Molecular Architecture of the *Saccharomyces cerevisiae* Kinetochore

by

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ABSTRACT

The kinetochore is a multiprotein complex capable of simultaneously binding centromeric DNA (*CEN*) and microtubules (MTs). In this capacity, the kinetochore is responsible for enabling sister chromatids to become attached to the mitotic spindle, thus ensuring proper chromosome segregation. Due to its small size and simple structure, the *Saccharomyces cerevisiae CEN* is particularly well suited for studying kinetochore assembly and function. Analysis of *S. cerevisiae* Ndc10p established the binding of this kinetochore protein to the CDEII element of the *CEN*, forming part of the DNA-Binding layer upon which the rest of the kinetochore is assembled. Alteration of the nucleotide bases deemed important for Ndc10p-*CEN* binding *in vitro*, have dramatic effects on chromosome segregation *in vivo*. Further characterization indicates that Ndc10p may also play a role in chromosome segregation at non-*CEN* locations.

A combination of live-cell imaging, biochemical and genetic techniques were used to identify eleven novel *S. cerevisiae* kinetochore subunits and elucidate their roles in microtubule attachment. Chromatin Immunoprecipitation (ChIP) Assays evaluated both the range of *CEN* DNA bound by these kinetochore proteins and determined the interdependencies required for their association with *CEN* DNA. Using this data, a preliminary model of the molecular architecture of the kinetochore is beginning to emerge. Dynabead-Kinetochore Reconstitution Assays suggest an interaction between the CBF3 Complex bound to *CEN* DNA and Ndc80p, Cse4p, Mif2p and Ame1p, adding further insight into kinetochore assembly. The spindle assembly checkpoint monitors proper bipolar attachment of sister chromatids to the mitotic spindle. Microscopy and ChIP demonstrate that the spindle checkpoint proteins Bub1p and Bub3p are recruited to the kinetochore as part of the normal cell cycle in budding yeast, whereas Mad1p and Mad2p are recruited only in response to damage to the *CEN*-kinetochore-MT connection. Analysis of specific *S. cerevisiae* kinetochore mutants indicates that attachment status is the signal recognized by the spindle checkpoint to allow the metaphase-to-anaphase transition to occur.

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Publications

Gillett ES *, Espelin CW * and Sorger PK (2004) Spindle Checkpoint Proteins and Microtubule-Kinetochore Attachment in Budding Yeast; *The Journal of Cell Biology,* Vol. 164 Number 4, 535-546 *(* authors contributed equally)*

Espelin CW *, Simons KT *, Harrison SC and Sorger PK (2003) Binding of the Essential *Saccharomyces cerevisiae* Kinetochore Protein Ndc10p to CDEII; *Molecular Biology of the Cell*, Vol. 14, Issue 11, 4557-4568. *(* authors contributed equally)*

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CHAPTER 1

Introduction to the Kinetochore

Accurate chromosome segregation requires proper assembly of the kinetochore, a multi-protein complex that simultaneously binds centromeric DNA (*CEN*) and microtubules (MTs). In this capacity, the kinetochore is essential for enabling chromosomes to become attached to the mitotic spindle, a necessary function for ensuring the equal distribution of genetic material between a mother and daughter cell during mitosis. Following DNA replication, one and only one kinetochore must assemble on each sister chromatid. Failure to form a functional kinetochore results in loss of a chromatid, while formation of more than one kinetochore on a single chromatid causes the chromosome to be simultaneously pulled in opposite directions and torn apart. The two sister kinetochores must form a bipolar attachment to the mitotic spindle, meaning one chromatid attaches to MTs emanating from one spindle pole body (SPB), while its sister becomes attached to MTs from the opposite SPB. The force generated by this bipolar attachment is sufficient to transiently separate the centromeres of sister chromatids from each other during metaphase (this is distinct from the complete separation of sister chromatids which occurs during anaphase). This ensures that each of the pairs of sisters will be pulled towards opposite poles of the spindle at the metaphase-anaphase transition, resulting in one copy of each chromosome in each of the resultant cells. It is essential that every chromatid pair establish bipolar attachment before separation of the sister occurs and it is the function of the spindle assembly checkpoint (spindle checkpoint) to halt progression through mitosis until these attachments have been properly formed. A complex of highly conserved checkpoint proteins monitors the *CEN*-kinetochore-MT connection and in response to damage, interacts with the cell cycle machinery to allow time for corrections to be made before proceeding.

The significance of the kinetochore in chromosome segregation and the implications of kinetochore defects for human disease cannot be overstated. Aberrant kinetochore function

results in aneuploidy and genome instability which are common features of most cancer cells and many human birth defects including Down's and Klinefelter's Syndromes (Cahill et al., 1998; Hassold and Hunt, 2001). Alteration of spindle checkpoint genes have been implicated in cancers including colorectal, breast, and lung cancer (Cahill et al., 1998; Lengauer et al., 1998; Lee et al., 1999; Michel et al., 2001). The exact cause and consequences of kinetochore and spindle checkpoint damage in cancer are not fully understood at this time, but the clinical correlations and theoretical consequences (such as loss of a tumor suppressor gene) suggest a strong link between errors in chromosome segregation and the occurrence of cancer. We are only now beginning to understand, on a molecular level, the key roles that the kinetochore and spindle checkpoint play in chromosome segregation, and with this knowledge we will hopefully begin to understand what goes wrong in disease states.

Despite recent advances in chromosome biology, there are many questions that require further investigation before we can fully appreciate how kinetochores establish the connection between chromosome and microtubule. Most fundamentally, what is the full set of kinetochore proteins? Answering this question will likely include the continued application of techniques such as protein purification and mass spectrometry which have proven successful thus far in identifying novel kinetochore proteins. The results will have implications for our ability to answer subsequent mechanistic questions about kinetochores. How do kinetochore proteins recognize the centromere and how are they organized on the DNA? How do the proteins of the kinetochore interact and relate with each other? How does the spindle checkpoint monitor proper bipolar attachment? Classical genetic methods in combination with recent advances in microscopy and molecular biology make *Saccharomyces cerevisiae* a powerful model organism in which to study the structure and function of the kinetochore. In an attempt to obtain answers

to some of the aforementioned questions, this thesis will focus on experiments carried out in *S. cerevisiae* but it is hoped that lessons learned in budding yeast will be applicable to other organisms. Comparisons between higher and lower eukaryotes will therefore be made where appropriate.

CENTROMERIC DNA

S. cerevisiae Centromere

The centromere is the region of the chromosome that specifies where binding of the kinetochore proteins occurs and is therefore the foundation for kinetochore assembly. The importance of the centromere can readily be appreciated in organisms such as budding yeast in which the centromere is bound by a single microtubule (Peterson and Ris, 1976; Winey et al., 1995). In *S. cerevisiae*, a 125 bp region of the chromosome has been determined to be necessary and sufficient to function as a centromere during mitosis and meiosis (Clarke and Carbon, 1980; Cottarel et al., 1989). The relatively small size of the budding yeast centromere presents a situation which is particularly amenable to studying both the centromeric DNA itself and *CEN* DNA-binding proteins. For example, single base mutations have been evaluated over large stretches of the *S. cerevisiae* centromere *in vitro* and *in vivo*, demonstrating that the DNA bases vary with regard to their importance for proper chromosome segregation (McGrew et al., 1986; Hegemann et al., 1988). Such an approach is currently impractical in most other eukaryotic organisms given the large size of their centromeres and apparent lack of sequence selectivity (see Other Centromeres below).

Comparison of all 16 *S. cerevisiae* centromeres has identified three conserved regions, denoted Centromere Defining Elements I, II and III (CDE I,II,III; Fig. 1-1A; Fitzgerald-Hayes et

al., 1982; Clarke and Carbon, 1985; Fitzgerald-Hayes, 1987). CDEI is an 8 bp imperfect palindrome which is bound by the Cbf1 protein and although neither the CDEI region nor Cbf1p are essential, disruption of either increases chromosome loss 10-fold, indicating a potential secondary or stabilizing role in kinetochore function (Bram and Kornberg, 1987; Hegemann et al., 1988; Baker et al., 1989; Cai and Davis, 1989). The sequence of CDEII varies somewhat between *S. cerevisiae* centromeres but is characterized by a length of 78-86 bp and high A-T composition (>90%; Clarke and Carbon, 1980; Fitzgerald-Hayes et al., 1982; Clarke and Carbon, 1985; Fitzgerald-Hayes, 1987). The conserved features of CDEII have been suggested to play two roles in centromere organization: the A-T composition has been predicted to create steric constraints based on the intrinsic bend of the DNA and the conserved length has been proposed to constitute a "spacer" between CDEI and CDEIII. CDEII is essential for centromere function and although point mutations are relatively inconsequential, large-scale deletions, insertions or replacements have a dramatic effect on chromosome segregation (Panzeri et al., 1985; Gaudet and Fitzgerald-Hayes, 1987). The data presented in Chapter 2 of this thesis shows that Ndc10p binds to CDEII, the first demonstration of direct interaction between a protein and this DNA element. CDEIII is an imperfect palindrome of 25 bp and point mutations in the central CCG of this element are capable of rendering the entire centromere nonfunctional *in vivo*, demonstrating the critical importance of this DNA element (McGrew et al., 1986; Ng and Carbon, 1987; Hegemann et al., 1988). Consistent with this absolute requirement, CDEIII is the binding site for the CBF3 Complex, which has been shown to be required for the binding of all other kinetochore proteins *in vivo* (Lechner and Carbon, 1991; Espelin et al., 1997; He et al., 2001; De Wulf et al., 2003). The sequence of the *S. cerevisiae* centromere not only determines which proteins bind to

the DNA, but also establishes their spatial relationships to each other and in doing so creates a proper foundation upon which the kinetochore is built.

Other Centromeres

The *S. cerevisiae* centromere is unique in its small size and simple structure relative to the *CEN* domains of other organisms. *S. pombe* centromeres encompass approximately 40-100kb and are composed of a nonconserved central core flanked by inner and outer repeat sequences (Clarke et al., 1986; Chikashige et al., 1989; Clarke and Baum, 1990; Clarke, 1998). Human centromeres are estimated to span megabases and contain numerous copies (1,500-30,000) of a 171 bp α-satellite DNA element, which is apparently neither sufficient nor necessary for centromere function (Fig. 1-1B and 1-1C; Depinet et al., 1997; Bjerling and Ekwall, 2002; Cleveland et al., 2003). It has been proposed that the *CEN* DNA of higher organisms may represent multiple repeats of the simpler *S. cerevisiae CEN*, and although this may be true at a protein level, DNA sequence data alone does not support such a model. The chromosomes of different organisms also differ with respect to the number of microtubules bound to the centromere: one per centromere in *S. cerevisiae*, 2-4 per *S. pombe* centromere and 30-40 per centromere in many higher organisms (Fig. 1-1D; Rieder, 1982; Ding et al., 1993; Winey et al., 1995; O'Toole et al., 1999). Another variation is the number of centromeres per chromosome as *C. elegans* and many plants are holocentric, meaning they contain multiple "centromeres" per chromosome. In *C. elegans* however, these multiple centromeres coalesce during mitosis into a single dot-like structure which is not much larger than a mammalian centromere and functions as a single unit (Fig. 1-1D; Dernburg, 2001; Moore and Roth, 2001). If one were simply to compare the centromeric DNA sequences of different organisms, it would

not be apparent that anything learned about the role of *CEN* organization and function in one model organism would be relevant to another. However, despite the differences in DNA sequence and structure, it is believed that the same objective exists for all centromeres-namely, facilitating attachment of chromosomes to the mitotic spindle. Based on this common function, it is hoped that lessons learned in budding yeast will aid in understanding the role of the centromere in other organisms, including humans.

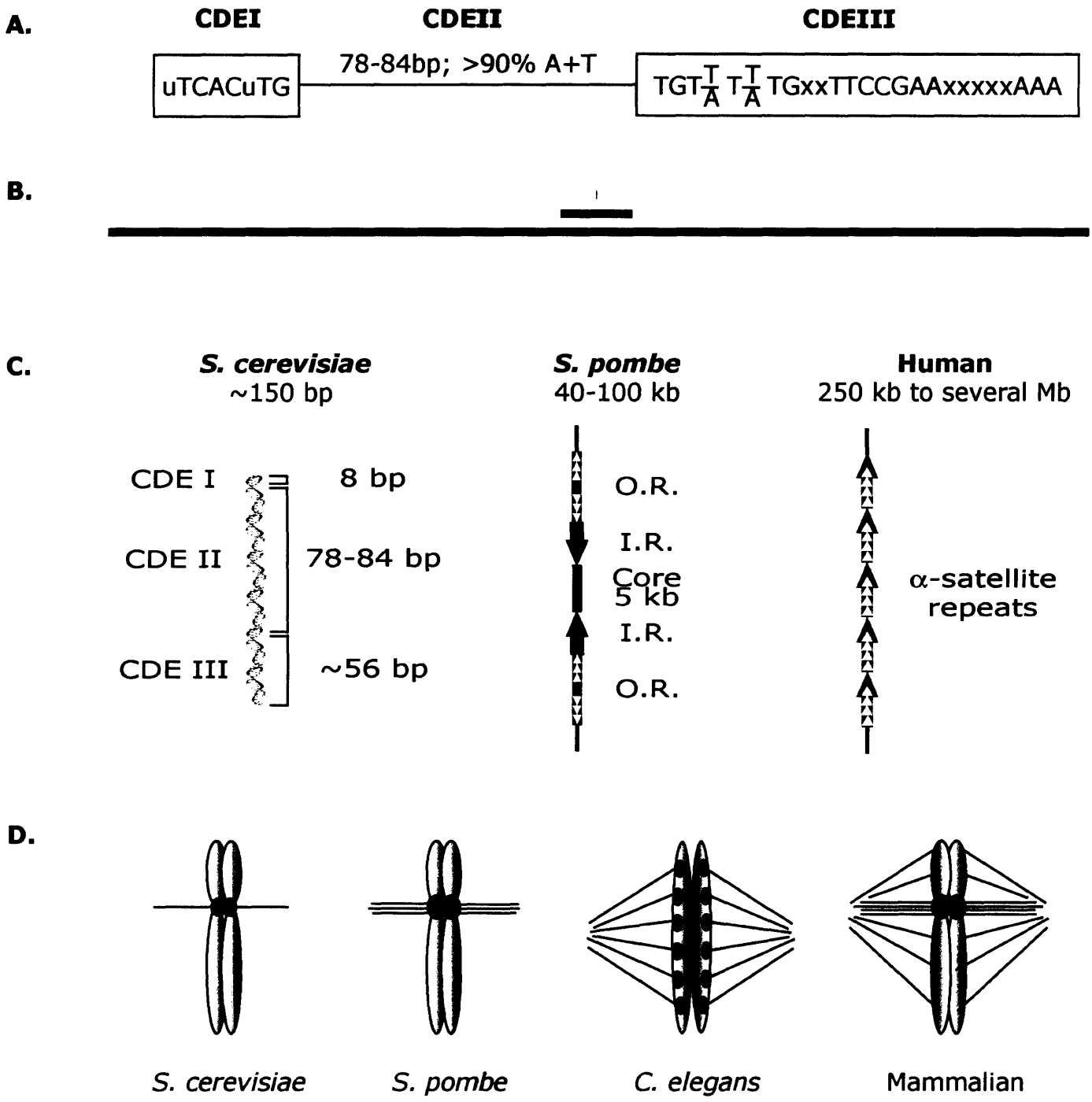


Figure 1-1 Centromere sequence and organization. (A) Consensus sequence from 16 *S. cerevisiae* centromeres demonstrating the conserved CDE I, II, III regions. x=any base; u=purine; the central CCG of CDEIII is highlighted in red. (B) Scale comparison of *S. cerevisiae* (top), *S. pombe* (middle) and human (bottom) centromeres. (C) Comparison of centromere organization in *S. cerevisiae*, *S. pombe* and humans. I.R.=inner repeat, O.R.=outer repeat (D) Comparison of MT-centromere attachment of *S. serevisiae*, *S. pombe*, *C. elegans* and mammalian chromosomes. Thin lines represent MTs attached to chromosomes via kinetochores (black circles) or chromosome arms (mammalian).

S. CEREVISIAE KINETOCHORE

CBF3

The kinetochore does not function solely as a rigid structural bridge, but must also sense and generate tension in conjunction with the microtubules and signal to the spindle checkpoint concerning attachment status. A kinetochore must therefore perform multiple functions suggesting that either a few proteins have multiple duties, or many proteins have distinct roles. A number of genetic screens initially identified genes that play roles in chromosome transmission fidelity, but it was the purification of the Centromere Binding Factor Complex (CBF3) by Lechner and Carbon that provided a major advance in our understanding of the *S. cerevisiae* kinetochore (Lechner and Carbon, 1991). Using a combination of classic biochemical purification and DNA affinity chromatography, the original purification identified a three protein complex (now known to contain four members) capable of specifically binding to *CEN* DNA. The CBF3 Complex consists of Ndc10p (p110/CBF3A/CTF14), Cep3p (p64/CBF3B), Ctf13p (p58/CBF3C) and Skp1p (p23). The genes for each of these proteins were subsequently identified and all determined to be essential for viability (Doheny et al., 1993; Goh and Kilmartin, 1993; Strunnikov et al., 1995; Connelly and Hieter, 1996). Mutations in any of the CBF3 genes, as well as mutations in CDEIII that interfere with CBF3 protein binding *in vitro*, are associated with dramatically increased chromosome loss rates *in vivo* (Jehn et al., 1991; Doheny et al., 1993; Goh and Kilmartin, 1993; Strunnikov et al., 1995; Connelly and Hieter, 1996). UV-crosslinking, bandshift assays (gel-retardation assays) and ChIP including the use of recombinant or yeast-purified CBF3, have demonstrated that Ndc10p, Cep3p and Ctf13p bind directly to *CEN* DNA *in vitro* and *in vivo* (Sorger et al., 1994; Espelin et al., 1997; Kaplan et al., 1997). However, only one of the CBF3 proteins-Cep3p, contains a DNA binding motif which is

recognizable by sequence analysis: an approximately 30 amino acid $Zn(II)_2Cys_6$ zinc cluster. This motif is typical of a class of transcriptional regulators that include Gal4p, Ppr1p and Hap1p, which bind as homodimers to direct or inverted CCG repeats (Marmorstein et al., 1992; Marmorstein and Harrison, 1994; Zhang and Guarente, 1994). Similarly, Cep3p has also been shown by UV crosslinking to bind the essential CCG of CDEIII (likely as a homodimer), but unlike the other members of the zinc cluster family, this binding requires additional members of the CBF3 complex and involves a single CCG (Fig. 1-2). Mutation of bases in the Cep3p zinc cluster that correspond to those required for DNA-binding in Gal4p and Prp1p result in increased chromosome loss *in vivo*, further supporting the importance of Cep3p's DNA binding ability in establishing a functional kinetochore (Lechner, 1994). Skp1p is required for the phosphorylation-dependent activation of Ctf13p, a function which may regulate the amount of active CBF3 complex in the cell (Kaplan et al., 1997; Rodrigo-Brenni et al., 2004). Skp1p is also a component of the ubiquitin-ligating SCF complex (Skp1-Cullin-F box), which targets proteins for degradation via the proteasome (Feldman et al., 1997; Skowyra et al., 1997). Although Ctf13p contains an F-Box motif, a requirement for association with the SCF, the exact role of Skp1p in mediating ubiquitin-dependent regulation of kinetochore components remains unclear.

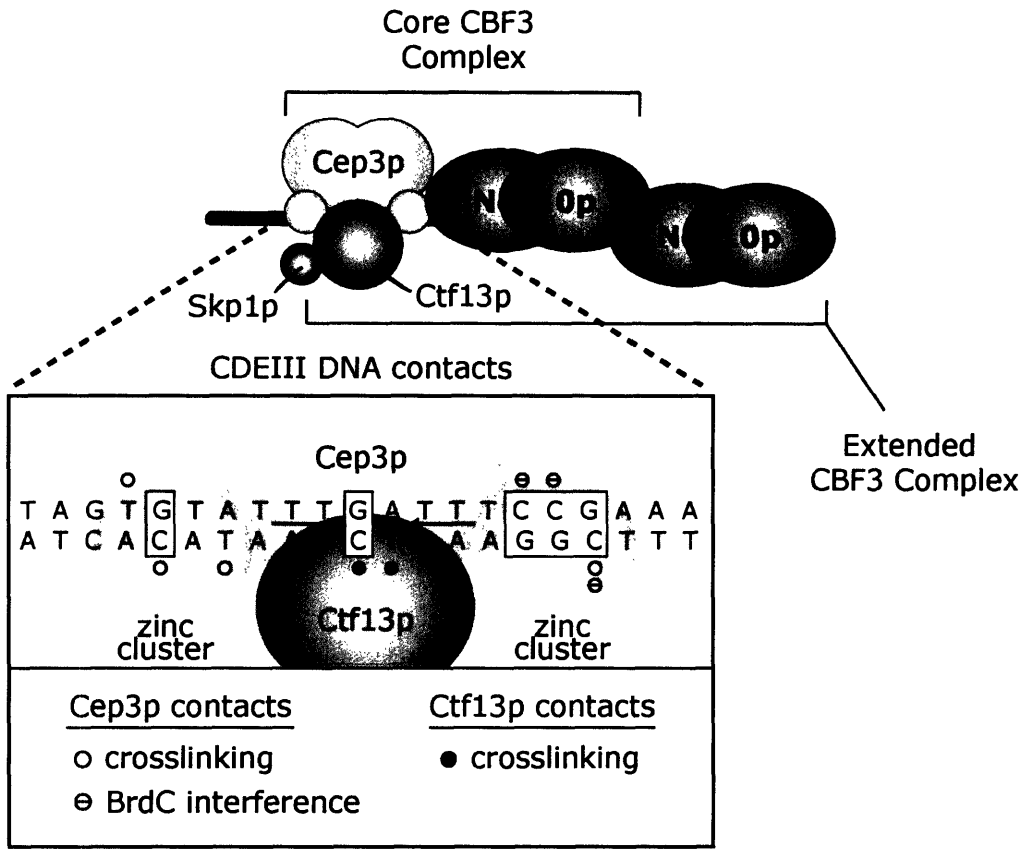


Figure 1-2 Binding of the CBF3 core to conserved bases in CDEIII of the *S. cerevisiae* centromere. Ctf13p, Cep3p and Ndc10p make contact with the major groove of *CEN* DNA. Inset shows the bases that crosslink to CBF3 subunits, or interfere with CBF3 binding when replaced by bromodeoxycytidine derivatives. The extended CBF3 complex containing an additional Ndc10p dimer assembles on *CEN* DNA by making contacts with bases proximal to the CDEIII core. Model based on results from Espelin et al., 1997.

Regulation of CBF3

Initial estimates of CBF3 protein levels in yeast extract suggested that there might be as little as one copy of CBF3 per centromere (Lechner and Carbon, 1991). Hydrodynamic analysis and UV-crosslinking experiments indicate that CBF3 exists as a complex consisting of a homodimer of Cep3p, a heterodimer of Ctf13p with Skp1p, and at least one homodimer of Ndc10p (Espelin et al., 1997; Russell et al., 1999; De Wulf et al., 2003). As has been mentioned before, the presence of multiple kinetochores along the chromosome increases the likelihood of the chromosome being torn as chromosome segregation proceeds, and tight regulation of CBF3 levels and activity may prevent this occurrence. Low CBF3 levels may increase DNA binding

specificity or as a function of stoichiometry, regulate the ability of other kinetochore proteins to associate with the centromere. Additional control of CBF3 is exerted by Skp1p's activation of Ctf13p which appears to be the limiting factor in assembling an active CBF3 complex (Russell et al., 1999). The combination of low protein levels, regulation of CBF3 activation and highly specific DNA affinity ensures that *S. cerevisiae* assembles one and only one kinetochore per chromosome.

MT Binding Capabilities

Proteins in *S. cerevisiae* whole cell extracts are capable of mediating the binding of *CEN*-coated beads to Taxol-stabilized MTs *in vitro*, while mutant extracts from cells carrying mutations in CBF3 cannot (Sorger et al., 1994). However, purified or recombinant CBF3 alone is not capable of mediating the same binding, indicating that CBF3 is necessary but not sufficient for MT binding (Sorger et al., 1994; K. Kaplan-unpublished observations). These results indicate that in addition to binding CDEIII, CBF3 is responsible for interacting (directly or indirectly) with additional kinetochore proteins including those that directly bind MTs. Chromatin Immunoprecipitation (ChIP; see Assays below) has shown that CBF3 is required for the recruitment of all other known kinetochore proteins to the centromere, and thus a reasonable assumption is that CBF3 initiates kinetochore formation. Verification of this model will require determination of the order of assembly for other kinetochore proteins, something that is currently underway. Our understanding of CBF3 has increased dramatically in the past 13 years, but many questions remain about this central player in kinetochore formation. Despite the identification of some protein-nucleotide interactions, we do not know the full extent of the CBF3-centromere interactions, including contact with CDEII and the relation between binding at CDEII and

CDEIII. What is the conformation of the *CEN*-CBF3 Complex-linear, bent, elliptical? Which proteins bind directly to the CBF3 proteins to form the next layer of the kinetochore? When does CBF3 bind to the centromere? It would seem likely that binding will occur during S-phase when a newly replicated centromere is first formed, but this has not been determined experimentally. Techniques and reagents required to answer many of these questions are currently available, and will hopefully be utilized in the near future to better understand how the direct *CEN*-CBF3 interaction forms the basis for assembly of the rest of the kinetochore.

Other Kinetochore Proteins Identified in Early Experiments

Cbf1p: Centromere binding factor 1 (CBF1, CBP1, CP1 and CPF1) is a DNA binding protein that contains a helix-turn-helix domain and binds to CDEI as a homodimer (Bram and Kornberg, 1987; Jiang and Philippsen, 1989; Cai and Davis, 1990). Deleting or mutating *CBF1* does not impair viability but increases chromosome loss rates 10-fold *in vivo*, indicating that Cbf1p is important, but not essential, for proper kinetochore function (Mellor et al., 1990; Foreman and Davis, 1993). Several experiments show that Cbf1p mediates its effects by binding to CDEI. *CEN* plasmids lacking CDEI are lost at an equivalent rate in wildtype and *cbf1Δ* backgrounds, whereas mutations in CDEI that decrease Cbf1p binding *in vitro*, increase the *in vivo* loss rates of plasmids or linear chromosome fragments containing those mutations (Baker et al., 1989; Cai and Davis, 1989). *In vivo* footprinting experiments demonstrate protection of CDEI from dimethyl sulfate methylation in wildtype cells, but not in *cbf1Δ* cells (Densmore et al., 1991). Taken together, these results show that Cbf1p binds CDEI and this interaction is important for correct chromosome segregation.

In addition to its role in chromosome segregation, Cbf1p plays a role in the transcriptional regulation of a variety of genes involved in amino acid metabolism. CDEI sequences are found in the upstream activating sequences (UAS) of the *MET16* and *MET25* genes, and *cbf1Δ* cells display methionine auxotrophy (Bram and Kornberg, 1987; Kent et al., 1994). A Cbf1p/Met28p/Met4p complex can bind CDEI in the UAS of *MET16* and is believed to clear nucleosomes from the DNA binding sites used by general transcription factors (Kent et al., 1994; O'Connell et al., 1995; Kuras et al., 1997). It remains unknown whether Cbf1p plays a similar role in nucleosome organization at the *S. cerevisiae* centromere.

Cse4p: *CSE4* was identified in a genetic screen for mutations that increase the loss rate of an endogenous chromosome containing deleted CDEII sequences (diploid strain; Stoler et al., 1995). Sequence comparison indicates that *CSE4* shares a region of significant homology with histone H3, and it is believed to be part of a specialized nucleosome that is bound to the *S. cerevisiae CEN* (Meluh et al., 1998). *cse4Δ* cells are inviable, while *cse4-1* temperature-sensitive mutants exhibit defects in chromosome segregation and provoke a mitotic checkpoint arrest (Stoler et al., 1995). In addition to genetic interactions between *CSE4* and CDEII DNA, both physical and genetic association with a number of other kinetochore proteins have also been reported, although the significance of these interactions remains undetermined (Stoler et al., 1995; Chen et al., 2000; Keith and Fitzgerald-Hayes, 2000; Westermann et al., 2003).

Although Cse4p has never been shown to bind DNA directly, largely due to the difficulties in producing a recombinant Cse4p-containing nucleosome, it is assumed that this specialized nucleosome behaves much the same as other nucleosomes, and directly contacts DNA. However, the position of the Cse4p-nucleosome with regard to the *CEN*, as well as the

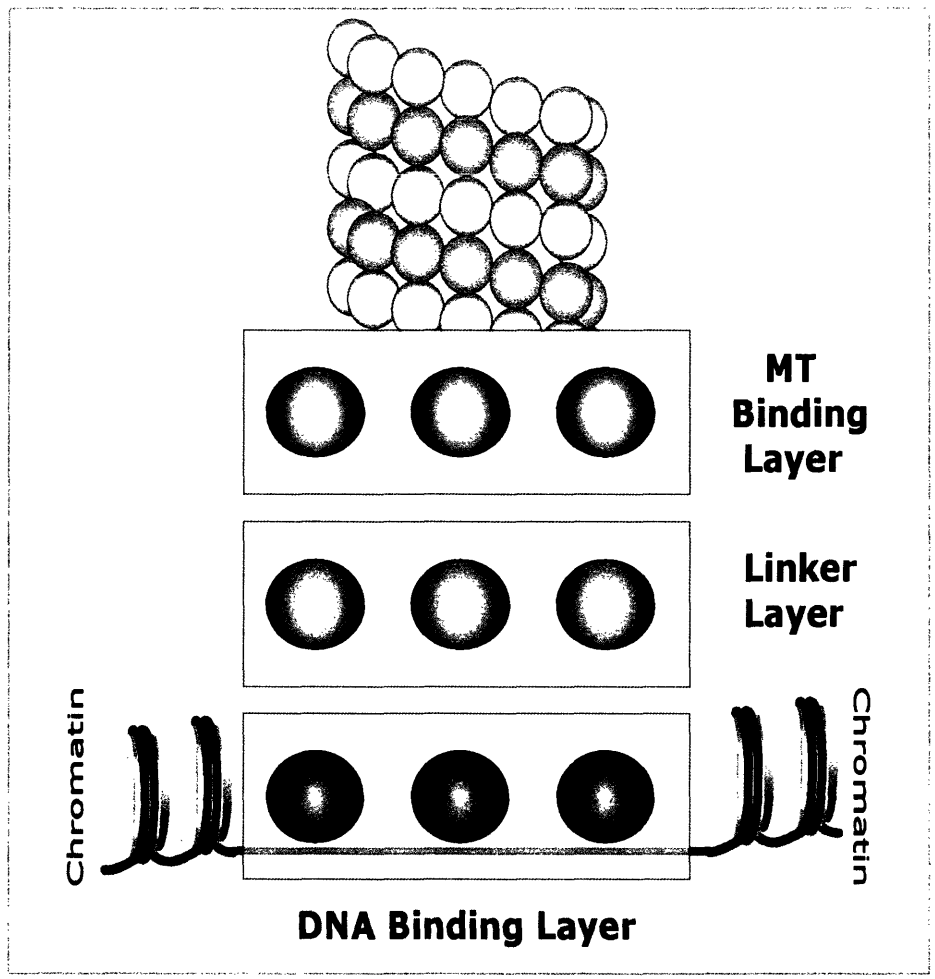
other kinetochore proteins remains undetermined. It is also unknown what features of Cse4p cause it to become selectively bound to centromeres, although the unique N-terminal tail is predicted to play a role.

Mif2p: *MIF2* was identified as a gene that increases chromosome loss when overexpressed. Loss of function mutations in *MIF2* demonstrate increased chromosome missegregation, impaired spindle integrity and a G2/M arrest; the null mutant is inviable (Meeks-Wagner et al., 1986; Brown et al., 1993; Meluh and Koshland, 1995; Meluh and Koshland, 1997). Mif2p localizes to *CEN* DNA in a CBF3-dependent manner *in vivo*, and synthetic lethality between mutants of *MIF2* and *NDC10* or *CEP3* indicate a potential interaction between Mif2p and the CBF3 Complex (Meluh and Koshland, 1995; Meluh and Koshland, 1997). Sequence analysis shows that *MIF2* contains an eight amino acid "A-T hook" motif which allows HMG-I(Y) proteins to bind the minor groove of A+T-rich DNA (Reeves and Nissen, 1993). This motif would seem to make Mif2p an attractive CDEII-binding partner, but deletion of the A-T hook region results in a protein that is functional *in vivo* (M. Jaffe-unpublished observation).

Recently Identified Kinetochore Proteins

The utilization of improved techniques such as mass spectrometry (described below) and an increase in the number of investigators in the *S. cerevisiae* kinetochore field has led to an explosion in the number of identified kinetochore components in the last 3-4 years to more than sixty. Thus, even the supposedly simple *S. cerevisiae* kinetochore is a very complex structure comparable in size and composition to other cellular organelles such as the nuclear pore complex and transcription machinery. It is of considerable interest to consider how the kinetochore is

assembled into a higher-order structure. Is it loaded onto the centromeric DNA as a single pre-assembled complex, as a number of subcomplexes or as individual proteins? Research from a number of groups has shown that kinetochore proteins are capable of existing both as members of discrete subcomplexes and as individual proteins (Ortiz et al., 1999; Cheeseman et al., 2001a; Janke et al., 2001; Wigge and Kilmartin, 2001; Cheeseman et al., 2002; Euskirchen, 2002; De Wulf et al., 2003). There appears to be a hierarchy of assembly as every kinetochore protein examined thus far requires the CBF3 Complex to associate with the centromere, while CBF3 does not require any other kinetochore protein to bind the same DNA (see ChIP in Assays). Based on our current knowledge about each kinetochore protein, we can classify kinetochore proteins as existing in three categories or "layers" (Fig. 1-3). The DNA-Binding layer is composed of proteins which bind directly to DNA and thus far includes Cbf1p and Cse4p (presumed), as well as Ndc10p, Cep3p and Ctf13p of the CBF3 Complex. The MT-Binding layer consists of those proteins which have been shown to bind directly to microtubules, and includes Stu2p, Bik1p, the DAM1 Complex and the motor proteins Cin8p, Kip1p and Kip3p. The middle category is the "Linker" layer and, for lack of better understanding at this time, includes all proteins (NDC80 Complex, MTW1 Complex, Mif2p, spindle checkpoint proteins, etc.) which do not fit in either of the other two categories. Our understanding of the exact organization of the kinetochore proteins in each of these layers, with regard to the DNA, MTs and each other, remains rudimentary at this point. However, the combined results from many researchers are slowly beginning to identify the manner in which these proteins come together to define the molecular architecture of the kinetochore.



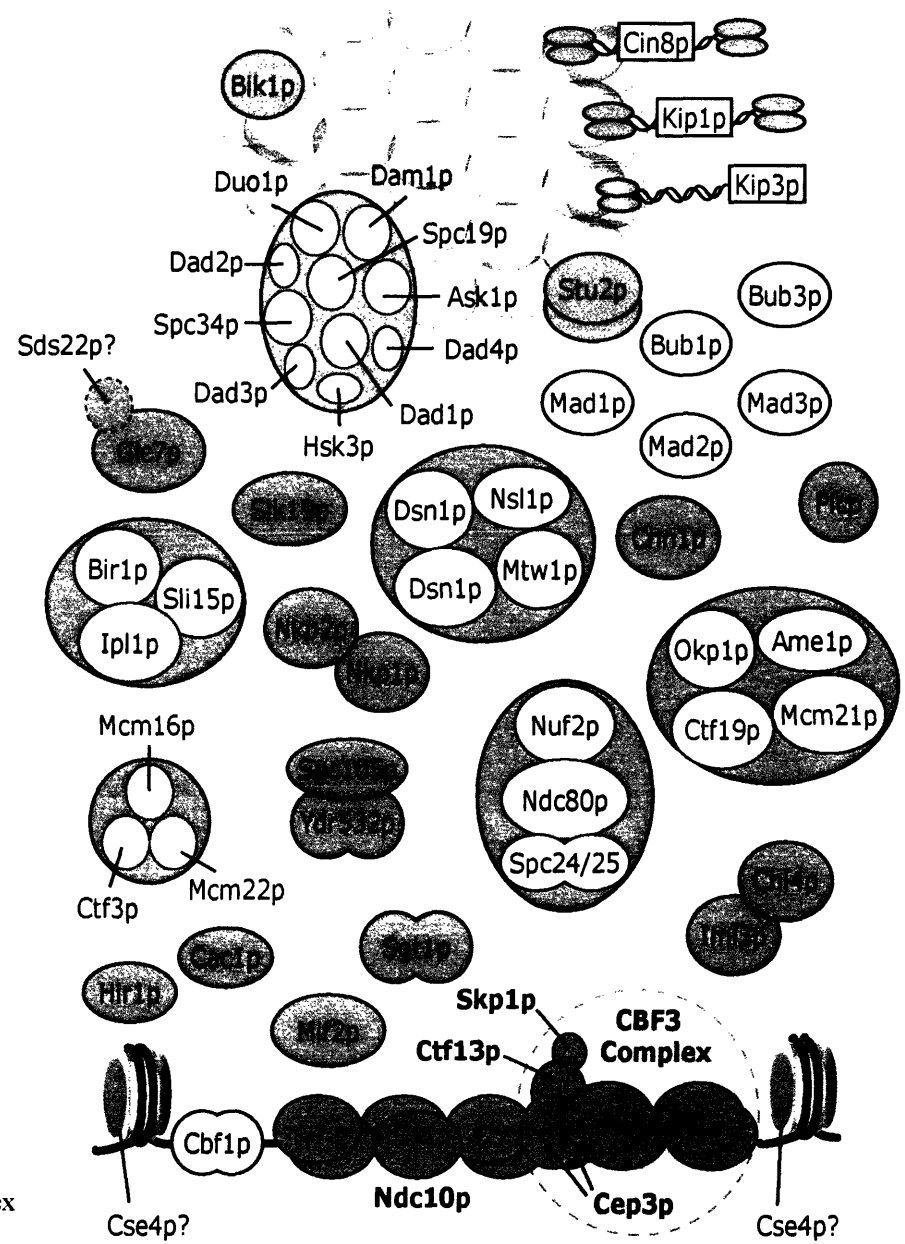


Figure 1-3 *Saccharomyces cerevisiae* kinetochore organization. (*Left*) Classification of kinetochore proteins into DNA-Binding, MT-Binding or Linker layers. (*Right*) Current model showing known kinetochore proteins with their designations as DNA-Binding (blue), MT-Binding (orange) or Linker (green). Spindle checkpoint proteins are in yellow. Protein names in red are non-essential. Proteins encompassed together or touching each other have been shown to exist as a complex, but no spatial relationships are inferred within the complex or between proteins and complexes. Figure is not drawn to scale.

NDC80 Complex: The NDC80 Complex is composed of four subunits-Ndc80p, Nuf2p, Spc25p and Spc24p-all of which are essential for viability and localize to the kinetochore *in vivo* (He et al., 2001; Janke et al., 2001; Wigge and Kilmartin, 2001). Mutations in any one of the components of the NDC80 Complex cause complete detachment of the chromosomes from the spindle, while mutations in Spc24p and Spc25p also eliminate association of Bub1p, Bub3p and Mad2p with the kinetochore, resulting in inactivation of the spindle checkpoint (Janke et al., 2001; Wigge and Kilmartin, 2001; Gillett et al., 2004). The NDC80 Complex requires the DNA-binding protein Ndc10p to associate with *CEN* DNA *in vivo* and in turn, the NDC80 Complex is required for the association of the MT-binding components Dam1p and Stu2p with the kinetochore (He et al., 2001; Janke et al., 2002). These results make the NDC80 Complex an attractive candidate to form a link between DNA-binding and MT-binding components of the kinetochore, as well as provide a site for interaction with the proteins of the spindle checkpoint.

MTW1 Complex: The MTW1 Complex is a four protein subcomplex composed of Mtw1p, Nsl1p, Nnf1p and Dsn1p, all of which are essential for viability in *S. cerevisiae*. Mutation of the proteins in this complex exhibit increased chromosome loss and arrest as large-budded cells with spindles of variable length (Goshima and Yanagida, 2000; Euskirchen, 2002; Nekrasov et al., 2003). *mtw1-1*, *nnf1-17* and *nsl1-5* mutants also demonstrate reduced transient sister separation, indicating a lack of tension being exerted across the sister chromatids despite apparent bipolar attachment to the spindle (Nekrasov et al. 2003; DeWulf et al., 2003; see Assays below). The biochemical basis of these phenotypes remains to be understood.

COMA Complex: The COMA Complex consists of Ctf19p, Okp1p, Mcm21p and Ame1p. The

members of this complex have been shown to localize to *CEN* DNA *in vivo* and are required for faithful chromosome segregation (Hyland et al., 1999; Ortiz et al., 1999; De Wulf et al., 2003). Okp1p and Ame1p are essential for viability whereas Ctf19p and Mcm21p are not. Ame1p and Okp1p can in fact form a stable complex without Ctf19p and/or Mcm21p, and may represent intermediates in the formation of the four protein COMA Complex (DeWulf et al., 2003). COMA mutants demonstrate increased sensitivity to benomyl and a G2/M delay, typical of a role in kinetochore function, whereas *okp1-5* and *ame1* degrons show reduced transient sister separation, indicating a lack of tension across sister chromatids (Hyland et al., 1999; Poddar et al., 1999; De Wulf et al., 2003; A. McAinsh-unpublished observation). Ctf19p has variously been proposed to interact with CBF3, Cse4p, Mif2p and members of the mitotic checkpoint based on genetic interactions, 2-hybrid screens and Co-IP results (Hyland et al., 1999; Ortiz et al., 1999; K. Simons-unpublished observations). Recent experiments indicate that Ame1p may bind CBF3, either directly or indirectly (C. Espelin-unpublished observations; see Chapter 5). Ctf19p has also been proposed to be a member of a larger 12 protein complex, but more accurate biophysical data using column chromatography and glycerol gradients indicates that this larger complex may represent a transient interaction between distinct subcomplexes that have been purified together (Cheeseman et al., 2002; DeWulf et al., 2003). Despite the lack of conclusive results thus far, the interactions which have been observed with members of the COMA Complex make it an interesting candidate for directly building upon the DNA-Binding layer.

DASH/DDD/DAM1 Complex: The DAM1 Complex is a 10 protein complex (see Fig. 1-3 for components), members of which have been shown to be capable of binding MTs *in vitro* and associate with kinetochores *in vivo* (Hofmann et al., 1998; Cheeseman et al., 2001a; Cheeseman

et al., 2001b; He et al., 2001; Cheeseman et al., 2002; Janke et al., 2002; Li et al., 2002). All members of this complex are essential for viability and temperature-sensitive mutants show chromosome segregation errors and a G2/M arrest. *dam1-1*, *dad2-9*, *spc19-4* and *spc34-3* cells exhibit monopolar attachment of both sister chromatids to a single SPB, while additional experiments with *spc34-3* mutants indicate that this monopolar phenotype may be the result of an inability to maintain, rather than form, a bipolar attachment to the spindle (He et al., 2001; Janke et al., 2002). The Ipl1p kinase has been proposed to regulate members of the DAM1 Complex by phosphorylation and indeed *ipl1-321* and *ipl1-2* cells show a similar monopolar phenotype to those of DAM1 Complex mutants (He et al., 2001; Cheeseman et al., 2002). The DAM1 Complex provides an attractive candidate for coupling MTs to the other proteins of the kinetochore.

Stu2p: Stu2p is a MT-associated protein that has been shown to localize to kinetochores and the cortical tips of cytoplasmic MTs *in vivo* (Wang and Huffaker, 1997; He et al., 2001). *stu2-277* and *stu2-279* mutants exhibit an interesting phenotype in that they seem to form proper bipolar attachment to the spindle, but appear to have reduced tension across their sister kinetochores, as detected by a lack of transient sister separation (He et al., 2001). Homologs of *STU2* in higher organisms (XMAP215 in *Xenopus*, chTOGp in humans) have been shown to regulate the dynamic behavior of microtubules, and Stu2p may perform a similar function at the plus-end of MTs in *S. cerevisiae* (Gard and Kirschner, 1987a; Gard and Kirschner, 1987b; Spittle et al., 2000).

Motors: It has long been thought that motor proteins may be involved in allowing chromosomes

to move along MTs during mitosis and although an early candidate, Kar3p, is most likely not directly involved, other candidates remain. Cin8p and Kip1p, two members of the BimC motor family, and Kip3p, a member of the MCAK/XKCM1/KinI family of kinesins, have been shown to associate with *S. cerevisiae* kinetochores by ChIP and microscopy (He et al., 2001; J. Tytell-unpublished observations). Cin8p and Kip1p have roles in spindle stability, whereas Kip3p is part of a family of kinesins that play a role in MT dynamics *in vitro* and increase catastrophe rates *in vivo* (Desai et al., 1999b; Goldstein and Philp, 1999; Walczak et al., 2002). It seems unlikely that Cin8p, Kip1p or Kip3p are solely responsible for movement of the chromosomes along MTs given that their deletions individually or in combination demonstrate little or no effect on chromosome-MT attachment (J. Tytell-unpublished observations). However, their ability to regulate MT dynamics and mitotic spindle stability *in vivo* and *in vitro*, coupled with their kinetochore localization, demonstrates a mechanism by which they may subtly regulate the segregation of chromosomes (Hoyt et al., 1992; DeZwaan et al., 1997; Desai et al., 1999b; Goldstein and Philp, 1999).

Ipl1p/Sli15p: Ipl1p is an Aurora B kinase that has been localized along with its partner protein Sli15p, to *S. cerevisiae* kinetochores by ChIP and microscopy (Biggins and Murray, 2001; He et al., 2001; Kang et al., 2001). *ipl1-321* and *sli15-3* mutants exhibit monopolar attachment of both sister chromatids to a single SPB, much like *dam1* mutants. However, unlike *dam1* mutants, *ipl1* cells are able to maintain bipolar attachments established before temperature shift, indicating that Ipl1p/Sli15p may play a role in establishing proper bipolar attachment rather than maintaining it (Tanaka et al., 2002). It has been proposed that Ipl1p/Sli15p act during the early stages of mitosis to resolve syntelic attachments (both sister kinetochores attached to a single SPB) by

briefly releasing the duplicated chromosomes from the MTs, thereby allowing an opportunity for the formation of a proper bipolar attachment (Tanaka et al., 2002). The exact mechanism by which Ipl1p-mediated release occurs is not known but is postulated to involve the phosphorylation of Ipl1p kinase targets such as Ndc10p, Ndc80p, Cse4p and/or members of the DAM1 Complex (Biggins et al., 1999; Cheeseman et al., 2002). By resolving improper connection of the chromosomes to the spindle, Ipl1p enables proper bipolar attachment which in turn silences the spindle checkpoint.

The Human Kinetochore-a brief comparison

When human chromosomes are stained, the *CEN*-kinetochore structure is readily observed by light microscopy as a constriction in the chromosome. Electron micrographs further show the human kinetochore to be a trilaminar proteinacious structure consisting of an inner plate, an outer plate and an interzone region (Rieder, 1982; Pluta et al., 1990; McEwen et al., 1993; Craig et al., 1999). This is comparable to the proposed "three-layer" organization of the *S. cerevisiae* kinetochore, and despite the evolutionary distance between budding yeast and humans, it is hoped that lessons learned in budding yeast will facilitate our understanding of the human kinetochore. Already, there are many indications that this may be the case. Of the more than 60 putative *S. cerevisiae* kinetochore proteins identified thus far, more than 50% have human orthologs and this number is likely to increase with improved database analysis (see Table 1). In a dramatic example of functional conservation, the human HEC1 protein is able to functionally substitute for Ndc80p in *S. cerevisiae* (Zheng et al., 1999).

A number of centromere proteins (CENPs) have been localized to human kinetochores using antibody staining and their positions within the kinetochore structure can be determined.

CENP-A (homolog of *S. cerevisiae* Cse4p) is part of a specialized nucleosome that has been localized to the inner plate of the human kinetochore and is found in close proximity to α-satellite DNA at active human centromeres (Vafa and Sullivan, 1997). Orthologs of CENP-A exist in many organisms and provide an attractive tool through which centromeres can be identified (reviewed in Mellone and Allshire, 2003). CENP-C (homolog of *S. cerevisiae* Mif2p) is a basic protein also localized to the inner plate of human kinetochores. Antibody micro-injections and conditional knockouts in cultured cells, as well as analysis of a CENP-C knockout mouse, have shown that CENP-C is essential for mitotic chromosome segregation and viability (Tomkiel et al., 1994; Fukagawa and Brown, 1997; Kalitsis et al., 1998). CENP-F is localized to the outer kinetochore and appears to interact with CENP-E, a MT motor protein also localized to mammalian kinetochores (Yen et al., 1991; Yen et al., 1992; Rattner et al., 1993; Chan et al., 1998). Dynein (homolog of *S. cerevisiae DYN1*), MCAK (homolog of *S. cerevisiae KIP3*) and CLIP170 (homolog of *S. cerevisiae BIK1*) are all human *CEN*-associated MT binding proteins but as in *S. cerevisiae*, their exact roles in mammalian kinetochores remains unknown. Consistent with observations in *S. cerevisiae*, there appears to be a hierarchy amongst human kinetochore proteins for their association with the centromere. CENP-A is required for the localization of CENP-C to the centromere, whereas hMis12 (homolog of *S. cerevisiae MTW1*) is recruited independently of CENP-A (Howman et al., 2000; Van Hooser et al., 2001; Goshima et al., 2003). Based on homologies and patterns of association with the centromere, it is therefore reasonable to hope that understanding the interdependencies between *S. cerevisiae* kinetochore proteins may shed light on the assembly and organization of their human counterparts. Despite the differences in *CEN* sequence and lack of identified orthologs of the *S. cerevisiae CEN*-binding proteins Ndc10p, Cep3p, and Ctf13p, it is apparent that many other kinetochore proteins

are highly conserved from yeast to humans (Table 1). The continued identification of kinetochore homologs throughout all organisms underscores the importance in conserving the critical components required for making the chromosome-to-MT connection.

Table 1. Evolutionary conservation of the *S. cerevisiae* kinetochore

		S. cerevisiae	*S. pombe*	*C. elegans*	*D. melanogaster*	*X. laevis*	*H. sapiens*	*Features*
DNA-BINDING	**CBF3 Complex**	*NDC10*						
		CEP3						Helix-loop-helix; zinc finger domain
		CTF13						F-box domain
		SKP1	*shp1+/ SPBC409.05*	SKR-1,2,3/ F46A9.5	SKPB1	Skp1	p19/SKP1A	Ctf13p-activation; SCF component
		CBF1						*CEN* binding; HLH domain; *MET* gene regulation
		CSE4	*cnp1+*	HCP-3/ CeCENP-A	Cid/ DmCENP-A		CENP-A	Histone H3-like; *CEN* binding
LINKER LAYER	**NDC80 Complex**	*NDC80*	*ndc10+*			xNDC80	HEC1/hNDC80	Coiled-coil domain
		NUF2	*nuf2+*	him-10		xNUF2	hNUF2R	
		SPC25				xSPC25	hSPC25	Coiled-coil domain
		SPC24	*spc24+*			xSPC24	hSPC24	Coiled-coil domain
	MTW1 Complex	*MTW1*	*mis12+*				hMIS12	
		NNF1	*spac30.08p*					
		NSL1	*spac688.02p*					
		DSN1	*spbc409.09c*					EF-hand/coiled-coil
	COMA Complex	*CTF19*						
		OKP1					CENP-F?	
		MCM21	*mal2+*					
		AME1						Coiled-coil domain
	CTF3 Complex	*CTF3*	*mis6+*				CENP-I	
		MCM16						
		MCM22						
	SPC105 Complex	*SPC105*						
		YDR532						
		MIF2		HCP-4/ CeCENP-C			CENP-C	AT-hook motif
		BIR1						
		PLC1	*plc1+*	PLC	PLC		PLC-δ1	Phospholipase C
		IML3						
		CHL4						
		NKP1						
		NKP2						
		CNN1						
		SLK19					CENP-F?	Coiled-coil domain

Table 1. (contd.)

		S. cerevisiae	*S. pombe*	*C. elegans*	*D. melanogaster*	*X. laevis*	*H. sapiens*	*Features*
MT-BINDING LAYER	DAM1 Complex	*DAM1*						
		DAD1	*Spac16a10.05*					
		DAD2						
		DAD3						
		DAD4						
		DUO1						
		SPC19						
		SPC34						
		HSK3						
		ASK1						
		CIN8	*cut7+*		KLP61F	Eg5	HKSP/hEg5	BimC kinesin-related protein
		KIP1	*cut7+*	KRP-85	KLP61F	Eg5	hEg5	BimC kinesin-related protein
		KIP3	*klp5+, klp6+*	CeMCAK	KLP98A/64D/67A	XKCM1	MCAK	
		STU2	*dis1+*	ZYG-9	minispindles	XMAP215	Ch-TOG	
		BIK1	*tip1+*		CLIP190		CLIP170	MT plus-end binding
REGULATORY		*IPL1*	*ark1+*	AIR-1	aurora b	XAIRK2	Aurora B	Protein kinase
		GLC7	*dis2+*	CeGLC-7A/B			PP1	Protein Phosphatase
		HIR1	*slm9+*		hira		HirA	WD40 domain; Chromatin assembly
		SGT1	*git7+*				SGT1	Ctf13 activation; SCF component
		SLI15	*pic1+*	CeINCENP	DmINCENP	XINCENP	INCENP	Targets Ipl1p
		CAC1			p180, p150	p150	p150	Chromatin assembly
		CDC5	*plo1+*	plk-1, plk-2	polo	Plx1	Plk1	Protein kinase
		BIR1	*bir1+*	BIR-1			Survivin	Chromosome passenger protein
SPINDLE CHECKPOINT		*MAD1*	*mad1+*	MDF-1		XMAD1	MAD1	Coiled-coil domain
		MAD2	*mad2+*	MDF-2		XMAD2	MAD2	
		MAD3	*mad3+*	CeMad3	BUBR1	XBUBR1	hBUBR1	
		BUB1	*bub1+*	CeBUB1	BUB1-like	XBUB1	BUB1	Ser/Thr protein kinase
		BUB3	*bub3+*		BUB3	XBUB3	BUB3	WD40 domain
		MPS1	*mph1+*		TTK	XMPS1	hMPS1	Ser/Thr protein kinase
		CDC20	*slp1+*	FZY-1	Fzy/cdc20	X-FZY	P55CDC	Activator of APC
		GLE2	*rae1+*				hRAE1	WD40 domain

DYNAMICS AND STRUCTURE OF THE MITOTIC SPINDLE

The association between *CEN* DNA and kinetochore proteins is critical for establishing a structure capable of binding MTs, but what are the key features of this connection? How do MTs and kinetochores find each other within the cell and what is involved in the movement of chromosomes during and after their attachment? There appear to be differences between organisms with regard to the mechanisms which are employed to establish this attachment. However, the evidence indicates that variations in these sub-processes do not change the eventual outcome, which is formation of a bipolar *CEN*-kinetochore-MT connection. This section briefly describes the MTs that capture the chromosomes, the dynamic process of establishing bipolar attachment of sister chromatids to the mitotic spindle, and the Microtubule-Associated Proteins (MAPs) and motors which are involved.

