The Spindle Checkpoint:
Bubs, Mads, and Chromosome-Microtubule Attachment in Budding Yeast

by

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ABSTRACT

The high fidelity of chromosome transmission in eukaryotes is achieved, in part, by the activity of the spindle checkpoint. This checkpoint monitors the status of chromosome-microtubule attachments and delays the onset of anaphase until all kinetochores have formed stable bipolar connections to the mitotic spindle. We have characterized the localization of the Bub and Mad spindle checkpoint proteins in *Saccharomyces cerevisiae*. In metazoan 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. We propose that differences in the behavior of spindle checkpoint proteins in metazoan cells and budding yeast are due primarily to evolutionary divergence in spindle assembly pathways.

The spindle checkpoint proteins Mad1p and Mad2p exhibit perinuclear localization in both budding yeast and metazoans. We find that the perinuclear localization of Mad1p is dependent on Myosin-like proteins Mlp1p and Mlp2p, two proteins that link nuclear pore complexes to the interior of the nucleus. Deletion of either *MLP1* or *MLP2* releases Mad proteins from the nuclear periphery and allows them to associate with kinetochores during early mitosis. Ectopic binding of Mad1p to kinetochores does not dramatically alter cell cycle progression, nor does loss of Mad1p from the nuclear periphery appear to impair spindle checkpoint activation. However, as the Mlps have been implicated in several cellular processes, such as the DNA damage response, we hypothesize that the perinuclear pool of Mad proteins may be required for spindle checkpoint-independent functions such as invoking metaphase arrest following DNA damage.

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...honor the
unique genes,
molecules that reproduce themselves, divide into

sets, the nucleic grain transmitted
in slow change through ages of rising and falling form,
some cells set aside for the special work, mind

or perception rising...

~ A.R. Ammons, "Mechanism"

Chapter 1

The Spindle Checkpoint

The Spindle Checkpoint

1.1 Introduction

Mitotic spindle assembly is a stochastic process reliant on an unpredictable series of chromosome-microtubule (MT) capture events. However, to maintain genomic stability, all chromosomes must become properly attached to the mitotic spindle prior to the onset of anaphase. The amount of time a given eukaryotic cell will require to complete spindle assembly is quite variable (Ault and Rieder, 1992). Therefore imposing a strict time limit on spindle assembly would not be sufficient to ensure proper chromosome segregation in all cases. Instead of a strict timer, eukaryotic cells have evolved a surveillance system called the spindle checkpoint that monitors chromosome-MT attachment and delays the onset of anaphase until spindle assembly has been completed. The spindle checkpoint is only one in a series of checkpoints that regulate passage through critical cell cycle transitions. As will be discussed in this chapter, the spindle checkpoint utilizes proteins that fulfill the classical definition of checkpoint components, as well as proteins that appear to have additional roles in mitotic timing and spindle assembly.

The process of spindle assembly has long intrigued cytologists. Chromosome movements during mitosis were painstakingly documented in drawings by the German anatomist Walther Flemming in the late 19th century (Paweletz, 2001), and chromosome segregation was postulated to be the basis of heredity by the German biologist Theodor Boveri and the American zoologist Walter Sutton at the beginning of the 20th century (Boveri, 1907; Sutton, 1903). Spindle assembly and chromosome segregation have proven to be rich and complex research topics, as scientists in the 21st century still remain actively engaged in unraveling the molecular details that underlie these processes in eukaryotes.

1.2 Cell Cycle Checkpoints

Successful mitosis requires that critical steps of the cell cycle such as DNA replication, chromosome segregation, and cytokinesis be executed in the appropriate order. Each period of the cell cycle is characterized by the presence of specific cyclins that bind to and activate cyclin-dependent kinases (Cdk's). The cell cycle machinery relies on proteolysis to regulate passage from one stage of mitosis to the next. In metazoan cells, cyclin B synthesis and cyclin B association with Cdk1 are essential for early mitotic events, while cyclin B degradation and deactivation of Cdk1 are essential for progression through anaphase and telophase (reviewed in Peters, 2002). The regulated depletion of critical proteins, such as cyclin B, imparts a strict directionality to mitotic events and makes each cell cycle transition a point of no return.

Passage from one phase of mitosis to the next is tightly regulated by a series of checkpoints. Each checkpoint requires surveillance components to monitor the status of a given process (such as DNA replication or mitotic spindle assembly) and effector components that interface with the cell cycle machinery to regulate mitotic progression (Hoyt et al., 1991; Li and Murray, 1991; Weinert and Hartwell, 1988). Cell cycle checkpoints were initially defined as non-essential surveillance systems required for survival only under unusual and adverse conditions. This definition stems from studies on the DNA damage response in *S. cerevisiae*. Eukaryotic cells with DNA damage normally arrest until damage has been repaired. Mutations in the checkpoint component *RAD9* eliminate G2/M cell cycle arrest and reduce viability following X-ray induced DNA damage; however, loss of Rad9p function does not dramatically alter the rate of cellular proliferation or viability under normal growth conditions (Weinert and Hartwell, 1988).

Interestingly, although *rad9* mutants allow mitosis to proceed without regard to the repair status of DNA damage, completion of DNA repair and viability can be restored if irradiated *rad9* cells are delayed in mitosis by alternate means, such as treatment with the MT-depolymerizing drug nocodazole. These data indicate that the primary role of Rad9p is to arrest the cell cycle in order to allow sufficient time for DNA repair to be completed (Hartwell and Weinert, 1989; Weinert and Hartwell, 1988). Although the behavior of Rad9p suggested that checkpoint proteins' main function is to regulate mitotic timing, recent studies have shown that some DNA damage checkpoint proteins, such as Rad24p, themselves participate in repair activities as well as checkpoint activation (Aylon and Kupiec, 2003; Aylon and Kupiec, 2004). The evolution of multifunctional proteins with both checkpoint and repair activities may increase efficiency and reduce the potential for competition at repair sites where both sensor and repair systems must be active (Aylon and Kupiec, 2004).

1.3 Chromosome Segregation and the Mitotic Spindle

Maintaining a stable genome through the process of mitotic division is essential for preserving cellular function and identity. The stability of the genome is dependent upon checkpoints that monitor the fidelity of newly replicated and repaired DNA sequences, as well as the spindle checkpoint that ensures that duplicate chromosomes are partitioned equally at anaphase. Failure to partition DNA correctly during mitosis or meiosis leads to aneuploidy. Although chromosome loss is generally lethal for unicellular organisms, aneuploid metazoan cells sometimes survive. Aneuploidy is responsible for a subset of birth defects in humans, such as Down Syndrome and Klinefelter Syndrome, and is also

characteristic of many tumor cells (Draviam et al., 2004; Hassold and Hunt, 2001; Jallepalli and Lengauer, 2001; Lowe et al., 2001; Storchova and Pellman, 2004).

Following DNA replication, duplicate sister chromatids remain paired together and chromosome segregation is subsequently accomplished via the mitotic spindle. This bipolar self-organizing array of MT polymers captures and maneuvers the sister chromatid pairs. MTs are nucleated by MT organizing centers (MTOCs) called spindle pole bodies (SPBs) in budding yeast and centrosomes in metazoans. In contrast to metazoan cells, yeast cells undergo a closed mitosis in which the nuclear envelope remains intact throughout the cell cycle. Following duplication, yeast SPBs separate and move to opposite sides of the nucleus. Spindle MTs that capture chromosomes and interpolar MTs that maintain spindle integrity emanate from a SPB's nuclear face, while astral MTs essential for nuclear positioning and division radiate from a SPB's cytoplasmic face.

MTs attach to chromosomes via kinetochores, multiprotein complexes that assemble on centromeric DNA. In metazoan cells, MTs are excluded from the nucleus during interphase; therefore, no chromosomes are attached to MTs during interphase or early mitosis. In contrast, budding yeast kinetochores remain closely associated with SPBs, and chromosomes are likely attached to spindle MTs throughout the entire cell cycle (Jin et al., 2000).

The bipolar symmetry of the mitotic spindle is essential for chromosome segregation. During spindle assembly, a given chromatid must become stably attached to MTs emanating from one and only one pole of the spindle, while its partner must become attached to MTs emanating from the opposite pole. Achieving such bi-orientation ensures that paired sister chromatids will migrate toward opposite poles of the spindle at anaphase. In budding yeast,

centromere duplication appears to precede maturation of the new SPB, and it has been postulated that duplicated sister chromatids are initially attached to a single SPB during early mitosis (Tanaka et al., 2002). This state of mono-orientation, or "syntelic" attachment, is typically resolved once the new SPB has matured and begun to nucleate MTs.

1.4 Search-and-Capture

All chromosomes in a cell must achieve bi-orientation to the spindle before mitosis can proceed. When unattached chromosomes are present, MTs search throughout the cell volume in order to capture them. This stochastic search-and-capture process is reliant on the dynamic nature of MT filaments. Free MTs transition unpredictably between periods of slow growth and rapid shrinkage (Desai and Mitchison, 1997). Chromosome capture stabilizes individual MTs, thereby enabling the formation of secure MT-chromosome attachments and facilitating the formation of a proper mitotic spindle (Hunt and McIntosh, 1998; Mitchison et al., 1986; Zhai et al., 1995).

MTs are assembled from αβ-tubulin heterodimers. Tubulin heterodimers initially assemble into linear protofilaments, 13 of which are then arranged in parallel orientation to form a hollow tubule ~25nm in diameter (Amos and Klug, 1974; Desai and Mitchison, 1997; Weisenberg and Deery, 1976). The asymmetry of the αβ-heterodimers imparts a structural polarity to the parallel protofilaments: α-tubulin subunits are exposed at less dynamic MT minus ends, while β-tubulin subunits are exposed at the faster growing MT plus ends. MT minus ends are embedded in MTOCs and therefore are more stable and less dynamic than MT plus ends *in vivo*.

GTP hydrolysis is responsible for the "dynamic instability" exhibited by MT filaments. Both the α- and β-tubulin subunits bind to GTP; however, only the β-tubulin subunit hydrolyzes and exchanges GTP (Desai and Mitchison, 1997; Spiegelman et al., 1977). Filament assembly is GTP-dependent as an αβ-heterodimer can only be added to the plus end of a MT when both subunits are bound to GTP (Desai and Mitchison, 1997). Polymerization catalyzes GTP hydrolysis and much of the β-tubulin within a MT fiber is GDP-bound (David-Pfeuty et al., 1977; MacNeal and Purich, 1978; Nogales et al., 2003). It is thought that GTP-bound heterodimers at the plus ends of MTs form caps that stabilize the MT lattice and prevent depolymerization. When a MT loses its GTP cap, the GDP-bound plus ends of the tubulin protofilaments then become curved and splay apart from one another, and the MT begins to disassemble (Mitchison and Kirschner, 1984; Nogales et al., 2003).

MT dynamics in metazoan cells change during the cell cycle with rates of MT growth and nucleation increasing significantly as cells enter early mitosis (Piehl and Cassimeris, 2003; Piehl et al., 2004; Rusan et al., 2001; Tirnauer et al., 2004; Tournebize et al., 2000). The fast turnover of MTs during mitosis allows for the swift completion of MT-chromosome capture and spindle assembly. In addition, dynamic instability provides part of the energy and force necessary to effect chromosome movements during mitosis (Dogterom and Yurke, 1997; Inoue and Salmon, 1995; McIntosh et al., 2002; Rieder and Salmon, 1998; Scholey et al., 2003). Motor proteins and MT associated proteins (MAPs) also contribute to force generation, in part by regulating MT dynamics (Hunter and Wordeman, 2000; Kosco et al., 2001; Severin et al., 2001; van Breugel et al., 2003). In budding yeast, MAPs such as Stu1p, Stu2p, and Slk19p, and a subset of the kinesin-like motor proteins (KLPs) contribute to the formation and stability of bipolar spindles by modulating MT dynamics, crosslinking MTs,

and, presumably, by attaching chromosomes to MTs (Hildebrandt and Hoyt, 2000; Kosco et al., 2001; Pasqualone and Huffaker, 1994; Saunders et al., 1997; Severin et al., 2001; Straight et al., 1998; Tytell and Sorger, submitted; Yin et al., 2002; Zeng et al., 1999).

1.5 The Spindle Checkpoint

During DNA replication, duplicate sister chromatids are glued together by cohesin complexes (Nasmyth, 2002). Once these cohesin complexes are dissolved and sister chromatids are separated at the onset of anaphase, there is no turning back. Interestingly, it was noted early on that when *S. cerevisiae* spindles are disrupted by treatment with anti-MT agents, such as the benzimidazole compounds benomyl or nocodazole, or the presence of certain tubulin mutations, cells arrest with large buds and a single, undivided nucleus (Huffaker et al., 1988; Jacobs et al., 1988). This suggested that a mechanism analogous to the DNA damage checkpoint might be involved in monitoring spindle assembly or other MT-dependent processes during cell division. Therefore, screens were performed in *S. cerevisiae* to identify mutants that failed to arrest when grown in the presence of the anti-MT drug benomyl.

Two separate genetic screens were initiated, and each yielded a unique set of mitotic checkpoint genes. The first screen searched for mutants that were unable to recover after 20hrs of growth on plates containing 70μg/ml of benomyl, a dose that is high enough to completely disrupt all visible MT structures (Hoyt et al., 1991). This screen yielded three genes: *B*udding *U*ninhibited by *B*enzimidazole (*BUB*) *1*, *2*, and 3. The second screen searched for mutants that were unable to survive when grown continuously on plates containing only 15μg/ml of benomyl. This screen yielded three different genes: *M*itotic

Arrest Deficient (*MAD*) *1*, *2*, and *3* (Li and Murray, 1991). Neither the *BUB* nor the *MAD* genes are essential under normal growth conditions, however deletion of *BUB1* or *BUB3* initially yields slow growing cells. *bub1*Δ and *bub3*Δ cells eventually overcome this slow growth phenotype in culture, presumably by accumulating one or more compensatory mutations (Hoyt et al., 1991; Roberts et al., 1994).

Subsequent characterization of these genes has revealed that *BUB1*, *BUB3*, and *MAD1-3* all participate in a checkpoint pathway that monitors chromosome-MT attachment and spindle assembly (reviewed in Lew and Burke, 2003), while *BUB2* is involved in a second checkpoint pathway that links spindle positioning to mitotic exit (Bardin et al., 2000; Li, 1999; Pereira et al., 2000). Another critical component of the spindle checkpoint is *MPS1*, a kinase that also has an essential role in SPB duplication (Weiss and Winey, 1996). *BUB1*, *BUB3*, *MAD1-3*, and *MPS1* have all been conserved through evolution, and spindle checkpoint components localize to kinetochores early during mitosis in metazoan cells and *S. pombe* (reviewed in Cleveland et al., 2003). Although the spindle checkpoint is not essential in budding yeast under normal growth conditions, it is essential in animal cells (Basu et al., 1999; Dobles et al., 2000; Kalitsis et al., 2000; Kitagawa and Rose, 1999). The reasons for this discrepancy are uncertain, but differences in the pathways of spindle assembly in each organism likely contribute.

1.6 The Anaphase Promoting Complex

In budding yeast, anaphase onset is characterized by separation and migration of sister chromatids toward opposite poles of the mitotic spindle, as well as spindle elongation

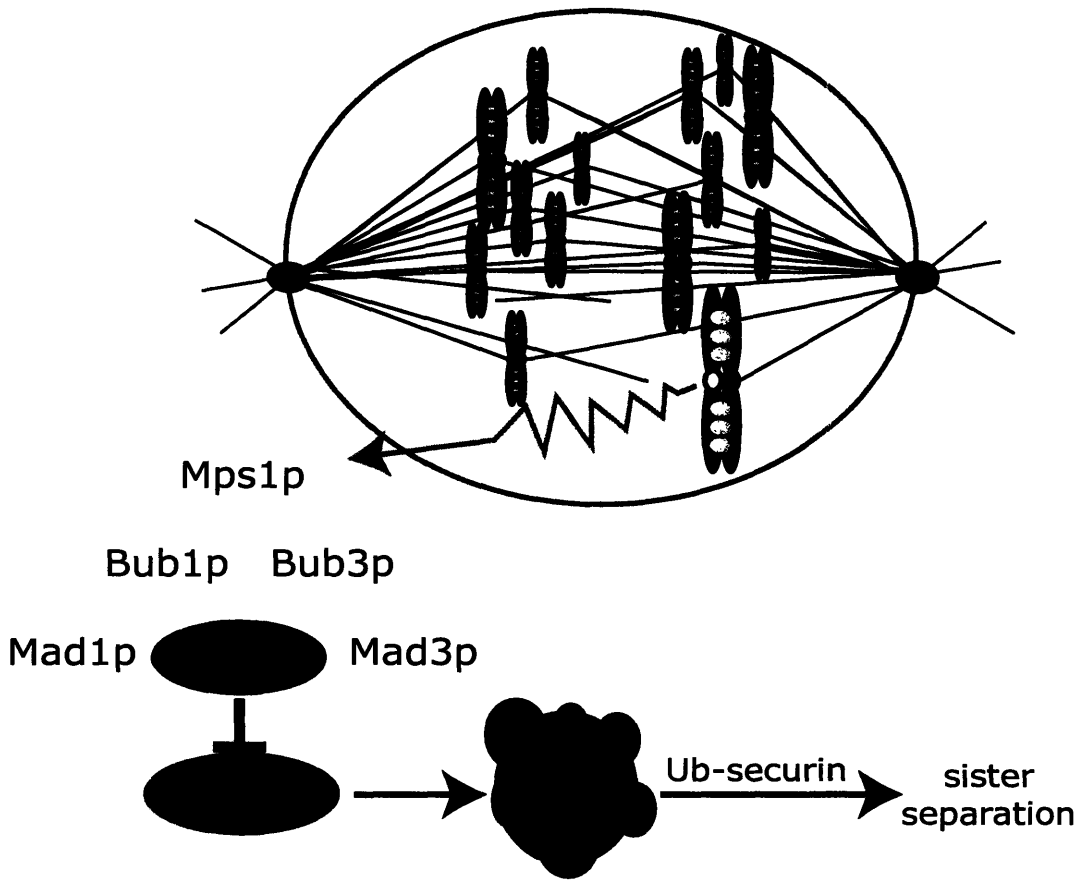

Figure 1.1 Spindle checkpoint components in budding yeast.
Unattached kinetochores signal to delay anaphase via Mps1p, Mad1-3p, Bub1p and Bub3p. Following checkpoint activation, Mad2p binds to Cdc20p and prevents the Anaphase Promoting Complex (APC) from ubiquitinating Pds1p/securin and other targets. Even the presence of a single unattached kinetochore is sufficient to engage the spindle checkpoint (Rieder et al., 1994).

through the bud neck. The anaphase promoting complex (APC), an E3 ubiquitin ligase, is required for passage through the metaphase-to-anaphase transition. The APC is phosphorylated and activated early during mitosis; however, it must also become associated with one of its two specificity factors, Cdc20p or Cdh1p, in order to ubiquitinate specific substrates (Peters, 1998; Schwab et al., 1997; Visintin et al., 1997). Ubiquitination of cyclins and other proteins targets them for destruction by the 26S proteasome (Glotzer et al., 1991; Hershko, 1991).

Interestingly, although loss-of-function mutations in APC components or *CDC20* cause metaphase arrest (Irniger et al., 1995; Tugendreich et al., 1995), expression of non-degradable mitotic cyclins does not arrest cells until telophase (Holloway et al., 1993; Surana et al. 1993). These data indicate that cyclins must not be the only targets ubiquitinated by APC^{Cdc20} at the metaphase-to-anaphase transition. An additional APC substrate called Pds1p/securin was isolated in screens for mutants defective in chromosome segregation (Yamamoto et al., 1996a; Yamamoto et al., 1996b). After replication, sister chromatids in budding yeast are held together by cohesins, tetrameric complexes consisting of Scc1p (also called Mcd1p), Scc3p, Smc1p, and Smc3p (reviewed in Nasmyth, 2002). Pds1p/securin binds to and inhibits separase/Esp1p, a cysteine endopeptidase. Once liberated from securin, Esp1p cleaves the cohesin subunit Scc1p, eliminating cohesion and allowing sister chromatid segregation to proceed (Ciosk et al., 1998).

Securin is the only APC^{Cdc20} substrate that must be degraded to allow passage through the metaphase-to-anaphase transition (Shirayama et al., 1999; Cohen-Fix et al., 1996; Yamamoto et al., 1996a; Yamamoto et al., 1996b). Spindle checkpoint proteins directly interact with and inhibit the activity of Cdc20p (Byers and Goetsch, 1974; Hwang et

al., 1998), thereby preventing premature destruction of securin. In budding yeast, Mad1p, Mad2p, and Mad3p co-immunoprecipitate with Cdc20p (Hwang et al., 1998; Sironi et al., 2001), and *cdc20* mutants that are unable to bind to Mad2p abrogate the spindle checkpoint response to MT-depolymerizing agents (Schott and Hoyt, 1998). In addition, interactions between Mad1 and p55CDC (the metazoan homolog of Cdc20), and between BubR1/Bub3 and p55CDC, have also been documented (Fang, 2002; Fang et al., 1998a; Fang et al., 1998b; Kallio et al., 1998; Tang et al., 2001).

