetochore complexes (Dam1) has been shown to bind microtubules directly (Cheeseman et al., 2001; Janke et al., 2002; Li et al., 2002), structural microtubule-associated proteins such as Stu2p, Bim1p and Bik1p (reviewed in Cheeseman et al., 2002b; He et al., 2001) and motor proteins such as Kar3p (Middleton and Carbon, 1994) are also known to function at the kinetochore to drive poleward movement of chromosomes during anaphase. In contrast, central kinetochore proteins are thought to not only have a role in the structural integrity of the kinetochore, which promotes chromosome-microtubule attachment, but also to provide a platform for regulation and checkpoint signaling (reviewed in Biggins and Walczak, 2003; Cleveland et al., 2003; Rieder and Salmon, 1998).

Defects in spindle assembly and kinetochore function trigger cell cycle arrest by activating the Mad2p-dependent spindle assembly checkpoint, while a second Bub2p-dependent checkpoint monitors proper spindle positioning and elongation into the bud to ensure that daughter cells receive an SPB before allowing exit from mitosis to occur (both described in detail in Chapter I) (reviewed in Amon, 1999; Musacchio and Hardwick, 2002). To date, a major distinction between checkpoint and kinetochore proteins is their response to damage; mutation of a checkpoint protein results in failure to establish the checkpoint, whereas mutation of some kinetochore proteins triggers the checkpoint response. However, kinetochore proteins must interact with checkpoint proteins and be an integral part of the "sensing" machinery. In mammalian cells, MAD2 and BUBR1 localize to kinetochores in cells in which the spindle assembly checkpoint has been activated (Green and Kaplan, 2003; Waters et al., 1998). In budding yeast, the CBF3 component Skp1p was shown to interact with Bub1p, providing evidence for a direct physical connection between the kinetochore and the spindle assembly checkpoint (Kitagawa et al., 2003). Yeast checkpoint proteins were also shown to localize to the kinetochore and interact with *CEN* DNA (Kerscher et al., 2003). In addition, Bub3p has been shown to co-purify with the central kinetochore protein Okp1p (De Wulf et al., 2003; Ortiz et al., 1999). However, the exact mechanism by which the

checkpoint response is established remains largely unknown. Finally, cells must have the ability to detect the status of kinetochore-microtubule attachment and consequently either maintain the checkpoint, or release from the arrest if the damage has been repaired. Kinetochore proteins are likely to play a role in both establishing and maintaining the arrest (Poddar et al. 2004).

As illustrated in Figure I.5, the last 5 years have greatly increased our knowledge of proteins involved in kinetochore function in budding yeast. Among the genome-wide approaches that have been developed in yeast, large-scale protein purification methods (Gavin et al., 2002; Ho et al., 2002) have allowed identification of many proteins that interact with known kinetochore components. Gavin et al. (2002) used tandem affinity purification (TAP) to isolate interacting partners of Iml3p, the kinetochore component characterized in Chapter II; *iml3*Δ mutants had at the time been shown to have an increased minichromosome loss phenotype (Entian et al., 1999), but no additional characterization had been undertaken. Iml3p-TAP interacted with known components of the Ctf19 and Ctf3 complexes, with some unknown proteins, and with a poorly characterized protein, Ame1p (Gavin et al., 2002). A subsequent TAP study using several kinetochore components of those complexes as baits proposed that Ctf19p, Mcm21p, Okp1p, Chl4p, Iml3p, Ctf3p, Mcm16p, Mcm22p, Nkp1p, Nkp2p, Mtw1p and Ame1p were part of one large Ctf19 complex (Cheeseman et al., 2002a). However, recent data indicates that some subunits of the Ctf19 complex are more tightly associated with each other, such as Ctf19p, Okp1p, Mcm21p and Ame1p, which form the 'COMA' complex (see Figure I.5.B); thus, previously described subcomplexes within the large Ctf19 complex (e.g. the Ctf3 complex) may be functionally meaningful (Measday et al., 2002; Ortiz et al., 1999). Moreover, Mtw1p has now been shown to interact tightly with three other proteins, Dsn1p, Nnf1p and Nsl1p, forming its own "Mtw1 complex", whereas the association of Mtw1p with the large Ctf19 complex was real, but weaker (De Wulf et al., 2003; Nekrasov et al., 2003; Pinsky et al., 2003; Scharfenberger et al., 2003; Westermann et al., 2003). The Mtw1 complex and another newly identified complex comprising Spc105p and an uncharacterized protein, Ydr532p, expanded the network of interactions among kinetochore subcomplexes, including more connections between the central kinetochore and the inner kinetochore through Mif2p (De Wulf et al., 2003; Nekrasov et al., 2003). Protein-protein interactions between chromatin assembly factors that had been previously reported to

have a role in centromere function, nucleosomal proteins including Cse4p, and Mif2p were also reported (Westermann et al., 2003). Figure I.5.B, while acknowledging the limitations of representing a 3D complex in 2D, attempts to give an overview of the current budding yeast kinetochore composition and the relationships among complexes. However, despite significant progress in the identification of kinetochore proteins, the exact role of many of these components in the process of microtubule attachment and its regulation is still unclear. A more thorough characterization of individual kinetochore proteins is thus expected to bring insights into these questions.

Having concentrated on a detailed characterization of some of the non-essential components of the large Ctf19 complex (Chapter II), it was of interest to study Ame1p, as it is one of only two essential proteins in that complex and is thus expected to have a crucial role in kinetochore function. *AME1/ARP100/YBR211C* ("Associated with Microtubules and Essential") was first identified in a large-scale protein localization screen where random transposon insertions throughout the genome created *GENE-LACZ* fusions (Burns et al., 1994). In that study an N-terminal fragment of Ame1p fused to the reporter localized to the SPB region and its associated short microtubules, suggesting a role for Ame1p in microtubule-dependent processes. Subsequently, Ame1p was found to interact with Okp1p, an essential component of the Ctf19 complex, by two-hybrid assays (Ito et al., 2001). Together with the protein purification studies described above, these data indicate that Ame1p is likely to function at the budding yeast central kinetochore; however its exact function remains largely unknown.

This chapter demonstrates that Ame1p is a functional component of the central kinetochore of budding yeast, as it has the hallmarks of a kinetochore protein: interaction with *CEN* DNA (by ChIP) and a kinetochore pattern of localization (by fluorescence microscopy). The chapter also describes the isolation of conditional *ame1* alleles and the analysis of chromosome segregation and spindle assembly checkpoint activation and maintenance in these mutants. This analysis reveals that Ame1p plays a crucial role in high-fidelity chromosome segregation. *ame1* mutants exhibit defects in kinetochore-microtubule attachment but not in sister chromatid cohesion, and show genetic interactions with mutants in genes encoding known components of the large Ctf19 complex. Mutations that either truncate the C-terminus (*ame1-31*) or result in mislocalization of the protein (*ame1-4*) show

severe chromosome instability. ame1p mutant proteins are also defective in interaction with Ctf19p. Combining these experimental results and recent data by De Wulf et al. (2003), Ame1p is proposed to be a protein located close to the CBF3 complex in combination with Okp1p in the overall kinetochore structure, serving as a link to *CEN* DNA for other Ctf19 complex components. Additionally, the spindle assembly checkpoint is activated in *ame1* mutants, resulting in a cell cycle delay, but does not appear to be maintained. Moreover, *ame1* mutants seem to undergo spindle elongation prior to sister chromatid separation, resulting in the accumulation of cells with properly positioned, elongated spindles in the mother cell. Overexpression of Okp1p rescues *ame1* defects in checkpoint maintenance but not premature spindle elongation or the chromosome loss phenotype, indicating that perhaps, only part of Ame1p's role is cooperative with Okp1p. Finally, strains compromised for *AME1* function are sensitive to overexpression of a microtubule-associated protein, Bik1p, that functions at the kinetochore. These data provide the first in depth analysis of Ame1p and demonstrate that Ame1p is a novel central kinetochore protein required for kinetochore function, that may also contribute to the maintenance of the spindle checkpoint signal.

Note: The work presented in this chapter involved the participation of Dr. Jackie Vogel, James Knockleby, Sonia Ah-Kye, and Nicolas Szapiel from Dr Vogel's laboratory, for the microscopy data and for overall experimental design and data interpretation (parts of Sections III.3.cd and III.4.abdfhij and Figures III.2.A, 3.D, 4.D, 6.ABC, 8.E, 9.AB and 10.ACDE). *ame1* mutants were first constructed by Dr. Jackie Vogel in the laboratory of Dr. Michael Snyder (Yale University) with the help of Gregory Liszt (Sections III.3.b and III.4.b).

Victoria Aneliunas (from Dr. Phil Hieter's laboratory) contributed to the constructions of many of the strains employed throughout this chapter, and participated in experiments described in sections III.3.ab and III.4.bcdehj (Figures III.5.BC, 8.AB and 9.B).

III.2. Materials and methods

III.2.a. Yeast strains, plasmids, media, suppressor screen and BIK1 overexpression

Yeast strains used for experiments in this chapter are listed in Table III.1. Unless otherwise stated, all strains are YPH499 derivatives (S288C background). Media for yeast strain growth and sporulation, as well as methods for yeast transformation, epitope tagging, synthetic lethality tests and the chromosome fragment loss assay were described in Chapter II. Epitope-tagged mutant strains are functional as judged from temperature sensitivity and chromosome loss phenotypes (data not shown). For the multicopy suppressor screen, a yeast genomic library (Connelly and Hieter, pers. commun.) carried on a 2μ *URA3*-marked vector was transformed into the *ame1-4* strain so as to provide five-fold genome coverage; the transformation mixture was plated on SC medium lacking uracil at 25°C for 1 day and then at 35°C for 5 days. Plasmids were rescued from colonies growing at 35°C, confirmed as suppressors by re-transformation into *ame1-4*, and sequenced at insertion points to identify the clones. *OKP1* was amplified from one of the rescuing clones by PCR, and subcloned into pRS426 (2μ *URA3*), giving pVA127. Cloning of *BIK1* into an overexpression vector was done by PCR using a forward primer located 44bp upstream of the *BIK1* ATG and containing a *Bam*HI restriction site at its 5'end, and a reverse primer located either directly downstream of the STOP codon (for the full length clone) or 40 amino acids upstream of the STOP codon (for the C-terminal truncation clone), and containing an *Eco*RI restriction site at its 5'end. Digested PCR products were cloned into the p415-GAL-L vector (Mumberg et al., 1994). Yeast strains transformed with the resulting plasmid (or empty vector control) were selected on SC medium lacking leucine. To assess growth upon overexpression of the *BIK1* clones, strains were first grown on selective medium containing raffinose and then streaked onto selective medium containing both raffinose and galactose (or galactose only) and grown at 25°C for 5 days.

III.2.b. Cloning, mutagenesis, and integration of ame1 alleles into the genome

A 1.5kb PCR fragment containing the *AME1* ORF plus 331bp of 5' promoter region and 187bp of 3' UTR, amplified with primers containing *Eag*I and *Kpn*I restriction sites, was subcloned into pUN15 (CEN *TRP1*) using *Eag*I/*Kpn*I digests, resulting in pGL1. pGL1 complemented the lethality of an *ame1*Δ*::HIS3* disruption. pGL1 was cut with *Spe*I and

*Bam*HI (which removed 155bp of 5' UTR and 0.9kb of the 1 kb *AME1* ORF) and the gapped plasmid was co-transformed into yeast with PCR products amplified from the *AME1* coding sequence under mutagenic conditions. Colonies containing gap-repaired plasmids were selected on SC medium lacking tryptophan. Gap-repaired plasmids were recovered from yeast, amplified in *E. coli*, re-transformed into yeast and screened for conditional growth defects using the plasmid shuffle method. Mutant alleles were confirmed by tetrad dissection.

In order to replace the wild type genomic copy of *AME1* with the mutant alleles, two methods were used (schematically depicted on Figure III.1). The first one involved amplifying each mutant allele from its parent plasmid by PCR, and integrating that PCR product into a wild type strain together with a second PCR product containing a nutritional marker, by homologous recombination. This method yielded only partial mutants that differed from the parent plasmid by 1) having integrated only part of the mutations due to recombination at a random site along the endogenous copy of the *AME1* gene; and 2) extra mutations due to PCR errors. The second method was designed to avoid these problems by cloning out the mutant alleles from the plasmids by restriction digests (instead of using PCR), and transforming the restriction fragments together with the nutritional marker PCR product into a strain deleted for *AME1* (to ensure insertion of the complete mutant sequence). Since deletion of *AME1* is lethal, it was covered by a *URA3*-marked shuffle plasmid containing a wild type copy of *AME1*, that was removed by plating on FOA medium after integration of the mutant allele. Mutations were identified by PCR; the resulting amino acid changes are listed in Table III.2.

III.2.c. Protein and chromatin immunoprecipitation assays

IPs and ChIP assays were performed as described in Chapter II. For quantification of the ChIP signal, diluted total and IP template DNA were used for multiplex PCR for each strain from three (for wild type and *ame1-4*) or two (for *ame1-31* and *ame1-5*) independent experiments. Amplifications were determined to be in the linear range for PCR. The resulting *CEN1*, *CEN3* and *PGK1* amplified products were quantified on ethidium bromide stained polyacrylamide gels using the Quantity One 4.2.1 software from Bio-Rad Laboratories (Hercules, CA). Local background was substracted from each band intensity,

and for each experiment band intensities from the IP sample were divided by band intensities from the template sample, and then averaged for the three experiments. Protein extractions and immunoblots were performed as described in Vogel et al. (2001).

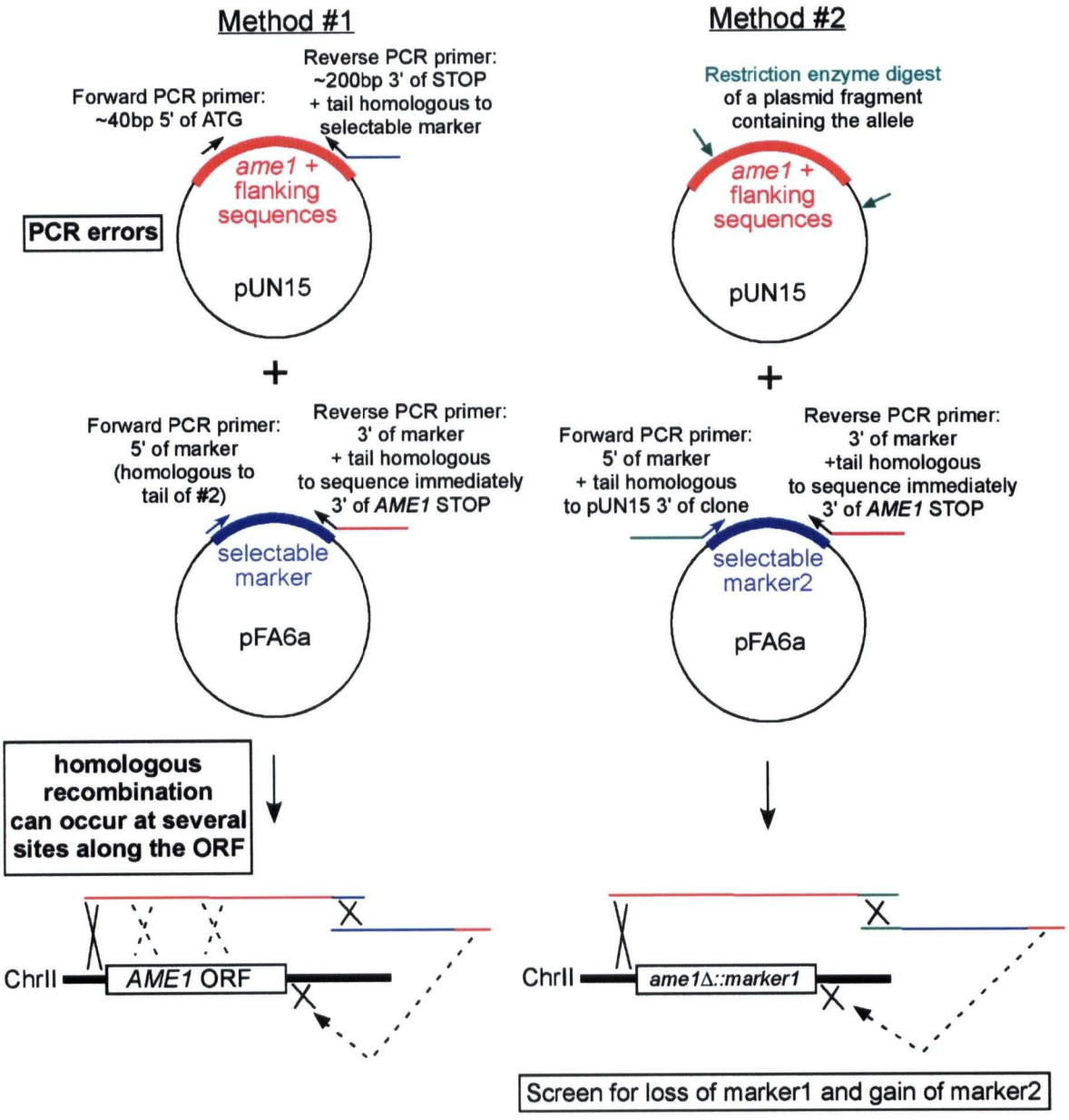


Figure III.1. Strategies employed to integrate mutant *ame1* alleles at the endogenous *AME1* locus

III.2.d. Light and fluorescence microscopy

All strains used for microscopy were grown in FPM (SC medium supplemented with adenine and containing 6.5g/L sodium citrate) to reduce auto-fluorescence. Cells were grown in FPM at 25°C to early log phase, rediluted in fresh FPM, grown for 90 min, and resuspended in pre-warmed FPM at 37°C (for the asynchronous time-course experiments) or incubated with α-factor as described previously (Vogel et al., 2001), until greater than 90% of the cells (~100 cells counted) had mating projections. α-factor-arrested cells were washed once in H_2O and resuspended in pre-warmed FPM at 37°C. For visualization of the GFP-LacI/*LacO:CEN* chromosome tag, cells were grown in FPM lacking histidine, rediluted in fresh FPM lacking histidine supplemented with 40mM 3-AT from Sigma (St Louis, MO) and incubated for 90 min at 25°C to induce the expression of the GFP-LacI fusion. Cells were then resuspended in fresh pre-warmed FPM and incubated at 37°C for the specified time. Cells were fixed in 4% EM grade methanol-free formaldehyde from Polysciences Inc. (Warrington, PA) for 5 min, washed in phosphate buffered saline (PBS) and mounted in a *Slowfade Light* anti-fade solution from Molecular Probes (Eugene, OR) in 50% glycerol according to the supplied protocol, or prepared for immunofluorescence as described (Vogel and Snyder, 2000). The anti-tubulin antibody (YOL134) and anti-rat FITC-conjugated antibody from Jackson Immunoresearch (West Grove, PA) were used at a 1/200 dilution in PBS/BSA.

