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SIGNALS AND COMPONENTS OF THE SPINDLE ASSEMBLY CHECKPOINT IN BUDDING YEAST

by

William Arnold Este Wells

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This thesis is dedicated to Julian Wells,

the most enthusiastic biologist I have ever known

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The spindle assembly checkpoint in budding yeast

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Abstract

Checkpoints delay the progression of the cell cycle until certain events are completed. The spindle assembly checkpoint is postulated to prevent the onset of anaphase until a bipolar spindle is assembled and all chromosomes are correctly attached. This thesis utilises the ability to create minichromosomes in *Saccharomyces cerevisiae* to gain evidence that kinetochores that fail to attach to the spindle can activate the spindle assembly checkpoint. Various mini-chromosomes result in mitotic delays, as seen in pedigrees of individual yeast cells, and these delays are abolished in the *mad* spindle assembly checkpoint mutants.

A screen for additional components of the MAD checkpoint has yielded new mutants with similarities and differences when compared to the original *mad* mutants. One mutant may identify a gene necessary to suppress G1/S specific events when cells are arrested in mitotis, although the mutant does not fully exit from mitosis as occurs in the *mad* mutants. Another mutation is an allele of TUB1, the major alpha-tubulin encoding gene in *S. cerevisiae*. The properties of this mutant when compared to other *tub1* mutants demonstrate that it is an unusual allele of TUB1. It may identify a second arm of the spindle assembly checkpoint which monitors the integrity of the spindle.

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Introduction

Introduction

The eukaryotic cell cycle is driven by the periodic activation of cyclin dependent kinases (cdk's). In simple embryonic cell cycles, the activation and inactivation of these kinases occur at regular intervals, which normally allows sufficient time for the completion of periodic events such as DNA replication and chromosome segregation (Cross et al., 1989). In more complex cell cycles, inhibition of these processes arrest the cell cycle. The systems which detect that these events are incomplete and then block transitions in the cell cycle machinery are called checkpoints (Hartwell and Weinert, 1989; Murray, 1992). Checkpoints contribute to the fidelity of the cell cycle; when they fail to function genetic errors arise much more frequently, even in the absence of cell cycle inhibitors (Weinert and Hartwell, 1990; Li and Murray, 1991). Mutation of checkpoints may be an early event in the genesis of cancer cells, allowing further genetic changes to accumulate more readily (Hartwell, 1992; Hartwell and Kastan, 1994). It may also be possible to use knowledge of checkpoints to combat cancer. Drugs which eliminate checkpoints increase the susceptibility of tumor cells to killing by other drugs which normally cause cell cycle arrests (Roberge et al., 1994; Russell et al., 1995; Powell et al., 1995; Fan et al., 1995). The selectivity of this approach can be high if the normal cells of the body still arrest, due to retention of additional checkpoints which the cancer cells have previously lost (Fingert et al., 1986).

This thesis concerns the spindle assembly checkpoint, which delays the onset of anaphase until a spindle is correctly assembled and all the chromosomes are attached to it. I will first discuss two related checkpoints which have been well studied in yeast that delay the onset of mitosis until damaged DNA is repaired and DNA replication is complete. I will not discuss a variety of less well characterised checkpoints such as those that detect failures in the condensation of chromosomes by topoisomerase II (Downes et al., 1994), the budding of *Saccharomyces cerevisiae* (Lew and Reed, 1995), the assembly of the flagellum

in *Salmonella typhimurium* (Hughes et al., 1993) and DNA replication in *Bacillus subtilus* (Ireton and Grossman, 1994).

I. The DNA Damage and Replication Checkpoints

The existence of a cell cycle checkpoint is defined by the ability to relieve a cell cycle block (Hartwell and Weinert, 1989). This block is usually caused by a chemical inhibitor or mutation, and the relief of the block can be caused by other chemicals. For example, caffeine leads to entry into mitosis in the absence of DNA replication (Schlegel and Pardee, 1986), without accelerating the cell cycle beyond its normal rate (Downes et al., 1990). The kinase inhibitor 2-aminopurine overcomes both replication and spindle assembly checkpoints (Andreassen and Margolis, 1992).

More commonly checkpoints have been studied by the isolation of mutants which relieve arrests. The first mutant of this type to be characterised was *rad9* from the budding yeast, *Saccharomyces cerevisiae*, which fails to arrest in response to radiation induced DNA damage (Weinert and Hartwell, 1988). Further studies in this organism have yielded three similar genes (*RAD17*: (Weinert and Hartwell, 1993), *RAD24* and *MEC3*: (Weinert et al., 1994)) and genes necessary for the DNA damage arrest as well as arrest in response to unreplicated DNA (*MEC 1*, and the protein kinase *MEC2/RAD53/SPK1*: (Weinert et al., 1994; Allen et al., 1994)). In the fission yeast, *Schizosaccharomyces pombe*, the genes necessary for the DNA damage checkpoint include *CHK1/RAD27*, a serine/threonine kinase (Walworth et al., 1993; Al-Khodairy et al., 1994) and two 14-3-3 proteins encoded by *RAD24* and *RAD25* (Ford et al., 1994). 14-3-3 proteins have been implicated in a wide variety of signal transduction pathways (Aitken et al., 1992). Again, some genes are required for arrest in response to both DNA damage and unreplicated DNA; in this group fall *RAD1*, *RAD3*, *RAD9*, *RAD17*, *RAD26*, *HUS1*, *HUS2*, *HUS3* and *HUS4* (Enoch et al., 1992; Al-Khodairy and Carr, 1992; Rowley et al., 1992; Al-Khodairy et al., 1994).

A checkpoint is expected to consist of three functional units. A sensor monitors the event (such as DNA damage) and generates a signal, which is passed on to a signal transduction cascade, eventually interacting with the cell cycle in order to halt it. Some attempts to rationalise the DNA replication and damage checkpoints in this manner have been made. There is substantial evidence that a functional DNA replication apparatus is required to act as a sensor for the DNA replication checkpoint. For example, the checkpoint defect in *cds1* mutants (see below) is only seen when DNA replication is partially, not fully, inhibited (Murakami and Okayama, 1995), as when replication is fully inhibited, no replication complexes remain to send a checkpoint signal. Other genes are required both for the initiation of S phase/DNA replication and to establish the DNA replication checkpoint. The fission yeast mutants *cdc18* (Kelly et al., 1993) and *cut5/rad4* (Saka and Yanagida, 1993) both fail to initiate DNA replication but enter a premature mitosis, suggesting that in this case the DNA replication checkpoint has never been established. Some budding yeast *cdc* mutants with defects in DNA replication normally arrest due to the DNA replication checkpoint, but tight alleles in which absolutely no initiation of replication occurs do not provide a template for the DNA replication checkpoint to sense, and thus the cell cycle is not stopped (J.H. Toyn and L.H. Johnston, pers. comm.). In non-arrested cell cycles, DNA synthesis is started well before this checkpoint is necessary, as the cells still require an active $p34^{cdc2/CDC28}$/cyclin B complex to enter mitosis.

Other systems may also help preserve the order of the cell cycle before establishment of the replication checkpoint in S phase. Sic1p, an inhibitor of $p34^{cdc2/CDC28}$/cyclin B complexes in budding yeast (Mendenhall, 1993), appears at the end of mitosis and is not destroyed by the ubiquitination enzyme Cdc34p until after START (Schwob et al., 1994). In fission yeast, the *RUM1* gene is necessary to prevent entry into mitosis in cells arrested before START (Moreno and Nurse, 1994).

A specific candidate for a part of the signalling machinery has been elucidated by

the isolation of the *dun2* mutant in *S. cerevisiae*. In response to incomplete DNA replication this mutant cannot induce the transcription of certain DNA damage-inducible genes, or arrest the cell cycle (Navas et al., 1995). *DUN2* is identical to *POL2*, which encodes DNA polymerase epsilon, one of three DNA polymerases essential for DNA replication, and possibly a repair enzyme (Nishida et al., 1988; Wang et al., 1993). Its proximity to DNA metabolism suggests it may act as a sensor of unreplicated DNA, or replication stalled due to chemical mutagenesis (but not radiation damage). Another connection is the Cds1 protein kinase in fission yeast (similar to the *RAD53/MEC2* gene in budding yeast), which is necessary to prevent mitotic entry from a replication arrest, and probably interacts with DNA polymerase alpha (Murakami and Okayama, 1995). Interestingly, the wild type gene also rescues the *rad1*, *rad3* and *rad9* mutants from their sensitivity to DNA replication inhibitors, but not irradiation. Finally, the Rad17 protein from budding yeast is reported to show homology with the DNA replication factor RF-C (Carr, A.M., cited in Murray, 1995).

As these checkpoints delay the entry into mitosis, the phosphorylations on $p34^{cdc2/CDC28}$ residues which inhibit entry into mitosis have been investigated as a possible endpoint of the pathways. These residues (threonine 14 and tyrosine 15 in *S. pombe*) are phosphorylated by the inhibitory Wee1 protein kinase, and dephosphorylated by the Cdc25 phosphatase (for a review see Coleman and Dunphy, 1994). In budding yeast, regulation of these phosphorylations is not necessary for arrest in response to damaged or unreplicated DNA (Amon et al., 1992; Sorger and Murray, 1992), but does play a role in the arrest in response to the failure to bud (Lew and Reed, 1995). It was previously reported that Wee1 was necessary for radiation mediated arrest in fission yeast (Rowley et al., 1992), but this does not appear to be the case (Barbet and Carr, 1993). Although the phosphorylation state of $p34^{cdc2/CDC28}$ affects the ability of fission yeast cells to arrest in response to damaged or unreplicated DNA (Enoch and Nurse, 1990; Al-Khodairy and Carr, 1992), neither Cdc25 or Wee1 are absolutely required for the arrest in

response to unreplicated DNA (Enoch et al., 1992).

Further evidence comes from the study of *Xenopus laevis* extracts. A replication checkpoint can be engaged in these extracts by the addition of a high concentration of nuclei and the DNA synthesis inhibitor, aphidicolin (Dasso and Newport, 1990). The phosphorylation state of $p34^{cdc2/CDC28}$ appears to be important in this system also (Kumagai and Dunphy, 1991; Smythe and Newport, 1992), but other reports argue for the presence of an inhibitor, as arrest can still occur with a $p34^{cdc2/CDC28}$ that cannot be phosphorylated (Kumagai and Dunphy, 1995). Thus it seems that although alteration of the phosphorylation state of $p34^{cdc2/CDC28}$ can accelerate mitotic entry and make restraint by the DNA replication checkpoint more difficult, this is not the normal mechanism by which the checkpoint works.

An alternative target for control has been suggested to be the destruction of cyclin A, which occurs before that of cyclin B, as a role for cyclin A in the DNA replication checkpoint has been demonstrated in frog extracts (Walker and Maller, 1991). A corresponding role for the S phase cyclin Clb5p in *S. cerevisiae* is being investigated (D. Germain and B. Futcher, pers. comm.). The isolation of the *HUS5* gene, a ubiquitin-conjugating enzyme homologous to the budding yeast gene *UBC9*, does not provide the expected clue to the end of the *S. pombe* DNA damage and replication checkpoints as *HUS5* is not required, as earlier reported, for cell cycle arrest (Al-Khodairy et al., 1995).

Can we get any other clues from other radiation checkpoints? *RAD9* is necessary for two additional radiation checkpoints in *S. cerevisiae*, which are in G1 (at START) and G1/S (after START but before DNA replication) (Siede et al., 1993; Siede et al., 1994). Its participation in so many checkpoints may reflect a role in processing damaged DNA. There is a prominent DNA damage checkpoint in G1 in many mammalian cell lines which requires the induction of p53 (Kastan et al., 1991; Kuerbitz et al., 1992). $p21^{CIP1/WAF1}$, an inhibitor of cyclin dependent kinases identified by several routes including as a downstream target of p53 (el-Deiry et al., 1993), is induced in p53

dependent G1 arrests (el-Deiry et al., 1994) and is necessary for the arrest (Deng et al., 1995). $p21^{CIP1/WAF1}$ binds to PCNA and inhibits processive DNA replication by DNA polymerase delta (Flores-Rozas et al., 1994).

The activity of p53 illustrates two possible responses to damage or errors in the cell. p53 can mediate cell cycle arrest, but it is also necessary for cancer cells to apoptose in response to certain anticancer agents (Lowe et al., 1993) and radiation (Lowe et al., 1994) and for T cells to apoptose in response to radiation (Lowe et al., 1993). Likewise in mitosis, microtubule disruption causes apoptosis in HL-60 leukemia cells (Martin and Cotter, 1990) and colcemid induces apoptosis in HeLa S3 cells (Sherwood et al., 1994), a cell type which also cell cycle arrests in mitosis very readily (Kung et al., 1990). p53 may be involved in these mitotic processes as well; a spindle assembly checkpoint dependent on the activity of p53 has been described in mice (Cross et al., 1995). Furthermore, the idea of apoptosis may be transferable to the unicellular yeasts (Murray, 1995); in budding yeast a double strand break in non-essential DNA can lead to a significant amount of cell death, largely independent of RAD9 function (Bennett et al., 1993).

Thus the study of these checkpoints has yielded a plethora of genes, but as yet relatively little mechanistic information. Closer study of the few components involved in sensing the defects is needed, while dominant activating alleles of genes whose products are in the pathways will help in both ordering the pathways and determining the identity of downstream components.

II. The Spindle Assembly Checkpoint

The first indication of the existence of a spindle assembly checkpoint came from the ability of mitotic inhibitors such as nocodazole, vinblastine and methylbenzylcarbamylate (MBC) to arrest cells in mitosis. These compounds all depolymerize microtubules, although it is now clear that doses which suppress microtubule dynamics but do not cause

depolymerization can also arrest the cell cycle (Jordan et al., 1992; Toso et al., 1993). The extent to which different organisms and cell types respond to these agents is extremely variable, ranging from essentially irreversible arrest to brief or non-existent arrest (Sluder, 1979; Kung et al., 1990). Evidence from the DNA damage checkpoint in yeast suggests that checkpoints can be downregulated in response to irreparable mistakes, saving the cell from a permanent, futile arrest (Sandell and Zakian, 1993).

In budding yeast, nocodazole prevents spindle formation by depolymerizing microtubules, and the cells arrest in mitosis (Jacobs et al., 1988) with high levels of cyclin B associated kinase activity (Amon et al., 1992). Two genetic screens for mutants which fail to arrest in similar drugs (benomyl or benzimidazole) have yielded six genes potentially involved in the spindle assembly checkpoint: *MAD1*, *MAD2* and *MAD3* (Li and Murray, 1991) and *BUB1*, *BUB2* and *BUB3* (Hoyt et al., 1991). In the presence of microtubule depolymerizing drugs, mutants in these genes fail to arrest and go through an abberant, error prone mitosis before entering the next cell cycle (as evidenced by rebudding and rereplication). In benomyl the mutants suffer enormous chromosome loss, but even in unperturbed cultures the *mad* mutants suffer increased chromosome loss, confirming the proposed surveillance function of this system (Li and Murray, 1991).

The sequences of the *MAD* and *BUB* genes indicate the existence of a signal transduction pathway. Bub3p is a substrate for the Bub1p protein kinase *in vitro* and also binds to and activates Bub1p (Roberts et al., 1994). Mad1p is a 90kD coiled-coil protein which becomes hyper-phosphorylated in response to microtubule depolymerization (Hardwick and Murray, 1995). This hyper-phosphorylation still occurs in *mad3* and *bub2* mutants, but not in *mad2*, *bub1*, and *bub3* mutants, suggesting that the latter mutants lie upstream of *mad1*, while *mad3* and *bub2* lie downstream or in a parallel pathway. The Mps1 protein kinase, encoded by an essential gene isolated in a screen for mutants with monopolar spindles (Winey et al., 1991), also has a defect in the spindle assembly checkpoint (Weiss and Winey, 1995). Unlike other mutants in spindle pole body assembly

such as *cdc31*, *ndc1* and *mps2*, *mps1* mutants fail to arrest either at the restrictive temperature (when the cell contains only a monopolar spindle) or in nocodazole. In these conditions Mad1p is not hyperphosphorylated (K. Hardwick, pers. comm.), suggesting that Mps1p also lies upstream of Mad1p.

Mad2p is a small (20kD) protein with no homology to known proteins; Mad3p is also unique, although it shares a short stretch of homology with Bub1p (K. Hardwick, pers. comm.). Bub2p is homologous to fission yeast Cdc16, which is necessary to maintain high $p34^{cdc2}$ kinase activity in response to a damaged spindle, and to suppress the formation of multiple septa (Fankhauser et al., 1993).

Studies in *Xenopus* extracts have identified a member of the MAP kinase family of protein kinases as a part of the signal transduction pathway of the spindle assembly checkpoint (Minshull et al., 1994). This possibility was raised by the study of the stable arrest of frog oocytes in metaphase II of meiosis, which is mediated by cytostatic factor (CSF). The proto-oncogene *mos* has CSF activity, and is necessary for the expression of CSF activity (Sagata et al., 1989). In lysates *mos* can activate MAP kinase (Shibuya and Ruderman, 1993) and a thiophosphorylated, activated MAP kinase can itself cause a CSF-like, metaphase arrest (Haccard et al., 1993). A spindle assembly checkpoint arrest, induced by the addition of a high density of nuclei and nocodazole, also requires the activity of a MAP kinase, p44ERK2 (Minshull et al., 1994). Unlike the destruction of cyclin after a CSF arrest (Lorca et al., 1991), the checkpoint arrest is not relieved by addition of calcium. The checkpoint is not activated by the microtubule polymerizing drug taxol, which does arrest other cell types im mitosis (Jordan et al., 1993). This raises the possibility that this checkpoint is detecting only one of two or more defects (such as free kinetochores and aberrant spindle structures) that can be detected in other cells. Mpk1p, a MAP kinase isolated as a component of the protein kinase C pathway (Lee et al., 1993), may play an analagous role in the spindle assembly checkpoint in budding yeast, as *mpk1* cells have some of the hallmarks of a spindle assembly checkpoint mutant (J. Minshull,

pers. comm.).

The sequences of the known yeast spindle assembly checkpoint genes give few clues as to the source and mechanism of generation of the signal that activates the checkpoint. The recent discovery that Mad2p is localised to kinetochores (R.-H. Chen, pers. comm.) suggests that kinetochores may be the feature of the spindle that is monitored. The induction of a mitotic delay by mutant, partially functional centromeres in budding yeast further implicates the kinetochore as a source of a checkpoint signal (Spencer and Hieter, 1992). Dicentric chromosomes also cause a cell cycle delay, which is dependent not only on the RAD9 gene, due to chromosome breakage, but also on the BUB2 gene, presumably due to the abberant interaction of the dicentric chromosome with the spindle (Neff and Burke, 1992).