1.7 Attachment versus Tension

Cytological studies have shown that the duration of spindle assembly in metazoan cells is highly variable, and that anaphase onset is significantly delayed in cells that contain unattached or mono-oriented chromosomes (Ault and Rieder, 1992; Rieder, 1990; Rieder, 1991). It was subsequently demonstrated that the time interval between nuclear envelope breakdown and anaphase onset is linearly related to the length of time unattached kinetochores persist in PtK_1 cells, and that the presence of even a single unattached kinetochore is sufficient to delay anaphase onset (Rieder et al., 1994). As kinetochores form the attachment sites for spindle MTs, it is generally thought that unattached kinetochores generate a diffusible "wait for me" signal that prevents the cell cycle machinery from initiating anaphase events until spindle assembly is complete. This idea gained further support when it was shown that laser ablating the kinetochores on the last misaligned chromosome inside a PtK_1 cell allows anaphase to proceed (Rieder et al., 1995) and that functional kinetochores are required for spindle checkpoint function in budding yeast (Gardner et al., 2001).

It is questionable, however, if a checkpoint that only monitors MT occupancy at kinetochores is sufficient to ensure accurate chromosome segregation. If sister kinetochores are configured in such a manner that it is unlikely for both of them to form attachments to MTs nucleated by a single spindle pole, then strictly monitoring attachment would be sufficient to ensure bi-orientation in most cases. During meiosis I in metazoan cells, however, homologous chromosomes are linked together by chiasmata and homologous kinetochores can move relatively independently from one another. In this situation, the kinetochores on homologous chromosomes attach to the same spindle pole quite frequently during early meiosis (Lew and Burke, 2003). However, these monopolar attachments are usually corrected prior to anaphase I, suggesting either that the spindle checkpoint recognizes mono-oriented chromosomes and delays anaphase until they have become bi-oriented, or that mono-oriented attachments are unstable enough to be released and corrected during most meioses.

It was initially proposed by McIntosh that one mechanism the spindle checkpoint might employ to distinguish kinetochores that are bi-oriented from kinetochores that are mono-oriented is to measure tension (Lew and Burke, 2003; McIntosh, 1991). In this case, a checkpoint tension sensor would activate the checkpoint and prevent anaphase onset in the presence of unattached and mono-oriented kinetochores, neither of which experience bipolar tension. Consistent with the tension hypothesis, using a micromanipulation needle to apply artificial tension across the last mono-oriented kinetochore in mantid spermatocytes allows anaphase to proceed (Li and Nicklas, 1995).

The tension hypothesis of spindle checkpoint regulation is elegant and appealing. However, the process of kinetochore-MT attachment turns out to be somewhat murkier than

this model suggests. It is complicated, for instance, by the fact that tension across kinetochores stabilizes MT attachments, allowing bi-oriented kinetochores to bind more MTs than mono-oriented kinetochores (Nicklas and Ward, 1994; King and Nicklas, 2000). Therefore, disentangling the effects of tension and MT attachment on spindle checkpoint signaling is difficult in systems where MT recruitment and the stability of kinetochore-MT attachments are modulated by tension. Theoretically, it may be easier to distinguish between the effects of tension and attachment in organisms such as budding yeast where each kinetochore recruits a single MT and chromosome-MT attachment may be an all-or-nothing event.

Interestingly, it does appear that the chemistry of kinetochores changes in response to tension. The 3F3 antibody, for instance, recognizes kinetochore-specific phosphoepitopes that are present on unattached kinetochores but absent on kinetochores that are under bipolar tension (Nicklas et al., 1995). Additionally, while associations of the metazoan spindle checkpoint proteins Mad1 and Mad2 are modulated by attachment status, kinetochore binding of the mammalian checkpoint protein BubR1 (the homolog of yeast Mad3p) appears to be sensitive to tension. Therefore, some have proposed that metazoan cells utilize a bifurcated checkpoint with a Mad2-dependent branch that monitors attachment and a BubR1-dependent branch that monitors tension (Skoufias et al., 2001).

1.8 Kinetochores in Budding Yeast

Centromeres were first defined as genetic loci required for stable chromosome transmission. Each wild-type chromosome contains one centromere, and each centromere serves as the assembly site for a single kinetochore (reviewed in Cleveland et al., 2003).

Centromeres in *S. cerevisiae* are well defined and each CEN region consists of a conserved stretch of 125 basepairs that is both necessary and sufficient to promote stable chromosome transmission during mitosis and meiosis (Clarke and Carbon, 1980; Cottarel et al., 1989). Haploid budding yeast cells have 16 chromosomes, and electron microscopy studies indicate that each kinetochore is captured by a single MT in this organism (Winey et al., 1995).

Centromeres and kinetochores in *Schizosaccharomyces pombe* and metazoans are considerably more complex than those found in *S. cerevisiae*. Human kinetochores, for instance, are assembled on stretches of DNA several megabases in length that contain numerous repeats of a conserved ~170 basepair α-satellite DNA element (Bjerling and Ekwall, 2002; Cleveland et al., 2003); however, these α-satellite repeats are not strictly required for centromere assembly as neo-centromeres sometimes form without detectable α-satellite sequences (Depinet et al., 1997; Wandall et al., 1998). In contrast to budding yeast kinetochores which recruit only a single MT, metazoan kinetochores each recruit between 15 and 30 MTs (McEwen et al., 1997). In an extreme case, *C. elegans* utilizes diffuse holocentric kinetochores that recruit MTs along the entire length of the chromosomes (Maddox et al., 2004).

Although budding yeast contain the best defined and simplest eukaryotic kinetochores characterized to date, assembly of a functional MT binding site on centromeric DNA still requires greater than 60 different proteins in this organism. These kinetochore components can be classified into three groups: DNA binding components, MT binding components, and linker components that bridge the DNA and MT binding layers (see Fig. 1.2; McAinsh et al., 2003). The extensive catalog of kinetochore components in budding yeast makes it an

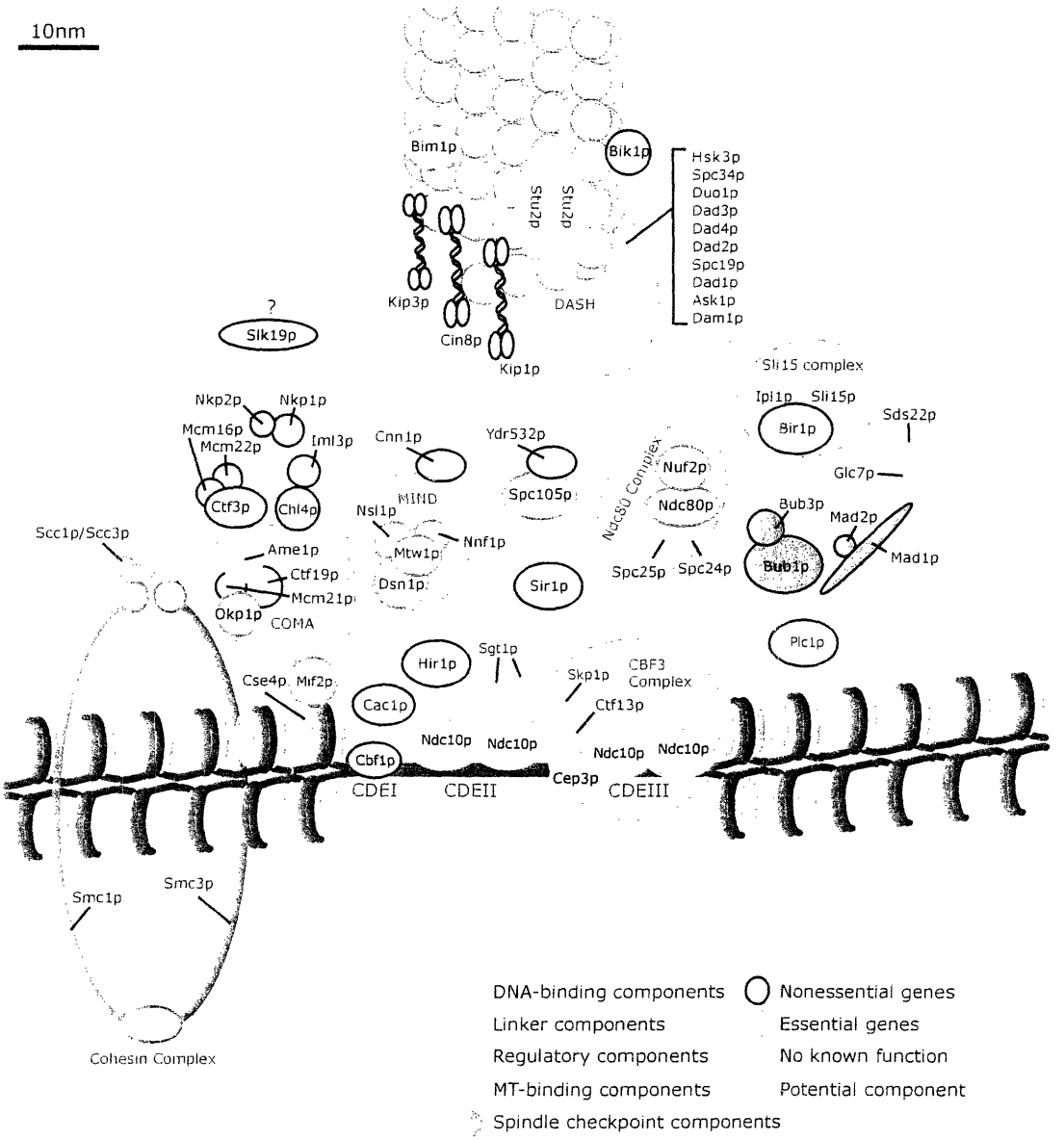

Figure 1.2 Architecture of a budding yeast kinetochore.
This figure depicts a speculative model for the organization of established kinetochore components. Known subcomplexes are enclosed in broken circles. Proteins are shown approximately to scale based on data from hydrodynamic analysis (when available). This schematic was adapted from a figure originally designed by Andrew McAinsh.

excellent system in which to characterize the interface between spindle checkpoint and kinetochore proteins. Checkpoint function appears to map to two kinetochore subcomplexes, the DNA-binding CBF3 complex and the linker NDC80 complex (Gardner et al., 2001; McCleland et al., 2004). As described below, temperature sensitive mutations in different kinetochore components also confer specific types of chromosome-MT attachment defects and can thus be used to analyze the process of spindle checkpoint activation.

1.8.1 DNA Binding Components

DNA binding kinetochore proteins associate with centromeric DNA and form the foundation of the kinetochore. DNA binding components include the CBF3 complex, which consists of Ndc10p, Cep3p, Ctf13p, and Skp1p (Connelly and Hieter, 1996; Doheny et al., 1993; Goh and Kilmartin, 1993; Lechner and Carbon, 1991; Strunnikov et al., 1995), specialized histones containing the histone H3 variant Cse4p, called CENP-A in metazoans (Meluh et al., 1998), and Cbf1p (Cai and Davis, 1990). All components of the CBF3 complex and *CSE4* are essential, whereas *CBF1* is nonessential but still required to achieve wild-type levels of chromosome transmission fidelity (Cai and Davis, 1990).

The 125bp budding yeast centromere can be separated into three conserved elements: CDEI, CDEII, and CDEIII (Fitzgerald-Hayes et al., 1982). CDEI and CDEIII are imperfect palindromes. Cbf1p binds to the CDEI element and CBF3 binds to the CDEIII element (Cai and Davis, 1990; Lechner and Carbon, 1991; Ng et al., 1986). The exact sequence of CDEII is quite variable, but this element maintains a consistent length of ~85bp and is always AT-rich (Clarke and Carbon, 1980). The CBF3 component Ndc10p is the only known protein to bind to CDEII *in vitro* (Espelin et al., 2003). Ndc10p is thought to bind to CDEII in the

absence of other CBF3 components, but other proteins such as the kinetochore components Mif2p (called CENP-C in metazoans) and the histone H3 variant Cse4p (CENP-A) have also been proposed to bind CDEII (Espelin et al., 2003). Notably, CBF3 function is required for kinetochore assembly and *ndc10-1* mutants completely disrupt MT attachment and centromere association of all other kinetochore components tested to date (Goh and Kilmartin, 1993; He et al., 2001).

Despite loss of chromosome-MT attachment, *ndc10-1* mutants do not activate the spindle checkpoint at non-permissive temperature (Gardner et al., 2001). Although it has been suggested that the CBF3 component Skp1p mediates Bub1p binding to kinetochores (Kitagawa et al., 2003), other data have shown that loss of the NDC80 linker complex (described below) also abrogates checkpoint activity (McCleland et al., 2004). As loss of NDC80 function does not disrupt the DNA binding layer, this result suggests that the CBF3 complex is necessary, but not sufficient, for checkpoint protein activation at kinetochores.

1.8.2 Linker Components

The central layer of *S. cerevisiae* kinetochores contains proteins essential for kinetochore function that do not have established DNA- or MT-binding activities. Linker components of the kinetochore include the NDC80 complex, the MIND complex, and the COMA complex. The NDC80 complex consists of four essential subunits—Ndc80p, Nuf2p, Spc24p, and Spc25p—all of which have homologs in metazoan cells (Bharadwaj et al., 2004; McCleland et al., 2003; McCleland et al., 2004; Wigge and Kilmartin, 2001). The MIND complex also consists of four essential proteins—Mtw1p, Nnf1p, Nsl1p, and Dsn1p—but homologs of these components are yet to be identified in metazoans (De Wulf et al., 2003;

Euskirchen, 2002; Nekrasov et al., 2003; Shan et al., 1997). The COMA complex contains two essential proteins—Okp1p and Ame1p—and two non-essential proteins—Ctf19p and Mcm21p—for which no metazoan homologs have been identified (De Wulf et al., 2003; Hyland et al., 1999; Ortiz et al., 1999; Poddar et al., 1999). The NDC80, MIND, and COMA complexes are not interdependent for centromere association, suggesting that the budding yeast kinetochore is assembled using a tripartite structure in which each linker complex forms an independent branch. Loss of functional NDC80 complex detaches chromosomes from MTs (He et al., 2001; Janke et al., 2001; Wigge and Kilmartin, 2001), while temperature sensitive mutations in components of the MIND and COMA disrupt chromosome dynamics and force generation but do not appear to cause complete loss of attachment (De Wulf et al., 2003).

An additional kinetochore complex whose role is less well defined is the Ctf3 complex which contains Ctf3p, Mcm15p, and Mcm22p (Measday et al., 2002). Components of the Ctf3 complex localize exclusively to kinetochores and are essential in metazoans, but nonessential in budding yeast (Goshima et al., 2003; Measday et al., 2002). Interestingly, the *S. pombe* homolog of Ctf3p, Mis6+, is required for recruiting the fission yeast Cse4p-like histone, Cnp1+, to centromeric DNA (Takahashi et al., 2000); however, the opposite dependency exists in *S. cerevisiae* and human cells (Goshima et al., 2003; Measday et al., 2002). It is currently hypothesized that the Ctf3 complex contributes to some as yet undefined aspect of centromeric chromatin assembly (McAinsh et al., 2003).

1.8.3 Microtubule Binding Components

Although it was initially postulated that a single motor protein might be sufficient to link budding yeast kinetochores to MTs, the reality has turned out to be much more complicated. Of the four nuclear KLPs in *S. cerevisiae*, three—Kip1p, Kip3p, and Cin8p—associate with kinetochores (Tytell and Sorger, submitted). No individual KLP is essential in budding yeast; however, *cin8Δ* is synthetically lethal with *kip1Δ* suggesting that there is some functional redundancy between these two KLPs (Hoyt et al., 1992; Roof et al., 1992). Interestingly, deletion of any single KLP has a unique effect with regard to anaphase movement. *cin8Δ* cells lack the rapid phase of anaphase B movement, *kip1Δ* cells have defects in the slow phase of anaphase B, and *kip3Δ* cells exhibit prolonged anaphase and impaired chromosome-to-pole movement during anaphase A (Straight et al., 1998; Tytell and Sorger, submitted). Whether or not these anaphase defects are due primarily to the roles that these KLPs play at the kinetochore is uncertain. Both Cin8p and Kip1p are BimC class motors that form homotetramers and can crosslink MTs (Hildebrandt and Hoyt, 2001); interestingly, both of these motors appear to help cluster kinetochores during metaphase (Tytell and Sorger, submitted). Both Cin8p and Kip1p also require the NDC80 complex to achieve wild-type levels of kinetochore association, whereas Kip3p does not (Tytell and Sorger, submitted). This suggests that Cin8p and Kip1p are likely outer kinetochore proteins, whereas Kip3p may be part of the inner kinetochore. As functional NDC80 complex is required for chromosome-MT attachment, Cin8p and Kip1p association with kinetochores may also be dependent on MTs. It has yet to be determined if any of the three KLPs require the COMA or MIND complexes for CEN association.

In addition to the KLPs, several MAPs associate with kinetochores including Stu2p, Bik1p, Slk19p, and the DASH complex (Cheeseman et al., 2001; He et al., 2001; Jones et al., 2001; Lin et al., 2001; Pellman et al., 1995). Stu2p is an essential protein homologous to Xenopus XMAP215 and human ch-TOG1 (Wang and Huffaker, 1997). Stu2p localizes to kinetochores and cortical tips, two sites where MT plus ends are concentrated, and also along the length of MTs (He et al., 2001). Although the majority of members of the TOG/XMAP215 family are thought to stabilize MTs (Gard and Kirschner, 1987; Tournebize et al., 2000; Vasquez et al., 1994), budding yeast Stu2p actually appears to destabilize them (van Breugel et al., 2003). Consistent with this, MTs seem to be less dynamic in *stu2* mutants than in wild-type cells (Kosco et al., 2001). Most kinetochores in *stu2* mutants maintain bi-orientation, however they appear to experience significantly less bipolar tension (He et al., 2001).

Bik1p binds to the plus ends of MTs and localizes to kinetochores and cortical tips (Berlin et al., 1990; He et al., 2001). Bik1p is homologous to human CLIP170 which plays a role in linking MT- and actin-based elements of the cytoskeleton (Brunner, 2002; Perez et al., 1999). Although deletion of *BIK1* does not have a dramatic effect on haploid or diploid cells, it is essential for kinetochore-MT attachment in polyploid yeast (Lin et al., 2001). Another MT plus end binding protein, Bim1p, is also a candidate kinetochore protein (Schwartz et al., 1997). Bim1p is the homolog of human EB1, a protein that binds to the adenomatous polyposis coli (AdPC) protein known to associate with the plus ends of MTs (Maekawa and Schiebel, 2004; Schwartz et al., 1997). Although it has not been demonstrated that Bim1p associates with kinetochores in budding yeast, EB1 is known to localize to kinetochores during metaphase in metazoan cells (Tirnauer et al., 2002a; Tirnauer et al., 2002b). As EB1

appears to be an important regulator of MT dynamics, it would not be surprising if Bim1p also plays a role at kinetochore-bound MTs in budding yeast.

SLK19 was isolated in a screen for genes that are synthetically lethal with a deletion of *KAR3*, a KLP motor located on the nuclear face of SPBs (Zeng et al., 1999). Slk19p localizes to kinetochores and, although it is nonessential, *slk19*Δ cells exhibit abnormally short spindles. *SLK19*'s genetic interactions with *KAR3* suggest that these two proteins have overlapping roles in maintaining spindle stability, despite the fact that they function at opposite ends of the MTs.

The DASH complex consists of at least nine essential subunits: Dam1p, Duo1p, Dad1p, Dad2p, Dad3p, Dad4p, Ask1p, Spc19p, and Spc34p (Cheeseman et al., 2002; Cheeseman et al., 2001; Enquist-Newman et al., 2001; Janke et al., 2002; Jones et al., 1999). The DASH complex is required for both establishment and maintenance of chromosome biorientation, as sister chromatids in *dam1-1* and *spc34-3* cells remain associated with a single SPB (He et al., 2001; Janke et al., 2002; Jones et al., 1999). The DASH complex binds to MTs *in vitro* (Hofmann et al., 1998), and DASH association with kinetochores is MT-dependent *in vivo* (Li et al., 2002), suggesting it is an outer kinetochore component.

1.8.4 Transient Kinetochore Components

While the majority of kinetochore components remain associated with centromeres throughout the cell cycle, several components only associate transiently during early mitosis. Of those already mentioned, Kip1p and Cin8p undergo degradation at the end of metaphase and mitosis, respectively (Gordon and Roof, 2001; Hildebrandt and Hoyt, 2001). Additional proteins that exhibit regulated kinetochore association include the Aurora-like kinase Ipl1p

(Buvelot et al., 2003), Slk19p (Zeng et al., 1999), the phosphatase PP2A regulatory subunits Tpd3p and Rts1p (Dobbelaere et al., 2003; Gentry and Hallberg, 2002), and the spindle checkpoint proteins.

The kinase Ipl1p exists in a complex with a second essential protein, Sli15p (Kim et al., 1999). Bir1p, a kinetochore protein with homology to the mammalian anti-apoptotic protein survivin, has also been reported to interact with Ipl1p (Cheeseman et al., 2002; Yoon and Carbon, 1999). The Ipl1p-Sli15p complex behaves in a manner similar to that of chromosomal passenger proteins, such as Aurora B, in metazoan cells. Ipl1p-Sli15p is present at kinetochores from S-phase to early mitosis in yeast, but re-localizes to the spindle midzone at the metaphase-to-anaphase transition (Buvelot et al., 2003); this suggests that the Ipl1p-Sli15p complex may play roles in multiple aspects of spindle function. Consistent with this idea, *ipl1* mutants have difficulty both achieving chromosome biorientation early in mitosis (Tanaka et al., 2002) and breaking down mitotic spindles late in mitosis (Buvelot et al., 2003).