Microtubules

Spindle microtubules are not static structures but instead are dynamic components of the mitotic machinery. Microtubules are composed of αβ tubulin heterodimers arranged longitudinally to form protofilaments, that in turn generate a hollow tube 25 nm in diameter, with an inherent polarity consisting of a less-active minus (-) end (slower rate of polymerization and depolymerization) and a more-active plus (+) end (Nogales, 1999; Nogales et al., 1999). The plus (+) end of the MT exhibits continuous depolymerization and polymerization of tubulin subunits (catastrophe and rescue), a property termed dynamic/directional instability which can give rise to rapid growth and shrinkage of the MT (Mitchison and Kirschner, 1984a; Mitchison and Kirschner, 1984b; Walker et al., 1988; Desai and Mitchison, 1997). The *S. cerevisiae* SPB (known as the centrosome or Microtubule Organizing Center (MTOC) in mammalian cells) is a

multiprotein, multi-layered structure which is embedded in the nuclear envelope and nucleates the microtubules of the mitotic spindle. *S. cerevisiae* undergoes a closed mitosis in which the nuclear membrane does not break down and the SPBs remain embedded in the nuclear membrane throughout mitosis. The SPB orients the MTs so the minus (-) end of the MT remains associated with the SPB, while the plus (+) end is distal to the pole (Oakley, 1992; Oakley, 2000). MT-associated motor enzymes such as members of the BimC family of kinesin-like proteins (KLPs), Kar3p and Dynein/dynactin are concentrated at various points along the MTs to assist the SPB in organizing the spindle. This includes the bridging of MTs and thus movement relative to one another, thereby arranging the polymers in parallel and forming asters of MTs with their plus (+) ends radiating out from the SPB (Merdes and De Mey, 1990; Verde et al., 1991; Gaglio et al., 1997). These motors may also play a role in MT dynamics by "reeling in" or destabilizing MTs at the poles. Three categories of MTs are present in *S. cerevisiae*: 1) kinetochore MTs (kMT), which are attached to the kinetochores, 2) pole MTs (pMT), which interact with MTs from the opposite SPB at the midzone of the spindle, and 3) cytoplasmic MTs (cMT), which project from the SPB into the cytoplasm and function to position the nucleus relative to the daughter bud. The combination of dynamic instability and MT orientation enhances the ability of the plus ends of the kMTs to efficiently probe a large area of the nucleus (or cell) in search of kinetochores. Once attached, it is evident that MT dynamics can generate force on the chromosomes, which in turn can be used to do mechanical work. As proof, it has been demonstrated *in vitro* that depolymerizing MTs can move an attached chromosome, even against a flow of buffer and in the absence of ATP (Koshland et al., 1988; Coue et al., 1991).

Capturing a Chromosome

Prometaphase in higher cells occurs when the nuclear envelope breaks down and MTs polymerize and depolymerize rapidly in a "search and capture" manner, allowing the plus-end of the microtubule to establish a connection with the kinetochore. Chromosomes commonly become attached to MTs emanating from one pole first (mono-oriented), resulting in stabilization of the plus (+) end of the attached MT. This initial attachment and MT stabilization does not eliminate movement of the sister chromatids as they seek attachment for the second kinetochore. Subsequently, the sister kinetochore captures MTs from the opposite pole resulting in bipolar attachment (bi-orientation) of the sister chromatids. Chromosomes with bipolar attachments are not sedentary but instead exhibit oscillations along the spindle axis, the speed and degree of which are variable among different organisms and even different chromosomes of a single cell (Rieder et al., 1986). Following bipolar attachment, the chromosomes undergo a process known as congression which results in alignment of all the chromosomes at the equator of the cell ("metaphase plate") in apparent preparation for their segregation at anaphase onset.

The situation in *S. cerevisiae* is slightly different from that of cells which utilize an open mitosis. From microscopic observations in *S. cerevisiae*, it appears that the chromosomes remain almost continually attached to MTs throughout the cell cycle (see Fig. 1-4). During G1, the chromosomes remain closely associated with the single SPB, indicating an attachment (Heath and Rethoret, 1980; Funabiki et al., 1993; Jin et al., 2000). This attachment is lost in *ndc10-1* cells, in which the kinetochore has been disabled (D. Rines-unpublished observations). During S phase, the chromosomes are duplicated and cohesion is established between the sister chromatids, ensuring that the two identical chromosomes will remain paired until anaphase (Michaelis et al., 1997). Presumably, the "old" kinetochore transiently releases from the

microtubule as the replication machinery passes along the DNA, although the status of the kinetochore during this process remains unknown. The order of assembly and timing required to establish a *de novo* kinetochore on the newly replicated chromosome remains an intriguing question. Also during S phase, a second, mature SPB develops near the "old" SPB and the proximity of this second SPB allows for the efficient joining of pMTs between the two SPBs. This proximity may also increase the efficiency of forming bipolar attachments to sister chromatids by reducing the area and time required to search for the sister kinetochore. Although the exact mechanism and physical constraints are not fully understood, it is apparent that the sister kinetochores become attached to both SPBs with the assistance of the Ipl1p kinase (Tanaka et al., 2002). With these MT-kinetochore attachments intact, the SPBs begin to separate with the "new" SPB moving along the nuclear envelope towards the daughter cell. Once the SPBs have reached either side of the nucleus (and possibly during SPB migration), tension is generated as the MTs pull in opposite directions on the "bipolarly" attached sister kinetochores, while the cohesin "glue" attempts to hold the sisters together. Establishment of the cohesin complex between the replicated chromosomes is required for this tension to occur (Tanaka et al., 2000). It has been shown in yeast and higher cells that sister chromatids transiently separate from each other in the region around the centromere as a result of the tension exerted by the spindle (transient sister separation), an observation that can be used to evaluate proper attachment (Shelby et al., 1996; Waters et al., 1996; Nicklas, 1997; Waters et al., 1998; Goshima and Yanagida, 2000; He et al., 2000; Tanaka et al., 2000; He et al., 2001; Skoufias et al., 2001; Zhou et al., 2002). Once the sister chromosomes have established bipolar attachment to the *S. cerevisiae* spindle and the SPBs have reached either side of the nucleus, they do not stop moving but instead continue to oscillate back and forth in association with the dynamic microtubules and

transient stretching. This movement produces a distinct bi-lobed pattern in *S. cerevisiae* that is equivalent to the metaphase plate in higher cells, and has been observed by tagging either the centromeres or kinetochore proteins with GFP (see Assays below). The unique bi-lobed pattern seen during yeast mitosis has been utilized to confirm the identity of a number of putative kinetochore proteins (Goshima and Yanagida, 2000; He et al., 2000; He et al., 2001; see Figs. 3-1 and 4-1).

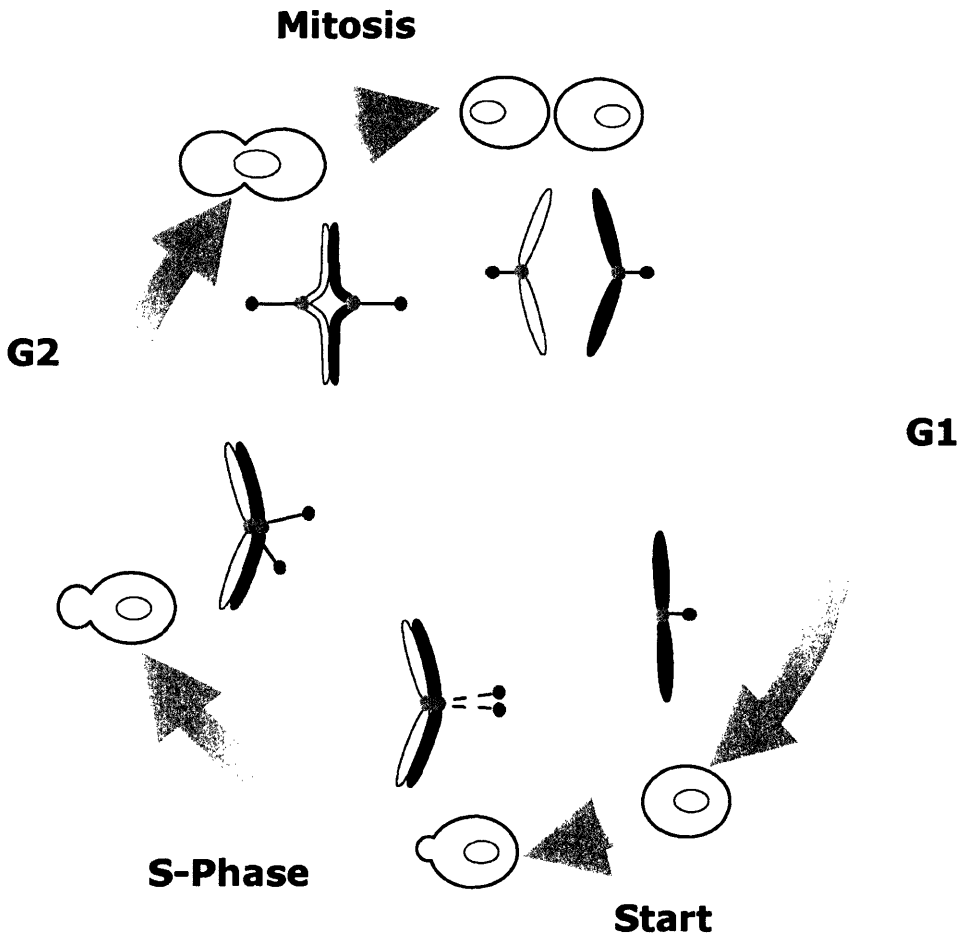


Figure 1-4 Analysis of a single chromosome with respect to the cell cycle of *S. cerevisiae* (nucleus indicated in yellow). G1 chromosomes (dark gray; blue = kinetochore) remain closely associated with the single spindle pole body (SPB; red). SPB duplication and DNA replication occur during S phase, at which time the kinetochore is presumably transiently released from the centromere as the replication machinery passes by (light gray = duplicated chromosome). Bipolar attachment of sister chromatids is achieved by attachment to two individual SPBs, which subsequently migrate to opposite sides of the dividing nucleus. This arrangement results in tension being exerted across the sister chromatids during metaphase and is observed as transient sister centromere separation. Following cleavage of Scc1p/Mcd1p (cohesin complex) at the metaphase-to-anaphase transition, the separated chromosomes are pulled into the resultant mother and daughter cells.

Motors and Microtubule Associated Proteins (MAPs)

Chromosome movement involves a number of other MT and kinetochore–associated proteins, but in most cases the function of these proteins in regulating movement remains obscure. Motor proteins are capable of transforming chemical energy into directed movement and are known to associate with kinetochores, chromosome arms, spindle poles and pole-initiated MTs. The interaction of a motor with a MT is capable not only of moving cargo along the MT, but also of regulating MT dynamics (Lombillo et al., 1995). These effects may occur as a result of increased/decreased MT stability, crosslinking MTs together or tethering organelles (including chromosomes) to the MTs. Three of the six kinesin-related motor proteins identified in the *S. cerevisiae* genome (Kip1p, Kip3p and Cin8p) have been localized to kinetochores, while Kip2p and Kar3p have been implicated in control of MT length and turnover at the SPB (Huyett et al., 1998; Cottingham et al., 1999; J. Tytell-unpublished observations). The exact role of the motors at kinetochores remains unclear in *S. cerevisiae* but directional motors such as CENP-E in vertebrates, and *klp5*$^{+}$*/klp6*$^{+}$ in *S. pombe* demonstrate substantial defects in chromosome movement when mutated (Wood et al., 1997; West et al., 2001). Differences exist in the requirement for motors at the SPB for MT organization and control, as depletion of dynein in vertebrate cells causes defects in congression and anaphase movement while deletion of the dynein heavy-chain in *S. cerevisiae* appears to have no effect on chromosome movement (Yeh et al., 1995; Sharp et al., 2000a; Sharp et al., 2000b). These results may reflect differences between open and closed mitosis as much greater mobility of both the chromosomes and MTs is likely required to establish an attachment in cells undergoing an open mitosis.

Some motors do not directly produce movement in the classic sense of carrying cargo, but instead bind and destabilize MT ends. Such is the case with the KinI family of kinesins

which are represented at kinetochores by MCAK in mammals, XKCM1 in *Xenopus* and *KIP3* in *S. cerevisiae*. The KinI family of proteins has been shown to play a role in mitotic-spindle assembly *in vitro* and to increase catastrophe rates *in vivo* (Desai et al., 1999a; Walczak et al., 2002). Other MAPs do not possess motor domains but also function to regulate MT stability. *S. cerevisiae* Stu2p is a conserved non-motor MAP with homologs in *S. pombe* (*dis1+*), *Xenopus* (XMAP215), *Drosophila* (Msps) and humans (ch-TOG1). The TOG/XMAP215 family of proteins has a direct effect on MT dynamics although the type of regulation appears to vary with species. XMAP215 promotes the polymerization of pure tubulin *in vitro* by increasing the growth and rescue rates of a MT (Vasquez et al., 1994). Stu2p on the other hand induces catastrophes by destabilizing MTs *in vitro*, consistent with *in vivo* results showing that MTs in *stu2* mutants are less dynamic than in wildtype cells (Kosco et al., 2001; van Breugel et al., 2003).

Lastly, proteins such as the DAM1 Complex are able to bind MTs but have not been shown to affect dynamics or MT organization. These proteins may serve as structural components which form the physical link between kinetochore and MT while other proteins regulate movement. It remains unclear whether kinetochore proteins, including those capable of binding MTs, assemble on *CEN* DNA independent of MTs, or if the connection involves a union of *CEN*-binding proteins with proteins that are bound to the plus-end of MTs. The use of tubulin mutants might address this issue by allowing for the identification of proteins that are still present at the kinetochore in the absence of a MT, although the question of establishment and maintenance might persist.

In summary, the combined actions of MT dynamics, MAPs and motors function to establish a mitotic spindle, which then interacts with the structural components of the

kinetochore to establish proper bipolar attachment to the duplicated chromosomes. Following attachment, tension is exerted across the sister kinetochores as the MTs, MAPs and motors exert force on the chromatids in preparation for the onset of the metaphase-anaphase transition. Cleavage of the cohesins which hold duplicated sister chromatids together releases the tension exerted by the MTs, and the individual chromosomes are free to move with the depolymerizing MTs in opposite directions, towards mother and daughter cell.

SPINDLE CHECKPOINT

The cell cycle is a series of processes which occurs in a defined spatial and temporal order: DNA replication must occur before chromosome segregation, chromosome segregation must occur before cytokinesis. Failure to adhere to this order of functions causes cell division to go awry. For example, starting cytokinesis before chromosome segregation has been completed can result in one cell receiving too many chromosomes and another cell receiving too few. Cells have therefore adapted mechanisms known as checkpoints which ensure the correct temporal order of cell cycle events is followed. The first demonstration of the role for a checkpoint was made in *S. cerevisiae* by Weinert and colleagues with the identification of the *RAD9* gene (Weinert and Hartwell, 1988; Weinert and Hartwell, 1989). Previous research had shown that low levels of X-ray exposure induced DNA damage and caused wildtype yeast to arrest during G2 phase. Weinert and colleagues showed that *rad9Δ* mutants fail to arrest in response to γ-irradiation and instead proceeded through mitosis with damaged DNA, resulting in decreased cell viability. *rad9Δ* cells exposed to γ-irradiation do arrest in response to nocodazole however, allowing time to repair their DNA. This indicates that the *RAD9* gene is not required for DNA repair *per se*, but instead acts to slow down cell division and ensure the opportunity to repair

damaged DNA before mitosis proceeds. Multiple distinct checkpoints are involved in making sure that cellular activities proceed correctly, including DNA replication, mitotic exit and chromosome segregation.

The Spindle Checkpoint and its Components

During mitosis, it is essential that all duplicated chromosomes make proper bipolar attachments to the mitotic spindle before the metaphase to anaphase transition, and it is the role of the spindle checkpoint to ensure that this occurs. Chromosome segregation is tightly monitored and very accurate as evidenced by the extremely low natural chromosome loss rate in *S. cerevisiae*, which is on the order of 1 loss event per 10^5 cell divisions (Hartwell et al., 1982; Hartwell and Smith, 1985). The presence of even a single unattached kinetochore or the addition of MT-altering chemicals such as nocodazole, taxol or benomyl is enough to halt progression through mitosis. The cell is in fact capable of responding to a variety of assaults on the formation of a proper bipolar attachment, including damage to the centromeric DNA, kinetochore proteins or MTs (Dustin, 1980; McIntosh and Hering, 1991; Pangilinan and Spencer, 1996; Tavormina and Burke, 1998; Downing, 2000). The spindle checkpoint is responsible for detecting these errors and providing time for the cell to remedy any attachment problems before proceeding, so that chromosome missegregation does not occur (Fig. 1-5A). The spindle checkpoint genes *MAD1-3* (mitotic arrest defective), *BUB1* and *BUB3* (budding uninhibited by benzamidazole) were first identified using genetic screens in *S. cerevisiae* to identify mutants that failed to arrest in response to the MT poison benomyl (Hoyt et al., 1991; Li and Murray, 1991). *MPS1* (monopolar spindle), a protein involved in SPB duplication, has also been shown to have a second role as part of the spindle checkpoint (Winey et al., 1991; Weiss and Winey,

1996). Moreover, based on results from *S. cerevisiae* and mammalian cells, it is also becoming apparent that the spindle checkpoint functions as part of the normal cell cycle, not only in response to errors, but to allow enough time for all of the duplicated chromosomes to be captured by MTs.

To understand the role of the spindle checkpoint, it is necessary to understand the genes involved in holding sister chromatids together and in regulating the metaphase-to-anaphase transition. Following DNA replication, duplicated chromatids are bound to each other by the cohesin complex (Scc1p (Mcd1p)/Scc3p, Smc1/3p) which is highly conserved through humans, and requires Ndc10p, Mif2p and Cse4p for localization to *S. cerevisiae CEN in vivo* (Guacci et al., 1997; Michaelis et al., 1997; Tanaka et al., 1999; Zachariae and Nasmyth, 1999). The protease Esp1p is bound by Securin (Pds1p in yeast) until the metaphase-to-anaphase transition, at which time Pds1p is targeted for ubiquitin-dependent degradation by the Anaphase-Promoting-Complex, an E3 ubiquitin ligase (APC; also known as the cyclosome; Fig. 1-5B). This frees Esp1p to cleave Scc1p, thus releasing the duplicated chromosomes from each other and allowing them to separate to opposite poles of the dividing cell (Fig. 1-5B; Ciosk et al., 1998; Uhlmann et al., 1999; Uhlmann et al., 2000; Hauf et al., 2001). In response to a signal sent from unattached or maloriented kinetochores, Mad2p binds to and inhibits Cdc20p, a positive regulator of the APC (Li et al., 1997; Hwang et al., 1998). It is the interaction between Cdc20p and the APC which regulates the degradation of Pds1p and therefore controls the metaphase-to-anaphase transition, and it is this interaction which is the ultimate downstream target of the spindle checkpoint (Li et al., 1997; Fang et al., 1998; Hwang et al., 1998; Kim et al., 1998). Although all of the spindle checkpoint proteins are required to properly arrest the cell, they appear to have differing functions, which result in variable severity with regards to their effects

on chromosome segregation (Warren et al., 2002). However, despite their different roles, the spindle checkpoint proteins function in a common cascade with Mps1p, Bub1 and Bub3p acting upstream of Mad2p, the downstream APC inhibitor (Fig. 1-5B). Bub3p, Mad3p/BubR1 and Mad1p have also been reported to combine with Mad2p in different combinations to regulate Cdc20p, although the function of the various complexes in cell cycle regulation is unknown (Chan et al., 1999; Wu et al., 2000; Fraschini et al., 2001; Skoufias et al., 2001; Sudakin et al., 2001; Millband and Hardwick, 2002). The role of phosphorylation in the spindle checkpoint also remains an interesting and complicated topic. Overexpression of the Mps1 kinase induces a mitotic arrest, Mad1p and Bub1p are hyperphosphorylated in response to nocodazole treatment (and other conditions) and kinase-dead Bub1p and BubR1p have been shown to be checkpoint-competent (Hardwick et al., 1996; Farr and Hoyt, 1998; Sharp-Baker and Chen, 2001; Warren et al., 2002). Multiple observations and conflicting results suggest that the significance of the various phosphorylation states remains to be determined.

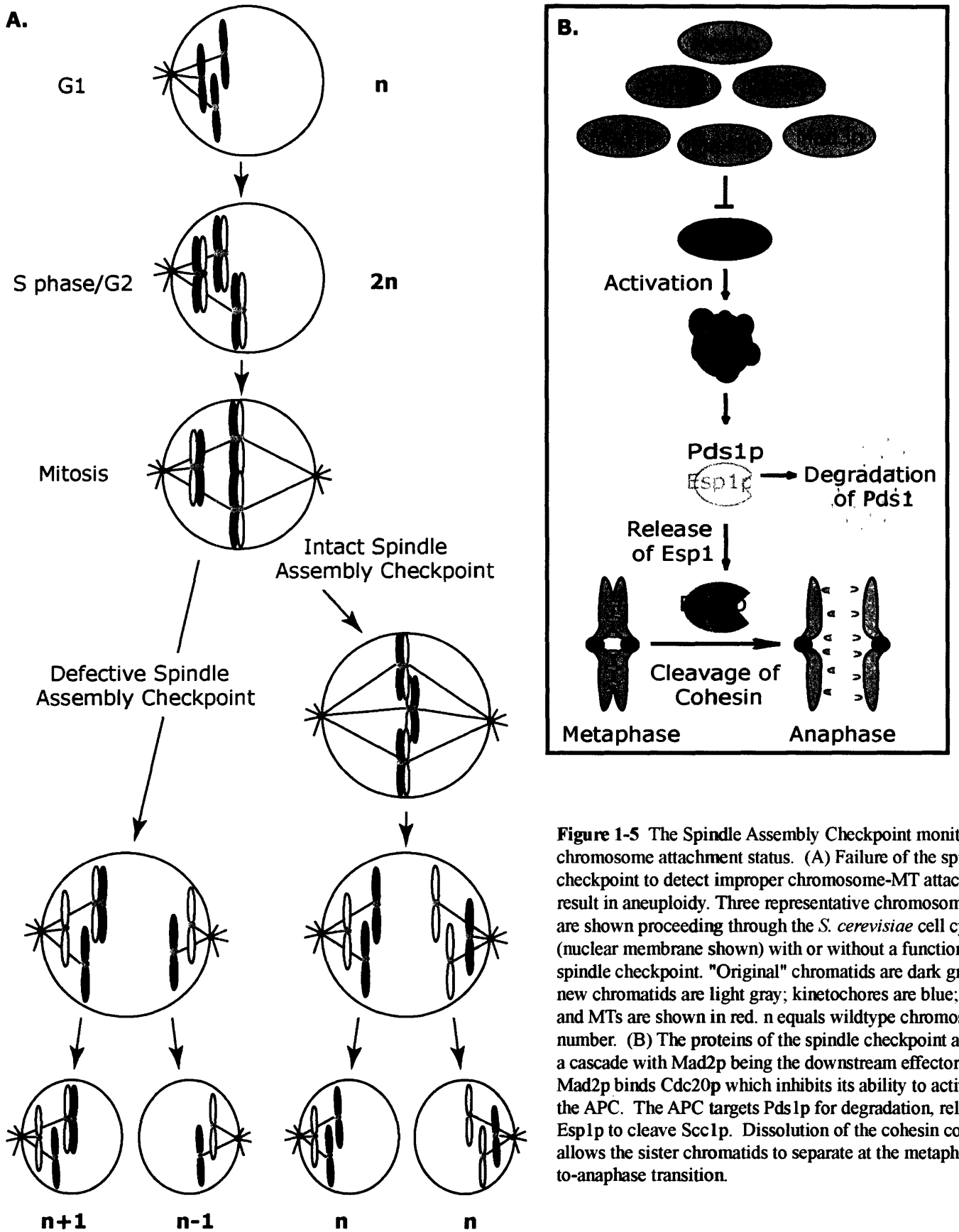


Figure 1-5 The Spindle Assembly Checkpoint monitors chromosome attachment status. (A) Failure of the spindle checkpoint to detect improper chromosome-MT attach can result in aneuploidy. Three representative chromosomes are shown proceeding through the *S. cerevisiae* cell cycle (nuclear membrane shown) with or without a functional spindle checkpoint. "Original" chromatids are dark gray; new chromatids are light gray; kinetochores are blue; SPBs and MTs are shown in red. n equals wildtype chromosome number. (B) The proteins of the spindle checkpoint act in a cascade with Mad2p being the downstream effector. Mad2p binds Cdc20p which inhibits its ability to activate the APC. The APC targets Pds1p for degradation, releasing Esp1p to cleave Scc1p. Dissolution of the cohesin complex allows the sister chromatids to separate at the metaphase-to-anaphase transition.

Spindle checkpoint Localization

Homologs of the spindle checkpoint proteins have been shown to localize to kinetochores in many organisms: *S. pombe* (He et al., 1997; Bernard et al., 1998; He et al., 1998); *Xenopus* (Chen et al., 1996; Chen and Murray, 1997; Chen et al., 1998); *Drosophila* (Basu et al., 1998; Basu et al., 1999); *C. elegans* (Kitagawa and Rose, 1999); *Mus musculus* (Taylor and McKeon, 1997; Martinez-Exposito et al., 1999); and humans (Li and Benezra, 1996; Cahill et al., 1998; Chan et al., 1998; Taylor et al., 1998; Chan et al., 1999; Jin et al., 1999). Chapter 4 of this thesis demonstrates that this is the case in *S. cerevisiae* as well. This localization indicates that the spindle checkpoint proteins interact with kinetochore proteins, and the nature of this biochemical interaction remains a point of great interest. Likely candidates for such an interaction would be kinetochore proteins which, when eliminated or mutated, are incapable of invoking a spindle checkpoint dependent delay. Mutations of most *S. cerevisiae* kinetochore proteins, including all non-essential proteins, induce a checkpoint-dependent arrest. However, two candidate complexes have emerged as being involved in the spindle checkpoint-the CBF3 Complex and members of the NDC80 Complex, neither of which cause a metaphase arrest when eliminated (Goh and Kilmartin, 1993; Gardner et al., 2001; Janke et al., 2001; Wigge and Kilmartin, 2001; McCleland et al., 2003). A functional kinetochore is required for recruitment of the spindle checkpoint proteins and in the case of loss of the CBF3 Complex, it appears that few, if any, kinetochore proteins are still present at the centromere (Ortiz et al., 1999; He et al., 2001; De Wulf et al., 2003; Nekrasov et al., 2003). Skp1p has been proposed to directly interact with and recruit Bub1p to the kinetochore (Kitagawa et al., 2003). However, the use of *SKP1* mutants in these experiments raises the question of whether CBF3 is functional *in vivo*, as it has previously been shown that *SKP1* is required as an activating factor of Ctf13p, a necessary step for the

assembly of CBF3 (Kaplan et al., 1997; Kitagawa et al., 2003). On the other hand, loss of the NDC80 Complex presents a situation in which some kinetochore proteins are still present (e.g. CBF3, Cse4p, Mtw1p) while many others such as the DAM1 Complex are lost, and the spindle checkpoint is inactive (He et al., 2001; Janke et al., 2001; Wigge and Kilmartin, 2001; Gillett et al., 2004). There is further distinction within the NDC80 Complex itself as *SPC24* and *SPC25* loss of function mutants disable the spindle checkpoint, while loss of *NDC80* or *NUF2* do not (Janke et al., 2001; McCleland et al., 2003). These results make the NDC80 Complex an interesting candidate for direct interaction with the proteins of the spindle checkpoint and biochemical experiments will hopefully explore this possibility in the near future. The identification of such an interaction would allow for directed experiments that should lead to a better understanding of how the spindle checkpoint monitors chromosome-kinetochore-MT attachment.

Comparison of Model Systems

The proteins of the spindle checkpoint have been highly conserved across phylogeny yet their role in different organisms seems to vary (Table 1). For example, the Bub and Mad proteins have been shown to be dispensable for viability in *S. cerevisiae*, while their elimination in mice results in embryonic lethality (Hoyt et al., 1991; Li and Murray, 1991; Dobles et al., 2000; Kalitsis et al., 2000; Wang et al., 2004). Also, the Mad2 protein appears to localize to the kinetochore of mammalian cells as part of the normal cell cycle, whereas it is only observed at *S. cerevisiae* kinetochores in response to damage (Howell et al., 2000; Gillett et al., 2004). These observations may reflect underlying differences in the process of spindle assembly. Chromosomes appear to remain attached to the spindle MTs throughout the cell cycle in *S.*

cerevisiae, whereas higher cells undergo nuclear envelope breakdown at which time unattached chromosomes must bind spindle MTs. Despite the apparent differences in requirements between *S. cerevisiae* and mammalian systems, the degree of conservation among the spindle checkpoint proteins throughout all organisms indicates a conserved role in monitoring the proper attachment of chromosomes to the mitotic spindle.

Tension vs. Attachment

What does the spindle checkpoint detect at unattached kinetochores and how is the signal regulated? Two models, which are not necessarily exclusive, have been proposed as the basis for sensing that all pairs of sister chromatids have formed proper bipolar attachments. The first is the "tension model", in which the exertion of proper tension across sister chromatids is proposed to be recognized by the spindle checkpoint (McIntosh, 1991; Li and Nicklas, 1995). The second is the "attachment model" which proposes that the attachment of all kinetochores to MTs fulfills an occupancy requirement indicating that anaphase is ready to proceed (Rieder et al., 1994; Rieder et al., 1995). In an elegant experiment, Li and Nicklas manipulated praying mantid spermatocytes to generate support for the theory that tension is the signal regulating the spindle checkpoint (Li and Nicklas, 1995). The biology of praying mantid spermatogenesis requires that three sex chromosomes be separated with one resultant sperm receiving the XX chromosomes and the other receiving the Y chromosome, a process which requires a tripartite attachment to the spindle. It was noticed that occasional bipartite attachments would form, resulting in the presence of a free, unattached X chromosome. The presence of this unattached chromosome caused delays in anaphase onset of many hours. Li and Nicklas (1995) used a microneedle to mechanically apply tension to the free X chromosome which resulted in rapid progression into

anaphase. The investigators interpreted these results as demonstrating that the imposition of proper tension on the paired chromatids is responsible for silencing the spindle checkpoint and allowing for anaphase onset. However, there are a number of issues which must be considered when evaluating the Li and Nicklas experiments. First and most significantly, it has subsequently been shown that increased tension results in an increase in the number of MTs attached to a chromosome, somewhat blurring the distinction between tension and occupancy of kinetochore attachment sites (Nicklas, 1997; King and Nicklas, 2000; Nicklas et al., 2001). Second, the Li and Nicklas experiments represent a meiotic process. It is not yet clear whether the *CEN*-kinetochore-MT attachment mechanism and the spindle checkpoint are the same in meiosis and mitosis. Lastly, there is a question of whether observations based on the unique biology of the praying mantid spermatids are broadly applicable. Other researchers have shown that mammalian cells treated with taxol, vinblastine or noscapine contain chromosomes which remain attached to the spindle without tension, resulting in a metaphase arrest (Waters et al., 1998; Skoufias et al., 2001; Zhou et al., 2002). Again, the interpretation is that a lack of proper tension across sister kinetochores invokes the spindle checkpoint. However, all of these drugs affect MTs and the exact status and number of MTs involved in the remaining spindle under these conditions remains difficult to determine.

In support of the attachment model, Rieder and colleagues used PtK1 (rat-kangaroo kidney epithelial) cells to show that laser-ablation of the last unattached kinetochore (on a mono-oriented chromosome pair) was sufficient to initiate entry into anaphase (Rieder et al., 1995). These experiments demonstrated that elimination of a lone unattached kinetochore, while not imposing tension on the last chromosome, was capable of down-regulating the spindle checkpoint. Caution must also be exercised when considering these experiments as it is not

known precisely what effect laser irradiation has on the remaining kinetochore and the cell as a whole. However, additional experiments in PtK1 cells and maize indicate that Mad2 staining at kinetochores, a sign of spindle checkpoint activation, depends on MT attachment and not tension (Waters et al., 1998; Yu et al., 1999).

Finally, it is possible that both tension and attachment regulate the checkpoint. Indeed, it has been proposed that individual components of the spindle checkpoint may play different roles in detecting tension and attachment. Animal cells treated with low levels of vinblastine arrest with attached chromosomes that are not under tension, with BUB1 and BUBR1 (Mad3p in fungi) observed at kinetochores, but not MAD2. In contrast, high levels of vinblastine cause detachment of the kinetochores from microtubules, and in this case MAD2 is recruited to the kinetochores in addition to BUB1 and BUB3 (Skoufias et al., 2001). These results have been interpreted as demonstrating that BUB1 responds to tension while MAD2 responds to attachment, although the same concerns regarding the effects of vinblastine on MTs remain.

Tension vs. Attachment in S. cerevisiae

The issue of tension versus attachment would appear to be more easily reconciled in *S. cerevisiae* where each kinetochore is bound by a single MT, and the complication of full versus partial occupancy of kinetochore MT binding sites is not a concern. Murray and colleagues have used a deletion of the Cdc6 replication initiator protein to support their claims that tension is the spindle checkpoint regulator (Biggins and Murray, 2001; Stern and Murray, 2001). *cdc6Δ* mutants fail to initiate DNA replication, bypass the DNA replication checkpoint and proceed into mitosis where they randomly segregate their single-copy chromosomes to either the mother or daughter of a dividing cell. Importantly, tension is absent from the mono-oriented chromatids as

they attach to spindle MTs. Deletion of *CDC6* in *S. cerevisiae* results in a *MAD2*-dependent delay in Pds1p degradation, an indicator of metaphase-to-anaphase progression (Stern and Murray, 2001). These results have been interpreted to show that lack of tension on the unreplicated chromosome is causing a spindle checkpoint-dependent delay, but much is not known about the status of the lone kinetochores in this situation. It is possible that individual kinetochores in these cells become transiently separated from the MT, and it is this lack of attachment which induces a delay. Supporting this interpretation is the work of Tanaka et al., who showed that the Ipl1p kinase may play a role in transiently releasing sister kinetochores that are attached to one SPB (syntelic attachment) early in metaphase, in order that they may then make a proper bipolar attachment (Tanaka et al., 2002). Based on this observation, the single kinetochores in a *cdc6Δ* cell may become transiently released from the MT either as a matter of course, or in response to a perceived lack of tension, and the unattached kinetochore might then be recognized by the spindle checkpoint and provoke a cell cycle delay. In contrast to *cdc6Δ* mutants, *cdc6Δ/ipl1-321* double mutants fail to delay Pds1p degradation, supporting the idea that Ipl1p transiently creates unattached kinetochores (Biggins and Murray, 2001). The authors interpret these results to mean that Ipl1p is responsible for detecting tension and is itself a component of the spindle checkpoint. An alternative scenario is that kinetochores are never released from the MT due to nonfunctional Ipl1p, and therefore the presence of an unattached kinetochore never occurs. The fact that nocodazole arrests *ipl1-321* cells (unlike *madΔ* or *bubΔ* cells) also makes the designation of *IPL1* as a spindle checkpoint component questionable. It is worth noting that the *cdc6Δ* phenotype is that of a slight delay, not an arrest; again consistent with a transient defect such as brief detachment which needs to be resolved, as opposed to an ongoing error such as lack of tension due to an absent chromosome.

S. cerevisiae kinetochore mutants that exhibit specific defects in attachment and tension generation provide a very powerful tool for examining the errors that are recognized by the spindle checkpoint. For example, based on the dynamics and spatial arrangement of their chromosomes (detected as GFP-tagged chromosomes or kinetochore proteins), distinctions can be made between mutants of *NDC10* and the NDC80 Complex (complete detachment of chromosomes), members of the DAM1 Complex (monopolar attachment of both chromosomes to one SPB) and mutants of *STU2* (bipolar attachment, but lack of tension). An examination of kinetochore mutants and the response of the spindle checkpoint is presented in Chapter 4 of this thesis. At present, the debate between attachment and tension remains unresolved both in higher cells and *S. cerevisiae*, and it is possible that this issue will remain unresolved if cells do monitor both attachment and tension.

ASSAYS TO STUDY THE KINETOCHORE

A wide variety of assays have been employed to understand the *S. cerevisiae* kinetochore including (but not limited to) plasmid/chromosome loss assays (Clarke and Carbon, 1980; Spencer et al., 1989; Spencer et al., 1990), 1- and 2-hybrid screens (James et al., 1996; Ortiz et al., 1999), synthetic lethal screens (Hyland et al., 1999), DNA affinity chromatography (Lechner and Carbon, 1991), bandshift assays (Lechner and Carbon, 1991; Sorger et al., 1994), UV crosslinking (Espelin et al., 1997) and MT-binding assays (Kingsbury and Koshland, 1991; Sorger et al., 1994). A brief description of techniques used or proposed in this thesis along with consideration of real or perceived caveats follows below.

Bandshift Assay: Bandshift or gel-retardation assays evaluate direct DNA-protein binding and provide an excellent method for studying the effects of mutations in DNA and proteins on complex formation. A DNA probe of desired size and constitution (often radiolabeled) is incubated with protein(s) under optimal binding conditions, and then run on a non-denaturing acrylamide gel to separate bound and unbound probe. DNA-protein complexes migrate more slowly (they are "retarded") as they run through the gel relative to unbound DNA. The motility of the DNA-protein complex is believed to reflect a number of factors including mass (MW), charge and overall shape of the complex (see Fig. 2-1). Titration of unlabeled DNA (either specific or nonspecific) into the binding reaction serves as a means to establish binding affinities and specificity by competition. Bandshift assays have been an excellent tool for studying the interaction of Cbf1p and CBF3 proteins with *CEN* DNA but identification of higher order complexes involving additional proteins has yet to be observed (Lechner and Carbon, 1991; Sorger et al., 1994; Espelin et al., 1997; Wieland et al., 2001). The lack of higher order complexes by bandshift assay has prevented the determination of which proteins bind to CBF3 to establish the next layer in kinetochore assembly. This failure may be due to biological reasons such as incorrect protein composition, regulation or modifications (phosphorylation, acetylation, etc.), or may represent technical issues such as the limited "pore size" of an acrylamide gel which may preclude very large complexes from entering the matrix. Nonetheless, the bandshift assay remains a gold standard for evaluating direct DNA-protein interactions and in combination with improved techniques such as identification of proteins by mass spectrometry from excised bands, may prove even more informative in the future.

Chromosome Loss Assay: Chromosome loss assays have many variations, but ultimately all evaluate how well a specific piece of DNA is maintained during rounds of cell division, making it a particularly useful assay for the study of all aspects of chromosome segregation. The basic principal involves use of an endogenous chromosome, an artificial chromosome or a plasmid that carries a scorable marker making it possible to ascertain whether the DNA has been maintained by the cell during mitosis or meiosis. The marker is typically an auxotrophic gene that is required for growth under minimal conditions, although the chromosome or plasmid which carries the marker is essentially disomic and contains no other genetic information that affects cell viability (Clarke and Carbon, 1980). For example, loss of a *URA* carrying plasmid can readily be observed as an ability to grow on complete media such as YPD, but not on selective media such as that lacking Uracil. In this manner, the ability to maintain the *URA* gene can be evaluated, which in turn is dependent on all of the components required for chromosome segregation including *CEN* composition and kinetochore protein function. In a very elegant experimental scheme, Hieter and colleagues developed a color assay to identify loss of an engineered chromosome fragment which carries a *SUP11* ochre suppressor gene (Hieter et al., 1985). When the *SUP11* suppressor is present in cells with an *ade2* mutation, yeast colonies are white. Loss of the *SUP11* suppressor results in a red colony due to buildup of a byproduct of the adenine biosynthesis pathway. In this manner, the ability to maintain a *CEN*-containing, *SUP11*-containing piece of DNA can readily be visually monitored under conditions that affect *CEN* or kinetochore function.

A number of aspects of kinetochore function influence how well a chromosome is maintained and include composition of the centromere, the status of kinetochore proteins and microtubule structure and function (as well as DNA replication, telomere function, etc.). These

factors, notably *CEN* composition and chromosome structure also determine the baseline chromosome loss rate and must be considered when using these systems. For example, plasmids have a much higher basal loss rate (about 1 loss per 100 cell divisions) relative to a full length chromosome, and thus would not be very effective for studying subtle chromosome segregation defects. Chromosome loss assays have been used to identify a large number of genes involved chromosome segregation and continue to provide an excellent means of evaluating all aspects of kinetochore function (Spencer et al., 1989).

Live and Fixed Cell Microscopy: For many years, the small size of the *S. cerevisiae* nucleus prevented the direct observation of chromosome dynamics until Straight and colleagues developed a repressor-GFP system capable of yielding data on single chromosome movements by fluorescence microscopy (Straight et al., 1996). An array of tetracycline operators (TetO) sequences is integrated along the length of a chromosome arm; for studies involving kinetochore motion the array is usually integrated 1-2 kb from the centromere. The cells simultaneously express GFP-tagged tetracycline repressor (TetR-GFP) integrated elsewhere in the genome, with the binding of repressor-GFP fusion protein to the operator sequence being visualized as a fluorescent spot (Michaelis et al., 1997). In the case of localization near the centromere, the fluorescent spot is closely associated with the kinetochore and appears as a single spot prior to chromosome segregation, transiently becomes two spots due to sister separation during metaphase (transient sister separation), and then completely separates into two distinct spots at anaphase (Goshima and Yanagida, 2000; He et al., 2000; Tanaka et al., 2000). Spc42p, a protein known to localize to the central SPB plaque, is tagged either with GFP or CFP and serves as a spindle pole marker to monitor the position of the chromosomes relative to the spindle axis

(Wigge et al., 1998; He et al., 2000). Observations can be made either in real time using live cells or in fixed cells after formaldehyde treatment, with multiple images taken through the Z plane and reconstructed to provide a 3D image of the spindle and kinetochores (Rines et al., 2002; Thomann et al., 2002). This system has been used to observe the stretching of *S. cerevisiae* centromeric DNA as well as provide a valuable assay to evaluate the effect of various kinetochore mutants on attachment and tension (Goshima and Yanagida, 2000; He et al., 2000; Tanaka et al., 2000; He et al., 2001).

Improved microscopy techniques have also allowed for the precise monitoring of GFP-tagged kinetochore proteins within the *S. cerevisiae* nucleus (He et al., 2001; Rines et al., 2002). GFP-fusions have been used to identify the cellular localization of many proteins and led to the observation by He et al. that *S. cerevisiae* kinetochores are distributed in a distinct bi-lobed pattern between the two SPBs during metaphase (He et al., 2000; He et al., 2001; De Wulf et al., 2003; Nekrasov et al., 2003). Using microscopy of GFP-tagged proteins, it is also possible to observe the effects of one kinetochore protein on another. Observing the localization of one kinetochore protein, in the presence of a second kinetochore mutant, allows for a determination of whether the bi-lobed pattern of the wildtype protein has been disrupted. As an example, the bi-lobed pattern of Ndc80-GFP disappears in an *ndc10-1* mutant at non-permissive temperature, indicating the dependence of Ndc80p on Ndc10p for proper kinetochore localization (Fig. 3-1; He et al., 2001). In this manner, it is possible to develop a series of interdependencies among kinetochore proteins, and thereby begin to build a picture of kinetochore assembly. Caution must be taken when using GFP-tagged proteins to assay kinetochore functions however, as microscopy is still quite subjective. The localization of kinetochore proteins to multiple structures in the cell is also a complication and because we do not know all of the roles of the

proteins involved in kinetochore function, verification of microscopic observations by other methods is critical.

Chromatin Immunoprecipitation (ChIP): Chromatin Immunoprecipitation is a technique capable of identifying whether a protein is directly or indirectly associated with a particular region of a chromosome *in vivo*. This powerful technique has been used to identify protein-DNA interactions in various biological systems including *S. cerevisiae*, *Drosophila* and mammalian cells (reviewed in Kuo and Allis, 1999; Orlando, 2000). Yeast are fixed with formaldehyde, lysed and sonicated to shear the chromosomal DNA to an average length of 200-500 bp. Immunoprecipitation is then used to isolate a desired protein along with any associated DNA. Crosslinks are reversed, DNA is recovered and PCR is used to identify the associated DNA. In the case of kinetochore proteins, *CEN* DNA primers are used to identify association with the centromere, while DNA primers for a region of the chromosome distal to the *CEN* serve as a negative control (as well as IP from a yeast strain without any tagged proteins). The use of ChIP has been a valuable tool not only for determining whether a protein is associated with *CEN* DNA *in vivo*, but by using a combination of mutants/deletions and ChIP, it has been possible to determine the protein interdependencies required for association with *CEN* DNA (Meluh and Koshland, 1997; Meluh et al., 1998; He et al., 2001). For example, if Ndc80p requires Ndc10p to associate with *CEN* DNA by ChIP, but the reciprocal is not true, we can postulate that Ndc10p lies between Ndc80p and *CEN* DNA. In the case of the CBF3 proteins, we know that they are in direct contact with *CEN* DNA, and indeed all kinetochore proteins examined thus far require CBF3 for association with *CEN* DNA (Espelin et al., 1997; He et al., 2001). By incorporating ChIP results involving a number of kinetochore proteins, it is possible to develop a network of

protein interdependencies which provide a rough view of kinetochore organization (see Chapter 6-Discussion for more details).

Conflicting results have been obtained by different researchers using ChIP and it would be a desirable, albeit formidable task for a single researcher to evaluate all combinations of kinetochore interdependencies using a standardized methodology. Further complications arise from partial dependencies, e.g. when the association of a protein with *CEN* DNA is reduced 50% in response to the elimination of another kinetochore protein. It is not clear what partial dependency means mechanistically. Another concern is the use of temperature-sensitive mutants, which may have hypomorphic phenotypes, thus making statements about dependencies between proteins difficult. The use of temperature-sensitive degron constructs which induce degradation of a target protein should provide "cleaner" results (Dohmen et al., 1994; Gardner et al., 2001; McCleland et al., 2003). Different proteins also seem to associate more or less strongly with *CEN* DNA, and these differences may be based on distance from the DNA (Ndc10p vs. Dam1p), antibodies used for IP (monoclonal, polyclonal, direct or to an epitope tag), variability of the protein tags themselves (myc, HA, GFP, etc.) or accessibility to the tag. Despite the issues involved with this method, ChIP provides a relatively easy method for evaluating *CEN*-association of proteins *in vivo* and has become widespread in its use for study of the kinetochore.

Protein Purifications Combined with Mass Spectrometry: The use of epitope tags has long provided a relatively simple means by which to purify proteins, but the identification of co-purifying proteins has until recently been quite tedious. However, complete sequencing of the *S. cerevisiae* genome, along with advances in mass spectrometry including the ability to detect

minute amounts of protein, have allowed for the rapid identification of protein complexes. The Seraphin Group has developed a method known as Tandem Affinity Purification (TAP) in which an epitope tagged protein is expressed at endogenous levels and associated proteins are recovered in their (presumed) native states (Rigaut et al., 1999; Puig et al., 2001). TAP utilizes two epitope tags, Protein A and Calmodulin Binding Peptide, separated by a TEV protease cleavage site. This construct allows for two rounds of protein purification to eliminate nonspecific contaminants, followed by release of the protein complex by addition of EGTA, a calcium chelator. The gentle elution conditions mean that the complexes purified using the TAP method may maintain their biological function. Eluted proteins are then run on SDS-PAGE and individual bands identified by mass spectrometry (the gel step may be skipped and the entire eluant analyzed directly). This method, and modifications of it, along with mass spectrometry have been used to purify a number of *S. cerevisiae* kinetochore subcomplexes including the NDC80 Complex (Janke et al., 2001; Wigge and Kilmartin, 2001), MTW1 Complex (De Wulf et al., 2003; Scharfenberger et al., 2003; Westermann et al., 2003), COMA Complex (Cheeseman et al., 2002; De Wulf et al., 2003; Nekrasov et al., 2003) and the DAM1 Complex (Cheeseman et al., 2002; Janke et al., 2002). The determination of what exactly constitutes a complex needs to be carefully considered when using ultra-sensitive mass spectrometry techniques. For example, Cheesman et al. (2002) have proposed a 12 member protein complex containing members of the MTW1 and COMA Complexes based solely on purification/mass spectrometry, while more accurate size exclusion chromatography and glycerol gradients have shown these to be two distinct complexes (Cheeseman et al., 2002; De Wulf et al., 2003). In the global view of the kinetochore, these distinctions may seem insignificant as the whole kinetochore must come together to function properly. However, it has been shown that each of the complexes may play

different roles in kinetochore function, and if we want to understand how this organelle assembles we need to understand the organization of its parts. The application of improved mass spectrometry techniques might also be applied to the identification of proteins responsible for *CEN*-MT attachment in MT binding assays (below), an option that was not available when these assays were first developed.

MT Binding Assays: The ultimate function of the kinetochore is to establish a connection between the centromeric DNA and microtubules. If we are to fully understand the kinetochore, it will be necessary to have a reliable MT-binding assay with which to test various aspects of the chromosome-MT attachment. Relatively straightforward experiments such as co-immunoprecipitation and spin-down assays have shown direct binding between MTs and a number of kinetochore proteins including Dam1p, Duo1p, Stu2p and Bim1p (Lee et al., 2000; Cheeseman et al., 2001a; van Breugel et al., 2003). These experiments involve the mixing of purified proteins with stabilized MTs and caution must be exercised when evaluating the specificities of such interactions. A more sophisticated approach is to assay the formation of a *CEN*-MT bridge. Using a MT spin-down assay, Kingsbury and Koshland showed that *CEN*-containing plasmids and chromosome fragments isolated from yeast extracts were capable of specifically associating with MTs *in vitro* (Kingsbury and Koshland, 1991; Kingsbury and Koshland, 1993). In this scheme, the isolated minichromosome must retain the ability to form the *CEN*-MT attachment although the exact composition of the proteins required to facilitate this interaction have never been identified. It was further shown by Kingsbury and Koshland using the same assay, that CDEII,III is necessary but not sufficient for binding to MTs (Kingsbury and Koshland, 1991). A more elaborate microscopy-based assay was developed by Sorger and

colleagues using Taxol-stabilized MTs adhered to a microscope slide (Sorger et al., 1994). Fluorescent beads were covalently bound to *CEN* DNA, mixed with yeast extracts and binding of the beads to the MTs observed and quantitated. Mutant *CEN* DNA and mutant kinetochore extracts serve as specificity controls. Although fractionation of yeast extracts was attempted in an effort to identify the specific components responsible for the MT binding observed in this assay, the exact composition of the fractions used by Sorger et al. (1994) also remained undetermined. In light of our increased understanding about the number of proteins which make up the kinetochore, it is not surprising that classic fractionation of kinetochore binding activity has proven a daunting task. It must also be kept in mind that most of these experiments employ MTs stabilized either by drugs such as Taxol or using GTP-analogs, and thus do not capture the true dynamic aspects of MTs which are present *in vivo*.

In Chapter 5 of this thesis, I will present an assay which uses a combination of *CEN* DNA, recombinant kinetochore proteins and yeast extracts in an attempt to establish which proteins are in association with CBF3. In brief, *CEN* DNA is covalently attached to magnetic Dynabeads (Dynal), mixed with recombinant CBF3, and either purified proteins or yeast extracts followed by use of Western Blotting to detect CBF3-associated proteins. This assay could readily be expanded to include identification of MT-binding proteins and ultimately MTs which are associated with *CEN* DNA via CBF3. An important goal of any *in vitro* MT-binding assay is to establish a system in which all of the components are known, and ultimately purified and quantified, so as to control all aspects of the assay. Incorporation of recombinant proteins and the advanced capabilities of mass spectrometry into an updated MT-binding assay should aid in our attempt to understand the requirements for *CEN*-MT binding. Once this has been established, alterations in the proteins and DNA can be characterized for their specific effects on

MT-binding. However, minimal requirements for *CEN*-MT attachment, and even whether *in vitro* reconstitution of the *CEN*-kinetochore-MT connection is possible, remain to be determined.