Do the components of the kinetochore give clues as to the source of the checkpoint signal? Although budding yeast does not have a morphologically visible kinetochore (Peterson and Ris, 1976), a short (125 bp) stretch of DNA can be defined as necessary and sufficient for centromere function (Clarke and Carbon, 1980; Cottarel et al., 1989). This sequence contains three elements, termed CDEI, II and III, with CDEIII the most important (Carbon, 1984). Proteins which bind these sequences have been isolated, including for CDEI, Cbf1p (Cai and Davis, 1990; Cai and Davis, 1989) and for CDEII, Mif2p (postulated only, by Brown et al., 1993). A complex of three proteins, collectively named the Cbf3 complex, binds to CDEIII (Lechner and Carbon, 1991). The components of Cbf3 are Cbf3a/Cbf2/Ctf14/Ndc10 (Goh and Kilmartin, 1993; Jiang et al., 1993), Cbf3b/Cep3 (Lechner, 1994; Strunnikov et al., 1995) and Cbf3c/Ctf13 (Doheny et al., 1993). Although it was initially reported that a CBF3 complex was sufficient for binding of centromeric DNA to microtubules and subsequent motility (Hyman et al., 1992), it now appears that a functional kinetochore requires more proteins. None of the isolated proteins show the sequence motifs of a microtubule motor, and further proteins are necessary for microtubule binding *in vitro* (Sorger et al., 1994).

As expected for a defect that activates the spindle assembly checkpoint, mutation of *CTF13* leads to a mitotic delay, but many alleles of *ndc10* do not delay, resulting in the segregation of all DNA to one pole (Doheny et al., 1993; Goh and Kilmartin, 1993). If in this situation the structure of the entire kinetochore is destroyed, the kinetochore may be incompetent to signal that there is a defect. Alternatively, Ndc10 may have a more specific role in the detection of chromosome attachment defects and the subsequent signalling to the checkpoint, or the non-arresting mutants may be leaky, partial loss of function mutants.

The mammalian kinetochore is an entirely different challenge. It is a large, trilaminar structure, with two parallel electron-dense plates sandwiching an electron-transluscent region (Rieder, 1982). Multiple microtubules can be seen interacting with a single kinetochore, in contrast with the single kinetochore microtubule in budding yeast. Tandemly repeated satellite DNAs are correlated with centromere function, and alpha satellite DNA can by itself provide some limited kinetochore function in primates (Haaf et al., 1992). The role of unique sequences is not ruled out, however, and a *Drosophila* centromere core of 220 kb of DNA contains significant amounts of complex DNA (Murphy and Karpen, 1995). Various protein components of the mammalian kinetochore have been identified using autoimmune sera and monoclonal antibodies to mitotic chromosome scaffold preparations. CENP-A is related to histone H3 (Palmer et al., 1991; Sullivan et al., 1994) as is the related Cse4 from budding yeast (Stoler et al., 1995). CENP-B binds a 17 bp sequence in alpha satellite DNA (Masumoto et al., 1989), CENP-C is a structural protein related to Mif2 from *S. cerevisiae* (Brown, 1995) that determines kinetochore size (Tomkiel et al., 1994), and CENP-E is a microtubule motor (Yen et al., 1992). If cells are injected with antibodies to CENP-C before mitosis, they go on to arrest in mitosis with small or non-existent kinetochores with few associated microtubules (Tomkiel et al., 1994).

Further evidence for the kinetochore as the originator of a checkpoint signal has been obtained using the superior cytology of oocytes and tissue culture cells. These

systems have previously allowed the determination of many details of the mechanics of chromosome segregation (Rieder and Alexander, 1990; Skibbens et al., 1993). Given that mitotic chromosome movement is at least generally similar in yeast (Palmer et al., 1989), lessons learnt in the related field of the spindle assembly checkpoint may also be transferable between systems.

Clear results from mitotic PtK_1 cells demonstrate that single unattached kinetochores can delay the cell cycle (Rieder et al., 1994). There are various possible explanations for this class of checkpoint arrest. Proximity to the centrosome (i.e. distance from the metaphase plate) has been suggested as a signal (Nicklas and Arana, 1992), although no evidence of this has been obtained. Free kinetochores with none or few microtubules attached could generate a signal. Finally, monoattachment will result in a lack of tension due to an opposing kinetochore fiber, which could be sensed either at the centrosome or, more likely, at the kinetochore (McIntosh, 1991).

Tension has previously been invoked to explain the stability of the attachments of bioriented chromosomes, as opposed to those of monooriented chromosomes, which frequently detach from the spindle and reorient. By exerting tension with a micromanipulating needle, a unipolar attachment of a bivalent chromosome can be made stable (Nicklas and Koch, 1969; Ault and Nicklas, 1989; Nicklas and Ward, 1994). The required tension can also be acheived by interlocking two bivalents, which are both unipolar, but oriented to opposite poles and hence pulling on each other (Henderson and Koch, 1970). These experiments are supported by observations of the kinetochores of oscillating chromosomes, which can be seen to alternately stretch and compress (Skibbens et al., 1993).

Some evidence in mitotic PtK_1 cells supports the idea that lack of tension in the occupied kinetochore of monoattached chromosomes is the signal for the spindle assembly checkpoint (Rieder et al., 1994). If taxol is added after the last kinetochore attaches, the cells delay still longer, even though the spindle remains bipolar, all kinetochores remain

attached and the chromosomes are aligned at the metaphase plate. The only visible effect of the taxol is the suppression of chromosome oscillations, suggesting that tension at the kinetochores may be reduced, leading to the delay. This effect has been seen with low concentrations of taxol in other systems (Jordan et al., 1993); a caveat to this explanation is that earlier studies using microtubule depolymerizing drugs also reported delays despite the absence of visible microtubule depletion (Jordan et al., 1992; Toso et al., 1993), but subsequent inspection using electron microscopy revealed that these doses do lead to the attachment of fewer microtubules to the kinetochore and subtle disruptions of centrosome structure (Wendell et al., 1993).

Further evidence for a role for tension at the kinetochore lies in the variable appearance of the 3F3 epitope. The 3F3 antibody was originally raised to thiophosphorylated frog extracts, and the phosphoprotein target in these experiments remains unknown. The antibody stains all kinetochores weakly (the extent varies in different cell lines) but stains unattached kinetochores strongly (Gorbsky and Ricketts, 1993). On recently bioriented chromosomes which are travelling towards the metaphase plate, the kinetochore nearest the plate is more strongly stained. The 3F3 protein could therefore be governing the activity of kinetochore associated microtubule motors in order to encourage chromosome congression. A second possible function for the 3F3 protein is as an indicator of correct biorientation of a chromosome; the strong staining on monooriented chromosomes may indicate a lack of tension and constitute the checkpoint signal. In light of this possibility, it is interesting that injection of 3F3 antibodies causes mitotic arrest, perhaps by locking the checkpoint on (Campbell and Gorbsky, 1995). Congression of the chromosomes is not affected by antibody injection, but all kinetochores continue to show bright staining of the 3F3 epitope until just before the delayed anaphase. In grasshopper spermatocytes chromosomes can be micromanipulated such that one chromosome has both kinetochores attached to one pole. The high 3F3 staining on these kinetochores, which are not under tension, can be dimished by exerting an opposing force on the chromosome with

a glass needle to create tension (Nicklas et al., 1995).

The most compelling evidence for the role of tension in mitotic timing comes from a direct experiment in mantid spermatocytes similar to that described above. In this system a monovalent X chromosome can delay anaphase onset in meiosis. This delay is prevented by applying tension to the chromosome with a glass needle in order to simulate attachment to the opposite spindle pole (Li and Nicklas, 1995).

In contrast, recent studies in mitotic PtK_1 cells using laser ablation of kinetochores identify unoccupied kinetochores as the source of an inhibitory signal halting the cell cycle (Rieder et al., 1995). If the unattached kinetochore of the last monoattached chromosome is destroyed, the cell no longer delays as usual, but rapidly enters anaphase. Mantid spermatocyes, in which the checkpoint signal appears to arise from lack of tension at occupied kinetochores, may represent a unique solution for a unique system (a meiosis involving trivalent sex chromosomes), or alternatively sufficient tension may be sensed by the PtK_1 kinetochore after ablation such that no checkpoint signal is generated. One possible source of tension in this experiment is a large "polar wind" in this cell type. The polar wind is postulated to arise from microtubules growing out rapidly from the centrosome and exerting force on chromosome arms. This theory arises from the observation that if chromosome arms are severed by laser microsurgery, the arms are expelled outwards, and monoattached chromosomes settle at an equilibrium position far closer to the pole to which they are attached (Rieder et al., 1986). Polar wind forces may aid in the normal positioning of monooriented chromosmes nearer to the metaphase plate, where the free kinetochore can be more easily captured by the other pole (Rieder and Salmon, 1994). In any case, the presence of a checkpoint signal before ablation of the free kinetochore suggests that free kinetochores constitute one, if not the only, checkpoint signal. The true answer may lie in a combination of the two models, in which a lack of tension is sufficient to generate some free microtubule binding sites in the kinetochore, and it is the latter which signals to the checkpoint.

Experiments in sea urchin zygotes suggest that other features may be detected in a checkpoint arrest. The creation of monopolar spindles in sea urchin zygotes leads to a significant delay in anaphase onset, but if an intact, bipolar spindle is also present in the same cytoplasm the cell cycle is no longer delayed (Sluder and Begg, 1983; Sluder et al., 1994). These cells have a positive requirement for a bipolar spindle, but no inhibitory effect from monooriented chromosomes.

The final aim of the spindle assembly checkpoint is to prevent the onset of anaphase and the exit from mitosis. Biochemically, the end of mitosis is marked by the inactivation of $p34^{cdc2/CDC28}$ via the degradation of its associated B type cyclins (Murray and Kirschner, 1989; Amon et al., 1994). Cyclin degradation occurs by ubiquitin mediated proteolysis, with the cyclin targeted via an N-terminal destruction box (Murray et al., 1989; Glotzer et al., 1991). Ubiquitination proceeds by the step-wise transfer of ubiquitin moieties from an E1 enzyme to an E2 enzyme and then, often with the help of an E3, to lysine residues of the target protein which is then targeted for degradation by the proteasome, a complex proteolytic machine (reviewed by Ciechanover, 1994). Cyclin degradation requires a novel E2 which is not active on other substrates (Hershko et al., 1994). The budding yeast gene UBC9 encodes an E2 which, when defective, stabilises both CLB5 (an S phase cyclin normally unstable throughout the cell cycle) and CLB2 (degraded only in G1) (Seufert et al., 1995).

The required E3 is only active during mitosis, although the interphase version can be activated by $p34^{cdc2}$ kinase after a lag period (Félix et al., 1990; Hershko et al., 1994; Sudakin et al., 1995; King et al., 1995). It is a large (20S) complex which contains the Cdc16 and Cdc27 proteins (King et al., 1995). It likely also contains the Cdc23 protein, as Cdc16, Cdc23 and Cdc27 (all TPR proteins) associate with each other (Lamb et al., 1994), and Cdc16, Cdc23 and Cse1 are necessary for degradation of B type cyclins in budding yeast (Irniger et al., 1995).

Where does destruction occur? Antibody staining localizes Cdc27 to the

centrosome and, to a lesser extent, the spindle (Tugendreich et al., 1995). This correlates with the localisation of a subset of cyclin B (Alfa et al., 1990; Pines and Hunter, 1991; Jackman et al., 1995) and p34$^{cdc2/CDC28}$ (Bailly et al., 1989), as well as the majority of their associated kinase activity (Kubiak et al., 1993). Various lines of evidence point to the spindle as a requisite site for cyclin destruction. During *Drosophila* embryogenesis, cytoplasmic cyclin levels do not fluctuate, yet these cycles are dependent on the destruction of cyclin, presumably in a local fashion around the spindle (Edgar et al., 1994). Studies using 2-aminopurine (which abrogates the spindle assembly checkpoint) have demonstrated that in the absence of checkpoint mechanisms, the cell remains dependent on spindle structure for mitotic exit (Andreassen and Margolis, 1994). Only cells in which some p34$^{cdc2/CDC28}$ remains associated with centrosomal microtubules can exit mitosis. In sea urchin eggs that fail to respond to multiple unattached kinetochores, the cell cycle is still delayed by nocodazole (Sluder et al., 1986), which may reflect the need for a spindle in order to destroy cyclin, or may reveal another checkpoint monitoring spindle integrity. Thus, while subtle defects in chromosome attachment can prevent mitotic exit by activating a checkpoint, the complete absence of a spindle may prevent the progression of the cell cycle by spatially separating cyclin B and the machinery which destroys it.

Must the spindle assembly checkpoint only prevent cyclin destruction? Cyclin destruction can be prevented experimentally by the expression of a mutant form of cyclin lacking the destruction box. This causes cell cycle arrest in mitosis, although chromosome separation occurs (Surana et al., 1993; Holloway et al., 1993). In contrast, inhibition of the ubiquitination system by antibodies (to Cdc27: Tugendreich et al., 1995), methyl-ubiquitin (Holloway et al., 1993), or mutation (*cdc16, cdc23 and cdc27* mutants in *S. cerevisiae*) causes a metaphase arrest. This suggests that the ubiquitination machinery also degrades a protein responsible for sister chromatid cohesion. Thus, when neither cyclin or the putative sister chromatid "glue" protein are degraded, the cell cycle halts at metaphase. This latter situation is also seen in mutants defective in components associated with the

budding yeast 26S proteasome such as *prg1, cim3* and *cim5*, which arrest with a single nucleus, short spindle (thus pre-anaphase) and replicated DNA (Ghislain et al., 1993; Friedman and Snyder, 1994). *cim3* and *cim5* also exhibit elevated Clb2 and Clb3 cyclin levels at the arrest, and the temperature sensitivity of *prg1* is suppressed by deletion of the CLB2 gene. The *ubc9* mutant also arrests after S phase and before anaphase, although the pre-anaphase arrest could result from the concommitant stabilization of Clb5p, rather than of a glue protein (Seufert et al., 1995).

The B type cyclins and the destruction machinery are therefore both possible targets of the spindle assembly checkpoint. The phenotype of *ubc9* favors individual cyclins as the target of the destruction machinery, as while CLB5 is being destroyed by a UBC9-dependent mechanism throughout the cell cycle, CLB2 destruction via UBC9 occurs only after mitosis. Similarly, cyclin A is destroyed earlier than cyclin B in unperturbed cell cycles (Minshull et al., 1989; Hunt et al., 1992) and is destroyed while cyclin B is protected in a checkpoint arrest (Minshull et al., 1989; Minshull et al., 1994). Some phosphorylation sites on frog cyclin B1 and B2 have been mutated; these mutants do not show any defects in $p34^{cdc2}$ activation or cyclin degradation, but possible effects on spindle assembly checkpoint arrest were not investigated and other phosphorylation sites were not mutated (Izumi and Maller, 1991). Of course it also remains possible that the destruction machinery can be modified to be inactive towards some substrates while still active towards others. If true, the checkpoint would have to prevent the destruction machinery from destroying not only cyclin B, but also any putative sister chromatid glue protein and perhaps the NimA protein, whose destruction is required for the exit from mitosis in *Aspergillus* (Pu and Osmani, 1995). In support of the destruction machinery as the target, in a cell free system destruction is dependent on the cell cycle state of the lysate not the cyclin (Luca and Ruderman, 1989).

Recent characterization of the *CDC55* gene in budding yeast has demonstrated an alternative means by which the cell can exit mitosis (Minshull et al., 1995). CDC55 is a

regulatory subunit of protein phosphatase 2A (PP2A) with a role in cell shape and morphogenesis at START (Healy et al., 1991). A *Drosophila* subunit of PP2A, however, shows defects in anaphase (Mayer-Jaekel et al., 1993), and *cdc55* mutants in budding yeast have some of the characteristics of *mad* mutants. They exit mitosis in the presence of nocodazole but, unlike the *mad* mutants (Hardwick and Murray, 1995), maintain high levels of Clb2p. The level of Clb2p associated H1 kinase activity does drop, concommitant with sister chromatid separation, and both events are prevented by a mutated *CDC28* gene in which Thr18 and Tyr19 are replaced with non-phosphorylatable residues. PP2A is known to promote the phosphorylation of $p34^{cdc2/CDC28}$ on these inhibitory residues via its action on the Wee1 kinase and the Cdc25 phosphatase (Coleman and Dunphy, 1994). In the absence of Cdc55p, the activity of PP2A may be increased, leading to $p34^{cdc2/CDC28}$ phosphorylation and inactivation, and exit of the cells from mitosis. It is unclear, however, if *CDC55* plays a role in the normal checkpoint arrest. It is not known if its activity changes in a checkpoint arrest or whether any of the known checkpoint genes feed through a pathway involving *CDC55*. The slippage out of checkpoint arrest in other systems is correlated with cyclin destruction rather than kinase inactivation (Minshull et al., 1989).

Many questions remain with regards to the spindle assembly checkpoint. Although recent studies have further implicated the kinetochore as the source of the checkpoint signal, it is not clear if this is the same system as that studied in yeast by the isolation of mutants, and it is not known what components are involved at the kinetochore. The signal transduction cascade is poorly defined and likely lacks some members. And finally, the possibility remains that there is more than one control over mitotic progression in response to spindle disruption.

This thesis is comprised of three parts. In the first chapter, the source of the checkpoint signal is examined. This work establishes that the kinetochore acts as a signal in yeast, and that the *MAD* gene products are part of this checkpoint. The second chapter

describes a screen for further *mad* mutants in an attempt to isolate more components of the checkpoint. Two mutants are described with both similarities and differences to the previous *mad* mutants. Finally, one other mutant from this screen is described in detail. This mutant is defective in Tub1p, the major alpha-tubulin in *S. cerevisiae*. The characterization of this mutant suggests the possibility of a second checkpoint pathway, partially overlapping with the MAD checkpoint, which monitors spindle structure.