Three other kinetochore proteins that move to the spindle midzone in a manner similar to that of the Ipl1p-Sli15p complex are Slk19p, Ndc10p, and Dam1p (Buvelot et al., 2003; Zeng et al., 1999). The MAP Slk19p is cleaved by Esp1p protease at the metaphase-to-anaphase transition and one of its cleavage products then re-localizes to the spindle midzone. This cleavage product appears to stabilize the mitotic spindle, thereby ensuring timely progression through mitosis (Sullivan and Uhlmann, 2003). Although a fraction of Ndc10p remains associated with centromeric DNA throughout the cell cycle, Ndc10p is also observed at the spindle mid-zone during anaphase and is thought to be a target of the Ipl1p kinase (Buvelot et al., 2003). Similarly, the MT binding component Dam1p relocalizes to the

midzone during anaphase and is also thought to be phosphorylated by Ipl1p (Kang et al., 2001).

In contrast to the other transient kinetochore components described above, the phosphatase PP2A regulatory subunits Tpd3p and Rts1p do not relocalize to the midzone, but instead move from kinetochores to the bud neck where they regulate septin dynamics and cytokinesis (Dobbelaere et al., 2003; Gentry and Hallberg, 2002). The function of these proteins at the kinetochore is not well understood. However, both *tpd3* mutants and budding yeast cells with mutations in the catalytic subunit of PP2A and its third regulatory subunit, *CDC55*, are nocodazole sensitive, suggesting that PP2A may have a role in spindle checkpoint function (Evans and Hemmings, 2000; Wang and Burke, 1997).

Prior to the experiments described herein, kinetochore localization of the spindle checkpoint proteins had only been characterized in metazoans and fission yeast. In metazoans, checkpoint proteins associate with kinetochores early during mitosis (review in (Cleveland et al., 2003)). A subset of spindle checkpoint components localize to centrosomes in metazoan cells, and some are thought to transit along the MTs from the kinetochore to the centrosomes following MT attachment in a dynein-dependent manner (Fisk and Winey, 2001; Gorbsky et al., 1998; Howell et al., 2000; Kallio et al., 2002). It remains to be proven whether or not the centrosomal localization of checkpoint proteins is important for activating or silencing checkpoint signaling.

1.9 Spindle Checkpoint Proteins

1.9.1 The Mad Proteins

Mad1p and Mad2p bind to one another (Chen et al., 1999) and a crystal structure of the tetrameric human Mad1-Mad2 complex has recently been solved (Sironi et al., 2002). The N- and C-termini of hMad1 consist primarily of coiled coils, but they are interrupted at the center by a hMad2 binding domain. hMad1 forms a dimer that binds to two molecules of hMad2. The C-terminus of hMad2 is mobile and acts as a "safety belt," folding over an elongated domain of hMad1 and latching hMad2 into place (Sironi et al., 2002). Mad1 is required for Mad2 binding to kinetochores in *Xenopus* (Chen et al., 1998). Treatment with the anti-MT drug nocodazole arrests cells in mitosis and results in hyperphosphorylation of Mad1p, as does GAL-driven overexpression of Mps1p (Hardwick et al., 1996). However, although overexpression of the mutant Bub1-5 protein also causes mitotic arrest, it does not lead to hyperphosphorylation of Mad1p (Farr and Hoyt, 1998), making the significance of Mad1p phosphorylation in checkpoint signaling uncertain.

Little is known about the structure of Mad3p and there is some debate as to its role in the spindle checkpoint response. Some studies have shown that *mad3*Δ cells have a less severe chromosome loss phenotype than do cells lacking *MAD1* or *MAD2*, and *mad3*Δ cells may also be less benomyl sensitive than *mad1*Δ and *mad2*Δ cells (Warren et al., 2002). It has also been shown that a deletion of *MAD3* is synthetically lethal with only a subset of those mutants known to be synthetically lethal with *MAD1* and *MAD2* deletions (Lee and Spencer, 2004), and although *MAD3* is required for the checkpoint response to unattached kinetochores, it does not appear to be required for the checkpoint response to unreplicated chromosomes that are attached but not under tension (Lee and Spencer, 2004). The closest

homolog of *MAD3* in metazoan cells is the kinase BubR1 (Taylor et al., 1998). The N-terminus of BubR1 is similar to Mad3p, but BubR1 also contains a C-terminal kinase domain similar to that of Bub1. Oddly, although Mad3p's role in the checkpoint response to tension is unclear, the BubR1 kinase appears to be recruited to kinetochores in response to loss of tension (Skoufias et al., 2001).

1.9.2 The Bub Proteins

BUB1 encodes a Ser/Thr kinase, whereas *BUB3* encodes a WD40-repeat β-propeller protein (Hoyt et al., 1991; Roberts et al., 1994; Taylor et al., 1998). Budding yeast Bub1p has been reported to have autophosphorylation activity and to catalyze phosphorylation of Bub3p (Roberts et al., 1994). The MT plus end binding protein AdPC is also a high affinity substrate of human Bub1 and the related kinase BubR1 (Kaplan et al., 2001). Bub1p and Bub3p bind to one another and Bub1p is thought to require Bub3p for kinetochore localization in human cells (Taylor et al., 1998). Although Bub3p has been reported to associate with kinetochores independently of Bub1p in PtK_2 cells (Howell et al., 2004), it may require Bub1p for kinetochore binding in *Xenopus* (Sharp-Baker and Chen, 2001).

Interestingly, although the kinase domain of Bub1p is conserved from yeast to metazoans, Bub1p's kinase activity is not absolutely required for checkpoint function in *Xenopus* or in budding yeast, suggesting that it may be important for other aspects of mitosis (Sharp-Baker and Chen, 2001; Warren et al., 2002). Initial reports showed that the kinase activity of *Xenopus* Bub1p is not required for mitotic arrest in response to nocodazole or for assembly of spindle checkpoint components onto kinetochores (Sharp-Baker and Chen, 2001); however, a more recent study suggests that the kinase activity of *Xenopus* Bub1p can

enhance the efficacy of the checkpoint in response to weaker stimuli, such as only one or two unattached kinetochores (Chen, 2004).

1.9.3 Other Spindle Checkpoint Kinases

Another kinase required for spindle checkpoint activity is Mps1p, an essential dual specificity kinase that plays a role in both checkpoint function and SPB duplication (Weiss and Winey, 1996). To date, the only known targets of the Mps1p kinase in yeast are the SPB components Spc42p (Castillo et al., 2002), Spc98p (Pereira et al., 1998), and Spc110p (Friedman et al., 2001), although genetic interactions between *MPS1* and the kinetochore component *DAM1* have also been reported (Jones et al., 1999). Mps1 kinase activity is required for kinetochore localization of Mad1 and Mad2 in *Xenopus* and humans (Abrieu et al., 2001; Liu et al., 2003). In budding yeast, overexpression of either Mps1p or a Bub1-5 mutant protein induces mitotic arrest in the absence of spindle damage (Farr and Hoyt, 1998; Hardwick et al., 1996). Mps1p is thought to be an upstream component of the spindle checkpoint as GAL-MPS1 overexpression requires all of the *BUB* and *MAD* genes to establish an arrest (Hardwick et al., 1996). Puzzlingly, although the GAL-MPS1 overexpression phenotype requires the Bub and Mad proteins, functional kinetochores do not appear to be required for this arrest (Fraschini et al., 2001; Poddar et al., 2004).

An additional kinase that may play a role both in kinetochore-MT attachment and checkpoint signaling is the Ipl1p kinase. *ipl1-321* kinetochores do not achieve bi-orientation at non-permissive temperature; however, they also do not invoke a spindle checkpoint arrest (Biggins and Murray, 2001). One interpretation of this result is that the Ipl1p kinase participates in the checkpoint signaling cascade. A second interpretation is that the structure

of kinetochores in *ipl1-321* cells is unrecognizable to the checkpoint machinery. Interestingly, *ipl1-321* cells do arrest in nocodazole at non-permissive temperature (Biggins and Murray, 2001) indicating that unattached kinetochores are still competent to activate the checkpoint in *ipl1-321* cells. It has been proposed that Ipl1p is required to activate the checkpoint specifically in response to lack of bipolar tension (Biggins and Murray, 2001). However, as Ipl1p is also postulated to facilitate MT turnover at kinetochores that have formed syntelic attachments (in which both kinetochores are attached to MTs emanating from the same SPB), it is also possible that Ipl1p activates the checkpoint by transiently detaching kinetochores from spindle MTs. Interestingly, although *dam1-1* cells have mono-oriented chromosomes similar to those in *ipl1-321* cells, *dam1-1* mutants are able to engage a spindle checkpoint response, perhaps due to the presence of active Ipl1p kinase (Jones et al., 1999). Future analysis of *ipl1-dam1* double mutants will hopefully lend insight into Ipl1p's role in spindle assembly and checkpoint signaling.

1.9.4 Differences between the Mads and Bubs

Several lines of evidence suggest that the Bub proteins may have roles in mitosis that the Mad proteins do not share. For instance, *bub1*Δ and *bub3*Δ cells have higher rates of chromosome loss than do deletions of *MAD1*, *MAD2*, or *MAD3* (Gardner et al., 2001). In addition, overexpression of Bub1p or Bub3p suppresses the kinetochore attachment defects of *tub1-729* cells in a manner that is independent of Mad2p checkpoint signaling (Abruzzi et al., 2002). Intriguingly, although spindle checkpoint activation does not absolutely require Bub1p kinase activity (Sharp-Baker and Chen, 2001; Warren et al., 2002), suppression of the

tub1-729 cold sensitive mitotic phenotype does require Bub1p's kinase domain (Abruzzi et al., 2002).

Mad proteins may also have roles that the Bub proteins do not share as recent work in animal cells also suggests that Mad2 has a Bub-independent role in regulating the basal timing of mitosis by preventing activation of the anaphase promoting complex (APC) prior to kinetochore assembly (Meraldi et al., 2004). Another property that appears to be unique to Mad1p and Mad2p is that both of these proteins associate with the nuclear periphery. This is true in animal cells during interphase and in budding yeast throughout the cell cycle (Campbell et al., 2001; Chen et al., 1998; Iouk et al., 2002). It has been reported that Mad1p associates with a subcomplex of nucleoporins containing Nup53p, Nup170p, and Nup157p, and that deletion of *MAD1* reduces nuclear transport rates by about two-fold (Iouk et al., 2002).

1.9.5 Spindle Checkpoint Proteins and Nuclear Pores

Intriguingly, there seem to be several connections between nuclear pore complexes (NPCs) and kinetochores. For instance, mutating the budding yeast nucleoporin *NUP170* leads to kinetochore and chromosome segregation defects (Kerscher et al., 2001), and nucleoporins such as hNup133 and hNup107 relocalize from NPCs to kinetochores during mitosis in human cells (Belgareh et al., 2001). In addition, the nuclear transport factor Ran-GTP plays a role in spindle and kinetochore assembly (reviewed in Di Fiore et al., 2004 and Salina et al., 2003) and several of its regulatory proteins associate with kinetochores following nuclear envelope breakdown in human cells (Arnaoutov and Dasso, 2003; Joseph et al., 2002). In addition, there is a significant degree of sequence similarity between the

spindle checkpoint protein Bub3 and the nuclear import factor Rael. Rael can functionally substitute for Bub3 in mice, and haploinsufficiency of murine RAE1 leads to chromosome missegregation and spindle checkpoint defects similar to those found in BUB3 heterozygotes (Babu et al., 2003).

1.10 Conclusion

Spindle assembly checkpoint activation in budding yeast requires functional kinetochores as well as the activities of Bub and Mad proteins, Mps1p, and Cdc20p. However, the molecular details of how unattached or mal-oriented kinetochores generate the "wait anaphase" signal remain uncertain. Budding yeast has many advantages as a model system for studying the dynamics of spindle checkpoint signaling. First, the spindle checkpoint is nonessential in this organism, therefore mutations and deletions in spindle checkpoint genes can be easily made. Second, this organism contains the simplest known and best characterized eukaryotic kinetochores known to date, making it an excellent system in which to characterize the interface between kinetochore components and spindle checkpoint proteins. Third, mutations in specific kinetochore components have been shown to produce distinct effects on MT-kinetochore attachment and chromosome dynamics in budding yeast; this allows one to probe the response of spindle checkpoint proteins to a variety of spindle defects including unattached kinetochores (as are found in *ndc80-1* mutants), mono-oriented chromosomes (as are found in *dam1-1* and *ipl1-321* mutants), and bi-oriented chromosomes with reduced tension (as are found in *stu2-279* mutants) (He et al., 2001). The conservation of the spindle checkpoint proteins through evolution also makes yeast a good system in which to test the behavior of rational mutants based on the observed

behavior of checkpoint components in animal cells. Studies of the spindle checkpoint components in budding yeast are likely to advance our understanding of spindle checkpoint signaling dynamics, illuminating both the types of lesions recognized by the checkpoint and how the Mad and Bub proteins execute mitotic arrest. Such data may shed light on the mechanisms responsible for generating aneuploid cells and may eventually suggest therapeutic strategies for targeting tumor cells that harbor checkpoint defects and aberrant numbers of chromosomes.

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Chapter 2

Spindle Checkpoint Proteins & Kinetochore-Microtubule Attachment

Spindle Checkpoint Proteins & Kinetochore-Microtubule Attachment

The following chapter was adapted, with permission, from Gillett et al. 2004. The chromatin immunoprecipitation experiments presented herein were performed by, or in collaboration with, Christopher W. Espelin. C.W.E. also performed the benomyl sensitivity assays on GFP-fusion strains found in Supplemental Figure 2.S1.

Gillett, E.S.*, C.W. Espelin* and P.K. Sorger. 2004. Spindle checkpoint proteins and kinetochore-microtubule attachment in budding yeast. *J. Cell Biol.* 164:535-46. *These authors contributed equally to this work.

2.1 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 *Saccharomyces 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, although 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.

ABBREVIATIONS: 3D, three-dimensional; BUB, budding uninhibited by benzimidazole; CEN, centromeric; CFP, cyan fluorescent protein; ChIP, chromatin immunoprecipitation; GFP, green fluorescent protein; MAD, mitotic arrest defective; MT, microtubule; SPB, spindle pole body.

2.2 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, which are multiprotein 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 (Rieder et al., 1994; Li and Nicklas, 1995).

Spindle checkpoint genes were first identified in budding yeast and include the mitotic arrest defective (MAD) genes *MAD 1–3* (Li and Murray, 1991) and the budding uninhibited by benzimidazole (BUB) 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 (for review, see Yu, 2002). At the metaphase to anaphase transition, Cdc20p activates the anaphase promoting complex, 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 (for review, see 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 (for review, see Cleveland et al., 2003), and functional kinetochores are required to generate the checkpoint signal in both animal cells and budding yeast (Rieder et al., 1995; Gardner et al., 2001). 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 wild-type levels of tension (Biggins et al., 1999; Kim et al., 1999; He et al., 2001; Janke et al., 2001; Wigge and Kilmartin, 2001; Janke et al., 2002; Tanaka et al., 2002).

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 *Saccharomyces cerevisiae* kinetochores. Although 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.

2.3 RESULTS

2.3.1 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 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. 2.S1). Spindle pole bodies (SPBs) were visualized by linking the SPB component Spc42p to CFP (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 three-dimensional (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. 2.1, A and B). 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. 2.1C; (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. 2.1, D and E). Binding was specific as neither protein cross-linked to DNA at the non-*CEN URA3* locus (unpublished data). 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. 2.1, D and E).

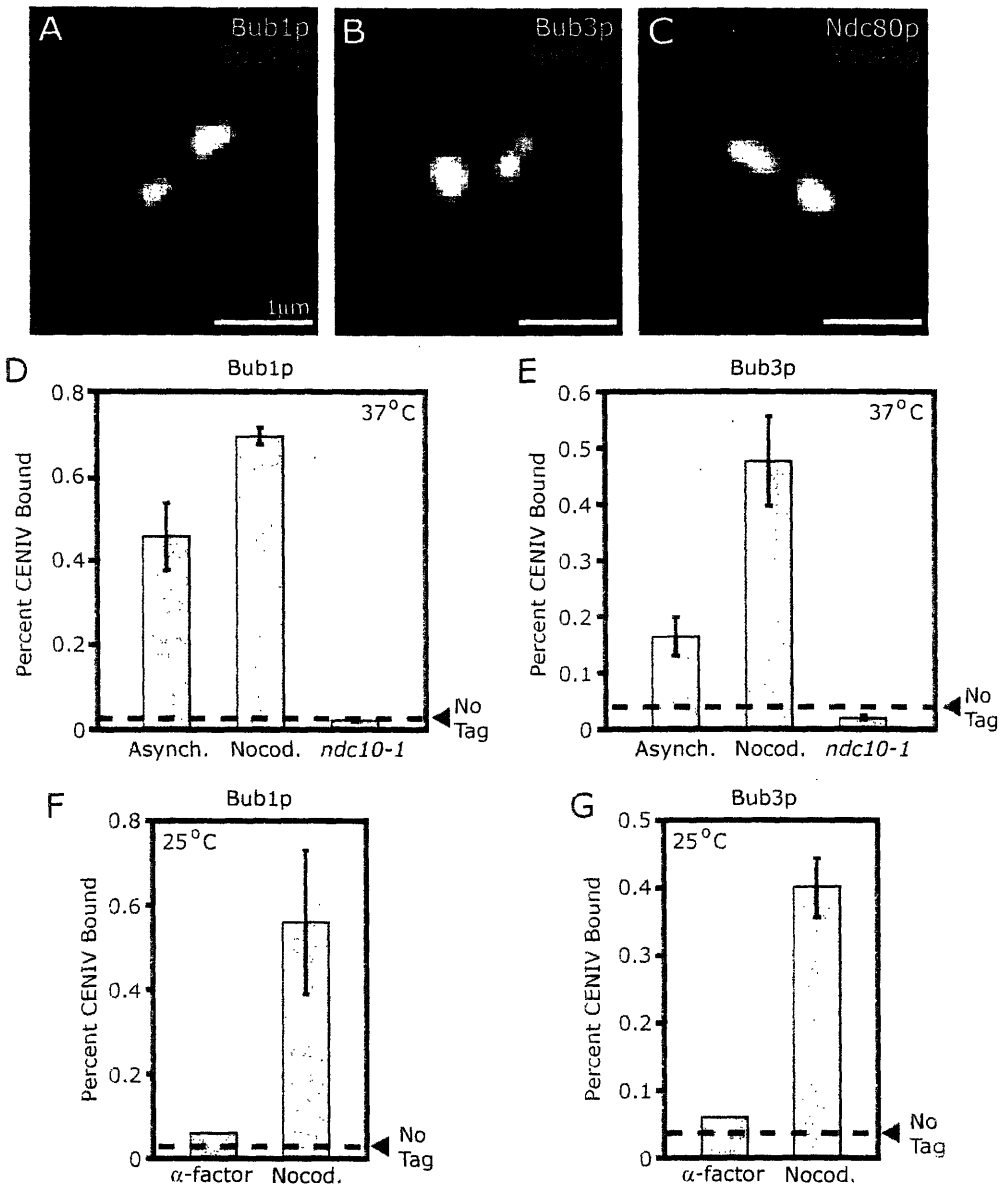


Figure 2.1 Bub1p-GFP and Bub3p-GFP are associated with kinetochores.
(A-C) Typical images of wild type mitotic cells expressing the SPB marker Spc42p-CFP (in red) and the spindle checkpoint proteins Bub1p-GFP or Bub3p-GFP (in green) or the kinetochore protein Ndc80p-GFP (in blue). Images represent 2D projections of 3D image stacks containing ten to fifteen 0.2μm sections. (D, E) ChIP of Bub1p-GFP and Bub3p-GFP at CENIV. Crosslinking of Bub proteins to CEN DNA was assayed in asynchronous wild type cells, nocodazole-treated wild type 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 hrs prior to 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 wild type cells (a negative control). Absolute differences in the amount of DNA precipitated among different panels are not considered to be meaningful. (F, G) ChIP of Bub proteins at *CENIV* is cell cycle regulated. Wild-type cells expressing Bub1p-GFP or Bub3p-GFP were grown to mid-log phase at 25°C and then treated with α-factor (5μg/mL final) or nocodazole (25μg/mL final) for 3 hrs prior to ChIP analysis.

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. 2.1, D and E). In contrast, in α-factor arrested G1 cells, ChIP signals for Bub1p and Bub3p fell to background levels (Fig. 2.1, F and G). 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.