OVERVIEW

Tremendous advances have been made in the study of the *S. cerevisiae* kinetochore, most notably in the identification of its constituents. What was once thought to be a "simple" structure has evolved into an organelle as complicated as any other in the cell. As with any good mystery, it is only now becoming apparent why our understanding of this structure has been so difficult. This thesis attempts to better understand the molecular architecture of the kinetochore. It begins with an analysis of the DNA-binding protein Ndc10p (Chapter 2) in an attempt to elucidate the structure of the DNA-Binding layer of the *S. cerevisiae* kinetochore-a platform upon which the rest of the kinetochore assembles. Chapter 3 provides evidence concerning the ability of proteins from all layers of the kinetochore to interact with each other, and the interdependencies required for association with *CEN* DNA. Localization of the spindle checkpoint proteins in *S. cerevisiae* and an examination of the kinetochore lesions to which the spindle checkpoint responds are explored in Chapter 4. Chapter 5 presents ongoing work into the identification of proteins which bind to CBF3 in a *CEN*-dependent manner, with an eye towards building out from CBF3 towards the MT. Overall, the work in this thesis provides data to further our overall understanding of how the kinetochore is organized, one step in understanding how it functions.

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CHAPTER 2

Binding of the Essential *S. cerevisiae* Kinetochore Protein Ndc10p to CDEII

The work presented in this chapter is adapted, with permission, from Espelin et al. 2003. This work was performed in equal collaboration with Kim Simons (Harrison Laboratory, Harvard Medical School). The CDEII pattern identification program was developed exclusively by Kim Simons.

Espelin CW*, Simons KT*, Harrison SC and Sorger PK (2003) Binding of the Essential *Saccharomyces cerevisiae* Kinetochore Protein Ndc10p to CDEII. *Molecular Biology of the Cell* Vol. 14, 4557-4568 *These authors contributed equally to this work.

ABSTRACT

Chromosome segregation at mitosis depends critically on the accurate assembly of kinetochores and their stable attachment to microtubules. Analysis of *S. cerevisiae* kinetochores has shown that they are complex structures containing 50 or more protein components. Many of these yeast proteins have orthologs in animal cells, suggesting that key aspects of kinetochore structure have been conserved through evolution, despite the remarkable differences between the 125 bp centromeres of budding yeast and the Mb centromeres of animal cells. We describe here an analysis of *S. cerevisiae* Ndc10p, one of the four protein components of the CBF3 complex. CBF3 binds to the CDEIII element of centromeric DNA and initiates kinetochore assembly. Whereas CDEIII binding by Ndc10p requires the other components of CBF3, Ndc10p can bind on its own to CDEII, a region of centromeric DNA with no known binding partners. Ndc10p-CDEII binding involves a dispersed set of sequence-selective and non-selective contacts over approximately 80 bp of DNA, suggesting formation of a multimeric structure. CDEII-like sites, active in Ndc10p binding, are also present along chromosome arms. We propose that a polymeric Ndc10p complex formed on CDEII and CDEIII DNA is the foundation for recruiting microtubule attachment proteins to kinetochores. A similar type of polymeric structure on chromosome arms may mediate other chromosome-spindle interactions.

INTRODUCTION

Accurate chromosome segregation depends on the attachment of microtubules to kinetochores, protein-DNA complexes assembled on centromeric DNA. During mitosis, each sister chromatid must assemble one and only one kinetochore. Chromatids lacking kinetochores

cannot be pulled into daughter cells at anaphase whereas those with two or more kinetochores are in danger of being pulled in two directions at once and becoming torn. Correct chromosome segregation therefore requires proper regulation of kinetochore assembly.

S. cerevisiae is an attractive organism in which to study kinetochore assembly because it contains particularly short centromeres. A 125 bp *CEN* sequence is necessary and sufficient to mediate accurate chromosome segregation during mitosis and meiosis (Clarke and Carbon, 1980; Cottarel *et al.*, 1989). In contrast, *S. pombe* centromeres are 40-100 kb in length, and human centromeres span megabases (Bloom, 1993). Despite the dramatic difference in the complexity of centromeric DNA among different eukaryotes, there is good evidence that many kinetochore proteins have been conserved through evolution (Dobie *et al.*, 1999). It is therefore reasonable to expect that lessons learned in yeast will be of general importance for the study of chromosome segregation in other organisms.

The sixteen centromeres in *S. cerevisiae* contain three conserved DNA elements: CDEI, CDEII and CDEIII (reviewed in Hegemann and Fleig, 1993). CDEI is not essential for centromere function, but both CDEII and CDEIII are. CDEI is the binding site for the Cbf1 protein (Cai and Davis, 1989; Jiang and Philippsen, 1989; Mellor *et al.*, 1990) and CDEIII is the binding site for the four-protein CBF3 complex (Ng and Carbon, 1987; Lechner and Carbon, 1991). Both CDEI and CDEIII contain highly conserved bases in which point mutations impair protein binding and centromere function (Niedenthal *et al.*, 1991; McGrew *et al.*, 1986; Ng and Carbon, 1987; Hegemann *et al.*, 1988). CDEII has no known binding partners but it has a conserved length (78-84 bases) and high A-T composition (>90%; Clarke and Carbon, 1980).

CBF3 is the most extensively studied kinetochore complex in budding yeast (Lechner and Carbon, 1991; Goh and Kilmartin, 1993; Sorger *et al.*, 1995; Strunnikov *et al.*, 1995; Connelly

and Hieter, 1996; Espelin *et al.*, 1997) and appears to be required for the initiation of kinetochore assembly (Kaplan *et al.*, 1997; Russell *et al.*, 1999). *S. cerevisiae* kinetochores are characterized by a hierarchy of protein-DNA and protein-protein contacts, and all known kinetochore proteins require CBF3 activity for *in vivo* association with centromeric DNA. CBF3 contains four protein subunits: Ndc10p, Cep3p, Ctf13p and Skp1p, all of which are necessary for DNA binding and for cell viability. Three CBF3 proteins, Ndc10p, Cep3p, and Ctf13p are in direct contact with DNA, as judged by DNA crosslinking *in vitro* (Espelin *et al.*, 1997). The fourth, Skp1p, mediates the phosphorylation-dependent activation of Ctf13p (Kaplan *et al.*, 1997).

Although CDEII is essential (Panzeri *et al.*, 1985; Gaudet and Fitzgerald-Hayes, 1987), it has no previously identified protein ligands. The experiments described here show that Ndc10p is a sequence-specific CDEII DNA binding protein. While CDEIII binding by Ndc10p requires the three other members of the CBF3 protein complex, CDEII binding does not. Linker-scanning mutagenesis of CDEII reveals that sequences across a roughly 80 bp region are involved in protein-DNA recognition with a particularly important site near the center. Mutations in this sequence significantly decrease the fidelity of chromosome segregation. We therefore propose that Ndc10p has two functions at kinetochores, one as an essential component of CBF3 and a second as a CDEII-binding factor.

RESULTS

Ndc10p binds to CDEII

To identify proteins that associate specifically with CDEII DNA, we performed *in vitro* binding experiments using wildtype and variant *CEN3* probes that cover different regions of the *S. cerevisiae* centromere (Fig. 2-1A). Both whole-cell lysates from *S. cerevisiae* and

recombinant kinetochore proteins expressed in insect cells were used as sources of potential centromere-binding proteins. Consistent with our previous findings, when insect cell extracts containing recombinant Ndc10p, Cep3p or Ctf13p-Skp1p were mixed and incubated with an 88 bp CDEIII probe (Probe 2), we observed a rapidly migrating CBF3 species and a second more slowly migrating species (Fig. 2-1B, lane 1; Espelin *et al.*, 1997). Both of these complexes contain at least three CBF3 subunits (Ctf13p, Cep3p and Ndc10p) but the fast migrating species has a single Ndc10p dimer whereas the slow migrating species has two dimers. As expected, we saw no complexes when extracts containing only one or two CBF3 proteins were present in the binding reaction (Fig. 2-1B, lanes 2-4; Kaplan *et al.*, 1997). A strikingly different result was obtained with a 184 bp probe spanning all of *CEN3* (Probe 1). When Ndc10p alone was mixed with Probe 1, several distinct DNA–protein complexes formed (Fig. 2-1C, lanes 7-9) but none was detected with Ctf13p-Skp1p alone or Cep3 alone (Fig. 2-1C, lanes 5 and 6). Moreover, complexes did not form when Ndc10p was incubated with a randomly selected fragment of pUC19 (Fig. 2-1C, lane 10), suggesting that the Ndc10p-Probe 1 complex is sequence-selective. To measure this sequence selectivity, mixed-sequence salmon sperm DNA was titrated into Ndc10p-Probe 1 binding reactions. Binding of Ndc10p to *CEN3* DNA (50 fM) was 50% inhibited at a salmon sperm DNA concentration of 20 mg/ml (our unpublished results). Thus, mixed sequence DNA can compete with probe DNA for Ndc10p binding only at very high concentrations. The microscopic sequence selectivity for Ndc10p binding to *CEN3*, relative to mixed sequence DNA, is about 2×10^5. This is 10-fold lower than that of CBF3 for CDEIII (Espelin *et al.*, 1997) but typical for many sequence-specific DNA binding proteins. From these data, we conclude that Ndc10p binds in a sequence-selective fashion to intact *CEN3* DNA in the

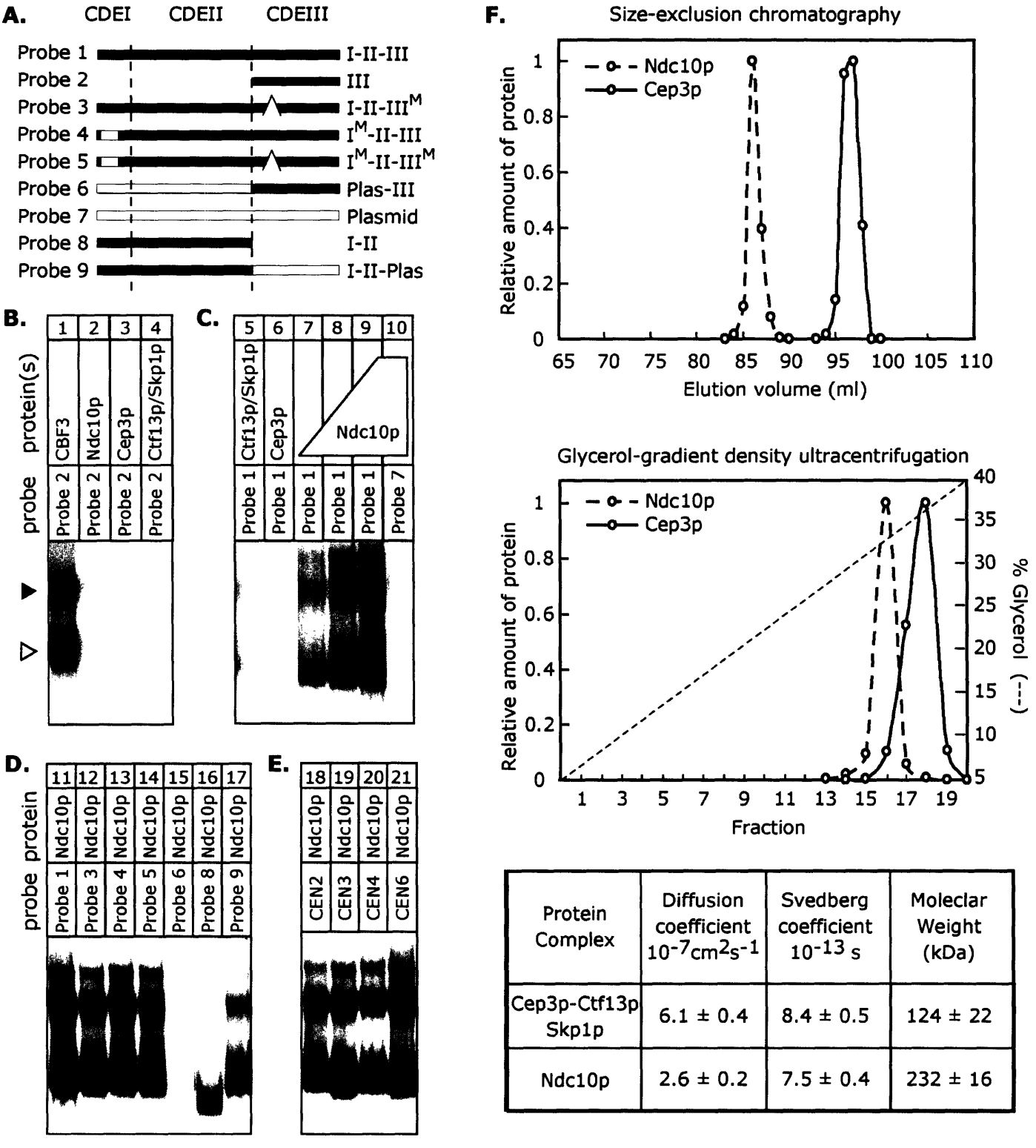
absence of other proteins, but does not bind to DNA containing only CDEIII, the binding site for CBF3.

Point mutations in CDEI or CDEIII disrupt association with Cbf1p and CBF3, respectively (McGrew *et al.*, 1986; Ng and Carbon, 1987; Baker and Masison, 1990; Cai and Davis, 1990; Neidenthal *et al.*, 1991). Incorporation of these mutations into *CEN3* either singly (Probes 3 and 4) or in combination (Probe 5) had no detectable effect on Ndc10p binding (Fig. 2-1D, lanes 12-14). However, when an 85 bp fragment of plasmid DNA was inserted in place of 85 bp of CDEII, Ndc10p binding was abolished (Fig. 2-1D, lane 15). This result suggests that Ndc10p binds to sequences in CDEII in a sequence-specific manner. To investigate whether CDEII is sufficient for Ndc10p binding, we used a 127 bp sequence comprising only wildtype CDEI + II of *CEN3* (Probe 8). Ndc10p bound to this DNA fragment, but more weakly than full-length *CEN3* (Probe 1). Suspecting that diminished Ndc10p binding might reflect the shorter length of Probe 8 relative to intact *CEN,* we generated a 184 bp probe (Probe 9) in which 88 bp of CDEIII were substituted with an equal length fragment of plasmid DNA while maintaining wildtype sequence for CDEI and CDEII. Ndc10p bound tightly to Probe 9 (complex formation was about 75% as efficient as for Probe 1), leading us to conclude that DNA flanking CDEII is involved in non-sequence specific Ndc10p binding. CDEII binding by Ndc10p is not a phenomenon limited to *CEN3* because a similar binding pattern was obtained with probes (Probe 1) derived from four different centromeres (Fig. 2-1E). Thus, binding to CDEII by Ndc10p appears to be a conserved feature of yeast kinetochores.

These findings with recombinant proteins indicate that Ndc10p is capable of existing independently of the other CBF3 components and that this Ndc10p is active in CDEII-binding. To demonstrate that this situation exists within a cell, whole-cell protein extracts were prepared

from wildtype yeast and subjected to hydrodynamic and bandshift analysis. Sizing columns and glycerol velocity gradients demonstrate that Ndc10p exists as a distinct species in yeast extract (Fig. 2-1F). When incubated with CDEII-containing DNA (Probe 9), these extracts gave rise to a set of complexes whose electrophoretic mobilities were indistinguishable from those of purified recombinant Ndc10p (our unpublished results). Combined with our analysis of recombinant proteins also showing that Ndc10p can exist both as a homodimer on its own and as part of CBF3 (Russell et al., 1999; unpublished observations), we conclude that unbound Ndc10p exists as a dimer and Cep3p is part of a 185 kD complex containing two Cep3p, one Ctf13p and one Skp1p subunit. From these observations we conclude that Ndc10p dimers represent the primary CDEII-binding activity that can be detected in yeast extracts using bandshift gels.

Figure 2-1 (next page) Binding of CBF3 and Ndc10p to CDEII and CDEIII centromeric DNA. Nuclear extracts from insect cells expressing recombinant proteins were incubated with radiolabeled DNA fragments and complexes resolved on non-denaturing bandshift gels (see Materials and Methods). Free probe is not shown. (A) Diagram of *CEN3*-derived probes used in this study and their names, as used in the text. Dashed lines denote approximate boundaries between CDEI, CDEII and CDEIII. "M" indicates a point or linker-scanning mutation as described in Materials and Methods. Probe 2 is 88 base pairs in length, probe 8 is 96 base pairs and all others are 184 base pairs.
(B) Binding of recombinant CBF3 proteins to CDEIII DNA (probe 2). "CBF3" (lane 1) denotes a binding reaction containing a mixture of three nuclear extracts from Hi5 cells expressing Ndc10p, Cep3p or Ctf13p/Skp1p. Binding reactions in lanes 2-4 contain only one extract with the proteins indicated. The open arrowhead marks the position of the CBF3-CDEIII core complex and the solid arrowhead the extended CBF3 complex (Espelin et al., 1997).
(C) Binding of recombinant CBF3 proteins to intact centromeric DNA (probe 1) and a plasmid control (probe 7). Ndc10p was purified from insect cell lysates by ion exchange chromatography and added at 0.4 pmol (lane 7), 1 pmol (lane 8) or 2 pmol (lane 9-10). Binding of 2 pmol of purified Ndc10p to the indicated probes (D) and to 184 bp fragments of *CEN2*, *CEN3*, *CEN4* and *CEN6* (E) that span CDEI-II-III (probe 1). (F) Analysis of whole-cell yeast extracts was performed using a Sephacryl S-500 HR column (Amersham Biosciences, Piscataway, NJ) and glycerol velocity gradients as described previously (Russell et al., 1999) followed by immuno-blotting with anti-Cep3p antibodies and anti-myc antibodies (for Ndc10p-myc6). The table shows estimated hydrodynamic properties (see Russell et al., 1999 for details).



Protein Complex	Diffusion coefficient $10^{-7}cm^2s^{-1}$	Svedberg coefficient 10^{-13} s	Moleclar Weight (kDa)
Cep3p-Ctf13p Skp1p	6.1 ± 0.4	8.4 ± 0.5	124 ± 22
Ndc10p	2.6 ± 0.2	7.5 ± 0.4	232 ± 16

Mapping Ndc10p binding sites in CDEII

To delineate the Ndc10p binding site in CDEII, we constructed a series of *CEN3* variants by substituting progressively larger regions of CDEII with plasmid DNA while maintaining the wildtype sequence of CDEI and CDEIII and the spacing between them (Fig. 2-2). When a substitution series from the left (starting at CDEI; LE1-7) was examined, Ndc10p-DNA binding decreased progressively (Fig. 2-2A). When substitutions from the right (starting at CDEIII; RE1-7) were examined, the most dramatic effects occurred with mutations covering the first 36 bp (Figure 2-2B; compare RE2 and RE3). From experiments with a set of linker-scanning mutations (S1-7), the CDEII region about 36 bp to the left of CDEIII appears to be the most critical for Ndc10p binding (Fig. 2-2C, S5), but no single 12 bp mutation abolished binding completely. We conclude from these data that Ndc10p-CDEII binding interactions are dispersed across the length of CDEII with particularly important contacts near the middle. We propose that Ndc10p makes extended DNA contacts and that multiple Ndc10p dimers participate in CDEII binding. The latter suggestion is consistent with the detection of more than one distinct Ndc10p-DNA complex in our experiments (Fig. 2-1C, for example). We have previously come to similar conclusions about the capacity of Ndc10p to form higher-order complexes on CDEIII, in association with CBF3 (Espelin *et al.*, 1997).

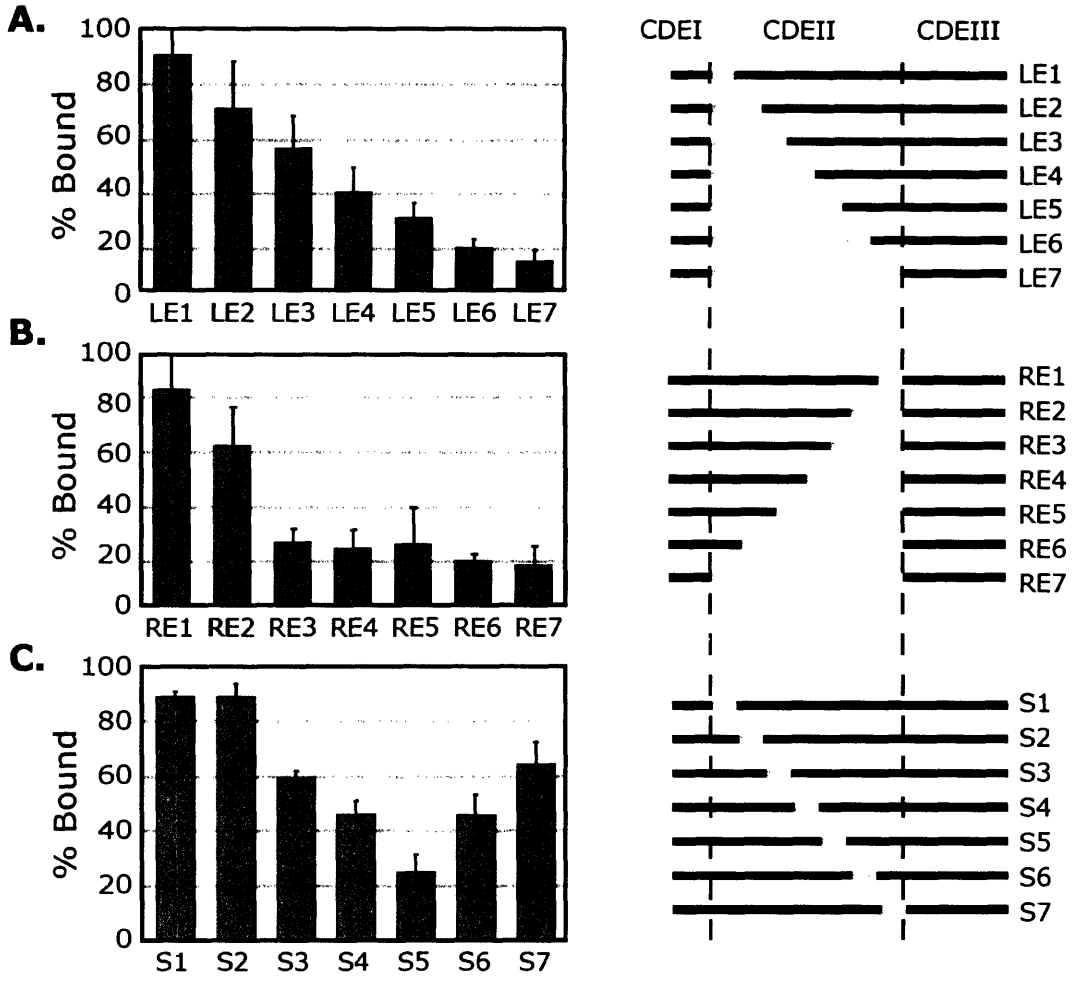


Figure 2-2 Mapping the Ndc10p binding site in CDEII by substitution and linker scanning mutagenesis. The amount of binding of each 184 base pair probe to 2 pmol purified Ndc10p is shown as a percentage of binding to a wildtype *CEN3* control. In the diagrams to the right of each bar graph, wildtype *CEN3* sequences are denoted in black and fragments of pBR322 or linker DNA with gray. (A) Substitutions from the left (CDEI; Left Extension) of *CEN3* in 12-base pair steps. (B) Substitutions from the right (CDEIII; Right Extension) side of *CEN3* in 12-base pair steps. (C) Linker-scanning mutations in successive 12-base pair steps across CDEII (Scanning).

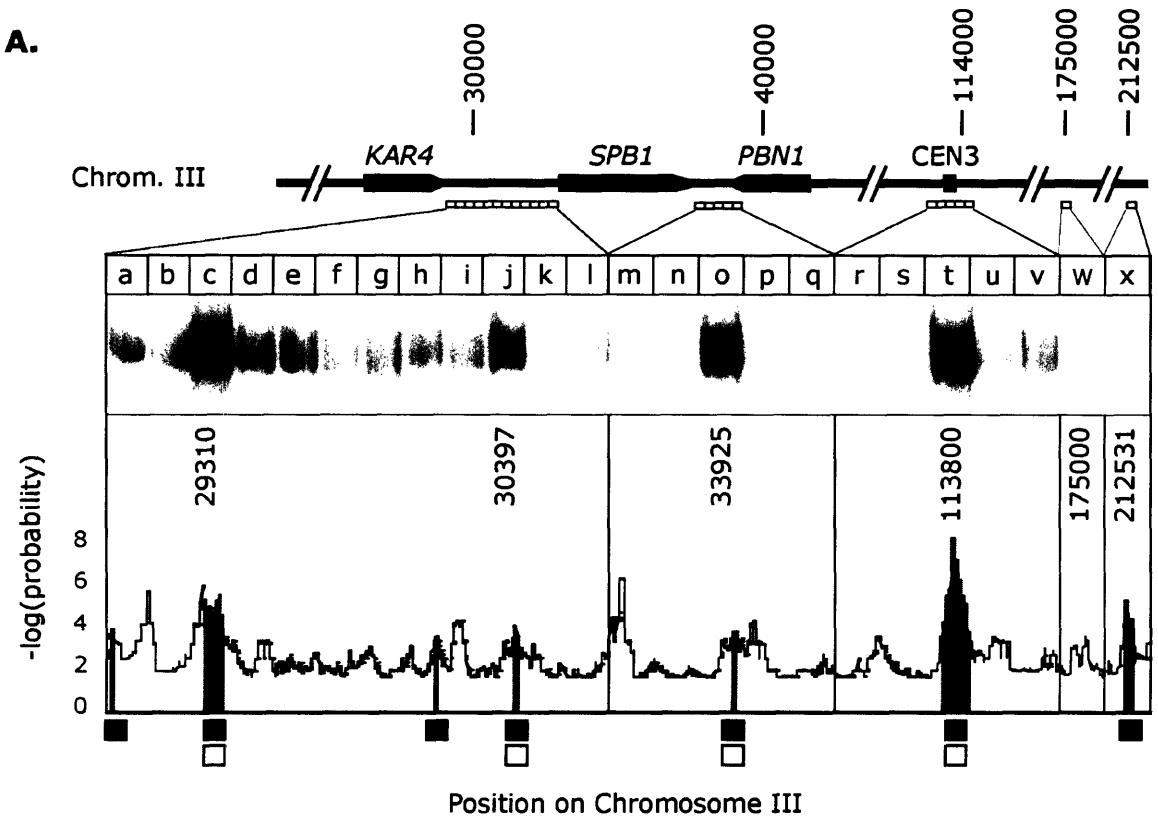
Properties of Ndc10p binding sites

Does CDEII contain a series of Ndc10p sites in tandem? The region of CDEII most critical for Ndc10p association (bases 49-60 of CDEII; Fig. 2-2C probe S5) does not represent a motif found elsewhere in CDEII. It therefore seems likely that Ndc10p binds to a family of related but non-identical AT-rich sequences. To capture this sequence selectivity computationally, we used a supervised learning algorithm to derive a pattern that extracts the

characteristic features of CDEII in all 16 *S. cerevisiae* centromeres. The yeast genome has an overall A+T composition of 61% but the distribution of A-T bases is non-random. When an 80 bp window (the approximate length of CDEII) is used to scan the genome, more regions with very high or very low A+T content are found than expected by chance (~100 times more frequent). Moreover, for any particular A-T composition, stretches of poly-adenine or poly-thymine are also more common than expected. The 16 CDEII sequences in *S. cerevisiae* are characterized both by high A-T content (averaging 92%), by the presence of 4-6 poly-A and poly-T stretches, and by a length of 78-84 bp.

Are there sequences at locations in the genome other than centromeres that match the CDEII pattern? When we scanned Chromosome III for matches to the CDEII pattern, 71 sites were found with a mean spacing of 4.5 kb (a similar density was found elsewhere in the genome, see Methods). A scan for the CDEIII pattern yielded only the single centromere present on each chromosome. Within the selected region of the Chromosome III arm shown in Figure 2-3, four matches to the CDEII pattern are found (including CEN3; Fig. 2-3A and 2-3B). These sites are characterized both by high A-T content (marked with blue squares) and poly-A/poly-T stretches (yellow squares). A highly AT-rich sequence is also present but it lacks poly-A and poly-T stretches (Fig. 2-3; fragment SGD 212531). To determine whether Ndc10p can bind to these non-centromeric sequences, we generated 25 successive 200 bp probes spanning the identified sequences and flanking DNA and analyzed Ndc10p binding to the probes on non-denaturing gels. Ndc10p associated efficiently with the three CDEII-like arm sequences (Fig. 2-3 lanes c, j and o) and to *CEN3*-CDEII (Fig. 2-3 lane t), but not to intervening DNA, a random genomic sequence (Fig. 2-3 lane w) or to the control AT-rich site (Fig. 2-3 lane x). In addition, the DNA-protein complexes formed on arm and CDEII sequences had similar electrophoretic mobilities

and the two classes of sequence (arms and *CEN*) cross-competed with one another (our unpublished results). Thus, the CDEII pattern generated by computer learning captures the DNA features responsible for Ndc10p binding, even though the pattern does not correspond to a simple consensus. In summary, our results support the conclusion that Ndc10p binds in the absence of other CBF3 proteins, specifically to a subset of AT-rich, poly-A and poly-T containing sequences similar to CDEII. The CDEII pattern is present considerably more often in the genome (~1000 times more frequent) than would be predicted by chance and Ndc10p binding sites may therefore be present along chromosome arms away from centromeres.



B.

	Position in Genome (SGD distance)		A+T%
AGGG**TTTT**GGATAG**TTTTTT**G**TTTT**CCAATTAGA**TTTTT**AGAAATA**TTTT**ATCATAG**TTTTTT**GTTTATTTATTTGTTTA	29310		81
TGG**TTTT**C**TTTTTTTTTTT**CAATCTTACTTAT**AAAA**TGAATATTTCAATTGATGAATAGCTATATATTGGATAC**AAAAA**T	30397		81
CTTGTATAATATTG**AAAA**TAAAGG**AAAA**GAAAGCAATAGATATATTATGTATACATAGAATTAATCAAATTATCATCAAA	33925		81
ATTTGA**TTTT**ATTATA**TTTTTAAAAAAA**GT**AAAAAA**T**AAAAA**GTAGTTTA**TTTTTAAAAAA**T**AAAA**TTT**AAAA**TATTAGT	113800	(CEN3)	94
AAGTGTTGTAGTATTCTGTACTTGACTTCAAATCTGATTTTAGTTCTTCAATTACAGTATCCCATACATTTCTTAGCTCT	175000		69
TCCTAGGTCTATATATATATCTATATACATATTTATATATATTATTAGAACTTTACAATATAGTATATACCATTCATTGT	212531		81

Figure 2-3 Binding of recombinant Ndc10p to sequences on Chromosome III arms and *CEN3*. (A) DNA probes in 200-base pair increments were generated across the indicated regions in the *KAR4-PBN1* interval and analyzed for binding to purified recombinant Ndc10p. Distances are indicated in nucleotides from the left telomere of Chromosome III. The graph shows the product of the log probability of the A-T composition in successive 80-base pair intervals and the log probability of the number of stretches containing four or more adenines or thymines in a row. To find potential Ndc10p binding sites, sequences were sought in which the A-T composition exceeds 80% (blue boxes) and the number of poly-A/poly-T stretches was significantly above average for the genome (yellow boxes). Ndc10p binding was predicted to occur on sequences in which both of these thresholds had been exceeded (green regions). (B) Sequences of six 80-base pair windows in Chromosome III analyzed for Ndc10p binding *in vitro*. Position refers to the number of the first nucleotide in the window relative to the left telomere of Chromosome III (Cherry et al., 1997) and "A+T" % to the percentage of adenine plus thymine. Stretches of A and T are emphasized in bold and underlined. Sequence 113800 is CDEII of *CEN3*; 175000 (lane w) represents a randomly chosen genomic location; 212531 is a site of high A-T content but lacking poly-A/poly-T stretches.

Increased chromosome loss associated with mutations in Ndc10p binding sites

To begin to establish a biological function for the binding of Ndc10p to CDEII, we asked whether mutations that affect Ndc10p-CDEII binding *in vitro* also affect centromere function *in vivo*. A subset of *CEN3* constructs containing wildtype or mutant sequences in combination with the *URA3* gene were recombined into Chromosome III in diploid cells, replacing the natural centromere on one chromosome with a modified sequence while maintaining the wildtype centromeric sequence on the other chromosome (Fig. 2-4A). We then determined the percentage of URA^+ cells by plating on SD-URA and YPD after zero, seven and thirteen generations of nonselective growth in YPD (Fig. 2-4B) and determined the loss rate of the mutated chromosomes (Fig. 2-4C). The loss rate of wildtype centromeres was essentially zero after 13 generations of nonselective growth (Fig. 2-4B and 2-4C). Alteration of bases '49-60' in the middle of CDEII, the mutation with the greatest reduction in Ndc10p binding *in vitro,* drastically decreased the mitotic stability of Chromosome III resulting in a loss rate of 2×10^{-2} per generation (Fig. 2-4B and 2-4C). When CDEII was replaced in its entirety with plasmid (Tet^r) DNA, mitotic chromosome segregation fidelity was again severely compromised, with an estimated loss rate of 2.6×10^{-2} per generation observed (Fig. 2-4B and 2-4C). Centromere function in Chromosome III could be substantially rescued, although not up to wildtype levels, by inserting a DNA sequence from an Ndc10p binding site from the Chromosome III arm while an AT-rich sequence that lacks Ndc10p binding activity *in vitro* rescued *CEN* function much less efficiently (our unpublished results). However, these data must be interpreted with some caution. The presence of chromosomes with CDEII mutations causes a substantial increase in cell-cycle doubling time (Fig. 2-4C), apparently as a consequence of a mitotic delay that is Mad2-dependent. Moreover, starting cultures appear to be quite aneuploid, probably as a

consequence of chromosome non-disjunction. Both of these problems are much more severe in our hands with CDEII mutations than with CDEIII mutations, and CDEII mutant chromosomes clearly warrant further investigation by live-cell analysis (He *et al.*, 2001). Nevertheless, our data do show that CDEII sequences which bind Ndc10p *in vitro* are important for centromere function *in vivo* and that Ndc10p-binding sites from chromosome arms can function at the centromere. We have therefore established a correlation between Ndc10p activity *in vitro* and CDEII function *in vivo* (see Discussion).

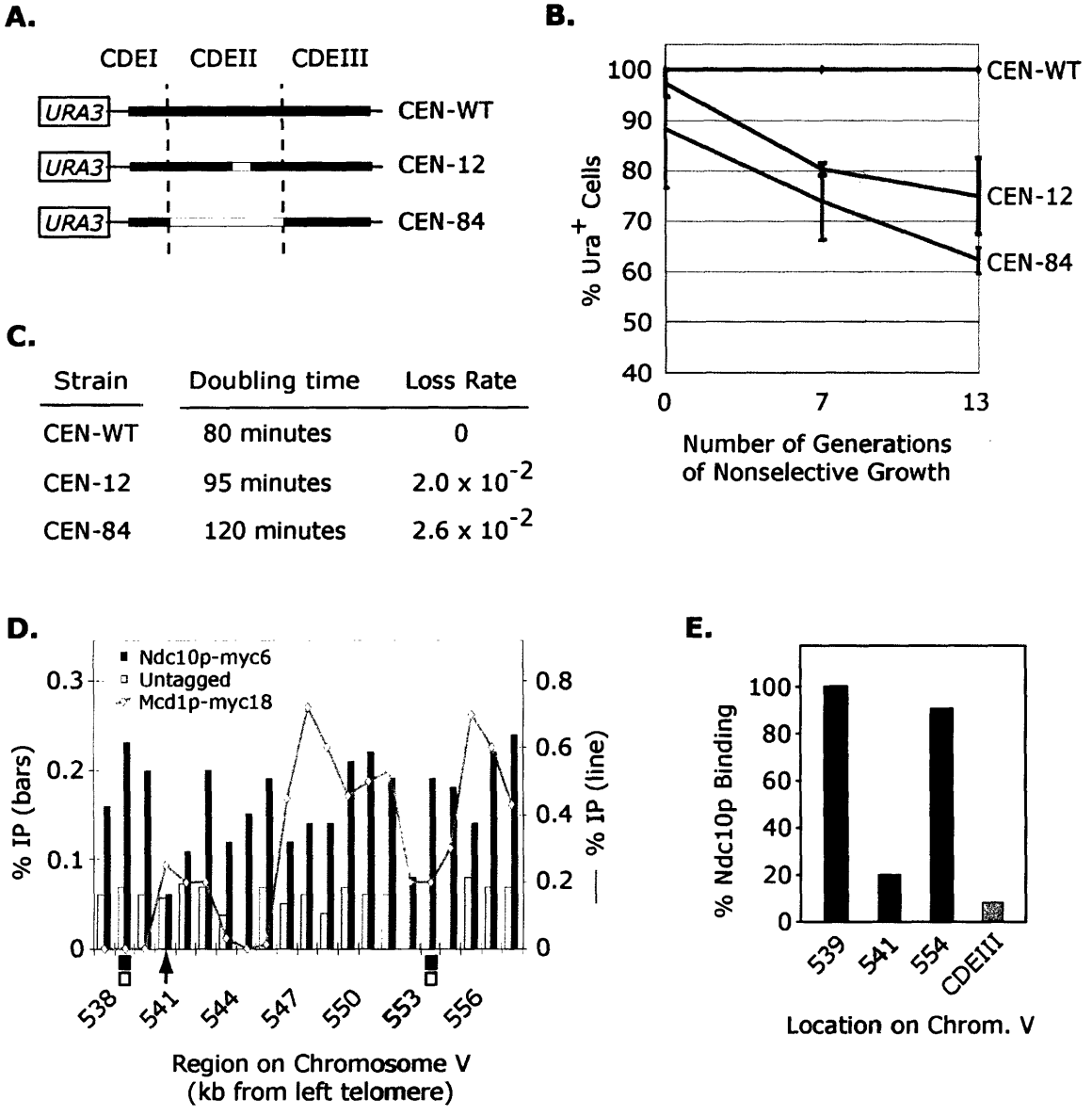


Figure 2-4 *In vivo* analysis of Ndc10p-DNA interactions. (A) Schematic of centromeric sequences carrying CDEII mutations and *URA3* used to evaluate chromosome loss. Wildtype regions are shown in black and mutant regions in gray. (B) Chromsome loss in cells carrying mutant centromeres on a *URA3*-marked chromosome after 0, 7, and 13 generations of nonselective growth in YPD. % *URA+* cells represents the ratio of colonies on SD-URA and YPD plates for each time point. Values represent the average of three individual cultures of each strain along with the SE of the mean. (C) Doubling time under nonselective conditions (YPD); chromosome loss rates determined from B. (D) Binding of Ndc10p and the cohesin subunit Mcd1p to a region of Chromosome V previously subjected to detailed analysis (Tanaka et al., 1999) by ChIP in 200-base pair DNA fragments spaced every 1 kb. A wildtype strain lacking a tagged protein (untagged) serves as a negative control for the level of background crosslinking. Percentage of crosslinking (relative to total input DNA) of Ndc10p-myc6 and the wildtype (Untagged) to Chromosome V are graphed against the right axis (vertical bars); percentage of crosslinking of Mcd1p-myc18 is graphed against the right axis (line; separate axes were used because absolute ChIP values differed). The sites indicated by the boxes and arrow correspond to sites used in the bandshift assay in E. (E) *In vitro* binding of Ndc10p to Chromosome V sites with high or low levels of ChIP crosslinking. CDEIII serves as a negative control.

Ndc10p association with chromosome arms

Does Ndc10p bind to chromosome arms *in vivo* as suggested by our *in vitro* data? To investigate this question, we performed Chromosome Immunopreciptation (ChIP) analysis on a region of Chromosome V that had previously been examined in detail for the binding of cohesins (Tanaka *et al.*, 1999). The cohesin proteins Mcd1p/Scc1p, Scc3p, Smc1p and Smc3p (Strunnikov *et al.*, 1993; Guacci *et al.*, 1997; Michaelis *et al.*, 1997; Skibbens *et al.*, 1999; Toth *et al.*, 1999) link sister chromatids together during metaphase and associate preferentially with AT-rich DNA (Blat and Kleckner, 1999; Megee *et al.*, 1999; Tanaka *et al.*, 1999). It therefore seemed possible that the cohesins and Ndc10p might bind to the same arm sequences. When the Chromosome V region was scanned for potential Ndc10p binding sites, several strong matches were identified. To examine the region for cohesin and Ndc10p binding *in vivo* by ChIP, yeast cells expressing either Mcd1p-myc18 or Ndc10p-myc6 in place of the wildtype proteins were crosslinked with formaldehyde, extracts prepared, DNA sheared to an average length of 500 bp, and the myc-tagged proteins isolated by immunoprecipitation. Extract from cells lacking a myc-tagged protein (Untagged) served as a negative control. Crosslinks were hydrolyzed and DNA sequences associated with Ndc10p-myc6 and Mcd1p-myc18 were detected by PCR (method of Megee *et al.*, 1999; Chrom. V oligos used by Tanaka *et al.*, 1999). We observed that Ndc10p-myc6 crosslinked strongly to *CEN5* DNA (not shown), as expected, and to different extents to DNA along the Chromosome V arms (Fig. 2-4D). The peaks of crosslinking were significantly above background levels observed with an untagged strain and corresponded to sequences that were positive for Ndc10p binding *in vitro* (Fig. 2-4E). The Ndc10p crosslinking peaks did not correspond (or alternate with) peaks of Mcd1p binding however, arguing against a connection between Ndc10p and cohesin (Fig. 2-4D). Furthermore, binding of Ndc10p to arm sites was at

best 3-4 fold above the untagged control and 5-10 fold lower than the level of Ndc10p crosslinking to centromeres. We have repeated this experiment with many variations using synchronized and asynchronous cultures and in each case obtained similar results: Ndc10p-myc6 crosslinks more strongly to some arm sites than others, crosslinking is well above background levels in untagged strains, but the absolute level of crosslinking is low. One explanation for the low but reproducible signal along arms is that the epitope tag used for Ndc10p immunoprecipitation is less accessible on arms than at centromeres. A second, more likely explanation, is low fractional occupancy of Ndc10p on arm sites. Consistent with this idea, the on-off rate of Ndc10p at CDEII *in vitro* is quite high (seconds/minutes as opposed to days for CBF3 on CDEIII; our unpublished results). In summary, ChIP data are consistent with non-centromeric binding by Ndc10p but short of conclusive.

We therefore sought, using high-resolution imaging, to obtain independent confirmation that Ndc10p is present at cellular locations other than centromeres. Asynchronous cultures of cells carrying Ndc10p-GFP were compared to cells in which known kinetochore proteins were similarly tagged. To determine the position of the spindle as well as verify cell cycle status, the spindle poles were marked with Spc42p-CFP and three-dimensional images were obtained from live and fixed cells using deconvolution microscopy. In other work from this laboratory, high-resolution imaging has shown that kinetochore proteins localize during metaphase to two lobes on either side of the spindle midzone (Goshima and Yanagida, 2000; He *et al.*, 2001). These lobes correspond to the average positions of congressed sister kinetochores, and thus to the budding yeast metaphase plate. The localization of one of these kinetochore-specific proteins, Mtw1p-GFP, is shown in Figure 2-5B and 2-5D (Goshima and Yanagida, 2000; He *et al.*, 2001). The apparent overlap of Mtw1p-GFP lobes and spindle poles is an artifact of projecting a three-

dimensional distribution onto two dimensions (see He *et al.*, 2000). Some kinetochore proteins have a more complex distribution than Mtw1p, being localized not only to lobes of congressed kinetochores, but also to nuclear microtubules, spindle pole bodies and cytoplasmic structures (e.g. Bik1p, Stu2p and Ipl1p: He *et al.*, 2001; Tanaka *et al.*, 2002).

Among the localization patterns we have observed for approximately 25 kinetochore proteins examined thus far, Ndc10p is unique. Ndc10p is found both in a region of the nucleus consistent with congressed kinetochores and is also present along much of the chromatin (Fig. 2-5A). In early anaphase spindles, Ndc10p-GFP fluorescence is visible in a broad distribution similar to that of DAPI-stained DNA (Fig. 2-5C; colocalization of Ndc10p and DAPI has not been possible because of the much greater brightness of the DAPI-stained DNA, but the pattern of Ndc10p localization is very similar). Somewhat later in anaphase, Ndc10p is also visible along the inter-pole microtubules, a pattern that has been noticed by others (our unpublished observations; Zeng *et al.*, 1999). In contrast, in control cells carrying only tagged spindle poles and imaged under identical conditions, neither chromatin nor microtubule fluorescence are visible, confirming that they are specific to Ndc10p-GFP (our unpublished observations). These data argue strongly that Ndc10p is present in nuclei not only at centromeres, but also at other structures that probably correspond to chromosome arms.

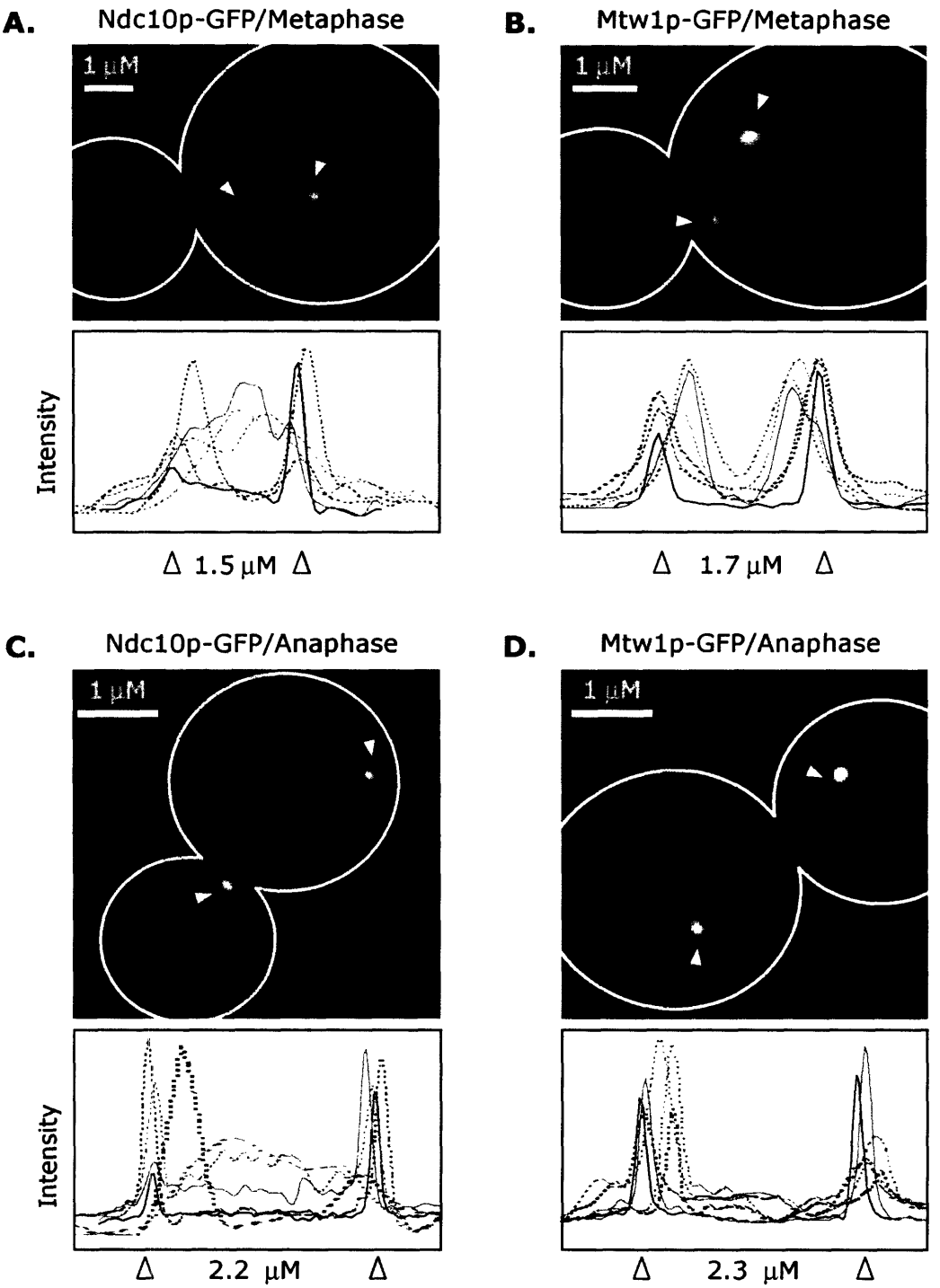


Figure 2-5 Localization of Ndc10p-GFP and Mtw1p-GFP in metaphase and anaphase cells. Ndc10p and Mtw1p, a known kinetochore protein (Goshima and Yanagida, 2000), were tagged with GFP (green) and the spindle pole component Spc42p was tagged with CFP (red; indicated by yellow arrowheads). Maximium intensity projections of three-dimensional image stacks containing ten to twenty 0.2-μm sections are shown representing typical images. The outline of the cell is indicated in yellow. All cells were exposed similarly and images have been adjusted to give the most accurate comparison. The graphs show the GFP and CFP fluorescence (raw pixel intensities) integrated along the spindle axis for three different representative cells with the solid line and indicated length representing data from the image shown.

DISCUSSION

Homologies between kinetochore proteins in higher and lower eukaryotes argue for substantial evolutionary conservation in kinetochore architecture. Centromeric DNA is strikingly different among different species, however, consisting of as few as 125 bp of specific DNA in budding yeast and as many as several Mb in humans. One of the central mysteries in mitosis is the molecular basis of this radical divergence in centromere organization within the context of substantial conservation in kinetochore composition. One way to clarify this mystery is to uncover the molecular principles of yeast centromere organization and to use these principles to study complex animal cell centromeres. The least well understood of the three sequence elements in *S. cerevisiae* centromeres is CDEII. Cbf1p has been shown to bind CDEI (Jiang and Philippsen, 1989; Cai and Davis, 1990; Mellor *et al.*, 1990) and CBF3 to bind CDEIII (Lechner and Carbon, 1991; Espelin *et al.*, 1997), but no CDEII-interacting proteins have been identified thus far. A key finding from a large number of studies is that the association of all known kinetochore proteins with centromeric DNA depends on functional Ndc10p (Ortiz *et al.*, 1999; He *et al.*, 2001; Janke *et al.*, 2001). In this work we propose that the critical role for Ndc10p is a consequence of its sequence-specific binding to both CDEII and CDEIII.

DNA binding activities of Ndc10p

Previous analysis of Ndc10p focused on its binding to CDEIII DNA as part of a CBF3 core complex containing a homodimer of Ndc10p, a homodimer of Cep3p, and a heterodimer of Skp1p and Ctf13p. When assembled into CBF3, Ndc10p, Ctf13p and Cep3p are in direct contact with CDEIII DNA, as judged by DNA-protein crosslinking, but none of these proteins can bind CDEIII on its own (Figure 6 of Espelin *et al.*, 1997). We now show that Ndc10p interacts with

CDEII in the absence of other CBF3 proteins. This binding includes an extended series of sequence-specific contacts over approximately 80 bp of CDEII as well as sequence-independent contacts with flanking DNA. In addition, three or more CDEII-Ndc10p complexes differing in electrophoretic mobility form *in vitro* (depending on Ndc10p concentration), probably representing different numbers of Ndc10p dimers on each DNA molecule. It is further notable that the *in vivo* loss rate of chromosomes carrying a specific 12 bp mutation in CDEII, which markedly reduces *in vitro* binding of Ndc10p, is similar to the loss rate observed by replacing all of CDEII with plasmid DNA. This suggests that we have disrupted a key function of CDEII, which we believe is the ability to bind Ndc10p. Together, these data suggest that Ndc10p forms a multimeric structure and makes dispersed contacts throughout the length of CDEII. A similar type of binding by Ndc10p also occurs in CDEIII. While there is only one Ndc10p dimer in the CBF3 core (Russell *et al.*, 1999), a second dimer associates with the core and flanking DNA to generate an extended CBF3 complex (Espelin *et al.*, 1997).

Our current model is that Ndc10p has multiple functions in kinetochore assembly involving three modes of DNA binding (Fig. 2-6): sequence-selective binding to CDEII in the absence of other proteins (this work), sequence-selective binding to CDEIII in the context of a CBF3 core complex (Espelin *et al.*, 1997), and sequence-independent but CBF3-dependent binding to centromere-distal sequences in the context of the extended CBF3 complex (Espelin *et al.*, 1997). We have generated a large series of Ndc10p truncation and point mutations and tested them for binding to both CDEII and CDEIII, but have found no separation-of-function mutations that retain one type of binding but eliminate another (data not shown). We therefore believe that all three binding modes involve a single DNA binding domain, rather than multiple distinct domains. There are many precedents for the binding of a protein to DNA in more than

one context. Transcription factors such as AP-1 can associate with a variety of other DNA-binding proteins on complex regulatory sites (Shaulian and Karin, 2002). The yeast transcription factors, Ste12p and Pho4p, can bind DNA as homo-multimers and bind as cooperative complexes with other proteins (Baur *et al.*, 1997; Magbanua *et al.*, 1997). Consistent with binding in several modes, Ndc10p exists as a free dimer in whole-cell yeast extracts.

Potential CDEII binding proteins

Our data argue that Ndc10p binds to both CDEII and CDEIII DNA *in vivo*, but proteins other than Ndc10p have previously been proposed to be CDEII-interactors. These proteins include ubiquitous AT-binding proteins and the kinetochore-specific proteins Mif2p, the yeast homologue of mammalian CENP-C (Meluh and Koshland, 1995; Meluh and Koshland, 1997), and Cse4p, the yeast homologue of the variant mammalian histone H3 CENP-A (Pluta *et al.*, 1995; Stoler *et al.*, 1995; Smith *et al.*, 1996; Meluh *et al.*, 1998). Although it is possible, and even likely, that more than one kinetochore protein has CDEII-binding activity, it is informative to compare the candidates. A variety of ubiquitous proteins bind to A-T tracts including, DATIN (Winter and Varshavsky, 1989), CMBF (Horn *et al.*, 1999), and HMG-I(Y) (Reeves and Nissen, 1993) but none have been shown to have a specific role in kinetochore function. DATIN specifically has been shown to be incapable of CDEII binding *in vitro* (Winter and Varshavsky, 1989). The discussion below therefore focuses on Ndc10p, Mif2p, and Cse4p. In considering the evidence discussed below it is important to note that none of these three proteins has been shown to be capable of binding to CDEII *in vivo* in the absence of a functional CDEIII sequence. It therefore appears that the binding of all kinetochore proteins (including the proposed CDEII-bound Ndc10p polymer) requires that CBF3 be associated with CDEIII. Based on our current

understanding of transcription, it seems likely that one important function of CDEIII is to disperse nucleosomes that might otherwise make centromeric DNA inaccessible to other binding factors.