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Chapter 1

Aberrantly segregating centromeres activate the spindle assembly checkpoint in budding yeast

Abstract

We have investigated the effects of mini-chromosomes on the cell division cycle in budding yeast by performing pedigree analysis. This method allowed us to observe the frequency and duration of cell cycle delays in individual cells. Short, centromeric linear mini-chromosomes, which have a low fidelity of segregation, cause frequent delays in mitosis. Their circular counterparts and longer linear mini-chromosomes, which segregate more efficiently, show a much lower frequency of mitotic delays, but these delays occur much more frequently in divisions where the mini-chromosome segregates to only one of the two daughter cells. Using a conditional centromere to increase the copy number of a circular mini-chromosome greatly increases the frequency of delayed divisions. In all cases the division delays are completely abolished by the *mad* mutants that inactivate the spindle assembly checkpoint, demonstrating that the Mad gene products are required to detect the subtle defects in chromosome behavior that have been observed to arrest higher eukaryotic cells in mitosis.

Introduction

Progress through the cell cycle depends on specific transitions caused by the activation and inactivation of cyclin dependent kinases. Activating the kinase activity of $p34^{cdc2/CDC28}$ -cyclin B complexes induces entry into mitosis. Ubiquitin-mediated proteolysis of cyclin B (Glotzer et al., 1991; Hershko et al., 1991) and other proteins (Holloway et al., 1993) is required to inactivate the kinase and allow anaphase and cytokinesis. To ensure the fidelity of chromosome segregation, anaphase can be delayed if the cell detects a problem in assembling the spindle or aligning the chromosomes on it. This control is an example of the growing number of cell cycle checkpoints that have been identified by mutations that can bypass cell cycle arrests induced by defects in the chromosome replication and segregation cycle (reviewed in Hartwell and Weinert, 1989; Murray, 1995; Murray, 1994; Hartwell and Kastan, 1994).

The prototypic checkpoint mutant is the budding yeast *rad9* mutant, which prevents cells from delaying their cell cycle in response to DNA damage (Weinert and Hartwell, 1988). The budding yeast *mad* (mitotic arrest deficient; Li and Murray, 1991) and *bub* (budding uninhibited by benzimidazole; Hoyt et al., 1991; Roberts et al., 1994) mutants define the spindle assembly checkpoint that monitors the structure of the mitotic spindle. In the presence of benomyl, a microtubule depolymerizing drug which prevents proper spindle assembly, these mutants fail to arrest in mitosis and thus suffer lethal damage. They also suffer increased rates of chromosome loss during normal vegetative growth, presumably due to a failure to detect occasional defects in chromosome attachment.

How does the spindle assembly checkpoint detect defects in the chromosome segregation machinery? One possibility is that the checkpoint monitors the interaction between microtubules and kinetochores, the protein complexes assembled on the centromeric DNA. In this scenario, chromosomes whose kinetochores are not attached to microtubules would generate the signal that the cell is not ready to exit mitosis. The observation that mammalian cells do not initiate anaphase until the last free kinetochore in

the cell attaches to the spindle (Rieder et al., 1994) supports this hypothesis, as do the mitotic delays caused by the injection of antibodies to kinetochore proteins (Bernat et al., 1990; Tomkiel et al., 1994).

Although the small size of budding yeast precludes the direct observation of individual chromosomes in living cells, the ability to manipulate chromosome structure in defined ways provides a powerful tool for analyzing mitosis. The short region of DNA which is necessary and sufficient for centromere function has been defined (Clarke and Carbon, 1980). Circular and linear mini-chromosomes can be created by combining centromeric sequences with DNA segments that act as origins of replication, telomeres, and encode selectable markers (Clarke and Carbon, 1980; Dani and Zakian, 1983; Murray and Szostak, 1983a). Using such artificial chromosomes we can perturb the process of chromosome segregation without the use of drugs or mutations and ask whether defects in the ability of mini-chromosomes to attach to the spindle engages the spindle assembly checkpoint. For example dicentric chromosomes induce a cell cycle delay that depends on both the G2 DNA damage and spindle assembly checkpoints (Neff and Burke, 1992), and the presence of certain mutant centromeric sequences induces mitotic delays (Spencer and Hieter, 1992).

Pedigree analysis in budding yeast allows the segregation of mini-chromosomes to be followed in defined cell lineages and correlated with defects in cell division (Murray and Szostak, 1983b). We have used pedigree analysis to follow the segregation of a variety of mini-chromosomes which carry a single wild type centromere, but whose segregation is compromised by their small size. We show that monocentric mini-chromosomes that carry wild type centromeres can induce mitotic delays, that these delays are correlated with errors in mini-chromosome segregation, and that the severity of the delay increases with the copy number of the mini-chromosomes. These mitotic delays are completely abolished by *mad* mutations, strongly suggesting that the spindle assembly checkpoint monitors kinetochore-microtubule interactions.

Materials and Methods

Yeast strains and media

Table 5 lists the strains used in this work. The original *mad* mutants were isolated in the A364A background (Li and Murray, 1991). Media were prepared and genetic manipulations were performed as described (Sherman et al., 1974).

Pedigree analysis

Pedigree analysis was performed as described (Murray and Szostak, 1983b). All pedigrees were started with unbudded cells, and the interval between observing cells in a given pedigree never exceeded 90 minutes. Pedigrees involving transfer from galactose- (YPGalactose) to glucose-containing medium (YPD) were performed on plates containing two segments of agar, YPGalactose and YPD, separated by a gap of 2-5 mm. Unbudded cells from stationary cultures grown in glucose-containing medium were placed on the YPGalactose segment of the plate, allowed to undergo from one to three divisions and then transferred to the YPD segment of the plate and followed for a further two to three divisions. For these experiments, cells were transferred from YPGalactose to the adjacent YPD slab as soon as possible after their last division. Incubation was at 30°C except for the time taken to inspect the cells, when they were at room temperature.

During pedigree analysis we scored divisions as delayed if they satisfied three criteria: the mother cell was unusually large at the time of cell division, the mother and daughter cells were of similar size at the time of division, and the division of the affected cell was delayed relative to other cells in the same pedigree. Although these criteria are somewhat subjective, the almost perfect correlation between delayed divisions and cells that are subsequently shown to contain the mini-chromosome argues that they are robust and reliable.

Immunofluorescence

Logarithmically-growing cells were treated with 10% formaldehyde for 1 hour at 22 degrees, then washed twice with 0.7M sorbitol, 0.1M KPO_4, pH 7.5. The cell walls were digested with zymolyase in 0.7M sorbitol, 0.1M KPO_4, pH 7.5 for 30-40 minutes at 37°C, before being attached to poly-lysine coated multi-well microscope slides. The slides were plunged into methanol (-20°C) for 5 minutes and acetone (-20°C) for 30 seconds and then allowed to air-dry. After washing with PBS, cells were blocked for 30 minutes with PBS with 4% milk and 0.1% Tween 20 (PBSTM) and washed with PBSTM. Staining was overnight at 4°C with primary rat anti-tubulin antibody diluted 1:200 in PBSTM. Cells were washed several times with PBSTM and then incubated for one hour at room temperature with FITC-labeled anti-rat secondary antibody at 1:50 dilution. After washing with PBSTM and then PBS, the DNA was stained with 1 mg/ml DAPI in PBS and cells were mounted in 90% glycerol, 1 mg/ml phenylenediamine, pH 9.0. Coverslips were sealed with clear nail polish and slides stored at -20°C.

Results

Short linear mini-chromosomes cause a *mad* dependent mitotic delay.

We investigated the relationship between the spindle assembly checkpoint and the segregation of linear mini-chromosomes. Although these small artificial chromosomes contain all of the elements required for normal chromosome function, their distribution is much less faithful than that of natural chromosomes (Murray and Szostak, 1983a; Dani and Zakian, 1983). Natural budding yeast chromosomes are present in a single copy in haploid cells and are lost at a frequency of approximately one division in 10^5, but linear mini-chromosomes have an average copy number of 15 and are lost at a frequency of 0.1 (Murray and Szostak, 1983a). The high copy number of short linear mini-chromosomes is likely to be a direct consequence of their poor segregation: a high frequency of segregation errors will create cells with increased copy numbers, and these cells will be favored by growth in selective medium, since the probability of generating two mini-chromosome bearing progeny increases with increasing copy number.

We asked if the presence of such poorly segregating linear mini-chromosomes perturbed passage through mitosis. These experiments were performed by pedigree analysis, in which a small lineage of cells is produced by separating the progeny of an individual cell, and each member of the pedigree is allowed to grow up into a colony of cells that can be tested for the presence of the selectable marker that the mini-chromosome carries. We followed cells harboring either the 16 kb linear mini-chromosome pVL106 (V. Lunblad, personal communication) or the 11 kb linear mini-chromosome YLp4 (Murray and Szostak, 1983a) (Figure 1). At each cell division, the size of the cell, the size of the bud (if any), the time of division and the relationship (mother versus daughter) of the cells were recorded. We scored divisions as delayed if they satisfied three criteria: the mother cell was unusually large at the time of cell division, the mother and daughter cells were of

similar size at the time of division, and the division of the affected cell was delayed relative to other cells in the same pedigree.

Cells lacking the mini-chromosome can arise by loss of the mini-chromosome in the cell divisions either before or during pedigree analysis. Since the pattern of cell divisions was observed before the pattern of chromosome segregation was determined, these experiments are intrinsically double blind. In cells lacking the mini-chromosomes the division time was quite uniform (approximately 90 minutes), cell division occurred before the bud grew as large as the mother cell, and mother cells always completed cytokinesis before the daughter they had produced in the preceding division had divided (Fig. 2a).

In contrast to the mini-chromosome-free divisions, 52-61% of divisions of the cells containing linear mini-chromosomes were delayed (Table 1). Figure 2b shows a representative pedigree in which cell division was delayed and the cells could not be separated even after the daughter cell had grown to be as large as the mother cell and both cells had rebudded. Such delays depend on the presence of the centromere of the mini-chromosomes. The divisions of cells containing the related acentric plasmids, pVL111 (V. Lunblad, personal communication) and YLp30 (Murray and Szostak, 1983a), are not delayed (Table 1). Acentric linear mini-chromosomes are present at roughly three times the copy number of centromeric linear chromosomes (Murray and Szostak, 1983a). Thus the division delays in cells containing centromeric mini-chromosomes are not due to the presence of excess telomeres or any other non-centromere element on the linear mini-chromosomes.

We determined whether the delay due to linear mini-chromosomes was due to the spindle assembly checkpoint by performing pedigree analysis on the *mad* mutants, which lack this control. When pedigrees were performed in *mad1*, *mad2* and *mad3* strains almost no delays were seen (0-4%, Table 1). Thus the division delay is induced by the engagement of the spindle assembly checkpoint, despite the presence of an apparently normal spindle as assessed by anti-tubulin immunofluorescence (data not shown).

To see where in the cell cycle the short linear mini-chromosomes are causing a delay, wild type or *mad1* cultures containing various mini-chromosomes were fixed and stained with anti-tubulin antibodies. The presence of the linear form of pVL106 in wild type cells results in a marked increase in the proportion of cells with short spindles as compared to cells that lack mini-chromosomes (Table 2). This observation indicates that the cell cycle delay induced by linear mini-chromosomes occurs after the onset of spindle assembly. FACS analysis also indicates an increase in cells with a 2C DNA content (data not shown). Control cultures with an acentric linear mini-chromosome (pVL111) or *mad1* cells carrying a centromeric mini-chromosome show the same distribution of spindle morphologies as mini-chromosome free cells. These comparisons show that the mitotic delay depends on the presence of the centromere and a functional spindle assembly checkpoint.

Rare errors in segregation correlate with a *mad* dependent cell cycle delay.

We wanted to know if the delay induced by the short linear mini-chromosomes is peculiar to these molecules. Circular mini-chromosomes segregate more faithfully than linear mini-chromosomes, but less faithfully than natural chromosomes: circular mini-chromosomes are present at 1-3 copies per cell and are lost at a frequency of about 10^{-2}. Examining the spindle morphology of cultures containing the circular form of the centromeric mini-chromosome pVL106 failed to reveal a consistent mitotic delay (Table 2). Because this analysis only provides information about a population of cells, we turned to pedigree analysis which follows the properties of individual cells. We followed the segregation of cells harboring the circular mini-chromosomes pGALCEN3 or A75p9 (whose structures are shown in Figure 1) as they divided on rich medium. A75p9 differs from YLp4 only by the absence of telomeric sequences allowing a direct comparison of linear and circular plasmids. In both cases division delays were observed in some cells that contained the

mini-chromosomes, but not in mini-chromosome-free cells. The frequency of cell cycle delays (6-9%) was much lower than that observed for cells containing linear mini-chromosomes (Table 3).

To ask whether the division delays reflected problems in segregating the mini-chromosomes, we correlated the occurrence of division delays with errors in chromosome segregation. A much larger proportion of the divisions which resulted in errors were delayed (50-100%; Figure 3) as compared to divisions in which both mother and daughter inherited the mini-chromosome (5-7%). This difference is highly statistically significant ($P < 10^{-10}$ by the χ^2 test). The observation that the cells that delay still mis-segregate mini-chromosomes suggests that the checkpoint can only produce a transient block to cell division. Like linear mini-chromosomes, the ability of circular molecules to delay the cell cycle depends on the spindle assembly checkpoint as *mad1*, *mad2* or *mad3* cells show delays in only 0-0.4% of divisions. No correlation between cell division delays and segregation errors occurred in cells containing linear mini-chromosomes (data not shown), probably because the presence of multiple copies of these molecules in a cell obscures segregation defects for any given pair of sister chromatids.

We quantified the fidelity of mini-chromosome segregation as the segregation frequency, the fraction of cell divisions in which only one of the two progeny receives the mini-chromosome (Table 3). The absence of cell cycle delays in the *mad* mutants does not improve circular mini-chromosome segregation. This strongly suggests that the segregation difficulties of the mini-chromosomes lead to delays rather than that cell cycle delays lead to aberrant mini-chromosome segregation.

The inability of *mad* mutants to delay in mitosis does not increase the frequency of errors in mini-chromosome segregation. The difficulty of measuring the distribution of mini-chromosome copy numbers within a population of cells makes rigorous interpretation of this finding impossible. One attractive explanation, however, is that many of the errors in segregation are due to defects that cannot be corrected once cells have entered mitosis. A

mitotic delay would not be expected to reduce the frequency of this type of error. The observation that circular centromeric mini-chromosomes show roughly five times as many 1:0 as they do 2:0 loss events (Hieter et al., 1985) is consistent with this possibility.

Linear mini-chromosomes segregate more faithfully and decrease in copy number as their length increases (Murray et al., 1986; Hieter et al., 1985). YLp21 is a 55kb linear mini-chromosome (Murray and Szostak, 1983a) which is present at single copy and segregates correctly in 98-99% of mitoses. In pedigrees, 2.6% of divisions with this artificial chromosome exhibited a delay (Table 3). As with the circular mini-chromosomes, a large number of divisions which resulted in errors in segregation showed a delay (10 of 11) whereas only 1% of symmetric divisions were delayed (Figure 3). Since YLp21 is maintained at a stable copy number and segregates normally in meiosis, we could determine the copy number of the artificial chromosome by producing diploid cells and examining the segregation of YLp21 in meiosis. Using this method we examined the copy number of cells whose sisters had failed to inherit the artificial chromosome to distinguish between chromosome loss (1:0 segregation) or non-disjunction (2:0 segregation) events. This analysis revealed that 10 of the 11 segregation errors events for YLp21 were 1:0 segregation events, suggesting that the primary cause of errors in segregation for this molecule is failure of replication or loss of a chromosome from the nucleus, rather than non-disjuction. Thus most dividing cells harbor two copies of the artificial chromosome and show no division delay, whereas almost all the cells that have a single copy do delay. This observation rules out the possibility that the division delays observed with artificial chromosomes are simply due to the presence of extra kinetochores in the cell.

Excess circular centromeric mini-chromosomes cause a *mad*-dependent cell cycle delay.

Previous attempts to determine the effect of multiple copies of circular mini-

chromosomes on the cell cycle have yielded conflicting results. By simultaneously selecting for several different mini-chromosomes bearing different selectable markers, Futcher and Carbon (Futcher and Carbon, 1986) concluded that the presence of excess kinetochores greatly delayed the cell cycle. An alternative method of raising mini-chromosome copy number uses the activation and inactivation of conditional centromeres, created by placing a strong, regulated promoter adjacent to the centromere. Transcription from the galactose (Hill and Bloom, 1987) or alcohol dehydrogenase II (Chelbowicz-Sledziewska and Sledziewska, 1985) promoters towards the centromere renders it incapable of directing segregation. This results in an increase in mini-chromosome copy number due to the bias of acentric plasmids to segregate to mother cells (Murray and Szostak, 1983b). Shutting off the promoter then reactivates the centromeres which are now present in multiple copies in a subset of the cells. Increasing copy number by this method has been variously reported as either toxic to the cell (Runge et al., 1991), possibly toxic (Hill and Bloom, 1987; Smith et al., 1990) or non-toxic (Chelbowicz-Sledziewska and Sledziewska, 1985).

All of the previous experiments examining the effect of excess centromeres suffer from the disadvantage that their conclusions are derived from analyzing populations of cells where the only known parameter is the average copy number. In addition, the selection of multiple differently marked mini-chromosomes in the same cell could lead to recombination events between the mini-chromosomes, resulting in the formation of dicentric molecules which are known to cause cell cycle delays (Koshland et al., 1987). To avoid these difficulties we exploited the combination of conditional centromeres, pedigree analysis, and the bias of acentric plasmids to segregate to the mother to create cells in which we could deduce the factor by which the mini-chromosome copy number has increased. We used pGALCEN3 (Hill and Bloom, 1987), a circular mini-chromosome with a conditional centromere. Due to transcription of the galactose promoter into the centromeric region, this plasmid segregates like an acentric ARS plasmid in cells growing on galactose, but has a

fully functional centromere when cells are exposed to glucose, which inhibits transcription from the *GAL1* promoter. We placed glucose-grown cells on galactose-containing medium, and performed pedigree analysis for three generations before transferring the cells to glucose-containing medium and continuing pedigree analysis for one to three additional generations, as shown in Figure 4. Since pGALCEN3 behaves as if it is acentric in cells on galactose, the mother cells will accumulate multiple copies of the plasmid. In particular, in those pedigrees where only the original mother cell inherits the plasmid we can deduce its minimum copy number. Thus a pedigree of three divisions on galactose that produces only one cell containing pGALCEN3 will have undergone three rounds of DNA replication without segregating the plasmid and the mother cell will contain $2^3 = 8$ times the number of mini-chromosomes as it did at the start of the experiment. Since circular mini-chromosomes fail to replicate in only 2% of cell divisions (Hieter et al., 1985), the chance of three successive replication failures in such a pedigree is negligible.