Cells were examined using a Nikon TE200U microscope equipped with a 100x 1.4 na objective mounted on PE peizo z-drive/Improvision Orbit controller, and Hamamatsu ORCA-ERG camera. Shutters and filter wheels were controlled with a MAC5000 controller from Ludl Electronics (Hawthorne, NY). Image stacks (0.5μm optical sections with 10-12 stacks per image) were acquired using OpenLab software, and deconvoluted with Velocity. All measurements (relative positions of GFP-tagged chromosomes and SPBs, and spindle lengths) were made using 3D reconstructions, and post-processed with Microsoft Excel. Images shown are the extended focus of a z-series of 0.5μm optical sections.

Table III.1. List of yeast strains used in experiments described in this chapter

Strain	Genotype	Reference
YPH499	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63*	P. Hieter
IPY1438	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-1-TRP1*	this study
IPY1441	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-3-TRP1*	this study
YPH1676	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-31-HIS3MX6*	this study
IPY1258	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-32-HIS3MX6*	this study
YPH1677	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-4-TRP1*	this study
IPY1255 & IPY1537[a]	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-41-kanMX6*	this study
YPH1678	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-5-TRP1*	this study
YPH1679	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 okp1-5-TRP1*	derived from YJL158 (Ortiz et al., 1999)
YPH1680	MATα *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 okp1-5-TRP1*	derived from YJL158 (Ortiz et al., 1999)
YPH1681	MATα *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 okp1-5-trp1::HIS3::trp1*	derived from YJL158 (Ortiz et al., 1999)
YPH971	MATa *ura3 lys2 ade2 his3 leu2 trp1 ndc10-1*	J. Kilmartin (JK421)
YPH1314	MATα *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ctf19Δ::HIS3*	(Hyland et al., 1999)
YPH1315	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ctf19Δ::HIS3*	(Hyland et al., 1999)
YPH1316	MATα *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ctf19Δ::TRP1*	(Hyland et al., 1999)
YPH1537	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 chl4Δ::HIS3MX6*	(Pot et al., 2003)
YVM111	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ctf3Δ::HIS3*	V. Measday
YVM112	MATα *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ctf3Δ::HIS3*	V. Measday
YVM996A	MATα *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ctf3Δ::his3::TRP1::his3*	V. Measday
YVM1448	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 spc24-8-kanMX6*	V. Measday
YVM1380	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 spc24-9-kanMX6*	V. Measday
YVM1363	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 spc24-10-kanMX6*	V. Measday
YPH1349	MATa *ura3-52 his3Δ200 leu2-3,112 dam1-1*	M. Winey (906) (Jones et al., 2001)
YPH1682	MATα *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 mad2Δ::HIS3*	V. Aneliunas & P. Hieter
YPH1683	MATα *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 bub2Δ::LEU2*	V. Aneliunas & P. Hieter
YPH1684	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-31-HIS3MX6 mad2Δ::HIS3*	this study
YPH1685	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-31-HIS3MX6 bub2Δ::LEU2*	this study

(continues)

Table III.1. (continued)

Strain	Genotype	Reference
YPH1686	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-4-TRP1 mad2Δ::HIS3*	this study
YPH1687	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-4-TRP1 bub2Δ::LEU2*	this study
YPH1688	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-5-TRP1 mad2Δ::HIS3*	this study
YPH1689	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-5-TRP1 bub2Δ::LEU2*	this study
IPY1978	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 okp1-5-TRP1 mad2Δ::HIS3*	this study
IPY1982	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 okp1-5-TRP1 bub2Δ::LEU2*	this study
YPH1124	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63* CFIII (*CEN3.L.* YPH982) *URA3 SUP11*	P. Hieter
IPY1659	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-1-TRP1* CFIII (*CEN3.L.* YPH982) *URA3 SUP11*	this study
IPY1663	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-3-TRP1* CFIII (*CEN3.L.* YPH982) *URA3 SUP11*	this study
YPH1690	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-31-HIS3MX6* CFIII (*CEN3.L.* YPH982) *URA3 SUP11*	this study
IPY1239	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-32-HIS3MX6* CFIII (*CEN3.L.* YPH982) *URA3 SUP11*	this study
YPH1691	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-4-TRP1* CFIII (*CEN3.L.* YPH982) *URA3 SUP11*	this study
IPY1223[b]	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-41-kanMX6* CFIII (*CEN3.L.* YPH982) *URA3 SUP11*	this study
YPH1692	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-5-TRP1* CFIII (*CEN3.L.* YPH982) *URA3 SUP11*	this study
YVM770A[c]	MATa *ade2 trp1-1 his3-11,15::GFP(pAFS144, thermostable)-LacI-HIS3 leu2-3,112 ura3-1 CEN15(1.8kb)-LacO-URA3 SPC29-CFP-kanMX6*	V. Measday and Goshima and Yanagida (2000)
YPH1693[d]	MATa *ade2 trp1 his3-11,15::GFP(pAFS144, thermostable)-LacI-HIS3 leu2 ura3 CEN15(1.8kb)-LacO-URA3 ame1-31-HIS3MX6 SPC29-CFP-kanMX6*	this study
YPH1694[d]	MATa *ade2 trp1 his3-11,15::GFP(pAFS144, thermostable)-LacI-HIS3 leu2 ura3 CEN15(1.8kb)-LacO-URA3 ame1-4-TRP1 SPC29-CFP-kanMX6*	this study
YPH1695[d]	MATa *ade2 trp1 his3-11,15::GFP(pAFS144, thermostable)-LacI-HIS3 leu2 ura3 CEN15(1.8kb)-LacO-URA3 ame1-5-TRP1 SPC29-CFP-kanMX6*	this study
YPH1640[c]	MATa *ade2-101 trp1-1 can1-100 his3-11,15::GFP(pAFS144, thermostable)-LacI-HIS3 leu2-3,112 ura3-1 CEN15(1.8kb)-LacO-URA3 chl1Δ::kanMX6*	(Mayer et al., 2004)
YPH1696	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 PDS1-13Myc-trp1::kanMX6::trp1*	this study
YPH1697	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-4-TRP1 PDS1-13Myc-trp1::kanMX6::trp1*	this study
YPH1698	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 AME1-13Myc-TRP1*	this study
YPH1699	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ctf19Δ::HIS3 AME1-13Myc-TRP1*	this study
YPH1700	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-31-13Myc-TRP1*	this study

(continues)

Table III.1. (continued)

Strain	Genotype	Reference
YPH1701	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-4-13Myc-kanMX6*	this study
YPH1702	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-5-13Myc-kanMX6*	this study
YPH1555	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 NDC10-13Myc-kanMX6*	(Pot et al., 2003)
YPH1703	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-4-TRP1 NDC10-13Myc-kanMX6*	this study
YPH1704	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 okp1-5-TRP1 NDC10-13Myc-kanMX6*	this study
YPH1705	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 CTF19-13Myc-HIS3MX6*	this study
YPH1706	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-4-TRP1 CTF19-13Myc-HIS3MX6*	this study
YPH1707	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 AME1-VFP-kanMX6 SPC29-CFP-hphMX4*	this study
YPH1708	MATa *ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-31-VFP-kanMX6 SPC29-CFP-hphMX4*	this study
YPH1709	*MATa ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-4-VFP-kanMX6 SPC29-CFP-hphMX4*	this study
YPH1710	*MATa ura3-52 lys2-801 ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ame1-5-VFP-kanMX6 SPC29-CFP-hphMX4*	this study

[a] IPY1255 was later found to have a secondary mutation leading to, for example, benomyl supersensitivity. IPY1537 is a derivative of IPY1255 where the secondary mutation was segregated away from the *ame1-41* mutation.
[b] IPY1223 contains the same secondary mutation as IPY1255.
[c] W303 background.
[d] Mixed W303 and S288C (YPH499) backgrounds.

Table III.2. List of amino acid mutations in *ame1* mutants[a]

ame1-1	*ame1-3*	*ame1-31*	*ame1-32*	*ame1-4*	*ame1-41*	*ame1-5*
E93G[b]	D20G	D20G		D23E		D21E
L150S	D25G	D25G		S45Y		N68S
F158L	L56P	L56P		S52P		E76D
K231E	V69A	V69A		D86V		Y79C
L263S	E76V	E76V		E93G[b]		E93G[b]
V296I[b]	Y79H	Y79H		S99R		K95R
	E93G[b]	E93G[b]		N116D		F178S
	D118E	D118E	D118E	I120T		M185V
	D149E	D149E	D149E	S127P		D215G
	N159G	N159G	N159G	Y171H	Y171H	N217S
	L189S	L189S	L189S	K172R	K172R	H237Q
	K213R	K213R	K213R	L216S	L216S	D238E
	Q219R	Q219R	Q219R	I221T	I221T	Q243L
	L246P	L239STOP	L246P	N253S	N253S	S282T
	V296I[b]		V296I[b]	I289V	I289V	V296I[b]
	I312T		I312T	F295L	F295L	D297G
	Q321R		Q321R	V296I[b]	V296I[b]	K308R
				Q321R	Q321R	S317P

[a] This list does not show silent mutations (mutations at the DNA level that do not lead to an amino acid change).
[b] This mutation was present in the template plasmid used in for PCR mutagenesis. Therefore, it is present in every mutant.

III.3. Results

III.3.a. Ame1p is an essential kinetochore protein

To determine the function of full length Ame1p, it was tagged with the GFP variant "Venus YFP" (hereafter referred to as "VFP") (Nagai et al., 2002), and imaged in a strain that also contained Spc29p-CFP to mark the location of the SPB (Elliott et al., 1999). The localization of Ame1p-VFP during the cell cycle was examined (Figure III.2.A). In unbudded and small budded cells, Ame1p-VFP was located close to SPBs (Figure III.2.A, *a*, *b*). In pre-anaphase cells (cells with a ~2μm spindle at/near the bud neck), Ame1p-VFP had a characteristic kinetochore localization, consisting of two foci located between the two SPBs (Figure III.2.A, *c, e*; He et al., 2001; Measday et al., 2002). In anaphase cells (cells with a >2.5μm-long spindle stretched across the bud neck), Ame1p-VFP co-localized with SPBs in mother and daughter cells (Figure III.2.A, *d, f*). In contrast to what had been reported (Burns et al., 1994), Ame1p-VFP was not detected on astral microtubules in pre-anaphase cells (Figure III.2.A, *a*, *b*) or on spindle microtubules of anaphase spindles (Figure III.2.A, *f*). This could be explained by an improved quality of microscopy since the first study, or by the fact that markers of organelles (such as the SPB marker) allow one to better distinguish the subcellular localization of proteins. Moreover, the construct that was reported to localize to the SPB and associated microtubules only represented an N-terminal fragment of Ame1p, due to the site of transposon insertion. Based on these results, I conclude that Ame1p is associated with kinetochores but not spindle or astral microtubule arrays.

Next, Ame1p was tested biochemically for hallmarks of a kinetochore protein. ChIP was performed with Myc-tagged Ame1p and probed for the presence of centromere sequences by PCR. The DNA that co-purified with Ame1p-Myc specifically contained centromere fragments (*CEN1*, *CEN3*) but not a non-centromeric control sequence containing the *PGK1* ORF (Figure III.2.B). These results are consistent with previously described kinetochore proteins and indicate that Ame1p is an integral component of the kinetochore (Measday et al., 2002; Pot et al., 2003 and others). The Ame1p-Myc–*CEN* DNA interaction did not require Ctf19p (Figure III.2.B), indicating that Ame1p is likely to reside closer to *CEN* DNA than Ctf19p. Finally, some kinetochore components (such as components of the Dam1 complex) have been shown to require intact microtubules for interaction with *CEN* DNA (Enquist-Newman et al., 2001; Janke et al., 2002; Li et al., 2002), while others (such as

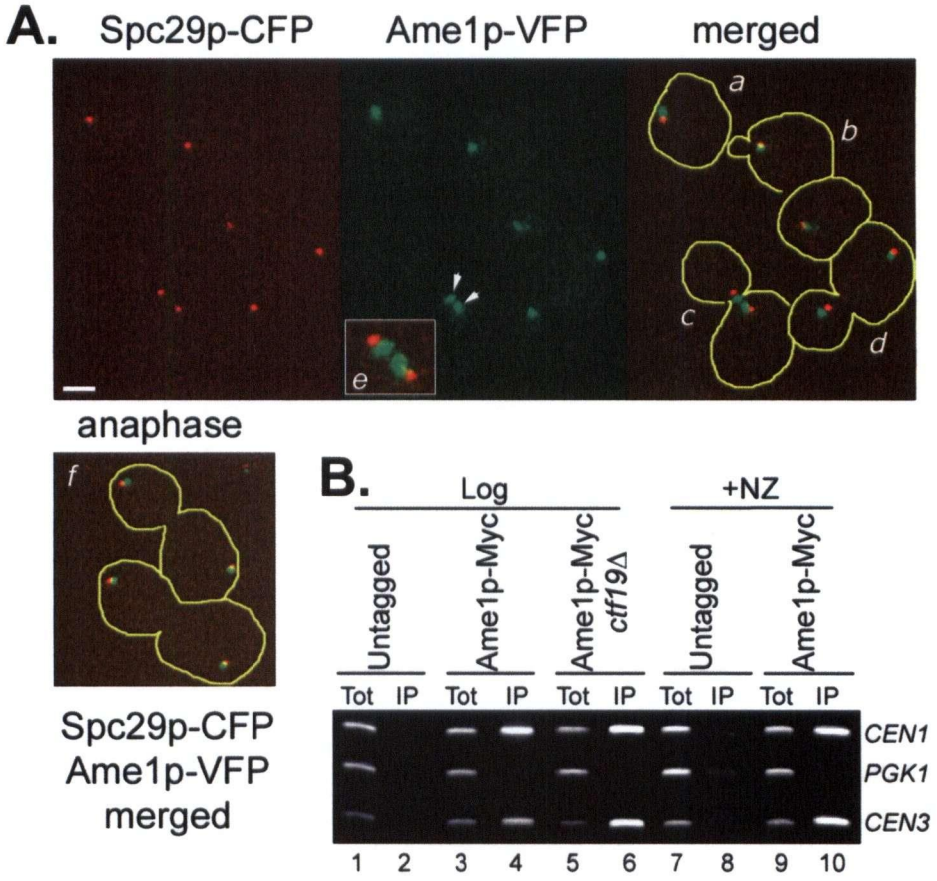


Figure III.2. Ame1p is a *bona fide* kinetochore protein.

A. Localization of Ame1p by fluorescence microscopy at several stages of the cell cycle. The Ame1p-VFP Spc29p-CFP strain was grown and imaged as described in materials and methods. Spc29p-CFP (red) is shown in the left panel, Ame1p-VFP signal (green) is shown in the middle panel; right and lower panels, and inset, merged signals. A-*a*, interphase cell; -*b*, small-budded cell; -*c*, pre-anaphase cell (enlarged in *e*); -*d*, early anaphase cell; -*f*, late anaphase cells. Bar, 2μm. B. ChIP assay of Myc-tagged Ame1p in wild type or *ctf19*Δ strains, or in a wild type strain after treatment with 15μg/ml NZ for 100 min, followed by multiplex PCR analysis, showing that Ame1p interacts with *CEN* DNA independently of Ctf19p and microtubules. Lanes 1-2 and 7-8 are controls performed with an untagged strain. Tot, total lysate; IP, immunoprecipitated fraction.

components of the large Ctf19 complex) do not (Hyland et al., 1999; Measday et al., 2002). To test whether the Ame1p-*CEN* DNA interaction required microtubules, ChIP was performed on cultures that had been treated with the microtubule-depolymerizing drug nocodazole (NZ) (Jacobs et al., 1988). The Ame1p-*CEN* DNA interaction was not disrupted in these conditions, indicating it does not require microtubules (Figure III.2.B). Together, the localization and *CEN* DNA interaction data indicate that Ame1p is a *bona fide* kinetochore protein that behaves similarly to previously characterized proteins of the Ctf19 complex (Hyland et al., 1999; Measday et al., 2002; Ortiz et al., 1999; Pot et al., 2003).

III.3.b. Ame1p sequence analysis and basic phenotypes of conditional ame1 mutants integrated at the endogenous AME1 locus

Although the 324 amino acid long (37.5kDa) Ame1p sequence has no obvious orthologs, BLAST analysis (Altschul et al., 1997) reveals that its C-terminus contains homology to the $p150^{glued}$ subunit of the dynactin complex, a regulator of microtubule dynamics and spindle positioning (Adames and Cooper, 2000), and to the SAP family of proteins that regulate the Sit4p phosphatase (Luke et al., 1996) (Figure III.3.A). The first 94 residues of the N-terminus are sufficient for SPB localization (Burns et al., 1994), but this region of the protein is not significantly similar to any other known protein (Figure III.3.A). Ame1p is predicted to have two regions of coiled coils (Lupas et al., 1991) within its C-terminal half (amino acids 199-262 and 302-319, Figure III.3.A). Coiled coils are generally accepted as domains likely to mediate protein-protein interactions, and are present in many structural proteins; they have been proposed to lead to unusual polymeric structures in SPB and centrosomal proteins (Wigge et al., 1998). Altogether, these observations suggest that these regions of Ame1p may mediate protein-protein interactions. However, the primary sequence of Ame1p does not contain any obvious functional domains.