We have obtained strong biochemical evidence that Ndc10p binds in a sequence-specific fashion to CDEII *in vitro* but cannot yet determine the precise function of this binding *in vivo.* By way of comparison, the function of the CDEIII-CBF3 interaction has been firmly established by multiple *in vitro* and *in vivo* experiments. CDEIII is a relatively short and well-conserved sequence in which single point mutations abolish activity. When a range of point mutations can be made in a sequence and activities *in vitro* and *in vivo* correlated, a strong argument for the biological function of a particular DNA-protein interaction can be made. In the case of CDEII, however, the distributed nature of a site in which no single linker-scanning block mutation eliminates binding, makes it hard to draw as tight a link between biochemical and cellular activities. However, we have uncovered some of the key features of Ndc10p-CDEII association. CDEII mutations that impair Ndc10p binding *in vitro* impair centromere function *in vivo.* An algorithm that extracts the sequence features of CDEII identifies non-centromeric DNA that is active in Ndc10p binding *in vitro,* and at least partially active in substituting for CDEII at centromeres *in vivo.* Additionally, we have attempted to use ChIP to map Ndc10p binding to CDEII and CDEIII in *vivo* and to evaluate the effects of CDEII mutations on Ndc10p-*CEN* association. However, the difficulty in resolving immediately adjacent sequences by ChIP has made it impossible to distinguish between binding to CDEII and to CDEIII. Nevertheless, we conclude that a biological role for Ndc10p in CDEII binding is likely.

The argument that Mif2p binds to CDEII is largely based on its homology to the mammalian centromere-binding protein CENP-C (Meluh and Koshland, 1995) and the presence

of an "AT-hook" motif proposed to assist in binding DNA (Brown *et al.*, 1993). However, the region responsible for DNA binding in CENP-C (Politi *et al.*, 2002) is not conserved in Mif2p and we have found that Mif2p lacking the "AT-hook" appears to be fully functional *in vivo* (data not shown). We have also examined recombinant Mif2p *in vitro*, but have not detected binding to *CEN* DNA, either in the presence or absence of CBF3. Furthermore, Mif2p has been shown to be incapable of binding CDEI+II DNA in *vivo* as determined by ChIP (Meluh and Koshland, 1997). Thus, while it is clear that Mif2p is bound to centromeric DNA (Meluh and Koshland, 1997), the evidence is not strong that this involves a specific interaction with CDEII DNA as opposed to association with other kinetochore components.

Cse4p, a specialized histone H3, has also been proposed to be a CDEII-binding protein. This proposal is based on interpretation of ChIP results (Meluh *et al.*, 1998) and the existence of genetic synergy between *cis*-acting mutations in CDEII and *trans*-acting mutations in *CSE4* (Smith *et al.*, 1996; Keith and Fitzgerald-Hayes, 2000), but no direct interaction has been demonstrated. Given the small size of the yeast centromere and the proximity of CDEII and CDEIII relative to the resolution of ChIP (about 200-500 bp), it is difficult, if not impossible to determine that Cse4p is binding CDEII using this method. Enhanced crosslinking of Cse4p to the center of *CEN* DNA (Meluh *et al.*, 1998) would be likely if Cse4p-containing nucleosomes flank the centromere on both sides. Furthermore, Ortiz et al. (Ortiz *et al.*, 1999) have shown that Cse4p can be crosslinked by ChIP to a *CEN3* fragment that includes CDEIII without CDEII, showing that CDEII is not necessary for Cse4p recruitment to centromeres. Classic experiments describing the nature of nucleosomes (Kornberg and Klug, 1981) dictate that changes in the number of nucleotide bases alter the phasing of DNA. Were core centromeric DNA sequences wrapped around a nucleosome, changes in the length of CDEII would have a dramatic effect on

the ability of kinetochore proteins to make specific DNA contacts and would thus alter their orientation and interaction with other kinetochore proteins. Previous data demonstrates binding of the kinetochore proteins Cep3p, Ctf13p, Ndc10p and Cbf1p (Espelin *et al.*, 1997; Cai and Davis, 1990) to both sides of the DNA, making it a steric improbability that kinetochore proteins could bind on the face of the nucleosome and not be affected by the length of CDEII (Bloom *et al.*, 1989; Meluh et al., 1998; Cheeseman et al., 2002). Nucleosomes containing Cse4p should also be similar in their binding specificities to conventional nucleosomes because the Cse4p-specific sequences, particularly the extended N-terminus, project away from the DNA (Luger *et al.*, 1997). Conventional nucleosomes are known to bind poorly to sequences containing stretches of Adenine followed by stretches of Thymine (Kunkel and Martinson 1981; Prunell 1982). While not excluded from nucleosomes, AT-rich DNA tends to be found at the ends of DNA wrapped around histones (Satchwell *et al.*, 1986), and CDEII would therefore appear to be a poor nucleosome binding sequence.

These considerations raise questions about the widely held assumption that Cse4p binds specifically to CDEII (Keith and Fitzgerald-Hayes, 2000; Cheeseman *et al.*, 2002). Instead, we put forward the hypothesis that CDEII is bound by a polymeric Ndc10p complex, that phased nucleosomes reside on either side of the central *CEN* DNA-kinetochore structure, as originally suggested by Bloom and Carbon (1982), and that it is these nucleosomes that contain Cse4p. The evidence in favor of this model is by no means definitive, but we believe it should be seriously considered as an alternative to the "nucleosome"-centric view.

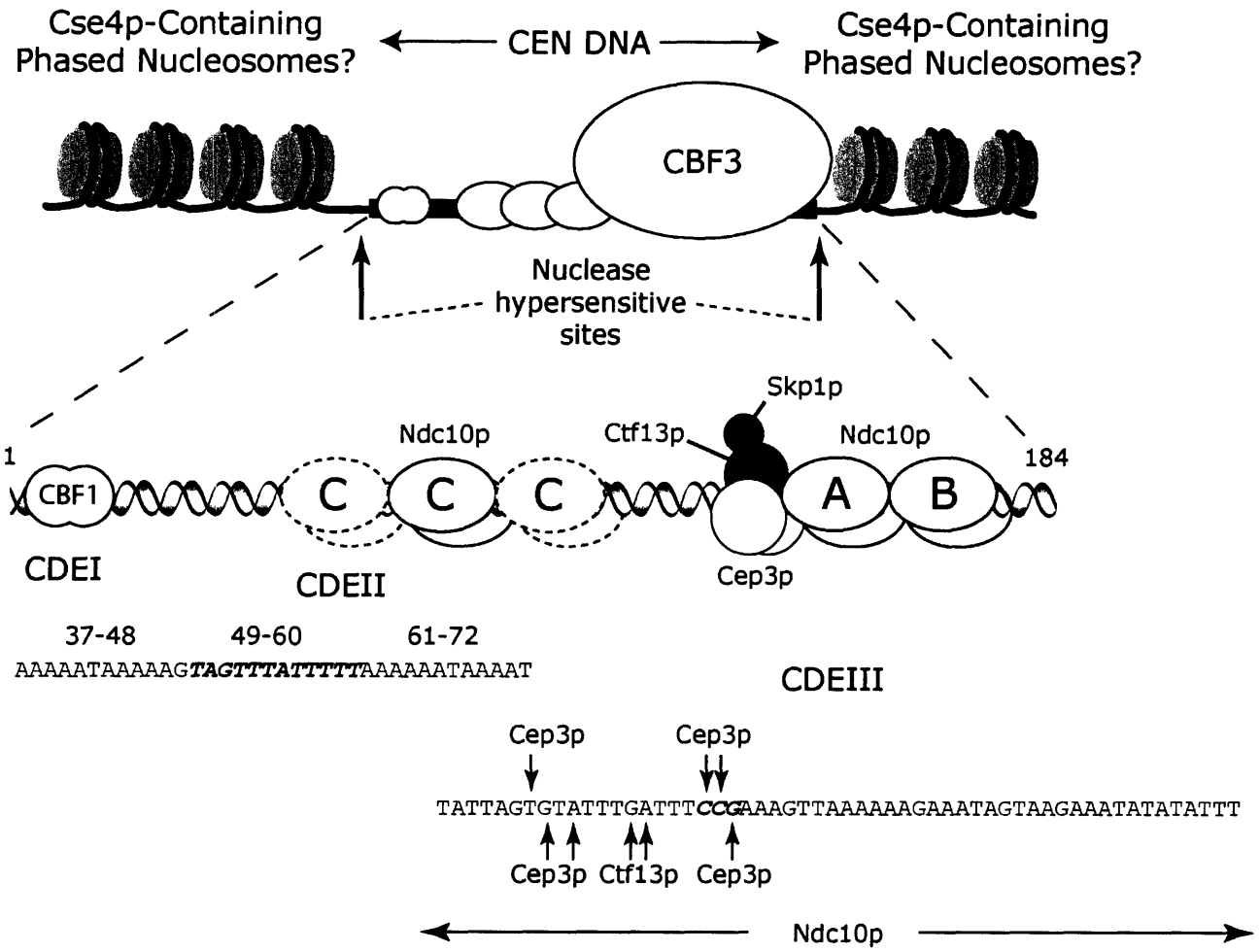


Figure 2-6 Speculative model for the interaction of Ndc10p, CBF3 and nucleosomes with budding yeast centromeres. Three different modes of Ndc10p-DNA interaction are shown. The extended CBF3 complex on CDEIII involves sequence-specific binding by one Ndc10p dimer (marked A) and sequence-independent binding by a second dimer (marked B; Espelin et al., 1997; Russell et al., 1999). We propose that multiple Ndc10p dimers bind sequence-selectively to CDEII (marked C), with particularly important binding by one dimer (C, solid outline) to positions 49-60 of CDEII (Figure 2). The sites of crosslinking by CBF3 subunits to bases in CDEIII are indicated by arrows (Espelin et al., 1997). This CBF3-Ndc10p-*CEN* DNA complex is proposed to be flanked by nuclease hypersensitive sites and embedded in a region of phased nucleosomes that contain the specialized H3 protein Cse4p (Bloom and Carbon, 1982; Bloom et al., 1983; Funk et al., 1989). No attempt has been made to speculate on the overall folding of the centromeric chromatin, but it seems likely from studies in other organisms that it adopts a special structure.

Non-centromeric roles for Ndc10p

Using the sixteen CDEII sequences in *S. cerevisiae* as a guide, we have identified sequences approximately every 5 kb along chromosome arms that match the CDEII pattern and are high affinity Ndc10p binding sites *in vitro*. The AT-rich, poly-A/poly-T pattern characteristic of these sites occurs 1000-fold more frequently in the yeast genome than would be expected by chance, implying a possible biological function. The Ndc10p-DNA complexes that form on arm sites are similar in electrophoretic mobility to complexes that form on CDEII, and arm sites cross-complete with CDEII for Ndc10p binding. Because the arm sites are not near CDEIII sequences, it is possible to probe the extent of Ndc10p binding *in vivo* using ChIP. Ndc10p is observed to crosslink to arm sites at levels well above background but below the levels observed at centromeres. The relatively low efficiency of crosslinking makes the result less than totally convincing but may reflect low occupancy of the sites *in vivo*. However, the localization of Ndc10p to structures other than kinetochores has been confirmed by 3D deconvolution microscopy (this study) and by other methods (Goh and Kilmartin, 1993; Zeng *et al.*, 1999). The function of extra-centromeric Ndc10p is not yet known, but may reflect the assembly of structures that transiently associate with microtubules, perhaps to generate a yeast analog of polar ejection forces (Rieder *et al.*, 1986).

SUMMARY

In summary, the data in this paper support the conclusion that, in addition to binding to CDEIII as an essential component of CBF3, Ndc10p also binds to CDEII and to sites along chromosome arms. We have not yet distinguished the functions of Ndc10p at CDEII and CDEIII, but it may not be meaningful to separate the kinetochore structure formed on CDEII

from the assembly on CDEIII because proteins bound to each region of DNA likely interact. In fact, inversion of CDEIII, relative to CDEII, results in complete loss of centromere function (Murphy *et al.*, 1991). ChIP experiments demonstrate that kinetochore proteins do not associate *in vivo* with CDEI or CDEII in the absence of a proximal CDEIII sequence, not even Cbf1p, which recognizes a discrete site at CDEI *in vitro* (Meluh and Koshland, 1995). A more detailed knowledge of Ndc10p is required to resolve these issues and we have therefore embarked on a high-resolution crystallographic analysis of Ndc10p complexes as well as electron microscopy of *CEN* DNA with the CBF3 protein complex, Ndc10p and Cse4p-containing nucleosomes. We nevertheless propose that kinetochores contain a polymeric Ndc10p assembly covering CDEII and CDEIII that forms a platform onto which the microtubule-binding components of kinetochores are recruited. The presence of an Ndc10p polymer may help to solve a compliance problem encountered in firmly anchoring 13 microtubule protofilaments to a single DNA sequence.

MATERIALS AND METHODS

Protein expression and purification

Ndc10p, Ctf13p/Skp1p and Cep3p-containing extracts were prepared from nuclear lysates of baculovirus-infected Hi5 insect cells (Kaplan *et al.*, 1997). These extracts were analyzed in bandshift assays with radiolabeled probes, as described (Sorger *et al.*, 1995). To purify Ndc10p, Hi5 cell lysates were prepared 36 hours post infection, centrifuged to remove particulate material and applied to a cation-exchange POROS HQ column (Perseptive Biosystems) in 150 mM KCl, 10 mM HEPES pH 8.0, 20 mM β-glycerophosphate, 20 mM NaF, 10% glycerol, 0.1% β-mercaptoethanol and eluted with 600 mM KCl in the same buffer. Fractions containing Ndc10p

were collected and the KCl concentration adjusted to 150 mM. The pooled fractions were then applied to an anion-exchange POROS HS column (Perseptive Biosystems) and eluted with 600 mM KCl. Fractions were again pooled on the basis of Ndc10p amount as judged by SDS-PAGE. Anti-myc antibody (9E10) was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA).

Plasmids and probes

Bandshift probes were generated as described (Espelin *et al.*, 1997) from subcloned fragments in plasmids or directly from genomic DNA. *CEN3* mutants were generated from pRN505 as follows: the $CDEI^{M}$ probe includes a CDEI mutation from CACATG to CATTATG and $CDEIII^{M}$ probes delete the central CCG of CDEIII (Sorger *et al.*, 1995). Probes for the experiments in Figure 2-2 were generated using PCR "mega-priming" with the final products subcloned into vectors and sequenced. A fragment of the tetracycline resistance gene from pBR322 was used as a source of "random" DNA in substitution and linker scanning mutations. Chromatin Immunoprecipitation (ChIP) was performed as described previously (Megee *et al.*, 1999; Tanaka *et al.*, 1999).

Strains and chromosome loss assay

Yeast strains were derived from W303 (*MATa, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL, psi+*). The strain harboring Ndc10p-myc6 was produced by transformation of W303 by NDC10-myc6::TRP1 (gift of S. Piatti, Universita degli Studi di Milano-Bicocca, Italy). The Mcd1p-myc18 strain was a gift of the Nasmyth laboratory (K6565: W303 *MATa, ade2-1, can1-100, leu2-3,112, GAL, psi+, MCD1-myc18::TRP1*). Strains for chromosome loss assays were generated as described (Clarke *et al.*, 1983) by transforming W303 haploid cells

with constructs containing variant *CEN* sequence and a linked *URA3* marker and subsequent mating with cells of the opposite mating type. Correct integrations were confirmed by PCR genotyping. The chromosome loss assay was performed as follows: cells were grown in selective media (SD-URA), then plated immediately (t=0) or grown under non-selective conditions for seven or thirteen generations in YPD before plating on SD-URA and YPD. Results shown are the average of three separate cultures and 200-400 colonies analyzed for each strain. Generations were monitored by OD_{600} and cell number. Cells were counted by hemacytometer for plating. The fraction of URA^+ cells was determined from the ratio of colonies obtained by plating on SD-URA and YPD. (Analysis was also performed using 5-fluoro-orotic acid (FOA) SD-complete plates with similar results. PCR analysis of several randomly chosen colonies from FOA plates confirmed loss of the chromosome carrying the CDEII mutation (our unpublished results).

Pattern identification program

To analyze CDEII sequences, a program was written that applies a sliding, non-overlapping window of 80 nucleotides (similar in length to CDEII) to the sequence of Chromosome III; this analysis uncovered 71 sites of high A-T composition (>80%). Four of these sites were selected at random for bandshift analysis (Fig. 2-3, lanes c, j, o, and x) along with a randomly selected chromosomal site as a control (Fig. 2-3, lane w). Bandshift probes were 200 bases in length and covered the 80 bp CDEII-matched-property window along with 60 flanking bases on both sides. The probability of a particular A + T composition is derived from the distribution observed in the yeast genome. Regions of DNA containing stretches of poly-A or poly-T were compared to each other using simulation: for a particular content of Adenine, the sequence was randomized and

number of stretches counted; from this distribution, probabilities were determined. We compute that CDEII-like sites occur on average every ~5 kb.

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CHAPTER 3

Molecular Analysis of Kinetochore-Microtubule Attachment in Budding Yeast

The work presented in this chapter is adapted, with permission, from He et al. 2001. The focus of my work in this chapter is the use of Chromatin Immunoprecipitation (ChIP) to evaluate *CEN*-localization of kinetochore proteins and determine the interdependencies required for their association with centromeric DNA (specifically Table 1, Figures 3 and 6).

He X*, Rines DR*, Espelin CW and Sorger PK (2001) Molecular Analysis of Kinetochore-Microtubule Attachment in Budding Yeast. *Cell* Vol. 106, 195-206 *These authors contributed equally to this work

ABSTRACT

The complex series of movements that mediates chromosome segregation during mitosis is dependent on the attachment of microtubules to kinetochores, DNA-protein complexes that assemble on centromeric DNA. We describe the use of live-cell imaging and chromatin immunoprecipitation in *S. cerevisiae* to identify ten kinetochore subunits, among which are yeast homologs of microtubule binding proteins in animal cells. By analyzing conditional mutations in several of these proteins, we show that they are required for the imposition of tension on paired sister kinetochores and for correct chromosome movement. The proteins include both molecular motors and microtubule associated proteins (MAPs), implying that motors and MAPs function together in binding chromosomes to spindle microtubules.

INTRODUCTION

The segregation of replicated sister chromatids into two equal sets at mitosis involves a complex series of movements mediated by kinetochores, DNA-protein complexes that assemble on centromeric DNA. Following microtubule attachment early in mitosis, paired sister chromatids exhibit directional instability and undergo oscillatory movements back and forth along spindle microtubules (Skibbens et al., 1993). Sister separation is delayed by a mitotic checkpoint comprising *MAD* and *BUB* genes that is silenced only when all pairs of chromatids have achieved bivalent attachment. Sister cohesion is then dissolved and chomatids begin their anaphase movement toward the spindle poles.

As structures that link centromeres to spindle fibers, kinetochores have both DNA and microtubule binding activities. The unusual compactness of *S. cerevisiae* centromeres (approximately 175 bp) has facilitated biochemical and genetic analysis of kinetochore-

associated DNA binding proteins. These include CBF3 and the specialized H3 histone Cse4p (Stoler et al., 1995). The assembly of kinetochores in *S. cerevisiae* appears to begin with the binding of CBF3, a four-protein complex, to the essential CDEIII region of centromeric DNA. Cells carrying temperature sensitive mutations in CBF3 subunits (Ndc10p, Cep3p, Ctf13p, or Skp1p) experience greatly elevated chromosome loss under semipermissive conditions (Hyman and Sorger, 1995). Several additional proteins have been identified that bind to yeast centromeres in a CBF3-dependent fashion (Stoler et al., 1995; Meluh et al., 1998; Hyland et al., 1999; Ortiz et al., 1999; Zheng et al., 1999). However, none of these proteins have been implicated directly in the attachment of chromosomes to microtubules or in the generation of force.

Historically, an important question about chromosome-microtubule attachment has been the identity of the kinetochore-associated motors. In animal cells, the kinesin-related motor proteins (KRPs) CENP-E and MCAK have been shown to function in kinetochore-dependent chromosome movement, as has dynein (for review see Rieder and Salmon, 1998); in yeast, it is not known which among the six KRP and dynein motors are kinetochore bound. Moreover, experiments in several organisms have shown that both the ATP-dependent sliding of motor proteins along microtubules and the GTP-dependent depolymerization of microtubule fibers are capable of generating sufficient force to move chromosomes (Hunter and Wordeman, 2000). Thus, nonmotor microtubule associated proteins (MAPs) may function to link kinetochores and microtubule plus ends during periods of polymer growth and shrinkage. The goal of a molecular analysis of yeast kinetochores is therefore to provide answers to the following general questions (1) how many different proteins are involved in chromosome-microtubule attachment and what are the relative roles of motors and MAPs, (2) do these proteins function only at kinetochores or

also in other cellular structures, and (3) do different proteins mediate different aspects of the complex pattern of metaphase and anaphase chromosome movement?

The identification of microtubule binding proteins in yeast kinetochores has been hindered by the absence of an assay to monitor chromosome-microtubule attachment. However, we and others have recently shown that force-generating processes at *S. cerevisiae* kinetochores impose sufficient tension on paired chromatids during metaphase to transiently separate centromeric chromatin toward opposite ends of the spindle (Goshima and Yanagida, 2000; He et al., 2000; Tanaka et al., 2000). Transient separations can pull sisters up to 1 μm apart for several minutes (a large movement relative to the 1.5 to 2 μm yeast spindle) and involve toward-the-pole separating forces and opposing cohesive forces. We reasoned that, by exploiting the phenomenon of transient sister separation to measure and analyze forces exerted on centromeres in wildtype and mutant yeast strains, a molecular analysis of microtubule attachment would be possible. In this paper, we identify as kinetochore components ten *S. cerevisiae* proteins previously thought to be involved in other mitotic processes. Some of these proteins, or their mammalian orthologs, are motors or microtubule binding proteins and mutations in several of these newly-identified kinetochore subunits impair force generation and chromosome movement *in vivo*. These data lead us to conclude that we have identified some of the proteins involved directly in the formation of microtubule attachment sites.

RESULTS

We have previously reported that kinetochore proteins in *S. cerevisiae* localize during metaphase to two lobes that lie on either side of the spindle midzone (He et al., 2000). The separation between these lobes is typically about half the separation between spindle pole bodies (SPBs). The distribution of kinetochore proteins changes subtly on a time scale of seconds

concomitant with fluctuations in the extent of overlap among the 32 centromeres in a mitotic haploid cell. Because the bi-lobed distribution of kinetochores was unexpected, we reasoned that it should be possible to identify additional kinetochore proteins based on this distinctive pattern and that some of these proteins might have been misidentified previously as spindle components. Of twenty or so spindle proteins whose functions were not well understood but for which some localization data were available, we found a total of ten (Table 1) that probably constitute structural kinetochore components and one kinase that appears to regulate kinetochore function.

To determine the localization of candidate kinetochore proteins, they were tagged with GFP at their extreme C termini and introduced into cells in the place of the wildtype gene using homologous recombination. The positions of the SPBs were determined by replacing the integral SPB protein Spc42p with a fusion to cyan fluorescent protein (Spc42p-CFP; Donaldson and Kilmartin, 1996; He et al., 2000). Time lapse and fixed-cell fluorescence microscopy were performed in two colors to visualize both GFP fusion proteins and spindle poles using optical sectioning microscopy followed by iterative deconvolution (on an Applied Precision DeltaVision Microscope). Cell cycle state in asynchronous cultures and synchrony-release experiments were determined by examining nuclear morphology and spindle length. All GFP (or CFP) fusions tested, including those involving essential genes (Table 1), supported wildtype rates of growth. To demonstrate that various proteins were indeed localized to kinetochores, we examined the CBF3-dependence of their localization by microscopy. GFP fusion proteins and Spc42p-CFP were introduced into *ndc10-1* cells and analyzed at 37°C, conditions that inactivate CBF3 and therefore disrupt kinetochore structure (Goh and Kilmartin, 1993).

As an independent assay for centromere association, we asked whether GFP-tagged proteins were bound to centromeric DNA *in vivo* as judged by formaldehyde crosslinking and

chromatin immunoprecipitation (ChIP). Wildtype and *ndc10-1* cells carrying different GFP-tagged proteins were treated with formaldehyde, cells were lysed, and DNA sheared by sonication to an average of 200–500 bp. Immune complexes were isolated using anti-GFP antibodies and the fraction of coprecipitating DNA determined by PCR using primers specific for *CENIV* and a negative control *URA3* sequence.

Table 1. Summary of the kinetochore proteins analyzed in this study

Protein[a]	Localization[b]					ChIP		Alleles Used in This Study	Homologues	Inter-actions[a,c,d]	Reported Functions[d]
	Kinetochores	Nuclear-MTs	SPB	Cortical-Tip	Cyto-MTs	In WT	In *ndc10-1*				
Ndc80p (E)	+	-	-	-	-	+	-	*ndc80-1*	HEC1	2H: *SPC24, SPC19*	Chromosome segregation
Nuf2p (E)	+	-	-	-	-	+	-	*nuf2-61; -457*	Nuf2R	2H: *CIN8, NDC80, SPC19*	Chromosome segregation
Spc24p (E)	+	-	-	-	-	+	-	none	*S.pombe* C336.08	2H: *SPC25*	Chromosome segregation
Spc25p (E)	+	-	-	-	-	+	-	none	N/A	2H: *SPC24*	Chromosome segregation
Dam1p (E)	+	-	-	-	-	+	-	*dam1-1; -9; -11*	N/A	2H: Ndc80, SPC34 SL: *cin8Δ*	Microtubule binding; Spindle integrity
Spc19p (E)	+	+	-	-	-	+	-	none	N/A	2H: *SPC34, NDC80*	Unknown
Spc34p (E)	+	+	-	-	-	+	-	none	N/A	2H: *SPC19*	Unknown
Stu2p (E)	+	+	+	+	-	+	-	*stu2-276; -27; -278; -279*	XMAP215	N/A	Microtubule binding
Bik1p (NE)	+	+	-	+	+	+	-	*bik1Δ:His3*	CLIP-170	2H: STU2 SL: *cin8Δ*	Spindle elongation
Cin8p (NE)	+	+	-	-	-	+	-	*cin8Δ:His3*	BimC kinesins	SL: *bik1Δ* SL: *dam1-1*	Spindle assembly and elongation; Chromosome segregation
Ipl1p (E)	+	+	-	-	-	-	-	*ipl1-2, -321*	Aurora-like kinases	SL: *cin8Δ*	Histone phosphorylation; Regulation of kinetochore-microtubule binding

[a]Abbreviations: E, Essential; NE, Non-essential; 2H, Two Hybrid; SL, Synthetic Lethality; SP, Suppression.
[b]Cellular localization shown by this study.
[c]Only interactions among proteins listed in this table are shown. Extensive genetic and biochemical interactions among Ndc80p, Nuf2p, Spc24p, Spc25p have also been demonstrated (Janke et al., 2001; Wigge and Kilmartin, 2001) and are not shown.
[d]References for interactions and reported functions are listed at the YPD database (Costanzo et al., 2000) and for 2-hybrid analysis in (Newman et al., 2000; Ito et al., 2001).

Proteins Localized Primarily to Kinetochores

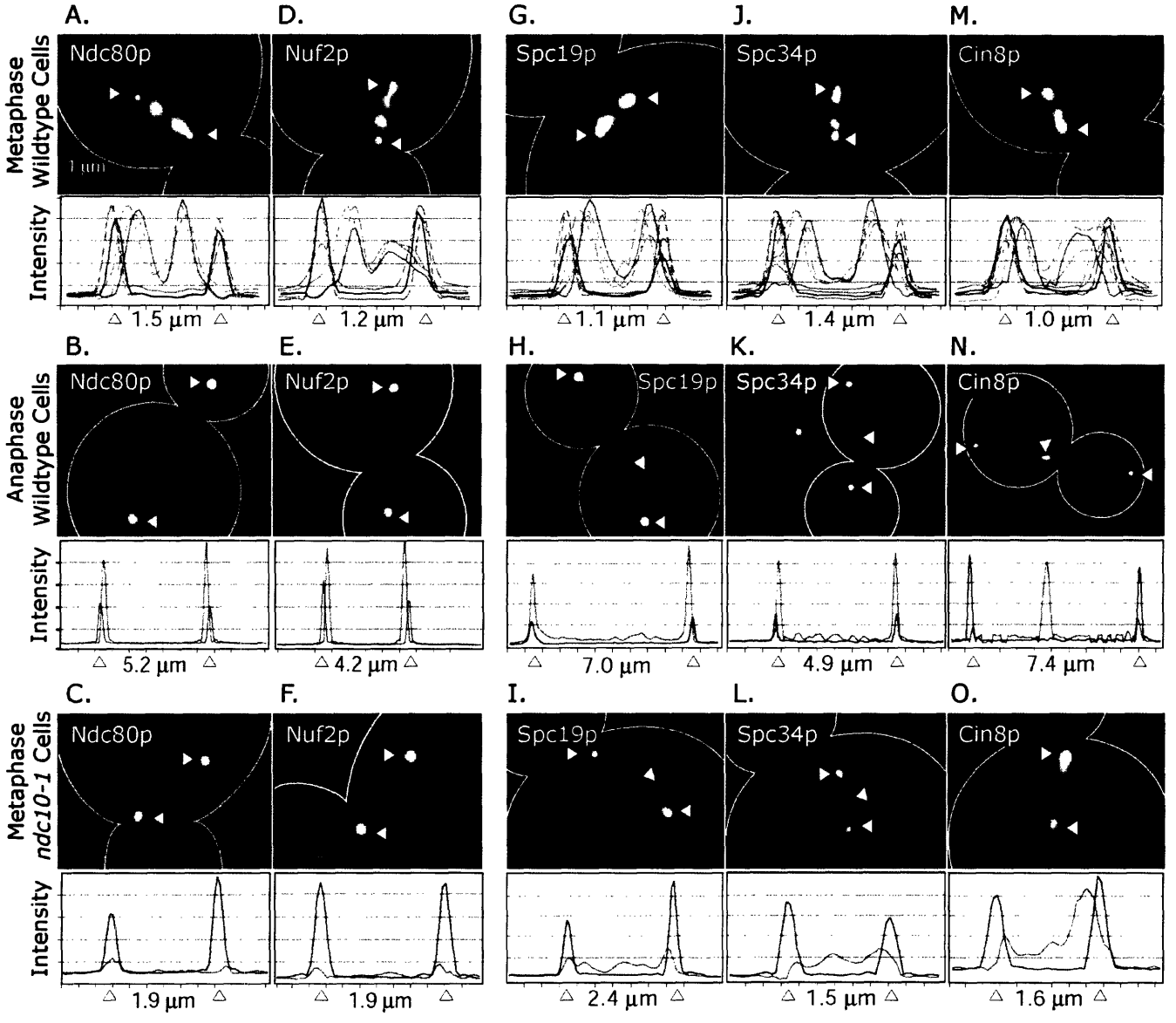
The first proteins we examined were Ndc80p, Spc24p, Spc25p, and Nuf2p. Ndc80p, Spc24p, and Spc25p were originally identified by MALDI-based microsequencing as proteins that cofractionate with yeast spindle pole bodies (SPBs) and localize, by immuno-EM, to the nuclear face of the SPB central plaque and to microtubules (Wigge et al., 1998). Nuf2p has been reported to be an SPB component based on its bi-lobed localization, but the small separation between the lobes seemed to us typical of a kinetochore protein (Osborne et al., 1994). When fixed and live cells were examined by 3D deconvolution microscopy, Ndc80p-GFP, Spc24p-GFP, Spc25p-GFP, and Nuf2p-GFP were seen during metaphase to localize to two lobes on either side of the spindle midzone and move toward the poles during anaphase (Figs. 3-1A to 3-1F, and data not shown). In cells that lack functional CBF3 (*ndc10-1* cells at 37°C) the bi-lobed pattern was abolished and replaced by dim, uniform nuclear fluorescence (Figs. 3-1C and 3-1F). The fluctuating bi-lobed distribution of Ndc80p-GFP, Spc24p-GFP, Spc25p-GFP, and Nuf2p-GFP was indistinguishable from that of Slk19p-GFP and Mtw1p-GFP, two bona fide kinetochore proteins that we and others have analyzed in some detail (Zeng et al., 1999; Goshima and Yanagida, 2000; He et al., 2000).

When assayed by ChIP, Ndc80p-GFP, Spc24p-GFP, Spc25p-GFP, and Nuf2p-GFP exhibited strong *NDC10*-dependent association to *CENIV* DNA but only background association with a negative control *URA3* fragment (Figs. 3-1P and 3-1Q). ChIP has previously been used to localize Cse4p, Mif2p, Slk19p, and other proteins to kinetochores (Meluh and Koshland, 1997; Meluh et al., 1998; Zeng et al., 1999), but we were concerned about possible nonspecific binding of spindle proteins to *CEN* DNA. As negative controls, we therefore performed ChIP with three non-kinetochore spindle proteins fused to GFP: Tub1p, which encodes α-tubulin, Tub4p, which

encodes the SPB-localized γ-tubulin, and Spc42p, a component of the SPB central plaque. None of these proteins associated to any significant extent with centromeric DNA, confirming the specificity of the ChIP reaction (Fig. 3-1R).

These findings confirm very recent reports that Ndc80p, Spc24p, Spc25p, and Nuf2p are components of an evolutionarily conserved multiprotein complex that associates with kinetochores in several eukaryotic organisms (Wigge et al., 1998; Janke et al., 2001). Careful examination of images of Ndc80p-GFP, Spc24p-GFP, Spc25p-GFP, and Nuf2p-GFP at different stages of the cell cycle with and without Spc42p-CFP to control for fluorescence bleedthrough showed only the bi-lobed localization typical of kinetochores and no association with spindle poles, spindle microtubules, or other nuclear structures (Figs. 3-1A and 3-1F; data not shown). We have found that Dam1p, a microtubule binding protein previously shown to be involved in spindle (Hofmann et al., 1998; Jones et al., 1999) and kinetochore function (Cheeseman et al., 2001) is a fifth protein that associates with *CEN* DNA in a *NDC10*-dependent fashion and that exhibits a localization in metaphase essentially identical to that of Ndc80p (data not shown). Overall, we conclude that Ndc80p, Nuf2p, Spc24p, Spc25p, and Dam1p associate with centromeres during mitosis in a CBF3-dependent manner and that kinetochores may be the only cellular structures with a significant level of these proteins as judged by GFP-tagging.

Figure 3-1 (next page) Analysis of proteins localized to kinetochores, or to kinetochores and spindle microtubules. Yellow arrows mark SPBs and white arrows spindle microtubules. (A, D, G, J and M) Typical images from metaphase wildtype cells carrying Spc42p-CFP (in red) and Ndc80p-GFP, Nuf2p-GFP, Spc19p-GFP, Spc34p-GFP or Cin8p-GFP (in green). Images represent projections of 3D image stacks containing ten to twenty 0.2μm sections. The graph shows the distribution of CFP and GFP signal intensities along the spindle axis (in arbitrary units) in several cells. The bold line is derived from the image shown after correction for bleed-through from the CFP to the GFP channel (see Experimental Procedures). (B, E, H, K and N) Typical images from anaphase wildtype cells or (C, F, I, L and O) metaphase *ndc10-1* cells. (P and Q) Crosslinking of proteins tagged with GFP to *CENIV* DNA in wildtype or *ndc10-1* cells at 37°C as assayed by Chromatin Immunoprecipitation (ChIP). DNA in immune complexes (IP) was amplified with primers specific for *CENIV* (CEN lanes) or, as a negative control, *URA3* (URA lanes) and compared to the amount of DNA in whole-cell lysates (TOTAL). (R) ChIP assays with control proteins not found at kinetochores. *TUB1* encodes α-tubulin, *TUB4* encodes γ-tubulin, and *SPC42*, an integral component of the SPB.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Metaphase Wildtype Cells
Ndc80p
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Spc19p
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Intensity
△ 1.5 µm △
△ 1.2 µm △
△ 1.1 µm △
△ 1.4 µm △
△ 1.0 µm △
B.
E.
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N.
Anaphase Wildtype Cells
Ndc80p
Nuf2p
Spc19p
Spc34p
Cin8p
Intensity
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△ 4.2 µm △
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△ 4.9 µm △
△ 7.4 µm △
C.
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O.
Metaphase ndc10-1 Cells
Ndc80p
Nuf2p
Spc19p
Spc34p
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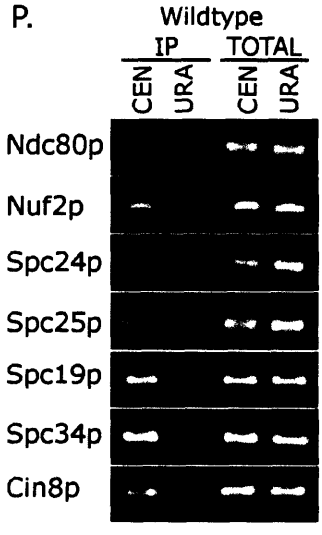
P.
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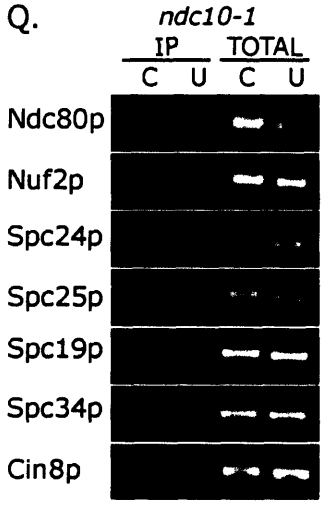
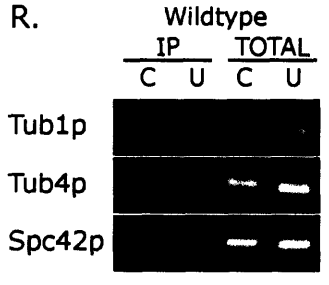
Spc34p
Cin8p


Q.
ndc10-1
IP
TOTAL
C
U
C
U
Ndc80p
Nuf2p
Spc24p
Spc25p
Spc19p
Spc34p
Cin8p


R.
Wildtype
IP
TOTAL
C
U
C
U
Tub1p
Tub4p
Spc42p

Proteins Bound to Kinetochores and to the Mitotic Spindle

Next, we examined three proteins that appeared, from careful examination of published images, to be at least partially localized to two nuclear lobes: Spc19p and Spc34p, proteins that copurify biochemically with SPBs (Wigge et al., 1998) and Cin8p, one of the six kinesin-like proteins in budding yeast (Hoyt et al., 1992; Roof et al., 1992). GFP fusions of all three proteins showed some *NDC10*-dependent kinetochore localization (Fig. 3-1) and specific binding to centromeric DNA by ChIP (Figs. 3-1P and 3-1Q). However, in contrast to Ndc80p, Nuf2p, Spc24p, Spc25p, and Dam1p discussed above, Spc19p, Spc34p, and Cin8p also localized to other microtubule-based structures in the cell.

In wildtype cells, Spc19p, Spc34p, and Cin8p were broadly similar in being localized to two kinetochore-like lobes as well as along the microtubules of the mitotic spindle (Figs. 3-1G and 3-1O). In anaphase, all three proteins retained their spindle localization while also concentrating at spindle poles, where centromeres are clustered. As described previously, Cin8p also has the interesting property of localizing to the spindle midbody late in anaphase (Fig. 3-1N; Hoyt et al., 1992). In *ndc10-1* cells, the bi-lobed components of Spc19p and Spc34p localization were abolished, whereas localization to spindle microtubules remained. Cin8p-GFP largely shifted to one pole or the other. The *NDC10*-dependence of localization is seen most clearly in intensity-distance plots that integrate the GFP and CFP signals along the spindle axis (see Figs. 3-1J and 3-1L in particular). In interpreting the plots and images, it should be noted that spindles in *ndc10-1* cells are about 25% longer than in wildtype cells, reflecting the loss of kinetochore-dependent pulling forces that shorten the spindle. In conclusion, although the localization patterns of Spc19p, Spc34p, and Cin8p are more complex than those of the five proteins discussed in the previous section, imaging and ChIP are consistent with the idea that a fraction of

Spc19p, Spc34p, and Cin8p is associated with kinetochores in metaphase yeast cells. Kinetochore association appears to be CBF3-dependent, whereas binding to spindle microtubules is CBF3-independent.

Proteins Bound to Kinetochores and a Variety of Microtubule-Based Structures

Next, we examined two microtubule binding proteins that are found in both the cytoplasm and the nucleus: Bik1p and Stu2p. Bik1p is homologous to the plus-end microtubule binding protein mammalian CLIP170 (Berlin et al., 1990) and Stu2p is a microtubule binding protein similar in sequence to *Xenopus* XMAP215 (Wang and Huffaker, 1997) and human TOGp (Spittle et al., 2000). Bik1p-GFP exhibited a complex localization to kinetochore-like lobes, to spindle microtubules, and to distinct spots in the cytoplasm that correspond to cortical attachment sites (Berlin et al., 1990), and only part of the localization appeared to be disrupted in *ndc10-1* cells at 37°C (Figs. 3-2A and 3-2C). Cortical capture sites are structures in the plasma membrane that bind the plus ends of microtubules that emanate from SPBs and function to orient the nucleus in the mother-bud neck late in metaphase (for review see Bloom, 2000). The localization of Bik1p to both kinetochores and cortical attachment sites is consistent with data that CLIP-170 binds selectively to the plus ends of microtubules. Stu2p-GFP was found in a pattern broadly similar to that of Bik1p and again, only a subset of the nuclear Stu2p-GFP appeared sensitive to *ndc10-1* inactivation (Figs. 3-2D and 3-2F). To determine whether the bright spots of Stu2p-GFP along the periphery of the cell might be cortical capture sites, we generated cells carrying α-tubulin-GFP (Tub1p-GFP) and Stu2p-CFP. In both metaphase and anaphase cells, cytoplasmic foci of Stu2p-CFP clearly lay at the extreme ends of cytoplasmic microtubule bundles, strongly suggesting that the foci were indeed cortical capture sites. Consistent with this localization, Stu2p and the well-characterized cortical site protein Kar9p

have recently been shown to interact by two-hybrid analysis (Miller et al., 2000). By ChIP, both Bik1p and Stu2p exhibited *NDC10*-dependent binding to centromeric DNA (Fig. 3-2J). From these data, we conclude that Bik1p and Stu2p are proteins that associate with a variety of microtubule-based structures including kinetochores and cortical capture sites, both of which bind to microtubule plus ends.

The final protein we examined was Ipl1p, an Aurora kinase that has been proposed to function in yeast kinetochore assembly (Biggins et al., 1999; Sassoon et al., 1999). It has previously been observed that Nuf2p is mislocalized in *ipl1-2* cells, a finding interpreted to reflect a role for Ipl1p in SPB formation (Kim et al., 1999). However, since Nuf2p is actually localized to kinetochores and not SPBs as previously assumed, we wondered whether Ipl1p might be a regulator of kinetochores. With Ipl1p-GFP, we observed a pattern consistent with kinetochore localization as well as with spindle binding, and a subset of the localization was *NDC10*-dependent but *CEN*-association was not detected by ChIP (Fig. 3-2J). The ChIP assay is a stringent criterion for kinetochore association and some kinetochore proteins may simply be too distant from DNA to be successfully crosslinked by formaldehyde. Our inclusion of Ipl1p in this analysis is justified by its clear role in chromosome movement (see below).

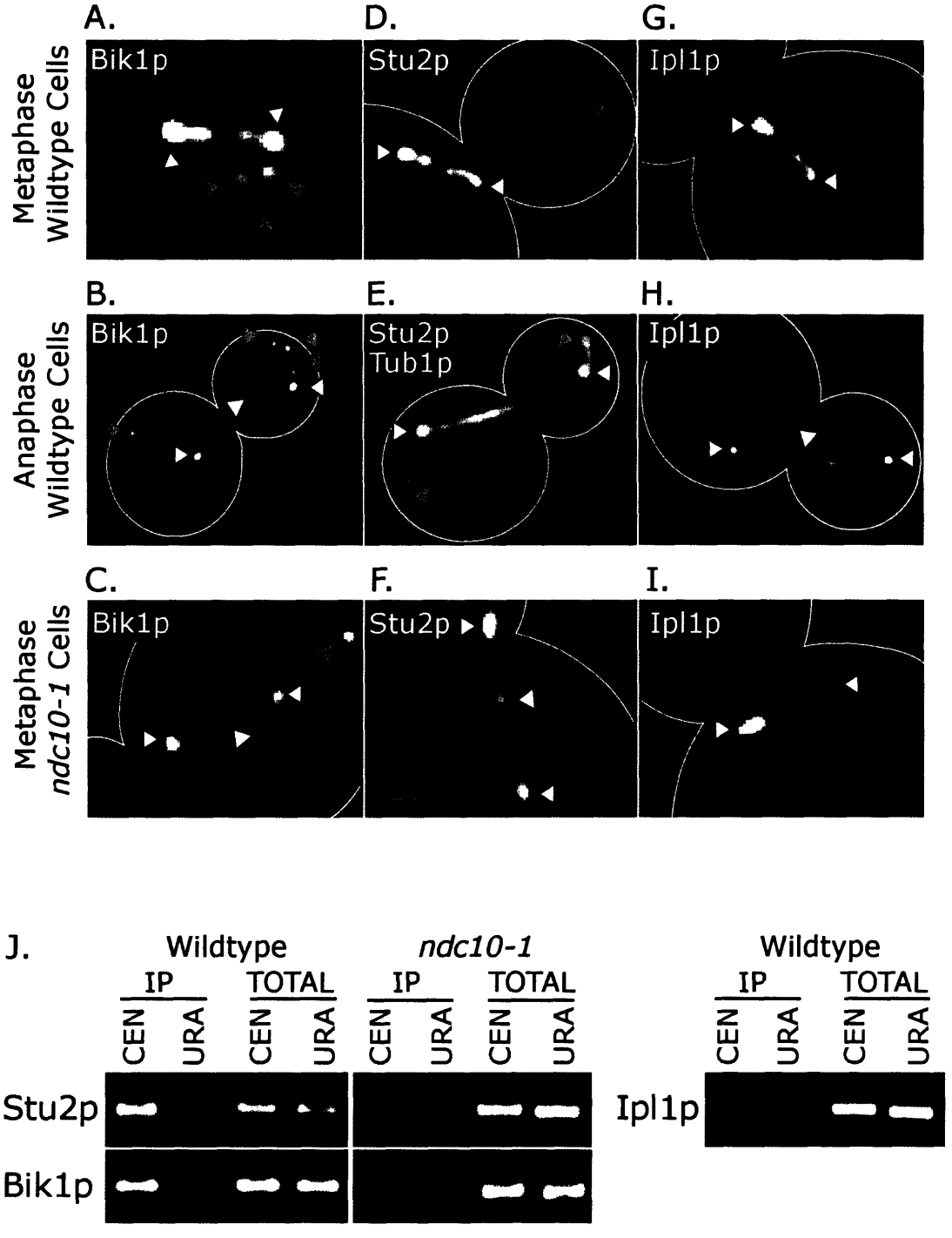


Figure 3-2 Analysis of proteins localized to kinetochores and to other microtubule-based structures. (A-I) Imaging of Bik1p, Stu2p and Ipl1p as described in Figure 3-1. Yellow arrows mark SPBs, white arrows mark spindle microtubules and pink arrows mark cortical capture sites. Note that in (E), the fluorophores have been changed so that Stu2p is fused to CFP (in red) and Tub1p (α-tubulin) to GFP (in green). (J) Crosslinking of Bik1p, Stu2p and Ipl1p to *CENIV* DNA as assayed by ChIP as described in Figure 3-1.

Mapping Chromosome Association to Centromeric DNA

We have previously proposed that centromeric chromatin in budding yeast spans nearly 20 kb of DNA centered on the centromere. This centromeric chromatin appears to be involved in the large-scale stretching that occurs during transient sister separation. It seemed possible that proteins we had localized to centromeres might be associated not with kinetochores themselves, but rather with an extended chromatin domain. This seemed particularly likely for Ndc80p, whose human homolog, Hec1, has been shown to complement an *NDC80* disruption in single copy and to interact biochemically and genetically with the Smc1p and Smc2p subunits of yeast cohesin and condensin (Zheng et al., 1999). In mitotic *S. cerevisiae* cells, cohesin is found both at centromeres and at discrete sites along chromosome arms, whereas condensin is ubiquitously distributed along chromatin (Megee et al., 1999; Tanaka et al., 1999; Freeman et al., 2000). To map the sequences to which Ndc80p binds, we used ChIP to quantitate its association to five successive 200 bp fragments of chromosomal DNA that span *CENIII*. We also examined Ndc80p binding to an 18 kb region on the arm of chromosome V that has previously been shown to contain a cohesin binding site (Tanaka et al., 1999). We observed that Ndc80p was present at high levels on the 200 bp fragment centered on *CENIII*, at much lower levels on sequences to the left and right of the centromere, and at only background levels at sites along chromosome V arms (Figs. 3-3A and 3-3B). The concentration of Ndc80p at centromeres was, if anything, tighter than that of Mif2p (Meluh and Koshland, 1997), the centromere-bound homolog of mammalian CENP-C, and clearly distinct from the broad distribution of Scc1p, a cohesin subunit (Megee et al., 1999; Tanaka et al., 1999). We therefore conclude that Ndc80p is tightly concentrated at centromeres and does not have the broader distribution along chromosomes characteristic of cohesin and condensin. Similar *CEN*-specific crosslinking was observed for

Spc19p, Spc34p, Cin8p, Bik1p, and Stu2p (Fig. 3-3C), confirming our conclusion that all of these proteins are specifically associated with kinetochores.

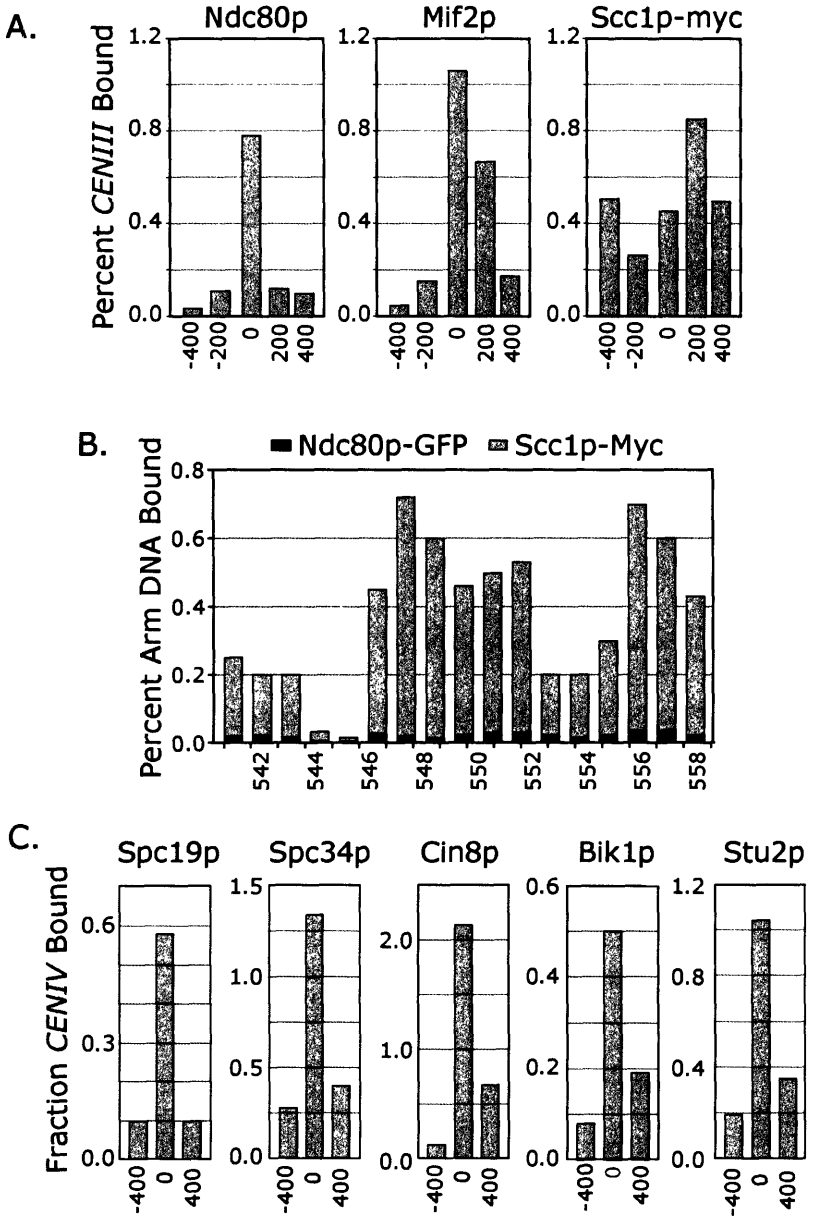


Figure 3-3 Quantitative Crosslinking Analysis by ChIP. (A) Quantitative ChIP analysis of the binding of Ndc80p, Mif2p and Scc1p to *CENIII* and flanking DNA. The horizontal axis denotes the position of 200 base pair fragments, relative to the center of *CENIII*, and the vertical axis the fraction of total DNA present in immune complexes as determined by PCR from serial dilution. Ndc80p was immunoprecipitated with anti-GFP polyclonal serum, Mif2p with rabbit polyclonal antiserum and Scc1p-myc12 with anti-myc polyclonal antibodies. (B) Quantitative ChIP analysis of Ndc80p and Scc1p crosslinking to sites along the arm of Chromosome V (Tanaka et al., 1999). Numbers on the horizontal axis refer to the position, in kilobases, from the left telomere as indicated by the Stanford Genome Database. (C) Quantitative ChIP analysis of Spc19p, Spc34p, Cin8p, Bik1p and Stu2p binding to *CENIII* and flanking DNA as described in (A).