We analyzed the segregation of the mini-chromosome and the fraction of cell division delays in these pedigrees. As expected, exposure to galactose caused rapid inactivation of the centromere leading to asymmetric segregation of the mini-chromosome in 45% of the cells undergoing their first division on galactose-containing plates. Centromere reactivation after transfer to glucose-containing medium was efficient as mini-chromosome loss was reduced to 13% in the first division and 3% in the second division on glucose (numbers derived from the pedigrees whose results appear in Table 4 and Figure 5). Mini-chromosome-free cells showed a very low frequency (less than 0.1%) of division delays, whether they arose from pedigrees started from a mini-chromosome-free cell or by segregation in a mini-chromosome-containing pedigree. Cells plated directly on glucose-containing medium without exposure to galactose showed a low level of delays (see previous data in Table 3). Mini-chromosome containing cells which had been on galactose for 2 or 3 generations, however, showed a high incidence of delays which was absent in *mad1*, *mad2* or *mad3* cells (Table 3).

The effects of altering mini-chromosome copy number on cell division delays are shown in Figure 5. The distribution of the delays is that predicted by the bias of acentric mini-chromosomes to segregate to the mother cell: for each mother-daughter pair the mother is at least twice as likely as the daughter to show a division delay on glucose-containing medium, and the initial cell used to start the pedigree, which has been a mother for three divisions on galactose, has a substantially higher frequency of mitotic delays than any other mother cell after transfer to glucose-containing medium (Figure 5a). The χ^2 test demonstrates that both of these differences have a probability of less than 0.001 of occurring by chance, strongly suggesting that the frequency of division delays increases as the mini-chromosome number rises.

We performed two tests to determine whether the division delays are due to slow or partial reactivation of the centromeres on glucose. First, in some experiments we followed the cells for three divisions after transfer from galactose- to glucose-containing medium (Figure 5b). There is still a substantial fraction of division delays in the third division on glucose-containing medium, when the fidelity of mini-chromosome segregation demonstrates that normal centromere function has been fully restored. Second, we tested the effects of allowing only a single division on galactose before transfer to glucose. These cells exhibited half the frequency of division delays on glucose-containing medium, compared to cells allowed to divide three times on galactose. Both of these tests strongly suggest that the strong mitotic delays after multiple divisions on galactose are due to the accumulation of excess centromeres over several generations.

Futcher and Carbon (Futcher and Carbon, 1986) suggested that the division delay induced by excess centromeres was due to the titration of a factor required for centromere function. If this explanation is correct, eliminating the division delay would be expected to induce anaphase before kinetochore assembly was complete on some chromosomes, leading to loss of both natural and artificial chromosomes, and cell death. To test this possibility, we followed the fate of *mad1*, *mad2*, and *mad3* cells containing pGALCEN3

after transferring cells from galactose to glucose. As stated above, the inactivation of the spindle assembly checkpoint eliminated the cell cycle delay seen after transferring cells from galactose to glucose, but led to no increase (*mad3*, 2% of divisions) or only a small increase (*mad1*, 11% of divisions; *mad2*, 4% of divisions) in the amount of cell death of mini-chromosome bearing cells on glucose. This result strongly suggests that the presence of excess mini-chromosomes delays mitosis because the mini-chromosomes themselves have difficulty interacting with the spindle, rather than because they interfere with the behavior of the natural chromosomes.

Discussion

We have used pedigree analysis to investigate the interaction between the spindle assembly checkpoint and the segregation of mini-chromosomes in budding yeast. Short linear mini-chromosomes, which segregate poorly and are present in multiple copies per cell, induce a high frequency of mitotic delays which are dependent on the presence of a functional centromere on the mini-chromosome. Circular and long linear mini-chromosomes, which segregate more faithfully and are present in 1-3 copies per cell, induce a lower frequency of mitotic delays and these are seen preferentially in cells which show aberrant segregation of the mini-chromosome. Increasing the copy number of circular mini-chromosomes increases the frequency of mitotic delays towards the value seen in cells with short linear mini-chromosomes. Finally, all of these delays are eliminated by mutations that abolish the spindle assembly checkpoint.

Although *mad* mutants abolish the cell cycle delays caused by circular mini-chromosomes, they do not increase the fidelity of plasmid segregation. This observation suggests that it is defects in mini-chromosome interactions with the spindle that cause the delays, rather than delays induced by some other mechanism leading to defects in mini-chromosome segregation. When the copy number of a circular mini-chromosome was increased by centromere inactivation and reactivation the frequency of delays (66% of divisions of original mother cells, Figure 5) increased to a value similar to that seen in strains containing linear mini-chromosomes (61% of divisions, Table 1). The simplest interpretation of this experiment is that each plasmid molecule in the cell behaves independently. Increasing the number of plasmids per cell raises the probability that at least one plasmid will experience problems in attaching correctly to the mitotic spindle, activate the checkpoint, and thus delay the onset of anaphase. The toxicity of excess centromeres seen in earlier experiments (Futcher and Carbon, 1986; Runge et al., 1991) is therefore most likely due to the ability of these poorly segregating molecules to activate the

checkpoint, rather than their ability to compete with natural chromosomes for limiting quantities of kinetochore components.

All of the delays described above are absent in *mad1*, *mad2*, and *mad3* mutants. Therefore, the defect in the association of these plasmids with the spindle is being detected by the spindle assembly checkpoint that was previously defined by the failure of mutants to arrest in response to microtubule depolymerization (Li and Murray, 1991). It is clear from our results and those of others (Neff and Burke, 1992; Spencer and Hieter, 1992) that defects that have no effect on overall spindle structure can activate the spindle assembly checkpoint. Observations of a wide range of cells in both mitosis and meiosis reveal that the most frequent spontaneous spindle defects are in the attachments of individual chromosomes to the spindle rather than in the overall morphology of the spindle. In particular, experiments on newt lung cells (Rieder et al., 1994) and insect spermatocytes (Li and Nicklas, 1995) demonstrate that the mis-orientation of a single chromosome is sufficient to delay cells in mitosis or meiosis, presumably via the spindle assembly checkpoint.

How would a checkpoint that monitored kinetochore-microtubule interactions detect other mitotic defects? Suppression of microtubule dynamics might produce unoccupied microtubule binding sites at the kinetochores or affect the tension exerted by microtubules on the kinetochore, whereas failures in spindle pole duplication will prevent up to half of the kinetochores from attaching to microtubules at all. Experiments in sea urchin eggs suggest that other mechanisms of detecting spindle defects must exist since although these cells cannot delay in mitosis in response to unattached chromosomes (Sluder et al., 1994) , they can still respond to spindle depolymerization (Sluder et al., 1986).

Most delayed cells do eventually divide, even though they mis-segregate mini-chromosomes. This suggests that cells can eventually override the checkpoint signal and proceed into anaphase. This interpretation is consistent with the observation that budding yeast cells containing damaged DNA eventually override the Rad9-dependent checkpoint

and pass through mitosis (Sandell and Zakian, 1993) and that many plant and animal cells treated with anti-microtubule drugs only arrest transiently in mitosis (Kung et al., 1990; Rieder and Palazzo, 1992; Rieder et al., 1994).

Why do linear mini-chromosomes segregate poorly and activate the spindle assembly checkpoint at a high frequency? Understanding these problems should yield valuable clues about the mechanism of chromosome segregation and the signal that activates the checkpoint. The possible explanations for the poor segregation of linear mini-chromosomes relative to their circular counterparts fall into two classes: those based on topological differences between linear and circular molecules (Murray and Szostak, 1985) and those based on effects of the nearby telomeres which have been observed to silence nearby genes (Aparicio et al., 1991) and delay the firing of nearby replication origins (Ferguson and Fangman, 1992; Raghuraman et al., 1994). Two observations suggest that the aberrant behavior of linear mini-chromosomes is due to topology rather than telomeres. First, manipulated versions of natural chromosomes that have one centromere very close to a telomere segregate normally (Murray and Szostak, 1986). Second, cells with mutations in the *SIR2* gene, whose product plays an essential role in telomeric silencing (Aparicio et al., 1991), show the same frequency of errors in mini-chromosome segregation and mitotic delays as wild type cells (data not shown).

Is there a common defect in the interaction of linear and circular mini-chromosomes with the spindle that explains why high copy numbers of both can activate the spindle assembly checkpoint? Both precocious sister chromatid separation and failures in chromosome replication produce kinetochores that are not linked to a partner that can attach to the opposite spindle pole. Experiments in mantid spermatocytes show that even though an unpaired meiotic chromosome remains attached to microtubules, it activates the spindle assembly checkpoint. Applying tension to this chromosome (which mimics the opposing force normally generated by a paired chromosome) allows the cell to enter anaphase, demonstrating that cells can monitor tension at the kinetochore (Li and Nicklas, 1995).

This observation suggests that premature separation of sister chromatids could induce mitotic delays either by removing tension at the kinetochore or by destabilizing kinetochore-microtubule interactions (Nicklas and Koch, 1969; Nicklas et al., 1995; Rieder et al., 1995). *In situ* hybridization shows that the two sister chromatids of a circular mini-chromosome remain associated with each other in cells arrested in mitosis (Guacci et al., 1994), although various technical limitations could conceal a small fraction of the population where the sisters had separated precociously. Because of their heterogeneous and high copy number, *in situ* hybridization of fixed cells cannot easily monitor when the sister chromatids of short linear mini-chromosomes separate. Thus precocious sister separation remains a possible mechanism for activating the spindle assembly checkpoint.

Small chromosomes, both circular and linear, may have problems capturing microtubules and aligning correctly on the spindle. In animal cells sister chromatid pairs move rapidly towards the pole that first captures them (Rieder and Alexander, 1990), but their approach is limited by a microtubule-dependent repulsive force referred to as the polar wind or astral exclusion force (Rieder et al., 1986; Rieder and Salmon, 1994; Cassimeris et al., 1994). Laser ablation of the chromosome arms allows them to be expelled from the spindle, while the remaining kinetochore fragment moves closer to the spindle pole (Rieder et al., 1986). If yeast spindle pole bodies exert a polar wind, mini-chromosomes would be largely immune to it and could approach very close to the pole they initially attached to. Such a position would constrain a mini-chromosome to be far from the opposite pole and would make it hard for the unattached kinetochore to capture microtubules from the distant pole. Polar wind forces in animal cells appear to play a role in restricting the oscillation of sister chromatid pairs that are attached to both poles to positions that are near the midpoint of the spindle (Skibbens et al., 1993). If such forces were ineffective on mini-chromosomes these molecules might engage in longer range oscillations that would destabilize their attachment to microtubules, and *in situ* hybridization should reveal that mini-chromosomes show a broader distribution along the length of the spindle than natural

chromosomes.

Regardless of the nature of the errors in mini-chromosome-spindle interactions, we observe a strong correlation between divisions in which delays are induced and divisions in which segregation errors occur. This correlation strengthens the assertion that the spindle assembly checkpoint detects nascent errors in chromosome segregation and delays cell cycle progression to give the cell a chance to correct the mistake. Increasing the copy number of a small circular plasmid presents the cell with more possible defects and so a delay is induced more often. The failure of *mad* mutants to delay in these circumstances provides an alternative screen for further *mad* mutants, and will allow us to test specific centromere elements and kinetochore proteins for their role in the spindle assembly checkpoint.

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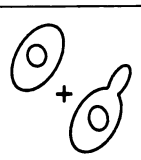
TABLE 1: CELL DIVISION DELAY BY LINEAR CENTROMERIC PLASMIDS

Checkpoint Genotype	Plasmid	Plasmid-bearing cells		Plasmid-free cells	
		% Delayed divisions	# Divisions observed	% Delayed divisions	# Divisions observed
MAD	pVL106 (CEN-linear)	61	147	1	233
mad1	pVL106 (CEN-linear)	2	330	3	38
mad2	pVL106 (CEN-linear)	1	284	2	54
mad3	pVL106 (CEN-linear)	4	400	0	42
MAD	pVL111 (acentric linear)	1	89	0	74
MAD	YLp4 (CEN-linear)	52	138	0	116
MAD	YLp30 (acentric linear)	0	80	0	25

Pedigree analysis of linear plasmids was performed on YPD plates and scored as described in Materials and Methods. Strains used were TBW2 (*MAD*, pVL106), TBW40 (*mad1*, pVL106), TBW18 (*mad2*, pVL106), TBW67 (*mad3*, pVL106), TA1165 (*MAD*, YLp4), and TA1186 (*MAD*, YLp30)

TABLE 2: DISTRIBUTION OF SPINDLE MORPHOLOGIES IN CULTURES CONTAINING SHORT LINEAR PLASMIDS

MAD Gentoype	Mini-chromosome			
MAD	No mini-chromosome	60	25	15
MAD	pVL106 (CEN-linear)	44	49	7
MAD	pVL106 (CEN-circle)	63	24	13
MAD	pVL111 (acentric linear)	59	27	14
mad1	pVL106 (CEN-linear)	58	31	11
mad1	pVL106 (CEN-circle)	55	30	15

Asynchronous cultures of wild type or *mad1* mutant cells harboring circular or linear derivatives of the plasmid pVL106 (or an acentric linear derivative, pVL111) were fixed and stained with anti-tubulin antibodies. Two hundred cells from each culture were scored for the presence of unseparated, short or long spindles. Linear and circular mini-chromosomes were produced as described in Figure 1. Applying the χ^2 test shows that the distribution of morphologies in the *MAD* strain containing the linear form of pVL106 is statistically significant from all other strains ($p < 0.002$ for all pairwise comparisons) but that none of the other strains show a statistically significant difference from each other ($p > 0.25$ for all pairwise comparisons). Strains used were BW30 (*MAD*), TBW1 (*MAD*, pVL106 circular), TBW2 (*MAD*, pVL106 linear), TBW40 (*mad1*, pVL106 linear), TBW39 (*mad1* pVL106 circular).

TABLE 3: RARE DIVISION DELAY BY LOW COPY CENTROMERIC PLASMIDS

Checkpoint Genotype	Plasmid	Plasmid-bearing cells		Plasmid-free cells		Segregation Frequency
		% Delayed divisions	# Divisions observed	% Delayed divisions	# Divisions observed	
MAD	pGALCEN3 (CEN circle)	9.2	523	0.6	162	0.054
mad1	pGALCEN3 (CEN circle)	0.4	566	0	171	0.034
mad2	pGALCEN3 (CEN circle)	0	427	0	47	0.044
mad3	pGALCEN3 (CEN circle)	0.4	520	0	107	0.027
MAD	A75p9 (CEN circle)	6.3	397	0	105	0.013
MAD	YLp21 (50kb linear YAC)	2.6	723	0	505	0.017

Pedigree analysis was performed on YPD plates and scored as described in Materials and Methods. The strains used were: TBW10 (*MAD*, pGALCEN3); TBW28 (*mad1*, pGALCEN3); TBW29 (*mad2*, pGALCEN3); TBW65 (*mad3*, pGALCEN3); TA624 (*MAD*, A75p9); DA248, DA249, DA250 and DA251 (*MAD*, YLp21).

TABLE 4: DIVISION DELAYS INDUCED BY HIGH COPY CIRCULAR CENTROMERIC PLASMIDS ARE *MAD*-DEPENDENT

Checkpoint Genotype	Plasmid	Plasmid-bearing cells		Plasmid-free cells	
		% Delayed divisions	# Divisions observed	% Delayed divisions	# Divisions observed
MAD	pGALCEN3 (CEN circle)	39	502	0.1	905
mad1	pGALCEN3 (CEN circle)	0	247	0.3	330
mad2	pGALCEN3 (CEN circle)	0.3	285	0	130
mad3	pGALCEN3 (CEN circle)	0	195	0	231

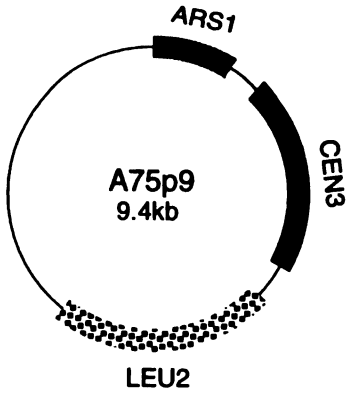
Pedigree analysis was performed for three divisions on YPGalactose plates followed by three divisions on YPD plates and scored as described in Materials and Methods. The strains used were: TBW10 (*MAD*, pGALCEN3); TBW28 (*mad1*, pGALCEN3); TBW29 (*mad2*, pGALCEN3); TBW65 (*mad3*, pGALCEN3).

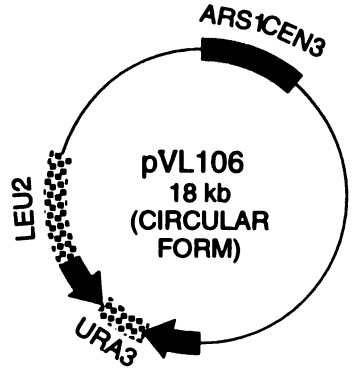
TABLE 5: Yeast Strains.