2.3.2 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 min. 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 Bub1-GFP prior to T= 45 min. (Fig. 2.2, A and C). Kinetochore binding by Bub1p then rose dramatically, peaking at T= 60 min., and fell again as mitosis progressed (Fig. 2.2C). In contrast, kinetochore binding by Ndc80p-GFP was apparent throughout the experiment, giving rise at early time points to a single GFP

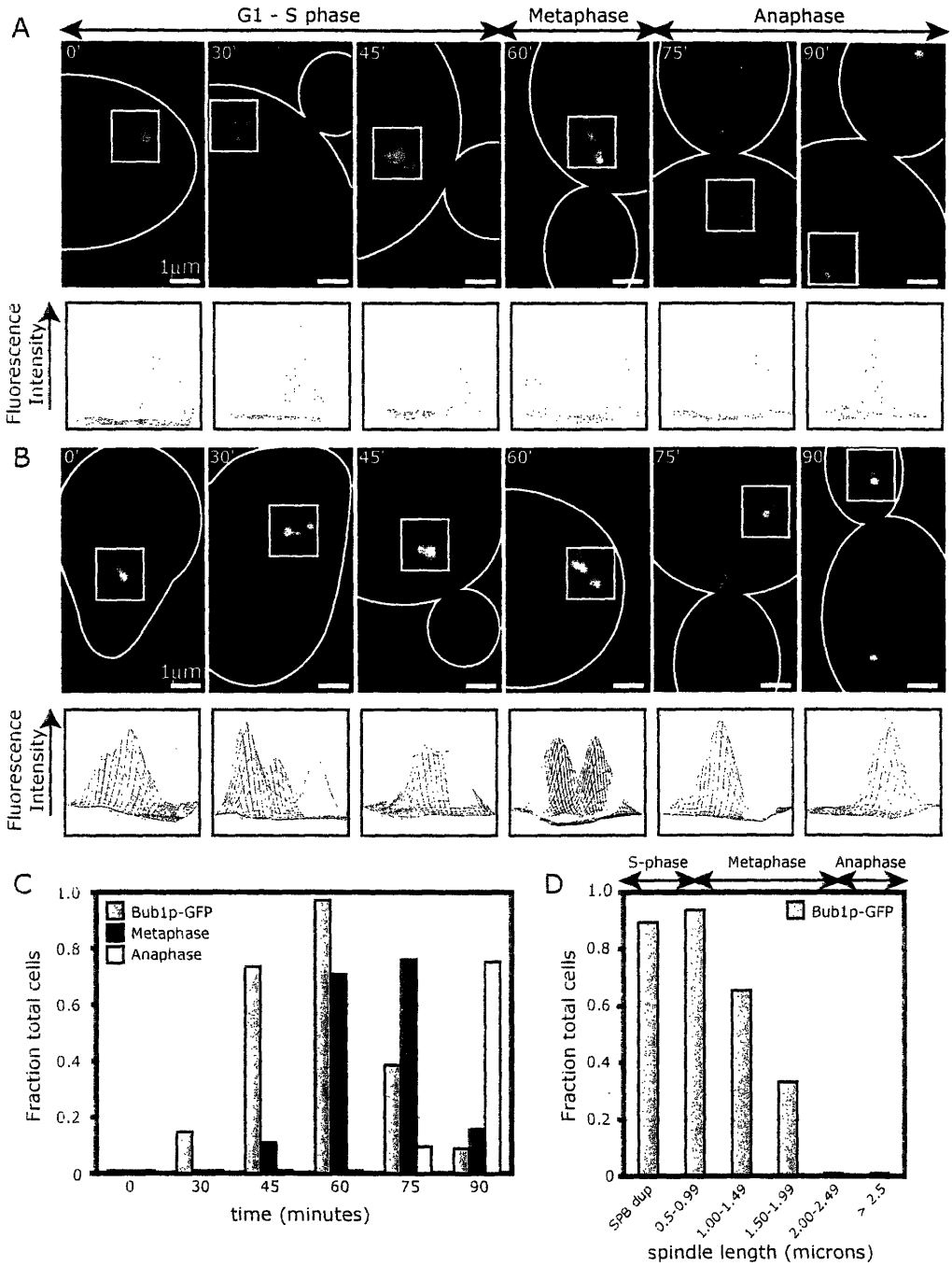


Figure 2.2 Kinetochore association of Bub proteins is cell cycle regulated.
(A) Wild type cells expressing Bub1p-GFP (green) and the SPB component Spc42p-CFP (red). Images are representative for each time point. The surface plot below each image depicts the distribution of GFP (green) and CFP (red) signal 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 Spc42-CFP (red). Images and graphs are as described for part A. (C) Fraction of total cells containing Bub1p-GFP kinetochore foci, metaphase spindles, and anaphase spindles versus time following -factor release at 25°C. Metaphase cells were those with spindle lengths between 0.8µm and 2.2µm and anaphase cells those with spindles > 2.2µm. At least 40 individual cells were scored at each time point. (D) Fraction of cells containing Bub1p-GFP kinetochore foci versus spindle length following α-factor release at 25°C (n = 281).

cluster in close proximity to the newly duplicated SPBs and subsequently resolving into a bi-lobed metaphase configuration (Fig. 2.2B).

Bub1p-GFP foci were first visible around the time of SPB duplication (during S-phase, at T= 30-45 min; Fig. 2.2, C and D). 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. 2.2C), indicating that Bub1p is released from kinetochores as metaphase proceeds. No Bub1p-GFP foci were seen in anaphase cells (Fig. 2.2A, 75 and 90 min; Fig. 2.2D). Bub1p was also absent from kinetochores arrested in metaphase by *cdc23-1* or *cdc20-1* mutations (unpublished data). 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 those of Bub1p-GFP (unpublished data). 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.

2.3.3 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. 2.3A, not depicted; Iouk et al., 2002). However, ChIP signals were high for both Mad1p-GFP and Mad2p-GFP in nocodazole-treated cells (Fig. 2.3A). The ChIP signal for Mad3p-GFP was consistently just above background levels in nocodazole-treated cells (Fig. 2.3A), but we have been unable to confirm kinetochore association by microscopy (not depicted). 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. 2.3B). However, most cells also contained 1 or 2 dim Ndc80p kinetochore foci $\geq$ 1 μm away from the SPBs (Fig. 2.3B, arrowheads). 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. 2.3, C-E). Some Mad1p-GFP also remained on the nuclear periphery (Fig. 2.3D; Iouk et al., 2002). 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.

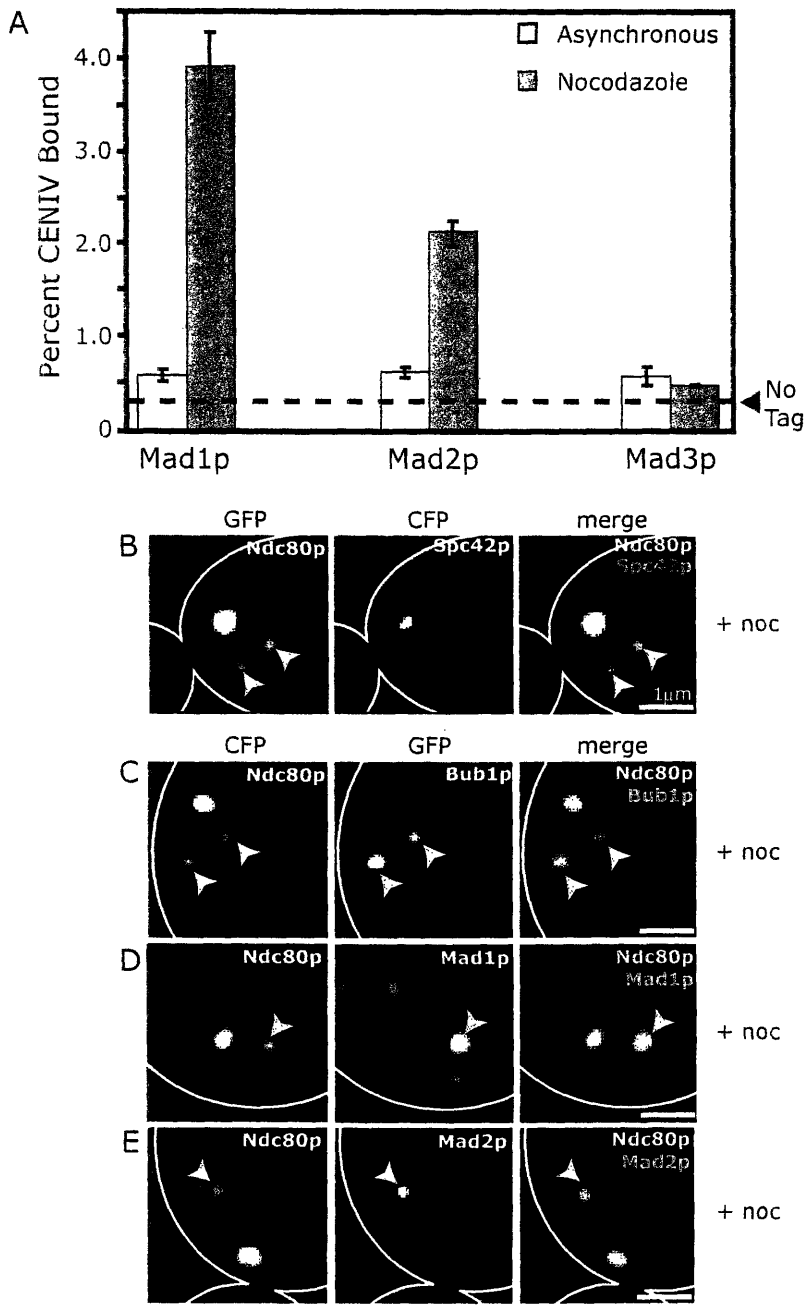
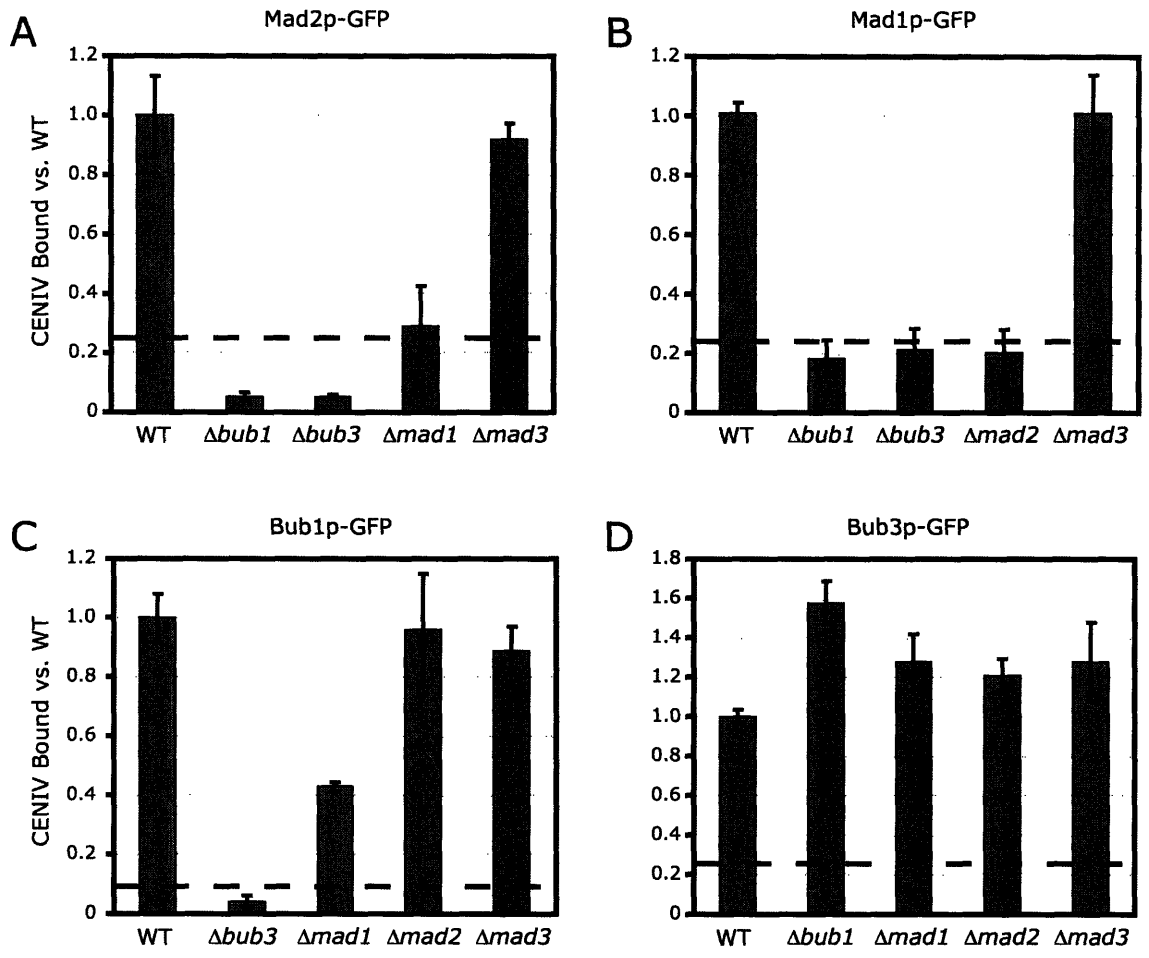


Figure 2.3 Kinetochore association of spindle checkpoint proteins in nocodazole-treated cells. (A) ChIP of Mad1p-GFP, Mad2p-GFP, and Mad3p-GFP at CENIV in cycling and nocodazole treated cells. Graphs are as described for Figure 1D-G. (B) Wild type cell co-expressing the SPB protein Spc42p-CFP and the kinetochore protein Ndc80p-GFP following treatment with 25μg/mL nocodazole for 1 hr at 25°C. Panels show Ndc80p-GFP alone; Spc42p-CFP alone; and Spc42p-CFP (red) merged with Ndc80p-GFP (blue). (C, D, E) Wild type cells co-expressing Ndc80p-CFP (blue) and Mad1p-GFP, Mad2p-GFP, or Bub1p-GFP (green) following nocodazole treatment. Panels are laid out as in (B). Orange arrowheads indicate the locations of unattached kinetochores.

2.3.4 A functional checkpoint pathway is required for kinetochore recruitment of Mad1p and 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 of Mad2p-GFP was abolished in *bub1Δ* and *bub3Δ* cells, as well as in cells lacking *MAD1*, but not in cells lacking *MAD3* (Fig. 2.4A). *CEN* association by Mad1p-GFP exhibited a similar set of dependencies, requiring *BUB1*, *BUB3*, and *MAD2*, but not *MAD3* (Fig. 2.4B). In contrast, both Bub1p-GFP and Bub3p-GFP associated with *CEN* DNA in cells lacking *MAD1*, *MAD2*, or *MAD3* (Fig. 2.4, C and D). Finally, although Bub1p-GFP did not bind to kinetochores in cells lacking *BUB3*, Bub3p-GFP could still be cross-linked to *CEN* DNA in *bub1Δ* cells (Fig. 2.4, C and D). In all but one case (Bub3p-GFP), results from imaging matched those from ChIP (Fig. 2.4E). High levels of autofluorescence in *bub1Δ* cells may have masked Bub3p-GFP kinetochore signals. Overall, our data show that kinetochore binding by checkpoint components is dependent on 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 2.4 Interdependencies of checkpoint proteins for kinetochore binding.
(A) ChIP of Mad2p-GFP at *CENIV* in wild type and checkpoint-delete cells in the presence of nocodazole (25μg/mL). (B) ChIP of Mad1p-GFP at *CENIV* in wild type and checkpoint delete-cells in the presence of nocodazole. (C, D) ChIP of Bub1p-GFP or Bub3p-GFP at *CENIV* in wild type and checkpoint delete cells. ChIP signals from deletion strains were normalized to the ChIP signal obtained from the wild type strain. Dashed line shows the amount of *CEN* DNA precipitated using untagged wild type cells (negative control). (E) Summary of the interdependencies of checkpoint protein kinetochore binding as assayed by imaging. Mad1p-GFP and Mad2p-GFP were examined in the presence of nocodazole, while Bub1p-GFP and Bub3p-GFP were examined in asynchronous cells.

2.3.5 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; Wigge and Kilmartin, 2001; McCleland et al., 2003). Consistent with this, neither Bub1p-GFP nor Mad2p-GFP is associated with *CEN* DNA in *spc25-7* cells at 37°C (Fig. 2.5, A and B). 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 this mutant (Fig. 2.5, A and B). In a control experiment, we observed that *CEN* binding by the Cep3p component of CBF3 was equally high in wild type, *spc25-7* and *ndc80-1* cells (Fig. 2.5C).

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). Although Bub1p-GFP and Nuf2p-myc could be cross-linked to *CEN* DNA in *ndc80-1* cells at permissive temperature, only Bub1p-GFP remained *CEN*-bound at 37°C (Fig. 2.5D and E). 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

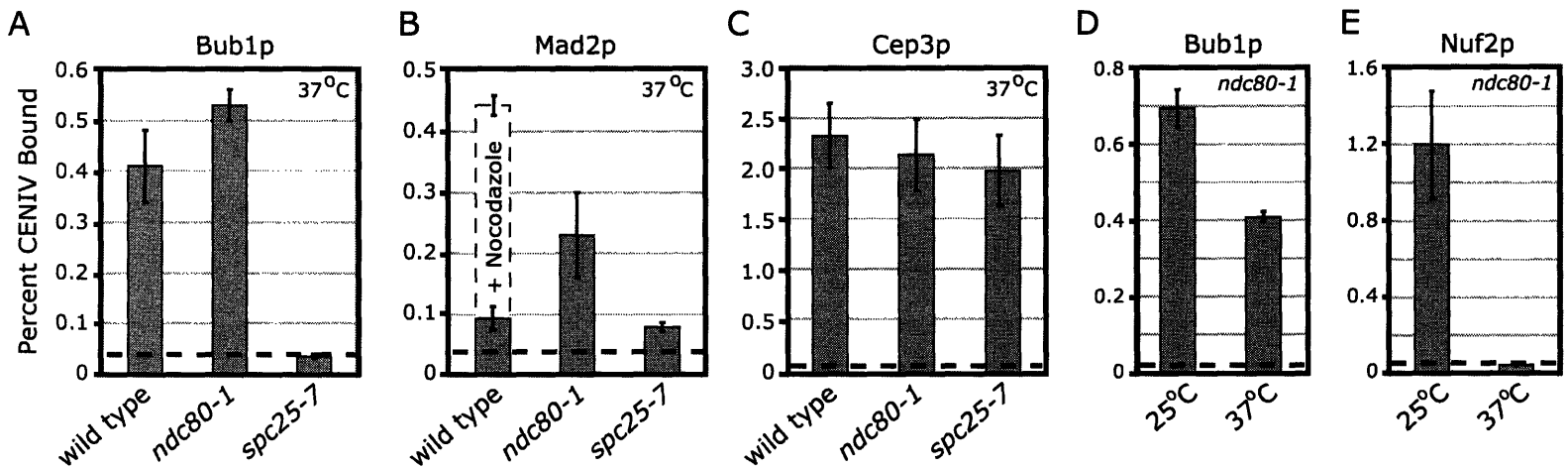


Figure 2.5 ChIP of Bub1p and Mad2p in NDC80 complex mutants.
(A-C) ChIP of (A) Bub1p-GFP , (B) Mad2-GFP (+/- nocodazole) and (C) Cep3p at CENIV in wild-type, *ndc80-1*, and *spc25-7* cells at 37°C. ChIP of (D) Bub1p-GFP and (E) Nuf2p-myc in an *ndc80-1* background at 25°C and 37°C. Graphs are as described in Fig. 2.1D.

subtle, and it is possible that Spc25p or other subunits of the Ndc80 complex may directly bind to Mad and Bub proteins.