Mutations in Kinetochore Proteins Reduce Transient Sisters Separation

To establish a function for proteins localized to kinetochores, we examined the extent of transient sister chromatid separation. We reasoned that mutations in proteins required for chromosome-microtubule attachment should interfere with the imposition of tension on sister centromeres and thereby decrease the frequency or extent to which sisters separate in metaphase. Nine of the eleven proteins we had localized to kinetochores are essential for vegetative growth but temperature sensitive mutants were available for only four (Table 1). Among the five genes without conditional mutations, it seemed most important to examine Stu2p, because it has a higher cell homolog (XMAP215) whose function is at least partially understood (Tournebize et al., 2000). We therefore generated 19 temperature sensitive alleles in the *STU2* gene using PCR mutagenesis and plasmid shuffling (see Experimental Procedures), and selected for further analysis alleles that arrested within one cell cycle of temperature upshift.

Centromeric DNA was visualized by integrating a TetO array 2 kb from *CENIV* (construct –2ChIV of He et al., 2000) in cells expressing TetR-GFP and a Spc42p-GFP. This generates cells in which both *CENIV*-proximal chromatin and spindle poles are marked with small green dots, permitting rapid single-color imaging. To quantitate sister separation in wildtype and mutant strains, they were synchronized at START using α-factor and then released into prewarmed medium at 37°C for 75–90 min. prior to fixation and imaging. When the extent of synchrony was assessed morphologically, more than 90% of cells were observed to have entered prometaphase and assembled bipolar spindles, and fewer than 5% were in anaphase. As positive and negative controls, we showed that the fraction of wildtype cells with separated sisters was 50%–60%, whereas in *ndc10-1* cells it was less than 1% (Fig. 3-4A; He et al., 2000). In *ndc80-1*, *dam1-1*, *nuf2-61*, and *stu2-277* cells, the extent of transient sister separation was 10-

to 20-fold lower than in wildtype cells, while in *ipl1-321* cells it was 4- to 5-fold lower (in about 15% of *ipl1-321* cells, centromeres appeared hyperstretched, with the TetO/TetR-GFP tag extended the full length of the spindle). Both *bik1*Δ and *cin8*Δ cells (which were assayed at 25°C, a temperature at which wildtype cells had fewer transient separations) exhibited a normal frequency of transient separation (Fig. 3-4B). In conclusion, these data show that Ndc80p, Nuf2p, Dam1p, Stu2p, and Ipl1p are required for transient sister chromatid separation, and thus, probably, for the imposition of normal tension on sister chromatids.

The proteins in this study fall into two classes: those that are found primarily at kinetochores (as judged by imaging GFP fusion proteins) and those that localize to both kinetochores and other microtubule-based structures in mitotic cells. To determine whether this distinction is also reflected in the functions of the proteins, we asked whether mutations that reduce tension across sister kinetochores also impair the migration of the nucleus into the mother-bud junction, an essential step in mitosis mediated by the interaction of cytoplasmic microtubules with cortical attachment sites. We observed that whereas nuclear migration was substantially perturbed in *stu2-276* cells, it appeared normal in *ndc10-1*, *ndc80-1*, *dam1-1*, *ipl1-321I*, and *nuf2-61* mutants (Fig. 3-4C; normal nuclear migration is observed in *bik1*Δ cells, a consequence of functional redundancy in proteins required for cortical attachment; Bloom, 2000). We conclude that kinetochore function is not required for nuclear migration and that the requirement for Stu2p probably reflects its localization to cortical capture sites. Additional non-kinetochore functions for proteins analyzed in this paper are summarized in Table 1.

Live Cell Analysis Reveals Three Types of Defect in Chromosome Movement

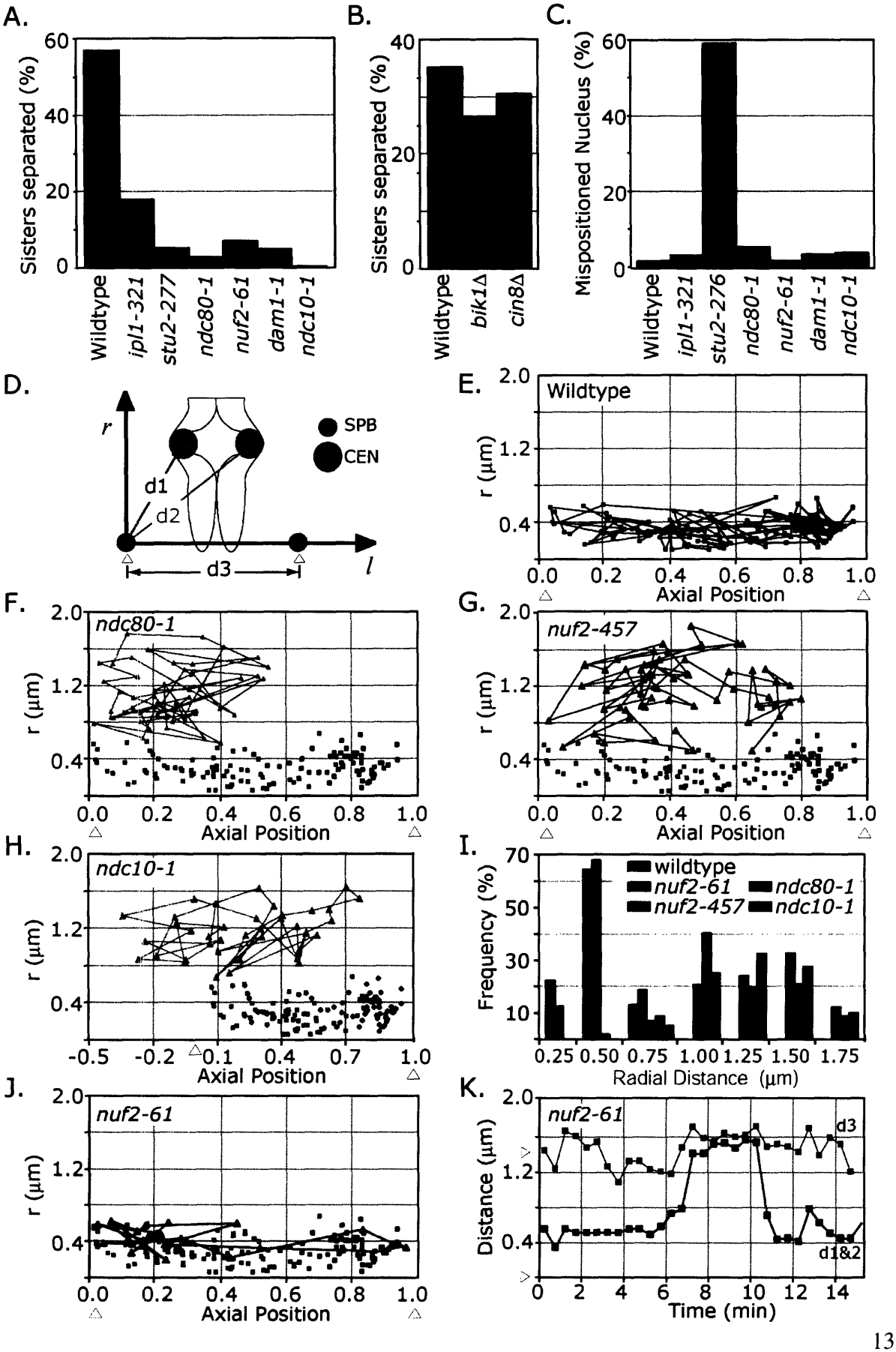
In principle, different kinetochore defects, ranging from complete failures of assembly to more subtle problems with force generation could reduce transient sister separation. To investigate the defects in *ndc80*, *nuf2*, *stu2*, *dam1*, and *ipl1* mutants, cells carrying GFP-tagged chromatids and SPBs were filmed at 37°C and the motion of centromeres determined relative to the spindle axis by manual and automated analysis of deconvolved 3D image stacks (Figs. 3-4D and 3-4E; He et al., 2000). In wildtype cells, metaphase chromosome movement is characterized by several superimposed motions along the spindle axis: rapid oscillations 10%–20% of spindle length of 0.2–0.5 μm, long duration oscillations 30%–60% of spindle length of up to 1.0 μm, and transient sister separations in which the two chromatids move independently for periods of 2–10 min. (He et al., 2000). In neither *ndc10-1*, *ndc80-1* (Fig. 3-4F and 3-4H, green and orange lines, respectively), or *nuf2-457* cells (Fig. 3-4G, blue lines; see figure legend for additional details) were any of these behaviors observed during metaphase and chromosomes remained exclusively in the mother cell at anaphase. Furthermore, whereas chromosomes stayed within 0.40 μm of the spindle axis in wildtype cells, in *ndc10-1*, *ndc80-1*, and *nuf2-457* cells, they appeared to detach completely from the spindle and were typically 1.0–1.5 μm from the spindle axis, the maximum distance possible in 2.5 to 3.0 μm nucleus (Fig. 3-4I). We conclude that loss-of-function mutants in *NDC80* and *NUF2*, like mutations in CBF3 genes, cause chromosomes to detach from spindle microtubules and move randomly within the mother cell.

In *ndc80-1*, *nuf2-457*, and *ndc10-1* cells, spindles undergo anaphase B elongation on approximately the same schedule as wildtype cells (data not shown), consistent with the conclusion by Janke et al. (2001) that the Ndc80p-Nuf2p complex is required for the mitotic checkpoint. We were therefore surprised to observe that at 37°C, *nuf2-61* cells arrested

homogeneously at the metaphase-anaphase transition in a Mad1-dependent fashion (data not shown), implying that the *nuf2-61* lesion engages the mitotic checkpoint (see also Janke et al., 2001). When *nuf2-61* cells were examined by live-cell microscopy, chromosomes were observed to associate with one pole for 5 to 10 min. and then suddenly jump to the other pole, binding to it for 5 to 10 min. before jumping again (Figs. 3-4J and 3-4K, red lines). In some cells, we observed up to four shifts between the poles in a 30 min. period. During these jumps, chromosomes remained as close to the spindle axis as in wildtype cells, supporting the notion that they were bound to microtubules, albeit aberrantly (Fig. 3-4I). Our interpretation of these findings is that whereas microtubule attachment sites are unable to assemble in *nuf2-457* cells, the attachments that form in *nuf2-61* mutants are metastable. In the complete absence of kinetochore-microtubule attachment, no checkpoint signal is generated whereas metastable kinetochore-microtubule attachments do signal the checkpoint and arrest cells at metaphase (see Discussion).

Next, we analyzed chromosome segregation in *stu2*, *dam1*, and *ipl1* cells. Because we were interested in gene-specific differences in phenotype rather than allele-dependent variation, multiple temperature sensitive mutants were examined for each gene. To identify loss-of-function mutants, alleles were ranked in severity based on the extent of transient sister separation, as judged using fixed-cell assays (as described in Fig. 3-4A). With many alleles, we observed a significant level of transient sister separation (approximately 20%–40% of chromatids were split), presumably representing a hypomorphic phenotype, but for each gene we were able to select two strong mutants.

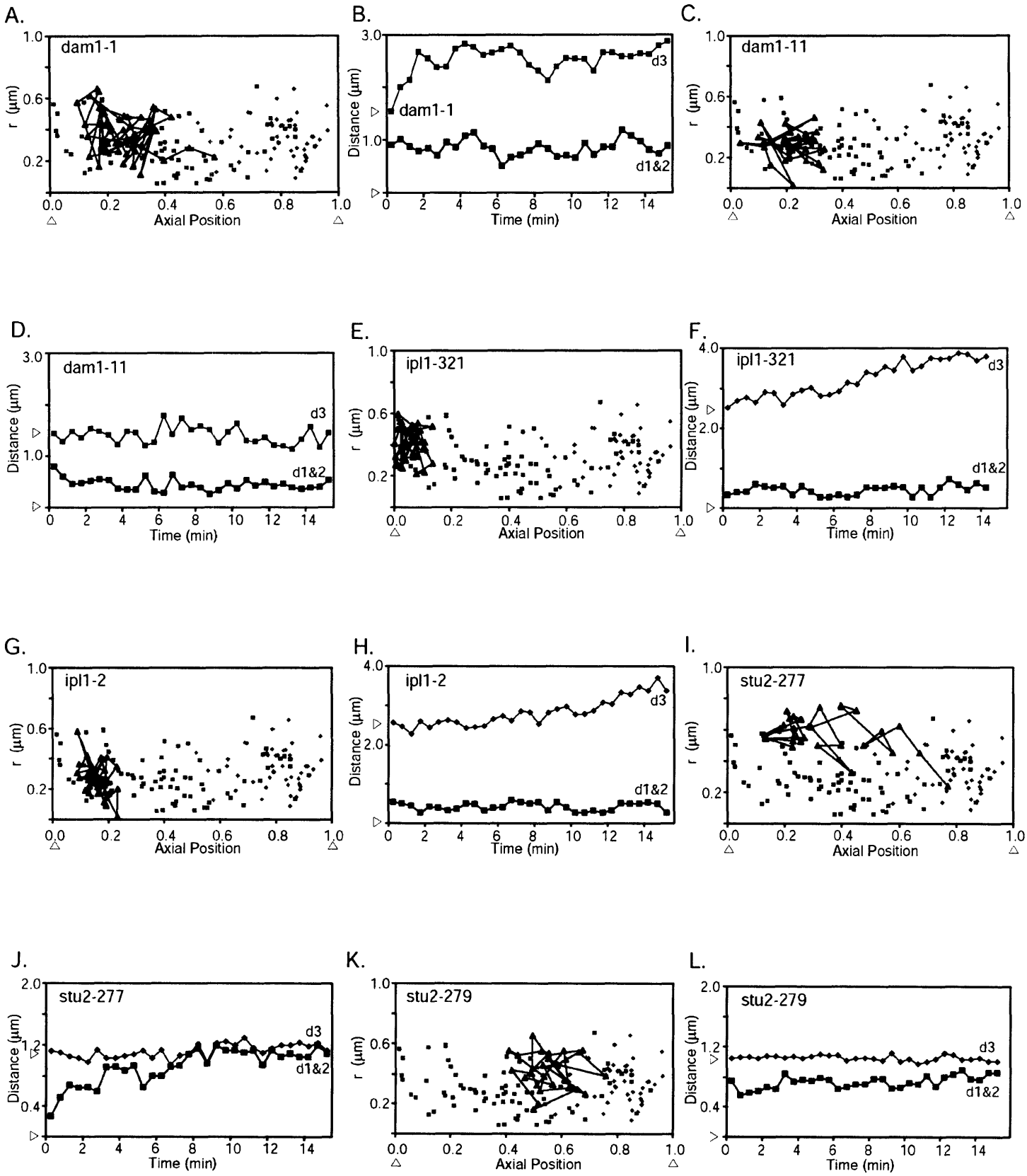
Figure 3-4 (next page) Effects of mutations in kinetochore proteins on transient sister separation, nuclear positioning migration and chromosome movement. (A) Mutations in essential kinetochore proteins reduce transient sister separation. Cells carrying Spc42p-GFP, the centromere-proximal-2ChIV chromosome tag (He et al., 2000), and the indicated temperature sensitive mutations were synchronized at START with α-factor at 25°C, and then released into prewarmed medium at the restrictive temperature of 37°C for 75 min. prior to fixation and analysis. The fraction of metaphase cells (n = 100), as judged by nuclear morphology, with separated sister chromosome tags was then scored. (B) Analysis of transient sister separation in wildtype, *bik1Δ::HIS3* and *cin8Δ::HIS3* cells performed at 25°C, as described in (A). (C) Analysis of nuclear positioning prior to anaphase. Pre-anaphase cells in which the nucleus had not migrated to the bud-proximal hemisphere of the mother cell were scored as mispositioned (Korinek et al., 2000). Note that the *STU2* allele used in this analysis was different from that in (A). We have observed partial separation of *STU2* function in these alleles, with *stu2-277,-279* having the greatest defect in transient sister separation and *stu2-276* having the greatest defect in nuclear positioning (by comparison 10% of *stu2-276* cells were transiently separated in a synchrony-release experiment). (D) Schematic of the -2ChIV GFP chromosome tag and Spc42p-GFP spindle pole tag, showing the spindle-centered polar reference system (with *r* and *l* coordinates) and three key parameters: *d1*, the distance from the reference spindle pole to the center of one chromatid tag; *d2*, the distance to the sister chromatid tag; and *d3*, the SPB-SPB distance. (E) Scatter plot of the positions of the -2ChIV chromosome tag over 15 min. in a time-lapse movie of a wildtype cell. The vertical axis is the absolute radial position (*r*) and the horizontal axis the relative axial position (*l/d3*). Dots represent the position of the tag at each time point; black and purple denote the independent paths of the two sisters during periods of transient sister separation. (F,G,H) Scatter plots of chromosome movement in *ndc80-1*, *nuf2-457* and *ndc10-1* cells compared to the wildtype movement in E (black and purple dots). Negative values of *l* arise when the *CEN* tag drifts so far from the SPBs that it is "behind" them. The phenotype of *ndc10-1* and *ndc80-1* cells are very homogenous, but 15-20% of *nuf2-457* cells exhibited behavior similar to that shown in (J) and (K). Synchrony-release experiments established that these cells are ones in which bipolar microtubule attachments had formed prior to temperature upshift. (I) Distribution of radial distances (*r*) between the chromosome tag and the spindle axis in wildtype, *nuf2-61*, *-457*, *ndc10-1* and *ndc80-1* cells. The data from sections (E-H) and (J) were used to calculate the values for *r*, which were then grouped in bins of 0.25 μm to generate a frequency distribution. Scatter (J) and distance (K) plots for *nuf2-61* cells at 37° C. Because no transient separation occurs, *d1* = *d2* (red line). Spindle length (*d3*) is shown in black.


A.
Sisters separated (%)
60
40
20
0
Wildtype
ipl1-321
stu2-277
ndc80-1
nuf2-61
dam1-1
ndc10-1
B.
Sisters separated (%)
40
20
0
Wildtype
bik1Δ
cin8Δ
C.
Mispositioned Nucleus (%)
60
40
20
0
Wildtype
ipl1-321
stu2-276
ndc80-1
nuf2-61
dam1-1
ndc10-1
D.
r
l
SPB
CEN
d1
d2
d3
E.
Wildtype
r (μm)
2.0
1.2
0.4
0.0 0.2 0.4 0.6 0.8 1.0
Axial Position
F.
ndc80-1
r (μm)
2.0
1.2
0.4
0.0 0.2 0.4 0.6 0.8 1.0
Axial Position
G.
nuf2-457
r (μm)
2.0
1.2
0.4
0.0 0.2 0.4 0.6 0.8 1.0
Axial Position
H.
ndc10-1
r (μm)
2.0
1.2
0.4
-0.5 -0.2 0.1 0.4 0.7 1.0
Axial Position
I.
Frequency (%)
70
50
30
10
wildtype
nuf2-61
ndc80-1
nuf2-457
ndc10-1
0.25 0.50 0.75 1.00 1.25 1.50 1.75
Radial Distance (μm)
J.
nuf2-61
r (μm)
2.0
1.2
0.4
0.0 0.2 0.4 0.6 0.8 1.0
Axial Position
K.
nuf2-61
Distance (μm)
2.0
1.2
0.4
d3
d1&2
0 2 4 6 8 10 12 14
Time (min)

In *dam1-1* and *dam 1-11* cells, we observed close association of tagged chromosomes with a single spindle pole (Figs. 3-5A and 3-5D). During this monopolar association, the chromosomes were found within 0.2 spindle diameters of the pole, but continued to oscillate rapidly (with an apparent velocity up to 1 μm/min.). An even more dramatic monopolar association was observed in *ipl1-321* and *ipl1-2* cells, in which chromosomes remaining within approximately 0.1 spindle diameters of the pole (Figs. 3-5E and 3-5H). This monopolar association was clearly distinct from the spindle detachment observed in *ndc10-1*, *ndc80-1*, or *nuf2-457* cells, and in none of the several dozen movies of *dam1* and *ipl1* cells were chromosomes further from the spindle axis than in wildtype cells (0.4–0.5 μm). Yet a third phenotype was observed in *stu2-277* and *stu2-279* cells (Figs. 3-5I and 3-5L). Chromosomes in *stu2* mutants moved to the middle of the spindle (congression), where they oscillated back and forth along the spindle axis (see especially Fig. 3-5I), apparently having achieved bipolar attachment and remaining at a wildtype radius from the spindle axis, but they covered a total distance 2- to 3-fold less than in wildtype cells and exhibited peak velocities at least 3- to 4-fold lower (0.3–0.4 μm/min.). This pattern of movement is consistent with diminished force generation at bivalently attached chromatid pairs.

In summary, live-cell analysis of chromosome dynamics in strains carrying mutant kinetochore proteins reveals at least three classes of defects: a complete failure of chromosome-microtubule attachment (in *ndc80*, *nuf2*, and *ndc10* cells), attachment to a single pole with a failure to undergo congression (in *dam1* and *ipl1* cells), and bivalent microtubule attachment with reduced rates of movement and reduced tension across sister centromeres (in *stu2* cells). The existence of distinct mutant phenotypes suggests that Ndc80p, Stu2p, Dam1p, Ipl1p, and Nuf2p have different functions in kinetochore-microtubule attachment.

Figure 3-5 (next page) Distinct defects in chromosome movement are observed in cells carrying mutations in different kinetochore proteins. (A, C, E, G, I and K) Scatter plots showing the radial (*r*) and relative axial positions (*l/d3*) of a -2ChIV chromosome tag over a 15 min. period of metaphase in various strains at 37°C (see Figure 3-4D for an explanation of the coordinates). The blue and purple dots show the wildtype positions. (B, D, F, H, J and L) Distance plots transforming the data to show distance between the sister chromatids and a reference SPB (red line; because no transient separation occurs, *d1* = *d2*) and the SPB-SPB distance (*d3*; black line). Multiple movies (3-6) were examined for each strain and the behaviors shown here are typical. The penetrance of the *ipl1-321* allele was only about 80% however, and some cells exhibited a sustained hyperstretching of the chromosome tag. The sampling rates used to collect this data are too low to permit the accurate measurement of rapid movements, but we have estimated the rates of long wavelength motions by smoothing the curves and then calculating a first derivative. On this basis, we find that centromeres in *stu2-277, -279* cells move at least 3-to 4-fold slower than in wildtype cells.


A.
dam1-1
r (μm)
Axial Position
B.
dam1-1
d3
d1&2
Distance (μm)
Time (min)
C.
dam1-11
r (μm)
Axial Position
D.
dam1-11
d3
d1&2
Distance (μm)
Time (min)
E.
ipl1-321
r (μm)
Axial Position
F.
ipl1-321
d3
d1&2
Distance (μm)
Time (min)
G.
ipl1-2
r (μm)
Axial Position
H.
ipl1-2
d3
d1&2
Distance (μm)
Time (min)
I.
stu2-277
r (μm)
Axial Position
J.
stu2-277
d3
d1&2
Distance (μm)
Time (min)
K.
stu2-279
r (μm)
Axial Position
L.
stu2-279
d3
d1&2
Distance (μm)
Time (min)

A Multilayer Structure at the Yeast Kinetochore

Among the chromosome segregation defects described in this paper, those caused by *ndc10-1*, *ndc80-1*, and *nuf2-457* are much more severe than any others. The severe phenotype of *ndc10-1*, is thought to arise because no kinetochore components, not even error-detecting checkpoint proteins, can associate with centromeric DNA in the absence of CBF3 (Goh and Kilmartin, 1993; Gardner et al., 2001). To explore the role of Ndc80p in kinetochore assembly, we used ChIP to determine interdependencies among various kinetochore subunits. As described above, the association of Ndc80p with kinetochores was dependent on functional CBF3 (Figure 3-6A), but the reciprocal was not true: both the Ndc10p (Fig. 3-6B) and Cep3p (data not shown) CBF3 proteins were centromere bound in *ndc80-1* cells at 37°C. However Nuf2p (Fig. 3-6C) and Stu2p (Fig. 3-6D) required both *NDC10* and *NDC80* function for kinetochore association. The association of the cohesin subunit Scc1p with centromeres requires *NDC10* (Tanaka et al., 1999), but Ndc80p and Scc1p bind independently to DNA (Figs. 3-6E and 3-6F). Thus, the assembly of *S. cerevisiae* kinetochores appears to involve a hierarchy of dependencies: CBF3 at the first level, Ndc80p at the second, and Stu2p at the third. We conclude that the severity of the *ndc80* phenotype probably reflects a requirement for Ndc80p in the association of several other proteins with centromeres.

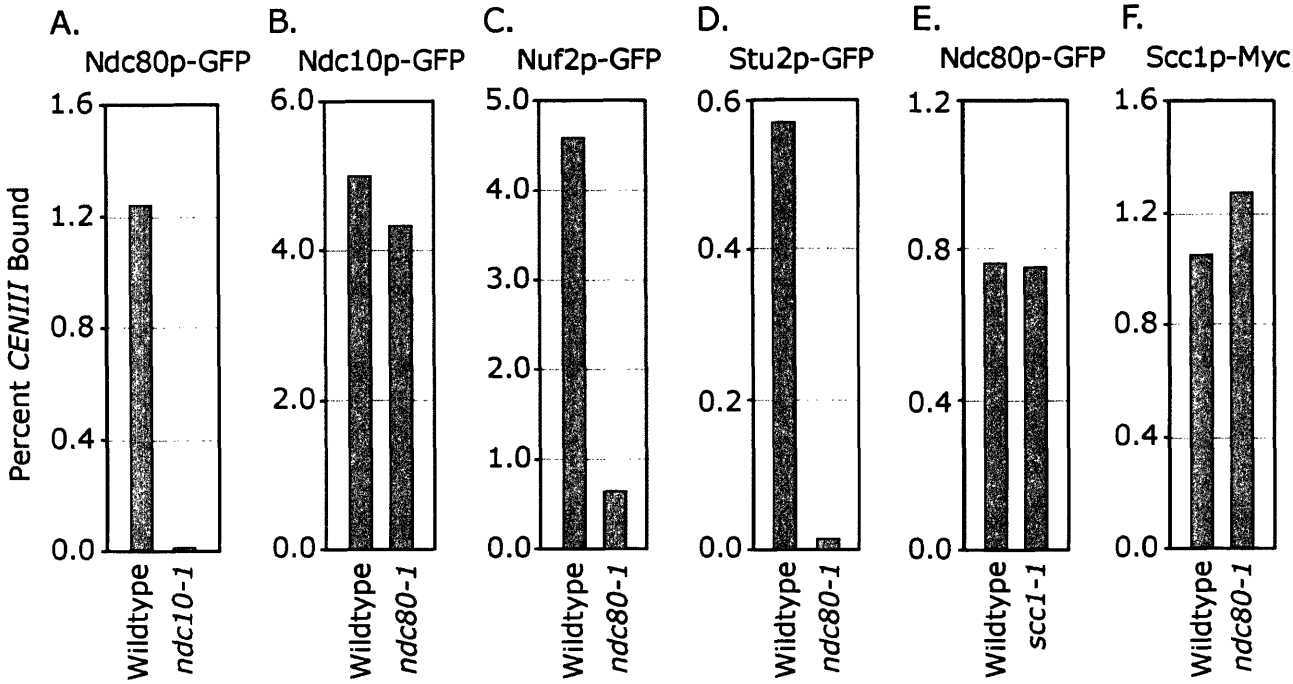


Figure 3-6 Hierarchy of dependencies in the association of kinetochore proteins with centromeric DNA. Quantitative ChIP was used to determine the effects of various mutations on the extent to which GFP-tagged protein crosslinked to *CENIII* DNA. Cells were grown at 25°C to mid-log phase and shifted to 37°C for 2.5 hours prior to analysis. The amount of the *CENIII* DNA in immune complexes (IP) is shown as the percentage of the DNA in the lysate (see Figure 3-3). Absolute differences in the amount of DNA precipitated between panels are not considered to be meaningful.

DISCUSSION

We have used three criteria to identify kinetochore proteins: (1) CBF3-dependent localization to the yeast "metaphase plate," a bi-lobed distribution that fluctuates over time as kinetochores move back and forth along the spindle axis, (2) CBF3-dependent association with centromeric DNA, as judged by chromatin immunoprecipitation, and (3) functional involvement in kinetochore-microtubule attachment, as judged by disruption of the normal pattern of transient sister separation and chromosome movement. By these criteria, we find that ten previously described spindle proteins of uncertain function are associated with *S. cerevisiae* kinetochores and an eleventh appears to be a kinetochore regulator. Among these kinetochore proteins are several that bind to microtubules, or have animal cell homologs that are microtubule binding.

Mutations in these proteins disrupt chromosome movement and appear to reduce or eliminate the tension that is normally imposed on sister centromeres. We hypothesize that the proteins are involved directly in the formation of microtubule attachment sites.

The mitotic spindle is a large multicomponent machine with long-range physical interactions among kinetochores, microtubules, and spindle poles. It is therefore important to inquire into the physical basis by which proteins might become associated with *CEN* DNA. We can imagine three possibilities. First, centromere association might be highly indirect, involving distant interactions mediated by microtubules. This seems unlikely because neither α-tubulin, γ-tubulin, nor the spindle pole component Spc42p detectably coprecipitated with *CEN* DNA by ChIP, and the available evidence suggests that ChIP is a reliable and highly selective cross-linking method (Meluh and Broach, 1999). Moreover, in cases in which *CEN* binding by ChIP is backed up by colocalization and functional data, our confidence in kinetochore association seems justified. Second, centromere association might involve binding directly to DNA, as in the case of CBF3, or binding to CBF3 in a multilayer kinetochore structure (Ortiz et al., 1999). In this case, a protein should be found associated with kinetochores independent of whether the kinetochores are linked to microtubules. Third, a protein that binds to microtubules might show centromere association through the attachment of microtubule plus ends to kinetochores. The human APC tumor suppresser protein, for example, localizes to kinetochores as a consequence of its binding to microtubule ends (Kaplan et al., 2001). Both of these latter possibilities are consistent with a protein's functioning in aspects of chromosome-microtubule attachment, but we have not yet distinguished between them experimentally.

Kinetochore-Associated Microtubule Binding Proteins

Among the eleven proteins discussed in this paper, four—Stu2p, Bik1p, Dam1p, and Cin8p—have been shown previously to bind microtubules and mutations in two of these—Stu2p and Dam1p—abolish the tension normally imposed on sister kinetochores. In principle, mutations that affect transient sister separation could act by diminishing the microtubule-mediated forces that pull centromeres apart or by increasing the cohesive forces that hold sister kinetochores together. Although our data do not conclusively distinguish between these possibilities, the abnormal chromosome movements we have observed seem most consistent with a failure to establish and maintain microtubule attachment, and not with an increase in sister cohesion. We speculate that the failure of deletions in the nonessential *CIN8* and *BIK1* genes to impair kinetochore-microtubule attachment is a consequence of functional redundancy. Previously reported redundancy in *CIN8* and *KIP1* during spindle assembly (Hoyt et al., 1992) and *BIK1* and *KAR9* during nuclear positioning (Miller and Rose, 1998) support this conclusion.

The higher cell homologs of Stu2p, XMAP215 in *Xenopus* and TOGp in humans, have been shown to bind to and modulate the dynamic behavior of microtubules (Gard and Kirschner, 1987; Vasquez et al., 1994; Tournebize et al., 2000). Kinetochore-associated Stu2p might therefore be expected to contribute to chromosome movement by altering the stability of microtubule plus ends. A similar function has been proposed for Dis1p, an *S. pombe* homolog of Stu2p that is required for correct chromosome movement and transient sister separation in fission yeast (Nabeshima et al., 1998). The human homolog of Bik1p, CLIP-170, localizes to microtubule plus ends, including those at kinetochores (Dujardin et al., 1998), and has also been postulated to regulate microtubule dynamics (Diamantopoulos et al., 1999). The *S. pombe* CLIP-170 homolog, Tip1p, functions to prevent catastrophic depolymerization of microtubule plus

ends, thereby promoting their capture at the cell cortex (Brunner and Nurse, 2000). We might therefore expect that Bik1p participates in plus-end microtubule capture at *S. cerevisiae* kinetochores. Yeast Dam1p has been shown to bind to microtubules, but its biochemical analysis has just begun (Hofmann et al., 1998), and, Dam1p has no obvious homologs in higher cells.

The observation that Cin8p localizes to yeast kinetochores is surprising. Cin8p is one of four kinesin-like proteins in yeast with a nuclear function, but it is most similar to the BimC class of motors thought to slide microtubules relative to each other (for review, see Hildebrandt and Hoyt, 2000). This is not an activity expected of a kinetochore protein although a plus-end-directed motor could generate a force that separates spindle poles, a well characterized activity of Cin8p (Hoyt et al., 1992; Gheber et al., 1999). Assuming that the association of Cin8p with kinetochores is not adventitious, then its function must be redundant with that of other proteins. We have looked for kinetochore association by the Kip1-3 motors using GFP-tagging and ChIP and have preliminary data that Kip3p, at least, may also localize to kinetochores. We must now undertake a careful analysis of chromosome dynamics and transient separation in cells lacking combinations of two, three, and four motor proteins.

General Implications for Kinetochore-Microtubule Attachment

Four implications of general significance for kinetochore biology can be drawn from the data in this paper. First, multiple proteins appear to be involved in the attachment of kinetochores to microtubules, including both motor proteins and MAPs, and these proteins play at least partially overlapping roles. Mutations in *NDC10*, *NUF2*, and *NDC80* cause a complete disruption of chromosome-microtubule attachment, apparently because they disrupt kinetochore assembly. In contrast, mutations in *STU2*, *IPL1*, and *DAM1* interfere with chromosome

movement but kinetochores retain some microtubule attachment. It therefore seems likely that multiple microtubule binding proteins contribute simultaneously to the formation of a fully functional attachment site.

Second, the formation of kinetochores with at least some microtubule binding activity, however aberrant, is required for mitotic checkpoint function. The genetic interaction between structural components of the kinetochore and the mitotic checkpoint is complex. Some mutations in kinetochore proteins, such as *ndc10* (Goh and Kilmartin, 1993; Tavormina and Burke, 1998), *spc24* and *ndc80* (Goh and Kilmartin, 1993; Tavormina and Burke, 1998; Wigge et al., 1998; Janke et al., 2001), are as effective as *mad2Δ* in disrupting checkpoint function. Other mutations however, appear to cause a checkpoint-dependent arrest, including *ctf13* (Doheny et al., 1993) and *cep3* (Strunnikov et al., 1995). This difference cannot be explained simply by postulating different biochemical functions for checkpoint-disrupting and checkpoint-engaging mutations: Ndc10p, Ctf13p, and Cep3p are all required for the DNA binding activity of CBF3. However, our data on *nuf2-61* and *nuf2-457* provide strong support for the hypothesis that it is the extent of kinetochore disruption that determines whether the checkpoint will function. When chromosome attachment is completely disrupted, as seen in *nuf2-457* cells, the checkpoint is abolished. However, when metastable attachments are generated, as in *nuf2-61* cells, the checkpoint is engaged. Similarly, the partially defective attachments generated in *dam1* and *stu2* cells arrest cells in a checkpoint-dependent fashion. These data fit well with the idea that kinetochores are the source of a checkpoint signal that acts to monitor the formation of fully functional microtubule attachment sites (Gardner et al., 2001).

Third, mutations in different proteins give rise to different defects in chromosome movement, including a complete loss of attachment (in *ndc10*, *ndc80*, and *nuf2*), slow movement

of chromatids that have apparently achieved bivalent attachment (in *stu2*), and close association with a single pole, presumably reflecting monopolar microtubule attachment (*dam1* and *ipl1*). This latter phenotype could arise either from a failure to duplicate kinetochores following DNA replication, or from a failure to develop microtubule attachments strong enough to oppose the splitting forces exerted on sisters during metaphase. A likely interpretation of these three phenotypic classes is that different proteins mediate different aspects of microtubule attachment and chromosome movement.

Fourth, many proteins involved in kinetochore-microtubule attachment also localize to other microtubule-based structures and appear to have more than one function in the cell. For example, Stu2p and Bik1p appear to function at both kinetochores and at cortical capture sites (and probably also at SPBs; Wang and Huffaker, 1997). Membrane-associated cortical capture sites bind cytoplasmic microtubules that emanate from the SPB and function to orient the nucleus into the mother-bud neck, a precondition for transporting chromosomes into the daughter during anaphase B (for review, see Bloom, 2000). The important similarity between cortical capture sites and kinetochores is that both bind to the plus ends of dynamic microtubules.

SUMMARY

In conclusion, the data in this paper suggest that yeast kinetochores contain several functional layers comprising DNA binding proteins such as CBF3, linker proteins such as Ndc80p, and microtubule binding components such as Stu2p, Dam1p, Cin8p, and Bik1p. The DNA binding and linker proteins seem to be highly specific to kinetochores, whereas several of the microtubule binding proteins have other functions in the cell. Although additional kinetochore subunits undoubtedly remain to be identified in yeast, our data have implications for

the fundamental question of whether it is motor or nonmotor proteins that play the primary role in microtubule attachment. Our findings clearly point to a critical role for nonmotor MAPs in chromosome-microtubule binding and force generation.

MATERIALS AND METHODS

Yeast Strains and Manipulations

All yeast strains used in this study were haploid and derived from W303 or S228C. Chromosomes and spindle poles were tagged with GFP as described (He et al., 2000). Proteins were tagged with GFP as follows: a 400–1000 bp C-terminal gene fragment was amplified with PCR and EGFP linked at the C terminus in the integrating vector pRS306. The endogenous gene was replaced with the tagged form in one-step gene replacement and correct integrants confirmed by PCR. Nineteen *stu2* ts mutants were generated by mutagenizing the *STU2* ORF *in vitro* using error-prone PCR, replacing *STU2* in the genomes with the library of mutagenized clones and then complementing the ts phenotype with wildtype *STU2* on a plasmid. Sequencing revealed the presence of multiple mutations in each ts allele. ChIP experiments were performed using standard methods (Meluh and Broach, 1999), and quantitation of PCR products by serial dilution. Anti-GFP polyclonal antibodies were from Clontech, anti-myc polyclonal antibodies from Santa Cruz Biotechnology, and anti-Mif2p polyclonal antibodies produced in house.

Microscopy and Image Analysis

Live cell imaging was performed using a Deltavision optical sectioning microscope on a Nikon TE200 base and Roper RTE camera essentially as described in He et al., 2000. A custom-built heated stage and a Bioptechs lens heater with feedback control were used to maintain the temperature at 37°C (details are available upon request). Dual-color fixed cell images of cells

carrying both CFP and GFP fusion proteins were collected using a Photometrics CH350 camera and Chroma 86002 JP4 (CFP) and 41018 (GFP) filters. The intensity plots in Figures 3-1 and 3-2 were corrected for 30% bleedthrough.

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CHAPTER 4

Spindle Checkpoint Proteins and Chromosome-Microtubule Attachment in *S. cerevisiae*

The work presented in this chapter is adapted, with permission, from Gillett et al. 2004 and represents an equal collaboration with Emily Gillett. The focus of my work in this chapter is the use of Chromatin Immunoprecipitation (ChIP) to determine *CEN* localization of the Spindle Checkpoint Proteins and analyze the interdependencies required for their association with centromeric DNA.

Gillett ES*, Espelin CW* and Sorger PK (2003) Spindle Checkpoint Proteins and Chromosome-Microtubule Attachment in Budding Yeast. *The Journal of Cell Biology* Vol. 164 (4), 535-546
*These authors contributed equally to this work.

ABSTRACT

Accurate chromosome segregation depends on precise regulation of mitosis by the spindle checkpoint. This checkpoint monitors the status of kinetochore-microtubule attachment and delays the metaphase to anaphase transition until all kinetochores have formed stable bipolar connections to the mitotic spindle. Components of the spindle checkpoint include the mitotic arrest defective (MAD) genes *MAD1-3* and the budding uninhibited by benzimidazole (BUB) genes *BUB1* and *BUB3*. In animal cells, all known spindle checkpoint proteins are recruited to kinetochores during normal mitoses. In contrast, we show that whereas *S. cerevisiae* Bub1p and Bub3p are bound to kinetochores early in mitosis as part of the normal cell cycle, Mad1p and Mad2p are kinetochore-bound only in the presence of spindle damage or kinetochore lesions that interfere with chromosome-microtubule attachment. Moreover, whereas Mad1p and Mad2p perform essential mitotic functions during every division cycle in mammalian cells, they are required in budding yeast only when mitosis goes awry. We propose that differences in the behavior of spindle checkpoint proteins in animal cells and budding yeast result primarily from evolutionary divergence in spindle assembly pathways.

INTRODUCTION

The spindle checkpoint ensures the fidelity of chromosome transmission by delaying anaphase until all chromatid pairs have formed proper links to the mitotic spindle. Sister chromatids attach to spindle microtubules (MTs) via kinetochores, multi-protein complexes that assemble on centromeric (*CEN*) DNA. During spindle assembly, a kinetochore must be captured by MTs emanating from one and only one pole of the mitotic spindle, whereas its partner must be captured by MTs emanating from the opposite pole. Sister pairs that have not formed bipolar

attachments will not segregate correctly at anaphase. The presence of even a single kinetochore pair that has not achieved bipolar attachment is sufficient to engage the spindle checkpoint and arrest cell cycle progression (Li and Nicklas, 1995; Rieder et al., 1994).

Spindle checkpoint genes were first identified in budding yeast and include the mitotic arrest defective genes *MAD 1–3* (Li and Murray, 1991) and the budding uninhibited by benzimidazole genes *BUB 1* and *BUB3* (Hoyt et al., 1991), all of which are well conserved among eukaryotes. The Bub proteins are thought to be upstream components of the checkpoint pathway whereas Mad2p and Mad3p (called BubR1 in animal cells) are downstream components that bind to and inhibit the regulatory protein Cdc20p (reviewed in Yu 2002). At the metaphase-to-anaphase transition, Cdc20p activates the anaphase promoting complex (APC), an E3 ubiquitin ligase, thereby promoting ubiquitination and degradation of the securin protein, Pds1p, and subsequent destruction of the cohesin complexes that tether sister chromatids together (reviewed in Morgan, 1999). Although the spindle checkpoint is not essential in budding yeast under normal growth conditions, it is essential in animal cells (Basu et al., 1999; Kitagawa and Rose, 1999; Dobles et al., 2000; Kalitsis et al., 2000).

Spindle checkpoint proteins have been shown to bind to kinetochores in animal cells and fission yeast (reviewed in Cleveland et al., 2003), and functional kinetochores are required to generate the checkpoint signal in both animal cells and budding yeast (Gardner et al., 2001; Rieder et al., 1995). However, the exact nature of the kinetochore lesions sensed by the spindle checkpoint remains uncertain. The first possibility is that it is the absence of tension across sister kinetochores that initiates checkpoint signaling (Stern and Murray, 2001), and the second is that it is a lack of MT attachment itself that is responsible (Rieder et al., 1995). Tension-based models are appealing because they link checkpoint silencing to an event that is absolutely

dependent on bipolar attachment. However, in higher eukaryotes, tension stabilizes individual kinetochore-MT attachments (King and Nicklas, 2000; Nicklas and Ward, 1994) and disentangling the effects of tension and MT attachment on checkpoint signaling is difficult.

Determining the nature of the events that initiate and silence spindle checkpoint signaling should be less complicated in organisms such as budding yeast in which each kinetochore recruits a single MT. Budding yeast also has the advantage of temperature-sensitive mutants defective in specific steps of kinetochore-MT attachment. Such lesions include mutations in subunits of the NDC80 Complex that cause chromosomes to detach from MTs, mutations in the MT binding component *DAM1* and the Aurora B kinase *IPL1* that prevent chromosomes from forming bipolar attachments, and mutations in the MT regulator *STU2* that allow chromosomes to form bipolar attachments but prevent them from establishing wildtype levels of tension (Biggins et al., 1999; He et al., 2001; Janke et al., 2001; Janke et al., 2002; Kim et al., 1999; Tanaka et al., 2002; Wigge and Kilmartin, 2001).

In budding yeast, only two known kinetochore complexes are required for spindle checkpoint function: CBF3 and NDC80 (Gardner et al., 2001; McCleland et al., 2003). The CBF3 complex binds directly to *CEN* DNA and is required for the assembly of all known kinetochore components on *CEN* DNA (for review, see McAinsh et al., 2003). In contrast, the NDC80 Complex is part of a set of "linker" proteins that do not bind directly to DNA or MTs but instead appear to link DNA-binding and MT-binding components. The NDC80 Complex consists of four essential proteins: Ndc80p, Nuf2p, Spc24p, and Spc25p. Among these, Ndc80p and Nuf2p are well conserved among eukaryotes (Wigge and Kilmartin, 2001) and human Ndc80 (Hec1) can functionally substitute for its yeast counterpart (Zheng et al., 1999). While loss of function mutations in *SPC24* or *SPC25* disable the spindle checkpoint (Janke et al.,

2001), mutations in *NDC80* or *NUF2* do not (McCleland et al., 2003). These and other data suggest that the NDC80 Complex may have an important role in relation to spindle checkpoint signaling.

In this paper, we report that four spindle checkpoint proteins—Bub1p, Bub3p, Mad1p, and Mad2p—associate with *S. cerevisiae* kinetochores. While Bub1p and Bub3p bind to kinetochores during normal mitoses, Mad1p and Mad2p are recruited only in the presence of spindle damage or checkpoint-activating kinetochore lesions. The kinetochore association of Bub1p and Mad2p requires the function of some, but not all, members of the NDC80 Complex. Our findings suggest that budding yeast kinetochores rarely, if ever, detach completely from MTs during normal cell division, and we propose that this aspect of spindle morphogenesis may explain why the checkpoint is not essential for mitosis in budding yeast under normal growth conditions. Our results also suggest that the release of the Bub proteins from kinetochores during normal spindle assembly is likely to be dependent upon a transition from immature to mature kinetochore-MT attachment rather than on the establishment of tension across sisters.

RESULTS

Bub1p and Bub3p are recruited to kinetochores during normal cell cycles

To localize spindle checkpoint proteins in *S. cerevisiae*, endogenous *MAD* and *BUB* genes were linked to green fluorescent protein (GFP) at their COOH-termini via homologous recombination. GFP tagging did not interfere with checkpoint function, as assayed by growth on plates containing the MT-depolymerizing agent benomyl (Fig. S1). Spindle pole bodies (SPBs) were visualized by linking the SPB component Spc42p to cyan fluorescent protein (Spc42p-CFP; Donaldson et al., 2001; He et al., 2001). Cells expressing GFP-tagged checkpoint proteins and

CFP-tagged Spc42p were observed using two-wavelength 3D deconvolution microscopy (Rines et al., 2002). Cell cycle state was determined from the length and position of the mitotic spindle.

When Bub1p-GFP and Bub3p-GFP were examined in early mitotic cells, a distinct pattern of two GFP lobes lying between the CFP-tagged SPBs was observed (Fig. 4-1A and 4-1B). This is the classic localization pattern of kinetochore proteins such as Ndc80p and reflects the metaphase clustering of budding yeast kinetochores into two lobes that lie along the spindle axis and between the spindle poles (Fig. 4-1C; He et al., 2000). To demonstrate the kinetochore association of Bub1p-GFP and Bub3p-GFP directly, we performed Chromatin Immunoprecipitation (ChIP) with primers specific for *CENIV* DNA. In asynchronous cultures, both Bub1p-GFP and Bub3p-GFP exhibited clear *CEN*-binding by ChIP (Fig. 4-1D and 4-1E). Binding was specific as neither protein crosslinked to DNA at the non-centromeric *URA3* locus (data not shown). Moreover, *CEN*-binding required the core kinetochore complex CBF3, as Bub1p-GFP and Bub3p-GFP ChIP signals were negligible in *ndc10-1* strains at 37°C (Fig. 4-1D and 4-1E).

When cells carrying Bub1p-GFP and Bub3p-GFP were treated with the anti-MT drug nocodazole to activate the spindle checkpoint, the ChIP signals for Bub1p and Bub3p at *CENIV* rose 1.5- and 3-fold, respectively, relative to untreated asynchronous cells (Fig. 4-1D and 4-1E). In contrast, in α-factor arrested G1 cells, ChIP signals for Bub1p and Bub3p fell to background levels (Fig. 4-1F and 4-1G). From these data, we conclude that Bub1p and Bub3p associate with *CEN* DNA during normal cell divisions, that this association requires functional kinetochores, and that it is cell cycle-regulated, being high in nocodazole-treated mitotic cells and low in G1. Our results with Bub1p in nocodazole-treated cells are consistent with those of Kitagawa et al.

(2003) and (Kerscher et al., 2003), but unlike Kitagawa, we conclude from imaging and ChIP that little to no Bub1p binds to kinetochores in α-factor arrested cells.

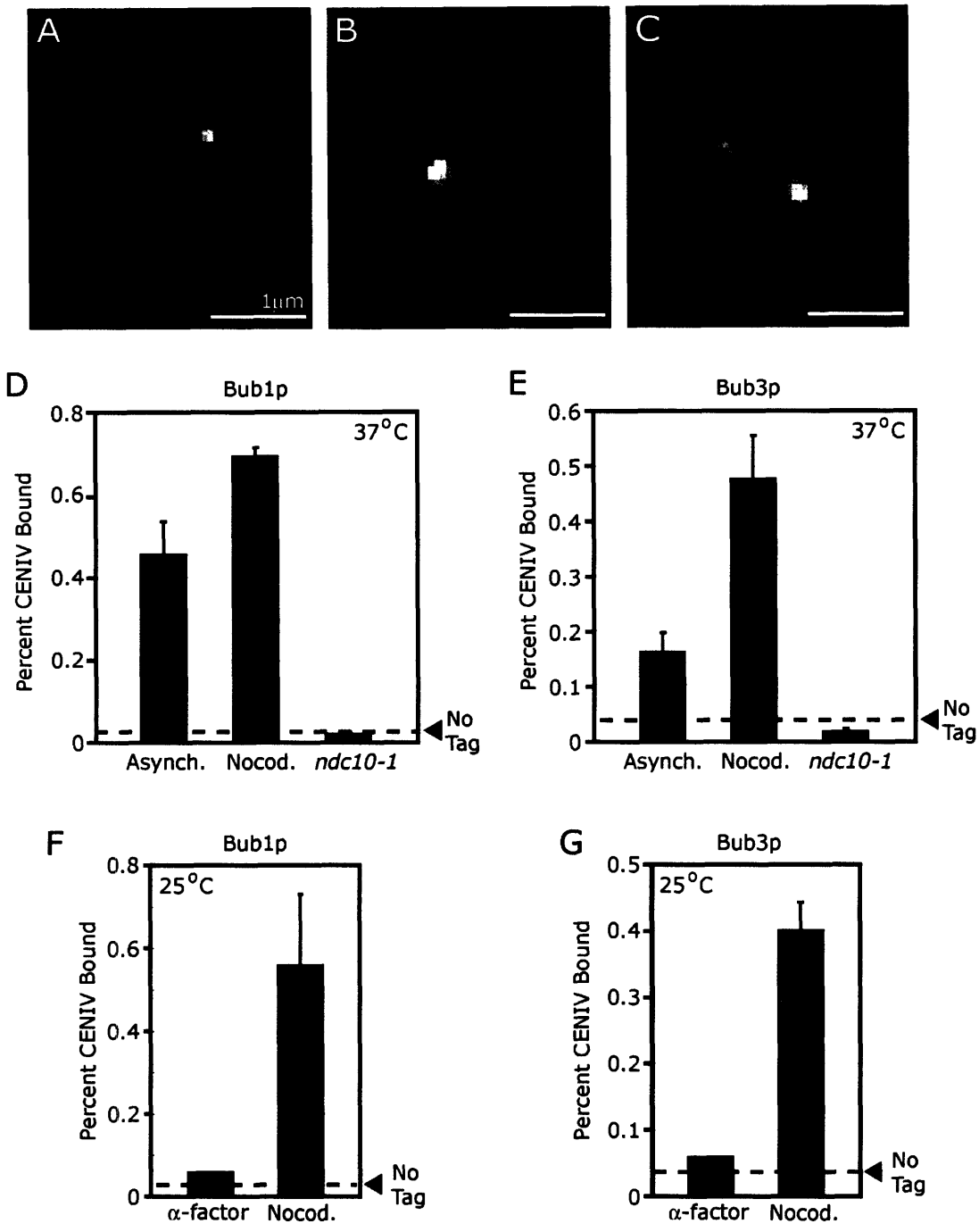


Figure 4-1 Bub1p-GFP and Bub3p-GFP are associated with kinetochores. (A-C) Typical images of wildtype mitotic cells expressing the SPB marker Spc42p-CFP (red) and the spindle checkpoint proteins Bub1p-GFP or Bub3p-GFP (green), or the kinetochore protein Ndc80p-GFP (blue). Images represent two-dimensional projections of 3D image stacks containing 10 to 15 0.2-μm sections. (D and E) ChIP of Bub1p-GFP and Bub3p-GFP at *CENIV*. Crosslinking of Bub proteins to *CEN* DNA was assayed in asynchronous wildtype cells, nocodazole-treated wildtype cells and *ndc10-1* cells at 37°C. All cells were grown to mid-log phase at 25°C, then shifted to 37°C for 3 hours before analysis. The amount of *CENIV* DNA recovered with immune complexes is shown as a percentage of the amount of *CENIV* DNA present in each total cell lysate. Dashed lines represent the percentage of *CENIV* DNA recovered with immune complexes from wildtype cells (negative control). Error bars show SD for two or more independent immunoprecipitations. Absolute differences in the amount of DNA precipitated among different panels are not considered to be meaningful. (F and G) ChIP of Bub proteins at *CENIV* is cell-cycle regulated. Wildtype cells expressing Bub1p-GFP or Bub3p-GFP were grown to mid-log phase at 25°C and treated with α-factor (5μg/ml final) or nocodazole (25μg/ml final) for 3 hours before ChIP analysis.