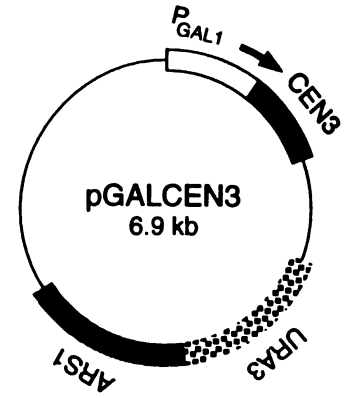
BW30	a	*ade3, ura3-52, leu2,3-112, his3-Δ200, met2*
TBW1	a	*ade3, ura3-52, leu2,3-112, his3-Δ200, met2, pVL106*
TBW2	a	*ade3, ura3-52, leu2,3-112, his3-Δ200, met2, pVL106 (linearised)*
TBW6	a	*ade3, ura3-52, leu2,3-112, his3-Δ200, met2, pVL111 (linearised)*
TBW40	a	*ura3-52, leu2,3-112, his3-Δ200, trp1, mad1-1, pVL106 (linearised)*
TBW18	a	*ura3-52, leu2,3-112, his3-Δ200, trp1, mad2-1, pVL106 (linearised)*
TBW67	a	*ade2, ura3-52, leu2,3-112, his3-Δ200, trp1, mad3-1, pVL106 (linearised)*
A281	α	*cir^o, leu2-3,112, his3-11,15, can1*
TA1165	α	*cir^o, leu2-3,112, his3-11,15, can1, YLp4*
YA1186	α	*cir^o, leu2-3,112, his3-11,15, can1, YLp30*
TBW10	a	*ura3, leu2,3-112, his3-Δ200, trp1, pGALCEN3*
TBW28	a	*ura3-52, leu2,3-112, his3-Δ200, trp1, mad1-1, pGALCEN3*
TBW29	a	*ura3-52, leu2,3-112, his3-Δ200, trp1, mad2-1, pGALCEN3*
TBW65	a	*ade2, ura3-52, leu2,3-112, his3-Δ200, trp1, mad3-1, pGALCEN3*
TA624	α	*cir^o, leu2-3,112, his3-11,15, can1, A75p9*
DA248	a/α	*leu2-3,112/leu2-3,112, his3-11,15/his3-11,15, trp1/trp1, ura3/ura3, ade1/ADE1, arg4/ARG4,YLp21*
DA249		same genotype as DA248
DA250		same genotype as DA248
DA251		same genotype as DA248
BW172	a	*ura3-52, trp1-Δ63, leu2-Δ1, lys2-Δ202, his3-Δ200*

Figure 1. Maps of the plasmids used for pedigree analysis

Schematic views of the linear and circular mini-chromosomes used in this work. The linear forms of pVL106, pVL111, and YLp30 are obtained by digestion of plasmid DNA with BamHI, which removes a stuffer fragment between two *Tetrahymena* telomeric fragments, followed by transformation of yeast with the linear DNA. The details of mini-chromosome construction can be found in the following references: YLp4, and YLp21 (Murray and Szostak, 1983a); and YLp30 (Murray and Szostak, 1983b);pGALCEN3 (Hill and Bloom, 1987); pVL106 and pVL111 (V. Lunblad, personal communication).


ARS1
CEN3
A75p9
9.4kb
LEU2


ARS1CEN3
LEU2
pVL106
18 kb
(CIRCULAR
FORM)
URA3


P GAL1
CEN3
pGALCEN3
6.9 kb
ARS1
URA3

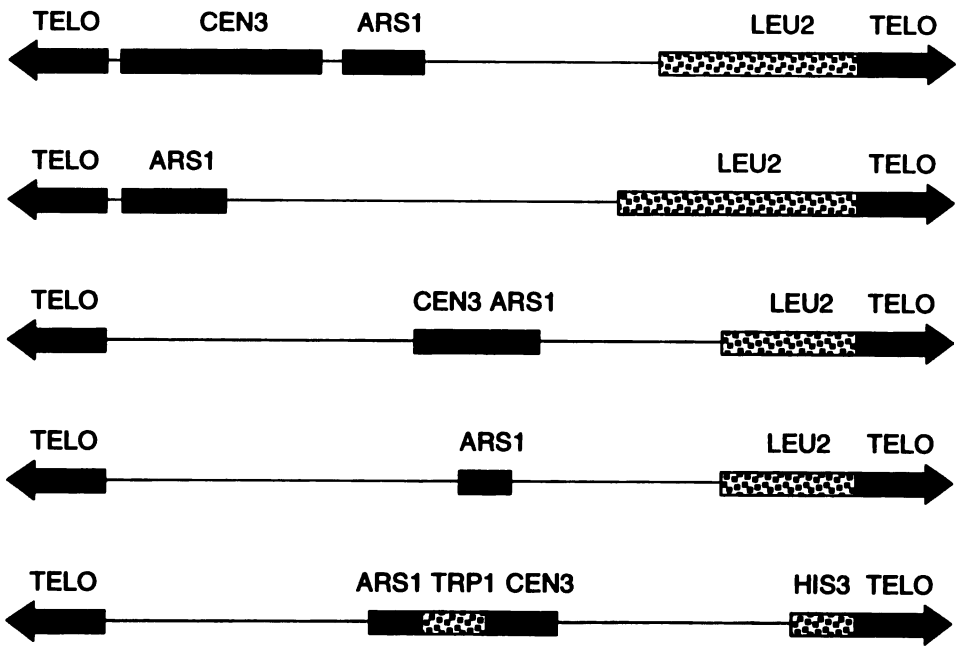
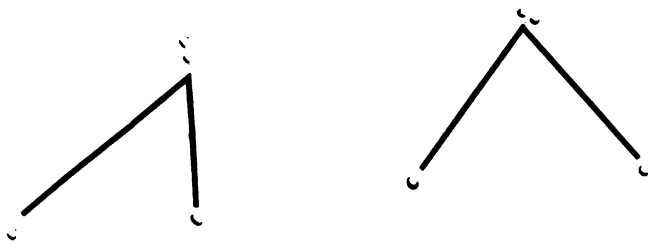

TELO CEN3 ARS1 LEU2 TELO
YLp4 11.2 kb
TELO ARS1 LEU2 TELO
YLP30 9.3 kb
TELO CEN3 ARS1 LEU2 TELO
pVL106 16 kb (LINEAR FORM)
TELO ARS1 LEU2 TELO
pVL111 15 kb (LINEAR FORM)
TELO ARS1 TRP1 CEN3 HIS3 TELO
YLp21 54 kb

Figure 2. Pedigree analysis of yeast cells containing the short linear artificial chromosome YLp4.

a) Two cell pairs from a section of a pedigree that does not contain the mini-chromosome. The cells are shown before and immediately after the mother and daughter cells were separated by micromanipulation. This pedigree came from the same experiment as the pedigree shown in b). Note that daughters can be separated when they are smaller than their mothers and that new buds are formed near the point at which mother and daughter cells were joined.

b) The figure shows photomicrographs of a pedigree starting from a single cell containing YLp4. The time in minutes after the start of the pedigree is shown in the left margin and the lines indicate the cell lineage. X, indicates a cell pair where the daughter has reached a size at which it would normally be possible to separate it from the mother cell. This pedigree contains several aberrant cell divisions: the initial cell pair failed to divide even after 90 minutes, when both mother and daughter cells had budded again. The two new buds on this cell were formed at opposite ends of the cell pair, rather than at the junction between mother and daughter; at 210 minutes only one of the new buds was separable and between this time and 310 minutes one member of the original cell pair died as indicated by a loss of refractility (more clearly visible at 390 minutes). Because of the aberrations in the pattern of cell divisions no attempt was made to distinguish mother and daughter cells at the time of cell separation. After the last time point, the cells were more widely separated, allowed to grow up into colonies and then tested for the inheritance of YLp4. All the cells in this pedigree inherited YLp4.

a



b

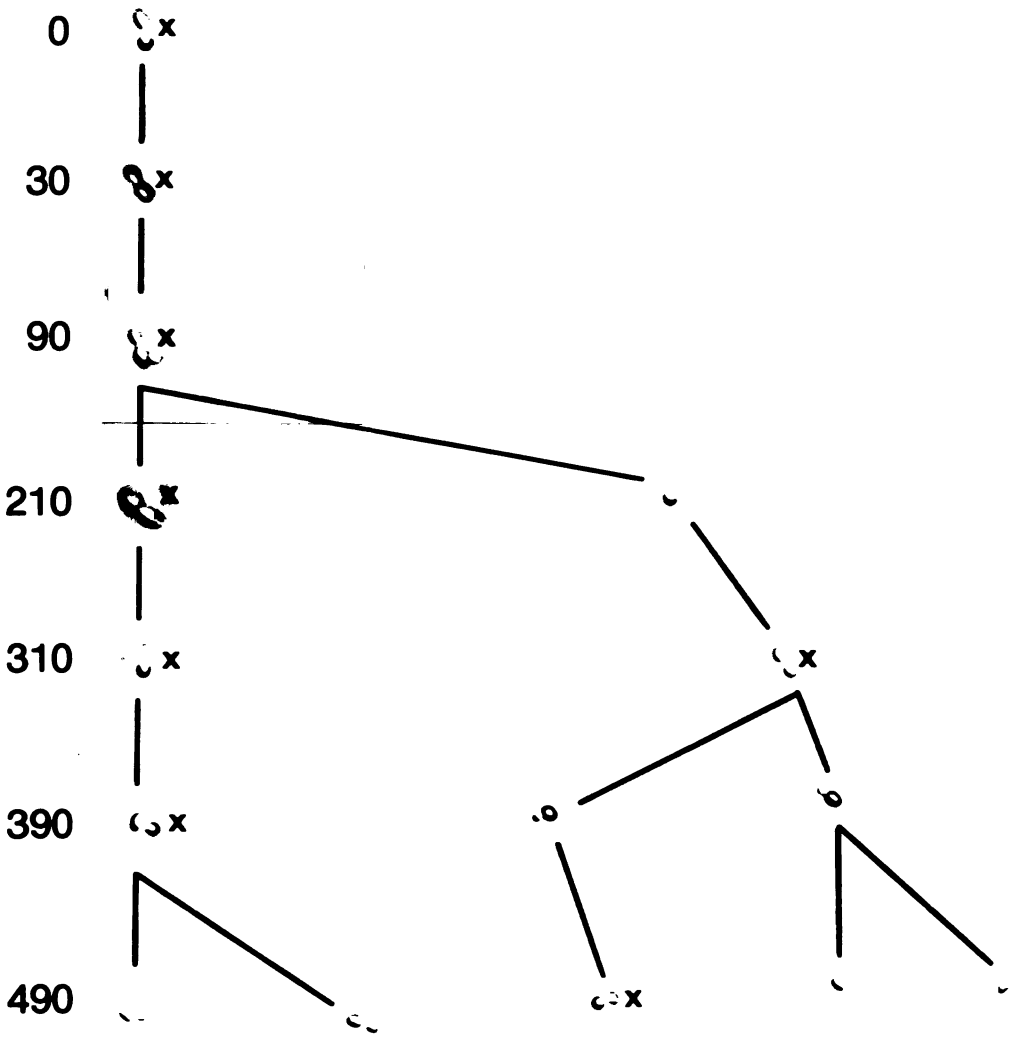


Figure 3. Errors in plasmid segregation correlate with mitotic delays. Divisions were scored as normal or delayed during pedigree analysis on glucose-containing medium for strains containing the indicated mini-chromosomes, and the pattern of mini-chromosome segregation was determined after the cells had grown into colonies. The null hypothesis that the frequency of delayed divisions is identical in divisions in which both progeny received the mini-chromosome compared to those divisions where only one cell received the mini-chromosome is rejected with a probability of less than 10^{-10} for both the 50 kb linear (YLp21) and the circular mini-chromosome (pGALCEN3). Strains used were: TBW10 (*MAD*, pGALCEN3); TBW28 (*mad1*, pGALCEN3); TBW29 (*mad2*, pGALCEN3); TBW65 (*mad3*, pGALCEN3); DA248, DA249, DA250 and DA251 (*MAD*, YLp21). The fractions of cell divisions that show asymmetric plasmid segregation are listed in Table 3 as the segregation frequency.

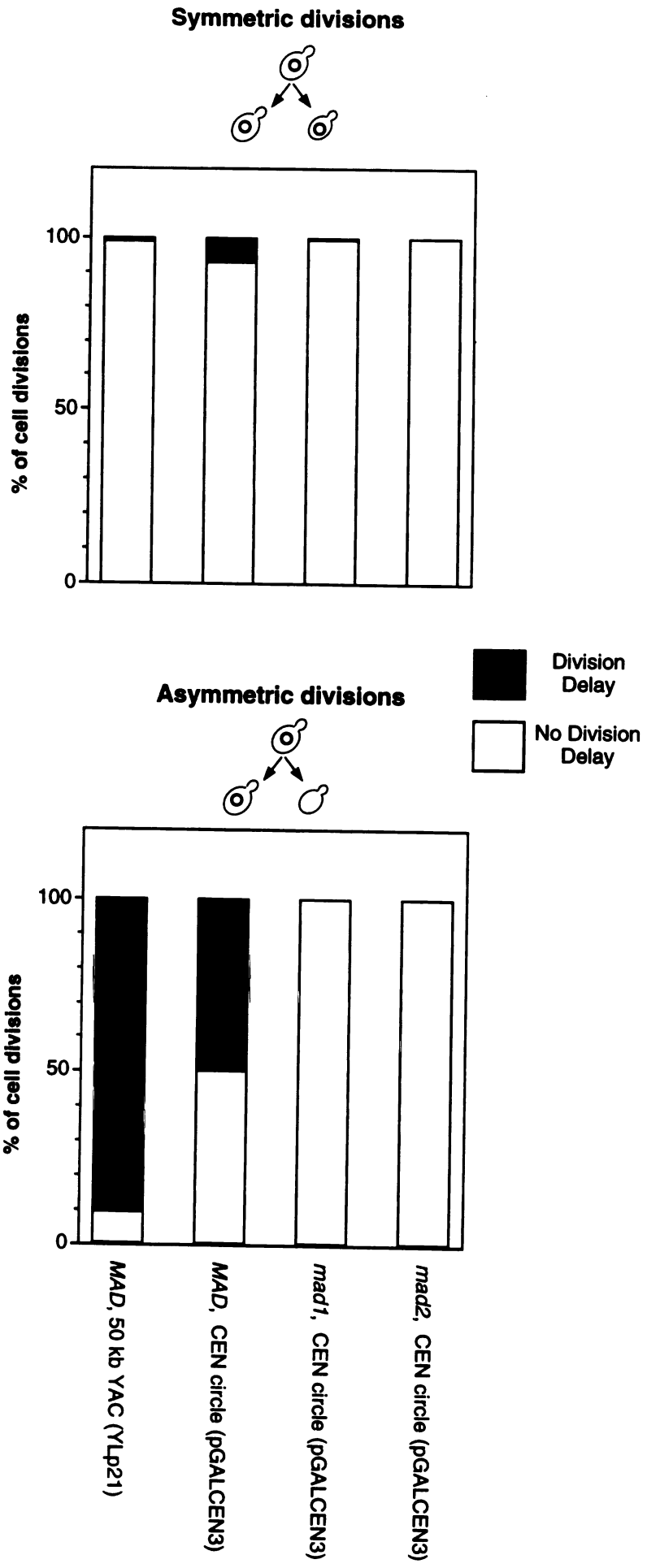

Symmetric divisions
100
50
0
% of cell divisions
Asymmetric divisions
Division Delay
No Division Delay
100
50
0
% of cell divisions
MAD, 50 kb YAC (YLp21)
MAD, CEN circle (pGALCEN3)
mad1, CEN circle (pGALCEN3)
mad2, CEN circle (pGALCEN3)

Figure 4. Use of a conditional centromere to increase the copy number of mini-chromosomes.

On galactose-containing medium the transcriptional activity of the *GAL1* promoter inactivates the centromere of pGALCEN3, and such functionally acentric mini-chromosomes are biased to segregate to the mother cell at cell division. Transferring cells to glucose-containing medium leads to inactivation of the *GAL1* promoter and restoration of centromere activity. In pedigrees, such as the one illustrated, where the plasmid segregates exclusively to the original mother cell, the copy number of the plasmid can be deduced.

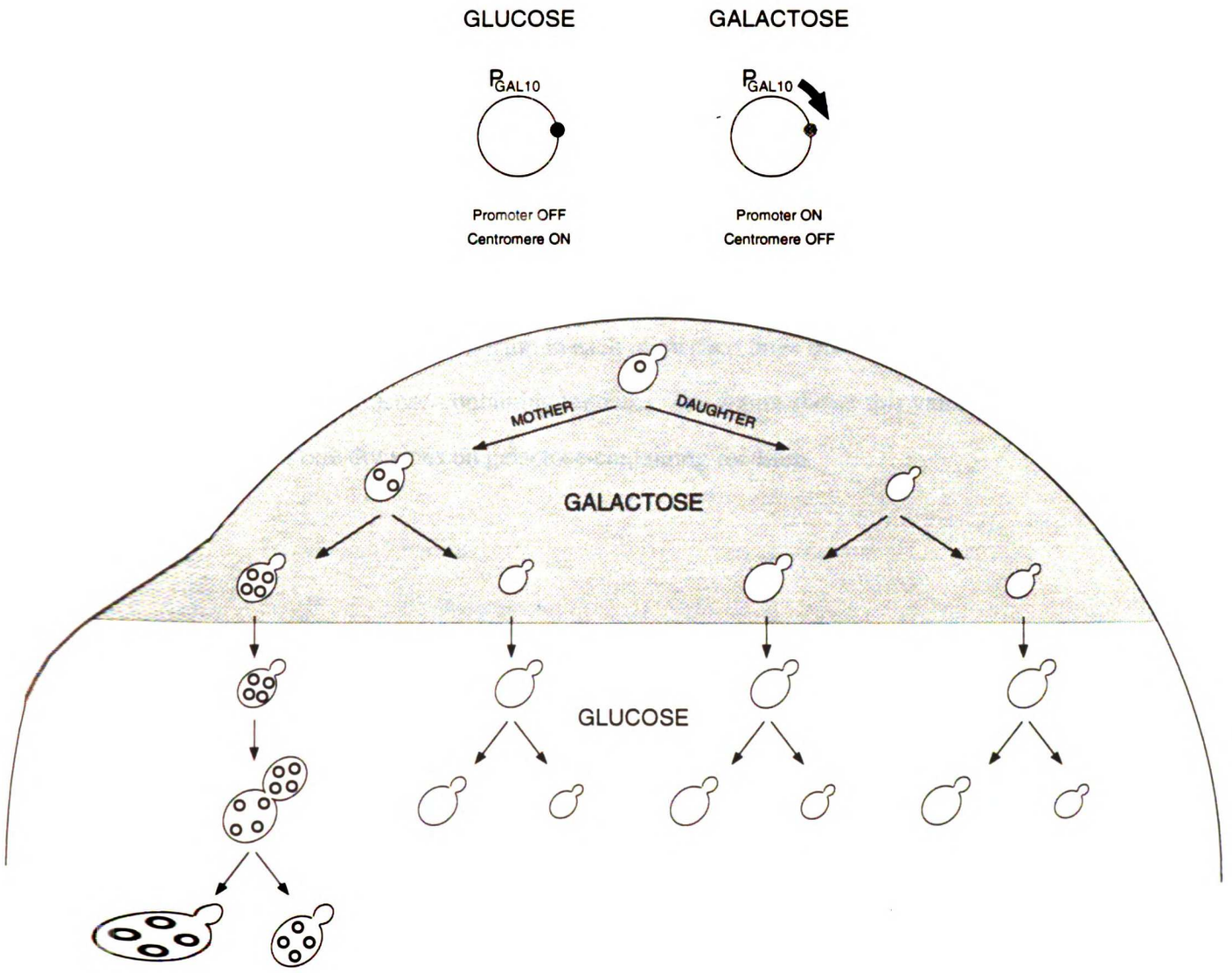
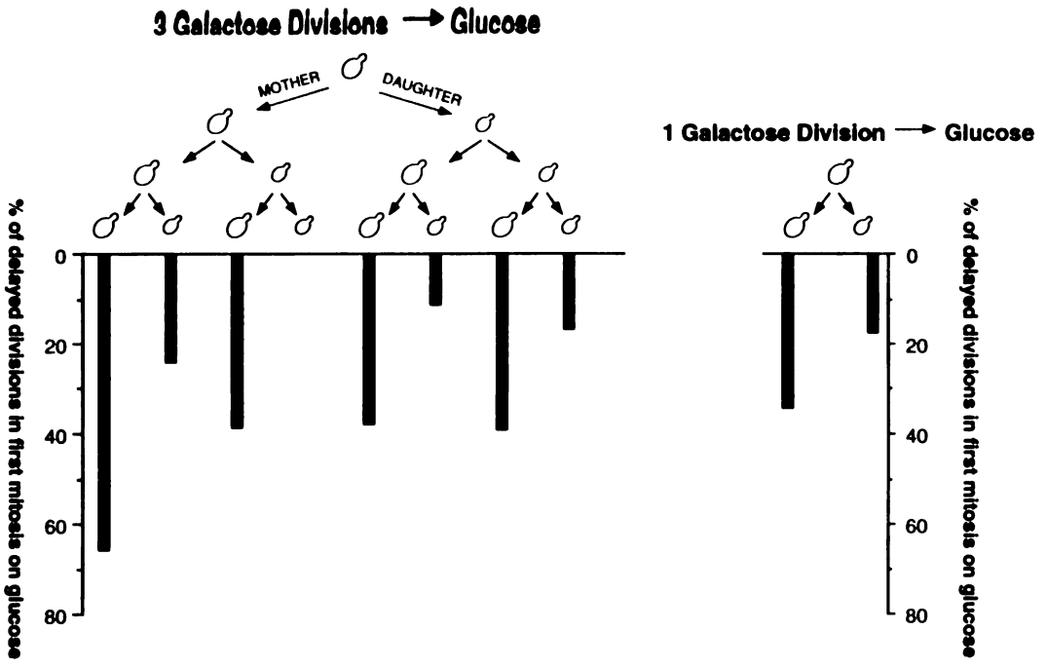

GLUCOSE
GALACTOSE
P GAL10
P GAL10
Promoter OFF
Centromere ON
Promoter ON
Centromere OFF
MOTHER
DAUGHTER
GALACTOSE
GLUCOSE

Figure 5. Mitotic delays induced by multiple copies of circular mini-chromosomes.