2.3.6 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; Kim et al., 1999; He et al., 2001; Janke et al., 2002; Tanaka et al., 2002). Interestingly, although *dam1-1* mutants engage the spindle checkpoint, *ipl1-321* mutants do not (Biggins and Murray, 2001; Cheeseman et al., 2001; He et al., 2001; Jones et al., 2001; Janke et al., 2002). 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. 2.6A). In contrast, the asymmetric distribution of kinetochores in *ipl1-321* cells was dramatic and consistent (Fig. 2.6D). By imaging, we found that Bub1p-GFP was present on kinetochores at non-permissive temperature in both *dam1-1* and *ipl1-321* cells (Fig. 2.6, B, E, G, and H). While Mad2p-GFP appeared to be kinetochore-bound in the majority of *dam1-1* cells after 1h at 37°C (Fig. 2.6, C and G), Mad2p-GFP was rarely detected

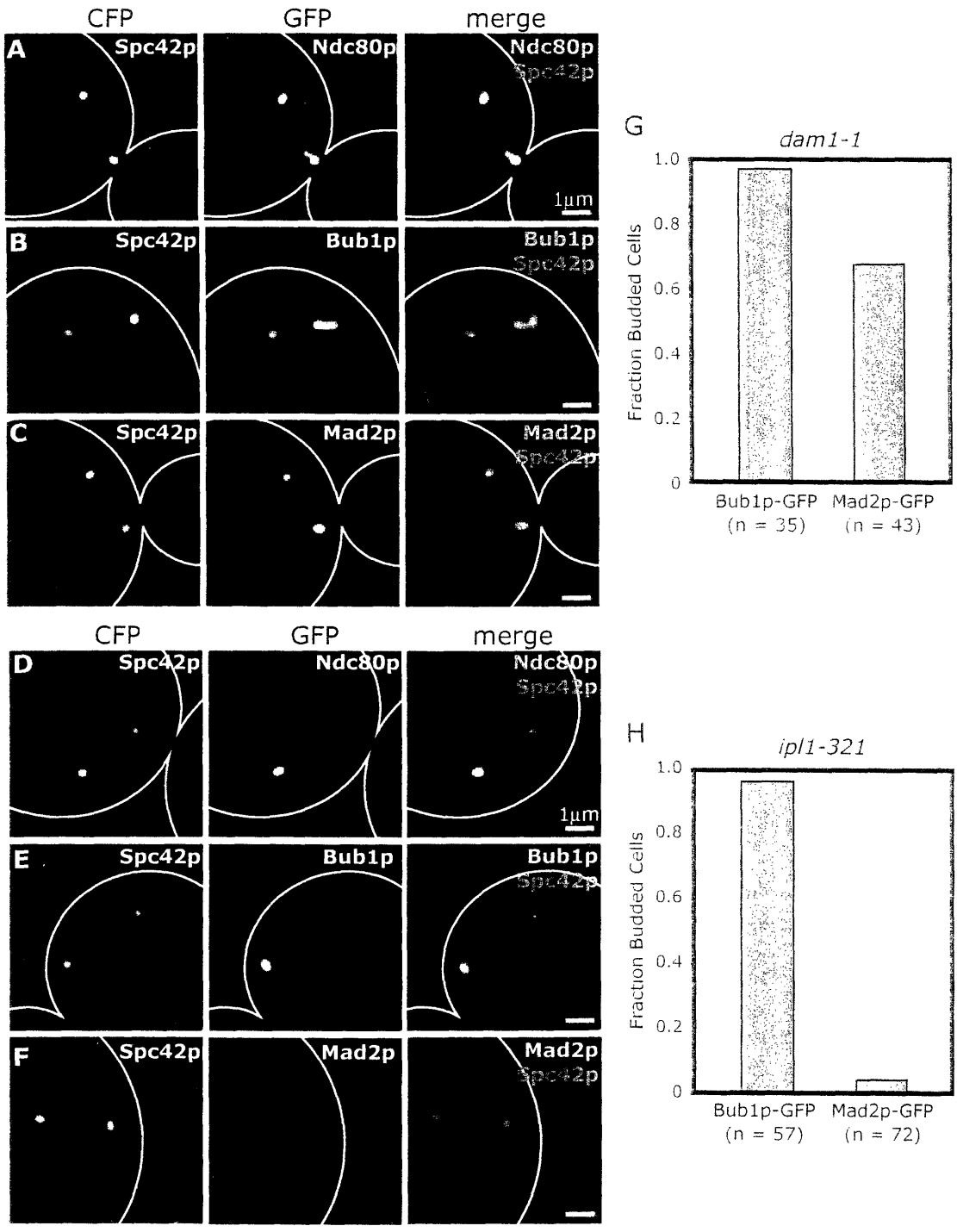


Figure 2.6 Bub1p and Mad2p localization in *dam1-1* and *ipl1-321* cells.
(A, B, C) *dam1-1* cells expressing the SPB protein Spc42p-CFP and Ndc80p-GFP, Bub1p-GFP or Mad2p-GFP at non-permissive temperature. Cells were grown at 25°C to mid-log phase and shifted to 37°C for 1 hr prior to fixation. Panels 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, E, F) *ipl1-321* cells expressing Spc42p-CFP and Ndc80p-GFP, Bub1p-GFP, or Mad2p-GFP at non-permissive temperature. Cells were arrested in α-factor for 2 hrs, shifted to 37°C for 10 min and then released at 37°C for 2 hrs prior to fixation. (G, H) Fraction of budded cells containing Bub1p-GFP and Mad2p-GFP for *dam1-1* cells after 1hr at 37°C and *ipl1-321* cells after 2hrs at 37°C following α-factor release. n = number of budded cells counted.

on kinetochores in *ipl1-321* cells at non-permissive temperature (Fig. 2.6, F and H). ChIP analysis confirmed these findings (unpublished data).

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 (unpublished data). 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.

2.3.7 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. 2.3, C-E). 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. 2.7, A and B) and both Mad2p and Bub1p were *CEN*-associated by ChIP (Fig. 2.7, F and G). However, almost all Mad2p-GFP and Bub1p-GFP foci lay $\geq$ 1μm from the spindle axis (Fig. 2.7, A and B), while the majority of kinetochores, as monitored by Ndc80p-GFP, lay between the SPBs (Fig. 2.7C). In most cells, one or two dim Ndc80p-GFP foci were also visible $\geq$ 1μm from the spindle axis (Fig. 2.7 C). 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. 2.7, D and E). 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).

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

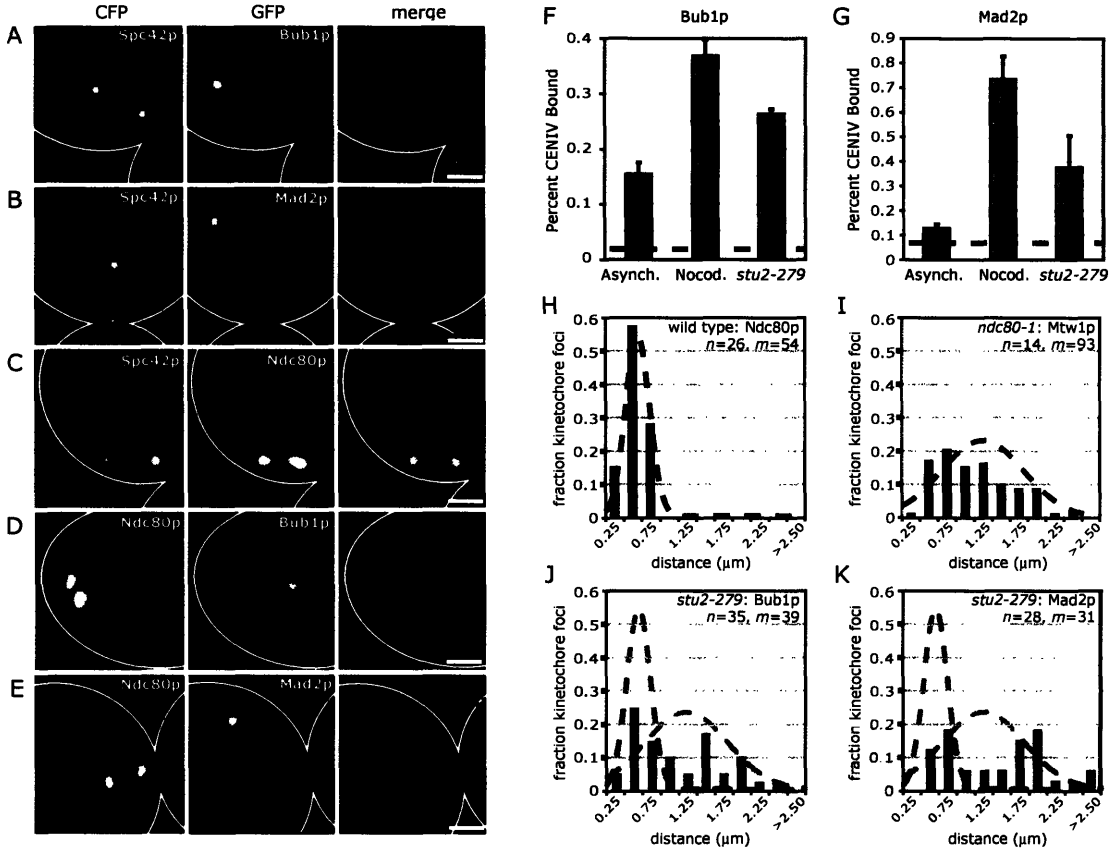


Figure 2.7 Bub1p and Mad2p localization in *stu2-279* cells.
(A, B, C) *stu2-279* cells co-expressing the SPB protein Spc42p-CFP and Bub1p-GFP, Mad2p-GFP, or Ndc80p-CFP. Panels show Spc42p-CFP alone; Bub1p-GFP, Mad2p-GFP, or Ndc80p-CFP alone; and Spc42p-CFP (red) merged with Bub1p-GFP (green), Mad2p-GFP (green), or Ndc80p-CFP (blue). Cells were grown at 25°C to mid-log phase and then shifted to 37°C for 2 hrs prior to fixation. Orange arrowheads denote unattached kinetochores. (D, 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 Fig. 1A. Red X's denote the inferred positions of SPBs. (F, G) ChIP of Bub1p-GFP and Mad2p-GFP at *CENIV* in asynchronous wild type cells, nocodazole-treated wild-type cells, and *stu2-279* cells, all at 37°C. (H-K) Spatial distribution of kinetochore protein foci for (H) Ndc80p-GFP in wild-type 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, μ=0.40 m, σ=0.15) or unattached kinetochores (green, μ=1.01μm, σ=0.52). The number of cells (n) and number of kinetochore foci (m) analyzed are listed on each graph.

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.R. 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 wild type 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. Although attached kinetochores exhibited a narrow distribution with a mean of 0.4μm (Fig. 2.7 H), unattached kinetochores showed a broad distribution with a mean of 1.0μm and a maximum of 2.3μm (Fig. 2.7 I). 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. 2.7, J and K). 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.

2.3.8 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 (unpublished data), 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 wild type and *mcd1-1* cells co-expressing Bub1p-GFP and Ndc80p-CFP revealed that Bub1p binding to kinetochores was very similar from 0-60 min after α-factor release (Fig. 2.8, A, B, and G). However, the dissociation of Bub1p from kinetochores was delayed ~15 min. relative to wild type cells (Fig. 2.8G). Interestingly, *mcd1-1* cells with longer spindles almost always contained a heterogeneous population of Bub1p-positive and -negative kinetochores (Fig. 2.8B, compare Ndc80p with Bub1p), 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.

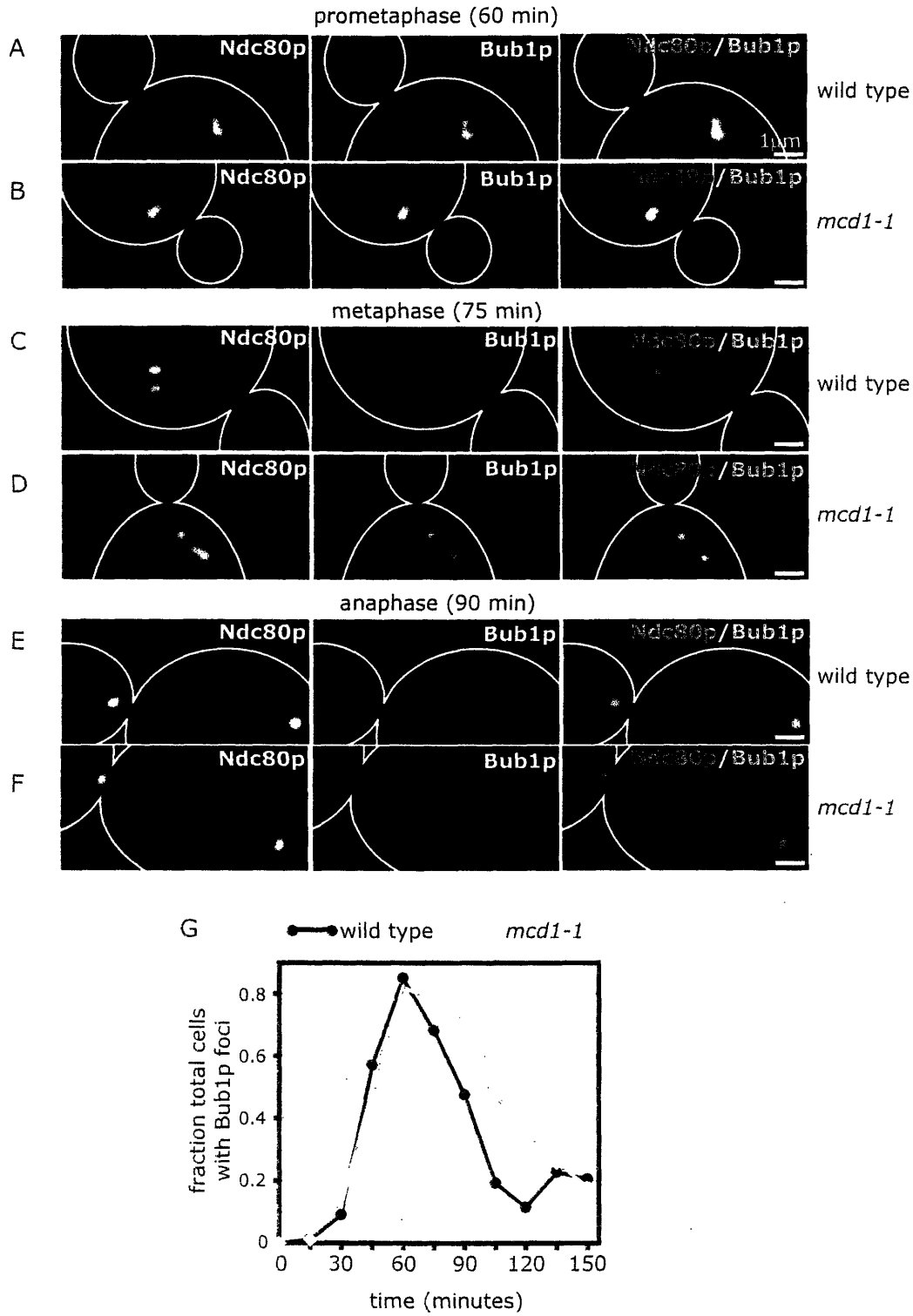


Figure 2.8 Bub1p localization in *mcd1-1* cells.
(A-F) Typical images of *mcd1-1* and wild type cells co-expressing Bub1p-GFP (green) and Ndc80p-CFP (red) at 65, 70 and 90 min after α-factor release at 37°C. (G) Fraction of *mcd1-1* and wild-type cells containing Bub1p kinetochore foci at 15 minute time points following α-factor release at 37°C. At least 60 individual cells were analyzed at each time point.

2.4 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.R. Rines, unpublished data). Although 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, which is 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), whereas 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, 2003), which implies that kinetochore binding by Mad2p might be a normal feature of meiosis. Therefore, it will be interesting to determine if Mad2p-positive chromosomes are generated during meiotic bouquet formation (Trelles-Sticken et al., 1999).

2.4.1 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 Spc24, Spc25p, Ndc80p and Nuf2p) have the special property of abolishing the checkpoint (Goh and Kilmartin, 1993; Gardner et al., 2001; Janke et al., 2001; McCleland et al., 2003). However, protein-protein and protein-DNA associations among kinetochore proteins are hierarchical; whereas 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 (He et al., 2001; Janke et al.,

2001; De Wulf et al., 2003). 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 (Fig. 2.5C, as measured using the CBF3 component, Cep3p). 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; Wigge and Kilmartin, 2001; McCleland et al., 2003). 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 (Doheny et al., 1993; Strunnikov et al., 1995; Connelly and Hieter, 1996; Tavormina and Burke, 1998; Gardner et al., 2001); 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, whereas 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.

2.4.2 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), whereas 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. Although 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, although 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.

2.4.3 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, although 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 wild-type cells (Warren et al., 2002).

We find selective binding of Bub proteins, but not Mad proteins, to kinetochores in three contexts: wild type 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 wild-type cells, but persist in *ipl1-321* cells (Tanaka et al., 2002). Although 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 wild-type 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.

2.4.4 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. Although 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.

2.5 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 (model 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 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), anti-Cep3p (Sorger Lab) antibodies. PCR amplifications of 200bp fragments of *URA3* and *CENIV* were performed on serial dilutions (to determine linearity) of two or more independent IPs; error bars show standard deviations.

Supplemental material

A summary of kinetochore localization by Spindle Checkpoint proteins can be found in Table 2.S1. Benomyl sensitivity assays of strains expressing GFP-tagged proteins are shown in Figure 2.S1.

2.6 ACKNOWLEDGEMENTS

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Supplementary Table 2.S1
Kinetochore Localization of Bub1p, Bub3p, Mad1p, and Mad2p.

BACKGROUND	KINETOCHORE PHENOTYPE	Bub1p	Bub3p	Mad1p	Mad2p
Wild-type	attached	+ [a]	+ [a]	-	-
Wild-type + α-factor (G1)	attached	-	-	-	-
Wild-type + nocodazole (M phase, checkpoint active)	unattached	++	++	++	++
	attached, no tension	-	-	-	-
ndc10-1	kinetochores not assembled	-	-	N.D.	-
ndc80-1	unattached	++	++	N.D.	+
spc25-7	unattached	-	-	N.D.	-
dam1-1	monopolar/monotelic attachment	++	N.D.	N.D.	++
ipl1-321	monopolar/syntelic attachment	++	N.D.	N.D.	-
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; N.D. = no data

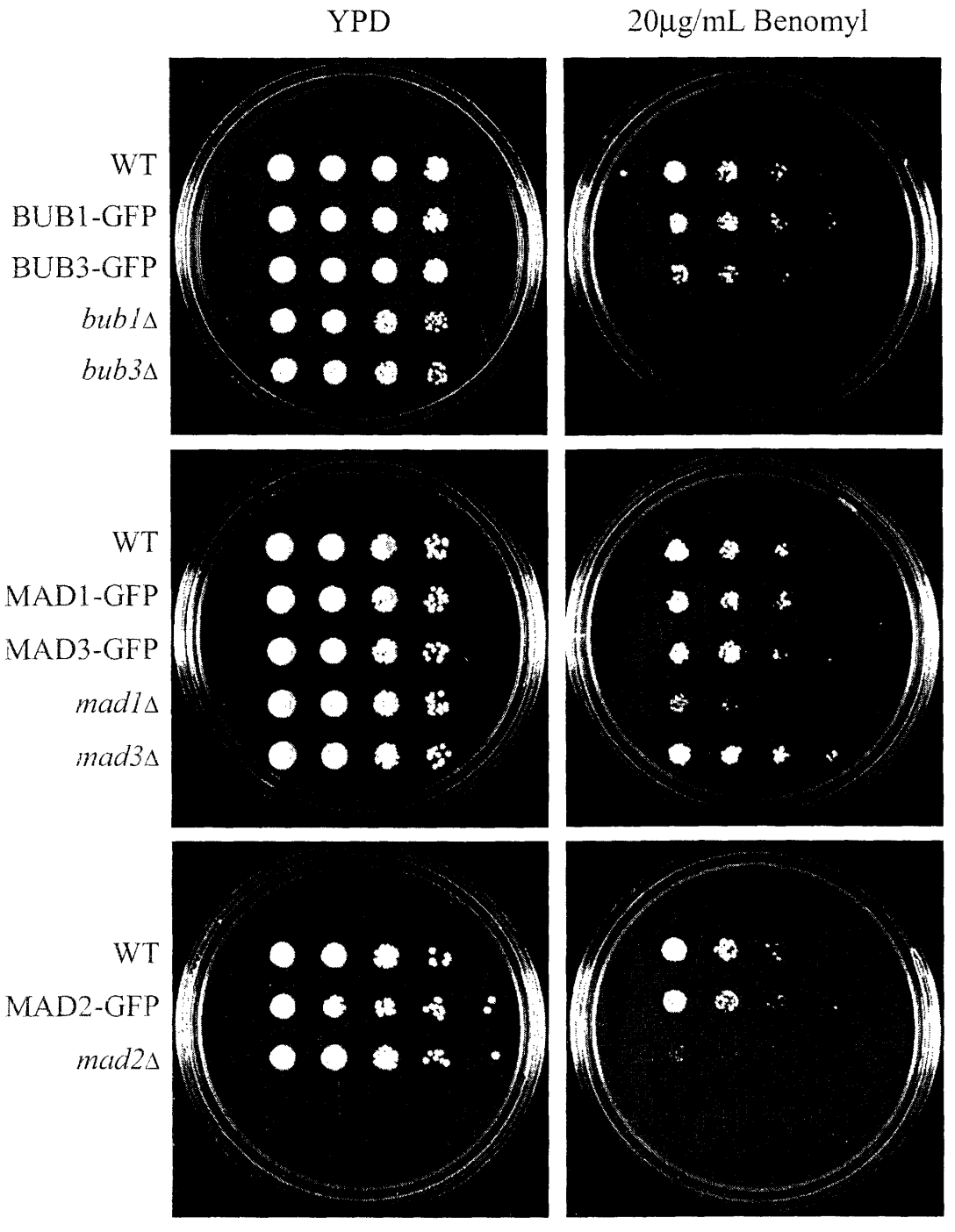

Figure 2.S1 Benomyl sensitivity of strains expressing GFP-tagged spindle checkpoint proteins.

Chapter 3

Mlps Facilitate Mad1p Binding to the Nuclear Periphery

Mlps Facilitate Mad1p Binding to the Nuclear Periphery

3.1 ABSTRACT

The spindle checkpoint monitors chromosome-microtubule attachment and delays the onset of anaphase until all chromosomes have attained bipolar links to the mitotic spindle. Two checkpoint components, mitotic arrest deficient (Mad) proteins 1 and 2, associate with the nuclear periphery and are not recruited to kinetochores in *Saccharomyces cerevisiae* during normal cell cycles. We show here that the perinuclear localization of Mad1p is dependent upon myosin-like proteins Mlp1p and Mlp2p which are thought to link nuclear pore complexes to the interior of the nucleus. Deletion of either *MLP1* or *MLP2* releases Mad proteins from the nuclear periphery and allows them to associate with kinetochores during early mitosis. However, ectopic kinetochore localization of Mad1p does not dramatically affect spindle assembly or mitotic timing, nor does loss of Mad1p at the nuclear periphery impair spindle checkpoint function. As the Mlps have been implicated in several cellular processes, such as the DNA damage response, we hypothesize that the perinuclear localization of Mad1p and Mad2p may be required for spindle checkpoint-independent functions such as invoking a metaphase arrest following DNA damage.