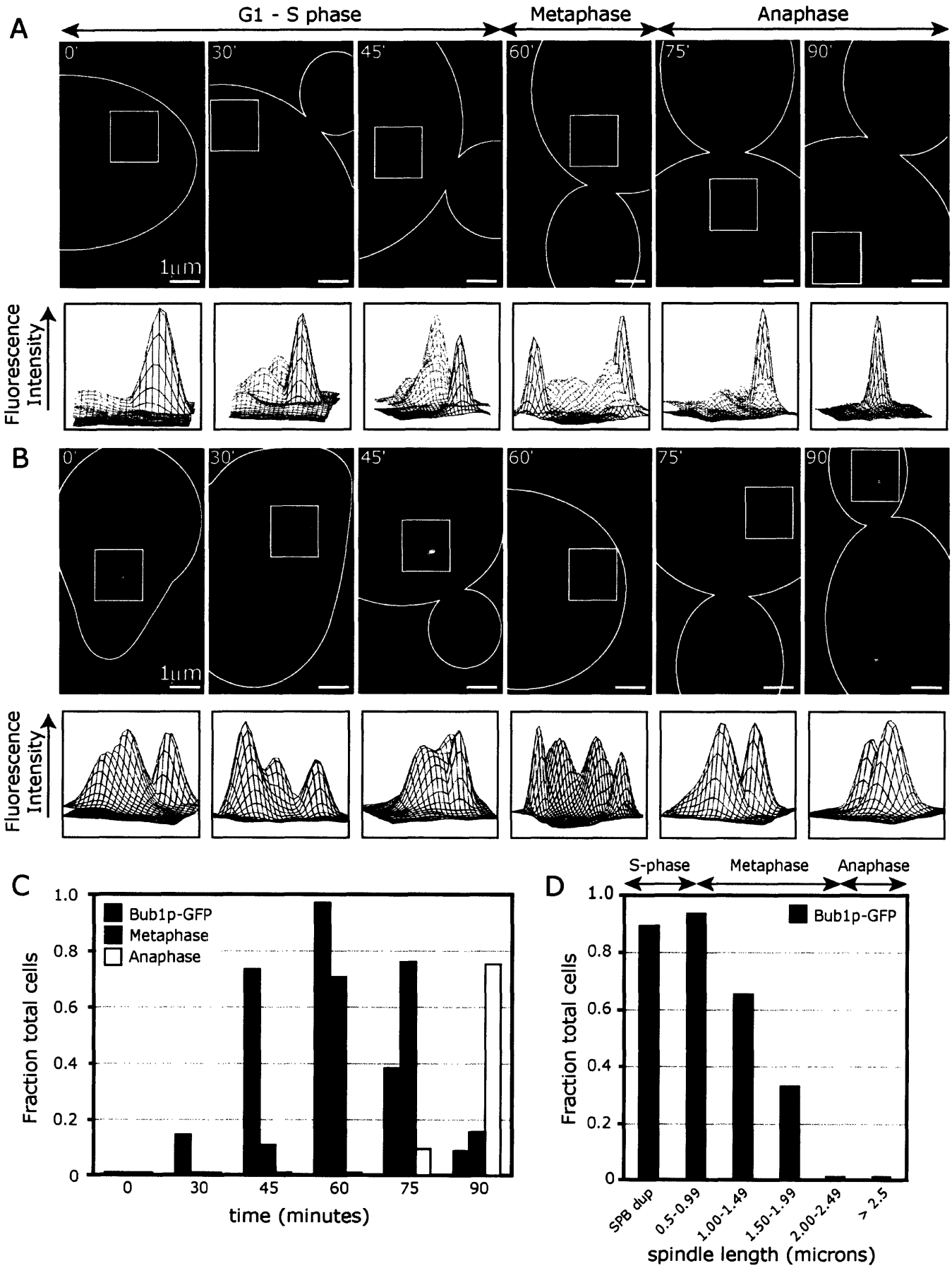
Kinetochore association by Bub1p occurs early in mitosis

To determine when during the cell cycle Bub proteins are recruited to kinetochores, the localization of Bub1p-GFP was compared to that of the kinetochore protein Ndc80p-GFP. Parallel cell cultures were synchronized using α-factor, released at 25°C, and samples withdrawn and fixed every 15 minutes. The percentage of cells containing Bub1p-GFP or Ndc80p-GFP foci was determined by analyzing at least 40 individual cells at each time point. Progression through the cell cycle was monitored by examining bud size, spindle length and spindle position (determined using Spc42p-CFP). In synchronous cultures released from α-factor, very few cells contained kinetochore-localized Bub1p-GFP prior to T= 45 min. (Fig. 4-2A and 4-2C). Kinetochore binding by Bub1p then rose dramatically, peaking at T= 60 min., and fell again as mitosis progressed (Fig. 4-2C). In contrast, kinetochore binding by Ndc80p-GFP was apparent throughout the experiment, giving rise at early time points to a single GFP cluster in close proximity to the newly duplicated SPBs and subsequently resolving into a bi-lobed metaphase configuration (Fig. 4-2B).

Bub1p-GFP foci were first visible around the time of SPB duplication (during S-phase, at T= 30-45 min.; Fig. 4-2C and 4-2D). At this point, the patterns of Bub1p-GFP and Ndc80p-GFP localization were very similar, suggesting that most if not all kinetochores were associated with Bub1p. The peak of Bub1p binding to kinetochores was observed at T= 60 min. in cells with spindles that averaged 0.8 μm in length. Cells at this point in the cell cycle contain duplicated SPBs, but kinetochores do not yet exhibit a bi-lobed metaphase configuration (as judged by Ndc80p-GFP). At T= 75 min., 71% of cells contained metaphase-length spindles, but only 38% contained Bub1p-GFP foci (Fig. 4-2C), indicating that Bub1p is released from kinetochores as metaphase proceeds. No Bub1p-GFP foci were seen in anaphase cells (Fig. 4-2A, 75 and 90

min.; Fig. 4-2D). Bub1p was also absent from kinetochores arrested in metaphase by *cdc23-1* or *cdc20-1* mutations (data not shown). Cells in asynchronous cultures exhibited a pattern of kinetochore association by Bub1p similar to that seen in synchronous cultures, showing that our findings were not an artifact of α-factor release. Moreover, the dynamics of Bub3p binding to kinetochores was indistinguishable from that of Bub1p-GFP (data not shown). From these results, we conclude that the Bub proteins first associate with kinetochores during S-phase when cells contain monopolar spindles, but dissociate from kinetochores as mature bipolar MT attachments are established early in mitosis.

Figure 4-2 (next page) Kinetochore association of Bub proteins is cell-cycle regulated. (A) Wildtype cells expressing Bub1p-GFP (green) and the SPB component Spc42p-CFP (red). Images are representative for each time point after α-factor release at 25°C. The surface plot below each image depicts the distribution of GFP (green) and CFP (red) signal intensities (in arbitrary units) across the boxed regions of each image. For the 30 min. time-point, we included an image representative of the 15% of cells that contained Bub1p-GFP foci. (B) Images of individual cells expressing the kinetochore protein Ndc80p-GFP (blue) and Spc42p-CFP (red). Images and graphs are as described for A. (C) Fraction of total cells containing Bub1p-GFP kinetochore foci, metaphase spindles and anaphase spindles versus time after α-factor release at 25°C. Metaphase cells were those with spindle lengths between 0.8 and 2.2 μm and anaphase cells those with spindles > 2.2 μm. At least 40 individual cells were scored at each time point. (D) Fraction of cells containing Bub1p-GFP kinetochore foci versus spindle length after α-factor release at 25°C (n = 281).


A
G1 - S phase
Metaphase
Anaphase
0'
30'
45'
60'
75'
90'
1μm
Fluorescence Intensity
B
0'
30'
45'
60'
75'
90'
1μm
Fluorescence Intensity
C
Bub1p-GFP
Metaphase
Anaphase
Fraction total cells
1.0
0.8
0.6
0.4
0.2
0
0
30
45
60
75
90
time (minutes)
D
S-phase
Metaphase
Anaphase
Bub1p-GFP
Fraction total cells
1.0
0.8
0.6
0.4
0.2
0
SPB dup
0.5-0.99
1.00-1.49
1.50-1.99
2.00-2.49
> 2.5
spindle length (microns)

Kinetochore recruitment of the Mad checkpoint proteins

Next, we examined the kinetochore association of Mad1p, Mad2p, and Mad3p in asynchronous and nocodazole-treated cells. We detected little or no kinetochore-bound Mad1p, Mad2p, or Mad3p in asynchronous cells by imaging or ChIP at any stage of the cell cycle (Fig. 4-3A and data not shown; Iouk et al., 2002). However, ChIP signals were high for both Mad1p-GFP and Mad2p-GFP in nocodazole-treated cells (Fig. 4-3A). The ChIP signal for Mad3p-GFP was consistently just above background levels in nocodazole-treated cells (Fig. 4-3A), but we have been unable to confirm kinetochore association by microscopy (data not shown). From these data we conclude that Mad1p, Mad2p, and Mad3p do not associate significantly with kinetochores in cycling cells but that Mad1p and Mad2p are kinetochore-bound in the presence of spindle damage.

Nocodazole treatment interferes with microtubule polymerization and causes mitotic spindles to collapse (Jacobs et al., 1988). When we imaged nocodazole-treated cells co-expressing Ndc80p-GFP and Spc42p-CFP, we found that the majority of kinetochores remained in a large cluster close to the collapsed SPBs (Fig. 4-3B). However, most cells also contained 1 or 2 dim Ndc80p kinetochore foci $\geq$ 1 μm away from the SPBs (arrowheads, Fig. 4-3B). Data from live-cell chromosome tracking experiments in nocodazole-treated cells suggest that these dim Ndc80p foci represent kinetochores that are detached from spindle MTs (D.R. Rines, unpublished data). Foci of Mad1p-GFP, Mad2p-GFP, and Bub1p-GFP co-localized specifically with the weaker Ndc80p kinetochore foci that were distant from SPBs (Fig. 4-3C to 4-3E). Some Mad1p-GFP also remained on the nuclear periphery (Iouk et al., 2002; Fig. 4-3D). From these data, we conclude that treating cycling cells with nocodazole causes some, but not all,

kinetochores to detach from spindle MTs and that spindle checkpoint proteins are recruited selectively to the detached kinetochores.

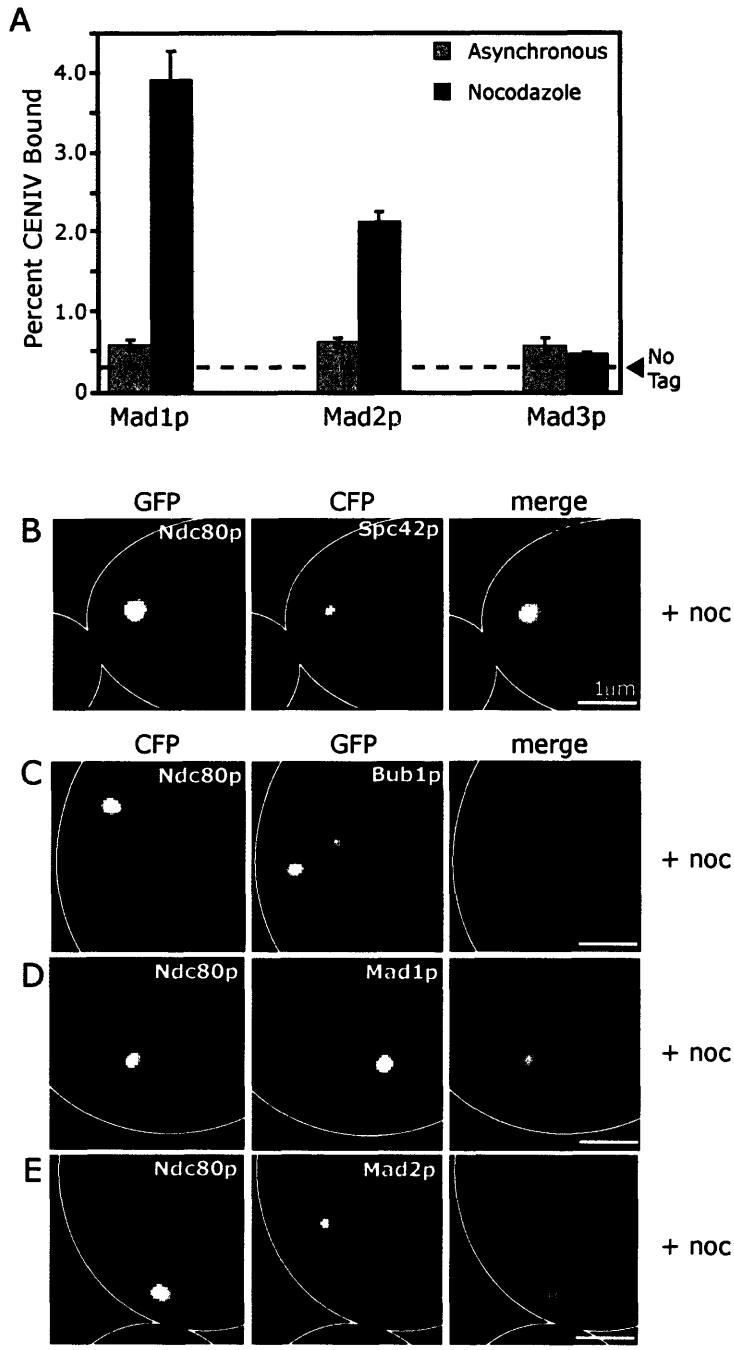
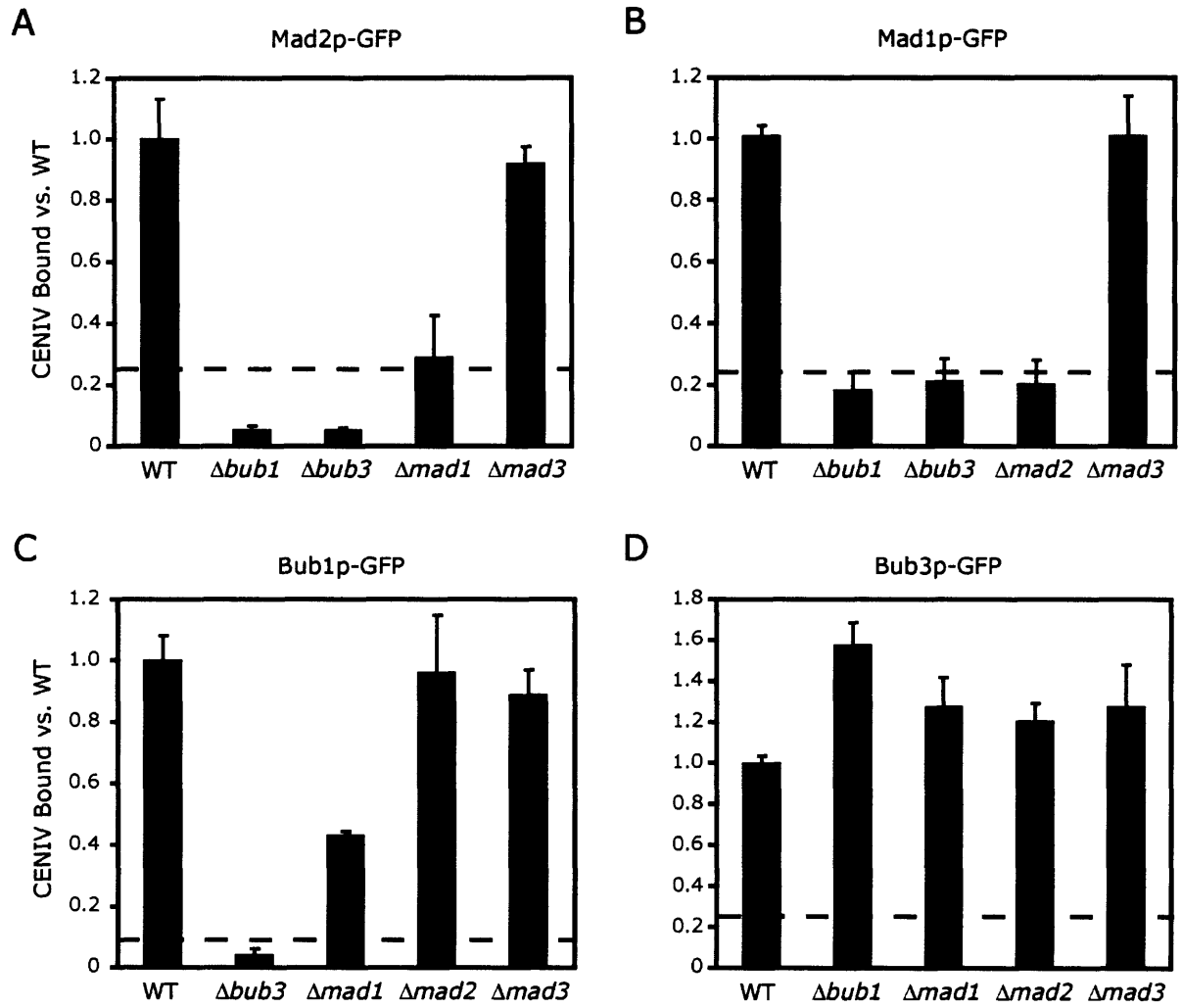


Figure 4-3 Kinetochore association of spindle checkpoint proteins in nocodazole-treated cells. (A) ChIP of Mad1p-GFP, Mad2p-GFP and Mad3p-GFP at *CENIV* in cycling and nocodazole-treated cells. Graphs are as described for Fig. 4-1 (D-G). (B) Wildtype cell co-expressing the SPB protein Spc42p-CFP and the kinetochore protein Ndc80p-GFP after treatment with 25 μg/ml nocodazole for 1 hour at 25°C. Panels show Ndc80p-GFP alone, Spc42p-CFP alone and Spc42p-CFP (red) merged with Ndc80p-GFP (blue). (C-E) Wildtype cells co-expressing Ndc80p-CFP (blue) and Mad1p-GFP, Mad2p-GFP or Bub1p-GFP (green) after nocodazole treatment. Panels show CFP channel alone, GFP channel alone and CFP merged with GFP. Orange arrowheads indicate the locations of unattached kinetochores.

A functional checkpoint pathway is required for kinetochore recruitment of Mad1p & Mad2p

Epistasis analysis has suggested that Bub1p and Bub3p are upstream components of the checkpoint pathway while Mad2p is a downstream effector (Farr and Hoyt, 1998). To determine if interdependencies for *CEN* binding by checkpoint proteins mirrored their proposed order in the checkpoint signaling pathway, ChIP of Mad1p, Mad2p, Bub1p and Bub3p was performed in cells deleted for other checkpoint components. *CEN*-association of Mad1p and Mad2p was assayed in cells treated with nocodazole, while that of Bub1p and Bub3p was assayed in asynchronous cells. We observed that *CEN*-association by Mad2p-GFP was abolished in *Δbub1* and *Δbub3* cells, as well as in cells lacking *MAD1*, but not in cells lacking *MAD3* (Fig. 4-4A). *CEN*–association by Mad1p-GFP exhibited a similar set of dependencies, requiring *BUB1*, *BUB3*, and *MAD2*, but not *MAD3* (Fig. 4- 4B). In contrast, both Bub1p-GFP and Bub3p-GFP associated with *CEN* DNA in cells lacking *MAD1*, *MAD2*, or *MAD3* (Fig. 4-4C and 4-4D). Finally, while Bub1p-GFP did not bind to kinetochores in cells lacking *BUB3*, Bub3p-GFP could still be crosslinked to *CEN* DNA in *Δbub1* cells (Fig. 4-4C and 4-4D). In all but one case (Bub3p-GFP), results from imaging matched those from ChIP (Fig. 4-4E). High levels of autofluorescence in *Δbub1* cells may have masked Bub3p-GFP kinetochore signals. Overall, our data show that kinetochore binding by checkpoint components is dependent upon the presence of proteins upstream in the signaling pathway: kinetochore binding by Mad1p and Mad2p requires *BUB1* and *BUB3* but not *MAD3*, Bub1p requires *BUB3* but not the *MAD* genes, and Bub3p is independent of all other checkpoint proteins.



E

	Δbub1	*Δbub3*	*Δmad1*	*Δmad2*	*Δmad3*
Bub1p		-	+	+	+
Bub3p	+		+	+	+
Mad1p + Noc.	-	-		-	+
Mad2p + Noc.	-	-	-		+

Figure 4-4 Interdependencies of spindle checkpoint proteins for kinetochore binding. (A) ChIP of Mad2p-GFP at *CENIV* in wildtype and checkpoint-delete cells in the presence of 25 μg/ml nocodazole. (B) ChIP of Mad1p-GFP at *CENIV* in wildtype and checkpoint-delete cells in the presence of nocodazole. (C and D) ChIP of Bub1p-GFP or Bub3p-GFP at *CENIV* in wildtype and checkpoint-delete cells. ChIP signals from deletion strains were normalized to the ChIP signal obtained from the wildtype strain. Dashed lines show the amount of *CEN* DNA precipitated using untagged wildtype cells (negative control). (E) Summary of the interdependencies of checkpoint protein kinetochore binding as assayed by microscopy. Mad1p-GFP and Mad2p-GFP were examined in the presence of nocodazole, whereas Bub1p-GFP and Bub3-GFP were examined in asynchronous cells.

Bub1p and Mad2p bind to kinetochores in ndc80-1 cells but not in spc25-7 cells

The structural proteins that recruit checkpoint components to kinetochores are unknown. The best candidates are those kinetochore components whose loss disables spindle checkpoint signaling. One such protein is Spc25p, a component of the NDC80 Complex. Kinetochores detach from spindle MTs in *spc25-7* cells but the spindle checkpoint is not activated (He et al., 2001; Janke et al., 2001; McCleland et al., 2003; Wigge and Kilmartin, 2001). Consistent with this, neither Bub1p-GFP nor Mad2p-GFP is associated with *CEN* DNA in *spc25-7* cells at 37°C (Fig. 4-5A and 4-5B). In contrast, kinetochores also detach from spindle MTs in *ndc80-1* cells at 37°C, but the checkpoint is engaged (Janke et al., 2001; McCleland et al., 2003) and we found that Bub1p-GFP and Mad2p-GFP are associated with *CEN* DNA in the *ndc80-1* mutant (Fig. 4-5A and 4-5B). In a control experiment, we observed that *CEN*-binding by the Cep3p component of CBF3 was equally high in wildtype, *spc25-7* and *ndc80-1* cells (Fig. 4-5C).

To confirm that the *ndc80-1* mutant was effectively disrupting kinetochore structure under our experimental conditions, we performed ChIP experiments using *ndc80-1* cells co-expressing Bub1p-GFP and myc-tagged Nuf2p, a protein known to require functional Ndc80p for *CEN*-association (He et al., 2001). While Bub1p-GFP and Nuf2p-myc could be crosslinked to *CEN* DNA in *ndc80-1* cells at permissive temperature, only Bub1p-GFP remained *CEN*-bound at 37°C (Fig. 4-5D and 4-5E). From these results, we conclude that the association of Bub1p and Mad2p with unattached kinetochores in budding yeast is dependent upon kinetochore components that assemble properly in *ndc80-1* cells but not in *spc25-7* cells. Differences between kinetochores in *ndc80-1* and *spc25-7* cells are likely to be quite subtle, and it is possible that Spc25p or other subunits of the NDC80 Complex may directly bind to Mad and Bub proteins.

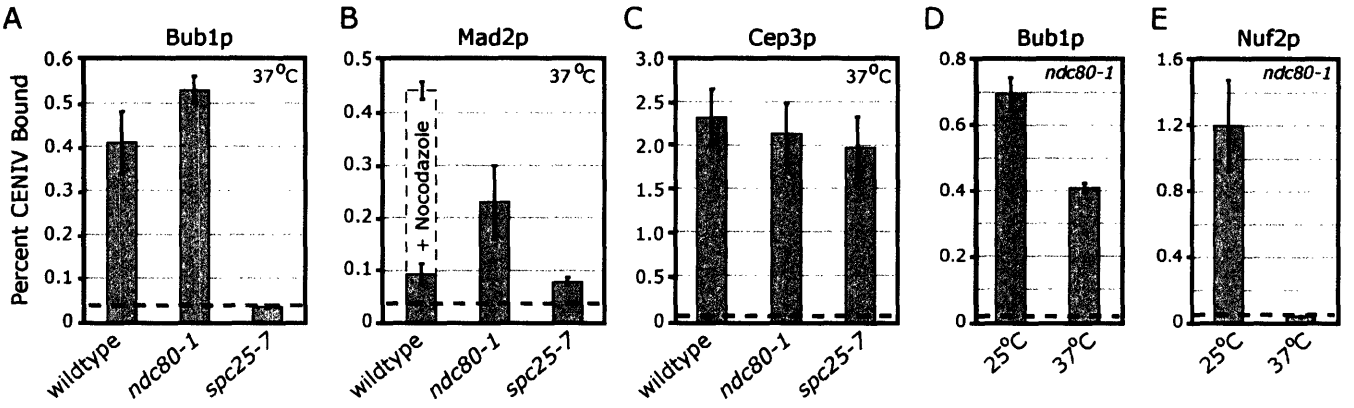


Figure 4-5 ChIP of Bub1p and Mad2p in NDC80 Complex mutants. (A-C) ChIP of (A) Bub1p-GFP, (B) Mad2p-GFP (+/- nocodazole) and (C) Cep3p at *CENIV* in wildtype, *ndc80-1* and *spc25-7* cells at 37°C. ChIP of (D) Bub1p-GFP and (E) Nuf2p-myc in an *ndc80-1* background at 25°C and 37°C. Graphs are as described in Figure 4-1D.

Mad2p is recruited to kinetochores in dam1-1 but not ipl1-321 cells

The existence of kinetochore mutants with distinct effects on chromosome dynamics affords an opportunity to investigate which types of lesions recruit checkpoint proteins to kinetochores. In *dam1-1* and *ipl1-321* cells, kinetochores cannot form stable bipolar attachments to spindle MTs, sister chromatid pairs each remain associated with a single SPB, and chromosome congression fails (Biggins et al., 1999; He et al., 2001; Janke et al., 2002; Kim et al., 1999; Tanaka et al., 2002). Interestingly, while *dam1-1* mutants engage the spindle checkpoint, *ipl1-321* mutants do not (Biggins and Murray, 2001; Cheeseman et al., 2001; He et al., 2001; Janke et al., 2002; Jones et al., 2001). To determine whether checkpoint proteins are recruited to kinetochores in *dam1-1* and *ipl1-321* mutants, we examined the localization of Ndc80p-GFP, Bub1p-GFP, and Mad2p-GFP in mutant cells co-expressing the SPB marker, Spc42p-CFP. Although it has previously been reported that kinetochores preferentially associate with the old SPB when subunits of the DAM1 Complex are inactivated (Janke et al., 2002), we find the asymmetric distribution of kinetochores in *dam1-1* cells to be somewhat variable. In

many cells, similar numbers of chromosomes were bound to each SPB (Fig.4- 6A). In contrast, the asymmetric distribution of kinetochores in *ipl1-321* cells was dramatic and consistent (Fig. 4-6D). By imaging, we found that Bub1p-GFP was present on kinetochores at non-permissive temperature in both *dam1-1* and *ipl1-321* cells (Fig. 4-6B, E, G, and H). While Mad2p-GFP appeared to be kinetochore-bound in the majority of *dam1-1* cells after 1hr. at 37°C (Fig. 4-6C and 4-6G), Mad2p-GFP was rarely detected on kinetochores in *ipl1-321* cells at non-permissive temperature (Fig. 4-6F and 4-6H). ChIP analysis confirmed these findings (data not shown).

Why do *dam1-1* kinetochores recruit Mad2p while *ipl1-321* kinetochores do not? One possibility is that Ipl1p is an upstream component of the checkpoint pathway required for the activity of Mad2p (Biggins and Murray, 2001). This is not strictly true, however, as Mad2p binding to *CEN* DNA could be detected by imaging and ChIP in *ipl1-321* cells treated with nocodazole (data not shown). A second possibility is that kinetochore-MT links in *ipl1-321* cells prevent Mad2p binding. It has been proposed that Ipl1p plays an essential role in releasing syntelic attachments that form early in the cell cycle when both kinetochores in a pair of sister chromatids bind to MTs emanating from the same SPB (Tanaka et al., 2002). We speculate that yeast Mad2p is not recruited to kinetochores in *ipl1-321* cells because they have syntelic MT attachments. In contrast, monotelic attachments (in which one kinetochore is attached, while its partner is unattached) likely predominate in *dam1-1* cells, and Mad2p is therefore recruited to the unattached kinetochore. By this reasoning, the inability of *ipl1-321* cells to engage the spindle checkpoint does not reflect a role for *IPL1* in checkpoint signaling, but rather the failure of *ipl1-321* cells to generate a kinetochore structure that the checkpoint can recognize.

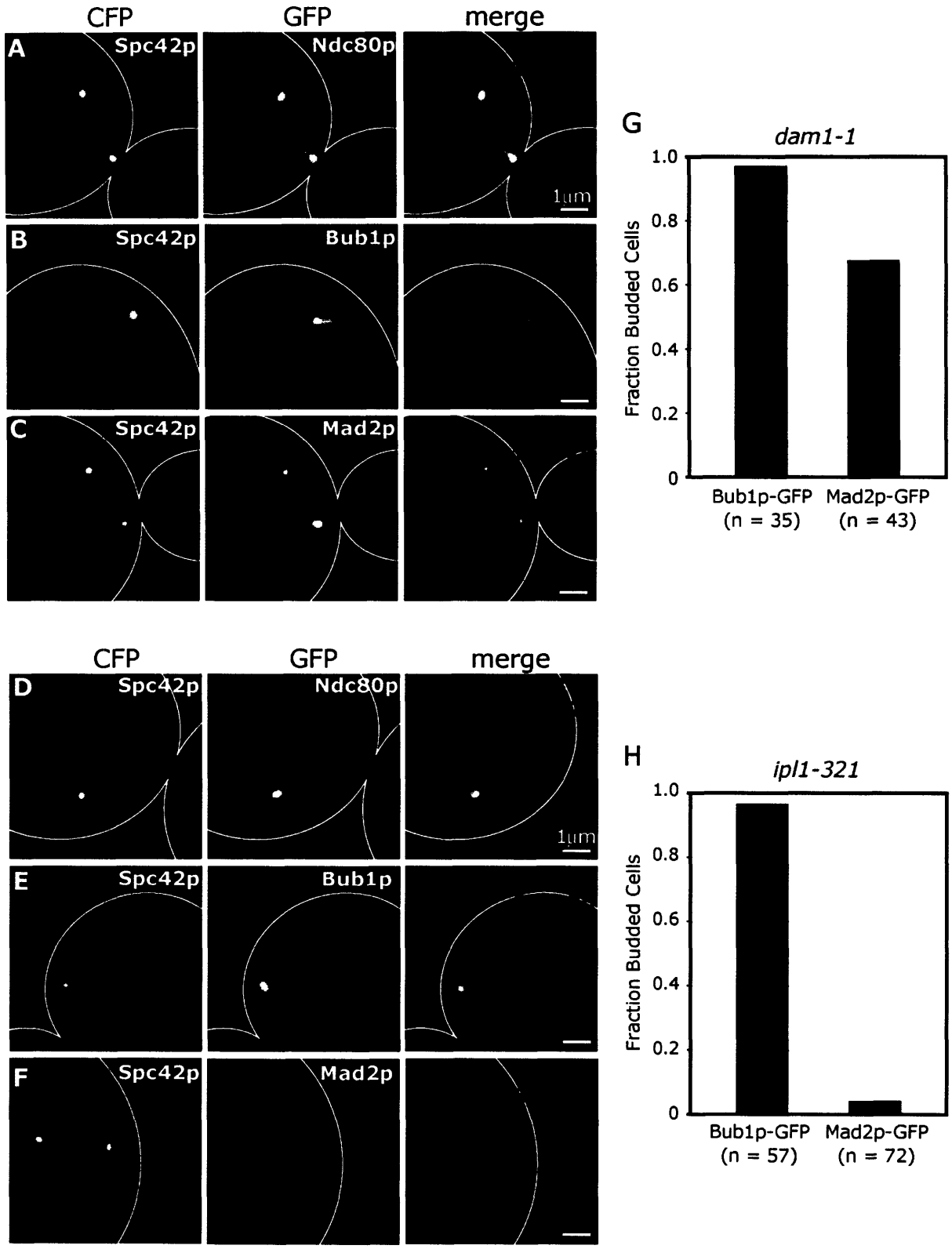


Figure 4-6 Bub1p and Mad2p localization in *dam1-1* and *ipl1-321* cells. (A-C) *dam1-1* cells expressing the SPB protein Spc42p-CFP and Ndc80p-GFP, Bub1p-GFP or Mad2p-GFP at nonpermissive temperature. Cells were grown at 25°C to mid-log phase and shifted to 37°C for 1 hour before fixation. Panels show Spc42p-CFP alone; Ndc80p-GFP, Bub1p-GFP or Mad2p-GFP; and Spc42p-CFP (red) merged with Ndc80p-CFP (blue), Bub1p-GFP (green) or Mad2p-GFP (green). (D-F) *ipl1-321* cells expressing Spc42p-CFP and Ndc80p-GFP, Bub1p-GFP or Mad2p-GFP at nonpermissive temperature. Cells were arrested in α-factor for 2 hours, shifted to 37°C for 10 min. and released at 37°C for 2 hours before fixation. (G and H) Fraction of budded cells containing Bub1p-GFP and Mad2p-GFP for *dam1-1* cells after 1 hour at 37°C and *ipl1-321* cells after 2 hours at 37°C following α-factor release. n = number of budded cells counted

Loss of tension is not sufficient to recruit Bub1p or Mad2p to kinetochores in stu2-279 cells

A major question in the study of mitosis is whether it is the absence of tension or the loss of MT attachment that is ultimately responsible for activating checkpoint signaling. Our data show that kinetochores that remain attached to collapsed spindles in nocodazole-treated cells do not recruit Mad and Bub proteins (Fig. 4-3C to 4--3E). As it is mechanically impossible for collapsed spindles to impose tension on chromatids, these results suggest that loss of tension does not recruit high levels of Mad or Bub proteins to kinetochores. To determine if checkpoint proteins are kinetochore-bound in cells in which tension has been eliminated by other means, we examined cells carrying mutations in the MT-associated protein Stu2p (He et al., 2001). *stu2-279* cells arrest in a checkpoint-dependent fashion with kinetochores that have bipolar attachments but are not under detectable tension (He et al., 2001; Severin et al., 2001a). When *stu2-279* cells co-expressing the SPB marker Spc42p-CFP and Ndc80p-GFP, Mad2p-GFP or Bub1p-GFP were examined by imaging and ChIP at non-permissive temperatures, one or two bright GFP foci were visible (Fig. 4-7A and 4-7B) and both Mad2p and Bub1p were *CEN*-associated by ChIP (Fig. 4-7F and 4-7G). However, almost all Mad2p-GFP and Bub1p-GFP foci lay ≥ 1μm from the spindle axis (Fig. 4-7A and 4-7B), while the majority of kinetochores, as monitored by Ndc80p-GFP, lay between the SPBs (Fig. 4-7C). In most cells, one or two dim Ndc80p-GFP foci were also visible ≥ 1μm from the spindle axis (Fig. 4-7C). The analysis of *stu2-279* cells co-expressing Ndc80p-CFP and either Bub1p-GFP or Mad2p-GFP made it clear that the dim Ndc80p-CFP foci distant from the spindle axis were coincident with the bright Bub1p-GFP and Mad2p-GFP foci (Fig. 4-7D and 4-7E). Thus, it appears that Bub1p and Mad2p are specifically recruited only to a subset of kinetochores in *stu2-279* cells. Similar results were obtained with a *stu2-277* mutant (data not shown).

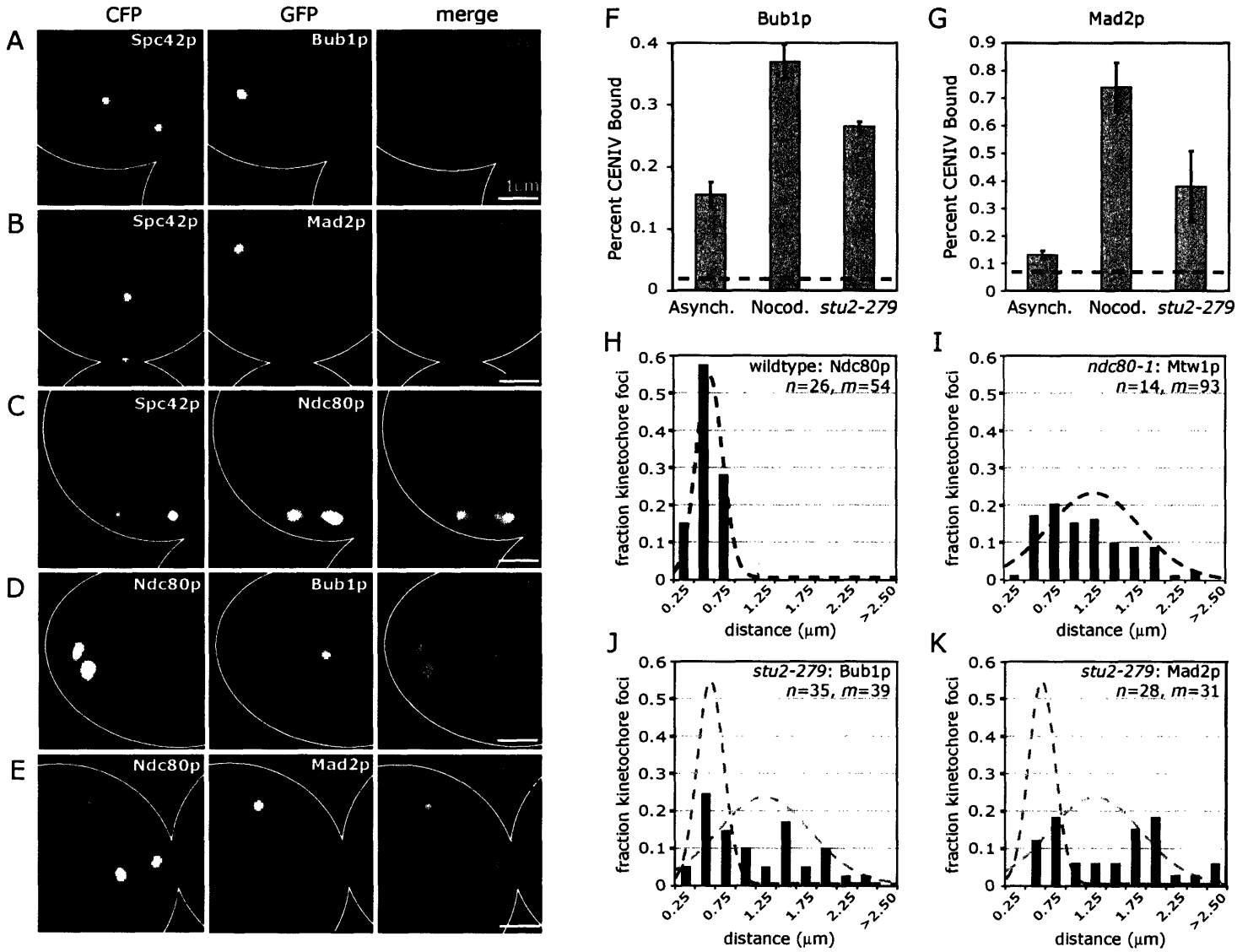


Figure 4-7 Bub1p and Mad2p localization in *stu2-279* cells. (A-C) *stu2-279* cells co-expressing the SPB protein Spc42p-CFP and Bub1p-GFP, Mad2p-GFP or Ndc80p-GFP. Panels show Spc42p-CFP alone; Bub1p-GFP, Mad2p-GFP or Ndc80p-GFP alone; and Spc42p-CFP (red) merged with Bub1p-GFP (green), Mad2p-GFP (green) or Ndc80p-GFP (blue). Cells were grown at 25°C to mid-log phase and shifted to 37°C for 2 hours before fixation. Orange arrowheads denote unattached kinetochores. (D and E) *stu2-279* cells co-expressing the kinetochore protein Ndc80p-CFP and Bub1p-GFP or Mad2p-GFP. Panels show Ndc80p-CFP alone; Bub1p-GFP or Mad2p-GFP alone; and Ndc80p-CFP (blue) merged with Bub1p-GFP (green) or Mad2p-GFP (green). Images are as described in Figure 4-1A. Red X's denote the inferred positions of SPBs. (F and G) ChIP of Bub1p-GFP and Mad2p-GFP at *CENIV* in asynchronous wildtype cells, nocodazole-treated wildtype cells and *stu2-279* cells, all at 37°C. Graphs are as described in Fig. 4-1 (D-G). (H-K) Spatial distribution of kinetochore foci for (H) Ndc80p-GFP in wildtype cells with attached kinetochores, (I) Mtw1p-GFP in *ndc80-1* cells with unattached kinetochores (at 37°C), and (J-K) Bub1p-GFP or Mad2p-GFP in *stu2-279* cells (also at 37°C). Distances were measured from each GFP focus to the center of the spindle. Spindle orientation and length was determined using Spc42p-CFP. Only cells with spindles between 0.75 and 1.50 μm were included. Lines represent normal distributions for attached (red, $\mu = 0.40$ μm; $\sigma = 0.15$ μm) or unattached kinetochores (green, $\mu = 1.01$ μm; $\sigma = 0.52$ μm). The number of cells (*n*) and number of kinetochore foci (*m*) analyzed are listed on each graph.

What distinguishes kinetochores that recruit Bub1p and Mad2p in *stu2* cells from those that do not? One possibility is that kinetochores that lie off of the spindle axis, and that bind to Bub1p and Mad2p, are not correctly attached to MTs. Although we had not anticipated that *stu2* cells would contain unattached kinetochores, MTs are known to be fewer in number and less dynamic in *stu2* mutants (Kosco et al., 2001) and it is likely that the spindle's ability to capture kinetochores and maintain kinetochore-MT attachments is compromised in these cells. Moreover, although we only detected attached chromosomes in our initial studies of *stu2* cells (He et al., 2001), recent live-cell data indicate that a subset of kinetochores do detach from spindle MTs in *stu2* mutants (D. Rines-unpublished data).

To better characterize the state of chromosome-MT attachment in *stu2* cells, we profiled the spatial distributions of Bub1p and Mad2p foci within the nuclei of these cells and compared them to the spatial distribution of kinetochores known to be attached (as determined from the positions of Ndc80p-GFP foci in metaphase wildtype cells) and those known to be unattached (as determined from the positions of Mtw1p-GFP foci in *ndc80-1* cells). In each case, spatial kinetochore distributions were profiled by measuring the distances from each GFP focus to the center of the spindle. While attached kinetochores exhibited a narrow distribution with a mean of 0.4 μm (Fig. 4-7H), unattached kinetochores showed a broad distribution with a mean of 1.0 μm and a maximum of 2.3 μm (Fig. 4-7I). Importantly, the distribution of Bub1p-GFP and Mad2p-GFP foci in *stu2-279* cells was very similar to that of unattached kinetochores, strongly suggesting that checkpoint proteins are recruited to kinetochores that have become detached from the spindle in *stu2-279* cells (Fig. 4-7J and 4-7K). We conclude that, in *stu2* mutants, the majority of kinetochores are attached to MTs and lack detectable Bub1p and Mad2p, despite a

lack of tension. However, a subset of kinetochores—perhaps one or two per cell—are not attached to MTs, and these kinetochores selectively recruit high levels of Bub1p and Mad2p.

Bub1p binds kinetochores in the absence of sister cohesion, but Mad2p does not

Another method by which tension across kinetochores can be eliminated is by inactivating sister cohesion. A temperature sensitive *mcd1-1* cohesin mutant disables sister pairing and allows chromatids to segregate independently of one another (Guacci et al., 1997). While *mcd1-1* cells experience a slight checkpoint-dependent cell cycle delay, they appear to undergo a morphologically normal anaphase (Biggins and Murray, 2001; Severin et al., 2001b). We were unable to detect Mad2p on kinetochores in *mcd1-1* cells by ChIP or imaging (data not shown), even though the cell cycle delay in *mcd1-1* cells is known to be *MAD2*-dependent. We cannot tell if this reflects an off-kinetochore function for Mad2p in response to lack of tension (Martin-Lluesma et al., 2002), or if Mad2p is present transiently at kinetochores below our limit of detection. However, it is clear that the lack of tension on kinetochores in *mcd1-1* cells is not sufficient to recruit the high levels of Mad2p seen on unattached kinetochores.

A comparison of wildtype and *mcd1-1* cells co-expressing Bub1p-GFP and Ndc80p-CFP revealed that Bub1p binding to kinetochores was very similar from 0-60 min. following α-factor release (Fig. 4-8A, B, and G). However, the dissociation of Bub1p from kinetochores was delayed ~15 min. relative to wildtype cells (Fig. 4-8G). Interestingly, *mcd1-1* cells with longer spindles almost always contained a heterogeneous population of Bub1p-positive and Bub1p-negative kinetochores (Fig. 4-8B), suggesting that Bub1p binding is likely to depend on the attachment status of individual kinetochores. From these data, we conclude that Bub1p is recruited properly to kinetochores in *mcd1-1* mutants early in mitosis and is then lost as mitosis progresses. Thus, bipolar attachment and tension are not absolutely required to release Bub1p

from kinetochores. At this point, it is not clear if delayed release of Bub1p from kinetochores in *mcd1-1* cells is a consequence of lack of tension *per se*, or rather of problems in establishing mature chromosome-MT attachments due to a lack of sister pairing.

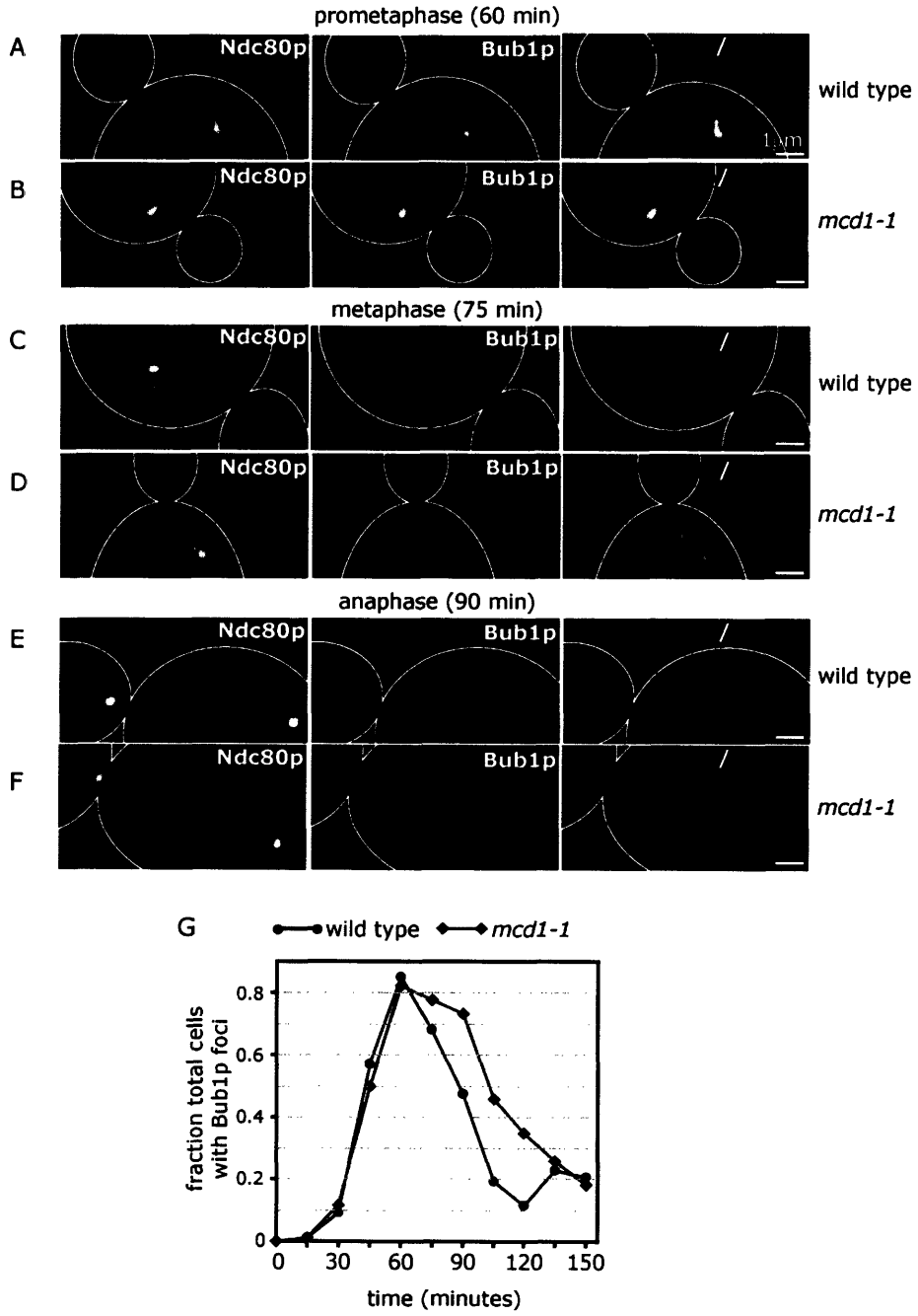


Figure 4-8 Bub1p localization in *mcd1-1* cells. (A-F) Typical images of *mcd1-1* and wildtype cells co-expressing Bub1p-GFP (green) and Ndc80p-CFP (red) at 60, 75 and 90 min. after α-factor release at 37° C. (G) Fraction of *mcd1-1* and wildtype cells containing Bub1p kinetochore foci at 15 min. time points after α-factor release at 37 °C. At least 60 individual cells were analyzed at each time point.

DISCUSSION

In this paper, we show that spindle checkpoint proteins in *S. cerevisiae* are recruited to centromeres in a kinetochore-dependent manner, just as they are in animal cells. Despite the high degree of conservation in Mad and Bub proteins through evolution, however, our data also show that interactions between kinetochores and spindle checkpoint proteins in yeast and animal cells differ in several significant ways. Budding yeast Bub1p and Bub3p are like their mammalian counterparts in that they bind to kinetochores during normal cell division. This binding is cell cycle regulated, being highest early in mitosis around the time of SPB duplication and falling as mitosis proceeds. In contrast, while mammalian Mad1 and Mad2 are bound to kinetochores during prometaphase in normally dividing cells, yeast Mad1p and Mad2p are kinetochore-bound only in cells in which chromosome-MT attachment is inhibited. We propose that organism-specific differences in the behavior of spindle checkpoint proteins are likely to reflect evolutionary divergence in the mechanics of spindle assembly rather than extensive differences in the pathways of checkpoint signaling.

Several key features distinguish spindle assembly in animal cells and budding yeast. Animal cells undergo an open mitosis and prometaphase chromosomes are initially free of spindle MTs following nuclear envelope breakdown. High levels of Mad and Bub proteins are present on these unattached kinetochores, but Mad1 and Mad2, in particular, dissociate as chromosome-MT attachments form (Waters et al., 1998). In contrast, budding yeast cells undergo a closed mitosis in which kinetochores remain closely associated with SPBs throughout the cell cycle (Jin et al., 2000; D. Rines-unpublished observations). While we find Mad1p and Mad2p on unattached *S. cerevisiae* kinetochores in cells with spindle damage or kinetochore lesions, yeast kinetochores do not recruit high levels of these proteins during normal mitosis,

consistent with the idea that yeast chromosomes are continuously linked to MTs. The maintenance of kinetochore-MT attachments throughout the yeast cell cycle may make spindle assembly more efficient, a property that could explain why yeast *MAD2* is not required for normal cell growth (Li and Murray, 1991) while murine Mad2 is essential (Dobles et al., 2000). Interestingly, yeast Mad2p appears to be important for chromosome bi-orientation during the first meiotic division (Shonn et al., 2000; Shonn et al., 2003) which implies that kinetochore-binding by Mad2p might be a normal feature of meiosis. It will therefore be interesting to determine if Mad2p-positive chromosomes are generated during meiotic bouquet formation (Trelles-Sticken et al., 1999).

The NDC80 Complex and spindle checkpoint signaling

An important issue in the study of spindle checkpoint signaling is determining how spindle checkpoint proteins bind to kinetochores. The best candidates for proteins that link Mad and Bub proteins to kinetochores are those whose inactivation disrupts checkpoint signaling without completely disrupting kinetochore assembly. Although mutations in almost all known kinetochore components engage the checkpoint (Gardner et al., 2001), loss-of-function mutations in subunits of the CBF3 complex (which consists of Ndc10p, Cep3p, Ctf13p, and Skp1p) and some subunits of the NDC80 Complex (which consists of Spc24p, Spc25p, Ndc80p and Nuf2p) have the special property of abolishing the checkpoint (Gardner et al., 2001; Goh and Kilmartin, 1993; Janke et al., 2001; McCleland et al., 2003). However, protein-protein and protein-DNA associations among kinetochore proteins are hierarchical: while loss of CBF3 function prevents all known kinetochore proteins from associating with *CEN* DNA (Goh and Kilmartin, 1993; He et al., 2001), loss of Ndc80 function disrupts the assembly of only a small subset of kinetochore

components (De Wulf et al., 2003; He et al., 2001; Janke et al., 2001). It has been suggested that the CBF3 subunit, Skp1p, mediates the binding of Bub1p to kinetochores (Kitagawa et al., 2003), but our data show that the *spc25-7* mutation prevents Bub1p and Mad2p from binding to kinetochores at non-permissive temperature without altering the level of *CEN*-bound CBF3 (as measured using the CBF3 component, Cep3p, Fig. 4-5C). This evidence strongly suggests that CBF3, and hence Skp1p, cannot be sufficient for the recruitment of Bub1p to kinetochores.