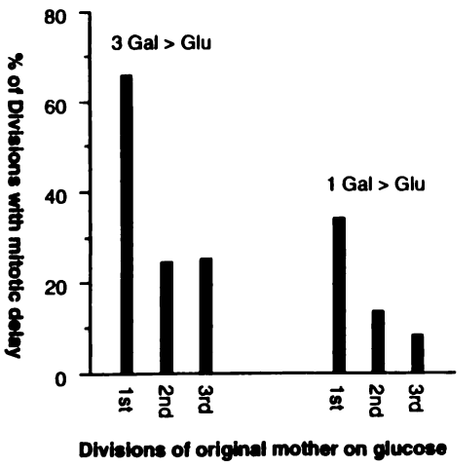
a) The fraction of delayed cell divisions of pGALCEN3-containing cells in the first division on glucose-containing medium after transfer from galactose-containing medium. The figure shows this value for cells at each of the positions of the cell lineages derived from either three or one divisions on galactose-containing-medium. The data is from multiple experiments involving 80 pedigrees with three divisions, and 181 pedigrees with one division on galactose-containing medium.

b) The fractions of delayed division in each of the first three divisions following transfer from galactose- to glucose-containing medium. The figure shows this value for cells from either three or one divisions on galactose-containing-medium.

a)


3 Galactose Divisions → Glucose
MOTHER
DAUGHTER
% of delayed divisions in first mitosis on glucose
0
20
40
60
80
1 Galactose Division → Glucose
% of delayed divisions in first mitosis on glucose
0
20
40
60
80

b)


% of Divisions with mitotic delay
80
60
40
20
0
3 Gal > Glu
1 Gal > Glu
1st
2nd
3rd
1st
2nd
3rd
Divisions of original mother on glucose

Chapter 2

Two mutants with a subset of the properties of mutants in the spindle assembly checkpoint in budding yeast.

Abstract

The spindle assembly checkpoint prevents the onset of anaphase when spindle structure is disrupted or chromosomes are not attached to the spindle. Previous screens to identify components of this checkpoint in budding yeast have defined 6 genes necessary for cell cycle arrest. The small number of alleles of these genes isolated suggest the screen is not saturated. Here we describe a screen, performed in a similar manner to those conducted previously, to isolate additional components of the checkpoint. The phenotypes of two mutants are described in detail. Mutant 155 fails to repress budding while arrested in nocodazole, but otherwise appears to arrest normally in mitosis. Mutant 261 is similar to previously identified checkpoint mutants, although minor differences indicate that the protein it normally encodes may function at the end of a bifurcated checkpoint pathway.

Introduction

The fidelity of events performed during the cell cycle is ensured by two mechanisms: error correction and checkpoints. Checkpoints detect the presence of an error and delay the progression of the cell cycle until it is corrected (reviewed by Hartwell and Weinert, 1989; Murray, 1992). In the budding yeast, *Saccharomyces cerevisiae*, a spindle assembly checkpoint detects the absence of a spindle and prevents the progression to anaphase and exit from mitosis (Li and Murray, 1991; Hoyt et al., 1991). Mutants in this checkpoint fail to respond to microtubule depolymerizing drugs and undergo an error-prone, lethal mitosis.

The two previous screens for spindle assembly checkpoint mutants defined 6 genes necessary for this process: *BUB1*, *BUB2* and *BUB3* (Hoyt et al., 1991) and *MAD1*, *MAD2* and *MAD3* (Li and Murray, 1991). Mps1p, an essential protein kinase necessary for spindle pole duplication (Winey et al., 1991), is also necessary for the spindle assembly checkpoint (Weiss and Winey, 1995). The sequences of these genes indicate the existence of a signal transduction cascade. Bub1p is a protein kinase which phosphorylates Bub3p; Bub3p also binds to and activates Bub1p (Roberts et al., 1994). Mad1p is a large coiled-coil protein which is hyperphosphorylated when microtubules are depolymerized (Hardwick and Murray, 1995). Mad1p hyperphosphorylation no longer occurs in *mad2*, *bub2*, *bub3*, and *mps1* mutants suggesting that these genes lie upstream of MAD1 (Hardwick and Murray, 1995). *mad3* and *bub2* mutants still show hyperphosphorylation of Mad1p; these genes likely lie downstream of *MAD1* or in a parallel pathway.

The two screens for spindle assembly checkpoint mutants resulted in the isolation of 8 alleles defining a total of 6 genes. It therefore seems likely that this screen is not saturated. In addition, the sequences of the known checkpoint components do not give an indication of the mechanism of generation of the checkpoint signal or the manner in which it prevents cell cycle progression, leaving open the possibility that mutants in the

components at either end of the pathway have not yet been isolated. To aid in the construction of a complete pathway, we have carried out a screen similar to that previously described (Li and Murray, 1991). We have isolated a mutant resembling previous checkpoint mutants (261; this chapter), and one which fails only to repress budding in the presence of nocodazole (155; this chapter), as well as a mutant defining a spindle integrity checkpoint (*tub1-171*; Chapter 3) and mutants defining an alternative means of exiting mitosis (Minshull et al., 1995).

Materials and Methods

Yeast strains and media

Table 3 lists the strains used in this work. *mad1* and *bub2-1* were backcrossed 3-4 times into the W303 background. Media were prepared and genetic manipulations were performed as described (Sherman et al., 1974).

Screen for checkpoint mutants

The screen was performed essentially as described (Li and Murray, 1991), but in the W303 background. Yeast were mutagenised by EMS and plated out for single colonies. These colonies were replica plated onto YPD and YPD benomyl plates, and 250 benomyl sensitive colonies were picked. Mutants were tested for rescue of benomyl sensitivity by the addition of 2.5mg/ml HU to plates with 7.5µg/ml benomyl. They were also tested in the microcolony assay (see text and Li and Murray, 1991).

Death assays

Yeast which had been growing exponentially overnight and had not exceeded an OD_{600} of 1.0 were resuspended to an OD_{600} of 0.3 in YPD with 15µg/ml nocodazole. 200µl of a 1:500 dilution of this culture was plated out for viability. The remainder of the culture was incubated at 22°C and plated for viability periodically.

Synchronous death assays were performed in a similar manner, but cultures were first incubated at an OD_{600} of 0.3 in YPD with 10µg/ml α-factor. After 3 hours at 22°C, cells were washed twice in 10mls of YPD and resuspended as above. Hydroxyurea was used at 10mg/ml.

Rebudding

1ml samples were withdrawn from death assay cultures, washed once in 50mM Tris, pH 7.5, and fixed in 70% ETOH for one hour at room temperature. After washing twice in 50mM Tris, pH 7.5, cells were resuspended in PBS and sonicated at maximum power for a few seconds. Cells were then scored microscopically as unbudded, small, medium, large or triply budded.

Clb2 Western blotting

0.5 ml samples were withdrawn from death assays, and yeast extracts prepared by bead beating as described (Hardwick and Murray, 1995). Samples were run on a 15% polyacrylamide gel, transferred to nitrocellulose by standard techniques and probed with an anti-Clb2 antibody at 1:500, as described (Hardwick and Murray, 1995).

Immunoprecipitations and H1 kinase assays

Pelleted, 1ml samples were lysed in 150µl of 50 mM Na-β-glycerophosphate, pH 7.4; 50mM Tris Cl, pH 7.5; 50 mM NaF; 500 mM NaCl; 2 mM EDTA; 2 mM EGTA; 0.1% Tween 20; and freshly added 2 mM $NaVO_4$ and 2 mM PMSF. Acid washed glass beads (0.5 mm diameter, cat # 11079105 from Biospec, PO Box 722, Bartlesville, OK 74005; 918-333-2166) were added and lysis acheived by bead beating for 2 minutes at 4°C. After a spin for 2 minutes at 14,000 rpm, 10-15 µl of supernatant was mixed with an equal volume of 2 x sample buffer for Westerns and protein level measurement. 100 µl supernatant was added to 25 µl of 2 M sucrose and frozen at -80°C.

An appropriate amount of sample (based on coomassie and western staining) was thawed and anti-Clb2 antibody added (1ml, undiluted) before incubation on ice for 30 minutes. An aliquot of protein A-Sepharose beads (Pharmacia Protein A-Sepharose 6MB catalogue # 17-0469-01), which had been equilibrated with two changes of lysis buffer, were added. The tubes were gently mixed at 4°C. The supernatant was then washed and resuspended

in 50 mM Tris-Cl pH 7.4; 50 mM NaF; 250 mM NaCl; 5 mM EDTA; 5 mM EGTA; 0.1% Nonidet P-40. The beads were transfered to a new tube and washed again. The beads were then washed with 80 mM Na-β-glycerophosphate pH 7.4; 15 mM $MgCl_2$; 20 mM EGTA. This buffer was removed and 15 µl of H1 kinase reaction mix added: this consists of the previous buffer plus DTT (final 1 mM), ATP (final 200 µM), 25 µg of histone H1 and10 µCi of g^{32}P-ATP. The reaction was stopped by the addition of 10 µl of 2x SDS sample buffer. 10 µl was loaded on a 15% polyacrylamide gel.

Fluorescence *in situ* hybridisation

A 1 ml sample from a death assay was resuspended in 18.5% formaldehyde, 0.1M potassium phosphate, pH 7.4, and incubated on a roller drum for 1 hour at 22°C. Two washes were performed in 0.1M potassium phosphate, pH 7.4, and then zymolyase added to .5 mg/ml. Spheroplasting was followed microscopically during a 20-30 minute incubation at 37°C. 30µl was placed on a polylysine coated slide and allowed to adhere briefly before washing the slide in PBS with 1% Tween 20 (PBST). Slides were incubated in PBST with 10µg/ml Protease K for 25 minutes at 37°C before washing with PBS for 3 minutes, 2 x SSCT for 30 minutes, and 50% formamide for 30 minutes. Labelled probe was then added to dried slides and coverslips sealed on with rubber cement. The slides were incubated on a 95°C heating block for 4 minutes and then in a humid chamber at 37°C for greater than 12 hours. The coverslip was removed and the slide washed twice in 2 x SSCT with 50% formamide for 1 hour each, three times in 2 x SSCT (10 minutes each), 10 minutes in DAPI (200µg/ml in 2 x SSCT), three times in 2 x SSC (5 minutes each) and once in water. After the addition of mount, slides were sealed and fluorescence observed.

The probe was cosmid number 9181 from the Yeast genetics center. 1µl of probe was added to 10µl of hybridization solution: 10% dextran sulfate, 50% formamide and 6 x SSC, before application to the slides.

Cloning

Attempts to clone the 155 and 261 genes were performed by LiAc transformation of a YCp50 based library as previously described (Hardwick and Murray, 1995).

Results

A screen for more *mad* mutants

Sensitivity to microtubule depolymerizing drugs such as benomyl can arise in two major ways. Mutants in the mitotic apparatus may be sensitive to benomyl due to increased destabilisation of components of the spindle. These mutants will arrest in benomyl as large budded cells due to the spindle assembly checkpoint and remain viable, resuming division upon release from the drug. Mutants in the checkpoint which normally restrains anaphase onset in the absence of a spindle will fail to detect that the drug is causing a problem and undergo mitosis without a spindle, resulting in massive chromosome loss and death (Li and Murray, 1991; Hoyt et al., 1991). Thus on release from the drug these cells will already be genetically dead.

250 benomyl sensitive mutants were generated by EMS mutagenesis of wild-type W303 cells. Mutants were tested for continued division in the presence of benomyl by microcolony assays. Briefly, α-factor arrested cells were separated on plates containing 12.5μg/ml benomyl and the number of buds produced recorded after 2, 4, 6 and 8 hours. Wild-type cells divide slowly, some benomyl-sensitive mutants do not divide at all, but checkpoint mutants divide more rapidly than wild-type before ceasing to divide due to death. All mutants positive for the microcolony assay were found to be rescued for growth on benomyl plates by the addition of a low concentration of the DNA synthesis inhibitor, hydroxyurea. Hydroxyurea provides an independent method of slowing the cell cycle until a spindle is constructed, allowing slowed but less error-prone growth for checkpoint mutants. Thus, of the remaining mutants, only those rescued by hydroxyurea were tested in the microcolony assay. This work was performed by Jeremy Minshull.

Thirty eight possible checkpoint mutants were tested for non-complementation of the existing *mad1*, *mad2* and *mad3* mutants and of each other by assaying diploids for

benomyl sensitivity on plates. This was not completely reliable, but screened out three alleles of *MAD1* (Table 1 lists the mutant numbers and identity). We tested the remaining 35 mutants in a rapid death assay. This involves exposing exponentially growing cells to a high concentration of nocodazole (10-15 μg/ml) for brief periods of time before plating for viability. Checkpoint mutants should lose viability rapidly due to cell cycle progression in the presence of the drug. Only 11 of the 35 mutants passed this test. Of these, one was mutant in *MAD3*, two in *BUB1*, and three in *BUB3*, while one was a non-mater. The remaining four mutants defined three genes. Two of the mutants (numbers 155 and 261) are discussed in this chapter, and another (171) in the next chapter.

We screened 80 more mutants which had been ambiguous in the microcolony assay by this rapid death assay. This yielded 7 additional mutants which were primarily characterised by Aaron Straight, including one more allele of *MAD1*, an allele of the MAP kinase gene *MPK1*, and two alleles of *CDC55* (Minshull et al., 1995). Thus our screen resulted in the isolation of 10 new alleles in 4 of the known checkpoint genes, as well as 7 alleles of 5 new genes under investigation. This compares to the total of 8 alleles of 6 genes isolated in the two previous screens. We did not isolate mutants in *MAD2* (probably because of its small size) or *BUB2*, but this particular version of the *mad* screen appears to be nearing saturation.

Phenotypic analysis of mutants 155 and 261

Figure 1 demonstrates the rapid death of mutants 155 and 261 in nocodazole, and the rescue from this death by hydroxyurea, demonstrating that the death phenotype is due to progression through the cell cycle. A lesser proportion of mutant 155 cells die in synchronous cultures; this may be due to a slow release from α-factor, as *mad1* cells also die poorly when α-factor is added back to the cultures after a one hour release (not shown). This is also clear from examination of the morphology of the cells as they are dying in

nocodazole (Figure 2). Both mutants as well as *mad1* put out a new bud indicating entry into the next cell cycle. Due to the absence of a spindle these cells do not undergo cytokinesis, but are identified as triply budded cells. This phenotype increases in degree above *mad1* levels for mutant 155 at later timepoints, but is less pronounced for 261 (Figure 2 and data not shown).

In other respects, mutant 261 behaves largely as the previous *mad* mutants do, but 155 is clearly different. Wild-type cells released from a G1 block into nocodazole accumulate high levels of the B type cyclin, Clb2, as they arrest in mitosis (Figure 3). The *mad1* mutant fails to arrest and destroys Clb2 protein as if no drug was present; the Clb2p is seen to transiently rise in mitosis before dropping nearer to G1 levels. Clb2 protein in nocodazole treated 261 mutant cells reaches a higher level than in *mad1* cells, but it is also degraded efficiently as the cells fail to arrest in mitosis. Mutant 155 accumulates high levels of Clb2p which are maintained as high as in wild-type cells.

Inactivation of $p34^{cdc2/CDC28}$ by inhibitory phosphorylation provides a mechanism by which cells can escape mitosis without destroying B type cyclins (Minshull et al., 1995). In nocodazole the kinase activity associated with $p34^{cdc2/CDC28}$/Clb2 complexes rises and then falls somewhat in mutant 155, although not as clearly as in the *cdc55* or *tub1-171* mutants (Figure 4 and Chapter 3). A mutant version of $p34^{cdc2/CDC28}$ which cannot be phosphorylated on inhibitory residues (due to substitution of Thr18 and Tyr19 with Val18 and Phe19 creating *cdc28-VF* (Booher et al., 1993)) prevents the inactivation of kinase activity by phosphorylation. It also halts the progression of the cell cycle of *cdc55* mutants grown in nocodazole (Minshull et al., 1995). When combined with the 155 mutant, however, *cdc28-VF* does not reduce the amount of rebudding in nocodazole (data not shown). Thus, it seems unlikely that these phosphorylations are mediating the innapropriate exit of 155 from mitosis.