To determine the function of Ame1p *in vivo*, a series of conditional alleles were created by random PCR mutagenesis of the *AME1* ORF. Mutated sequences were subsequently integrated into the genomic *AME1* locus (see materials and methods, section III.2.b for details). Because of the initial method of genomic integration used (see Figure III.1), three partial alleles were obtained in addition to the 4 alleles initially carried on plasmids. One of these partial mutants, *ame1-31*, is an 86 amino acid C-terminal truncated form of *ame1-3* that

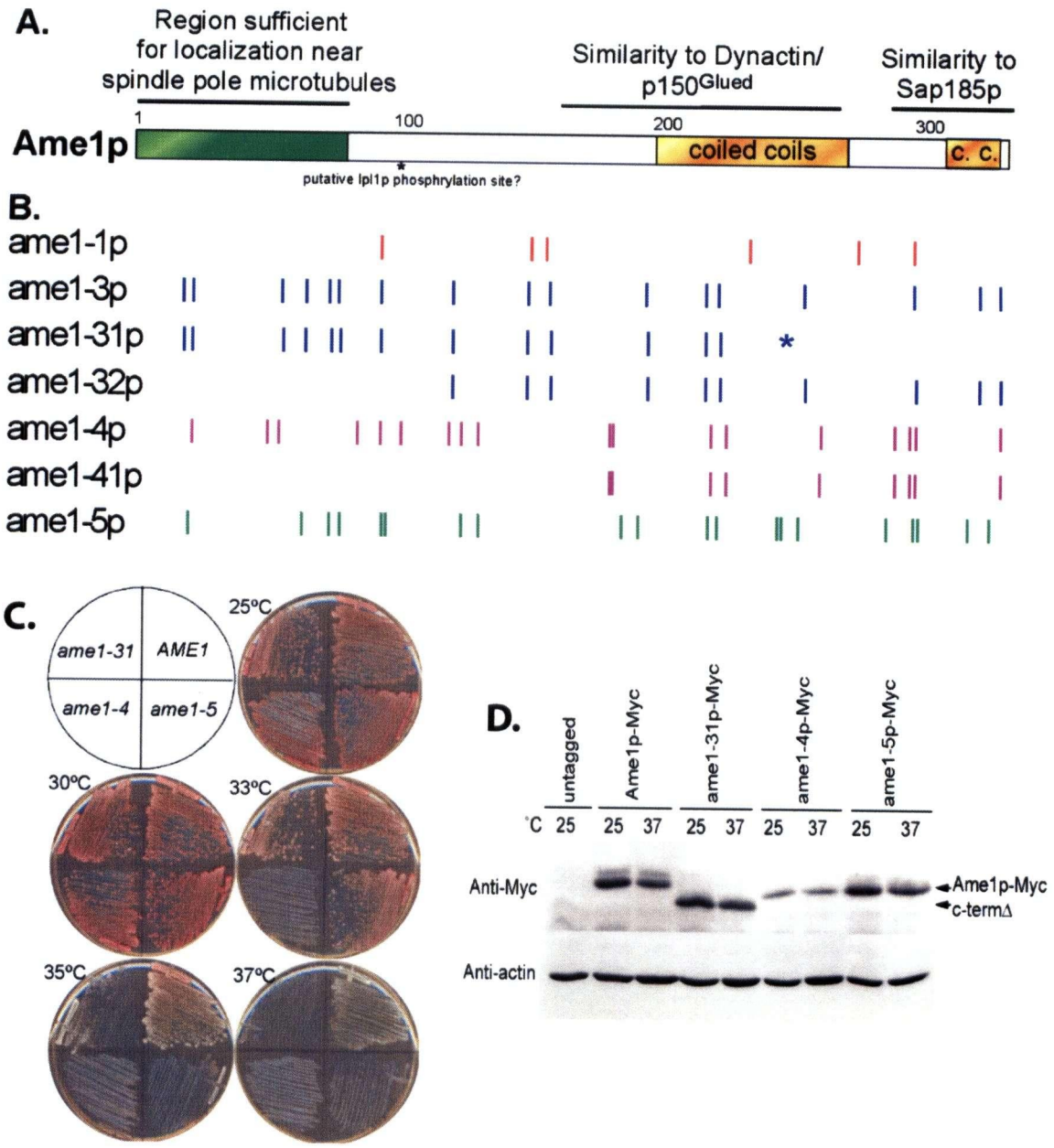


Figure III.3. Ame1p sequence analysis and basic *ame1* mutant phenotypes.

A. Schematic representation of the Ame1p 324 amino acid protein showing domains of homology to the dynactin ($p150^{Glued}$) family of proteins and to yeast Sap185p, a regulator of the Sit4p phosphatase; also shown are the locations of the putative N-terminal localization domain and the coiled-coil (CC) domains. B. Positions of non-conservative amino acid substitutions in ame1p mutants as compared to the diagram presented in A. ame1-31p, ame1-32p and ame1-41p are partial mutants derived from ame1-3p and ame1-4p, with an extra mutation leading to premature termination (ame1-31p) or for which not all the original mutations integrated into the genome (ame1-32p, ame1-41p). Colors group partial mutants with the parent mutant they were derived from. Bars indicate missense mutations; the asterisk indicates a nonsense mutation. C. Temperature sensitivity of three of the mutants strains (*ame1-31*, *ame1-4* and *ame1-5*) compared to wild type (*AME1*) on YPD medium. D. Protein stability assay. Total protein extracts were prepared from strains expressing either untagged Ame1p or Myc-tagged wild type or mutant Ame1p proteins grown at the permissive temperature (25°C) or shifted to restrictive temperature (37°C) for 4 hours. The Ame1p fusion proteins were detected on Western blots using anti-Myc antibodies. Actin acts as a loading control.

has a premature STOP codon at residue 239, disrupting the C-terminal coiled-coil domain (Figure III.3.B). Two other partial mutants (*ame1-32* and *ame1-41*) contain the original mutations in the C-terminal half of the protein, but are wild type in the N-terminal half (Figure III.3.B). Integrated *ame1* mutants were sequenced and shown to contain a large number of non-conservative mutations spanning the entire ORF (Figure III.3.B and Table III.2). Unfortunately, because the original "wild type" plasmid that was used for mutagenesis already contained two mutations, all the alleles also contained these two mutations (this "wild type" plasmid was able to rescue an *ame1*Δ strain at all temperatures, and the variant *AME1* gene behaved as wild type in all basic assays when inserted in the genome; data not shown). The large number of mutations circumvents any informative clues about the role of specific amino acids in the sequence, but the mutants can still be used to investigate the role of *AME1* by evaluating phenotypes of the conditionally functional alleles.

Mutants were screened for conditional growth defects at 25°C, 30°C, 33°C, 35°C and 37°C (Figure III.3.C and Table III.3). Of all mutant strains, *ame1-4* was the most compromised for growth, as it had the lowest restrictive temperature (33°C) and grew slowly at permissive temperature (25°C). *ame1-5* failed to grow above 35°C while *ame1-1*, *ame1-3* and *ame1-31* remained viable up to 36°-37°C. Most of the subsequent analysis was carried out with three of the alleles, *ame1-31*, *ame1-4* and *ame1-5*, which showed the most interesting and severe phenotypes. The stability of the resulting three mutated proteins at restrictive temperature was examined. Protein extracts of wild type and mutant Myc-tagged strains grown at permissive (25°C) and restrictive (37°C) temperatures were analyzed by Western blots (Figure III.3.D). ame1-31p-Myc and ame1-5p-Myc were present at levels similar to Ame1p-Myc at both temperatures (Figure III.3.D). The overall amount of ame1-4p-Myc was reduced relative to that of Ame1p, but did not vary with temperature (Figure III.3.D). Thus, defects observed at restrictive temperature in the mutant strains (Figure III.3.C) cannot be attributed to a reduction in the stability or amount of mutated protein.

III.3.c. ame1 mutants interact with mutations in subunits of the Ctf19 complex

The large Ctf19 central kinetochore complex is composed mainly of non-essential subunits, many of which can be removed in combination without leading to reduced fitness (Measday et al., 2002; Pot et al., 2003). As Ame1p is one of only two essential proteins in

Table III.3. Summary of basic *ame1* mutant phenotypes

Mutant Allele	Temperature sensitivity[a]	Return to growth[b]	Chromosome Loss (by sectoring assay)[c]	FACS profile[d]
ame1-1	37°C	yes	+	slight G2/M delay at restrictive temperature
ame1-3	37°C	yes	– (<37°C)	slight G2/M delay at restrictive temperature
ame1-31	36°C	n/d[e]	++	Strong G2/M delay at permissive and restrictive temperatures
ame1-32	37°C[f]	n/d	–	slight G2/M delay at restrictive temperature
ame1-4	33°C	no	–[g]	Strong G2/M delay at permissive and restrictive temperatures
ame1-41	37°C[f]	n/d	–	slight G2/M delay at restrictive temperature
ame1-5	35°C	no	± (<33°C); ++ (>33°C)[h]	Strong G2/M delay at restrictive temperature

[a] The temperature sensitivity value represents the restrictive temperature at which a strain is unable to grow.
[b] For return to growth, plates that had been incubated at 37°C for 5 days were re-incubated at 25°C for a few days and growth of mutant strains was recorded.
[c] –, no sectoring; ±, almost no sectoring; + some sectoring; ++, strong sectoring.
[d] FACS profiles were collected for samples that had been incubated at restrictive temperature (37°C) for 3 hours and compared to samples incubated at permissive temperature (25°C) and to a wild type control.
[e] n/d: not determined.
[f] The strain sometimes shows some residual growth at this temperature.
[g] Sectoring was difficult to assess in this mutant due to its poor growth; another technique was therefore used to assess its chromosome missegregation phenotype (see Figure III.4.D).
[h] Sectoring occurred only at temperatures above 33°C in this mutant.

this complex (the other being Okp1p), genetic interactions between *ame1* mutants and mutants in other genes encoding members of the Ctf19 complex were investigated. The *ame1* allele strains were mated to *okp1-5*, *ctf19*Δ and *ctf3*Δ mutants, the resulting diploids were sporulated, and synthetic growth defects in double mutant spores of the dissected tetrads were assessed at a range of temperatures. All *ame1* mutants were synthetically lethal in combination with *okp1-5*, indicating that simultaneously compromising the function of these two essential genes is not tolerated (Table III.4). In contrast, double mutants of *ame1* with *ctf19*Δ or *ctf3*Δ displayed a range of synthetic phenotypes (summarized in Table III.4).

Most notably, while *ame1-4* was inviable in combination with both *ctf19Δ* and *ctf3Δ*, *ame1-5* was synthetically lethal only with *ctf19Δ* at 25°C; *ctf3Δ* lowered the *ame1-5* restrictive temperature but the double mutant was viable at 25°C (Table III.4). Other combinations of mutants led to conditional synthetic lethality, where the double mutant spores grew at 25°C but became inviable at a lower temperature than the restrictive temperature of the single *ame1* mutant (Table III.4). The genetic interactions observed between *ame1* alleles and mutations in genes of the large Ctf19 complex are consistent with these genes having a function in a common process.

Table III.4. Genetic interactions between *ame1* mutants and Ctf19 complex subunits

Allele	*okp1-5*	*ctf19Δ*	*ctf3Δ*	p*OKP1* (2μ)[a]
ame1-1	SL[b]	viable[c]	viable	n/d[d]
ame1-3	SL	strong CSL[e]	weak CSL[f]	n/d
ame1-31	SL	strong CSL	strong CSL	SUP[g] (≤37°C)
ame1-32	n/d	n/d	n/d	n/d
ame1-4	SL	SL	SL	SUP (≤35°C)
ame1-41[h]	SL	strong CSL	strong CSL	n/d
ame1-5	SL	SL	strong CSL	SUP (≤35°C)

[a] The 2μ plasmid containing *OKP1* is pVA127.
[b] SL: synthetic lethality: individual mutations do not cause inviability on their own, but a strain containing both mutations is inviable at 25°C.
[c] viable: no difference in growth is observed at all temperatures tested, between a strain containing the two mutations and one containing only the *ame1* mutation.
[d] n/d: not determined.
[e] Strong CSL: conditional synthetic lethality: double mutant spores are viable at 25°C but die at a temperature that is significantly lower than the restrictive temperature of the single *ame1* ts mutant.
[f] Weak CSL: conditional synthetic lethality: double mutant spores are viable at 25°C but show slightly reduced fitness as compared the single *ame1* ts mutant; for example, double mutant spores may appear sick at a temperature at which the single *ame1* mutant grows at a normal rate.
[g] SUP: suppressor of the growth defect up to the indicated temperature.
[h] The *ame1-41* strain used in these crosses was later shown to contain an unlinked mutation that gave rise to benomyl sensitivity (data not shown). Therefore, the effect shown here may not be due solely to the compromised *ame1*.

III.3.d. Functional Ame1p is required for chromosome segregation

To determine whether *ame1* mutants were defective in chromosome segregation, the qualitative color-based chromosome loss assay described in Chapter II, in which sectoring colonies attest for chromosome instability, was performed (Koshland and Hieter, 1987). Because this assay was carried out during the preliminary characterization of the alleles, all alleles were tested at a range of temperatures. Results are summarized in Table III.3. The *ame1-31* mutant exhibited high CF loss at 25°C; *ame1-31* colonies were primarily red or sectored, whereas the wild type colonies were mostly white (Figure III.4.A, B). In contrast, the *ame1-5* mutant strain stably retained the CF at 25°C but exhibited a severe CF loss phenotype at 33°C (Figure III.4.C). It was difficult to assess CF loss in the *ame1-4* strain due to its poor growth even at permissive temperature. In the course of introducing the CF in *ame1-4*, it also became apparent that this strains's fitness was further reduced in colonies carrying the CF as opposed to those not carrying it (see Figure III.5.A). This observation was investigated further (see section III.3.e below). Finally, the *ame1-3* mutant, which contains identical point mutations as *ame1-31* but does not have a truncated C-terminus, did not show sectoring ≤35°C (Table III.3). This result indicates that the C-terminus of Ame1p may be crucial for chromosome stability.

In order to assay for chromosome segregation defects in the *ame1-4* strain, the segregation of sister chromatids was observed by fluorescence microscopy in a strain containing the GFP-LacI/*LacO:CEN* chromosome tagging system (Goshima and Yanagida, 2000), which marks the centromeres of chromosome XV with GFP through the interaction of the LacI repressor (fused to GFP) with LacO repeats (integrated next to *CEN15*). *AME1* and *ame1-4* strains were grown and imaged as explained in materials and methods. After 4 hours at 37°C, the vast majority of wild type anaphase cells had properly segregated sister chromatids (Figure III.4.D); in contrast only 8% of *ame1-4* cells had undergone a normal anaphase (GFP-tagged sister chromatids located near the SPBs in mother and bud). The majority of *ame1-4* cells with elongated spindles (>2.5μm regardless of position) had missegregated chromosomes. Of these, 12% retained their spindle in the mother cell and had two foci of GFP located inbetween SPBs; however, the relative position of GFP signals showed one of the chromatid lagging near the mid-zone of the spindle, while the other was closely associated (≤1μm) with its SPB (that is, one chromatid was 50% further from the SPB than

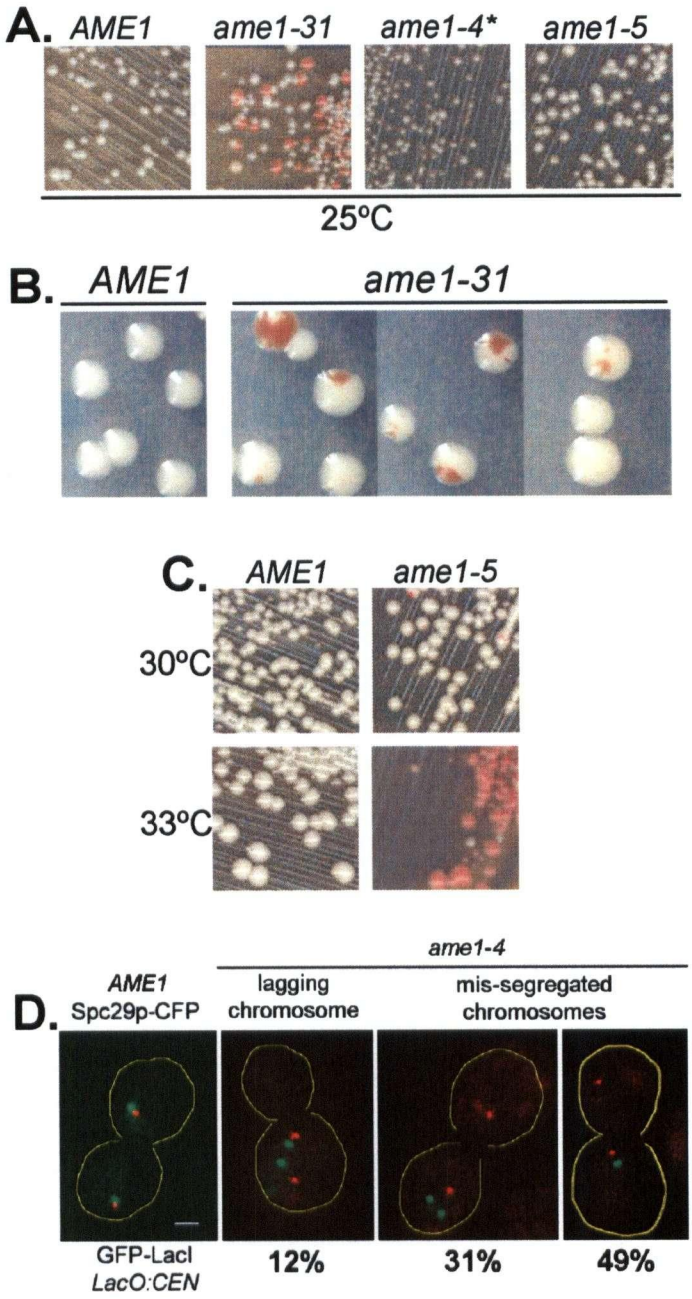


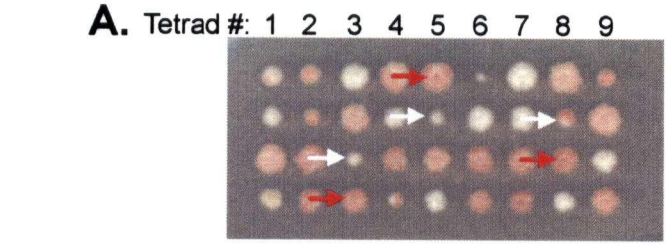
Figure III.4. Chromosome loss phenotype of *ame1* alleles.

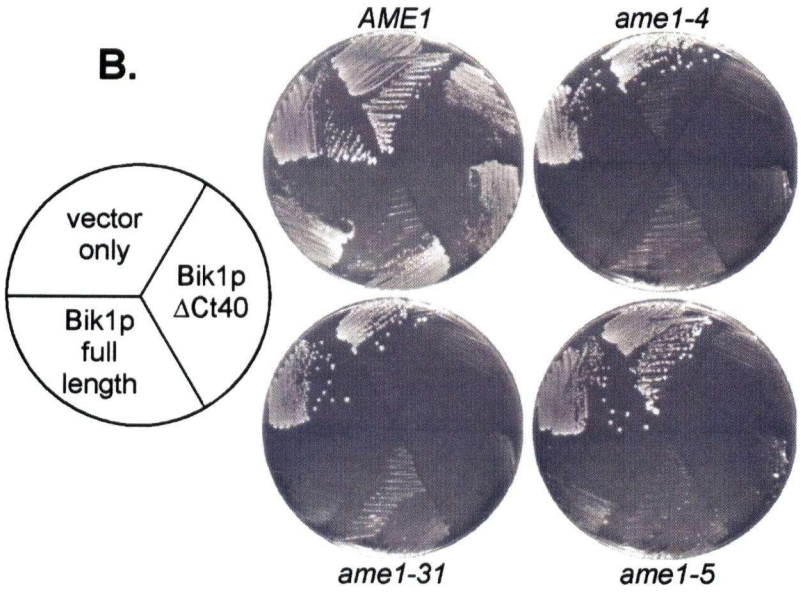
A-C. Chromosome loss sectoring assay. A. Wild type and *ame1* mutant strains. The *AME1*, *ame1-31*, *ame1-4* and *ame1-5* strains all contain the non-essential chromosome fragment CFIII[*CEN3.L.* YPH982]; *the *ame1-4* CF-containing strain grows poorly. B. Close up of the sectoring phenotype in the *ame1-31* mutant, as compared to wild type. Strains were plated on minimal medium with low adenine content to enhance the visualization of color in sectoring colonies. C. The wild type and *ame1-5* CF-containing strains streaked on YPD from SC medium lacking uracil for 3 days at 30°C and 33°C demonstrate the dramatic shift in CF stability in the *ame1-5* mutant. D. Chromosome missegregation in the *ame1-4* mutant. Wild type and *ame1-4* strains carrying *LacO* repeats at 1.8kb from *CEN15* and a GFP-LacI fusion (GFP-LacI/*LacO:CEN*) (green) as well as the SPB marker Spc29p-CFP (red), were grown as described in materials and methods and imaged as in Figure III.2.A; the percentage of cells showing lagging or missegregated chromosomes, or where only one GFP signal was visible (either due to chromosome loss or to unseparated sister chromatids) was determined. Images of representative merged signals are shown. Bar, 2μm.

the other chromatid was to the opposite SPB) (Figure III.4.D). In 31% of cells, the SPBs were located in both cells, but two foci of GFP remained associated with one of the SPBs (Figure III.4.D). In 49% of cells only one focus of GFP was observed (Figure III.4.D), indicating either a failure in DNA replication or sister chromatid separation, or chromosome loss. Based on these results, lack of functional Ame1p results in severe defects in chromosome segregation, and proper kinetochore-microtubule attachment may require the C-terminal domain of Ame1p.