3.2 INTRODUCTION

Successful cell division requires that sister chromatids are partitioned equally between daughter cells following DNA replication. In eukaryotes, chromosome segregation is accomplished via the mitotic spindle, a self-organizing bipolar array of microtubules (MTs). MTs attach to chromosomes via kinetochores, multiprotein complexes that assemble on centromeric DNA. During spindle assembly, a chromatid attaches to MTs emanating from one and only one pole of the spindle, while its partner attaches to MTs emanating from the opposite pole. The spindle checkpoint contributes to chromosome transmission fidelity by delaying the onset of anaphase until all chromosomes have attained bipolar links to the mitotic spindle.

Although all known spindle checkpoint proteins are recruited to unattached kinetochores during prometaphase in metazoan cells (Cleveland et al., 2003), kinetochores in *S. cerevisiae* recruit only a subset of spindle checkpoint components during normal mitoses. Bub1p and Bub3p are consistently recruited to kinetochores during early mitosis, but Mad1p and Mad2p are not (Gillett et al., 2004; Iouk et al., 2002; Kerscher et al., 2003). In contrast to animal cells, budding yeast kinetochores remain closely associated with spindle pole bodies (SPBs) and attached to spindle microtubules (MTs) throughout the cell cycle (Jin et al., 2000). As Mad proteins are specifically recruited to unattached kinetochores in metazoan cells (Waters et al., 1998), this disparity may be due to differences in the spindle morphogenesis pathways employed in each organism.

Two spindle checkpoint components, Mad1p and Mad2p, localize to the nuclear envelope in *S. cerevisiae*, *Homo sapiens*, and *Xenopus* (Campbell et al., 2001; Chen et al., 1998; Iouk et al., 2002). Budding yeast undergo a closed mitosis, and Mad1p remains

associated with the nuclear envelope throughout the cell cycle in this organism (Iouk et al., 2002). Mad1p co-immunoprecipitates with a subcomplex of nucleoporins that includes Nup53p, Nup157p, and Nup170p, and deletion of *MAD1* reduces nuclear transport rates by about two-fold (Iouk et al., 2002).

Several intriguing connections exist between kinetochores and components of the nuclear pore complex (NPC). For instance, mutating the budding yeast nucleoporin *NUP170* leads to kinetochore and chromosome segregation defects (Kerscher et al., 2001), and nucleoporins such as hNup133 and hNup107 relocalize from NPCs to kinetochores during mitosis in human cells (Belgareh et al., 2001). In addition, the Ran-GTP regulatory proteins RCC1, RanBP2, and RanGAP1 also move to kinetochores following nuclear envelope breakdown in human cells (Arnaoutov and Dasso, 2003; Joseph et al., 2002) and Ran-GTP plays an important role in spindle and kinetochore assembly (reviewed in Di Fiore et al., 2004 and Salina et al., 2003). In addition, there is a significant degree of sequence similarity between the spindle checkpoint protein Bub3 and the nuclear import factor Rael. Rae1 can functionally substitute for Bub3 in mice, and haploinsufficiency of murine RAE1 leads to chromosome missegregation and spindle checkpoint defects in a manner similar to that of BUB3 (Babu et al., 2003).

All of the spindle checkpoint components are required for checkpoint arrest in response to spindle damage or kinetochore lesions. Bub1p and Bub3p are thought to be upstream components of the checkpoint pathway, while the Mad proteins are thought to be downstream components. Following checkpoint activation, Mad2p binds and inhibits Cdc20p, a specificity factor for the anaphase promoting complex (APC). The APC is an E3 ubiquitin ligase, and binding of Mad2p to Cdc20p prevents Cdc20p from directing the APC

to targets such as Pds1p/securin which must be ubiquitinated and degraded by the 26S proteasome in order for sister chromatids to separate and anaphase to proceed (reviewed in Nasmyth, 2002).

Here, we examine more closely the factors responsible for sequestering Mad proteins at the nuclear periphery. We find the perinuclear localization of Mad1p is dependent upon Myosin-like proteins Mlp1p and Mlp2p, two proteins implicated in multiple cellular processes. Mlps are thought to be the yeast homologs of metazoan Tpr proteins that link NPCs to the interior of the nucleus (Strambio-de-Castillia et al., 1999; Cordes et al., 1997; Krull et al., 2004). Release of Mad1p from the nuclear periphery allows it to bind to kinetochores early during mitosis. However, this mislocalization does not have a significant impact on cell cycle progression and does not impair spindle checkpoint activation in the presence of unattached kinetochores. We propose that the perinuclear pool of Mad proteins may be important for spindle checkpoint-independent activities, such as executing mitotic arrest following DNA damage.

3.3 RESULTS AND DISCUSSION

3.3.1 Mad1p Co-localizes with Mlp1p and Mlp2p

To localize proteins at the nuclear membrane in *S. cerevisiae*, endogenous *MAD*, *NUP*, and *MLP* genes were linked to GFP or CFP at their COOH termini via homologous recombination. Cells expressing GFP- and CFP-tagged proteins were observed using two-wavelength three-dimensional (3D) deconvolution microscopy (Rines et al., 2002). Although Mad1p is reported to associate with a subset of nucleoporins (Iouk et al., 2002), we find that Mad1-GFP does not co-localize completely with Nup49-CFP (Fig. 3.1A) or with other nucleoporins such as Nic96p or Nup170p (unpublished data). As a control, we also examined cells co-expressing two nucleoporins, Nup170-GFP and Nup49-CFP, and these two proteins co-localize well under our experimental conditions (Fig. 3.1B). In contrast to nucleoporins, which are distributed relatively evenly across the entire surface of the nuclear envelope, Mad1-GFP frequently exhibits a crescent-shaped fluorescence pattern at the nuclear periphery (Fig. 3.1A). This suggests that Mad1p is, at most, recruited to a subset of NPCs.

Two additional perinuclear proteins, Mlp1p and Mlp2, are also known to exhibit a crescent-shaped pattern and partial co-localization with NPCs (Galy et al., 2004; Strambio-de-Castillia et al., 1999). The Mlps are putative coiled-coil proteins thought to link nuclear pore complexes (NPCs) to the interior of the nucleus. It has been reported that Mlps participate in a variety of pathways, including those that regulate telomere length and clustering, transcriptional silencing, double strand break repair, and mRNA export (Feuerbach et al., 2002; Galy et al., 2004; Galy et al., 2000; Hediger et al., 2002a; Hediger et al., 2002b; Kosova et al., 2000). Although the mechanisms that restrict their localization is

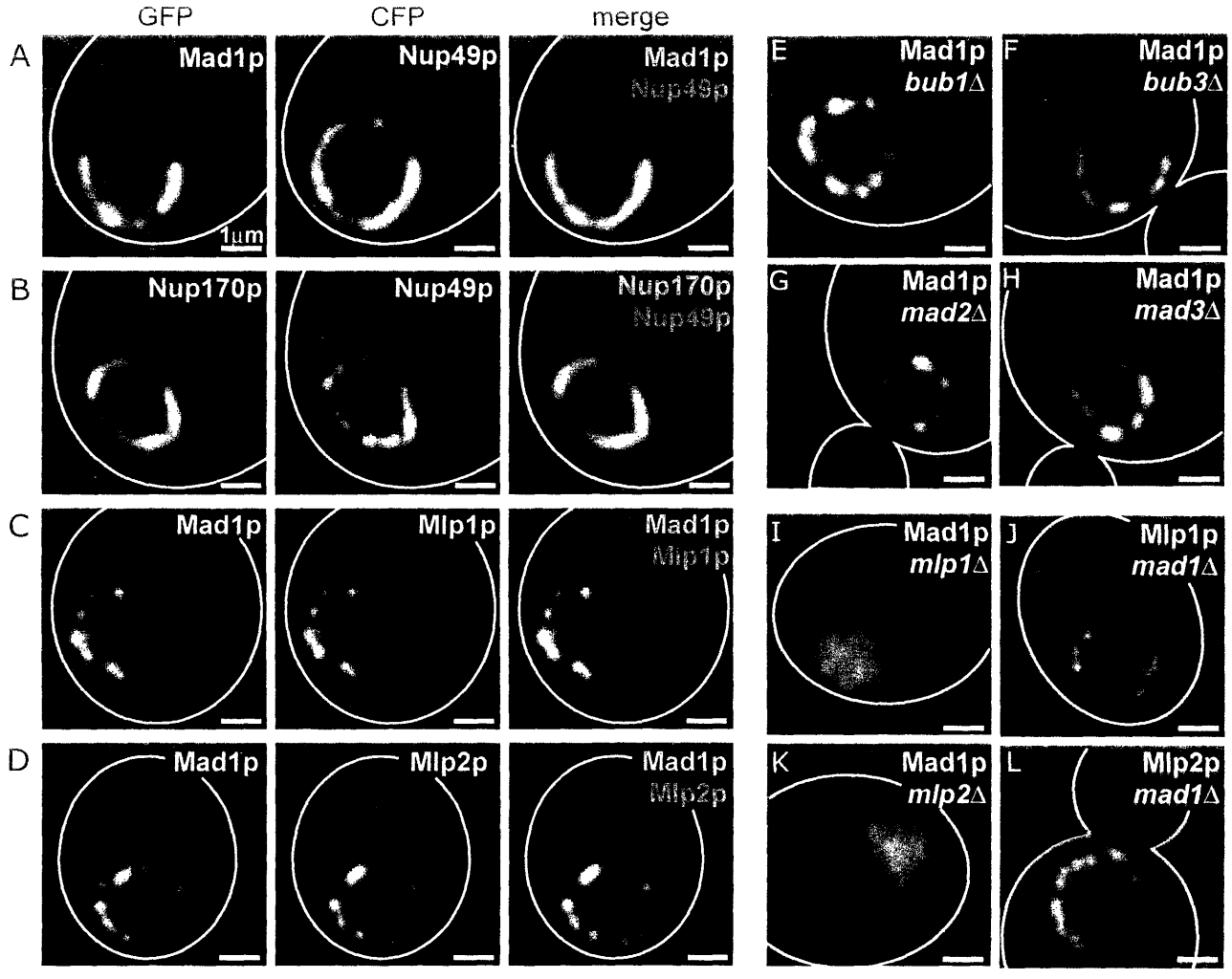

Figure 3.1 Perinuclear localization of Mad1-GFP is *MLP*-dependent.
(A-D) Single optical sections from 3D image stacks of typical cells co-expressing Mad, Nup, and Mlp proteins fused to GFP or CFP. GFP channel is shown in the first column, CFP channel in the second column, and merged images with GFP (in green) and CFP (in red) are shown in the third column. (E-F) Mad1-GFP localization in cells deleted for *BUB* and *MAD* checkpoint genes. (I, K) Mad1p in Δ*mlp1* and Δ*mlp2* cells, respectively. (J, L) Mlp1-CFP and Mlp2-CFP localization in Δ*mad1* cells.

unknown, these proteins are consistently seen on the opposite side of the nucleus from the nucleolus (Galy et al., 2004). We find that Mad1-GFP co-localizes well with Mlp1-CFP and Mlp2-CFP (Fig. 3.1, C and D).

3.3.2 Perinuclear Localization of Mad1p is Mlp-Dependent

In budding yeast, Mad1p requires the upstream checkpoint proteins Bub1p and Bub3p, as well as its binding partner Mad2p, to bind to unattached kinetochores (Gillett et al., 2004). In contrast, we find that none of the other *BUB* and *MAD* checkpoint components, nor the upstream kinase *MPS1* (Weiss and Winey, 1996), are required to maintain the perinuclear localization of Mad1p (Fig. 3.1, E-H; unpublished data, (Iouk et al., 2002)). However, deletion of either *MLP1* or *MLP2* is sufficient to disrupt Mad1p's perinuclear localization (Fig. 3.1, I and K). The reverse is not true, as *MAD1* is not required for the perinuclear localization of Mlp1p or Mlp2p (Fig. 3.1, J and L). Although deletion of *NUP53* reportedly reduces the intensity of Mad1p foci at the nuclear periphery (Iouk et al., 2002), we find that the Mad1p nuclear signal is bright but diffuse in *mlp1*Δ and *mlp2*Δ cells. Thus, our data show that Mad1p co-localizes with the NPC-associated proteins Mlp1p and Mlp2p, and that Mad1p's association with the nuclear envelope is dependent on both *MLP1* and *MLP2*.

3.3.3 Loss of Perinuclear Tethering Allows Mad1p to Bind Kinetochores

We have previously shown that the Bub and Mad spindle checkpoint proteins exhibit distinct behaviors during normal cell cycles. Although Bub1p and Bub3p bind to kinetochores early during normal mitosis, Mad1p and Mad2p are only recruited to kinetochores following spindle checkpoint activation (Gillett et al., 2004). Interestingly,

deletion of *MLP1* or *MLP2* not only releases Mad1p from the nuclear periphery, it also allows Mad1p to bind to kinetochores during early mitosis (Fig. 3.2, A and B). Kinetochore association of Mad1p is cell cycle-regulated in these mutants, being evident in small budded S- and early M-phase cells, but absent in unbudded G1 and large-budded anaphase cells (Fig. 3.2, A and B). Mad2p exhibited a similar pattern of kinetochore localization in *mlp1*Δ and *mlp2*Δ cells, consistent with the fact that Mad1p requires *MAD2* to bind kinetochores (unpublished data; (Gillett et al., 2004)).

Mad1p association with centromeric DNA in *mlp1*Δ and *mlp2*Δ cells was verified using chromatin immunoprecipitation (ChIP) at *CENIV*. As Mad1p requires spindle activation for binding to kinetochores in wild-type cells, ChIP signals were quantified and normalized to that of Mad1-GFP in nocodazole-treated wild-type cells. The ChIP signal for Mad1-GFP in cycling *mlp1*Δ and *mlp2*Δ cells was significantly greater than the background signal detected for Mad1-GFP in cycling wild-type cells, but not as great as that of Mad1-GFP in nocodazole treated wild-type cells (Fig. 3.2 C). This decrease may be due to the fact that nocodazole treated cells are arrested in mitosis and have a higher percentage of Mad1p-positive cells than do asynchronous *mlp1*Δ and *mlp2*Δ cells. Alternatively, kinetochores in *mlp1*Δ and *mlp2*Δ cells may each recruit lower levels of Mad1p proteins than do unattached kinetochores in nocodazole treated wild-type cells. The kinetics of Mad1p kinetochore association in *mlp1*Δ and *mlp2*Δ cells (Fig. 3.2C) are very similar to those of Bub1p in wild-type cells, consistent with the fact that *BUB1* is required for Mad1p binding to kinetochores in wild-type cells (Gillett et al., 2004).

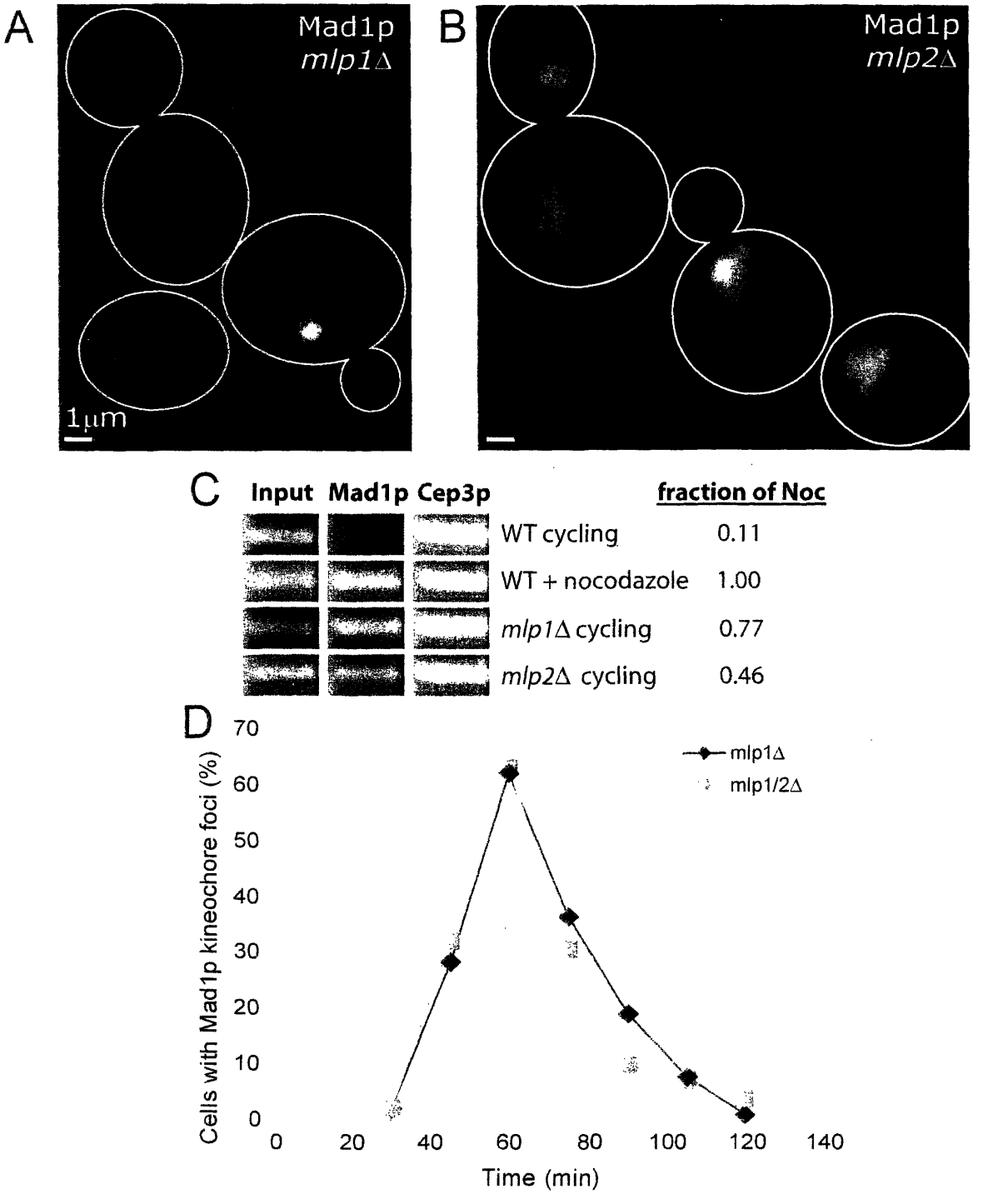

Figure 3.2 Mad1p associates with kinetochores during early mitosis in *mlp1*Δ and *mlp2*Δ cells. (A, B) Mad1-GFP localization in *mlp1*Δ and *mlp2*Δ cells during G1, early M, and anaphase. (C) Chromatin immunoprecipitation (ChIP) of Mad1-GFP at *CENIV* in wild-type cycling, wild-type nocodazole treated, cycling *mlp1*Δ, and cycling *mlp2*Δ cells. ChIP signals were quantified using serial dilutions and values normalized to the Mad1p nocodazole signal. (D) Kinetics of Mad1p kinetochore association in *mlp1*Δ and *mlp1*Δ/*mlp2*Δ cells at 25°C. X-axis shows time following α-factor release. n>100 individual cells were counted for each time point.

3.3.4 Mitotic Timing and Spindle Checkpoint Activity in *MLP* deletes

Why is Mad1p sequestered at the nuclear periphery in the absence of unattached kinetchores? One possibility is that Mad1p binding to kinetochores is somehow disadvantageous during normal cell cycles. Sequestration is used as to inhibit other proteins during mitosis such as the phosphatase Cdc14p which is held in the nucleolus prior to anaphase to prevent premature dephosphorylation of Cdks and other targets (Shou et al., 1999; Visintin et al., 1999). If kinetochore binding by Mad1p and Mad2p transiently activates the spindle checkpoint, we reasoned that *mlp1*Δ and *mlp2*Δ cells might experience a slight mitotic delay. We therefore examined the mitotic progression of α-factor synchronized *mlp1*Δ, *mlp2*Δ, and wild-type cells by monitoring spindle length and position. However, progression through mitosis in *mlp1*Δ and *mlp2*Δ cells is not significantly delayed compared to wild-type cells (Fig. 3.3 A).

As Mad1p bound to kinetochores in *mlp1*Δ and *mlp2*Δ cells does not cause an extended checkpoint delay, we next examined if the spindle checkpoint was functional in these cells. To elicit a spindle checkpoint response, we treated *mlp1*Δ and *mlp2*Δ cells co-expressing Mad1-GFP and the kinetochore marker Ndc80-CFP with 25 μg/mL nocodazole, a MT depolymerizing drug that inhibits spindle assembly and activates the spindle checkpoint. *mlp1*Δ and *mlp2*Δ cells treated with nocodazole arrested in mitosis similarly to wild-type cells, and Mad1p was recruited to unattached kinetochores in *mlp* deletions indicating that the spindle checkpoint is functional these cells (Fig. 3.3, B-D).