Mutant analysis suggests the link between checkpoint signaling and mutations in subunits of the NDC80 Complex is fairly complex: *spc24-2* and *spc25-7* mutants abrogate the checkpoint whereas *ndc80-1* and *nuf2-457* mutants engage the checkpoint (He et al., 2001; Janke et al., 2001; McCleland et al., 2003; Wigge and Kilmartin, 2001). We have found that these functional differences are reflected in the extent to which Mad and Bub proteins are recruited to kinetochores. Gene and allele-specific differences among *spc24, spc25, ndc80* and *nuf2* mutations may be a simple consequence of differences in allelic strength: in the case of CBF3, Burke and colleagues have elegantly demonstrated that hypomorphic alleles engage the checkpoint whereas complete loss-of-function mutations inactivate it (Connelly and Hieter, 1996; Doheny et al., 1993; Gardner et al., 2001; Strunnikov et al., 1995; Tavormina and Burke, 1998), and the results of McClelland et al. (2003) suggest that *ndc80-1* may indeed be a hypomorphic allele. Alternatively, it is also possible that some subunits of the NDC80 Complex are required for the recruitment of Mad and Bub proteins to kinetochores while other subunits are not. Either way, the requirement for a functional NDC80 Complex in checkpoint signaling and the evolutionary conservation of the NDC80 Complex (human Ndc80/HEC1 can functionally substitute for yeast *NDC80*; Zheng et al., 1999) are suggestive of important functional connections between the NDC80 Complex and the spindle checkpoint.

Attachment, tension, and the spindle checkpoint in budding yeast

Two main hypotheses exist regarding what features of kinetochore-MT attachment are monitored by the spindle checkpoint. The tension hypothesis posits that the checkpoint monitors tension across paired sister kinetochores (Stern and Murray, 2001) while the attachment hypothesis suggests that the checkpoint monitors the occupancy of kinetochore-MT attachment sites (Rieder et al., 1995). In budding yeast, Mad1p, Mad2p, Bub1p and Bub3p are recruited to unattached kinetochores in *ndc80-1* cells and to kinetochores with monopolar attachments in *dam1-1* cells. However, in no context have we observed high levels of checkpoint proteins bound to kinetochores that have achieved bipolar attachment but lack tension. While cells carrying a mutation in the kinetochore-associated MAP, Stu2p, contain attached tension-free kinetochores as well as unattached kinetochores, high levels of Bub1p and Mad2p are recruited only to the latter. Similarly, while a few kinetochores detach from spindle MTs in cells treated with the anti-MT drug nocodazole, the majority of kinetochores remain attached to very short MTs and in close proximity to the collapsed SPBs. Although the collapsed spindles in nocodazole-treated cells cannot generate tension across sister kinetochores, Bub1p and Mad2p are found only on unattached kinetochores. Finally, Mad2p is not detectable on kinetochores in *mcd1-1* cells that lack sister cohesion and bipolar tension. Thus, the absence of tension on paired sister chromosomes is not sufficient to recruit high levels of Mad or Bub proteins to kinetochores. Overall, our data are most consistent with the attachment hypothesis, but it remains possible that lack of tension may cause the transient binding of Bub and Mad proteins to kinetochores at levels that are below our limit of detection.

Role of the Bub proteins during normal spindle assembly

High levels of Bub1p and Bub3p, but not Mad1p or Mad2p, are recruited to kinetochores during normal mitosis, suggesting that Bub1p and Bub3p play a role in spindle assembly that the Mad proteins do not share. Several additional pieces of evidence support this hypothesis. First, budding yeast cells deleted for *BUB1* or *BUB3* experience much more severe chromosome loss than do cells deleted for *MAD1*, *MAD2*, or *MAD3* (Warren et al., 2002). Second, extra copies of *BUB1* or *BUB3* suppress the chromosome-MT attachment defects generated by *tub1-729* mutant, independent of *MAD2*-dependent signaling (Abruzzi et al., 2002). Third, while the conserved kinase domain of Bub1p is not required for nocodazole arrest in yeast (Sharp-Baker and Chen, 2001; Warren et al., 2002) or the recruitment of downstream checkpoint proteins to kinetochores in *Xenopus* (Sharp-Baker and Chen, 2001; Warren et al., 2002), it is required for suppression of attachment defects in *tub1-729* cells (Abruzzi et al., 2002) and for accurate chromosome transmission in wildtype cells (Warren et al., 2002).

We find selective binding of Bub proteins, but not Mad proteins, to kinetochores in three contexts: wildtype cells early in mitosis, *ipl1-321* cells, and *mcd1-1* cells. Early during spindle assembly, kinetochores are thought to form transient syntelic attachments in which both sister kinetochores are linked to the old SPB. Syntelic attachments resolve to bipolar attachments early in spindle assembly in wildtype cells, but persist in *ipl1-321* cells (Tanaka et al., 2002). While Bub1p is recruited to kinetochores with syntelic attachments in *ipl1-321* cells, it is also recruited to kinetochores in *mcd1-1* cells which are necessarily unpaired and therefore unable to form syntelic attachments. What feature is common to *ipl1-321* and *mcd1-1* chromosome-MT attachments as well as to wildtype attachments early in the cell cycle? It is known that kinetochores in animal cells initially bind to the sides of MTs during spindle assembly (Merdes

and De Mey, 1990), and MT binding assays have demonstrated that reconstituted budding yeast kinetochores form "lateral" attachments to the sides of MTs *in vitro* (Sorger et al., 1994). We therefore propose that Bub proteins are recruited in yeast to kinetochores that have attached to the sides rather than the ends of MTs, as well as to kinetochores that lack MT attachment altogether.

SUMMARY

In summary, our analysis of spindle checkpoint proteins in budding yeast reinforces the idea that Bub1p and Bub3p have a role during spindle assembly that Mad1p and Mad2p do not share. While the Bub proteins appear to respond to changes in chromosome-MT attachment that occur during the course of normal spindle assembly, Mad proteins respond primarily to chromosome-MT detachment, a condition that does not exist in normally growing yeast cells. Our data help to explain why the spindle checkpoint is non-essential in budding yeast as well as why deletions of *BUB1* or *BUB3* have more dramatic effects on cell growth and chromosome loss than do deletions of *MAD1-3*. More broadly, our findings support the hypothesis that it is changes in the state of chromosome-MT attachment rather than in tension across sister kinetochores that is responsible for recruiting checkpoint proteins to kinetochores and, presumably, for initiating checkpoint signaling.

MATERIALS AND METHODS

Yeast Strains and Manipulations

Strains were derived from W303 or S288C parental stocks. Proteins were tagged with GFP or CFP by linking a 300–800 bp C-terminal PCR gene fragment to the coding sequence for EGFP or ECFP in pRS306 or pRS304. Endogenous genes were replaced using one-step gene replacement and correct integrants were verified by PCR.

Microscopy Analysis

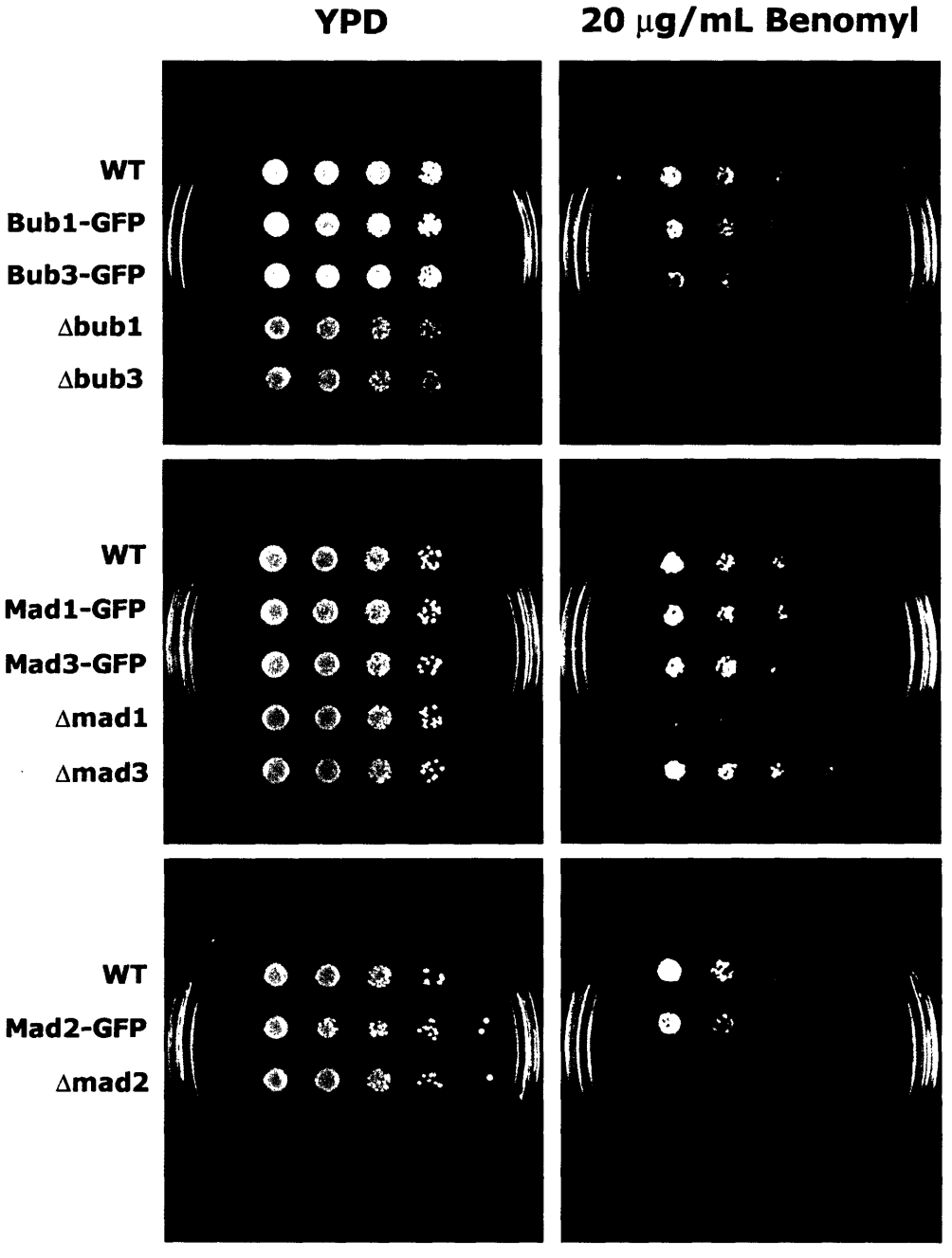
Images of fixed cells carrying CFP and GFP fusion proteins were collected at room temperature using a fluorescence microscope (Deltavision with Nikon TE200 base), Plan Apo 100X/1.40 oil objective, and a camera (CoolSnap HQ; Photometrics) with Chroma 86002 JP4 (CFP) and 41018 (GFP) filters. 3D image acquisition, deconvolution, and maximum intensity 2D projections were done using softWoRx software. Fixed cells were treated with 2% formaldehyde (final) for 5-10 min. followed by 0.1 M phosphate buffer (pH 6.6) for at least 5 min. and imaged at RT.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described (Megee et al.,1999) except that cells were crosslinked with formaldehyde for 2 hours at RT, lysed using glass beads in a Bio101 FastPrep FP120, sonicated until DNA was an average of 200-500 bp in length and centrifuged to remove cellular debris. Immunoprecipitations were performed using anti-GFP (Clontech), anti-myc (Santa Cruz) and anti-Cep3p (Sorger Lab) antibodies. PCR amplifications of 200 bp fragments of *URA3* and *CENIV* were performed on serial dilutions (to determine linearity) of two or more independent IPs; error bars show standard deviations.

Online supplemental material

Benomyl sensitivity assays of strains expressing GFP-tagged proteins are shown in Figure S1. A summary of kinetochore localization by spindle checkpoint proteins can be found in Table S1.



Supplementary Figure 4-1 Wildtype, C-terminally GFP-tagged and deletion strains (as indicated) were spotted in fivefold (*BUB1* and *BUB3*) or tenfold (*MAD1-3*) dilutions on YPD and YPD + 20 μg/ml benomyl and incubated at 30°C for 3 days. These results indicate that the GFP-tagged strains are functional.

Table S1. Kinetochore Localization of Bub1p, Bub3p, Mad1p, and Mad2p

BACKGROUND	KINETOCHORE PHENOTYPE	Bub1p	Bub3p	Mad1p	Mad2p
wildtype	attached	+ [a]	+ [a]		
wildtype + α–factor (G1)	attached				
wildtype + nocodazole (mitosis; chkpt. activated)	unattached	++	++	++	++
	attached, no tension				
ndc10-1	kinetochores not assembled			N.D.	
ndc80-1	unattached	++	++	N.D.	+
spc25-7	unattached	++	N.D.	N.D.	
dam1-1	monopolar attachment			N.D.	++
ipl1-321	syntelic attachment	++	N.D.	N.D.	
ipl1-321 + nocodazole	unattached	++ [b]	N.D.	N.D.	++ [b]
	attached	++ [b]	N.D.	N.D.	+ [b]
stu2-279	unattached	++	N.D.	N.D.	++
	bipolar attachment, no tension		N.D.	N.D.	
mcd1-1	unpaired chromatids, no tension	++	N.D.	N.D.	

gray = spindle checkpoint disabled; [a] = kinetochore binding during early mitosis; [b] = data not shown
N.D. = no data

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CHAPTER 5

In vitro Dynabead-Kinetochore Reconstitution Assay

The proteins that bind to *S. cerevisiae CEN* DNA include the CBF3 Complex (CBF3), along with Ndc10p on its own, Cbf1p and presumably Cse4p (Jiang and Philippsen, 1989; Cai and Davis, 1990; Lechner and Carbon, 1991; Espelin et al., 1997; Meluh et al., 1998; Espelin et al., 2003). In the 13 years since the identification of these *CEN* DNA-binding proteins, many additional kinetochore proteins have been discovered. However, we still do not know which proteins assemble on the DNA-Binding layer of the kinetochore, and in particular which proteins associate specifically with CBF3. Chromatin Immunoprecipitation and microscopy have established the central role of the CBF3 Complex for the association of all other kinetochore proteins with *CEN* DNA, but these methods cannot establish whether interactions are direct or indirect. Determining which proteins directly interact with CBF3 will be essential for understanding how the kinetochore assembles as well as for determining its overall molecular organization.

Previous attempts have been made to identify CBF3-interacting proteins using 1- and 2-hybrid screens, synthetic lethal screens, co-immunoprecipitation experiments and purification of the individual CBF3 components. Ctf19p, Okp1p, Mcm21, Ame1p (COMA Complex), Cbf1p, Bir1p and Phospholipase C have all been proposed to interact with members of the CBF3 Complex based variously on results from one or more of the aforementioned techniques (Ortiz et al., 1999; Yoon and Carbon, 1999; Hemmerich et al., 2000; Lin et al., 2000; De Wulf et al., 2003; K. Simons-unpublished observations). The 1-, 2-hybrid results and the few co-immunoprecipitation experiments that have been performed to support the 1-, 2-hybrid experiments are intriguing. However, these IPs use crude yeast extracts, allowing for the possibility of intermediate proteins mediating the observed interaction between CBF3 proteins and COMA. Purification of individual CBF3 components followed by mass spectrometric

analysis of associated proteins has only identified interactions amongst member proteins of the CBF3 complex (Westermann et al., 2003). There are a number of potential biological and technical explanations for our inability to identify CBF3-interacting proteins by purifying the individual components. Although it has been shown that some aspects of kinetochore function can be reconstituted *in vitro*, it is not known whether the multiprotein kinetochore complex remains intact through multistep purification processes, particularly under the stringent conditions required to ensure specificity (Sorger et al., 1994). It is also not known whether CBF3 is able to remain bound to *CEN* DNA through the purification, a likely requirement for maintaining interactions between CBF3 and other kinetochore proteins.

This chapter describes the development of an assay to identify proteins that interact with CBF3 and Ndc10p bound to *CEN* DNA. The premise of the assay is based on the MT-binding assay developed by Sorger and colleagues briefly described in Chapter 1 (Sorger et al., 1994). In their MT-binding assay, fluorescent beads are covalently bound to *CEN* DNA, mixed with yeast extracts and evaluated by microscopy for their ability to associate with Taxol-stabilized MTs *in vitro*. The implication of the observations made using this assay is that all of the kinetochore proteins required to build a *CEN*-MT connection are present. However, because yeast extracts were used, the exact identity of the proteins involved in building microtubule attachment sites *in vitro* remain undetermined. I have modified the methods of Sorger et al. (1994) as a basis for an assay in which magnetic Dynabeads are covalently bound to *CEN* DNA and mixed with recombinant CBF3. The utilization of Dynabeads allows for more stringent purification conditions throughout the assay than the latex beads in Sorger et al. (1994), thereby increasing the potential specificity of protein-protein interactions. The use of recombinant CBF3 establishes an initial DNA-binding complex which should then be competent for the subsequent

binding of other kinetochore proteins. Following the binding of rCBF3 to *CEN* DNA, the goal is to identify CBF3-interacting proteins from yeast extracts, fractionated extracts and/or recombinant kinetochore proteins. Positive results will subsequently be tested *in vivo* and in functional assays *in vitro*.

The results presented here are preliminary. Proper controls have not been fully established in some cases, and a number of experimental issues (including some that plagued earlier work) remain to be worked out, and will be addressed in the Discussion. However, initial results are quite promising and suggest I have developed an effective method for identifying proteins that interact with CBF3 and help establish a connection to MTs. My experiments show that the kinetochore proteins Cse4p, Mif2p, Ndc80p and Ame1p specifically interact with centromeric DNA in a CBF3-depenendent manner. In contrast to results published by Ortiz et al., I have not observed association of Ctf19p with CBF3, although this may reflect differences in the experimental methods used (Ortiz et al., 1999). Experiments to date have involved the use of yeast extracts and establishing whether the interactions are direct or indirect will require further characterization including the use of purified or recombinant components. Nevertheless, these preliminary data provide a promising starting point for identifying proteins that interact with the DNA-Binding layer, the next step in establishing a connection to the MT.

RESULTS

Recombinant CBF3 binds specifically to CEN DNA

To identify proteins that interact with CBF3, I first constructed probes using magnetic Dynabeads (DYNAL, Oslo, Norway) bound to either wildtype *CEN* (184 bp CDEI,II,III;

Dynabead A), mutant *CEN* (181 bp *CEN* with 3bpΔ in CDEIII; Dynabead B), random plasmid DNA (184 bp; Dynabead C) or no DNA (Dynabead D). See Figure 5-1.

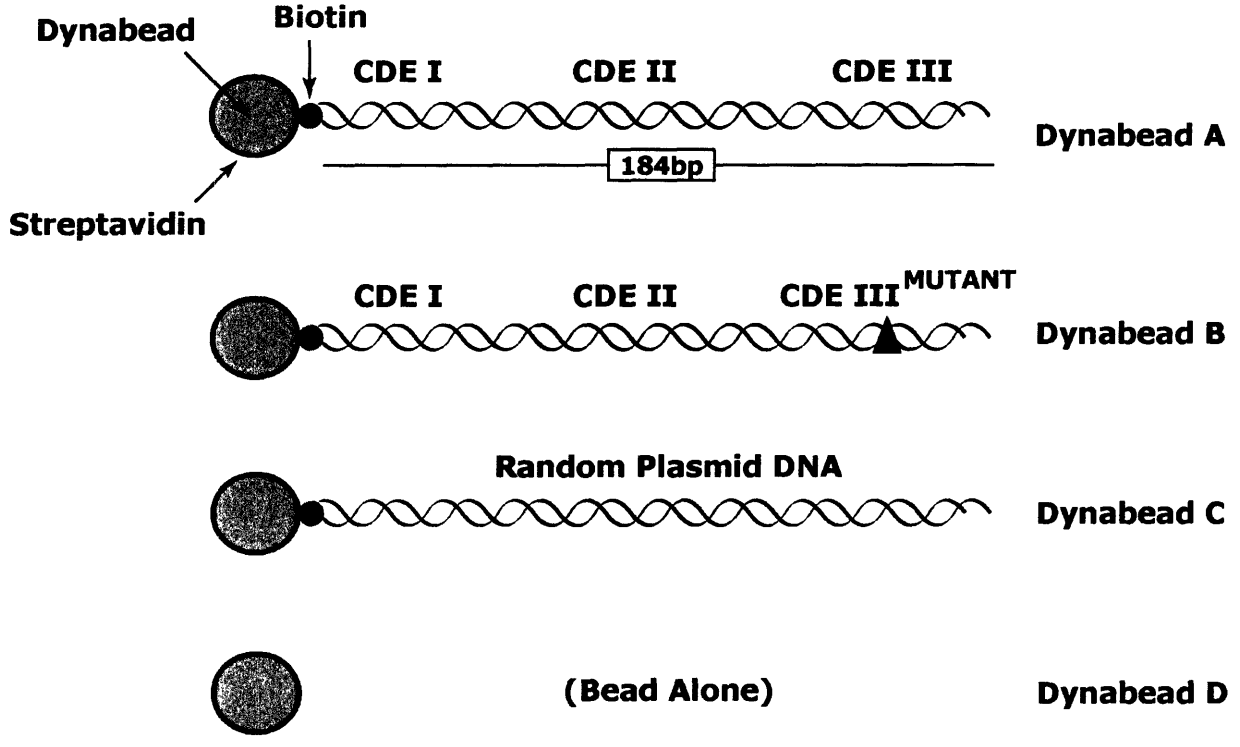


Figure 5-1 Dynabead-DNA constructs. Biotinylated DNA was bound to streptavidin-coated Dynabeads. Dynabead A is bound to 184 bp WT *CEN3*; Dynabead B is bound to 181 bp mutant *CEN3* (3bpΔ in CDEIII); Dynabead C is bound to 184 bp random plasmid DNA; Dynabead D contains no DNA. Figure is not drawn to scale.

As a source of CBF3, I co-expressed the four CBF3 subunits (Ndc10p, Cep3p, Ctf13p and Skp1p) in insect cells and purified the four-protein complex using 6xHis-tagged Cep3p (Figure 5-2, lane 1; see Materials and Methods for details). Equivalent amounts of rCBF3 were incubated with each of the Dynabeads, after which the supernatant was recovered (FT) and both the beads and FT evaluated for the presence of CBF3 proteins by SDS-PAGE and Coomassie

staining. Ndc10p, Cep3p and Ctf13p were bound to Dynabead A (Fig. 5-2, lane 2), but exhibited 10 to 12-fold reduced binding to Dynabeads C and D (Fig. 5-2, lanes 4 and 5). Skp1p was not readily identifiable by Coomassie staining in any of the binding reactions. This is not unexpected as Skp1p can be removed from the CBF3 Complex, following activation of Ctf13p, without affecting the ability of the remaining members of the CBF3 Complex to bind *CEN* DNA *in vitro* (Kaplan et al., 1997). When Dynabead B was mixed with rCBF3, binding of Ndc10p, Cep3p and Ctf13p was reduced 4 to 6-fold relative to Dynabead A + rCBF3 (Fig. 5-2, lane 3). The binding of CBF3 to Dynabead B was a bit unexpected as mutations in the central CCG of CDEIII render the chromosome essentially acentric *in vivo*, and very little interaction with MTs was observed using the *in vitro* assay of Sorger et al. (Ng and Carbon, 1987; Hegemann et al., 1988; Sorger et al., 1994). It is possible that mutant CDEIII maintains reduced CBF3 binding capability which is observed *in vitro*, while drastically affecting chromosome segregation *in vivo*. Complete elimination of CDEIII using a CDEI,II-plasmid construct should establish the specificity of the binding seen with Dynabead B unambiguously. Regardless, the difference in binding observed with Dynabead A (WT *CEN*) relative to Dynabead C (plasmid DNA) demonstrates that rCBF3 specifically binds WT *CEN* bound to Dynabeads.

The binding of CBF3 proteins to Dynabead B makes it difficult to know whether Ndc10p is also bound at CDEII, as would be predicted by the results presented in Chapter 2. The addition of Ndc10p alone to CDEI,II-plasmid DNA will be pursued in the future to evaluate this possibility, as such an interaction would provide a reagent for identifying kinetochore proteins that rely on Ndc10p at CDEII for kinetochore assembly. Previous data demonstrating that CBF3 is required for the association of all other kinetochore proteins with the centromere, coupled with the fact that it is able to bind DNA on its own, makes it reasonable to presume that CBF3 arrives

at the *CEN* before all other proteins. Therefore, it is hoped that rCBF3 bound to *CEN* DNA is favorable for the subsequent binding of additional kinetochore proteins.

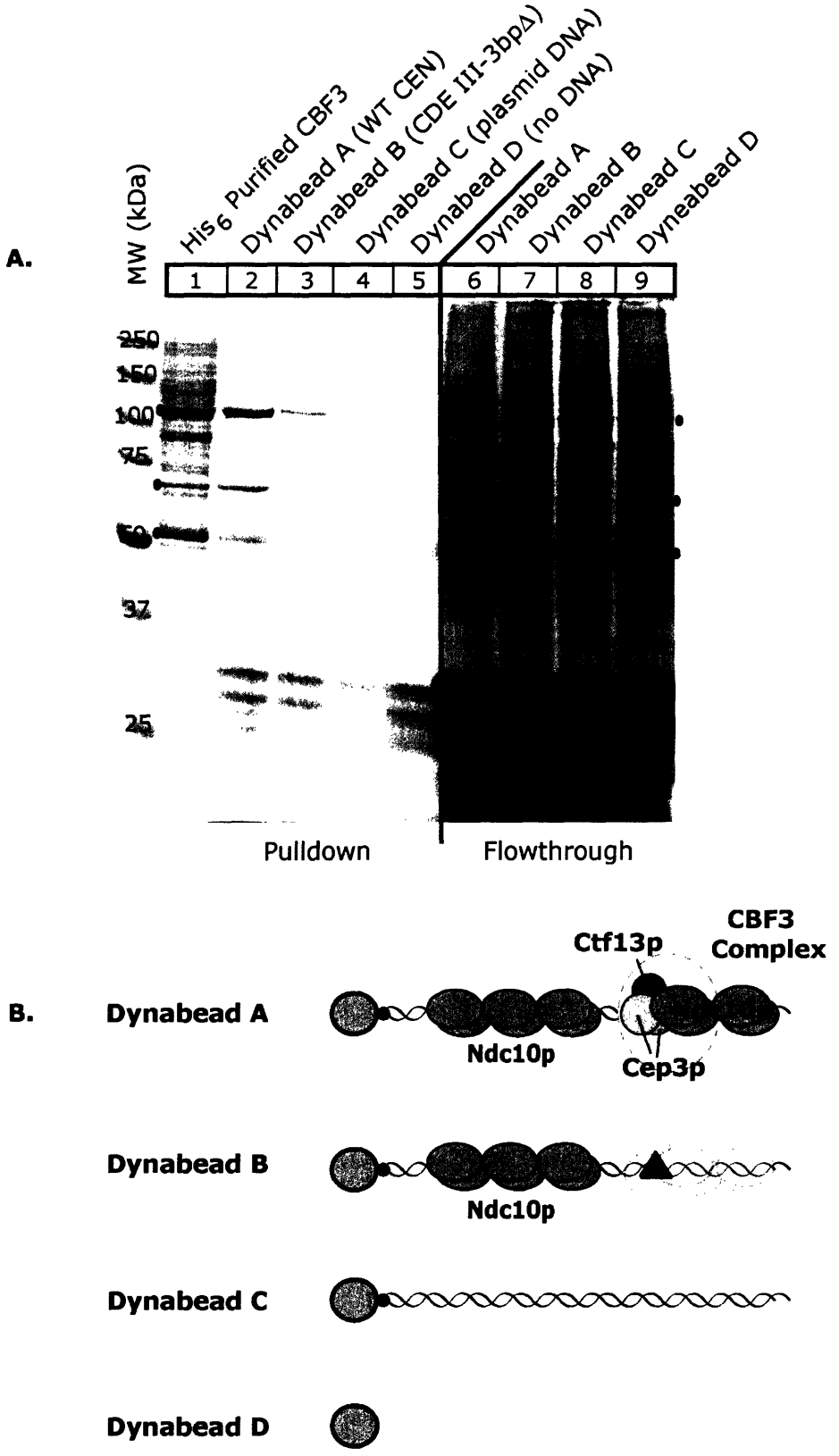


Figure 5-2 Binding of recombinant CBF3 (rCBF3) to Dynabead-DNA constructs. rCBF3 Complex was purified using 6xHis-tagged Cep3p and metal-chelate chromatography (lane 1; see Materials and Methods). Red dots indicate the positions of the CBF3 components Ndc10p (110kD), Cep3p (64kD) and Ctf13p (58kD). Dynabeads coupled to WT *CEN* DNA (lanes 2, 6), mutant CDEIII (lanes 3,7), random plasmid DNA (lanes 4,8) or no DNA (lanes 5,9) were incubated with rCBF3, beads isolated (Pulldown; lanes 2-5) and supernatant (Flowthrough; lanes 6-9) recovered. Pulldown and Flowthrough fractions were separated on a 10% discontinuous SDS-PAGE and observed by Coomassie staining. (B) Schematic of proposed DNA-protein complexes in lanes 2-5 of (A). Dynabead constructs are as in Figure 5-1. The core CBF3 Complex is circled.

Cse4p, Mif2p, Ndc80p and Ame1p associate with CBF3 bound to CEN DNA

rCBF3 was bound to Dynabead A as described above and then independently mixed with crude *S. cerevisiae* extracts containing TAP-tagged kinetochore proteins (TAP: Tandem Affinity Purification; C-term tag at endogenous loci; described in Chapter 1). As negative controls, Dynabead A without rCBF3 and Dynabead C alone were independently mixed with the same yeast extracts (Dynabeads B and D were omitted from these experiments, but will be included in the future). Eight extracts containing (separately) the following tagged proteins: Ame1p-TAP, Cse4p-TAP, Ctf19p-TAP, Mif2p-TAP, Mtw1p-TAP, Ndc80p-TAP, Cin1p-TAP (negative control) and untagged extract (negative control), were separately incubated with each of the three Dynabead preparations. Following incubation of the Dynabeads with the individual yeast extracts, the beads were washed with binding buffer and evaluated by Western blot for the association of TAP-tagged proteins using α-PAP antibodies (Sigma, St. Louis MO) which recognize the Protein A component of the TAP tag.

TAP-tagged Cse4p, Mif2p, Ndc80p and Ame1p associated with Dynabead A plus rCBF3 but not with Dynabead C (Fig. 5-3A, lanes 2 and 4; Fig. 5-3B, lanes 6 and 8; and data not shown). Reduced binding of each of these proteins to Dynabead A without added rCBF3 was also observed, consistent with the presence of endogenous CBF3 in the extracts (Fig. 5-3A, lane 3; Fig. 5-3B, lane 7). Future experiments will include the use of *ndc10-1* mutants to eliminate endogenous CBF3 activity, as well as addition of rCBF3 to Dynabead C, in order to further verify the CBF3-dependence of kinetochore proteins for their *CEN* association. Binding of Ctf19p-TAP, Mtw1p-TAP and Cin1p-TAP to Dynabead A (with or without rCBF3) and Dynabead C was at a low and equal level, implying that interactions between these proteins and centromeric DNA were not specific (Figure 5-3C, compare lanes 12, 15 and 17; Figure 5-3D,

compare lanes 21, 24 and 27; data not shown). Quantitation of Cse4p and Mif2p indicate approximately 15-20% of the TAP-tagged protein present in the added yeast extracts is being bound by Dynabead A + rCBF3, although these values have caveats which are addressed in the Discussion.

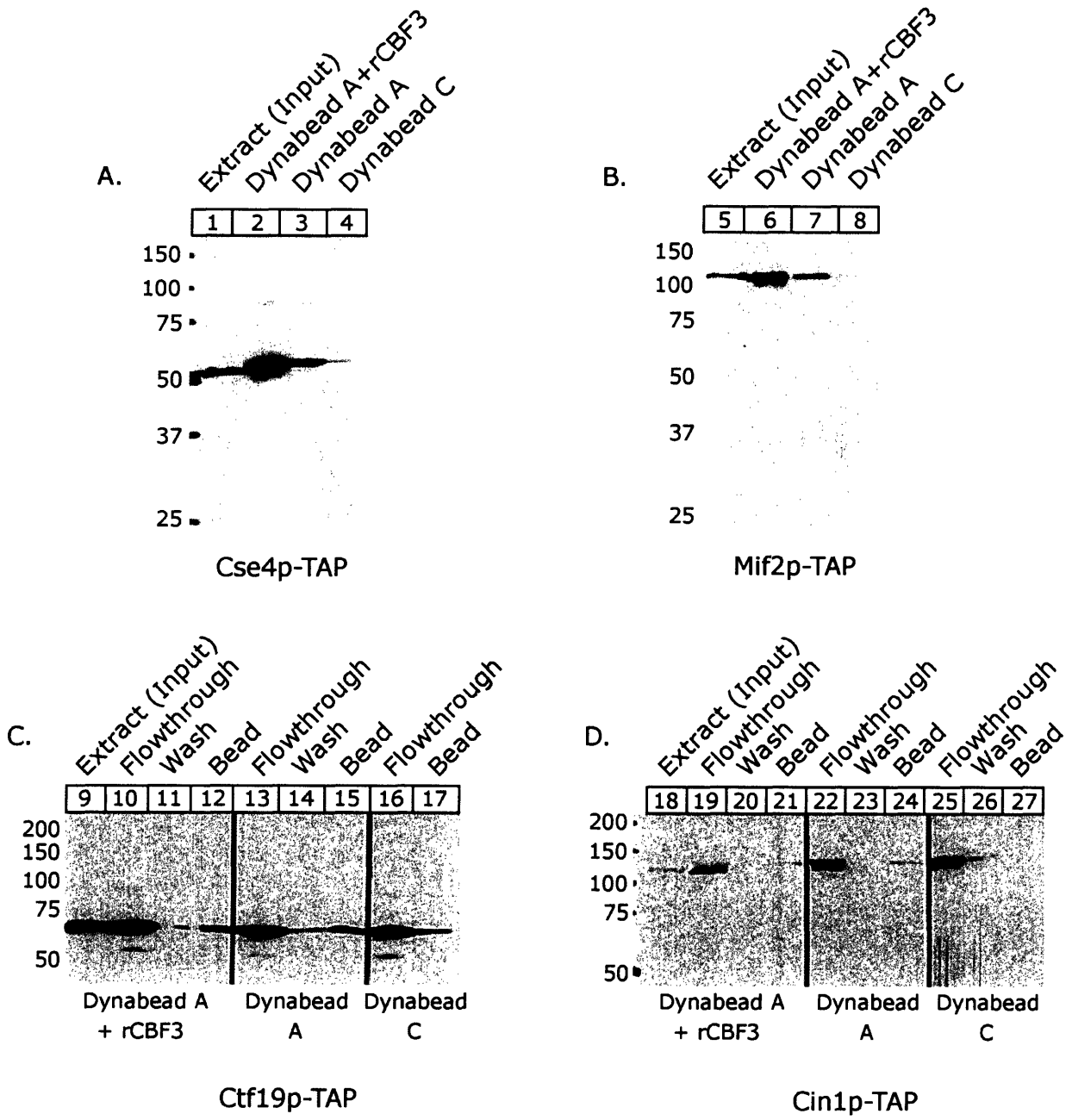


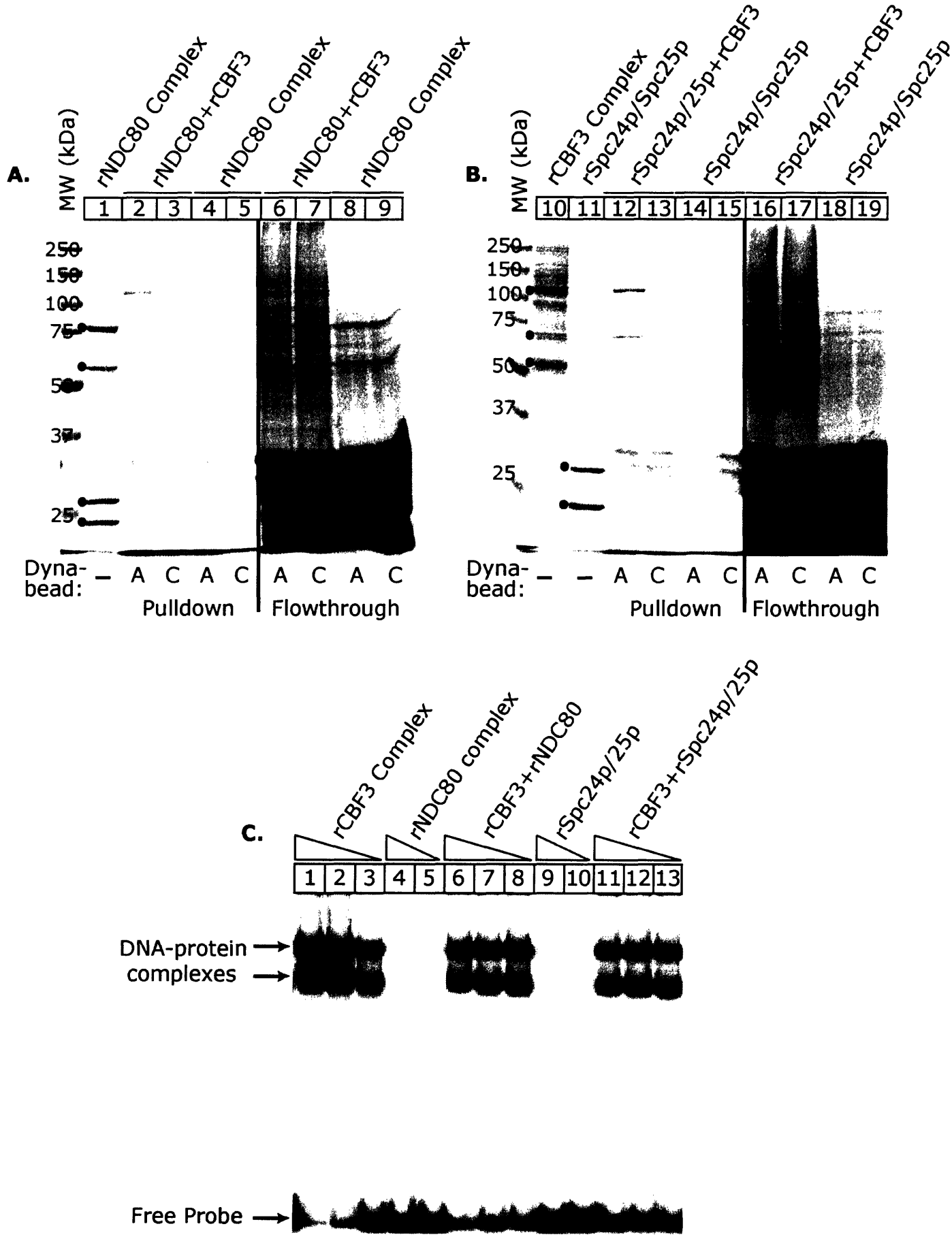
Figure 5-3 Pulldown of TAP-tagged proteins from *S. cerevisiae* extracts using Dynabead-DNA probes. Dynabead A (+/- rCBF3) and Dynabead C were individually mixed with *S. cerevisiae* extracts containing Cse4p-TAP (A) or Mif2p-TAP (B). Following incubation, beads were isolated, supernatant (Flowthrough) was recovered, beads were washed with binding buffer (Wash) and then evaluated for the presence of associated TAP-tagged proteins by separation on a 10% discontinuous SDS-PAGE gel followed by Western blot analysis using α-PAP antibody. Only the beads are shown in (A) and (B). Ctf19p-TAP (C) or Cin1p-TAP (D) yeast extracts were incubated with the indicated Dynabeads, and treated as in A and B. Beads, Flowthrough and Wash are shown in (C) and (D).

rNdc80 Complex does not associate with rCBF3 bound to CEN DNA

The use of crude yeast extracts allows for the possibility that intermediate proteins are responsible for the interaction between CBF3 bound to *CEN* and the TAP-tagged kinetochore proteins. To examine this possibility, I expressed the NDC80 Complex as a whole (Ndc80p, Nuf2p, Spc25p and Spc24p; rNDC80) and Spc24p/25p alone (rSpc24p/25p) in insect cells and purified them using metal-chelate chromatography (Fig. 5-4A, lane 1 and Fig. 5-4B, lane 11, respectively; see Materials and Methods). I then individually mixed Dynabeads A and C with rCBF3, along with purified rNDC80 Complex or rSpc24p/25p, and assayed bead binding by SDS-PAGE and Coomassie staining. When the NDC80 Complex was mixed with Dynabead A + rCBF3, binding of the CBF3 Complex to beads was observed, but association of the NDC80 Complex with beads was not detected (Fig. 5-4A, lane 2). Neither rCBF3 nor rNDC80 Complex bound Dynabead C, as expected (Fig. 5-4A, lane 3). Similarly, when rSpc24p/25p was mixed with Dynabead A or C + rCBF3, no interaction of Spc24p/25p with the Dynabeads was observed (Fig. 5-4B, lanes 12 and 13). Western blot analysis would likely be more sensitive for detecting interactions than Coomassie staining, but additional results from bandshift gels give no indication that such an association between the two complexes exists under current experimental conditions. Specifically, nondenaturing gel analysis (bandshift) does not detect the presence of supershifted bands in the lanes where rNDC80 or rSpc24p/25p are simultaneously present with the CBF3 complex, as would be expected for the binding of additional proteins to *CEN*-CBF3 (Fig. 5-4C, lanes 6-8, 11-13). Furthermore, neither the NDC80 Complex nor Spc24p/25p specifically bound Dynabead A on their own (Fig. 5-4A, lanes 4 and 5; Fig. 5-4B, lanes 14 and 15), consistent with bandshift experiments, indicating that these proteins do not bind directly to *CEN* DNA (Fig. 5-4C, lanes 4, 5 and 9, 10). Given the data in Figure 5-3 that Ndc80p in yeast

extracts associates with Dynabead A plus CBF3, the implication is either that additional protein(s) present in yeast extract are necessary for the association between the NDC80 and CBF3 Complexes, or the NDC80 Complex must need to be activated in some manner. The next step in evaluating these possibilities will be to add yeast extracts to rNDC80 + rCBF3 to determine if the recombinant Ndc80p is then capable of binding to beads. Thus, the combination of observations using yeast extracts and recombinant proteins may lead to the discovery of novel factors, or determination of a role for known kinetochore proteins, which is required to allow these complexes to interact.

Figure 5-4 (next page) Interaction between the NDC80 and CBF3 Complexes. (A) Recombinant NDC80 Complex (rNDC80) was co-expressed in insect cells and purified using 6xHis-Nuf2p and metal-chelate chromatography (lane 1; red dots indicate Ndc80p (80kD), Nuf2p (53kD), Spc25p (25kD) and Spc24p (24.5kD); see Materials and Methods for details). rNDC80 Complex was mixed with Dynabead A +/- rCBF3 (lanes 2, 4) or Dynabead C +/- rCBF3 (lanes 3, 5). Dynabeads were isolated by magnet, Flowthrough recovered (lanes 6-9), beads washed and associated proteins (Pulldown; lanes 2-5) separated by SDS-PAGE and visualized by Coomassie staining. (B) Co-expressed recombinant Spc24p/Spc25p (lane 11; purified using 6xHis-Spc24p and metal-chelate chromatography; marked with red dots) was mixed with Dynabead A +/- rCBF3 (lanes 12 and 14) or Dynabead C +/- rCBF3 (lanes 13 and 15). Associated proteins identified as in (A). (C) Nuclear extracts from insect cells expressing the indicated recombinant proteins were mixed with radiolabeled 88bp CDEIII DNA and resolved on nondenaturing bandshift gels. Lanes 1-3, 6-8, and 11-13 each recieved 1μL, 0.5μL, and 0.25μL of rCBF3 nuclear extract, respectively. rNDC80 Complex was added to lanes 4 and 6 (1μL), 5 and 7 (0.5μL) and lane 8 (0.25μL). rSpc24p/25p was added to lanes 9 and 11 (1μL), 10 and 12 (0.5μL) and lane 13 (0.25μL). Triangles depict the relative amount of protein added to binding reactions; for lanes 6-8 and 11-13, the triangle applies to both complexes. The observed DNA-protein complexes represent the CBF3 core and extended complexes as previously described (Espelin et al., 1997).


A.
MW (kDa)
rNDC80 Complex
rNDC80+rCBF3
rNDC80 Complex
rNDC80+rCBF3
rNDC80 Complex
1 2 3 4 5 6 7 8 9
250
150
100
75
50
37
25
Dyna-bead: – A C A C A C A C
Pulldown
Flowthrough
B.
MW (kDa)
rCBF3 Complex
rSpc24p/Spc25p
rSpc24p/25p+rCBF3
rSpc24p/Spc25p
rSpc24p/25p+rCBF3
rSpc24p/Spc25p
10 11 12 13 14 15 16 17 18 19
250
150
100
75
50
37
25
Dyna-bead: – – A C A C A C A C
Pulldown
Flowthrough
C.
rCBF3 Complex
rNDC80 complex
rCBF3+rNDC80
rSpc24p/25p
rCBF3+rSpc24p/25p
1 2 3 4 5 6 7 8 9 10 11 12 13
DNA-protein complexes
Free Probe

DISCUSSION

We have known about the kinetochore foundation for a number of years but the rest of structure has become an ever-increasing pile of materials with no blueprint to let us know how it is assembled. The CBF3 Complex and Ndc10p bind directly to *CEN* DNA, and this interaction is essential for the subsequent addition of the rest of the kinetochore. However, we have thus far been unable to determine which proteins interact with CBF3 and Ndc10p to form the next layer of the kinetochore. Results using the Dynabead-Kinetochore Reconstitution (DKR) Assay described in this chapter suggest that Cse4p, Mif2p, Ndc80p and Ame1p may interact specifically with *CEN* DNA in a CBF3-dependent manner. Further experimentation will be required to determine whether these interactions are direct and to rigorously demonstrate specificity. However, the DKR Assay represents the development of a tool which combines our knowledge of the DNA-Binding layer of the kinetochore using recombinant proteins and *CEN* DNA, with the ability to probe yeast extracts for candidate interacting proteins. Once potential proteins have been identified, we will attempt to "re-incorporate" these proteins as purified, recombinant reagents into the assay. As was mentioned at the start of this chapter, these results are preliminary and much of the following Discussion will focus on controls and future experiments.

Potential CBF3-binding Proteins

Of the more than sixty identified *S. cerevisiae* kinetochore proteins, I have evaluated six for their ability to associate with CBF3 bound to *CEN* DNA. It is reassuring that Cse4p and Mif2p, two proteins proposed to be in close proximity to *CEN* DNA, are able to specifically interact with Dynabeads containing wildtype *CEN* and rCBF3. The binding of Cse4p is

particularly intriguing given that it is proposed to be part of a specialized nucleosome and is dependent on CBF3 for its localization to the *S. cerevisiae* centromere *in vivo* (Tanaka et al., 1999). Specialized nucleosomes are found at centromeres in nearly every organism, but the mechanism by which they are localized remains unknown and Cse4p-nucleosomes have not been shown to possess sequence-specific DNA binding capabilities. It remains to be determined whether other nucleosome components (H2A, H2B, H4) are also present in the Cse4p pulldown, or if they are assembled subsequent to Cse4p localization to the kinetochore. Determining how this localization occurs in *S. cerevisiae* would be a major advance in understanding how centromeres are recognized by specialized nucleosomes, and may help elucidate the means by which these important chromatin factors are recruited to centromeres in other organisms. The N-terminal tail domain of Cse4p is distinct from that of H3 and it is postulated that this domain is responsible for its interactions with other kinetochore proteins. To further characterize the association between CBF3 and Cse4p, future experiments will include altering of the Cse4p N-term tail (the region of the protein which is particular to the H3 variant proteins) and competition with a Cse4p N-term peptide, to assess whether the interaction is disrupted. A number of kinetochore proteins rely on Cse4p for their localization to the *CEN*, and disrupting the ability of Cse4p to interact with CBF3 using selective mutants should provide further insight into kinetochore assembly.

The presence of Ndc80p is also notable as only members of the NDC80 and CBF3 Complexes have been shown to exhibit a chromosome detachment phenotype when mutated or eliminated *in vivo*. It should be noted however that the observed phenotypes of many mutant kinetochore proteins in *S. cerevisiae* are based on temperature-sensitive alleles, which in some cases may represent hypomorphic alleles. The chromosome dynamics of *CSE4* and *MIF2*

mutants have been evaluated using temperature-sensitive alleles, but not null mutants, and in the complete absence of functional protein, detachment may be observed as well. Thus, the detachment phenotype may not be exclusive to mutants of the NDC80 and CBF3 Complexes, but this does not diminish the fact that the NDC80 Complex is essential for the connection between *CEN* and MT. A simple explanation for the different results obtained for association of Ndc80p with CBF3 + *CEN* using yeast extracts and recombinant proteins, is the presence of a protein(s) in crude yeast extracts which is required to directly mediate their interaction. Another possibility is that the NDC80 Complex must be activated in some way. It is possible that Mif2p and/or Cse4p are required for interactions between CBF3 and the NDC80 Complex and we will evaluate this possibility using recombinant proteins. If the addition of yeast extract to recombinant NDC80 plus CBF3 rescues the ability of the recombinant complexes to interact with each other, it will serve to verify the presence of a factor(s) in extracts which mediates the interaction. In this way, the use of extracts and recombinant proteins can be used in a complimentary fashion to advance our understanding of the relationships among the kinetochore proteins.

Caveats and Concerns

A number of issues, both technical and biological, need to be addressed before conclusions can be drawn from the results presented in this chapter. Foremost is the preparation of the Dynabeads and binding of rCBF3 to them. I prepared the beads based on careful quantitation of the biotinylated DNA and the manufacturer's suggestion regarding the capacity of the streptavidin beads for biotinylated DNA. This involved a crude titration of DNA to bead, but a more thorough analysis will be required to establish a complete binding curve and further

consideration will need to be given to the desired DNA:bead ratio. Nevertheless, all beads were prepared identically and the amount of bound DNA was equivalent in each case (wildtype *CEN*, mutant *CEN*, plasmid). Future experiments may also include the incorporation of other variant centromeres, such as plasmid-CDEIII or CDEI,II-plasmid as well as longer or shorter *CEN* sequences, to further refine our results.

The amount of rCB3 bound to the beads will require a proper titration of rCBF3 versus Dynabead-wildtype *CEN,* and is also dependant on the amount of DNA on the beads. At the moment, I do not know what percentage of the DNA molecules on the beads capable of binding CBF3, are actually bound, as it is difficult to quantitate the protein bands present in the flowthrough (FT) stained by Coomassie (Fig. 5-2, lanes 6-9). Western blot analysis could be employed to better evaluate the binding efficiency of rCBF3 to *CEN*. Qualitatively, there is however an abundance of TAP-tagged protein (Cse4p-TAP, Mif2p-TAP, etc.) in the flowthrough following incubation with Dynabead-*CEN*-rCBF3 (data not shown), indicating that there is likely an excess of TAP-tagged protein relative to available *CEN*-bound CBF3 molecules. Although we don't want to induce nonspecific binding, we do want to ensure that maximum specific binding between CBF3 and any interacting proteins is possible. Thus, the 15-20% recovery of TAP-tagged protein from yeast extracts is encouraging, but may actually be an underestimate of the binding capacity of rCBF3-*CEN* for these proteins.

The initial plan for this assay was to incorporate a combination of recombinant proteins (CBF3, NDC80 Complex, etc.) and TAP-purified proteins from yeast extracts to determine which proteins would interact with CBF3 bound to *CEN* DNA, and to then determine the interdependencies amongst these proteins. In theory, this approach could lead to *in vitro* reconstitution of the connection between centromeric DNA and MT using well characterized

protein components. The TAP purification method uses a "Protein A-TEV protease site-CBP" moiety which allows for endogenous expression of the protein to be purified, followed by two rounds of purification under gentle conditions. The advantage of this scheme is that proteins are able to retain their native protein-protein interactions and possibly their functions, a likely requirement for kinetochore reconstitution. The TAP method has previously been used for purification of proteins on a large scale (upwards of 10L), such that visualization of the final purified proteins was possible by Silver staining and in some cases even by Coomassie (Rigaut et al., 1999; Puig et al., 2001; Cheeseman et al., 2002; De Wulf et al., 2003; Westermann et al., 2003). I have been able to routinely purify kinetochore proteins from less than 500 mL volumes using the Protein A tag and detected by Western Blot using α-PAP antibodies. However, despite repeated attempts using a variety of approaches, I am unable to detect the CBP epitope before or after TEV cleavage (which removes the Protein A epitope). This has made incorporation of these purified proteins into the DKR Assay following TEV cleavage futile, as they cannot be followed by Western Blot analysis, and other options will eventually need to be sought. Reversing the Protein A-IgG interaction would require harsh conditions (low pH, urea, etc.) which are likely to alter protein structure and function, making them undesirable for reconstitution assays. One unappealing option is purification of the proteins on a scale at which they can be incorporated into the DKR Assay and observed by Silver or Coomassie staining. Probably the best option is to generate yeast strains with a modified TAP tag that is more amenable to Western blotting, such as incorporating a myc or HA epitope into the TAP construct. These options will need to be weighed, but will definitely follow an initial survey of the TAP-tagged strains already available.