III.3.e. Overexpression of BIK1 exacerbates the ame1 defect and causes lethality in ame1 mutants

As mentioned in section III.3.d, the *ame1-4* strain displayed a synthetic growth defect when carrying the CF used in the sectoring assay (CFIII [*CEN3.L.* YPH982] *URA3 SUP11*) (Figure III.5.A). In order to determine if that effect was due to cellular stress caused by the presence of an extra chromosome in a strain compromised for kinetochore activity, or due to a gene that is present on CFIII, a different CF (CFVII [*RAD2.d.* YPH256] *URA3 SUP11*), which contains a fragment from another chromosome, was introduced in the *ame1-4* strain. With CFVII, *ame1-4* showed no reduced fitness as compared with the CF-lacking strain (data not shown), indicating that the effect was CF-specific. Thus, increased dosage of a gene present on CFIII might be further compromising the *ame1-4* strain. CFIII contains about 110kb of sequence corresponding to the left arm of chromosome III. Any of the genes present on this CF could be responsible for the synthetic dosage effect on *ame1-4*. However, an informed guess pointed to *BIK1* as a candidate gene, due to some aspects of its proposed function. *BIK1*, the yeast homolog of the human CLIP-170 gene, is involved in regulation of microtubule dynamics at the plus-end of microtubules, and has recently been shown to function at the kinetochore as well (Berlin et al., 1990; Lin et al., 2002). In order to determine if increased dosage of *BIK1* affects a strain compromised for *AME1* function, the *BIK1* sequence was cloned into an overexpression vector under the control of an attenuated GAL promoter (GAL-L) (Mumberg et al., 1994), and transformed into the *ame1-4* strain. Induction of *BIK1* caused lethality in the *ame1-4* strain, while a wild type control was still able to grow (Figure III.5.B). Interestingly, being able to observe the interactions described above required the growth media to contain both raffinose and galactose as carbon source, as





C.

Strain	pGAL-L vector only	pGAL-L BIK1	pGAL-L BIK1ΔC40
wild type	+	+	+
ame1-4	+	–	–
ame1-31	+	–	–
ame1-5	+	–	–
okp1-5	+	–	–
ndc10-1	+	–	–
ctf19Δ	+	+	+
ctf3Δ	+	–	–
chl4Δ	+	–	–
spc24-8	+	–	–
spc24-9	+	–	–
spc24-10	+	+	+
dam1-1	+	+*	+*

Figure III.5. Overexpression of *BIK1* is deleterious to kinetochore mutants

A. *ame1-4* is sensitive to the presence of CFIII. Shown is a set of 9 tetrads dissected from the cross of an *ame1-4* CF^- strain with a wild type $CFIII^+$ strain. Red arrows indicate *ame1-4* CF^- colonies and white arrows indicate *ame1-4* CF^+ colonies. B. Overexpression of *BIK1*, or a truncated version which removes 40 amino acids of the C-terminus, from the pGAL-L vector, in *AME1*, *ame1-4*, *ame1-31* and *ame1-5* strains. Strains were grown on minimal medium selecting for the plasmid and containing 2% raffinose/2% galactose as a carbon source, at 25°C for 5 days. Two independent isolates are shown for each transformation. C. Summary of the effect of overexpressing *BIK1* in various kinetochore mutants, grown as in B. +, growth; -, no growth; *, growth of this strain upon overexpression of *BIK1* was slightly less robust than in the wild type.

none of the strains (including the wild type) transformed with the *BIK1* overexpression plasmid was able to grown on media containing solely galactose. *BIK1* overexpression had been previously reported to cause a growth defect, but the experiment had been conducted in a diploid strain containing a homozygous mutation in *BIK1*, and the effect on a wild type strain had not been assessed (Berlin et al., 1990). Taken together, these data suggest that yeast strains are very sensitive to small changes in the amount of Bikp (even those caused by a slight change in media composition), and that a strain compromised for a microtubule-dependent process may not be able to tolerate increased amount of Bik1p.

To determine whether this effect was specific to this *ame1* allele, or to *ame1* mutants as opposed to mutants in other genes, *BIK1* was overexpressed in *ame1-31* and *ame1-5*, as well as a panel of kinetochore mutants that represent most of the kinetochore subcomplexes: *ndc10-1* (CBF3 complex) (Goh and Kilmartin, 1993); *okp1-5* (Ortiz et al., 1999), *ctf19*Δ (Hyland et al., 1999), *chl4*Δ (Pot et al., 2003) and *ctf3*Δ (Measday et al., 2002) (large Ctf19 complex); *spc24-8*, *-9* and *–10* (V. Measday, unpublished data) (Ndc80 complex); and *dam1-1* (Jones et al., 1999) (Dam1 complex). These results are summarized in Figure III.5.C. *ame1-31* and *ame1-5* were affected by overexpression of *BIK1* to a similar degree as *ame1-4* (Figure III.5.B). A number of other kinetochore mutants, but not all, were sensitive to *BIK1* overexpression (Figure III.5.C). Alleles of *spc24* were differentially affected. Thus, while the *BIK1* overexpression sensitivity phenotype is not specific to *AME1*, it does seem to have some selectivity among kinetochore mutants, at least in view of the effect on the specific point mutants (in the case of essential genes) that were tested here.

Bik1p is part of a family of proteins that are thought to act as linkers between microtubules and a cargo, while not having a motor function (Lin et al., 2002; Pierre et al., 1994). Bik1p interacts with *CEN* DNA in a manner that depends on its C-terminal domain, since a C-terminal truncated, yet properly localizing form of Bik1p loses the ability to interact with *CEN* DNA (Lin et al., 2002). The genetic interaction of *ame1* mutants with overexpressed *BIK1* suggests that Ame1p may represent a cargo for Bik1p and that Bik1p's interaction with the kinetochore could be mediated by Ame1p. Under this hypothesis, if Ame1p bound Bik1p through Bik1p's cargo-binding domain, and a mutant ame1p exhibited a looser interaction with the rest of the kinetochore than wild type Ame1p, then an excessive amount of Bik1p could titrate Ame1p away from the kinetochore, thus leading to lethality.

This hypothesis predicts that overexpressing C-terminal truncated Bik1p (without the cargo-binding domain) would not be able to exert the same effect as full length Bik1p on *ame1* mutants. However, *ame1* mutants behaved similarly whether the truncated form or the full length Bik1p was overexpressed (Figure III.5.B, summarized in C). Similarly, there were no differences in the effect of increased dosage of Bik1p between the full length or truncated form in the other kinetochore mutants tested (Figure III.5.C).

Altogether, these data indicate that overexpression of *BIK1* affects strains with compromised kinetochore function, perhaps through the combined effects of altered microtubule dynamics and weaker microtubule attachment. Differential sensitivities to *BIK1* overexpression among kinetochore mutants will allow further investigation of the mechanism by which this phenotype occurs.

III.3.f. ame1 mutants exhibit defects in kinetochore-microtubule attachment

ame1 mutants display phenotypes consistent with a defective kinetochore. To differentiate whether defective chromosome segregation in *ame1* mutants was due to the protein malfunctioning at the kinetochore, or simply to loss of protein localization to the kinetochore, the localization of VFP-tagged ame1p's (i.e. Ame1p mutated proteins) at restrictive temperature (37°C) was examined in strains expressing the tagged SPB marker Spc29p-CFP using fluorescence microscopy (see materials and methods). All mutant proteins localized at permissive temperature (25°C); either between the SPBs in budded cells or near the SPB in unbudded cells (data not shown). At 37°C, Ame1p-VFP localized with the expected bilobed distribution between the two SPBs in pre-anaphase cells (Figure III.6.A, *a*; 4.7% of cells were in that stage of the cell cycle). ame1-31p-VFP and ame1-5p-VFP localized near the SPBs in the majority of cells (Figure III.6.A, *b and d, red arrows*). ame1-31p-VFP was occasionally mislocalized relative to Spc29p-CFP (10% of cells), and was not detected in ~20% of cells (n>100 cells counted). In cases where ame1-31p-VFP was mislocalized, it was primarily distributed throughout the length of the spindle, between the two SPBs (Figure III.6.A, *b, yellow arrows*). In ame1-5p-VFP cells a minor signal was detected along the spindle but the bulk of protein was properly localized (Figure III.6.A, *d*). Unlike ame1-31p-VFP and ame1-5p-VFP, ame1-4p-VFP was rarely detected, even in cells where the Spc29p-CFP signal was clearly visible (Figure III.6.A, *c*). In most cases where

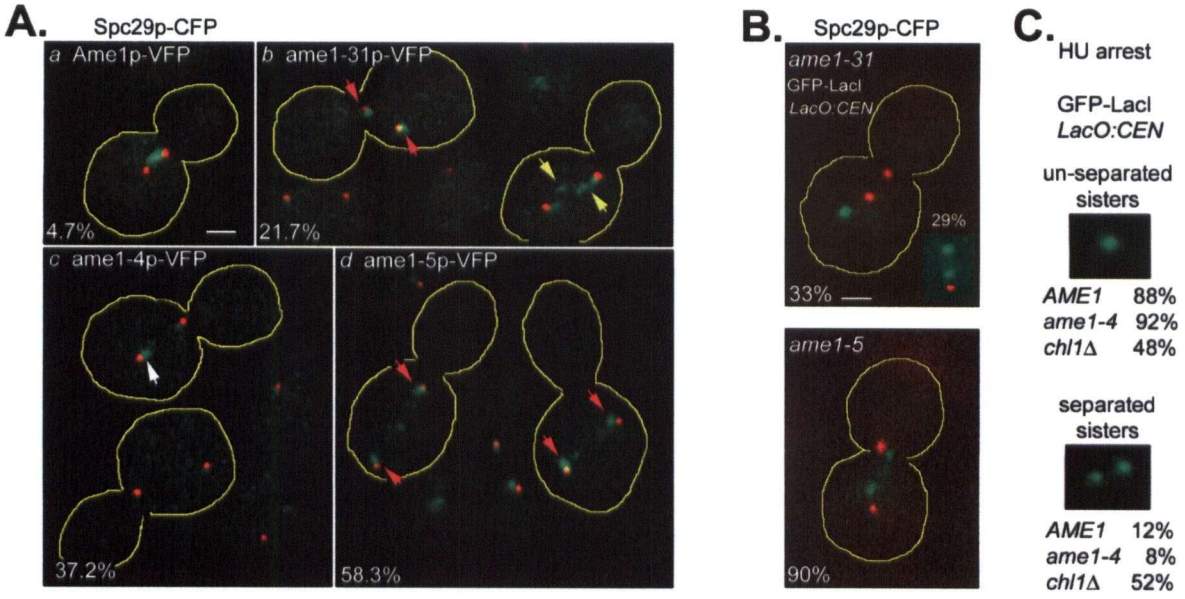


Figure III.6. Altered protein localization and defects in chromosome segregation in *ame1* mutants are not due to defective sister chromatid cohesion.

A. Localization of wild type and mutant Ame1p at 37°C. Strains grown to early log phase at permissive temperature (25°C) were shifted to restrictive temperature (37°C) for 4 hours, and imaged by fluorescence microscopy (see materials and methods) as in Figure 1A. All strains contain Spc29p-CFP (red) as a marker for the SPB. *a*, Ame1p-VFP (green) localizes normally at 37°C; *b*, ame1-31p-VFP is found associated with the SPBs (red arrows), or diffuse throughout the length of the spindle (yellow arrows); *c*, ame1-4p-VFP is not detected in the majority of cells, but is sometimes detected at one of the SPBs (white arrow); *d*, ame1-5p-VFP is localized at the SPBs of elongated spindles in the mother cell (red arrows). Percentages represent the fraction of cells within each population that are at the particular stage of the cell cycle shown on the image. B. *ame1* mutants show chromosome segregation defects, as detected using the GFP-LacI/*LacO:CEN* chromosome tag system. In *ame1-31* mutants, chromosome missegregation is observed in the majority of cells. In *ame1-5* mutant cells, 90% of cells exhibit an anaphase spindle with lagging chromosomes. Percentages represent the fraction of cells within each population that have the phenotype shown on the image. C. Abnormal chromosome segregation observed in *ame1* mutants is not due to defects in cohesion. As assayed with the GFP-LacI/*LacO:CEN* tag, the majority of sister chromatids in *AME1* (88%) and *ame1-4* (92%) cells remain as one distinct GFP focus as compared to the cohesin mutant *chl1*Δ (48% unseparated signal). Bar, 2μm.

ame1-4p-VFP was detected the majority of the signal was associated with a single SPB (Figure III.6.A, *c*, *white arrows*).

During imaging of the mutant proteins, it was noticed that very few short pre-anaphase spindles (such as the one shown for wild type in Figure III.6.A, *a*) were observed in *ame1* mutants incubated at the restrictive temperature (*ame1-31*, 2.1%; *ame1-4*, 5.3%; *ame1-5*, 11.1%). An accumulation of cells with short spindles would be expected if the observed defects in chromosome segregation in these mutants (Figure III.4 and Table III.3) activated

the spindle assembly checkpoint. Instead, the majority of spindles in mutant cells at this stage of the cell cycle were elongated (>2.5μm in length) and positioned in the mother cell (*ame1-31*, 21.7%; *ame1-4*, 37.2%; *ame1-5*, 58.3%). Such elongated spindles in the mother cell were rarely observed in *AME1* cells (<1%). Interestingly, many of the elongated spindles were properly oriented toward to the bud neck but had not entered the bud (Figure III.6.A). Thus, unlike kinetochore mutants that trigger the spindle assembly checkpoint, *ame1* mutants do not arrest with a short (<2.5μm) pre-anaphase spindle.

Given the difference in localization between ame1-4p and the two other ame1p mutants, the next question was whether sister chromatids would separate in *ame1-31* and *ame1-5* mutants, similar to *ame1-4* (Figure III.4.B). This analysis was carried out using the GFP-LacI/*LacO:CEN* tag system, and revealed that in *ame1-31* cells with elongated spindles, 33% of cells had a single focus of GFP associated with one SPB (indicating either chromosome loss or unseparated sister centromeres), while 29% had two foci of GFP associated with one SPB (Figure III.6.B, *upper panel*). These results are consistent with defects in bipolar attachment. The remainder of cells (38%) had two GFP foci located between the two SPBs with one GFP focus lagging near the spindle mid-zone (data not shown). In *ame1-5* cells with elongated spindles, two GFP foci were located between the SPBs in ~90% of cells (Figure III.6.B, *lower panel*); in 50% of those cells one GFP focus lagged in the mid-zone. Thus, the majority of cells in *ame1* mutants have separated sister centromeres.

Since separation of sisters might arise from a defect in sister chromatid cohesion, the separation of GFP-LacI/*LacO:CEN* tagged centromeres was compared among *AME1, ame1*, and a *chl1*Δ strain in which cohesion is defective (Mayer et al., 2004). Strains were incubated in 100mM hydroxyurea (HU) at 37°C until >90% of cells were large budded. Sister centromeres separated in the majority of HU-arrested *chl1*Δ cells (52%), which was expected due to the known cohesion defect in this mutant. In contrast, ~90% of HU-arrested *AME1* and *ame1-4* cells had properly cohesed sister centromeres (Figure III.6.C). Similar results were obtained for *ame1-31* and *ame1-5* (data not shown). This analysis indicates that *ame1* mutants are defective in kinetochore-microtubule attachment, but do not have a general defect in sister chromatid cohesion.

Taken together, these data indicate that *ame1* mutants do not arrest in metaphase at restrictive temperature, but instead initiate anaphase with chromosomes improperly attached to microtubules, which results in defective chromosome segregation.

III.3.g. Functional Ame1p is required for the stability of the Ctf19 complex

Given the localizations of the three ame1p mutant proteins, their association with *CEN* DNA at restrictive temperature was next investigated using ChIP. Myc-tagged mutant proteins were immunoprecipitated from formaldehyde-crosslinked lysates prepared from strains grown at permissive (25°C) or restrictive (37°C) temperature for 3 hours, and the presence of *CEN* DNA sequences in the immunoprecipitated fraction was probed by PCR (Figure III.7.A). The amount of PCR product in each strain was quantified as explained in materials and methods (Figure III.7.B). At permissive temperature, all mutant ame1p proteins interacted with *CEN* DNA like the wild type protein. Interestingly, while ame1-4p-Myc showed a large decrease in *CEN* DNA interaction at restrictive temperature, ame1-5p-Myc did not, indicating that the mutations in these two alleles affect Ame1p differently (Figure III.7.A, B); in addition, ame1-31p-Myc was still partially able to interact with *CEN* DNA at restrictive temperature (Figure III.7.A, B).