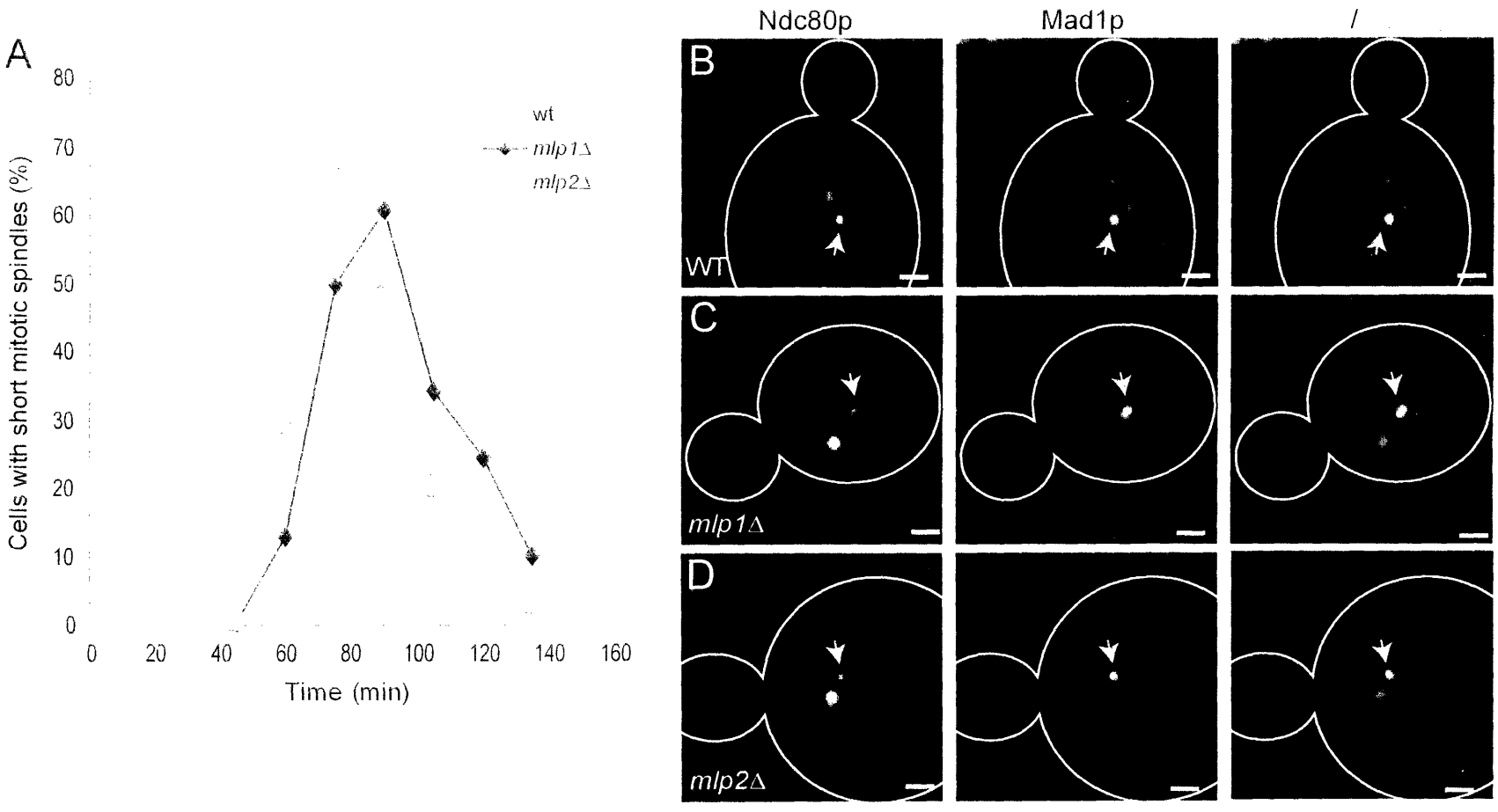

Figure 3.3 Progression through mitosis and mitotic arrest are not significantly perturbed in *mlp1*Δ and *mlp2*Δ cells.
(A) Progression through mitosis was monitored by examining spindle length and position in cells expressing the SPB marker Spc42-CFP. Cells were cultured at 25°C. X-axis shows time following α-factor release. At least 100 cells were counted per time point. Cells were classified as having short mitotic spindles if SPB separation was evident but the spindle did not extend through the bud neck. (B-D) Co-localization of Mad1-GFP and Ndc80-CFP in cells treated for 1hr with 25 μg/ml nocodazole. Panels in the first column show Mad1-GFP, second column shows Ndc80-CFP, third column shows merged images of Mad1-GFP (in green) and Ndc80-CFP (in red). Orange arrowheads indicate the positions of unattached kinetochores.

3.3.5 Mlp localization in *nup60Δ* and nocodazole treated cells

Two proteins, Nup60p and Nup145p, are required to maintain the proper distribution of Mlp1p and Mlp2p on the nuclear envelope (Feuerbach et al., 2002; Galy et al., 2000). Deletion of Nup60p or truncation of Nup145p causes Mlp1p and Mlp2p molecules to mislocalize at 37°C. Although deletion of *NUP60* does not affect the localization of integral nucleoporins such as Nsp1p and Nup49p (Dilworth et al., 2001), Mlps cluster tightly at the nuclear periphery in *nup60Δ* cells (Fig. 3.4, A and B; (Feuerbach et al., 2002)). Mad1p's association with Mlp-dependent attachment sites at the nuclear periphery must be relatively strong, as Mad1p and Mad2p are similarly mislocalized in *nup60Δ* cells (Fig. 3.4, C and D). The Mad1p foci in *nup60Δ* cells are present throughout the cell cycle and thus are distinct from the cell-cycle regulated Mad1p kinetochore foci found in *mlp1Δ* and *mlp2Δ* cells. The close association between Mad1p and the Mlps at the nuclear envelope prompted us to examine if Mlp1p and Mlp2p might be recruited to unattached kinetochores following spindle checkpoint activation. However, no Mlp1p or Mlp2p is evident at unattached kinetochores in nocodazole-treated cells (Fig. 3.4, E and F) indicating that Mad1p is released from its Mlp-associated complex during checkpoint activation.

3.3.6 Summary and Conclusions

Our previous studies of Bub1p and Mad2p localization suggested that budding yeast kinetochores have at least three states of attachment. In wild-type cells, unattached kinetochores recruit Bub1p and Mad2p, kinetochores with mature attachments in G1 and late mitosis recruit neither Bub1p nor Mad2p, and kinetochores with immature attachments during early mitosis recruit Bub1p but not Mad2p (Gillett et al., 2004). The loss of Mlp-

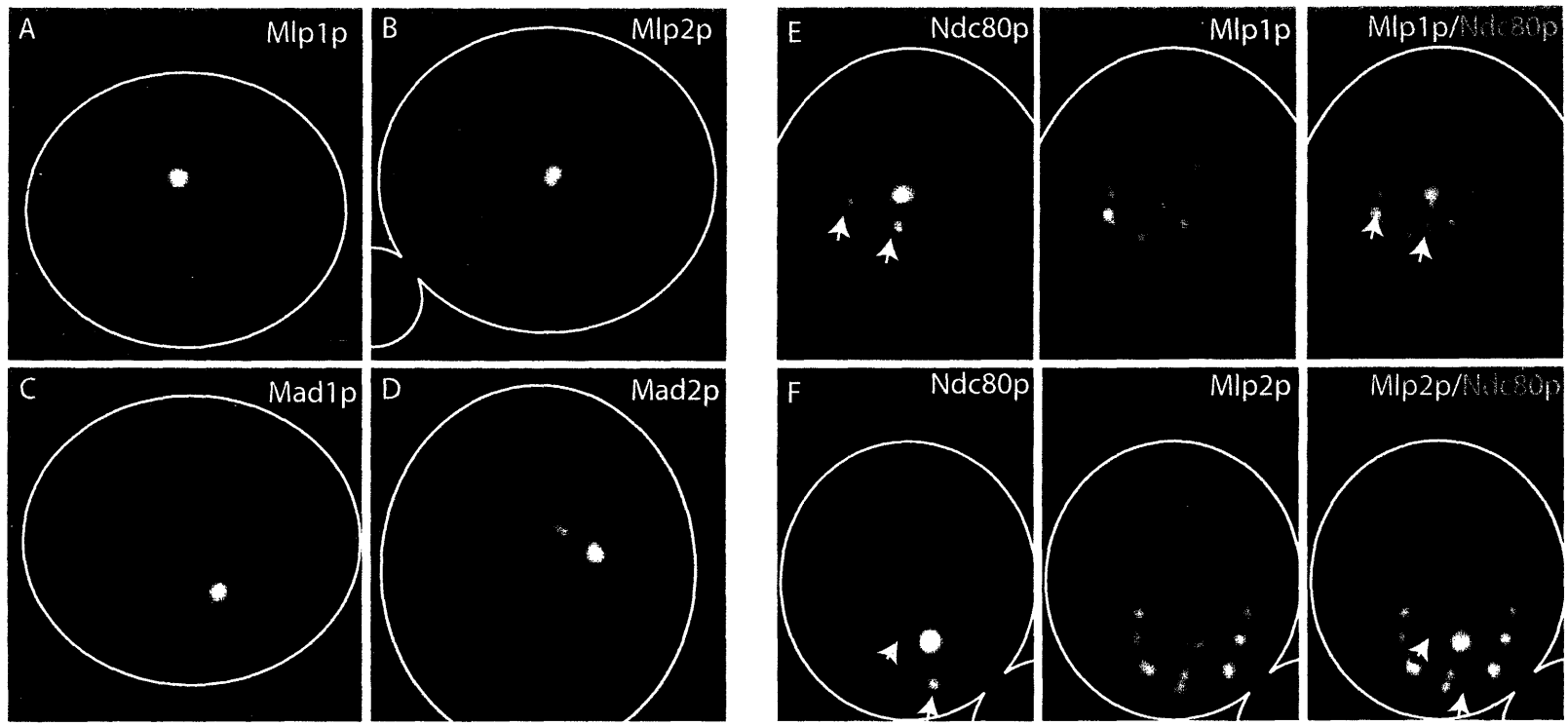

Figure 3.4 Mlp localization in *mup60*Δ and nocodazole treated cells.
(A-D) Mlp and Mad proteins are similarly mis-localized in *mup60*Δ cells at 37°C. Images of (A) Mlp1-GFP, (B) Mlp2-GFP, (C) Mad1-GFP, and (D) Mad2-GFP are 2D projections of 3D image stacks. (E, F) Mlp proteins are not recruited to unattached kinetochores in nocodazole treated wild-type cells. Panels in the first column show the distribution of all kinetochore in each cell as measured by Ndc80-CFP localization, panels in the second column shows Mlp1-GFP and Mlp2-GFP localization, and the third column are merged images of CFP (in red) and GFP (in green). Orange arrowheads indicate the positions of unattached kinetochores.

dependent tethering of Mad1p to the nuclear periphery allows Mad1p and Mad2p to bind to kinetochores during early mitosis. Although Mad kinetochore localization could be indicative that kinetochores are being detached from spindle MTs in *mlp1*Δ and *mlp2*Δ cells, spindle assembly and chromosome dynamics appear relatively normal in these cells (unpublished data). Thus, it seems more likely that Mad proteins are being recruited to kinetochores that have not wholly detached from spindle MTs. However, Mad localization to these kinetochores in *mlp1*Δ and *mlp2*Δ cells is not sufficient to induce a prolonged spindle checkpoint arrest. In fact, Mad proteins are displaced from kinetochores as mitosis progresses and mature bipolar attachments are formed. Thus, either the Mad proteins bound to kinetochores in *mlp1*Δ and *mlp2*Δ cells are not bound in the proper context to activate the checkpoint, or additional factors, such as Cdc20p, are not properly configured to allow the formation of inhibitory complexes that block APC activation and anaphase onset.

Mad1p binding to kinetochores early during mitosis in *MLP* deletions can be easily explained if the Mad1p binding sites at kinetochores with immature microtubule attachments are of lower affinity than the Mad1p binding sites at the nuclear periphery. If *MLP*-dependent binding sites for Mad1p at the nuclear periphery are of higher affinity than are the binding sites for Mad1p on kinetochores with immature attachments, Mad1p will preferentially bind to the nuclear periphery throughout the cell cycle. However, in this case, Mad1p must either have an even greater affinity for unattached kinetochores than it does for the nuclear periphery, or spindle checkpoint-dependent modifications, such as phosphorylation events, must release Mad1p from the nuclear periphery in order to allow it to bind unattached kinetochores in wild-type cells. Such a release mechanism for Mad1p may be required as fluorescence recovery after photobleaching (FRAP) experiments suggest

that Mad1 and Mad2 molecules are stably bound at the nuclear envelope in PtK_2 cells (Shah et al., 2004). Although spindle checkpoint activation appears to function normally in *mlp1*Δ and *mlp2*Δ cells, it is also worth investigating if down regulation of the spindle checkpoint signal is slower when perinuclear Mad1p binding sites are unavailable.

Another possibility consistent with our data is that Mad1p plays a role at the nuclear periphery that is independent of its role in spindle checkpoint signaling. Previous data have shown that loss of *MAD1* reduces the efficiency of nuclear transport (Iouk et al., 2002). However, as both the Mads and Mlps have also been linked to the DNA damage response, another possibility is that Mad1p at the nuclear periphery is important for invoking a metaphase arrest in response to DNA damage. Mlp2p is required to physically tether both Mad1p and the DNA damage response protein Yku70p to the nuclear periphery, and deletion of *MLP2* decreases the efficiency of double strand break (DSB) repair (Galy et al., 2000). Mad proteins are partially required for the G2/M arrest of cells in response to DNA damage (Garber and Rine, 2002; Maringele and Lydall, 2002; Scott and Plon, 2003) and also for the eventual arrest of cells with DSBs in which the Rad24p checkpoint protein is defective (Aylon and Kupiec, 2003). It is appealing to imagine that Mlp attachment sites may serve as auxiliary activation centers for the formation of Mad2p-Cdc20p inhibitory complexes in response to DNA damage or other stimuli that induce cell cycle arrest. Interestingly, deletion of *YKU70* prevents cells with persistent DSBs from resuming growth, causing a permanent G2/M arrest (Lee et al., 1998). As Mlp2p is required to tether both Yku70p and Mad1p to the nuclear periphery, it may serve as an integration site linking the repair response and cell cycle arrest, possibly regulating the transition from prolonged cell cycle arrest to adaptation in the presence of persistent damage.

3.4 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 pRS304, pRS303, or pRS306. Endogenous genes were replaced using one-step gene replacement and correct integrants were verified by PCR and microscopy.

Microscopy Analysis

Images of live and 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 (model 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 for 5-10 min. followed by 0.1 M phosphate buffer (pH 6.6) for at least 5 min and imaged at RT. Live cells were imaged in SD media.

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) and anti-Cep3p (Sorger Lab) antibodies. PCR amplifications of 200bp fragments of *URA3* and

CENIV were performed on serial dilutions (to determine linearity) of two or more independent IPs.

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Chapter 4

Conclusions & Future Directions

Conclusions & Future Directions

Robust genetics and simple, well characterized kinetochores make budding yeast a powerful system in which to analyze spindle checkpoint function. Spindle checkpoint genes were first identified in budding yeast more than ten years ago (Hoyt et al., 1991; Li and Murray, 1991); however, before I began this work, little was known about the localization and behavior of spindle checkpoint proteins in this organism. Spindle checkpoint proteins are expressed at very low levels, and the experiments described herein were only recently made possible by technological advances in fluorescence microscopy and the development of deconvolution microscopes (Wallace et al., 2001). My work provides a basic foundation for future cell biological analyses of spindle checkpoint function in budding yeast and, as will be described below, my results serve both to confirm certain aspects of checkpoint behavior in budding yeast, and to suggest areas in which additional levels of complexity may exist.

4.1 Locating kinetochores in budding yeast

In contrast to metazoan cells, budding yeast kinetochores do not assemble into a proper metaphase plate configuration. Instead, budding yeast kinetochores cluster during mitosis and kinetochore proteins appear as two distinct lobes located between the SPBs (He et al., 2000). Although individual kinetochores can be easily distinguished in metazoan cells, the clustering of budding yeast kinetochores prevents individual chromosomes from being resolved during normal spindle assembly. Thus far, analyses of single chromosomes in budding yeast have primarily employed fluorescent tags on single chromosomes generated by integrating arrays of DNA binding sites close to a centromere and then expressing the appropriate DNA binding protein fused to GFP (Michaelis et al., 1997; Straight et al., 1997). These types of experiments have illuminated certain aspects of chromosome dynamics during

spindle assembly, such as transient sister separation, and have been useful in characterizing MT attachment defects in various kinetochore mutants.

Prior to my work presented here, it was not known if the fluorescence signals from GFP-tagged kinetochore components, such as Ndc80p, would be bright enough to allow the detection of single kinetochores in budding yeast. Assuming the amounts of Ndc80p bound to unattached and attached kinetochores are relatively similar, integration of signal intensities has indicated that the weak foci present in my images of *stu2* cells likely represent one set, or at most two sets, of paired kinetochores (unpublished data). Thus, my work shows for the first time that Ndc80-GFP can be used to detect individual budding yeast kinetochores that have become detached from the mitotic spindle. As the presence of unattached kinetochores had previously been missed in the analysis of the *stu2* mutants, my experiments also demonstrate that using GFP-tagged kinetochore proteins to survey the distribution of kinetochores within a cell can reveal heterogeneity that is less easily detected using single chromosome tracking methods. Examining kinetochore distributions complements information gained from the experiments probing the dynamics of individual chromosomes.

4.2 Three states of attachment?

Following DNA replication, budding yeast nuclei contain 32 kinetochores and an average of ~35 potential kinetochore MTs, suggesting that each kinetochore captures a single MT during spindle assembly (Winey et al., 1995). Although metazoan kinetochores recruit multiple MTs and kinetochore-MT attachment is a gradual process in animal cells, it has long been assumed that chromosome-MT attachment in budding yeast is an all-or-nothing event. In contrast, my data suggest that at least three states of kinetochore-MT attachment may exist

in budding yeast. One model consistent with my checkpoint protein localization data is that unattached kinetochores recruit both Bub and Mad checkpoint proteins, kinetochores with mature MT attachments recruit no checkpoint proteins, and kinetochores with immature MT attachments recruit Bub proteins but not Mad proteins. Should early kinetochore-MT attachments differ from mature kinetochore-MT attachments in structure or configuration, however, it is still difficult to say if these early attachments evolve to stable, mature Bub-negative structures, or if kinetochores release their initial early MT attachments and form novel attachments to new MTs as spindle assembly progresses. It is also possible that both of these processes may occur.

My experimental results also suggest something about what the nature of these immature attachments might be. In addition to being recruited to wild-type kinetochores during early mitosis, high levels of Bub proteins are found at kinetochores in *ipl1-321* and *mcd1-1* cells at non-permissive temperature, while high levels of Mad proteins are not. It is possible that Bub proteins may be responding to a physical trait of kinetochores that is similar in all three of these situations. In *ipl1-321* cells, chromosomes are thought to have syntelic attachments in which both sister chromatids are attached to a single SPB (Tanaka et al., 2002). In contrast, in *mcd1-1* cells, cohesin complexes are non-functional and sister chromatids segregate independently (Guacci et al., 1997; Michaelis et al., 1997). What property might kinetochores in early mitotic cells, *ipl1-321* cells, and *mcd1-1* cells have in common? One possibility is that each of these kinetochores may be forming attachments to the lateral sides of MTs rather than their plus ends. It is well documented that kinetochores in metazoan cells frequently form initial attachments to the lateral sides of MTs (Merdes and

De Mey, 1990), and *in vitro* experiments have demonstrated that budding yeast kinetochores are also capable of making lateral attachments to MTs (Sorger et al., 1994).

As kinetochores are invisible to current electron microscopy techniques, proving the existence of lateral kinetochore-MT attachments in budding yeast is not feasible at present. However, irrespective the nature of the kinetochore attachments that recruit Bub and not Mad proteins, our experiments definitely suggest that single kinetochore-MT attachment events in budding yeast may be more complicated than originally suspected. This potential complication should be taken into account when interpreting experiments designed to test the spindle checkpoint response to loss of tension between sisters. To date, virtually all of the experiments probing this issue have employed cells containing unpaired sister chromatids generated either by inactivation of cohesin (in *mcd1-1* cells; Guacci et al., 1997; Michaelis et al., 1997; Biggins and Murray, 2001) or inhibition of DNA replication (in *cdc6*Δ cells; Piatti et al., 1995; Biggins and Murray, 2001; Stern and Murray, 2001). My work with these two mutants suggests that individual kinetochores in these cells lose Bub1p association during spindle elongation, despite the fact that they never experience bipolar tension. This suggests that unpaired kinetochores still transition from a state of immature attachment that recruits Bub1p to a state of mature attachment in which Bub1p is not bound. This transition appears to be somewhat less efficient in the absence of partner kinetochores, but independent of tension as bipolar attachments are never established under these conditions. This kinetochore transition may be similar, in principle, to the MT-dependent, but tension independent, maturation process exhibited by kinetochores in PtK_1 cells (Hoffman et al., 2001).

Although it will be difficult to define the exact nature of the kinetochore-MT attachments that recruit Bub, but not Mad, proteins, my work indicates that characterizing

Bub and Mad proteins localization can expose subtle differences between kinetochore mutants with similar phenotypes. This was certainly true in the case of the *dam1-1* and *ipl1-321* mutants. Differences between the monopolar attachment phenotypes in these two mutants had previously been suggested by data from studies of chromosome dynamics (He et al., 2001). Our observation that Mad2p is recruited to kinetochores in *dam1-1* cells but not *ipl1-321* cells reinforces the idea that the kinetochore-MT attachments in these two mutants are significantly different.