The question of specificity must also be considered to determine whether CBF3 bound to *CEN* DNA has pulled out a large kinetochore "mass", consisting of many kinetochore proteins stuck together. It is formally possible that CBF3 bound to DNA on one Dynabead is able to interact with proteins bound to DNA on another Dynabead. This is a particular concern in the case of Cse4p which could be wrapped around naked DNA on one Dynabead, and then interact with rCBF3 bound to a second Dynabead. Preliminary results showing that Mtw1p and Ctf19p do not bind beads suggest that intact kinetochores are not being pulled out in my assay, but additional characterization will be required to address these concerns.

Future Directions

Immediate experiments will involve the verification of results showing that Cse4p, Mif2p, Ndc80p and Ame1p bind *CEN*-rCBF3 and resolution of the technical issues mentioned above. Additional experiments such as a time course of binding to Dynabead-*CEN*-rCBF3, may help define the temporal requirements and interdependencies required for Cse4p, Mif2p, etc. to bind to the kinetochore. Drawing on the results obtained using yeast extracts, incorporation of recombinant Cse4p-nucleosomes and Mif2p (courtesy of K. Simons-Harrison Lab) along with the recombinant NDC80 and CBF3 Complexes already produced, will help us pursue potential interactions using recombinant reagents. Firmly establishing the interaction between these core proteins and *CEN* DNA will represent a major step forward in our understanding of the heart of the kinetochore.

Utilizing the large bank of yeast strains containing TAP-tagged versions of most known kinetochore proteins already available in our lab (generated by P. De Wulf), I will continue to evaluate the ability of individual kinetochore proteins to interact with CBF3 bound to *CEN*

DNA. This should produce a subset of kinetochore proteins which are capable of interacting with *CEN*-CBF3 and produce attractive prospects to pursue further. It will also be possible to evaluate whether the proteins bound from yeast extracts to Dynabead-*CEN*-rCBF3 are capable of interacting with MTs *in vitro*. Exogenous, stabilized MTs will be added to the assay and evaluated for the ability to associate with Dynabead-*CEN*-rCBF3 + yeast extract. The exogenous MTs will be labeled (rhodamine) allowing them to be evaluated for interaction with the Dynabead by Western blot analysis, as well as discern them from endogenous tubulin. The incorporation of Dynabeads into a MT-binding assay will allow for stringent washing conditions, and in combination with multiple DNA moieties and the use of recombinant CBF3 should serve as a rigorous assay for MT-binding. Previous MT-binding assays (Chapter 1) have set a precedent for this type of experiment, and it is hoped that the combination of recombinant and purified proteins identified using the Dynabeads will provide an advance in identifying the components involved in the *CEN*-MT connection.

Another powerful technique that may be combined with the DKR Assay is mass spectrometry. The improved ability of mass spectrometry to detect minute amounts of protein should make it possible to identify proteins which are bound to the various Dynabead constructs. Experiments can be scaled up to provide enough material for identification by Silver or Coomassie staining and subsequent mass spectrometry analysis, thus allowing for comparison between proteins bound to wildtype *CEN* DNA and plasmid DNA. At a minimum, mass spectrometry should be capable of identifying proteins which are bound to wildtype *CEN* + rCBF3, relative to *CEN* alone, thus identifying kinetochore proteins that specifically interact with CBF3. On a more ambitious level, if a connection can be made between *CEN* DNA and a MT

using the DKR Assay, mass spectrometry may be able to identify the proteins which are responsible for making this interaction possible.

SUMMARY

In summary, I have presented an assay which is capable of detecting proteins that specifically interact with CBF3 and/or Ndc10p bound to *CEN* DNA. Cse4p, Mif2p, Ndc80p and Ame1p are proposed to interact with *CEN* in a CBF3-dependent manner, although the exact biochemical links remain to be determined. This assay represents a means to utilize our knowledge about the DNA-Binding layer of the kinetochore to identify the proteins which make up the next layer of this multiprotein complex. Interactions which are identified will subsequently be verified using recombinant or purified proteins in an attempt to fully know the identity of all the components involved in the assay, which will hopefully represent the connection between *CEN* DNA and MT.

MATERIALS AND METHODS

Dynabead-DNA Preparation

Wildtype *CEN3* sequence is derived from pRN505, *CEN3* CDEIII-3bpΔ from pSF137 (conserved CCG of CDEIII deleted) and nonspecific DNA from pUC19 (Ng and Carbon, 1987; Sorger et al., 1994). An 184 bp fragment of wildtype *CEN3* was amplified by PCR using a biotinylated "left-hand" primer (biotin-GTACAAATAAGTCACATGATGATATTTG) that anneals 10 bp to the left of CDEI and a "right-hand" primer (CCACCAGTAAACGTTTCATATATCC) that anneals 68 bp to the right of the central CCG of CDEIII. The same primers were used to generate the *CEN3* CDEIII-3bpΔ with pSF137,

resulting in an 181 bp fragment. PCR using a biotinylated primer corresponding to pUC19 sequence 1-20 (biotin-TCGCGCGTTTCGGTGATGAC) and a second primer corresponding to the sequence 162-184 (TGGTGCACTCTCAGTACAATCTG) generated an 184 bp fragment of random DNA. For each DNA fragment, 60 independent PCR reactions were pooled, gel purified and quantitated.

Purified DNA was linked to M-280 Streptavidin Dynabeads (uniform, superparamagnetic, polystyrene beads with streptavidin covalently attached to the bead surface; DYNAL, Oslo, Norway). The beads were prepared as follows. An aliquot of beads from the stock vial was isolated in a magnetic rack; supernatant was aspirated and the beads washed 3 times in 1X B&W Buffer (5mM Tris-HCl $_{pH\ 7.5}$, 0.5mM EDTA, 1M NaCl). The washed beads were resuspended as a 50% slurry in 1X B&W Buffer and incubated with the purified DNA for 30 minutes at RT on a Nutator. Bead-DNA complexes were isolated on a magnetic rack and the supernatant was aspirated, removing unbound DNA. The beads were then washed 3 x 5 minutes with 1X B&W Buffer and resuspended as 50% slurry with TE Buffer.

Protein Preparation

Recombinant CBF3 Complex, NDC80 Complex and Spc24p/25p were obtained from High Five insect cells (GIBCO BRL, Gaithersburg, MD). Cells were independently co-infected to express the four components of the CBF3 Complex, the four components of the NDC80 Complex or *SPC24* and *SPC25* together. The construction of baculovirus vectors and protein recovery from insect cells is described elsewhere (GIBCO BRL; Bac-to-Bac Baculovirus Expression Systems Manual). Each infection contained one His-tagged component (6xHis-Cep3p, 6xHis-Nuf2p or 6xHis-Spc24p) which was used to purify the complexes using metal-chelate chromatography.

Insect cell nuclear lysates in binding buffer (10mM $HEPES_{pH8.0}$, 50mM β-glycerophosphate, 500mM KCl, 5mM $MgCl_2$, 10% glycerol, 10μg/ml leupeptin, pepstatin and chymotrypsin, 1mM PMSF; 10mM imidazole) were incubated in batch with Ni-NTA resin (Qiagen) for 45 minutes while rocking on a Nutator at 4°C. Protein-bound resin was washed twice with 1X binding buffer and eluted in 1X binding buffer containing 150mM imidazole.

For whole-cell yeast extracts, cultures were grown to 2-5 $x10^7$ cells/ml, washed by pelleting in breakage buffer (100mM bis-tris-Propane, 50mM β-glycerophosphate, 5mM EDTA, 5mM EGTA, 5mM NaF, 150mM KCl and 10% glycerol), resuspended in a minimal amount of breakage buffer with protease inhibitors (1mM PMSF, 10μg/ml each of leupeptin, pepstatin and chymotrypsin), frozen in liquid nitrogen and ground in a chilled mortar and pestle (see Sorger et al., 1995 for further details). Cell debris was removed by centrifugation at 15,000g for 15 minutes, supernatant was recovered, and protein concentration determined by BioRad Assay.

Binding Assays

Purified recombinant proteins were mixed with Dynabead-DNA constructs in 1X Bandshift Binding Buffer (10mM $HEPES_{pH8.0}$, 6mM $MgCl_2$, 10% glycerol, and adjusted to 150mM KCl final) containing 0.3μg/ml casein and .05 μg/ml sheared salmon sperm DNA for 45 minutes, rocking at RT. Yeast extracts or recombinant proteins were then added to the Dynabead-rCBF3 mixture, Binding Buffer was adjusted to 1X and incubated for 2 hours at RT while rocking on a Nutator. Dynabeads were isolated using a magnet, supernatant (FT) was recovered and beads were gently washed with 1X Bandshift Binding Buffer + 150mM KCl. Beads were again isolated using a magnet, the supernatant (Wash) recovered and beads were resuspended in 1X SDS-PAGE Buffer with 5% β-mercaptoethanol and boiled for 5 minutes. To evaluate direct

binding of recombinant proteins to Dynabead-DNA, the addition of yeast extracts or additional recombinant proteins and ensuing 2 hour incubation was eliminated from the procedure.

All fractions were evaluated by electrophoresis on 10% discontinuous SDS-PAGE gels. Recombinant proteins were observed by Coomassie staining. Identification of TAP-tagged proteins from yeast extracts was performed by Western blot analysis using α-PAP antibody (P1291; Sigma, St. Louis, MO). Bandshift assays were performed as previously described (Sorger et al., 1995; Espelin et al., 1997)

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CHAPTER 6

Conclusion

The kinetochore is a multiprotein complex that is essential for mediating the attachment of chromosomes to MTs, a necessary prerequisite for proper chromosome segregation. When I began the work presented in this thesis (1998), there were only 7 bona fide *S. cerevisiae* kinetochore proteins: Cbf1p, the four proteins of the CBF3 Complex (Ndc10p, Cep3p, Ctf13p, and Skp1p), Cse4p and Mif2p. It had been determined that these proteins alone were not sufficient to mediate the *CEN*-MT attachment, at least *in vitro*, and I naively thought there might be one or two missing proteins which would complete the connection once they were discovered (Sorger et al., 1994). The kinetochore field has changed dramatically since that time and in excess of sixty kinetochore proteins are now known. It is apparent that these proteins are involved in a wide variety of functions including structure, tension sensing and force generation, and signaling to the spindle checkpoint. This increased complexity has created a great deal of excitement among researchers and has provided many opportunities for study of the kinetochore in years to come. It is now obvious that a lot more than a single protein was missing back in 1998.

This thesis presents data which advances our understanding of the molecular organization of the *S. cerevisiae* kinetochore. Using bandshift assays combined with *CEN* mutation analysis and chromosome loss assays, I have shown that Ndc10p binds to the CDEII region of the centromere. This interaction, along with the CBF3 complex bound at CDEIII, is believed to form the foundation that recruits all other kinetochore proteins, including those which bind directly to MTs. Chromatin Immunoprecipitation and microscopy also suggest Ndc10p may bind structures other than kinetochores, such as chromosome arms, although a biological role for this binding has not yet been determined. Following the identification of eleven additional kinetochore subunits by microscopy and ChIP, the interdependencies required for their ability to

associate with *CEN* DNA was evaluated. Combining this interdependency data with microscopic analysis of kinetochore mutants, we have begun to develop an elementary picture of how kinetochore proteins are positioned with regard to each other and the centromeric DNA. This information is important not only for our basic understanding of kinetochore organization, but also for making predictions about the effects of one kinetochore protein on another and for understanding kinetochore mutants at a mechanistic level.

The kinetochore is essential for the bipolar attachment of sister chromatids to the mitotic spindle and it is the function of the spindle checkpoint, a set of proteins which are highly conserved from yeast to humans, to monitor whether this connection has been properly established. Spindle checkpoint proteins have previously been localized to the kinetochore in a number of organisms including *S. pombe*, *X. laevis*, *D. melanogaster* and humans, and the work presented in this thesis demonstrates that the same is true in *S. cerevisiae* (Chen et al., 1996; Li and Benezra, 1996; He et al., 1997; Basu et al., 1998; Chan et al., 1998; Chen et al., 1998; He et al., 1998; Taylor et al., 1998; Basu et al., 1999; Chan et al., 1999; Jin et al., 1999; Gillett et al., 2004). Bub1p and Bub3p are found at the *S. cerevisiae* kinetochore as part of the normal progression of the cell cycle, while Mad1p and Mad2p are localized to the kinetochore only in response to errors in the *CEN*-kinetochore-MT attachment. Analysis of kinetochore mutants which exhibit specific spindle defects provides a means for evaluating how the spindle checkpoint responds to kinetochore damage. Our data supports attachment status as the primary kinetochore signal recognized by the spindle checkpoint, a point of debate for many years. Lastly, preliminary data from the ongoing Dynabead-Kinetochore Reconstitution (DKR) Assay indicate that Cse4p, Mif2p, Ndc80p and Ame1p specifically interact with CBF3 bound to *CEN*

DNA. This represents a first step in understanding how the next layer of the kinetochore builds upon the platform established by the binding of CBF3 and Ndc10p directly to *CEN* DNA.

Ndc10p Binds CDEII

The demonstration that Ndc10p binds CDEII of the *S. cerevisiae* centromere represents the first evidence of direct binding between a protein and this DNA element. Binding is dispersed across approximately 48 bp of CDEII with a particular stretch of bases (49-60) being more important than others. The lack of a single CDEII mutation capable of completely eliminating Ndc10p binding is consistent with multiple contacts being made by Ndc10p along the length of CDEII. Size exclusion chromatography and glycerol gradients demonstrate that Ndc10p exists as a homodimer in solution, and although the presence of multiple complexes on bandshift gels is consistent with the existence of multiple Ndc10p homodimers, we do not yet know the exact stoichiometry or orientation of these homodimers on *CEN* DNA. Analysis by others has shown that large deletions, insertions or mutations of the CDEII region can have a deleterious effect on chromosome segregation, but the exact nature of these changes with regard to protein binding have never been investigated (Gaudet and Fitzgerald-Hayes, 1987). These analyses involved the use of relatively large fragments of DNA inserted or removed using naturally occurring restriction sites and would not have been precise enough to detect the specific CDEII bases that we have determined to be important for Ndc10p binding. We have shown that within a cell, mutation of the CDEII nucleotides found to be important for Ndc10p binding *in vitro* cause chromosome loss equivalent to that seen by total replacement of CDEII with random DNA. This indicates that the bases which we have identified as being important for *in vitro* binding of Ndc10p are also important *in vivo*, presumably for the same reason.

Interaction between CDEII and CDEIII

The binding of Ndc10p to CDEII appears to be different from binding of Ndc10p to CDEIII *in vitro*, in that Ndc10p is able to bind CDEII on its own whereas binding to CDEIII requires the presence of the other members of the CBF3 complex. Despite the different modes of DNA binding, proteins bound at CDEII and CDEIII likely share interactions in order to establish a functional kinetochore. Supporting this notion, inverting CDEIII relative to CDEI,II renders the centromere nonfunctional *in vivo*, evidence that the relationship between the proteins at CDEII and CDEIII is important (Murphy et al., 1991). Although the correlation between binding of Ndc10p to CDEII *in vitro* and the deleterious consequences on chromosome segregation of altering these same bases *in vivo* is highly suggestive of a similar role, it is short of conclusive. We therefore sought to obtain direct *in vivo* evidence of Ndc10p-CDEII binding using ChIP. Unfortunately, our attempts to discern the biological function of Ndc10p bound to CDEII and CDEIII have proven difficult for a number of technical and biological reasons. In theory, mutating CDEII while leaving CDEIII undisturbed should allow the role of CDEII-bound Ndc10p to be determined. In practice, altering CDEII engages the spindle checkpoint, which causes changes in growth rates, cell number and cell cycle status, making comparison to a control wildtype strain very difficult. The use of ChIP has been very helpful for identifying the presence or absence of kinetochore proteins at the centromere both under wildtype conditions and in the presence of kinetochore mutants. However, because of the immediate proximity of the CDEII and CDEIII elements it is extremely difficult, if not impossible, to discern between binding at these two locations using this method. It is also not immediately obvious whether ChIP would be capable of detecting a potentially subtle decrease in Ndc10p association at the centromere as a result of mutating CDEII (loss of Ndc10p at CDEII, while maintaining CBF3 at

CDEIII), especially without knowledge about the number of Ndc10p molecules present at each location. It may be possible to overcome this issue using real-time PCR and multiple experimental values to ensure statistical significance though, and may be pursued in the future in an attempt to further discern the role of Ndc10p at CDEII *in vivo*.

Our knowledge regarding the DNA-Binding layer of the kinetochore has continued to evolve over the years: Cbf1p binds CDEI, Ndc10p binds across the CDEII region and the CBF3 Complex is bound to CDEIII. Continued effort will be required to further identify exact protein-DNA contacts and methods such as UV crosslinking, footprinting, electron microscopy and ultimately X-ray crystallography should aid in this effort.

Building upon the DNA-Binding Layer

An important next step in understanding the molecular architecture of the kinetochore is identification of the proteins that are directly interacting with the DNA-binding protein Ndc10p and members of the CBF3 Complex. Although it has repeatedly been stated that all kinetochore proteins require CBF3 for their localization to the centromere, all researchers (ourselves included) have used the *ndc10-1* mutant to evaluate dependency. Considering our results showing Ndc10p forms a "platform" across a large region of the centromere, and the fact that the CDEII region is essential for centromere function, the possibility exists that some proteins may depend on Ndc10p alone for their association with the kinetochore. This distinction may be subtle because, as was mentioned before, binding of Ndc10p at CDEII is likely coupled to CBF3 binding at CDEIII *in vivo*. Nonetheless, if we wish to have a complete picture of the kinetochore, this aspect of organization should be evaluated. The use of CDEII DNA (without CDEIII) in the DKR Assay (described in Chapter 5) should allow for identification of

kinetochore proteins which depend exclusively on Ndc10p at CDEII to associate with *CEN* DNA. Another option for identifying proteins which bind Ndc10p might include the use of modified chromosomes. Other investigators have engineered chromosomes to contain secondary *CEN* sequences, which are located on the chromosome arms and silenced by a GAL promoter (conditional *CEN*), in order to identify the DNA and protein requirements for localization of kinetochore proteins and the cohesin complex (Bloom et al., 1989; Hill and Bloom, 1989; Tanaka et al., 1999). In a similar manner, evaluation of kinetochore protein localization to CDEI,II sequences on the chromosome arm may allow for the identification of proteins which require Ndc10p for their localization. It is fairly certain that additional kinetochore proteins interact with Ndc10p as it binds along CDEII, as it would seem unlikely that all subsequent kinetochore protein associations extend solely from the CBF3 Complex while the rest of the *CEN*-Ndc10p structure remains uninvolved.

Localization of a Cse4p-containing nucleosome

A major question regarding the organization of the kinetochore is the function of specialized nucleosomes, which have been identified at the centromeres of nearly all organisms (reviewed in Sullivan et al., 2001). *S. cerevisiae* Cse4p is an H3-like histone believed to be part of a specialized nucleosome found only at the centromere, and the Cse4 protein has been localized to the centromere *in vivo* using ChIP (Meluh et al., 1998). Genetic interactions between mutations in *CSE4* and CDEII further indicate a possible physical connection (Stoler et al., 1995). Based on these results along with the publicity of numerous reviews, the positioning of Cse4p at CDEII has become accepted as fact despite an absence of evidence that such a direct interaction occurs (Kitagawa and Hieter, 2001; Cheeseman et al., 2002; Cleveland et al., 2003).

There is little doubt that Cse4p is part of a nucleosome and as such, is presumably in direct contact with DNA. However, the position of this nucleosome with regards to *CEN* DNA and the other kinetochore proteins remains in question. The resolution of the ChIP published for Cse4p is on the order of 200-500 bp considering the degree of sonication of genomic DNA and the DNA primers used to evaluate localization. Based on this data alone and the relatively small size of the *S. cerevisiae* centromere (125 bp), it is difficult if not impossible to establish that Cse4p is truly bound to CDEII alone (Meluh et al., 1998). Additionally, the practice of combining mutations in *CEN* DNA and kinetochore proteins is a useful genetic technique to identify potential interactions, but it is quite a stretch to interpret these results as demonstrating a direct physical interaction between Cse4p and CDEII, as there are many potential intermediate proteins involved (Keith and Fitzgerald-Hayes, 2000).

I provide evidence in Chapter 2 of this thesis that Ndc10p directly binds the CDEII element of the *S. cerevisiae* centromere. It has previously been shown that Cse4p requires Ndc10p for its localization to the centromere *in vivo*, while CBF3 does not require Cse4p, indicating that the Ndc10p/CBF3 Complex determines Cse4p's position at the kinetochore (Tanaka et al., 1999; Measday et al., 2002). This allows for the possibility that Ndc10p binding is disrupted by a mutation in CDEII, and this in turn affects the ability of Cse4p to localize properly to the kinetochore, causing increased chromosome loss. There are also structural constraints which should, in theory, be imposed by the positioning of a nucleosome at CDEII. It would seem improbable that the CBF3 Complex, which has been shown to bind to both sides of the DNA, as well as Ndc10p bound along CDEII and a Cbf1p homodimer are able to coexist with a nuclesosome all within approximately 200 bp of DNA (Jiang and Philippsen, 1989; Espelin et al., 1997; Meluh et al., 1998; Espelin et al., 2003). Further, DNA containing stretches

of A's and T's, as exist in CDEII, have been shown to be poor sites for nucleosome binding due to the intrinsic bending of such DNA (Kunkel and Martinson, 1981; Prunell, 1982). We therefore prefer a model in which CBF3 directs the positioning of the Cse4-containing nucleosome to flanking DNA, thus determining the perimeter of the kinetochore domain, and establishing the phasing of nucleosomes around the centromere. Intriguingly, early *in vivo* footprinting experiments are consistent with this possibility (Bloom and Carbon, 1982; Bloom et al., 1983). As future experimental evidence, ChIP combined with increased sonication of genomic DNA and multiple primer pairs across the *CEN* region might be used to produce a better resolution map of the binding of Cse4p along CDEI,II,III. It should at least be possible to determine if Cse4p is peripheral to Ndc10p and/or the CBF3 Complex. This same approach has been used to define the extent of the histone domain targeted for deacetylation in promoter regions, which in turn regulate transcription (Kadosh and Struhl, 1998). Other options for refining the location of the Cse4p-nucleosome might include the use of electron microscopy or footprinting, which could establish the relative positions of the Cse4p-nucleosome, Ndc10p and the CBF3 Complex.

Ndc10p at non-Centromere Locations

ChIP shows that Ndc10p may bind to DNA at chromosome arms *in vivo*, and the distinctive localization of Ndc10p-GFP (relative to the bi-lobed pattern seen for many other kinetochore proteins), supports the potential binding of Ndc10p to sites other than kinetochores. The biological significance of this binding remains undetermined although we can speculate about possible functions based on observations from other organisms. Multiple microtubules bind to chromosomes in other organisms and in some cases these attachments are facilitated by

proteins known as chromokinesins (Afshar et al., 1995; Funabiki and Murray, 2000). These additional connections are believed to assist the chromosome in forming proper attachments to the mitotic spindle as well as aiding chromosome oscillations and congression (Levesque and Compton, 2001). Ndc10p has been proposed to associate with spindle MTs and although it has not been possible to observe individual MTs in budding yeast by light microscopy, Ndc10p may transiently perform a similar function as chromokinesins on chromosome arms in *S. cerevisiae* (Goh and Kilmartin, 1993; Goshima and Yanagida, 2000).

Neocentromeres are regions of a chromosome capable of becoming functional if the primary centromere becomes disabled, thus enabling chromosome segregation to proceed under non-ideal conditions. Activation of these "new centromeres" in humans has been associated with a number of cancers and developmental problems, largely as a result of chromosomal duplications and rearrangements (Weiss, 1996; Gisselsson et al., 1999a; Gisselsson et al., 1999b). These DNA sequences have been shown to exist in humans and can be experimentally created in *Drosophila*, but their existence in *S. cerevisiae* remains speculative at this time (Voullaire et al., 1993; Williams et al., 1998). In human cells, the CENP-B protein has also been shown to bind to both active centromeres and chromosome arms (potential neocentromeres), although no sequence homology has been identified between CENP-B and Ndc10p (Earnshaw et al., 1989; Saffery et al., 2000). Despite a lack of evidence for neocentromeres in *S. cerevisiae*, the presence of sequences which bear resemblance to CDEII along the arms allows for speculation about the formation of rudimentary kinetochores at these locations.

Molecular Organization based on Chromatin Immunoprecipitation

Chromatin Immunoprecipitation has become a standard means for the identification of kinetochore proteins (Meluh and Koshland, 1997; Ortiz et al., 1999; He et al., 2001). This method can also be used to determine the protein interactions required for a protein of interest to associate with the centromere. As depicted in Figure 1-3 of the Introduction, our understanding of the spatial relationships between proteins of the Linker layer began as that of an amorphous mix, but a sense of order is beginning to develop as a result of interdependencies determined through ChIP. In Chapter 3, I describe the use of ChIP and live-cell imaging to identify eleven new kinetochore proteins based on three criteria: 1) bi-lobed pattern of protein-GFP staining between two SPBs, 2) association with *CEN* DNA *in vivo*, as determined by ChIP and 3) CBF3-dependency for *CEN* association as evaluated by ChIP in an *ndc10-1* background. As stated previously, Ndc10p, Cep3p and Ctf13p of the CBF3 Complex bind directly to *CEN* DNA and we can use this information to begin assembling our organizational model from this starting point. Using DNA primers spanning the central 200 bp of the centromere and moving 200 and/or 400 bp beyond in both directions, distinct patterns of association can be observed for the individual kinetochore proteins by ChIP analysis, thus providing additional information about their localization. Ndc80p and Spc19p show a tight association with the middle of the centromere, while Spc34p, Cin8p, Bik1p and Stu2p are also largely found at the center but additionally show a moderate preference for the CDEIII-side of the *CEN*. Mif2p interacts with the center of the centromere but also demonstrates a strong preference for the CDEIII side (Figure 3-3). On the other hand, Scc1p shows uneven binding throughout the *CEN* region, consistent with previous reports (Megee et al., 1999; Tanaka et al., 1999). The resolution obtained using primers separated by hundreds of base pairs on either side of the centromere are modest. However, a

more detailed ChIP analysis using increased sonication to minimize chromosomal DNA fragment size combined with primers that span much smaller segments of DNA (i.e. 30-50 bp) as described above, might provide increased resolution allowing for better determination of the relative positions of individual kinetochore proteins.

Determining Interdependencies using Kinetochore Mutants

An example of the conclusions that can be drawn based on ChIP interdependencies is illustrated by Ndc80p (Figure 3-6). Like all other kinetochore proteins, Ndc80p requires Ndc10p to associate with the centromere but Ndc10p does not require Ndc80p for its own association, thus establishing the relationship: *CEN*-Ndc10p-Ndc80p. Using an *ndc80-1* mutant, we can establish that Nuf2p requires Ndc80p to associate with the centromere and thus we have *CEN*-Ndc10p-Ndc80p-Nuf2p. The NDC80 Complex has recently been observed by electron microscopy and the organization of the subunits support the ChIP results published here and elsewhere (R. Wei-unpublished observations; He et al., 2001; Janke et al., 2001). Namely, Spc24p/Spc25p exists as an apparent heterodimer at one end of the complex, with Ndc80p and Nuf2p as a heterodimer at the opposite end of the same complex. As was mentioned previously, Scc1p requires Ndc10p but not Ndc80p to associate with the centromere, and Ndc80p does not require Scc1p. In this manner, a network of interactions can be built up and a sense of how the kinetochore proteins relate to each other has begun to evolve. Although a simple relationship can be predicted between Ndc10p, Ndc80p and Nuf2p, all kinetochore proteins do not appear to be arranged in a linear manner such that every protein depends on another like segments in a column. It must also be kept in mind that intermediary proteins, known or unknown to us, may also be involved in these relationships.

Nonetheless, many researchers have used the same ChIP methodology to establish dependencies among kinetochore proteins and we can combine these results to form a working model of kinetochore organization (Figure 6-1).

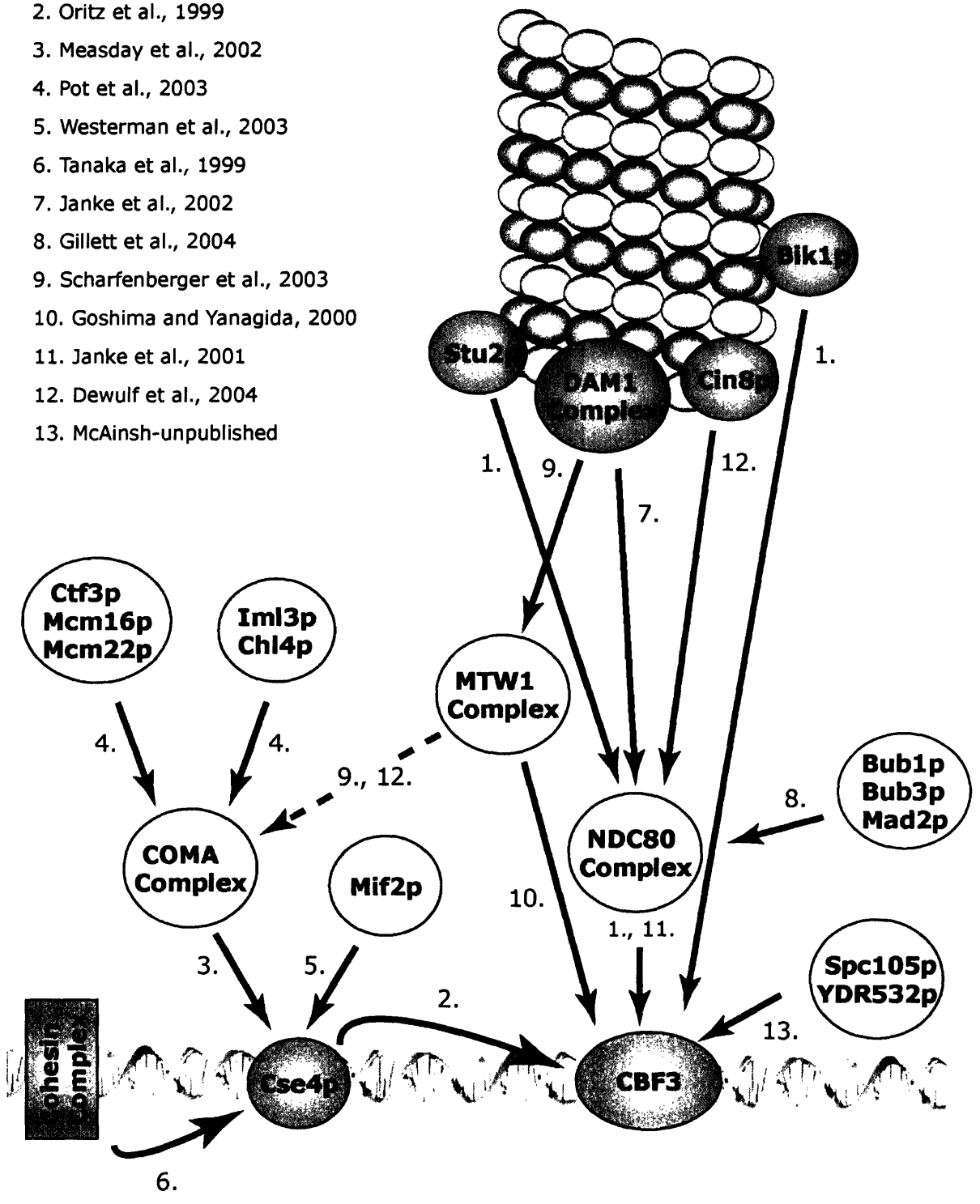

Figure 6-1 Interdependencies among kinetochore proteins for association with *CEN* DNA as determined by Chromatin Immunoprecipitation (ChIP). Arrows indicate dependence on protein (pointed towards) for association with *CEN* DNA. Dashed arrow indicates discrepancy in the literature. Secondary dependencies are implied, i.e. Cse4p depends on CBF3, and the COMA Complex requires Cse4p, therefore the COMA Complex requires CBF3. Numbers correlate with references that support data (in some cases, multiple researchers have demonstrated the same result-earliest reference is listed).

As was mentioned in Chapter 1, there are a number of issues which can arise when multiple researchers perform ChIP experiments without a standardized protocol (there would likely be discrepancies even with a standardized protocol). Errors are even possible within one lab: Ipl1p was published as negative for *CEN*-association by ChIP (although positive by microscopy), but has subsequently been shown to be positive by other researchers (He et al., 2001; Kang et al., 2001). Fortunately, discrepancies have been few and the dependence of the MTW1 Complex on the COMA Complex is currently the only major point of contention. Hopefully continued evaluation of additional proteins from each of these complexes will sort this issue out (De Wulf et al., 2003; Scharfenberger et al., 2003). It is essential to incorporate data from other methods such as microscopy and biochemical interactions in order to corroborate the ChIP results. As an example, it has been reported that the DAM1 Complex, Stu2p and Cin8p require Ndc80p for their association with the kinetochore (He et al., 2001; Janke et al., 2002; De Wulf et al., 2003). However, the phenotype of the *ndc80-1* mutant is detachment of chromosomes from MTs, and because the DAM1 Complex, Stu2p and Cin8p are all MT binding proteins, it is unclear whether their dependence on NDC80 for *CEN* binding is a result of a direct biochemical interaction or the more global effect of detachment of the kinetochore from the MT. For reasons such as these, we have insisted on at least three criteria for determining whether a protein should be considered a member of the kinetochore. Continued results using a combination of ChIP, microscopy and biochemistry should provide confidence in the conclusions used to establish an architectural model of the kinetochore.

Order of Assembly of the Kinetochore

Although we know that Cbf1p, Ndc10p and the CBF3 Complex bind centromeric DNA, little is known about the order of assembly of the kinetochore. It is presumed that the *CEN* DNA-binding proteins first identify the centromere and initiate kinetochore formation, but which proteins arrive next? Do they bind simultaneously or in a pre-determined order? When do the MT binding proteins join the kinetochore-before the MT or as a result of MT association? Once again, the use of ChIP in combination with the conditional-*CEN* constructs previously described may prove useful in determining the order of assembly of the kinetochore (Bloom et al., 1989; Hill and Bloom, 1989; Tanaka et al., 1999). Minute-by-minute analysis of DNA association by transcription factors using ChIP has demonstrated the potential to evaluate assembly of a complex on DNA very precisely, and a similar approach could be taken to determine the order of kinetochore assembly (Katan-Khaykovich and Struhl, 2002; Bryant and Ptashne, 2003; Wolner et al., 2003). Following "activation" of the conditional-*CEN*, samples would be taken periodically and evaluated for the presence of an individual kinetochore protein. Following analysis of multiple kinetochore proteins in an identical manner, the data for all evaluated proteins could be overlayed to produce a temporal map of arrival at the centromere, and comparisons made to establish the order of assembly at a nascent centromere.

It is also unknown when during the cell cycle the kinetochore is established, although we assume it occurs during S phase following DNA replication. A possible means for determining this assembly could involve synchrony/release of cells, followed by ChIP performed throughout the cell cycle to establish when individual proteins are binding to the centromere. This would establish the arrival of kinetochore proteins at the centromere both relative to the cell cycle and each other. The order of assembly should provide further information about the organization of

proteins and may also answer questions regarding the temporal relationship between DNA replication and kinetochore formation.

Localization of the Spindle Checkpoint in *S. cerevisiae*

We have shown that Bub1p, Bub3p, Mad1p and Mad2p are capable of localizing to the *S. cerevisiae* kinetochore *in vivo*, while Mad3p was not observed at the centromere under any experimental conditions. Preliminary evidence also indicates that Cdc20p and Mps1p, two additional components of the spindle checkpoint, may be localized to the *S. cerevisiae* kinetochore *in vivo*, although further characterization will be required to confirm these results (C. Espelin-unpublished observations). Bub1p and Bub3p appear at the *CEN* during S phase and remain there until metaphase (as detected by ChIP and microscopy), much like their mammalian counterparts. In addition to their function as members of the spindle checkpoint, Bub1p and Bub3p appear to have additional roles during the normal *S. cerevisiae* cell cycle which are not shared by the Mad proteins. Warren et al. have shown that deletions or mutations of Bub1p and/or Bub3p cause dramatic effects on chromosome segregation and cell growth rates which are not seen with alteration of the Mad proteins (Warren et al., 2002). In contrast to Bub1p and Bub3p, we did not observe Mad1p or Mad2p at the kinetochore as part of the normal *S. cerevisiae* cell cycle, but instead these proteins localized to kinetochores only in the presence of damage to *CEN*-MT attachments. This is in contrast with mammalian cells in which MAD2 is present at kinetochores during normal cell division and may reflect biological differences between open and closed mitoses. The demonstration that spindle checkpoint components are present at the *S. cerevisiae* kinetochore represents the first evidence that these proteins localize similarly to their counterparts in higher organisms, and provides an opportunity to combine the

powerful genetic capabilities of *S. cerevisiae* with an ability to monitor spindle checkpoint function.

The power of this combination is particularly evident when considering the specific phenotypes exhibited by kinetochore mutants in budding yeast (Fig. 6-2). For example, *ndc80-1* mutants exhibit complete detachment of sister chromatids from MTs, *dam1-1* mutants have monopolar attachment of both sister chromatids to a single SPB and *stu2-279* mutants show bipolar attachment to both SPBs but lack tension across their sister chromatids. In the case of *ndc80-1* and *dam1-1* mutants, we observe Bub1p and Mad2p localized to the unattached kinetochores. *stu2* mutants on the other hand, provide a unique opportunity to evaluate tension and attachment defects as the majority of their chromosomes maintain bipolar attachment without any tension, while in the same cell, a few chromosomes become completely detached from MTs. Importantly, Mad2p is localized to the unattached chromosomes but not to those which have maintained their bipolar attachments but lack tension. A similar situation arises in cells treated with the MT poison nocodazole which causes the spindle MTs to collapse towards a single SPB, thus eliminating tension on the attached sister chromatids. Under these conditions, the occasional chromosome also becomes detached from the MT resulting in Mad1p and Mad2p localization to that chromosome, but not to those which have remained attached and follow the MTs back to the SPB. Taken together, our data supports the theory that attachment status is the signal to the spindle checkpoint that bivalent connections have been achieved by the chromosomes.

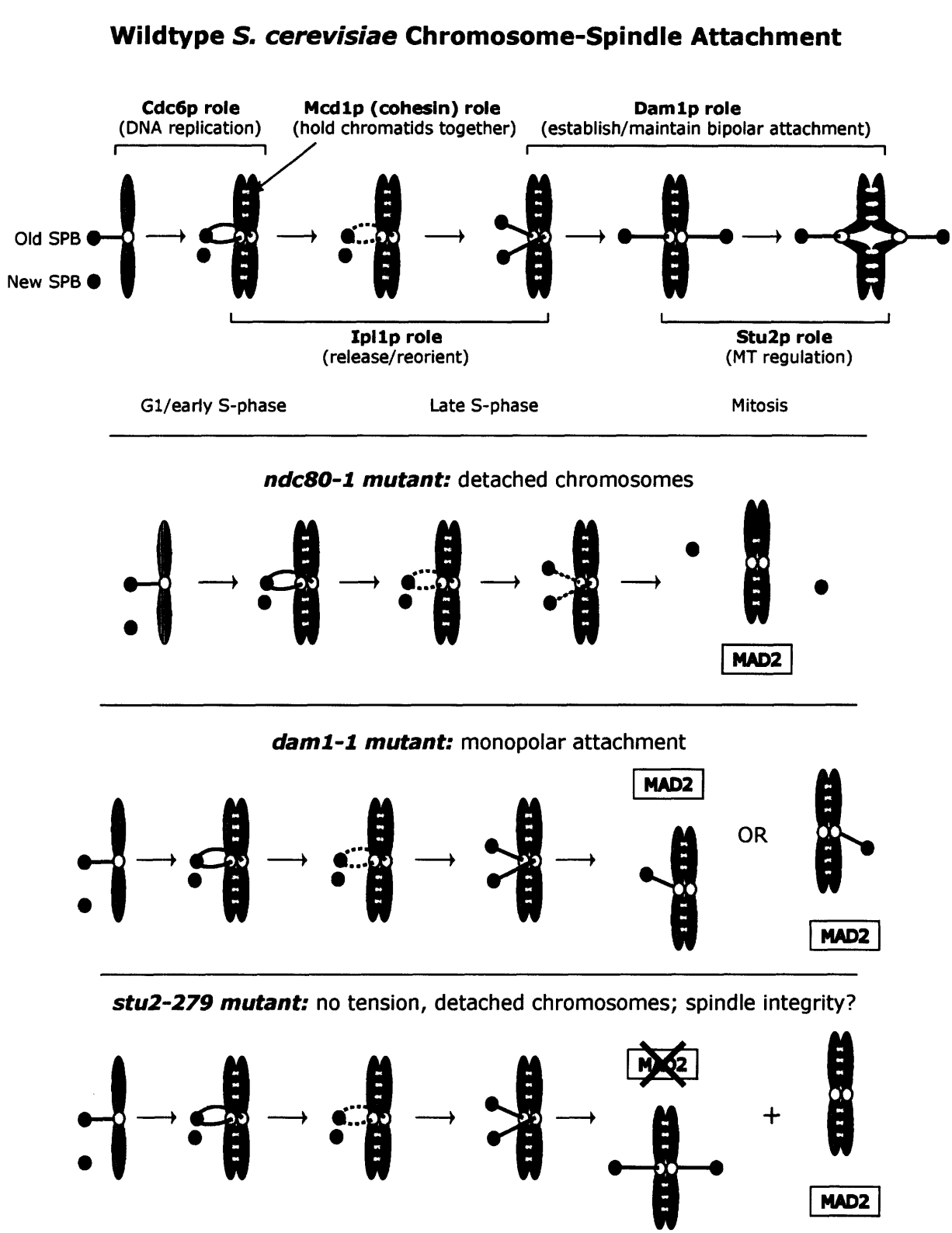

Figure 6-2. Comparison of chromosome-spindle attachment in wildtype and kinetochore mutants through the *S. cerevisiae* cell cycle. Single chromosomes remain attached to the "old" SPB during G1 and into S phase when a second "new" SPB develops. Following DNA replication, Ipl1p is proposed to release the duplicated chromosomes from the single SPB, allowing establishment of bipolar attachment. SPBs separate during S-phase/mitosis and apply tension across sister chromatids during metaphase, resulting in transient sister chromatid separation. *ndc80-1* mutants demonstrate detachment of the duplicated sister chromatids from the spindle. *dam1-1* mutants fail to maintain bipolar attachment and the duplicated chromosomes randomly remain attached to either of the SPBs. *stu2-279* mutants form bipolar attachments but do not exhibit proper tension across sister chromatids, as demonstrated by a lack of transient sister separation. Some chromosomes also become detached in the same *stu2* mutant cells that exhibit a lack of tension. Boxed MAD2 indicates presence of Mad2p and crossed box indicates Mad2p not present on the indicated chromosomes, based on microscopy.

The Spindle Checkpoint-Kinetochore Connection

The search for a physical connection between the proteins of the spindle checkpoint and the kinetochore remains a point of great interest. The identification of this biochemical link would open the door to a variety of experiments capable of improving our understanding of how the spindle checkpoint recognizes problems at the kinetochore, as well as how it functions during the normal cell cycle. Specific kinetochore lesions which eliminate the ability of the spindle checkpoint proteins to bind the kinetochore while not disrupting the structural connection between *CEN*-kinetochore-MT would prove a very valuable tool. Deleting members of the CBF3 Complex eliminates the ability of the spindle checkpoint to detect errors, but at the same time eliminates the binding of all other kinetochore proteins resulting in a nonfunctional kinetochore. On the other hand, alteration of members of the NDC80 Complex also eliminates spindle checkpoint function, but still leaves a number of kinetochore proteins including the CBF3 Complex, the COMA Complex, the Ctf3 Complex, Mtw1p, Cse4p and Mif2p present at the centromere (Measday et al., 2002; Pot et al., 2003; Westermann et al., 2003). The NDC80 Complex is composed of four proteins-Ndc80p, Nuf2p, Spc25p and Spc25p, and while *ndc80-1* and *nuf2-61, -457* mutants engage the spindle checkpoint, *spc25-7* and *spc24-2* mutants do not (He et al., 2001; Janke et al., 2001; Wigge and Kilmartin, 2001). This puts particular focus on Spc24p and Spc25p, or proteins that rely on these two for their kinetochore localization, as potential sites for binding of the spindle checkpoint proteins. These observations, along with a high degree of evolutionary conservation among spindle checkpoint proteins and members of the NDC80 Complex, make this connection particularly intriguing. It is of further interest to note that the human homolog of *NDC80* is HEC1, or Highly Expressed in Cancer, and a far-reaching

implication of the physical relationship between the spindle checkpoint and the kinetochore might be the understanding of the role of these complexes in cancer.

CEN-CBF3 recruits Cse4p, Mif2p, Ndc80p and Ame1p

Developing an assay to test a biological function is a critical step in evaluating what the requirements are for that function and in the case of kinetochore activity, attempts have previously been made at developing *CEN*-MT binding assays (described in Chapter 1). The shortcoming of these assays has been the inability to identify the specific proteins involved in the observed MT binding events, and it is only recently that we have begun to appreciate why this has been so difficult-namely, the large number of kinetochore proteins involved. *In vitro* reconstitution of MT binding ideally includes knowledge of the identity of all components involved, and provides a means for deciphering their individual roles. *In vitro* reconstitution from fully purified proteins is likely many years in the future, especially considering that we are not even certain of all the components at this time.

Purification of members of the CBF3 Complex using low stringency Tandem Affinity Purification (TAP), or similar purification schemes, has thus far identified only CBF3 components interacting with each other (Westermann et al., 2003). These schemes involve isolation of the individual proteins using epitope tags, and a reason these methods have failed to identify proteins which interact with CBF3 may be that *CEN* DNA is required. The DKR Assay is not immediately intended to recapitulate the entire kinetochore, but instead is primarily meant to identify those proteins which are most closely interacting with *CEN*-bound CBF3. The next step will be to determine the interdependencies among these proteins, and then expand past the proteins that are binding to CBF3 with the hope of establishing a functional attachment to MTs.

This may seem like a lofty goal but from the analysis of kinetochore mutants, only alteration of the CBF3 Complex and the NDC80 Complex have been demonstrated thus far to cause detachment of the chromosomes from the MT. Establishing a connection between *CEN* and MT may be much simpler *in vitro* without the requirement for many of the tension sensing and generating components of the kinetochore, and it is formally possible that the combination of rCBF3, rNDC80 and a few additional kinetochore proteins along with a MT-binding protein/complex such as the DAM1 Complex or Stu2p may provide the minimal requirements for MT attachment.

I have presented an *in vitro* Dynabead-Kinetochore Reconstitution Assay in Chapter 5 of this thesis which represents an initial step in building the kinetochore out from the *CEN* DNA, with the eventual goal of establishing attachment to the MT. An 184bp CDEI,II,III *CEN* fragment bound to magnetic Dynabeads is capable of specifically binding recombinant CBF3, thus providing an excellent reagent for identifying CBF3-interacting proteins. Recombinant CBF3 bound to *CEN* is able to specifically bind Cse4p, Mif2p, Ndc80p and Ame1p from yeast extracts, while the kinetochore proteins Mtw1p and Ctf19p as well as Cin1p (negative control), were not specifically bound. Although the use of yeast extracts allows for the possible involvement of intermediate factors in binding, these preliminary results have identified proteins that are attractive candidates for purification or expression followed by re-evaluation in the DKR Assay. It is also reassuring that the proteins which have been identified as interacting with CBF3 + *CEN* DNA are those which have been speculated to be close to CBF3 based on other experimental data. Future experiments will continue to evaluate additional kinetochore proteins for their ability to interact with CBF3 as well as identify the interdependencies between these proteins. For example, does Mif2p require Cse4p to associate with CBF3 *in vitro*?

Incorporation of purified and recombinant proteins into the DKR Assay will also be pursued in an attempt to better define all components involved.

Localization of a Cse4p-containing Nucleosome:

Interpretations from an in vitro Dynabead-Kinetochore Reconstitution Assay

The recurring question of Cse4p-containing nucleosome localization and assembly may also be revisited using the DKR Assay. We have established that CBF3 and Ndc10p bind CDEII,III, and yet this association along an 184 bp piece of DNA is capable of specifically binding Cse4p. It remains difficult to imagine how this piece of DNA is simultaneously bound to CBF3 and Ndc10p (and likely Cbf1p), wrapped around a nucleosome, and all the while remaining attached to a Dynabead. An immediate question which arises is whether Cse4p is part of a fully assembled nucleosome when it interacts with *CEN*-CBF3. Ongoing experiments are aimed at evaluating whether the Cse4p identified in the DKR Assay is part of a complete nucleosome, a subcomplex alone (i.e. Cse4p + H4) or is present on its own. The core histone-fold domains (HFD) of Cse4p and H3 are highly conserved and it is these regions which are involved in DNA contact, although they are not believed to confer sequence specificity. So how does Cse4p localize at the centromere? One possibility is that there is competition between "regular" H3-containing nucleosomes and "specialized" Cse4p-containing nucleosomes throughout the genome, and it is only because of additional contacts between Cse4p and kinetochore proteins at the centromere, that the specialized nucleosome is localized to the *CEN* region. However, if binding to DNA occurs through regions of the nucleosome which are highly conserved, there would not seem to be any energetic preference between "regular" nucleosomes and "specialized" nucleosomes at non-*CEN* locations, yet Cse4p-nucleosomes are only detected

at centromeres. Another possibility is that Cse4p (+/- H4) initially interacts with components of the kinetochore, and the other members of the nucleosome (H2A/H2B) are subsequently assembled, followed by binding to nearby DNA. This would provide the specificity required for localization of a Cse4p-nucleosome to the centromere, and once established, may direct the phasing of "regular" nucleosomes in the region around the centromere. Continued work incorporating recombinant nucleosomes and their subunits into the DKR Assay will hopefully provide additional information regarding establishment of specialized nucleosomes at the centromere.

Lessons for Human Kinetochores

Despite the apparent lack of conservation in centromere sequence, it is becoming increasingly clear that many similarities exist between the composition and role of the kinetochore in *S. cerevisiae* and humans. The molecular mechanisms involved in proper chromosome segregation, such as transient sister chromatid separation, appear to be highly conserved making analysis of the "simple" kinetochore of *S. cerevisiae* a potentially valuable tool for understanding human kinetochores. Analysis of ever-expanding genomic databases has identified homologs of many kinetochore proteins in both yeast and humans, with identification of further orthologs likely to continue. This not only provides confidence that key components of the kinetochore are conserved, but also increases the likelihood that observations made using genetically tractable *S. cerevisiae* cells will translate into practical experiments on human kinetochores. A notable example of conservation is the presence of specialized nucleosomes at active centromeres in *S. cerevisiae*, *S. pombe, C. elegans, D. melanogaster,* mice and humans despite the divergent centromeric DNA exhibited by these organisms. Understanding the

deposition of this nucleosome at the *S. cerevisiae* centromere will hopefully shed light on a function which is apparently conserved from yeast to humans. The general organization of the kinetochore also appears to be similar, as *S. cerevisiae* and human kinetochore proteins exist in different "layers", although this designation is conceptual in *S. cerevisiae* at this point while it has been observed by microscopy in humans (Rieder and Salmon, 1998). This arrangement lends itself to dependencies between the different proteins for their ability to associate with the centromere, and again it is hoped that lessons learned in *S. cerevisiae* can be extended to human kinetochores. However, one significant difference which remains is the lack of CBF3 equivalents in higher cells and it remains to be seen whether homologs just haven't been identified yet, or a separate mechanism has developed for centromere identification in other systems.

Altered expression of spindle checkpoint genes has been implicated in a number of cancers and although the distinction between cause and effect has yet to be firmly established, a better understanding of the spindle checkpoint proteins should provide insight into both cancer development and potential therapeutics. The high degree of evolutionary conservation among these proteins is cause for hope that their function has also been maintained despite adaptation to accommodate biological differences between organisms. Identification of the biochemical connection between the spindle checkpoint and the kinetochore in *S. cerevisiae* would immediately lead to revealing experiments in human cells.

SUMMARY

In summary, the data presented in this thesis has increased our overall knowledge of the molecular organization of the *S. cerevisiae* kinetochore on a number of levels, and this work

represents an attempt to better understand one of the fundamental questions of biology: how does chromosome segregation occur? We have shown that Ndc10p specifically binds to the CDEII region of the *S. cerevisiae* centromere and functions along with the CBF3 Complex bound at CDEIII, to form the initial DNA-Binding layer upon which the rest of the kinetochore is assembled. The Dynabead-Kinetochore Reconstitution Assay has provided preliminary results indicating that Cse4p, Mif2p, Ndc80p and Ame1p are associated with CBF3 bound to *CEN* DNA. Experimental evaluation of interdependencies among these proteins will determine whether interactions with CBF3 are direct or indirect, allowing us to better understand how the next layer of kinetochore proteins is assembled upon an initial DNA-Binding layer. Chromatin Immunoprecipitation (ChIP) makes it possible to evaluate relationships among kinetochore proteins of the Linker layer, and between these proteins and the DNA-Binding and MT-Binding proteins. Using this data, we can begin to develop a model illustrating the spatial relationships between the kinetochore proteins as well as provide a means for better explaining the phenotypes of various mutations in kinetochore subunits. Finally, we have shown that the spindle checkpoint is localized to the *S. cerevisiae* kinetochore to monitor proper bipolar attachment of sister chromatids to the mitotic spindle. Bub1p and Bub3p localize to the kinetochore as part of the normal cell cycle, while Mad1p and Mad2p are recruited only in response to damage. This is in contrast to mammalian cells in which MAD1 and MAD2 are localized to the kinetochore, along with BUB1 and BUB3, during every cell cycle and may represent differences between open and closed mitoses. The simultaneous analysis of specific kinetochore mutants and the response of the spindle checkpoint support the hypothesis that it is the status of MT attachment by kinetochores that is monitored by the spindle checkpoint.

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