Another marker for the end of mitosis is the separation of sister chromatids. We and others (Guacci et al., 1994; A. Rudner and A. Dernburg, unpublished results) have

developed a technique to visualize sister chromatid separation by fluorescence *in situ* hybridization (FISH). Although sister chromatids are not correctly segregated as *mad* cells exit mitosis in the presence of nocodazole, they can be seen to separate, giving rise to two signals per cell rather than one (J. Minshull, A. Dernburg and A. Rudner, unpublished results). We used this technique to ask if the 155 and 261 mutants were separating their sister chromatids in nocodazole. Separation similar to that seen for *mad1* occurs for mutant 261, but mutant 155 does not separate its sister chromatids significantly and appears like wild type (Figure 5). Thus mutant 261 is exiting mitosis in nocodazole as measured by cyclin degradation, sister chromatid separation and rebudding, but mutant 155 is only rebudding in the presence of continuing high Clb2p associated H1 kinase activity.

Previous work has shown that short linear mini-chromosomes can delay cells in mitosis, and that this delay is absent in *mad* cells (Wells and Murray, 1995). These mini-chromosomes segregate with low fidelity, and it is likely that it is their difficulty in attaching to the spindle correctly that induces the checkpoint-dependent delay. The mini-chromosomes therefore provide a means of testing whether the checkpoint detecting unattached kinetochores is intact, without disrupting the spindle. Cultures grown exponentially for greater than 12 hours under selection for the mini-chromosome were fixed and stained for tubulin immunofluorescence. The proportion of cells with short spindles is increased if wild type cells contain a linear, centromeric minichromosome due to a delay in mitosis (Figure 6). *bub2-1*, a previously isolated checkpoint mutant, and mutant 155 also have a greater proportion of cells with short spindles in the presence of the mini-chromosome. *mad1* and mutant 261 do not, however, show a delay when compared to strains carrying a circular derivative of the linear mini-chromosome. This suggests that *mad1* and mutant 261 are incompetent to detect the problems that the linear mini-chromosome has attaching to the spindle, but that *bub2-1* and mutant 155 can still sense this defect and delay the cell cycle.

kar3 mutants were originally identified as defective both in karyogamy and an

undefined step of mitosis due to mutation of a gene with homology to the microtubule motor, kinesin (Meluh and Rose, 1990). Kar3p is a minus end directed microtubule motor which may crosslink microtubules (Endow et al., 1994) or play a role at the kinetochore (Middleton and Carbon, 1994). Strains deleted for *KAR3* are viable but delay in G2/M. If this delay is mediated by the *MAD* mitotic checkpoint it may be absent in *kar3Δ/mad* double mutants. If the delay is essential to allow the cell to overcome a deficiency in Kar3p, such double mutants may not be viable. The latter is true for *mad2-1* (R. Li, pers. comm.), *bub1-1* and *bub3Δ* , which are all synthetically lethal with *kar3Δ*, although not for *bub2-1* (Meluh, 1992). We wanted to see if the *kar3Δ* strain was synthetically lethal with 155 or 261 mutants. The deletion plasmid pMR868 was cut with NruI, BamHI and NcoI and the large NruI/BamHI fragment isolated and transformed into *mad2-1*, *bub1-242*, *cdc55-215*, 155 and 261 mutants. All the mutants were covered by their respective rescuing plasmids; in the case of 155 and 261 a clone of *TUB1* was used (see below). Transformants obtained on double dropout plates were innoculated into non-selective media (2-3 transformants picked per transformation) and grown for four days with dilution into fresh non-selective media to allow loss of the rescuing plasmids. Cells were then plated out for single colonies and tested for loss of the rescuing plasmid. All colonies for all strains were *LEU*$^+$ due to the *kar3Δ*. For *mad2-1*, *bub1-242* and *cdc55-215* all the colonies were also *URA*$^+$ due to the rescuing plasmid, demonstrating that these mutants could not coexist with the *kar3Δ* without the presence of a rescuing plasmid. In contrast, approximately one third of the 155 and 261 mutant colonies were *URA*$^-$, indicating that they could lose the plasmid without becoming inviable. Thus while mutants in *MAD2*, *BUB1*, *BUB3* and *CDC55* are synthetically lethal with *kar3Δ*, this is not the case for mutants 155 and 261. This is the only major phenotypic difference identified between mutant 261 and *mad1-1*.

Attempts to clone the 155 and 261 genes

A YCp50 based genomic library (Hardwick and Murray, 1995) was transformed into both 155 and 261 and URA$^+$ transformants selected. These colonies were then scraped off in YPD medium and plated onto YPD plates with 10μg/ml benomyl to select for phenotypically MAD$^+$ cells. A total of 163 potential positives were picked for the two mutants; of these 27 retested as positive and the corresponding plasmids were rescued. On retransformation into the mutants, 6 clones showed good rescue (2 for 155, 4 for 261). (Of 22 potential positives from a cDNA library under the control of the inducible GAL1 promoter only one rerescued, and this encoded the Yap1/Snq3 transcriptional activator known to confer multi-drug resistance (Hussain and Lenard, 1991)).

The clones were restriction mapped and the rescuing regions defined (Figure 7). Filters containing lamda clones covering the entire *Saccharomyces cerevisiae* genome were probed with labelled inserts to determine the location of the rescuing regions. A combination of these techniques and DNA sequencing identified the rescuing clones as *SNQ2* (a multi-drug transporter; (Servos et al., 1993)), *TUB1* (isolated three times) and a C-terminal portion of *SWI4* (isolated twice). All clones rescued 155 and 261 equally when transformed into the other mutant. *TUB1* and the portion of *SWI4* also rescued *bub2-1*, although all other *mad* and *bub* mutants were not rescued. Analysis of 6 more clones that rescued less well revealed that all were rescuing due to a small gene next to *BUD5*. This gene, YCR037c, is of unknown function, but may have a homolog on chromosome X named JO336 (70% DNA sequence identity), also of unknown function.

Neither mutant is linked to the *TUB1*, *SWI4* or *BUD5* loci. The *SWI4* sequence begins at a SauIIIA site at amino acid 837; there is a methionine codon 27 bp downstream which has the potential to be the start of a protein of 249 amino acids. This exact fragment of *SWI4* has been isolated before in a screen involving suppression of a multicopy phenotype of the cAMP dependent protein kinase *TPK1* (Daniel, 1993). The mutants are

not rescued by a full *SWI4* clone (pJO69) or by multiple additional copies of *SWI6* (pJO66), *SWI4*'s partner in the transcription factor that activates genes at G1/S (Andrews and Herskowitz, 1989). It is possible that the *SWI4* clone isolated is acting as a dominant negative by binding *SWI6* and thereby blocking the action of this complex. This does not seem likely, however, as overexpression of *SWI6* does not prevent rescue of the mutants by the *SWI4* clone.

To identify candidate genes we attempted to map the mutants to a chromosome. Integration of 2 micron plasmid sequences into yeast chromosomes causes chromosome breakage and loss due to recombination events (Falco et al., 1982) and can be used for mapping mutations (Falco and Botstein, 1983). A multiply marked cir^0 strain (which lacks the 2 micron recombination enzymes) with 2 micron DNA integrated near the centromere of one chromosome is crossed to the cir^+ mutant strain. The cir^+ strain provides the recombination enzymes which act on the DNA substrates near the centromere of the chromosome from the cir^0 strain. Unequal recombination events lead to frequent loss of the centromere and therefore of the chromosome. The loss can be monitored by testing for loss of the $URA3^+$ gene associated with the tester chromosome. If the chromosome lost is the one which carries the wild-type version of the gene which is altered in the mutant strain, the mutation will be uncovered and the mutant phenotype expressed. Repeating this procedure with a different cir^0 strain for every chromosome allows the location of all mutants. A candidate chromosome was not identified for 155 using this method. This may reflect errors in the tester strains, or a low rate of loss of this particular chromosome or chromosome arm.

The mutation in 261 was identified as being on chromosome VII (22 of 30 $ura3^-$ colonies benomyl sensitive). The identity of the test strain was checked by testing for the uncovering of the *ndc10* mutant, also on chromosome VII (7 of 8 $ura3^-$ colonies temperature sensitive). Chromosome VII contains such candidate genes as *CDC55*, *CSE1*, *CLB1*, *CLB6*, *ESP1*, *BUB1* and *NDC10*. 261 is not closely linked to *CSE1*, *BUB1*,

NDC10 or *CDC20*. Unfortunately, chromosome VII is the equal longest chromosome of the *S. cerevisiae* genome (1.15 Mb), making linkage analysis a challenge, and will not be sequenced until 1996. This sequencing may uncover a homolog of a clone from a *Xenopus laevis* cDNA library, isolated by J. Minshull in a screen for *Xenopus BUB2* homologs. This gene, encoding a unique 670 amino acid protein, rescues the *bub2-1* and 261 mutants, but not any other *mad*-like mutants (including 155). A very homologous gene exists in humans, defined by three incomplete cDNA sequences (Accession numbers R38426 (42/49 identical amino acids); R27052 (101/111); D30950 (19/24)).

Discussion

A screen for mutants in the spindle assembly checkpoint

We have undertaken an extensive screen for further mutants in the spindle assembly checkpoint. Two mutants in previously unidentified complementation groups have been characterised phenotypically. Mutant 155 shares few properties with the original *mad* mutants and appears to be defective only in repressing rebudding when arrested in mitosis. Mutant 261 resembles the other *mad* mutants more closely, although some differences remain. The properties of these two mutants and other mutants isolated in this and other screens are listed in Table 2.

The screen has identified 10 alleles of 4 of the 6 *MAD* and *BUB* genes previously described (Li and Murray, 1991; Hoyt et al., 1991) and 7 alleles in 5 other genes which are under investigation; 155 and 261 (this Chapter), *tub1-171* (Chapter 3), *cdc55* (Minshull et al., 1995) and *mpk1* (J. Minshull and A. Straight, unpublished results). There have now been a total of 18 alleles of the 6 original spindle assembly checkpoint genes isolated in three similar screens. Thus the screen may not yet be saturated, but it seems that alleles for most of the genes identifiable in this way have been isolated.

Characteristics of mutant 155

The primary criterion for this mutant hunt is that cells show signs of cell cycle progression when their spindle is depolymerized, and that this cell cycle progression results in death. The isolation of mutant 155 makes clear that not all of the cell cycle must progress for a mutant to pass this test. Mutants such as *mad1* fail to maintain high Clb2p in nocodazole and therefore exit mitosis, separating their sister chromatids (presumably a lethal event) and entering the next cell cycle, as evidenced by the production of a new bud

(properties of the various mutants are summarised in Table 2). Mutant 155 maintains high Clb2p in nocodazole and sister chromatids do not disjoin. A slight decrease in Clb2 associated H1 kinase activity does not appear sufficient to result in exit from mitosis, and probably reflects a slow decline of H1 kinase activity secondary to some other events occuring. Mutant 155 does enter the next cycle in one sense, however, as a new bud is produced. This defect is specific to mitosis; no rebudding or death occurs if cells are arrested in the DNA synthesis inhibitor, hydroxyurea (data not shown). The rapid death of 155 in nocodazole suggests that this rebudding or an as yet unidentified event must be lethal. Rebudding is likely to be temporally close to spindle-pole body duplication, as it is in a normal cell cycle (Pringle and Hartwell, 1981). The presence of more than two spindle pole bodies might make correct segregation of chromosomes on recovery from nocodazole treatment impossible. If spindle pole body duplication is the lethal event for mutant 155 it may explain the slightly slower rate of death of mutant 155 as compared to *mad1*, whose presumed lethal event (sister chromatid separation) occurs earlier in the cell cycle. This difference is more pronounced in lower concentrations of nocodazole (data not shown). Spindle pole body duplication should also be prevented by α-factor. Mutant 155 shows a poorer death profile when released from α-factor (Figure 1b) and the extent of death decreases further (although not completely) when α-factor is added back after a one hour release into nocodazole (not shown).

Mutant 155 displays two further characteristics which are different from *mad1*; it delays correctly in mitosis in response to poorly segregating short linear mini-chromosomes, and is not synthetically lethal with *kar3Δ*, which likely reflects its ability to delay mitosis in this mutant. Given all these differences, what is defective in mutant 155 and what is the cause of this defect? The only thing which is clearly defective is the regulation of budding, which occurs despite high kinase activity associated with $p34^{cdc2/CDC28}$/Clb2 complexes. One important parameter to define is whether budding is the only process which is perturbed or if many G1/S events are occuring (such as CLN

transcription and DNA replication). If only budding is occuring it is likely that regulation of the budding process is awry due to mutation of a polarity establishment gene or regulators such as the *SIT4* phosphatase. *SIT4* is necessary for the G1 to S transition (Sutton et al., 1991), and also for budding, even if *CLN2* is provided by other means (Fernandez-Sarabia et al., 1992). The normal function of 155 may be to regulate the activation of budding as cells exit mitosis. It does not seem unreasonable that the regulation of G1/S events such as budding could normally occur around the time of exit from mitosis. For example the Cdc6 protein, which assists in the formation of pre-replication complexes for DNA replication initiation (Liang et al., 1995), is synthesized in telophase at a *cdc15* cell cycle block, and in cycling cells it appears 15 mins before *CLN1* (Zwerscke et al., 1994).

If multiple G1/S events are occuring in mutant 155, it is more likely that a common regulator upstream of these events is defective. Mutant 155 may bypass the requirement for G1 cyclin activity as occurs in mutants in the *BYC1* gene (Epstein and Cross, 1994), although these mutations are dominant, which is not the case for 155. Alternatively, the transcription of the G1 cyclins may be occuring in mutant 155, allowing budding and the initiation of DNA synthesis. $p34^{cdc2/CDC28}$ in association with the G1 cyclins Cln1, Cln2 or Cln3 is responsible for the polarization of the actin cytoskeleton to the bud site, whereas $p34^{cdc2/CDC28}$/Clb2 activity causes depolarization in G2 (Lew and Reed, 1993; reviewed in Lew and Reed, 1995). The normal product of the 155 gene may transduce the signal from $p34^{cdc2/CDC28}$/Clb complexes which represses *CLN* transcription (Amon et al., 1993); this would predict prolonged expression of *CLN* message after S phase in the 155 mutant. This repression of *CLN* transcription may involve the Swi4 protein, which with the Swi6 protein activates transcription in G1/S by binding to SCB elements in the promoters of the HO (Andrews and Herskowitz, 1989) and CLN1 and CLN2 genes (Ogas et al., 1991; Nasmyth and Dirick, 1991). Swi4p associates with Clb2p, and is a substrate for $p34^{cdc2/CDC28}$/Clb complexes (Amon et al., 1993). If

this phosphorylation is inhibitory for Swi4p DNA binding or transcriptional activation, its failure could result in a phenotype like that of mutant 155: the premature occurence of G1/S events in mitosis.

Mutants 155 and 261 are rescued by a C-terminal fragment of *SWI4*, but not the full length clone. This is the region of Swi4p involved in binding Swi6p (Andrews and Moore, 1992; Sidorova and Breeden, 1993); by itself it may act as a dominant negative inhibitor of SWI6 function, thus preventing cell cycle progression. Overproducing Swi6p does not, however, reverse the rescue by the fragment of SWI4. Perhaps the protein being poisoned is Swi4p itself, as in the absence of Swi6p, Swi4p can form heterogeneous complexes on SCB elements (Sidorova and Breeden, 1993). In this case, overexpression of wild type *SWI4* should reverse the rescue by the truncated *SWI4*. The truncated *SWI4* may also act by binding and somehow altering the function of Clb2p, preventing its destruction (mutant 261) and/or the inactivation of Clb2p associated H1 kinase by other means (Chapter 3).

Is mutant 155 a checkpoint mutant? The discussion above concentrates on the regulation of budding by the 155 gene product, but it remains possible that the spindle assembly checkpoint feeds into this pathway to prevent budding or other G1 events while cells are arrested in mitosis. The rescue of mutant 155 by the *TUB1* and partial *SWI4* clones that rescue mutants 261 and *tub1-171* (Chapter 3) suggests that mutant 155 may be involved with these checkpoint components (see Chapter 3 for details). If this is the case, it is defective in only one of the functions which this checkpoint appears to perform; while rebudding occurs, Clb2 associated kinase remains high and sister chromatids do not separate. It may represent a downstream effector of the checkpoint, devoted only to preventing G1 events.

Characteristics of mutant 261

Mutant 261 is similar to *mad1* in many respects; in nocodazole it fails to maintain high levels of Clb2p and so exits mitosis, separating its sister chromatids and rebudding. It also fails to delay mitosis in the presence of short linear mini-chromosomes, indicating that a perturbed spindle is not necessary for the wild type protein to produce a delay. Since a single signal (unattached kinetochores) is transduced by both 261 and the established *MAD* proteins, 261 probably lies in the same spindle assembly checkpoint pathway.

Mutant 261 differs from *mad1* in several phenotypes. It is rescued by genomic clones of *TUB1* and *SWI4* (a C-terminal portion only) which do not rescue *mad1*, but do rescue a mutant defining a possible spindle integrity checkpoint (Chapter 3). Perhaps the protein encoded by the 261 gene interacts with (although does not participate in - see below) a checkpoint apparatus which checks for bipolarity of the spindle (see Chapter 3). Mutant 261 is also viable with *kar3Δ*, and it accumulates slightly higher levels of Clb2p than *mad1*. These properties could be explained by leakiness of the 261 mutation. Mutant 261 does not, however, die more slowly than *mad1* in nocodazole or appear less benomyl sensitive on plates, although a weaker allele of 261 (mutant 185) does have these properties. It is also possible that the inviability of *mad2-1*, *bub1-1* and *bub3Δ* with *kar3Δ* reflects a defect in these mutants other than a failure to arrest; a defect which mutant 261 does not have.