Next, the effect of *ame1* mutations on kinetochore structure and/or stability was examined. Interaction of Ame1p with *CEN* DNA and proper localization of Ame1p do not require Ctf19p (Figure III.2.B and De Wulf et al., 2003), suggesting that Ame1p lies proximal to *CEN* DNA relative to Ctf19p. Since Ame1p and Okp1p are the only essential components of the large Ctf19 complex, it is plausible that they represent a bridge between the CBF3 complex (which is made up of essential proteins) and central kinetochore complexes. However, it is also possible that these proteins function in parallel to the CFB3 complex and provide direct connection to *CEN* DNA, or that they influence the core kinetochore structure. To test this latter hypothesis, ChIP was performed with Ndc10p-Myc strains that contained mutations in either *AME1* or *OKP1*. In both cases, co-immunoprecipitation of *CEN* DNA by Ndc10p-Myc was as efficient in the mutants as in the wild type at both permissive and restrictive temperatures (Figure III.7.C). Since ame1-4p has defective *CEN* DNA binding at restrictive temperature (Figure III.7.A, B), and Okp1p had been shown to require Ndc10p for interaction with *CEN* DNA (Ortiz et al., 1999), these data suggest that binding of components

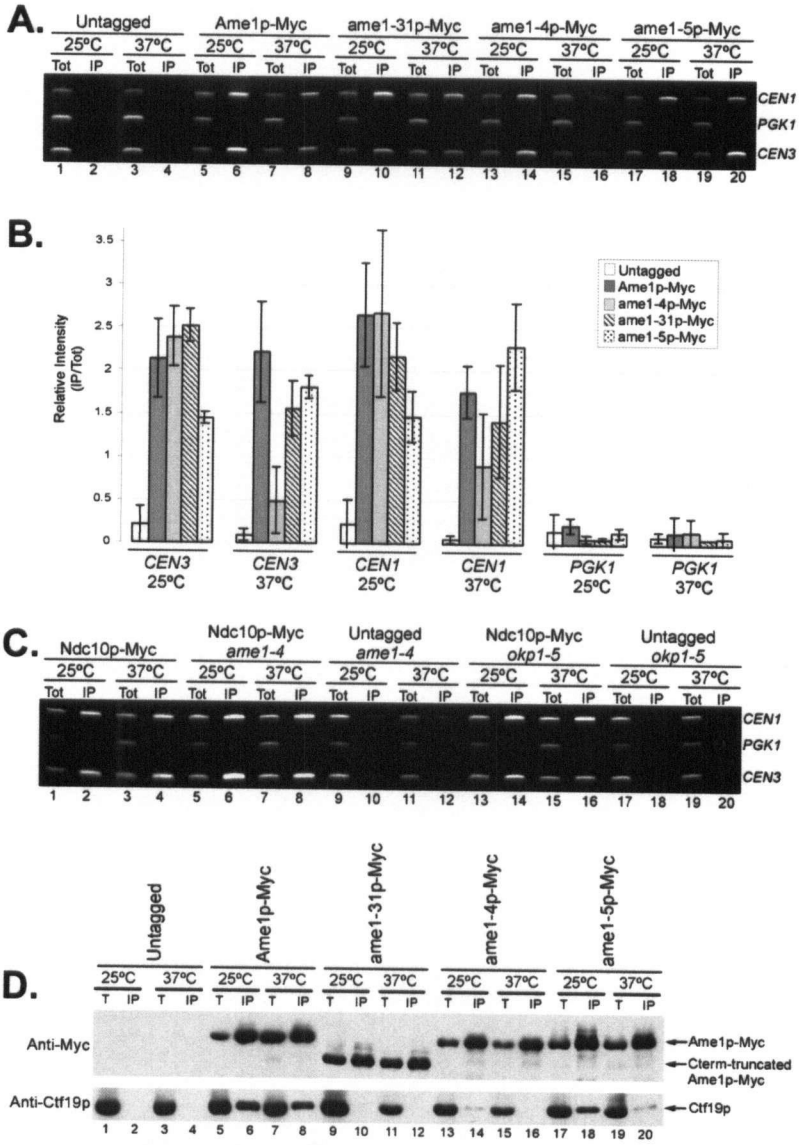


Figure III.7. Ame1p is located between the inner kinetochore and Ctf19p.
A. ame1-4p has reduced *CEN* DNA interaction at restrictive temperature. ChIP assay performed as in Figure III.2.B using Myc-tagged wild type and mutant Ame1p followed by multiplex PCR analysis. Each strain was grown at both permissive (25°C) and restrictive (37°C) temperature for 3 hours from log phase cultures. B. Quantification of A (see materials and methods). C. The inner kinetochore structure is not disrupted by COMA mutants. ChIP assay performed as in A using wild type, *ame1-4* or *okp1-5* strains containing Ndc10p-Myc (or untagged controls), followed by multiplex PCR analysis. D. Interaction with Ctf19p is disrupted in ame1p mutants. Anti-Myc IPs performed in strains containing Myc-tagged wild type or mutant Ame1p and an untagged control strain, followed by Western blot analysis with anti-Myc and anti-Ctf19p antibodies. In A, C and D, Tot, total lysate; IP, immunoprecipitated fraction.

of the inner kinetochore to the centromere does not require Ame1p or Okp1p and that these two proteins interact with the centromere via CBF3.

The large Ctf19p complex (Cheeseman et al., 2002a) consists of several sub-complexes in which specific subunits are more tightly associated, such as COMA (De Wulf et al., 2003). Ame1p-Myc immunoprecipitations contained Ctf19p (Figure III.7.D, lane 6). Given the hypothesis that Ame1p serves as a bridge for the interaction of components of the Ctf19 complex with the core kinetochore, and since ame1p mutants are not completely defective in interaction with *CEN* DNA, some of the phenotypes observed in *ame1* mutants could be due to impaired interaction of ame1p mutants with other components of the large Ctf19 complex. This hypothesis was tested by examining the Ame1p–Ctf19p interaction in *ame1* mutants. Co-immunoprecipitation of Ctf19p with ame1p-Myc mutant proteins performed at permissive and restrictive temperatures showed that while Ctf19p was present in the immunoprecipitate of ame1-5p-Myc at permissive temperature (Figure III.7.D, *lane 18*), the interaction between Ctf19p and ame1-5p-Myc was disrupted at restrictive temperature (Figure III.7.D, *lane 20*). Ctf19p was not detected in immunoprecipitated ame1-31p-Myc at either temperature (Figure III.7.D, *lanes 10 and 12*), and the interaction between ame1-4p-Myc and Ctf19p was greatly reduced at permissive temperature and not detected at restrictive temperature (Figure III.7.D, *lanes 14 and 16*). Thus, mutations in Ame1p disrupt interactions within the COMA complex, and the Ame1p C-terminus appears to be especially important for interaction with Ctf19p.

III.3.h. ame1 mutants break through a Mad2p-dependent G2/M arrest

The analysis of spindle length and sister chromatid separation in *ame1* mutants suggested that these cells do not arrest with short pre-anaphase spindles despite severe defects in achieving proper chromosome-microtubule attachment. Preliminary data indicated that all *ame1* alleles incubated at restrictive temperature showed a G2/M delay in cell cycle progression as assessed by FACS analysis (see Table III.3). The *ame1-31, ame1-4* and *ame1-5* strains were then examined in more detail. After 2 hours at restrictive temperature, these mutants showed a clear accumulation of cells with 2N DNA content, indicative of a mitotic delay, while the wild type strain had an approximately equal number of cells with 1N and 2N DNA content (Figure III.8.A). However, after 4 hours at restrictive temperature, the

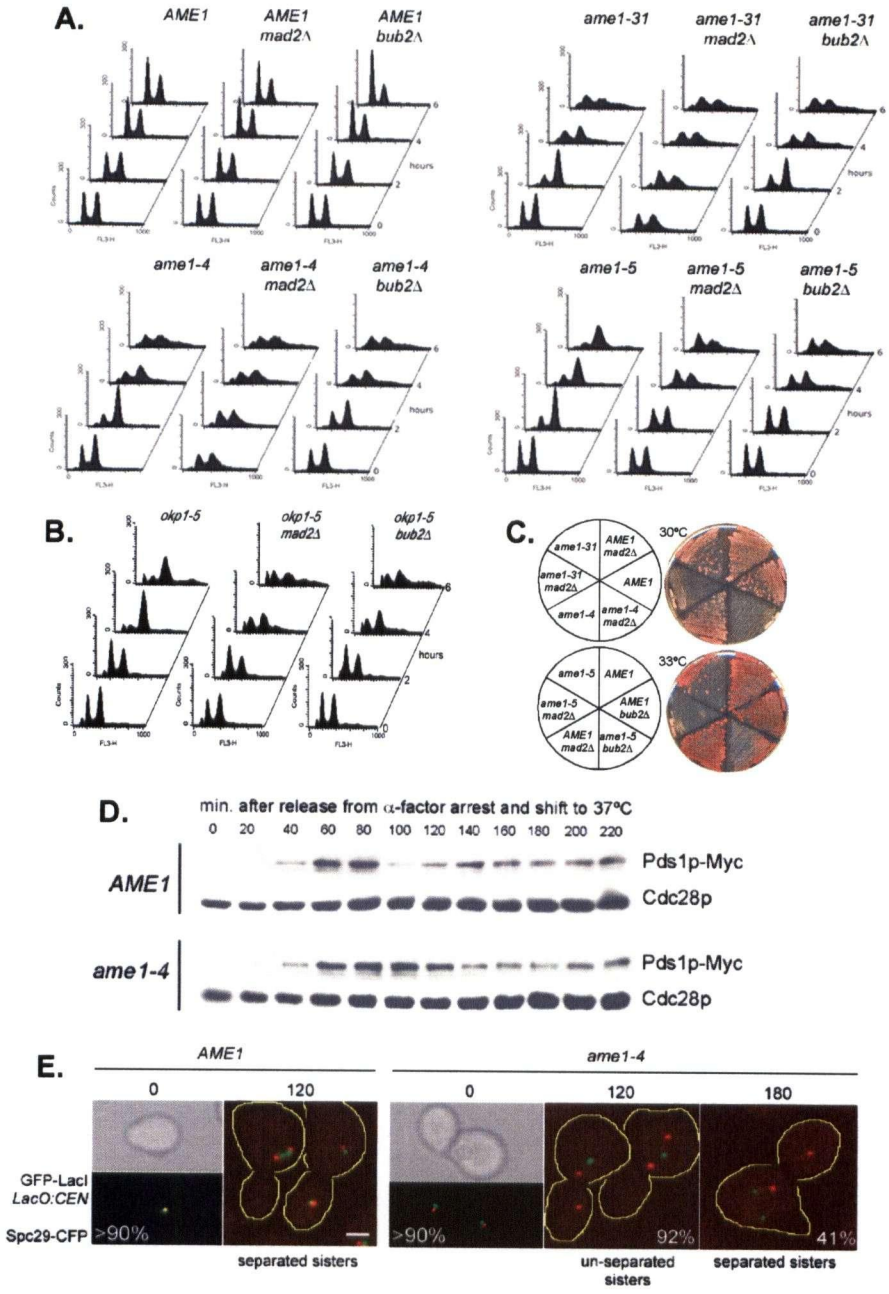


Figure III.8. *ame1* mutants break through a Mad2p-dependent G2/M arrest.

A. FACS analysis of wild type and *ame1* mutant strains in the presence or absence of functional spindle assembly (Mad2p) and mitotic exit (Bub2p) checkpoints. Strains grown to early log phase at permissive temperature (25°C) were shifted to restrictive temperature (37°C). Samples were collected at 0, 2, 4 and 6 hours after the shift and processed for FACS analysis. B. FACS analysis of the *okp1-5* mutant strain. Cells were grown and processed as in A. C. *ame1* mutants show reduced fitness in the absence of functional checkpoints. Strains were grown on YPD at the indicated temperature. D. The securin Pds1p breaks down in *ame1-4* cells, indicating onset of anaphase. Wild type and *ame1-4* strains were grown to early log phase at permissive temperature (25°C), synchronized in α-factor, and shifted to restrictive temperature (37°C). Samples were collected at 20min intervals and processed for immunoblotting using anti-Myc antibody. Anti-Cdc28p is used as a loading control. E. Separation of sister chromatids parallels Pds1p-Myc breakdown in *ame1-4* cells, and metaphase spindles (120 minutes post-release) are unusually long in *ame1* strains. GFP-LacI/*LacO:CEN AME1* and *ame1-4* strains containing Spc29p-CFP were grown as in D and samples were fixed at the indicated times and imaged as in Figure III.6 (see materials and methods). Percentages represent the fraction of cells within the population that show the indicated sister centromere separation state at the indicated time. Bar, 2μm.

ame1-31 and *ame1-4* strains (but not *ame1-5*) started to lose a clear G2/M accumulation and contained an increased number of G1 cells; by 6 hours these strains displayed cells with 1N, 2N and >2N DNA content (Figure III.8.A). In contrast, the *ame1-5* mutant arrested at restrictive temperature with 2N DNA content and maintained this arrest for 6 hours (Figure III.8.A). The same trends were more clearly observed when cells were first synchronized in G1 at permissive temperature using α-factor, and then released into the cell cycle at restrictive temperature (data not shown). By comparison, the G2/M delay in the *okp1-5* mutant was only apparent after 4 hours at restrictive temperature, but was maintained similarly to *ame1-5* (Figure III.8.B and Ortiz et al., 1999). Thus, all three *ame1* mutants initially delay mitosis, but two of them (*ame1-31* and *ame1-4*) do not seem to maintain this arrest past 3 hours at restrictive temperature.

To determine if the G2/M accumulation was dependent on either the spindle assembly or the spindle positioning checkpoints, the experiment was repeated with strains lacking either Mad2p or Bub2p, respectively. The mitotic delay in *ame1* and *okp1* mutants was not observed in *mad2*Δ strains, indicating that this delay results from activation of the spindle assembly checkpoint (Figure III.8.A, B). Lack of Bub2p did not have a pronounced effect on the early delay of *ame1-31* and *ame1-4* strains, but abrogated the G2/M delay in *ame1-5* and *okp1-5* (Figure III.8.A, B). Since the sister chromatids are separated in the majority of *ame1-5* cells after 4 hours at restrictive temperature (Figure III.6.B), but the majority of *ame1-5* cells still accumulate with 2N DNA content even after 6 hours at restrictive temperature, it is likely that this mutant initially triggers the spindle assembly checkpoint but maintains a G2/M arrest through the activity of the spindle positioning checkpoint. Similarly, *okp1-5* appears to trigger both mitotic checkpoints.

Interestingly, the three *ame1* mutations caused conditional synthetic lethality when combined with *mad2*Δ (and to some extent, *bub2*Δ for *ame1-5*) (Figure III.8.C). That is, the permissive temperature of *ame1* mutants was reduced by ~1-2°C in the presence of the checkpoint mutation. These observations strengthen the notion that in strains compromised for *AME1* function, a requirement for the spindle checkpoint becomes more important in order to survive, to alleviate the effects of a defective kinetochore. Similar effects were observed for other kinetochore mutants lacking *MAD2*, indicating that these mutants activate the checkpoint (Gardner et al., 2001).

Degradation of the securin Pds1p is required for sister chromatid separation and is a hallmark of the onset of anaphase (Cohen-Fix et al., 1996). Thus the level of Pds1p can be used as a marker of cell cycle progression, because after accumulating to high levels at metaphase, it gets completely degraded at the onset of anaphase. A Pds1p-Myc fusion protein was introduced in the *AME1* and *ame1-4* strains, in order to determine biochemically when these cells initiate anaphase. The strains were arrested in G1 using α-factor and released at restrictive temperature (37°C). Protein samples were collected every 20 minutes for ~4 hrs. In *AME1* cells, Pds1p levels increased until 80 minutes post-release and then decreased, signaling anaphase onset (Figure III.8.D). Cells returned to a 1N DNA content shortly thereafter (data not shown). In contrast, Pds1p-Myc levels in the *ame1-4* strain remained high until 120 minutes post-release, then decreased and remained low for a further 40 minutes (Figure III.8.D). The decrease in Pds1p-Myc is consistent with the timing of sister chromatid separation in the *AME1* and *ame1-4* cells (Figure III.8.E). At 120 minutes post-release, when Pds1p-Myc levels in the *AME1* strain are low, cells have either separated their sisters or are in late anaphase (Figure III.8.E). In contrast, Pds1p-Myc levels are high at 120 minutes in the *ame1-4* strain, and the majority of cells have cohesed sisters (Figure III.8.E). Interestingly, the spindle lengths in these cells are unusually long for metaphase cells (>2.5μm) (data not shown). At 180 minutes post-release, Pds1p-Myc levels are low in the *ame1-4* strain, and 41% of cells have separated sisters (Figure III.8.E). Taken together, these data indicate that *ame1* mutants initially arrest in metaphase via the Mad2p-dependent checkpoint, but do not maintain the arrest; and although it cannot be ruled out that the delay in Pds1p cycling observed in the *ame1-4* mutant is due to slow growth (rather than an initial checkpoint activation), the data still show that Pds1p levels are not maintained in this mutant, unlike what would be expected if the checkpoint was continuously active. Furthermore, spindle elongation appears to precede sister chromatid separation in *ame1* mutants, suggesting that these two mitotic events have been uncoupled.

III.3.i. ame1 mutants have elongated metaphase spindles

The cell cycle progression analysis raised the possibility that the spindle assembly checkpoint might not remain active in *ame1* mutants. This observation could be explained by two mechanisms: either the checkpoint sensing machinery stops detecting the defects of

ame1 mutants, or alternatively Ame1p contributes to the sensing mechanism that will promote a sustained checkpoint activation. If the latter is true, sister chromatid separation (indicative of cell cycle progression and lack of checkpoint arrest) would be expected to occur even when the checkpoint has been artificially triggered. The Mad2p-dependent arrest was thus extraneously induced using NZ (Jacobs et al., 1988) in cells carrying the strongest *ame1* allele (*ame1-4*) and the response was compared to wild type. *AME1* and *ame1-4* cells containing the GFP-LacI/*LacO:CEN* tag and Spc29p-CFP were synchronized in G1 using α-factor, released into media containing NZ, and incubated at restrictive temperature (37°C). After 2 hours, both *AME1* and *ame1-4* cells exhibited a large-budded phenotype with cohesed sister chromatids, indicative of NZ-induced G2/M arrest (data not shown). Thus, the initial checkpoint response is intact in those cells. After 4 hours in NZ, cells started to break through the arrest; however, sister chromatid separation occurred in 48% of *ame1-4* mutant cells, compared to only 22% of *AME1* cells (Figure III.9.A, *arrows*). Thus, *ame1-4* mutants seem to fail to maintain the metaphase checkpoint arrest induced by NZ to a greater extent than wild type cells, and to allow sister chromatid separation to occur even in the presence of an intact checkpoint system.