4.3 Illuminating the Interface between Spindle Checkpoint Proteins and Kinetochore Components

The NDC80 complex appears to have a unique role in relation to spindle checkpoint binding to kinetochores. Before I began this work, it was assumed that because *ndc80-1* kinetochores detach from spindle MTs at non-permissive temperature, most kinetochore components are likely to be disrupted in these cells. Therefore, it was initially surprising to us that both Bub1p and Mad2p could be detected at kinetochores in *ndc80-1* cells. Interestingly, mutating a second member of the complex, *SPC25*, prevents checkpoint protein binding, suggesting that *ndc80-1* allele may not completely deactivate NDC80 complexes. Data from other groups has also implicated the NDC80 complex in spindle checkpoint responses (McCleland et al., 2003) and human Ndc80 and Nuf2 are required for high levels of Mad1 and Mad2 to accumulate on kinetochores (Meraldi et al., 2004). In contrast to many other budding yeast kinetochore components, the NDC80 complex has been conserved through evolution, which makes it a good candidate for interacting directly with conserved spindle checkpoint components. Further studies probing the role of the NDC80 complex in

recruiting checkpoint proteins to kinetochores may lend insights into how the checkpoint senses kinetochores with improper attachments and what elements of the kinetochore modulate its ability to activate checkpoint signaling during the attachment process.

An important question in the checkpoint field is whether or not checkpoint proteins must be associated with kinetochores in order to invoke a cell cycle arrest. In some instances, Mad2-dependent delays have been described in cells where Mad2 was not detectable at kinetochores (Martin-Lluesma et al., 2002; Shannon et al., 2002). As even a single unattached kinetochore recruiting Mad2 is sufficient to activate the spindle checkpoint, this is an extremely tricky issue to resolve. One interesting observation made from studies of budding yeast is that GAL-MPS1 overexpression can cause a checkpoint arrest in *ndc10-1* cells at non-permissive temperature when kinetochores are not properly assembled (Fraschini et al., 2001a; Poddar et al., 2004). Although GAL-MPS1 overexpression may not be a physiologically relevant manner in which to activate the spindle checkpoint, examining the localization of Bub and Mad checkpoint proteins, as well as chromosome dynamics, in GAL-MPS1 overexpressing cells may still increase our understanding of checkpoint signaling or alternative mechanisms of checkpoint activation. A second piece of evidence that Mad2p may affect mitotic timing without being visible at kinetochores comes from *mcd1-1* cells. Although these cells are reported to experience a Mad2p-dependent checkpoint delay (Stern and Murray, 2001), my data show that *mcd1-1* kinetochores recruit high levels of Bub1p but no visible Mad2p. This suggests either that Mad2p has an off-kinetochore function, or that the levels of Mad2p associated with kinetochores in these cells are below our limits of detection. Another interesting characteristic of Mad2p is that it appears to cause a delay in mitotic progression prior to completion of kinetochore assembly in mammalian cells

(Meraldi et al., 2004). This supports the idea that Mad2p may not always require kinetochore association in order to invoke a mitotic delay.

4.4 Exploring Differences between the Roles of Bub and Mad Proteins

My localization studies reinforce the idea that Bub and Mad checkpoint proteins have distinctive roles during mitosis in budding yeast. One future approach that may contribute to our understanding of these differences is to find and compare mutants that are synthetically lethal with deletions of the *BUB* and *MAD* genes. As the Bub1p kinase domain may not be absolutely required for checkpoint signaling (Sharp-Baker and Chen, 2001; Warren et al., 2002), comparing mutants that are synthetically lethal with kinase dead Bub1p versus those that are synthetically lethal with a total deletion of *BUB1* may also be informative.

Examining the chromosomes dynamics in cells depleted of Bub and Mad proteins could also expose further differences between the various checkpoint proteins. Deletion of either *BUB1* or *BUB3* causes an initial slow growth phenotype that reverts as cells adapt and secondary, unidentified mutations accumulate (Hoyt et al., 1991; Roberts et al., 1994). This suggests that in order to examine the full consequences of Bub depletion, an inducible system must be employed. One possibility is to design degron alleles of the *BUB* genes which, when invoked, will cause rapid degradation of the Bub proteins (Dohmen et al., 1994). An alternate approach to test the immediate effects of specifically inhibiting Bub1p kinase activity is to employ the strategy designed by Kevan Shokat in which the ATP binding pocket in a protein's kinase domain is modified to accommodate an ATP-analog which, when added to cells, specifically inhibits only the modified kinase (Bishop et al., 1998).

Identifying what types of changes occur in *bub1*Δ and *bub3*Δ cells during adaptation may also help us understand the roles these proteins play during spindle assembly. The easiest approach to this issue might be to utilize gene chip technologies to profile the mRNA expression levels across the genome in wild-type cells and adapted *bub1*Δ or *bub3*Δ cells. Depending on whether compensatory mutations are dominant or recessive, more classical gene mapping and cloning techniques may be appropriate. Examining the behavior of Bub and Mad proteins in the context of diploid cells, polyploidy cells, and meiotic cells may also be informative. Work from the Pellman lab has shown that deletion of some genes that do not produce a strong phenotype in haploid or diploid cells, such as *BIK1*, can cause lethality in polyploid cells (Lin et al., 2001; Storchova and Pellman, 2004). Although the majority of the work on spindle checkpoint function in budding yeast has been done in haploid cells, wild strains of this organism most often grow as diploids. It will therefore be interesting to learn if checkpoint protein localization, or the behavior of chromosomes during mitosis, differs significantly in diploids or polyploids. Studies examining relationships between the spindle checkpoint and polyploidy could inform our understanding of tumorigenesis, as cancer cells frequently contain excessive numbers of chromosomes and exhibit chromosomal instability.

4.5 Defining Kinase Contributions to the Spindle Checkpoint and Spindle Assembly

Identifying the consensus target sequences and direct substrates of the Bub1p, Mps1p, and Ipl1p kinases at kinetochores is certain to increase our understanding of the roles that these proteins play in spindle assembly and checkpoint response. It is intriguing that both Bub1p and Ipl1p are only present at kinetochores early during mitosis in budding yeast. My

preliminary results suggest that while Mps1p is associated with the SPBs throughout the cell cycle in budding yeast, Mps1p also associates with kinetochores only during early mitosis (unpublished data). Temperature sensitive mutants of these proteins exist and, as described earlier, employing Shokat alleles is a second way in which to probe the activities of each of these kinases (Bishop et al., 1998). It will be interesting to learn how disabling each of these kinases separately, and in combination, affects spindle assembly and checkpoint signaling.

4.6 Biochemistry of Spindle Checkpoint Complexes

Although several binding interactions have been identified between different spindle checkpoint components, how checkpoint complexes change during checkpoint activation and inactivation is uncertain. Some have suggested that the checkpoint proteins in mammalian cells form a large complex called the mitotic checkpoint complex (MCC) which consists of hBubR1, hBub3, Cdc20, and Mad2 (Sudakin et al., 2001). Thus far, biochemical characterizations of complexes in budding yeast have suggested that the checkpoint proteins likely form multiple different complexes, and that the spindle checkpoint is unlikely to behave as a linear signaling pathway (Brady and Hardwick, 2000; Fraschini et al., 2001a; Fraschini et al., 2001b). Recent work using velocity sedimentation gradients and gel filtration techniques have greatly expanded our understanding of the budding yeast kinetochore and its subcomplexes, and it seems likely that applying similar techniques to analyzing spindle checkpoint components will help us to understand which complexes are critical for generating the "wait anaphase" signal, and how they interact with one another.

An additional technique that has begun to reveal new aspects of checkpoint signaling dynamics is fluorescence recovery after photobleaching (FRAP). (Howell et al., 2004; Shah

et al., 2004). These studies have indicated that there is both a pool of Mad2 that turns over rapidly at the kinetochore, and a pool that turns over more slowly. As others have previously proposed that checkpoint function requires both free Mad2 and Mad2 bound to Mad1 (Chung and Chen, 2002), one idea that is emerging is that Mad2 bound stably to Mad1 at the kinetochore may serve as a platform to activate additional Mad2 molecules which then inhibit Cdc20. Although FRAP at kinetochores would be technically difficult in *S. cerevisiae*, budding yeast may be a good system in which to rapidly test the localization and checkpoint activity of mutant proteins based on the data gained from FRAP studies and other experiments done in metazoan cells.

4.7 Links to the MLP Proteins and DNA Damage

Mad1p and Mad2p localize to the nuclear envelope in both yeast and metazoans, suggesting that they may have important functions at this location. My work on the perinuclear localization of Mad1p has revealed a novel connection between the Mad proteins and Mlp proteins. Mlp proteins are thought to link NPCs to the interior of the nucleus and have been implicated in several processes, including the DNA damage response. As several groups have reported that Mad2p contributes to cell cycle arrest following DNA damage, it will be interesting to examine the localization of the Mad proteins in cells following DNA damage and to determine if Mad2p-dependent DNA damage responses require functional kinetochores. Much more work remains to be done in characterizing the Mad-Mlp connection, including determining whether or not the interactions between these proteins are direct or indirect.

4.8 Summary

Overall, my work has contributed to the spindle checkpoint field by providing a comprehensive overview of the behavior of Bub1p, Bub3p, Mad1p, and Mad2p in wild-type and mutant budding yeast cells. It has both laid the foundation for future studies on the checkpoint proteins in yeast and suggested several interesting avenues for future research, including the maturation of kinetochore-MT attachments and potential interactions between the spindle checkpoint and DNA damage checkpoint pathways.

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Appendix

Gillett and Sorger, 2001

The following review is reprinted, with permission, from E.S. Gillett and P.K. Sorger. 2001. Tracing the pathway of spindle assembly checkpoint signaling. *Dev. Cell.* 1: 162-4.

Tracing the Pathway of Spindle Assembly Checkpoint Signaling

ABSTRACT

Most current models of spindle assembly checkpoint signaling involve inhibition of the Cdc20-APC by Mad2 protein. Interestingly, a paper from Hongtao Yu and colleagues in this issue of *Developmental Cell* suggests that the Cdc20/APC can also be inhibited in a Mad2-independent manner by a complex of proteins that includes BubR1.

DISCUSSION

During eukaryotic cell division, accurate transmission of the genome is essential for survival and is ensured both by intrinsic properties of the cell cycle machinery and by a series of checkpoints. The entry into mitosis is controlled by checkpoints that monitor DNA damage and the replicative state of DNA while the exit from mitosis is controlled by checkpoints that monitor assembly and position of the mitotic spindle. The spindle assembly checkpoint links chromosome-microtubule attachment to anaphase onset and is particularly intriguing because it serves as a link between the mechanical and regulatory aspects of mitosis.

Chromosomes bind to microtubules of the mitotic spindle via kinetochores, multiprotein complexes assembled on centromeric DNA. The search and capture process that drives microtubule-kinetochore attachment is stochastic; therefore, cells rely on the spindle

assembly checkpoint to monitor when all kinetochores have become attached to the spindle. Several components of the spindle assembly checkpoint have been identified, but the manner in which these proteins sense microtubule-kinetochore attachment, their order of action in the checkpoint signaling pathway, and the exact nature of the "wait" signal they produce are still under investigation.

Genetic experiments in *Saccharomyces cerevisiae* identified seven spindle checkpoint genes: BUB1, BUB2, and BUB3 (Budding Uninhibited by Benzamidazol); MAD1, MAD2, and MAD3 (Mitotic Arrest Deficient); and MPS1 (Monopolar spindle 1) (for references, see Amon, 1999). Subsequent work has established that Bub1p, Bub3p, and Mad1-3p are all essential for spindle assembly checkpoint function while Bub2p helps monitor spindle positioning as part of the mitotic exit network. Mps1p participates in both spindle pole duplication and the spindle assembly checkpoint pathway. The complicated pattern of biochemical interactions between spindle assembly checkpoint components supports the hypothesis that the checkpoint signal propagates via a nonlinear signaling network, reliant on several multiprotein complexes (Burke, 2000).

Orthologs of the budding yeast spindle assembly checkpoint genes exist in many organisms, including *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Xenopus*, mice, and humans, suggesting that the basic components of the checkpoint are well conserved among eucaryotes. Two recent papers have shown that the roles of mouse and *Xenopus* Mps1 appear to be analogous to those of Mps1p in budding yeast (Abrieu et al. 2001 and Fisk and Winey 2001). However, despite this conservation, different organisms are more or less dependent upon spindle assembly checkpoint function

during cell division. In budding yeast, spindle checkpoint activity is only required when spindle assembly is disabled by microtubule depolymerizing agents or other factors, while the complexity of spindle assembly in higher eucaryotes appears to necessitate checkpoint function even under normal conditions.

Kinetochores not only link chromosomes to microtubules, they also serve integral roles in transducing the spindle assembly checkpoint's "wait" signal. Checkpoint activation by unattached or tension-free kinetochores leads to inhibition of the anaphase promoting complex (APC). The APC is an E3 ubiquitin-protein ligase that targets key cell cycle regulators for degradation by the proteosome. At the metaphase-to-anaphase transition, the association of APC with Cdc20/p55Cdc/Fizzy activates its ubiquitination activity; the APC then triggers dissolution of the cohesin complexes that secure sister chromatids together and subsequently promotes the destruction of M phase cyclins, thereby enabling mitotic exit (Morgan, 1999).

The spindle assembly checkpoint protein Mad2 has long been thought to block activation of the APC by inhibiting its association with Cdc20 (Shah and Cleveland, 2000). In budding yeast, Mad2p, Mad3p, and Bub3p are believed to form a complex that binds to Cdc20p (Shah and Cleveland, 2000). It was therefore not surprising when human Cdc20 was discovered to interact with BubR1, a vertebrate protein homologous to Mad3p but with a kinase domain similar to that of Bub1p (Wu et al., 2000). Interestingly, in this issue of *Developmental Cell*, Tang et al. (2001) suggest that although both vertebrate Mad2 and BubR1 can bind to Cdc20, they are unlikely to do so in a single, isolatable complex. This paper begins with the characterization of a mitosis-specific complex containing BubR1,

Bub3, and Cdc20, but not Mad2, suggesting that vertebrate Mad2-Cdc20 and BubR1-Cdc20 complexes are independent.

Using in vitro assays, Tang et al. demonstrate that both BubR1 and Mad2 can sequester Cdc20 from APC and inhibit APC-mediated ubiquitination. In addition, the authors show that recombinant BubR1 inhibits Cdc20/APC at much lower concentrations than does recombinant Mad2. Although BubR1 can phosphorylate Cdc20 in vitro (Wu et al., 2000), the data from Tang et al. indicate that BubR1's kinase activity is not required for it to inhibit Cdc20/APC. Most research on APC activation has focused on the interactions between Cdc20 and Mad2. Therefore, Tang et al.'s suggestion that the output of the spindle assembly checkpoint pathway bifurcates into separate Mad2- and BubR1-dependent branches is very intriguing.

If BubR1 and Mad2 can each function independently to inhibit the APC, it is surprising that loss of either one abrogates spindle checkpoint arrest in vivo (Chan et al. 1999 and Dobles et al. 2000). Tang et al. make a number of suggestions for reconciling the results of genetic and biochemical experiments (see Figure 1). Mad2, which has a half-life at kinetochores of 24–28 s, may act as a diffusible Cdc20 inhibitor, while BubR1 may act as a local inhibitor at kinetochores (Shah and Cleveland, 2000). Alternatively, BubR1 may recruit Cdc20 to kinetochores and facilitate its association with Mad2. A third intriguing possibility supported by additional data from Skoufias et al. is that Mad2 and BubR1 may block Cdc20/APC activation as part of separate signaling systems, the first acting in response to loss of microtubule attachment and the second in response to a lack of tension across kinetochores (Skoufias et al., 2001). Consistent with this third model, BubR1 associates with

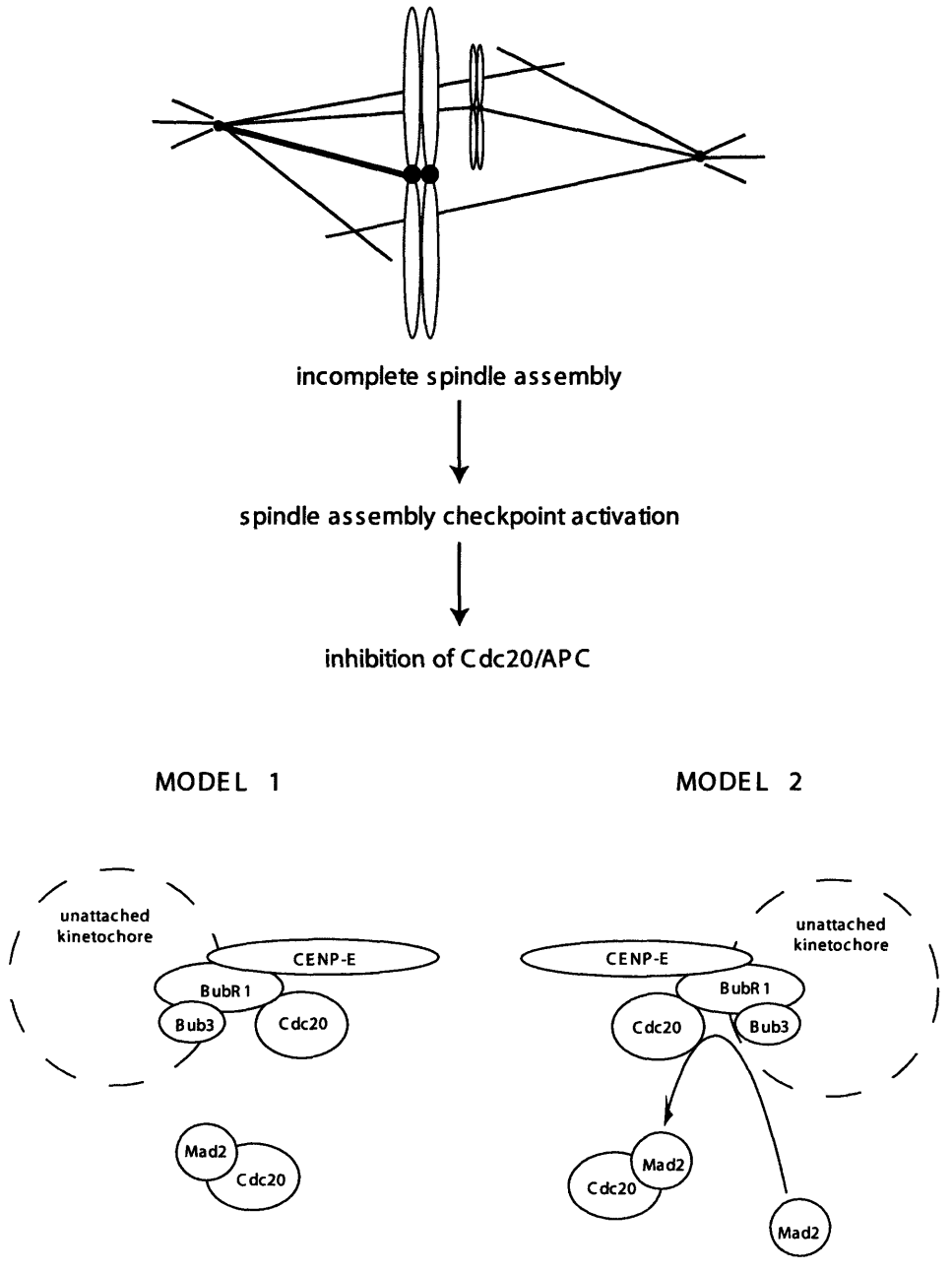

Figure 1. Proposed models for Mad2- and BubR1-dependent inhibition of Cdc20.
In Model 1, Mad2 and BubR1 form independent complexes with Cdc20. In this case, Mad2 could act as a diffusible inhibitor of Cdc20 while BubR1 acts as a local inhibitor at kinetochores. However, another possibility to explain the presence of separate Mad2-Cdc20 and BubR1-Cdc20 complexes in Model 1 is that Mad2 inhibits Cdc20 in response to loss of microtubule attachment while BubR1 inhibits Cdc20 in response to lack of tension across kinetochores (perhaps detected by the CENP-E motor). In Model 2, binding of BubR1 to Cdc20 instead facilitates the formation of an inhibitory Mad2-Cdc20 complex.

CENP-E, a kinetochore-localized kinesin-like motor whose interaction with microtubules could alter BubR1 activity in response to changes in kinetochore tension (Chan et al., 1999).

Although its exact mechanism remains uncertain, two requirements of the spindle assembly checkpoint signal are that it be diffusible to ensure that all sister chromatids separate in concert and that it decay relatively rapidly to allow anaphase to proceed in a timely fashion. Biochemical experiments provide invaluable information about kinetically stable complexes formed between checkpoint components, but higher order complexes formed through transient interactions can be missed. Experiments using live cell fluorescence microscopy will be necessary to map the dynamics of checkpoint signaling in greater detail. Biochemical analyses such as those presented by Tang et al. will remain essential, however, as they reveal to us which molecular interactions should be examined.

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The man of science is nothing
if not a poet gone wrong.
~ George Meredith