The distance between SPBs was also observed to be abnormally long in *ame1-4* cells treated with NZ as compared to that in NZ-treated *AME1* cells (Figure III.9.A). Therefore, spindle microtubules in *AME1* and *ame1-4* cells containing Spc29p-CFP and grown as in Figure III.9.A in the presence or absence of NZ, were examined by indirect immunofluorescence of α-tubulin (Tub1p). Astral and spindle microtubules were present in untreated *AME1* and *ame1-4* cells; however, astral microtubules were lost in the presence of NZ (Figure III.9.B, *arrows*). In *AME1* cells treated with NZ, spindles collapsed to less than 2μm in length (Figure III.9.B). In contrast, elongated spindles were observed in 90% of *ame1-4* mutant cells in the presence of NZ (Figure III.9.B). It is unlikely that microtubules are generally stabilized in these cells, as *ame1* mutants do not display a benomyl resistance phenotype (data not shown). The response of wild type cells to NZ at 37°C (i.e. the presence of a short spindles) indicates that the drug may not be as effective at high temperature as is normally observed at lower temperatures; experiments are in progress to test conditions that circumvent this problem. However, because of the difference in response observed between wild type and *ame1-4* cells, these results still indicate that in *ame1* mutants, spindle

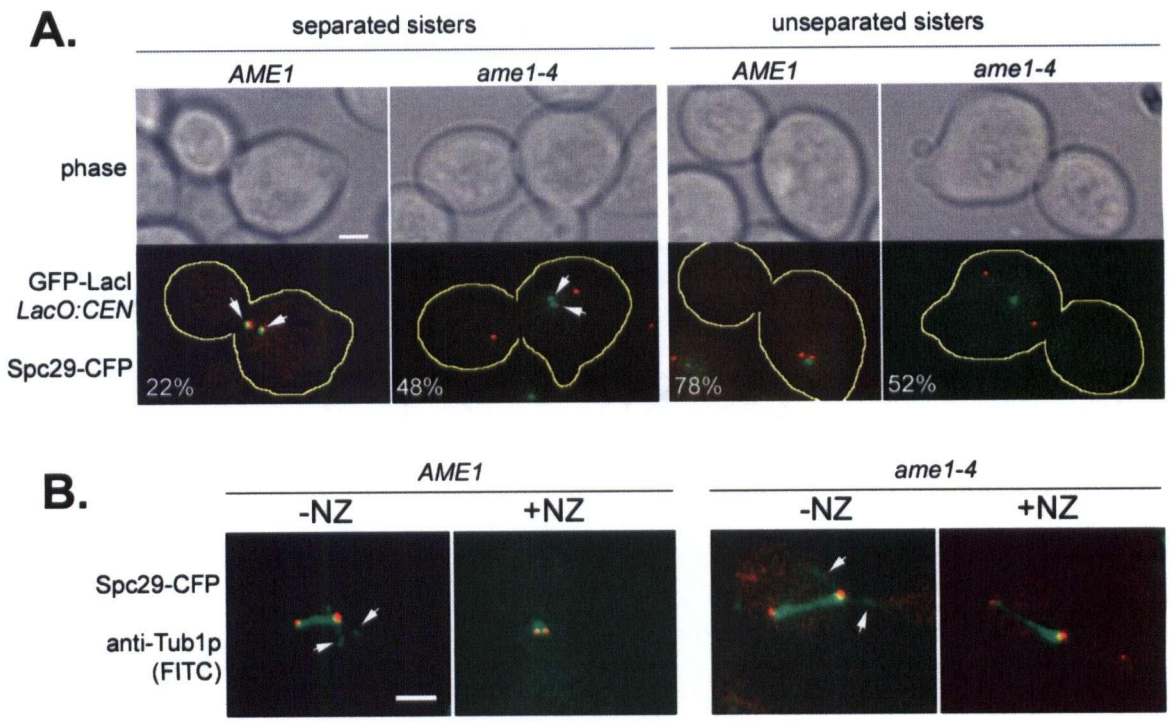


Figure III.9. *ame1-4* bypasses NZ-induced arrest.
A. Separation of sister chromatids (green) occurs more frequently in *ame1-4* cells (48%) relative to wild type (22%) when Mad2p-dependent arrest is induced by incubation of synchronized cells in 15μm/mL nocodazole (NZ) for 4 hours at 37°C. Chromosomes in NZ-treated *ame1-4* cells frequently lose their association with the SPBs (Spc29p-CFP, red) and exhibit elongated spindles. B. Microtubules are present in the elongated spindles observed in *ame1-4* cells. Both astral (white arrows) and spindle microtubules are observed in untreated cells, but astral microtubules are not detected in cells treated with NZ for 4 hours as in A. Microtubules were imaged by indirect immunofluorescent detection of Tub1p with the YOL134 antibody. Bar, 2μm.

microtubules might be stabilized, and that the onset of spindle elongation could be uncoupled from sister chromatid separation.

III.3.j. Overexpression of OKP1 restores metaphase arrest in ame1 mutants

To further identify genes that interact with *AME1*, a screen for high copy suppressors of *ame1-4* was performed at 35°C (i.e. slightly above the restrictive temperature of this mutant) (see materials and methods). Two independent clones able to suppress the temperature sensitivity of *ame1-4* were recovered. In both cases, the library insert contained the *OKP1* ORF. After *OKP1* was confirmed to be the suppressor gene, it was subcloned by PCR into a 2μ vector and the resulting plasmid was transformed into the *ame1-4* strain. *ame1-4* cells

carrying the *OKP1*-containing 2μ plasmid (p*OKP1*) were able to grow at restrictive temperature, but not with vector alone (Figure III.10.A and Table III.4). Similarly, overexpression of *OKP1* rescued the growth defects of *ame1-31* and *ame1-5* (Table III.4). Thus, increased dosage of Okp1p can alleviate the growth defects caused by loss of Ame1p function in all *ame1* mutants.

To determine the mechanism by which overexpression of *OKP1* suppressed *ame1-4* mutant defects, cell cycle progression was examined in *ame1-4* cells in the presence or absence of p*OKP1* (Figure III.10.B). Overexpression of *OKP1* extended the G2/M arrest of *ame1-4* cells from 2 hours to >4 hours (Figure III.10.B), indicating that increased dosage of Okp1p can rescue the failure to maintain checkpoint arrest. The localization of ame1-4p-VFP at 37°C in the presence of p*OKP1* or vector alone was also examined; Ame1p-VFP was used as a control (Figure III.10.C). As expected, after 4 hours at 37°C, asynchronous cultures showed fewer than 10% of mutant cells with ame1-4p-VFP localized at both SPBs in the vector control; in contrast, this localization pattern occurred in ~30% of mutant cells overexpressing *OKP1* (Figure III.10.C). In general, spindles with localized ame1-4p-VFP were >2.5μm in length and had discrete ame1-4p-VFP foci near the SPBs (Figure III.10.C, arrows). Next, sister chromatid separation was examined in GFP-LacI/*LacO:CEN*, Spc29p-CFP *ame1-4* cells overexpressing *OKP1* grown at restrictive temperature for 4 hours and imaged as in Figure III.2. The majority (>90%) of *ame1-4* cells had a single focus of GFP, indicating that they were in pre-anaphase (Figure III.10.D). These cells consisted of two categories: one in which the spindle was abnormally long (57%) and another in which the spindle was ~2μm in length (43%) (Figure III.10.D). Thus, while overexpression of *OKP1* partly rescues the defects in checkpoint maintenance and protein localization, the formation of elongated pre-anaphase spindles still takes place.

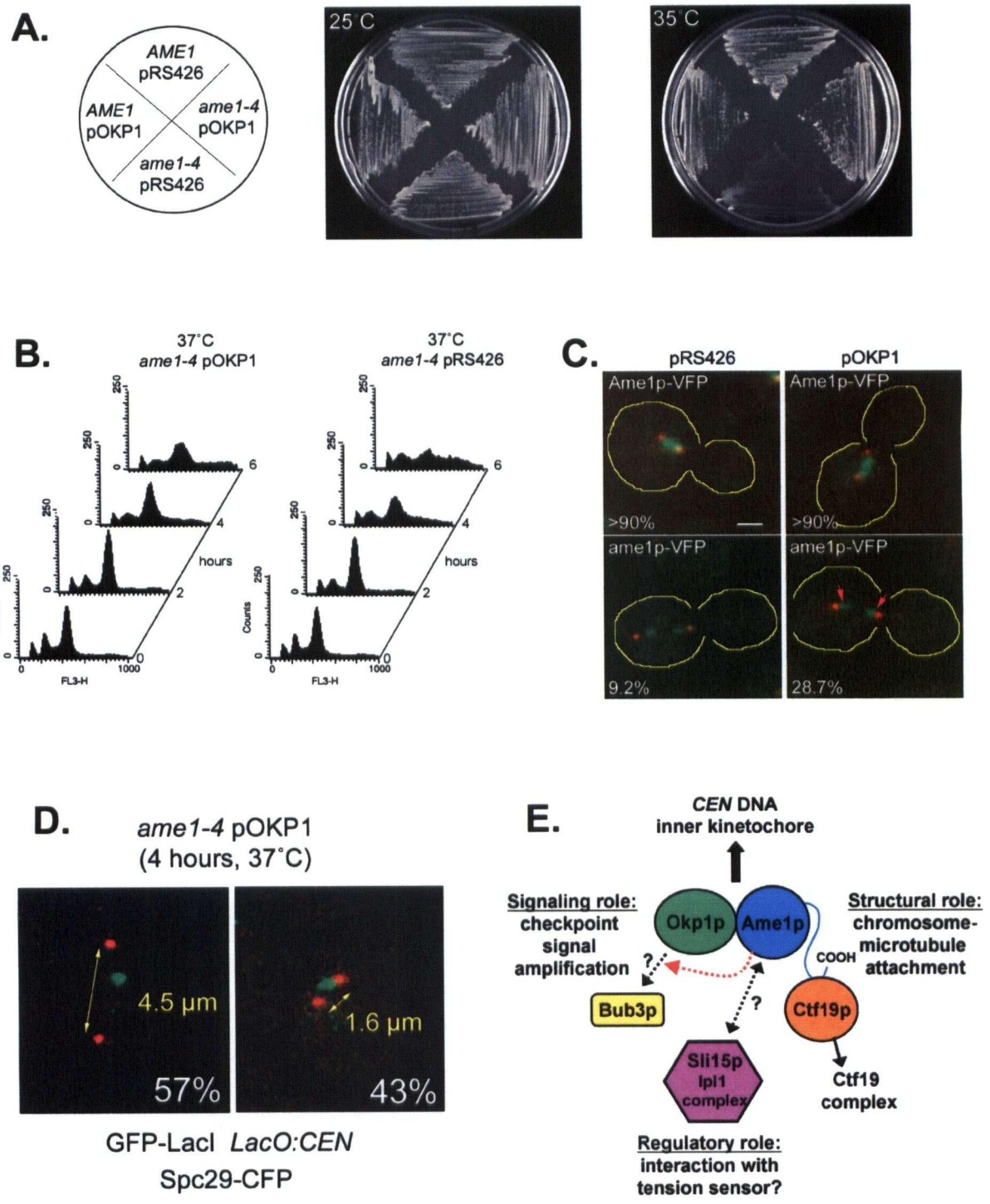


Figure III.10. Overexpression of *OKP1* suppresses *ame1* phenotypes.

A. Overexpression of *OKP1* rescues the temperature sensitivity of *ame1-4* mutants. *AME1* and *ame1-4* strains were transformed with the *OKP1*-carrying 2μ plasmid (p*OKP1*) or vector alone (pRS426) and incubated at permissive (25°C) and restrictive (35°C) temperatures. B. Overexpression of *OKP1* in *ame1-4* cells allows maintenance of the checkpoint arrest beyond 2 hours at restrictive temperature. C, D. Overexpression of *OKP1* enhances localization of ame1-4p-VFP at restrictive temperature (C), but metaphase spindles (cells with paired sister centromeres) are still elongated in *ame1-4* cells (D). Percentages represent the fraction of cells within the population that exhibit proper kinetochore localization in early anaphase spindles (C) or the fraction of cells with unseparated sister centromeres that show long (>2.5μm) or short (≤2μm) spindles (D). E. Model for the function of Ame1p at the kinetochore. Note that the Sli15p connection is discussed in Chapter IV.

III.4. Discussion

The yeast kinetochore is composed of at least 50 proteins, and principally acts as a linking bridge between chromosomes and microtubules of the mitotic spindle. A small number of its constituent proteins have been demonstrated to directly bind either *CEN* DNA or microtubules. However, the function of the majority of central kinetochore proteins remains unclear. The kinetochore also coordinates the chromosome cycle with the cell cycle. For example, some kinetochore proteins participate in signaling a failure of proper chromosome-spindle attachment to the cell cycle machinery, resulting in cell cycle delay that allows for repair of the failed attachment. Exactly how the signal is first activated and then maintained is still unclear, but it is likely that a number of kinetochore proteins play roles in this process. In this chapter, the functional characterization of an essential kinetochore protein, Ame1p, was carried out. This analysis provides evidence that Ame1p functions in maintaining faithful chromosome segregation, by promoting the structural integrity of the kinetochore complex and mediating proper kinetochore-microtubule attachments. Additionally, the data suggest that Ame1p might also promote the maintenance of a cycle cell delay to allow for repair of defective chromosome-microtubule attachments.

Ame1p was previously shown to interact with proteins of the large Ctf19 complex (Cheeseman et al., 2002a; De Wulf et al., 2003; Gavin et al., 2002). The data presented here confirm that Ame1p localizes to the kinetochore and interacts with *CEN* DNA. They also show that Ame1p does not depend on Ctf19p for its interaction with the centromere. Consistent with this result, Ame1p has recently been shown to remain localized in a *ctf19Δ* mutant (De Wulf et al., 2003). Thus, Ame1p is clearly located at the inner edge of the Ctf19 central kinetochore complex.

To investigate the function of Ame1p *in vivo*, several conditional *ame1* alleles were isolated. Of the seven original mutants, three were studied in more detail as they presented the most interesting and severe phenotypes. These three mutant ame1p proteins are stable at restrictive temperature, indicating that they are not encoded by null alleles, and they also differ in their ability to localize to the kinetochore and bind to *CEN* DNA at restrictive temperature: ame1-31p and ame1-5p localize to both the kinetochore and to *CEN* DNA at the restrictive temperature, but ame1-4p does not. However, localization of the protein is not sufficient for high fidelity of chromosome segregation, since *ame1-5* cells exhibit severe

chromosome instability despite proper localization of the protein at restrictive temperature. The data also show that *ame1* mutants are defective in binding to Ctf19p, and that mutants with a high chromosome instability phenotype, such as *ame1-31*, are most impaired in their interaction with Ctf19p. In contrast, the *ame1-5* mutant is able to interact with Ctf19p at the permissive temperature and does not show chromosomal instability under these conditions. Thus, the interaction of Ame1p with other components of the Ctf19 complex is critical for faithful chromosome segregation.

Non-essential Ctf19 complex components such as Ctf3p and Ctf19p are also demonstrated here to be indispensable for the viability of *ame1* temperature sensitive mutants at their permissive temperature. Thus, while many of these non-essential components can be removed from cells in combination without affecting viability (Measday et al., 2002; Pot et al., 2003), they do not tolerate coincident compromised Ame1p function. The molecular organization of the large Ctf19 complex was also probed further here by analyzing phenotypes of *ame1* mutants. For example, the *ame1-4* mutant does not disturb the core kinetochore structure (Ndc10p's ability to interact with *CEN* DNA), but that it impairs the interaction of Ctf19p with the centromere (my unpublished data). Recent studies on the molecular localization of Okp1p have demonstrated that this protein is necessary for other Ctf19 complex members, including Ame1p, to bind *CEN* DNA (De Wulf et al., 2003); the data presented here show that the *okp1-5* mutant, similar to *ame1-4*, does not disturb the inner kinetochore (Figure III.7.C). Moreover, Okp1p is crucial for the structural integrity complex of the COMA complex (De Wulf et al., 2003); it is possible that lack of Ame1p also affects the stability of Ctf19 complex components (see Figure III.7.D, compare lane 9 to 11, and lane 13 to 15). Conditional alleles of *ame1* were used here to demonstrate that uncompromised Ame1p is essential in the *okp1-5* mutant. The observed synthetic genetic defect supports the idea that these two essential components of the Ctf19 complex represent a major functional unit within the kinetochore. Taken together, the data presented here and those of De Wulf et al. (2003) demonstrate that the COMA complex is localized distal to the inner kinetochore and is likely to serve as a bridge between the inner kinetochore and other components of the Ctf19 complex.

Analysis of the phenotypes of *ame1* alleles indicates that Ame1p may play multiple roles in mitosis (Figure III.10.E). The cell cycle progression data indicate that *ame1* mutants fall

into two classes; in both classes the cells exhibit an initial mitotic arrest that delays progression through the cell cycle due to the Mad2p-dependent spindle assembly checkpoint. The first class of mutants (*ame1-31* and *ame1-4*) then appear to break through the G2/M arrest, as suggested by the observation that an increased number of cells have separated sister chromatids, at a time that coincides with Pds1p destruction; by comparison, unseparated sister chromatids and high Pds1p levels would be expected to be maintained in checkpoint-arrested cells. The checkpoint dependency of Pds1p levels in these mutant alleles needs to be further examined in strains mutated for checkpoint components. In sharp contrast, cells in the second class of mutants (*ame1-5*) progress through part of anaphase but maintain the G2/M accumulation of cells with elongated spindles, with ame1-5p-VFP located next to the SPBs, and with separated sister chromatids. This phenotype, together with the lack of G2/M accumulation in the absence of Bub2p in this strain, suggests that the maintained arrest occurs through the activity of the Bub2p-dependent spindle positioning checkpoint. Interestingly, the *okp1-5* mutant also maintains G2/M arrest for >6 hours; and although its G2/M accumulation occurs slightly later than in *ame1* mutants, it is dependent on both mitotic checkpoints, similar to *ame1-5*. Thus, in addition to a role in maintaining the stability of the interaction between DNA and microtubules, one attractive hypothesis is that Ame1p also plays a role in the maintenance of the checkpoint response. The phenotypes observed in *ame1* mutants are reminiscent of some phenotypes observed in vertebrate cells depleted of the mitotic kinesin CENP-E. This kinetochore protein is required not only for stable kinetochore-microtubule attachments, but also for establishing and maintaining checkpoint response in cells challenged with NZ (Abrieu et al., 2000). Since the absence of CENP-E does not lead to cell cycle arrest in the presence of improperly attached kinetochores, despite recruitment of some of the checkpoint components (such as MAD2), it is thought that CENP-E acts as a sensor of kinetochore-microtubule attachment, and that its role is to amplify the checkpoint signal to a level that will lead to sustained mitotic arrest (Abrieu et al., 2000; Putkey et al., 2002; Weaver et al., 2003). Therefore, one possibility is that Ame1p functions cooperatively with other proteins (such as Okp1p) to enhance signaling of defective kinetochore-microtubule attachments to checkpoint proteins, which will result in maintenance of the mitotic delay. However, further experiments are required to determine whether Ame1p does play an active role in checkpoint signaling, or whether the

breakthrough from checkpoint arrest in *ame1* mutant cells occurs because the alleles studied here are "leaky" (i.e., they don't cause a defect that is strong enough to be continuously detected by the checkpoint machinery, and thus the cells resume cell cycle progression). Nevertheless, the defects caused by *ame1-31* and *ame1-4* are strong enough to lead to extensive chromosome missegregation and death (cells rapidly lose viability at restrictive temperature; V. Aneliunas, unpublished data); it is thus difficult to assess with certainty whether these are partial alleles. One way to address the issue of allele strength is to use constructs of Ame1p that can be conditionally turned "off", such as degron-tagged alleles. For example, it was shown that point mutants in genes encoding some components of the CBF3 complex were not defective in checkpoint function, whereas the degron-tagged version showed a complete lack of checkpoint activity (Gardner et al., 2001). Thus, further work will be required to determine whether Ame1p actively contributes to checkpoint signaling.

The observed phenotype of *ame1-5* suggests a further role for Ame1p in microtubule organization and/or microtubule motor function. In all *ame1* mutants, but especially so in *ame1-5*, elongated spindles were observed. One reason for the premature elongation could be breakthrough from the spindle checkpoint arrest; however, the fact that the elongation occurs prior to sister chromatid separation seems to argue against that simple explanation. Alternatively, spindles poles might appear further apart due to the decreased inward force that is normally caused by attachment to cohesed sister chromatids. Elongated and aberrant spindles were observed in some other kinetochore mutants, such as *ask1* (Li et al., 2002); therefore, a quantified comparison of spindle elongation phenotypes between *ame1* and some of these other mutants will demonstrate whether the *ame1* phenotype is unique. A third possibility is that *ame1* mutants have elongated spindles because they are defective in a microtubule destabilization function of Ame1p (see below). Interestingly, the majority of elongated spindles in mother cells in *ame1* mutants are properly oriented, yet fail to enter the bud. Yeast strains with a mutation in the microtubule-associated protein Bik1p also have a significant proportion of cells where anaphase occurs within the mother cell, suggesting a defect in microtubule-dependent spindle migration into the bud (Berlin et al., 1990). Thus, the presence of elongated spindles in mother cells observed in *ame1* mutants could be an indication that Ame1p participates in dynamic microtubule processes impinging on spindle migration. Further experiments will need to be conducted to address these hypotheses.

Moreover, since the initiation of spindle elongation appears to occur prior to sister chromatid separation, there might be an uncoupling of these two events in *ame1* mutants. This novel phenotype suggests that Ame1p could have a role in coordinating two aspects of mitosis: sister separation and spindle elongation.

A puzzling finding is that unlike many other kinetochore mutants, including mutants in most genes whose products are part of the large Ctf19 complex (see Figure II.1.B and Ghosh et al., 2001; Hyland et al., 1999; Ortiz et al., 1999; Poddar et al., 1999; Roy et al., 1997), *ame1* mutants do not have high sensitivity to the microtubule-depolymerizing drug benomyl (my unpublished data). This observation suggests that Ame1p could have a role in microtubule destabilization. In support of this hypothesis, an *AME1/ame1*Δ heterozygote can suppress the benomyl sensitivity of a *TUB4/tub4*Δ heterozygote (Jackie Vogel, unpublished data). Tub4p is the yeast γ-tubulin that functions in nucleation of microtubules at the SPB (see section I.3). Thus, while Ame1p seems to function close to the inner kinetochore, it is possible that its role also involves regulation of microtubules, which could be achieved through interacting partners. An interesting possibility is that Ame1p's role in microtubule dynamics involves Bik1p, as overexpression of *BIK1* is not tolerated in the three *ame1* mutants studied here in detail. The stoichiometry of Bik1p has previously been shown to be important, as overexpression of *BIK1* does affect microtubule structure and cell cycle progression (Berlin et al., 1990). Bik1p and its mammalian homologue CLIP-170 are thought to have two independent roles: one as a microtubule plus-end tracking protein, that participates in microtubule formation and stabilization; and another, as a linker protein that anchors targets, such as vesicles or kinetochores, to microtubules (Coquelle et al., 2002; Lin et al., 2002; Pierre et al., 1994). The observed increased Bik1p dosage sensitivity phenotype of *ame1* mutants presented here is however unlikely to reflect a specific Ame1p-Bik1p role in kinetochore-microtubule connections, since the phenotype is not restricted to *ame1* mutants; and it is unlikely that Ame1p is a cargo for Bik1p, since overexpression of the cargo-binding domain mutant of Bik1p still affected *ame1* mutant to a similar degree as full length Bik1p. Also, Ame1p does not seem to interact with Bik1p, at least by co-immunoprecipitation and in the experimental conditions used here (data not shown, and see Chapter IV). Thus, it is more likely that many kinetochore mutants (and perhaps other mutants in genes involved in the mitotic process, which weren't tested here) don't survive

upon overexpression of *BIK1* due to an imbalance effect in the microtubule dynamics aspect of Bik1p's function, such as by titrating out key proteins involved in microtubule attachment to the kinetochore.

Data in this chapter also show that increasing the dosage of *OKP1* allows cells to live in the absence of fully functional *AME1*. This suggests that an increased amount of Okp1p either makes up for the defects of malfunctioning Ame1p, or that Okp1p has a partly redundant function with Ame1p. Interestingly, Okp1p and Ame1p were found not only as part of COMA but also as a stable dimer; this dimer, but not COMA, was isolated from G1 cells, indicating that it forms the basic unit of the complex (De Wulf et al., 2003). One possibility is that increased amount of Okp1p stabilizes its interaction with mutant forms of Ame1p; ame1-4p-VFP is more likely to localize as discrete foci at both SPBs when *OKP1* is overexpressed. Furthermore, overexpression of *OKP1* stabilizes the Mad2p-dependent arrest, as shown by the FACS analysis and by the accumulation of spindles with paired sister chromatids. However, the fact that these spindles are still elongated demonstrates that increasing the amount of Okp1p does not correct the premature spindle elongation in these cells, which suggest that this phenotype is reflective on another aspect of Ame1p function that is independent of Okp1p.

If Ame1p has an active role in promoting the checkpoint response, it could do so in several ways. Purifications of Okp1p were shown to contain Bub3p (De Wulf et al., 2003), indicating that a transient interaction between this spindle assembly checkpoint protein and COMA was possible (see also Chapter IV). *bub3*Δ and *bub1*Δ strains show a higher rate of chromosome missegregation than strains containing deletions of the *MAD* genes, perhaps due to differences in the structural, sensing, or signaling role of these proteins in the context of kinetochore defects (Warren et al., 2002). Bub3p has also been demonstrated to localize to kinetochores even in the absence of checkpoint induction, and to be required for *CEN* DNA interaction and kinetochore localization of the other spindle assembly checkpoint proteins (Bub1p, Mad1p, Mad2p and Mad3p), suggesting that Bub3p is the upstream component of the signaling pathway (Gillett et al., 2004; Kerscher et al., 2003); thus Bub3p likely interacts with kinetochore proteins. Additionally, Bub3p localization to the kinetochore is enriched in NZ-treated cells or in cells with defective centromeres (Kerscher et al., 2003), and murine BUB3 was shown to be associated with lagging chromosomes during metaphase, with a

significant enrichment in microtubule poison-treated cells, indicating that BUB3 functions in sensing proper microtubule attachment (Martinez-Exposito et al., 1999). Since in the present study, lagging chromosomes were observed in *ame1* mutants but checkpoint arrest did not appear to be maintained, one possible model is that Ame1p participates in checkpoint maintenance by modifying an interaction of Okp1p with Bub3p, or by acting co-operatively with Okp1p to connect to the checkpoint, for example by promoting localization of Okp1p to the kinetochore (preliminary results indicate that indeed, Okp1p localization is disturbed in *ame1-4* mutants; James Knockleby and Jackie Vogel, pers. commun.).

In summary, this chapter proposes that Ame1p is positioned at the center of an interaction group within the COMA complex that is structurally essential for chromosome segregation but might also have additional roles, possibly in checkpoint maintenance (Figure III.10.E). The results presented here are consistent with an interaction between the C-terminus of Ame1p and Ctf19p being crucial for chromosome segregation. A hypothesis discussed here proposes that defects in chromosome segregation could be signaled to Okp1p and Bub3p in an Ame1p-dependent manner. In the absence of Ame1p function, either as a result of mislocalization (*ame1-4*) or lack of function (*ame1-31*, *ame1-5*), Okp1p might not be able to maintain a signal to the checkpoint due to either an instability of its interaction with Bub3p, or its delocalization from the kinetochore. Further investigation into the role of Ame1p at the kinetochore will be required to determine whether hypotheses presented here are valid; some aspects of further work undertaken with Ame1p are discussed in Chapter IV.

CHAPTER IV

CONCLUSIONS AND FUTURE PROSPECTS

IV.1. Future prospects in chromosome segregation research

The aim of this thesis work was to identify and characterize new components of the budding yeast kinetochore. At the time the project started, relatively few components had been identified and the mechanisms by which microtubules were attached to the kinetochore complex were still very elusive. As illustrated in Figure I.5, the number of proteins comprising the kinetochore has now greatly increased in parallel with our knowledge of mechanisms for microtubule attachment, checkpoint signaling, and regulation of the kinetochore. Despite these advances, the functions of many kinetochore components, other than as a structural scaffold for the complex, remain unclear. Thus, the challenge in the next few years will be to shift from simple identification to thorough functional characterization of kinetochore components. Many questions remain to be answered: why are there so many proteins at the kinetochore, especially when the centromere is as simple as in the budding yeast? What do all of these proteins do? Although a few proteins have been assigned specific roles due to the presence of a conserved domain (e.g. the Ipl1p kinase), or to their ability to bind specific structures (e.g. the CBF3 complex binding DNA, the Dam1 complex binding microtubules), the functions of the remainder of kinetochore components remain to be explained (Mellone and Allshire, 2003).

Another aspect of kinetochore function that will have to be examined is the differentiation between proteins that have a core, conserved kinetochore function, and those that play organism-specific roles. Indeed, while several yeast kinetochore proteins and most spindle assembly checkpoint components have homologs in other eukaryotes, a number of yeast kinetochore components have homologs only in other fungi, or they may have no sequence homologs at all. Interestingly, the two structures connected by the kinetochore, centromeric chromatin and the spindle, are made up of proteins that are very conserved across all eukaryotes. For example, histone H4, a component of nucleosomes, and ß-tubulin, a component of microtubules, have over 70% identity across species and are conserved throughout their length; in comparison, even highly conserved kinetochore components show much less similarity between homologs (Tyler-Smith and Floridia, 2000) – perhaps this difference in the level of conservation is due to the fact that histones and tubulins are involved in so many basic and essential processes in cells. Major regulatory proteins with a

role in chromosome segregation, such as the Aurora/Ipl1p kinase, are also relatively well conserved. Thus, it is intriguing that there should be a divergence in the components of the kinetochore, even if the variation in *CEN* DNA composition can probably account for some of the protein diversity. Some answers to these questions may arise with the completion of more genome sequences, and an improvement in the ability to compare protein structures beyond sequence-based information.

It should also be noted that, as new data become available, the schematic representation of the yeast kinetochore as it is depicted in Figure I.5 is likely to change. One good example stems from recent studies with the inner kinetochore protein Ndc10p. These studies suggest that the classic view of *CEN* DNA being wrapped around a centromere-specific Cse4p-containing nucleosome may not be accurate. Instead, it is proposed that the 125bp *CEN* region is nucleosome-free, and that Ndc10p forms polymers in that region (in addition to its role within the CBF3 complex), while Cse4p-containing phased nucleosomes would reside in regions immediately flanking the core *CEN* DNA (Espelin et al., 2003). That chromatin at *CEN* DNA and its surrounding region is less compact than in other nucleosome-bound regions was also proposed to allow chromatin stretching upon forces applied when kinetochores are bipolarly attached to the spindle and was therefore suggested to have a role in proper chromosome segregation (Pearson et al., 2001).

The next phase in understanding kinetochore function and the chromosome segregation process in a broader sense will also involve characterizing other proteins than the core kinetochore components. For example, as was discussed in Chapter I, components of chromatin have now been associated with proper centromere function (Sharp et al., 2002). Recently, a necessary role for chaperones (proteins that prevent unspecific aggregation of nascent polypeptides and promote their correct folding) was demonstrated for the activation of the CBF3 complex components Skp1p and Ctf13p, leading to proper CBF3-*CEN* complex formation (Stemmann et al., 2002). Tubulin chaperones also show genetic interactions with many of the non-essential kinetochore components (Tong et al., 2004), and several genes involved in tubulin folding in yeast were first identified in a screen for mutants that displayed a chromosome instability phenotype (*cin1*, *cin2* and *cin4*; Hoyt et al., 1997; Hoyt et al., 1990; Stearns et al., 1990). Moreover, regulated protein folding and complex formation, as well as

complex disassembly and protein unfolding followed by proteolysis, all contribute to ensuring that functional proteins and complexes are present at the right concentration at a particular time of the cell cycle (Zhang et al., 2002). Thus, it is not unreasonable to expect that more chaperones will be found to have a role in proper chromosome segregation.

Spindle assembly checkpoint components have also been shown to interact with the nuclear pore, perhaps as a mechanism to sequester them until they need to be activated (Campbell et al., 2001; Iouk et al., 2002). Conversely, some nuclear pore components (nucleoporins, NUPs) were shown relocalize to the kinetochore and the SPB at mitosis (Belgareh et al., 2001; Salina et al., 2003). A relocalizing mammalian NUP was shown to have a role in kinetochore assembly, spindle organization and chromosome congression and segregation (Salina et al., 2003). Moreover, mutants in a component of the Mtw1 complex, Nnf1p, were originally described to have phenotypes similar to nuclear pore proteins and nucleocytoplasmic transport mutants (Shan et al., 1997). In organisms where the nuclear envelope breaks down, the use of nuclear pore components to regulate the kinetochore might ensure that nuclear envelope disassembly and kinetochore-dependent events proceed in a timely and ordered fashion (reviewed in Stukenberg and Macara, 2003). Moreover, mammalian presinilins, mutations of which cause aneuploidy and have been linked to Alzheimer's disease, were shown to reside in the nuclear membrane and to co-localize with kinetochores; presinilins were then proposed to act as receptors for kinetochore proteins that would help anchor chromosomes during interphase, and to have a general role in chromosome segregation (Li et al., 1997a). Thus, several nuclear membrane-associated proteins appear to affect chromosome transmission.

Kinetochore and spindle checkpoint regulation might occur through control of the activity of the Ran GTPase, which has been shown to participate in several cell cycle processes in addition to its well-characterized role in nucleocytoplasmic transport; these include DNA replication, accurate chromosome segregation, exit from mitosis and nuclear envelope assembly, and microtubule polymerization (see Chapter I) (Arnaoutov and Dasso, 2003; Moore, 2001; Salina et al., 2003). The apparent involvement of Ran in several of these processes is likely to reflect its role in the transport of key substrates in and out of the nucleus (e.g. import of Cdks and Ddks into the nucleus resulting in G1/S progression; import of mitotic Cdks and mammalian CDC25 at the G2/M transition). However, Ran – that is, the

ratio of GTP-bound Ran to GDP-bound Ran – may have more direct effects that are independent from nucleocytoplasmic transport, such as influencing the stability or activity of cell cycle progression regulators, or directly regulating microtubule assembly (Moore, 2001). Ran-GTP has also been shown to release the MAP TPX2, that is required for microtubule assembly, from sequestration at the nuclear pore complex to allow it to relocalize to the spindle at mitosis (Gruss et al., 2001). Furthermore, the relative concentration of Ran-GTP was shown to affect the kinetochore localization of mammalian checkpoint proteins (MAD2, CENP-E, BUB1 and BUB3), although whether the effect is direct or indirect remains unclear (Arnaoutov and Dasso, 2003). Thus, Ran could act by modulating the interaction of proteins required in mitosis with sequestering/inhibitory molecules such as nuclear pore-associated components.

In summary, despite the fact that a much more complete picture of kinetochore function has been achieved recently, there are still many unanswered questions, and the function of kinetochore components will have to be investigated in the context of other subcellular structures that might regulate their function.

IV.2. Further probing the role of Chl4p and Iml3p

Chapter II described the characterization of two non-essential components of the kinetochore, Chl4p and Iml3p. This thorough analysis not only confirmed that the two proteins were part of the kinetochore, but also provided some insight into the molecular organization of the complex. The data suggest that Chl4p and Iml3p form a subcomplex within the large Ctf19 central kinetochore complex. Interestingly, this Chl4p-Iml3p complex seems to form a separate "branch" of the complex from the Ctf3 complex (Ctf3p-Mcm16p-Mcm22p), downstream of Ctf19p, since both Chl4p and Ctf3p depend on Ctf19p for interacting with *CEN* DNA, but interact with *CEN* DNA independently of each other. However, the fact that Chl4p seems to mediate the Ctf19p-Ctf3p interaction, and the different requirements for proper Ctf3p localization in cells with short versus long spindles, demonstrate that these complexes cannot be described as simple linear entities. The data emphasize the fact that the large Ctf19 complex must be thought of as a 3D entity that is probably dynamic over the cell cycle. Thus, further experiments into interdependencies

within the complex at various cell cycle stages are required for a full understanding of its function.

One puzzling observation is that among all kinetochore complexes, only the large Ctf19 complex contains non-essential components (with the exception of the newly described non-essential component Ydr532p that is not part of this complex). All other structural kinetochore components are essential (see underlined proteins in Figure I.5). Many of the non-essential proteins of the Ctf19 complex can be deleted simultaneously without causing inviability of the strain (Measday et al., 2002; Pot et al., 2003), suggesting that there might be some redundancy in the role of this complex with other complexes at the kinetochore. Another explanation for this observation is that while these components are not required during mitosis in laboratory conditions, they might be required during other cellular processes or in non-standard conditions of growth. Additional assays, beside a simple assessment of growth ability, may also be required for a full understanding of the function of these proteins. For example, *chl4*Δ mutants were shown to have a defect in sister chromatid cohesion during mitosis (Warren et al., 2004). Chl4p and Iml3p were also recently demonstrated to have a role in meiotic cohesion (Marston et al., 2004): a screen of the yeast non-essential gene deletion strain collection for decreased sporulation efficiency and defective chromosome segregation during meiosis identified *chl4*Δ and *iml3*Δ as deletions that prematurely lost Rec8p-dependent centromeric cohesion, which is required for faithful chromosome segregation in meiosis (see Chapter I). The similar mutant profiles of these two genes in this assay supports the idea that they might work as a complex within the kinetochore; furthermore, a role in centromeric cohesion appears to be specific to this "branch" of the Ctf19 complex as deletion of the Ctf3 complex components did not lead to the same phenotypes (Marston et al., 2004). Thus, even though the exact function(s) of Chl4p and Iml3p have not yet been found, the participation of these proteins in proper sister chromatid cohesion, as well as a potential role for Chl4p in *de novo* centromere specification (Mythreye and Bloom, 2003) and the interaction of Chl4p with Mif2p, a protein believed to reside close to *CEN* DNA (Pot et al., 2003), suggest that Chl4p and Iml3p may have a role in a DNA/chromatin-dependent process that influences the integrity of chromosome segregation.

IV.3. Exciting prospects for a role of Ame1p at the kinetochore

In Chapter III, another component of the large Ctf19 complex was examined in detail, the essential protein Ame1p. While this investigation was in progress, it was demonstrated that the large Ctf19 complex may in fact be composed of several subcomplexes within which proteins are more tightly associated (De Wulf et al., 2003), validating previous findings describing the Ctf19 complex, the Ctf3 complex and a possible Chl4p-Iml3p complex (Measday et al., 2002; Ortiz et al., 1999; Pot et al., 2003). Ame1p was hypothesized to be in a tight complex with the previously described Ctf19 complex, forming COMA (De Wulf et al., 2003; Ortiz et al., 1999). The data presented here demonstrate that Ame1p indeed is a kinetochore protein and probe its function further using conditional mutants. As expected for a kinetochore protein, *ame1* mutants have a strong defect in chromosome segregation, and the difference in chromosome loss phenotype and Ctf19p interaction data between a C-terminal truncated mutant (*ame1-31*) and its full length equivalent (*ame1-3*) suggests that the C-terminal domain is important in mediating attachment to downstream components of the Ctf19 complex. This aspect of Ame1p function, similar to the non-essential components of the large Ctf19 complex, can be viewed as structural (Figure III.10.E). However, the data presented here, together with recent data published by other groups, suggest that the role of Ame1p may go further. For example, Ame1p could participate in a signal that triggers checkpoint systems to detect defects in kinetochore-microtubule attachments, leading to a sustained metaphase arrest to allow for repair of these defects. The lack of Ame1p is also hypothesized here to lead to premature spindle elongation and defective spindle migration into the bud, although the mechanim behind this defect and its link to a possible checkpoint maintenance role of Ame1p are still unclear.

As mentioned in Chapter III, the checkpoint component Bub3p has been shown to co-purify with Okp1p (De Wulf et al., 2003). Since Ame1p is tightly associated with Okp1p, one hypothesis is that Ame1p might influence the Okp1p-Bub3p interaction, for example by transmitting information about the state of microtubule attachment of the kinetochore. To test this hypothesis, experiments are in progress to examine Bub3p localization in *ame1* and *okp1* mutants, and the stability of the Ame1p-Okp1p interaction when Ame1p is not fully functional. As mentioned in Chapter III, Ame1p is important for the proper localization of Okp1p, and since the reverse is also true, kinetochore localization of these two proteins can

be viewed as interdependent (James Knockleby and Jackie Vogel, pers. commun.; De Wulf et al., 2003). These observations suggest that the two proteins function as a unit.

However, another component of the kinetochore that co-purifies with Ame1p is Sli15p (De Wulf et al., 2003), a finding that gives a clue to a possible role for Ame1p at the kinetochore. INCENP/Sli15p is a regulator of the Aurora/Ipl1p kinase (Carmena and Earnshaw, 2003; reviewed in Katayama et al., 2003; Kim et al., 1999); the two proteins exist in a complex with Survivin/Bir1p (Altieri, 2003; Yoon and Carbon, 1999). Ipl1p is involved in promoting biorientation of sister kinetochores (attachment of sisters to opposite poles) by sensing tension and causing high turnover of kinetochore-microtubule attachments as long as sisters are not under tension (Biggins and Murray, 2001; Biggins et al., 1999; Tanaka et al., 2002). As mentioned in Chapter I, kinetochores are linked to the SPB for most of the cell cycle, and thus biorientation of duplicated kinetochores requires detachment from the old SPB and reattachment to both the old and new SPBs, which will create tension; the Ipl1 complex is thought to have a major role in regulating kinetochore-microtubule dynamics so that proper bipolar attachment can be achieved. How exactly tension is sensed by the Ipl1 complex is still unclear, but recent experiments in yeast have shown that tension arising from a link between two sister kinetochores (by cohesin or other means) was sufficient to promote bipolar attachments (Dewar et al., 2004).

Ipl1p is a kinase that phosphorylates a number of kinetochore components, including its own regulator Sli15p, and Ndc10p, Dam1p (and several other Dam1 complex components), Ndc80p, as well as the chromatin components histone H3 and CENP-A (Biggins et al., 1999; Cheeseman et al., 2002a; Hsu et al., 2000; Kang et al., 2001; Zeitlin et al., 2001). Mammalian BUBR1, a component of the spindle assembly checkpoint, has also been proposed to be an Aurora substrate (Lens and Medema, 2003). Ipl1p action is thought to be antagonized by the phosphatase Glc7p (Hsu et al., 2000; Sassoon et al., 1999). The Dam1 complex, which binds microtubules, is thought to have an Ipl1p phosphorylation-dependent kinetochore affinity (Shang et al., 2003). Thus, the absence of tension would lead to release of the Dam1 complex-microtubule entity from the kinetochore; an exposed unattached kinetochore would then activate the spindle assembly checkpoint (summarized in Courtwright and He, 2002). Microtubule attachment regulation may also occur concomitantly with regulation of microtubule stability. In fact, vertebrate Aurora was

demonstrated to regulate disassembly of kinetochore microtubules until bipolar attachment was achieved (Lampson et al., 2004). Moreover, a novel vertebrate inner centromere protein, ICIS, was shown to be able to stimulate the microtubule depolymerization activity of the KinI family MCAK/XKCM1 catastrophe inducer. ICIS localizes similarly to Aurora/Ipl1p and INCENP/Sli15p prior to anaphase, at the inner centromere, and ICIS also physically interacts with Aurora/Ipl1p and INCENP/Sli15p. It was then hypothesized that ICIS-MCAK activity, possibly regulated by Aurora/Ipl1p phosphorylation, might contribute to the correction of improper kinetochore-microtubule attachments (Ohi et al., 2003). Distinct Aurora/Ipl1p-dependent mechanisms between yeast and vertebrates might arise because of the difference in the number of microtubules attached to each kinetochore: having more than one microtubule connected to a single kinetochore adds a source of incorrect attachment (one kinetochore can attach to both poles) and is likely to require additional levels of regulation.

Several kinetochore proteins, including the Ipl1 complex, relocalize to the spindle midzone in late anaphase; although the meaning of this localization is still unclear, the Ipl1 complex has been hypothesized to play a role in spindle disassembly, independent of its role in monitoring bipolar attachment (Buvelot et al., 2003). Interestingly, this aspect of Ipl1 complex function is regulated by Cdc14p-dependent dephosphorylation of Sli15p (Pereira and Schiebel, 2003).

The contribution of Sli15p to Ipl1p function is to stimulate its kinase activity and also to bind substrates of Ipl1p (Kang et al., 2001). Thus, Ame1p binding to Sli15p could be interpreted in several ways: Ame1p could be a substrate of Ipl1p (discussed below), and could thus (at least in part) mediate the destabilizing action of Ipl1p on microtubule-kinetochore attachment; alternatively, Ame1p could act upstream of Ipl1p, by signaling a defect in microtubule-kinetochore attachment (accompanied by lack of tension) to Ipl1p; finally Ame1p could be instrumental in mediating the proper kinetochore localization of the Ipl1p complex. If that last interpretation is true, lack of functional Ame1p is expected to result in mislocalization of components of the Ipl1 complex; Ipl1p function may then be impaired, and microtubule-kinetochore attachments would be stabilized. However, this would only be one aspect of the role of Ame1p, as *ame1* mutants do not have the same phenotypes as *ipl1* mutants. Experiments are currently in progress to address these ideas.

Could Ame1p be a possible phosphorylation target of Ipl1p? As seen in Figures III.3.D and III.7.D, Ame1p-Myc runs on Western blots as one main faster migrating band and a number of slower migrating bands present in lower amounts. This migration pattern is indicative of possible post-translational modifications. To investigate whether the slower migrating forms were due to phosphorylation, extracts of Ame1p-Myc, some of which had been treated with phosphatase or phosphatase + inhibitor, were analyzed by two-dimensional gel electrophoresis; preliminary results indicate that Ame1p is multiply phosphorylated; data also shows that levels of the slower migrating (phosphorylated) bands fluctuate over the cell cycle (data not shown; James Knockleby and Jackie Vogel, pers. commun.). These observations contradict earlier claims that none of the large Ctf19 complex components were modified by phosphorylation (Cheeseman et al., 2002a). Whether Ipl1p is the kinase responsible for modifying Ame1p is still unknown. The consensus sequence for Ipl1p phosphorylation was defined as R/K-x-T/S-L/V/I, where T/S is the site of phosphorylation (Cheeseman et al., 2002a). Ame1p contains one site that partially matches the consensus sequence (NKSL; see Figure III.3.A) and is predicted to be phosphorylated (Jackie Vogel, pers. commun.). Interestingly, the mutants analyzed here showed an altered pattern of post-translational modification, with *ame1-4* being the most severe (see Figure III.7.D); however, none of the mutations in *ame1-4* directly affect a phosphorylatable residue. Phosphorylation may affect Ame1p either by altering its structure, localization, interaction with other proteins, or by regulating its function. Experiments are now in progress to characterize the phosphorylation sites, define the kinase(s) involved, and subsequently analyze the role of these phosphorylations.

Although the main binding partners of Ame1p have now been defined from protein purifications analyzed by mass spectrometry, which clearly places Ame1p in the large Ctf19 kinetochore complex (Cheeseman et al., 2002a; De Wulf et al., 2003; Gavin et al., 2002), it cannot be excluded that Ame1p binds other proteins more transiently. As a clue to other possible partners, various large-scale two hybrid screens have yielded Rpt3p (a component of the proteasome), Did4p (a vacuolar sorting factor that showed interaction with other kinetochore components and chromatin remodelling factor components), Ies3p (a putative component of a chromatin remodelling factor), and Pac1p (a protein required for chromosome segregation and nuclear orientation, working in the dynactin pathway) (Ito et

al., 2001; Newman et al., 2000; Uetz et al., 2000). These putative interactors have not yet been confirmed by any other methods, but they might constitute the starting point for further investigation.

Finding new interacting partners for Ame1p may help to understand Ame1p's function and regulation at the kinetochore better. Two approaches were undertaken to investigate other possible partners of Ame1p, and will only be briefly presented here, as they have so far not yielded informative results. First, a number of strategically chosen HA-tagged proteins available in the Hieter laboratory, including Kar3p, Ase1p, Bim1p, Bik1p and Mad2p, were introduced in the Ame1p-Myc strain and co-immunoprecipitations were performed. The earlier report of Ame1p being associated with short microtubules at the SPB prompted the investigation of a possible connection between microtubule-associated proteins (Bim1p, Bik1p) or microtubule-associated motors (Kar3p) and Ame1p. Additionally, a reported two-hybrid interaction between Sap4p and Mad2p suggested that Ame1p may interact with Mad2p, since Ame1p has some homology to the SAP family of protein (see Chapter III). Mad2p is part of the spindle assembly checkpoint (see Chapter I); since the association of checkpoint proteins with the kinetochore is highly dynamic and responsive to the status of kinetochore-microtubule attachment, it is possible that a Mad2p-Ame1p interaction would only be observed in conditions which activate the checkpoint. Thus the Ame1p-Mad2p co-immunoprecipitation was also tested in the presence of NZ. Unfortunately, none of the directly tested proteins showed an interaction with Ame1p by co-immunoprecipitation under the conditions used (data not shown).

The second approach to look for additional partners of Ame1p was to perform a large scale immunoprecipitation of the Ame1p-Myc protein to obtain an amount of purified protein that was suitable for analysis by tandem mass spectrometry (Aebersold and Mann, 2003). The mass spectrometry analysis was performed by Dr. Mark Flory in Dr. Ruedi Aebersold's laboratory at the Institute for Systems Biology in Seattle, WA. The list of peptides generated by this analysis contained a large number of Okp1p hits, indicating that Okp1p was a definite partner of Ame1p and that it was potentially its closest partner (data not shown), in tune with data that was subsequently published by the Sorger laboratory (De Wulf et al., 2003). Two other members of the large Ctf19 complex, Ctf19p and Nkp1p, were represented by only one peptide. The hit list also included a number of other potential candidate interactors, but none

of them were represented by more than one or two peptides, and the level of confidence in the results varied (data not shown). To date none of these candidates have been investigated further, except for Ase1p, which was tested for co-immunoprecipitation as indicated above but did not reconfirm to be an interactor of Ame1p under the conditions used. In view of the lack of successful results from these attempts to isolate new partners of Ame1p, the search was not pursued further, although it does not exclude that protein interactors remain to be found. Different techniques, or different conditions with the two techniques described above, might need to be used.

Lastly, a clue to Ame1p's function might be obtained from further analysis of its sequence homologies. There are currently no obvious sequence homologs of Ame1p in other organisms; as mentioned above, one reason for the divergence between yeast kinetochore components and those in other organisms might be that these proteins are specialized to interact with the yeast sequence-specified short centromere, as opposed to centromeres mostly defined by other mechanisms such as chromatin structure and epigenetic modifications. For example, most components of the CBF3 complex, which is directly associated with *CEN* DNA, do not have homologs; the exception being Skp1p which functions in another conserved complex (see Chapter II). Thus, the inner layer of the kinetochore might differ, while complexes residing closer to the outer face of the kinetochore would show more conservation. A hypothesis that Ame1p lies very close to the inner kinetochore would thus predict that Ame1p is part of the yeast-specific kinetochore core. However, Ame1p does have a limited homology to a component of the dynein regulator complex dynactin (see Chapter III), which may be of some significance, especially in light of the proposed interaction with Pac1p, a yeast protein that interacts with dynactin/dynein (Lee et al., 2003). Yeast dynein functions mostly in spindle positioning and elongation (see Chapter I), whereas in mammals dynein appears to affect many processes (Karki and Holzbaur, 1999). Thus, the functions of vertebrate dynein may be accomplished by more than one protein in yeast, part of which could be achieved by a complex containing Ame1p.

IV.4. Chromosome segregation research and disease therapy

The value of this thesis work, beside contributing to an understanding of the fundamental principles that govern proper chromosome segregation, is its potential to provide avenues to

treat and prevent syndromes and diseases that occur through faulty chromosome transmission. It has been demonstrated that the uncontrolled proliferation of most cancer cells is due to alteration of many cell cycle progression regulators, including cyclin-Cdks and other kinases such as Aurora (Malumbres and Carnero, 2003). The conservation of many of these regulators renders yeast an attractive model system to understand their function and the consequences of their altered function; as mentioned in Chapter I, it is in yeast that checkpoint proteins were first described (Hoyt et al., 1991; Li and Murray, 1991). The validity of studies of cell cycle progression in model organisms has been recognized in the 2001 Nobel Prize for Physiology or Medicine, received by three researchers, Lee Hartwell, Tim Hunt and Paul Nurse, who pioneered this kind of research. Thus, it is likely that characterization of yeast kinetochore components and kinetochore function in general will lead to greater understanding of the process in all eukaryotes.

Moreover, knowledge of the specific functional mechanisms of these proteins that was gained over the past few years will provide a basis upon which to understand the molecular differences between normal and diseased cells, and allow the development of more targeted drugs with less side-effects. For instance, a major problem in the fight against cancer is the lack of specificity of drug action toward cancer cells as opposed to normal cells. Many of these drugs target the proliferative aspect of cell function, and thus will affect other normally highly proliferative cells such as those in the gut, hair follicles, and bone marrow, leading to undesirable side effects (e.g. diarrhea, hair loss, and decreased immune response). This toxicity to normal cells also limits the dose of the drug that can be used, resulting in less than 100% killing of the unwanted cells. To improve treatment, two solutions have been proposed (see below). One involves inducing an arrest in cell proliferation, and then targeting cells that are not able to arrest (such as cancer cells); another one is to selectively enhance treatment toxicity toward diseased cells (e.g., by targeting a protein that is mutated or deregulated specifically in those cells, or whose activity is essential in those cells and not in normal cells) (Keyomarsi and Pardee, 2003; for examples, see Reeves and Beckerbauer, 2003; Yoshida et al., 2003). Additionally, understanding the differences between viral, prokaryotic or lower eukaryotic, and mammalian cell cycle machineries would allow to target pathogens specifically (Hammarton et al., 2003).

As mentioned above, one property of cancer cells is that they divide at a high rate. Several drugs that are commonly used to treat cancers are part of a group of compounds that target microtubule dynamics, which is essential for the proper progression of mitosis. Examples of these compounds include colchicine, NZ and the vinca alkaloids (such as vinblastine), which inhibit microtubule polymerization, taxanes such as paclixitel and doctaxel, which promote microtubule stability, and noscapine and its derivatives; many of these agents interact directly with tubulin (Hadfield et al., 2003; Zhou et al., 2003). For example, paclitaxel (trade name Taxol) was shown to prevent the "breathing" of centromeric regions that depends on alternative cycles of polymerization and depolymerization of microtubules attached to kinetochores, and to shorten the average distance between sister centromeres that occurs when centromeres are under tension. Thus, the checkpoint remained activated and cells were blocked at the metaphase-to-anaphase transition (Kelling et al., 2003).

Only a subset of microtubule dynamics-interfering compounds are used for treatment, due to toxicity and lack of efficacy; development of resistance against the drugs by cancer cells has also been a problem (Zhou et al., 2003). Moreover, microtubules are involved in a variety of cellular processes beside cell division (e.g. cell cytoskeleton, axonal transport, etc.), and thus these drugs lack selectivity towards cancer cells. Better compounds might therefore be obtained if they target proteins that are solely involved in the mitotic process. For example, targeting kinetochore proteins or mitotic checkpoint proteins may result in chromosome missegregation, which in many cases leads to cell death. Possible compounds include specific kinase or motor protein (ATPase) inhibitors, or compounds that disrupt protein-protein interactions between kinetochore proteins, disrupting kinetochore assembly; the fact that many of these proteins are conserved throughout eukaryotes makes it possible to use yeast to screen compounds quickly. Additionally, increased sensitivity of cancer cells to treatment may be achieved by using combinations known anti-microtubule drugs and inhibitors of a specific kinetochore protein or other proteins involved in chromosome segregation, such as microtubule-associated proteins and motors (Jablonski et al., 2003; Miyamoto et al., 2003). The value of a search for drugs that affect microtubule-dependent pathways of cell division is not restricted to the fight against cancer; for example, the drug griseofulvin, used to treat fungal infections, was found to act by binding to MAPs and

therefore disrupt the mitotic cycle and prevent cell division; several other drugs to treat pathogenic fungi act by targeting their cell cycle (Bordon-Pallier et al., 2003).

Although anti-microtubule drugs will affect cancer cell growth by inducing a mitotic block through the spindle checkpoint, they might not always be effective as many tumor cells are checkpoint-defective and do not arrest upon treatment with these drugs. However, this observation can actually provide an advantage in the design of anti-cancer drugs. Indeed, while a high dose of the drug would affect both cancerous and non-cancerous cells, a lower dose might induce normal cells to activate the checkpoint until the drug effect is compensated, whereas cancer cells would continue through mitosis and misseggregate chromosomes, resulting in an aneuploidy which in the majority of cases would be detrimental and lead to cell death; similar effects might occur in cells defective for the DNA damage checkpoint treated with ionizing radiation, that would proceed through mitosis with detrimental DNA lesions (Jallepalli and Lengauer, 2001; Wassmann and Benezra, 2001). Additionally, the checkpoint deficiency of cancer cells can be used in a therapeutic strategy that targets a component of the chromosome segregation apparatus that is normally not absolutely necessary for cell survival but becomes essential if the checkpoint is defective. For example, depletion of mammalian HEC1, a homolog of the yeast kinetochore component Ndc80p, has catastrophic effects when MAD2 is concurrently inactivated (Martin-Lluesma et al., 2002). Other properties of cancers cells may also be exploited for treatment. For example, it was shown that the yeast homolog of the microtubule-interacting protein CLIP-170, Bik1p, was non-essential in haploid and diploid cells but became essential in polyploid cells (Lin et al., 2002). Since cancer cells often have altered ploidy, targeting of genes such as CLIP-170 may selectively affect cancer cells.

In summary, the fight against diseases such as cancer is likely to involve a combination of drugs that specifically target several pathways and takes advantage of the differences between normal and diseased cells to protect normal cells against the effect of the drugs and enhance susceptibility of the diseased cells to the drug (Keyomarsi and Pardee, 2003). With the aid of model organisms, the first step toward these goals is to identify and gain a thorough understanding of the normal mechanisms and pathways that sustain cell growth and division, for example those that govern the proper transmission of the genetic material.

IV.5. Conclusion

The work presented in this thesis aimed to further our understanding of kinetochore function in budding yeast, and to make a contribution to our knowledge of factors that mediate and regulate chromosome segregation in eukaryotes. Three proteins were studied in detail, and the characterization of their function is ongoing. Together with data from other groups, the data presented here allowed a more detailed description of the molecular organization of the yeast kinetochore and of the way in which microtubule attachment is regulated and monitored.

It is my hope that ultimately this work may provide, beside basic knowledge of cellular function, a tiny piece of the puzzle that will contribute to a faster, more streamlined and targeted development of treatments and prevention strategies in diseases where chromosome missegregation plays an important role.

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