Alternatively, these properties may be a clue that 261 normally functions on only one arm of the *MAD* checkpoint; that which prevents cyclin proteolysis. If the other arm is active, perhaps correctly detecting the absence of a bipolar spindle in mutant 261 in nocodazole, the exit from mitosis may be delayed by some other means just long enough for *kar3Δ* to remain viable and for a slight accumulation of Clb2p to take place. Such a bifurcation is likely to be at the bottom of the pathway, as the majority of the spindle assembly checkpoint mutants do not show these properties of mutant 261. Additionally, in

mutant 261 (as in mutant 155), Mad1p is still hyperphosphorylated in nocodazole (K. Hardwick, pers. comm.), suggesting the upper portion of the pathway is intact.

In the course of these studies we have also found that mutants in the *BUB2* gene (point and deletion mutants) show an important difference when compared to *mad* mutants; they can arrest the cell cycle in response to short linear mini-chromosomes (Figure 6). *bub2-1* also shares properties common to mutants 155, 261 and *tub1-171*, but not other *mad* and *bub* mutants: it is rescued by the *TUB1* and partial *SWI4* clones, and it is viable with a *kar3Δ*. Thus *BUB2* may be a component of the checkpoint assessing spindle integrity (Chapter 3) rather than kinetochore attachment. Information on the status of Clb2p and sister chromatid separation will help to clarify the relation of *BUB2* to the newer checkpoint mutants.

In summary, we have described two mutants which die rapidly in nocodazole. Mutant 155 enters the next cell cycle as evidenced by rebudding, but otherwise does not progress out of mitosis. It may represent a downstream effector of part of a mitotic checkpoint, or merely reflect deregulation of budding control. Mutant 261 appears similar to previously isolated mutants in the spindle assembly checkpoint. It is likely to be downstream of *MAD1*, and possibly interacts with the spindle at a bifurcation of the checkpoint pathway.

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Table 1. A list of all mutants identified in this screen which die rapidly in microtubule depolymerizing drugs.

21 mutants were identified which divide rapidly in micocolony assays, are rescued by hydroxyurea and die rapidly in benomyl/nocodazole. 10 are mutant in identified checkpoint genes.

Identification of mutants which die rapidly in benomyl

Mutant isolation number	Plasmid rescue	Allelic by cross	Comments
151	MAD1	N/D	
192	MAD1	N/D	
248	MAD1	N/D	
181	MAD1 (AFS)	Yes (AFS)	Mad1p not present on blot
142	MAD3	Yes	More ben. sens. than original *mad3-1* allele; equal to *mad1-1*.
242	BUB1	Yes	
245	BUB1	N/D	
235	BUB3	N/D; Non-mater	
247	BUB3	Yes	
305	BUB3 (AFS)	Yes	
155	TUB1	No	Unknown gene; see text
171	TUB1	Yes	See Chapter 3
185	TUB1	No	Weak allele of 261
261	TUB1	No	Unknown gene; see text
169	None (AFS)	N/D; Non-mater	
215	CDC55 (JM)	Yes (JM)	
236	CDC55 (AFS)	Yes (AFS)	
222	TUB1 (AFS)	No (AFS)	Does not rebud
157	TUB1 (AFS)	N/D; Non-mater	
131	MPK1 (AFS)	Yes (AFS)	
286	CND1 (AFS)	Yes (AFS)	Dies due to osmotic problems in mitosis

Table 2. A summary of the properties of the mutants discussed in this Chapter.

The properties of various mutants when grown in nocodazole is shown (derived from Chapters 2 and 3, Hoyt et al. (1991), Hardwick and Murray (1995) and Minshull et al. (1995)). Properties differing from those of the prototypic checkpoint mutant, *mad1*, are shown in bold type. Unknown properties are blank. 261 is the most similar to *mad1*; 155 the most different.

Summary of mutant properties

Strain / Property in noc	WT	mad1	155	171	cdc55	261	bub2
Rapid death	No	Yes	Yes	Yes	Yes	Yes	Yes
Rebud	No	Yes	Yes	Yes	Yes	Yes	Yes
Clb2	High	Low	**High**	**High**	**High**	Low	
Clb2 asoc kinase	High	Low	**Med**	Low	Low	Low	**Med**
Sister separation	No	Yes	**No**	Yes	Yes	Yes	
Rereplicate	No			Yes			Yes
Retain MTs	Yes	Yes		Yes			
kar3 del lethal	No	Yes	**No**	**No**	Yes	**No**	**No**
Short linear delay	Yes	No	**Yes**	**Yes**	No	No	**Yes**

Mutant properties which differ from those of *mad1* are in **bold**.

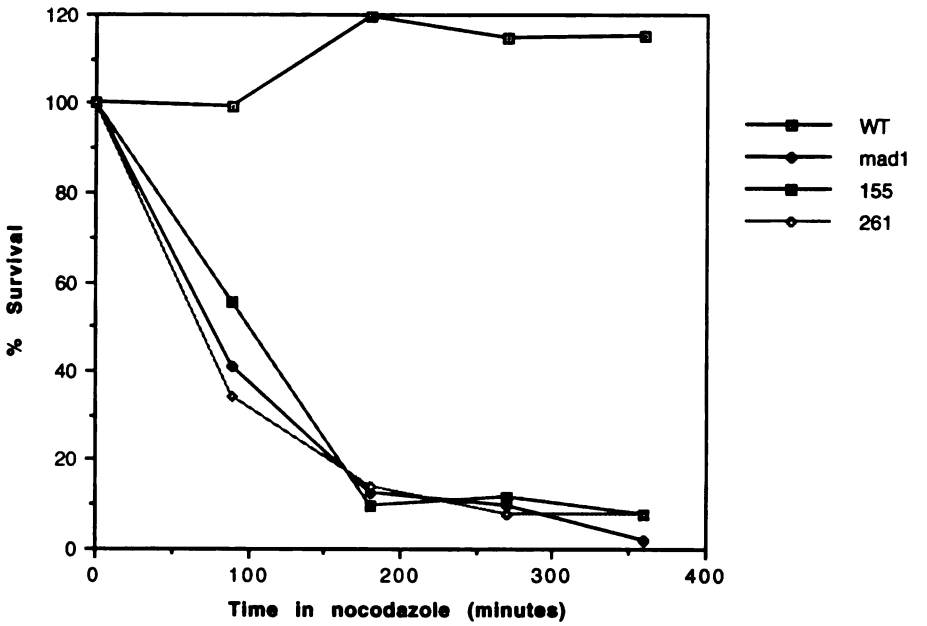
TABLE 3: Yeast Strains.

AFS34	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100*
BW128	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100, mad1Δ::HIS3*
BW159	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100, mad2Δ::URA3*
BW130	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100, mad3Δ::LEU2*
BW146	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100, mutant 155*
BW144	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100, mutant 261*
BW132	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100, bub1Δ::HIS3*
BW257	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100, bub2-1*
BW133	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100, bub2Δ::URA3*
BW53	a	*ura3-52, lys2-801, leu2,3-112, his3-Δ200, bub3Δ::LEU2*
BW168	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100, cdc55-215*
B-7599 to 7614	α	*cir^0, ura3-52, leu2-3,112, trp1-289, met2, his3-1*, YEp24 at centromeres of chromosomes I - XVI (from Yeast Genetic Center).
TBW136	as AFS34 but with linear pVL106	
TBW195	as AFS34 but with circular pVL106	
TBW207	as BW146 but with linear pVL106	
TBW208	as BW146 but with circular pVL106	
TBW210	as BW144 but with linear pVL106	
TBW211	as BW144 but with circular pVL106	
TBW205	as BW168 but with linear pVL106	
TBW206	as BW168 but with circular pVL106	
TBW173	as BW257 but with linear pVL106	
TBW108	as BW133 but with circular pVL106	

Figure 1. Rapid death of mutants 155 and 261 in nocodazole.

Exponentially growing (a) or a-factor arrested (b) cells were grown in YPD at 22°C with 15μg/ml nocodazole, and samples withdrawn at the indicated times and tested for viability by plating on YPD. Where indicated, the DNA synthesis inhibitor, hydroxyurea (HU), was added at the same time as the nocodazole at 10mg/ml. Viability can increase above 100% due to cell division.

a)


120
100
80
60
40
20
0
% Survival
0
100
200
300
400
Time in nocodazole (minutes)
WT
mad1
155
261

b)

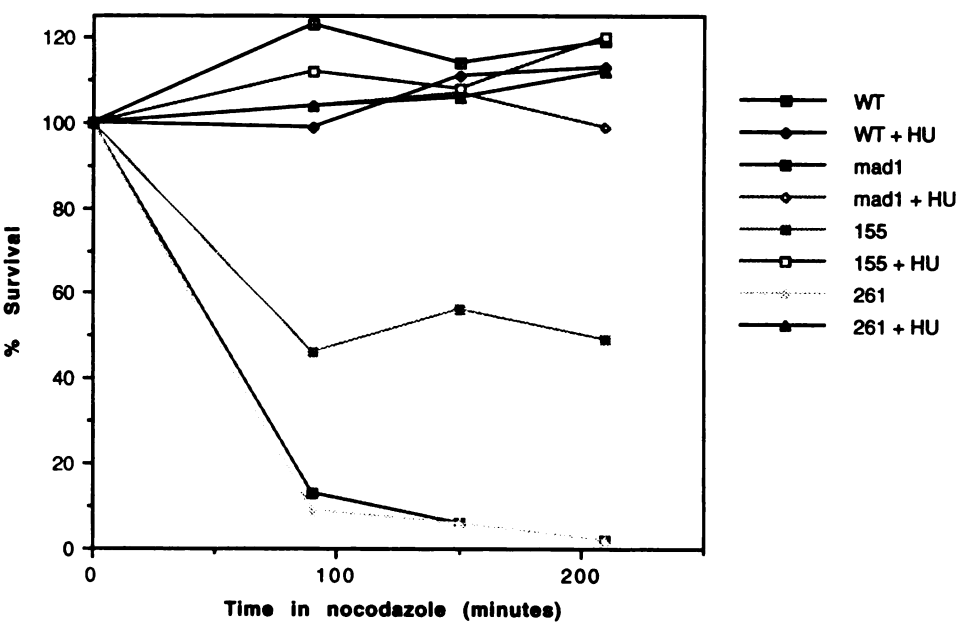

120
100
80
60
40
20
0
% Survival
0
100
200
Time in nocodazole (minutes)
WT
WT + HU
mad1
mad1 + HU
155
155 + HU
261
261 + HU

Figure 2. Rebudding of mutants 155 and 261 in nocodazole.

Exponentially growing cells were grown in YPD at 22°C with 15μg/ml nocodazole, and samples withdrawn at the indicated times, fixed and the cell morphology determined. A minimum of 200 cells were counted for each strain and timepoint. At the first timepoint >95% of cells were unbudded, and at later timepoints >90% of cells were large or triply budded cells. The figure shows the proportion of large budded cells which rebudded.

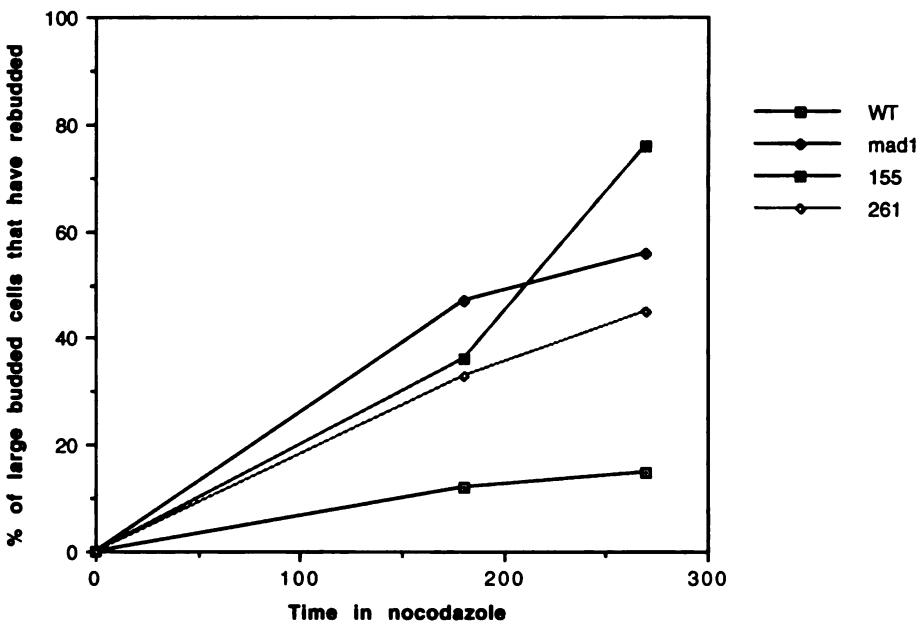

100
80
60
40
20
0
% of large budded cells that have rebudded
0
100
200
300
Time in nocodazole
WT
mad1
155
261

Figure 3. Clb2p levels of mutants 155 and 261 in nocodazole.

Cells arrested in α-factor were released into YPD with 15μg/ml nocodazole at 22°C. Samples were withdrawn at the indicated times and protein extracts made. The extracts were run on a gel and probed for Clb2p. Clb2p levels rise and stay high in wild-type and mutant 155, but fall in *mad1* and mutant 261.

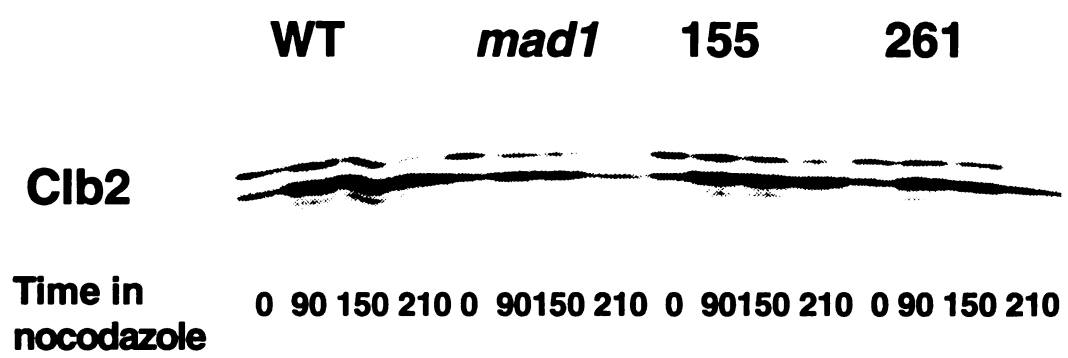

WT
mad1
155
261
Clb2
Time in nocodazole
0 90 150 210 0 90150 210 0 90150 210 0 90 150 210

Figure 4. Clb2p-associated H1 kinase levels in mutants 155 and 261 in nocodazole.

Cells arrested in α-factor were released into YPD with 15μg/ml nocodazole at 22°C. Samples were withdrawn at the indicated times and protein extracts made. The extracts were run on a gel and probed for Clb2p and immunoprecipitated with anti-Clb2p antibody before assaying for H1 kinase activity. Clb2p levels rise and stay high in wild-type, *cdc55*, and mutant 155 (a). H1 kinase activity stays high in wild-type, but drops significantly in *cdc55* and slightly in mutant 155 (b).

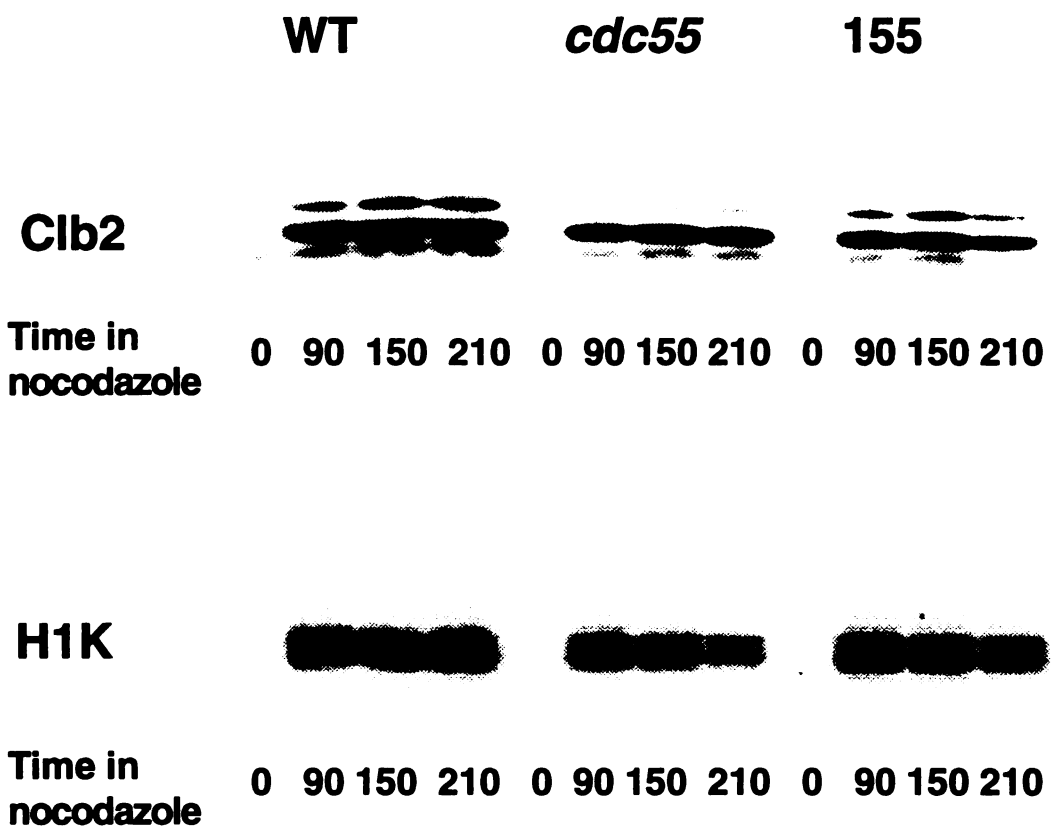

WT
cdc55
155
Clb2
Time in nocodazole
0 90 150 210 0 90 150 210 0 90 150 210
H1K
Time in nocodazole
0 90 150 210 0 90 150 210 0 90 150 210

Figure 5. Sister chromatid separation in mutants 155 and 261 in nocodazole.

Cells arrested in α-factor were released into YPD with 15μg/ml nocodazole at 22°C. Samples were withdrawn at the indicated times and fixed, spheroplasted and adhered to slides. The slides were probed with a fluorescent cosmid probe, which was visualized directly. Although proper segregation of chromosomes does not occur, sister chromatid separation can be easily visualized as the appearance of two dots in the cell where once there was one. A fraction of the cells have closely apposed dots, which are scored here as "1.5" *in situ* signals. *mad2* and mutant 261 show an increased proportion of cells with two *in situ* signals, indicating that sister chromatid separation is occuring.

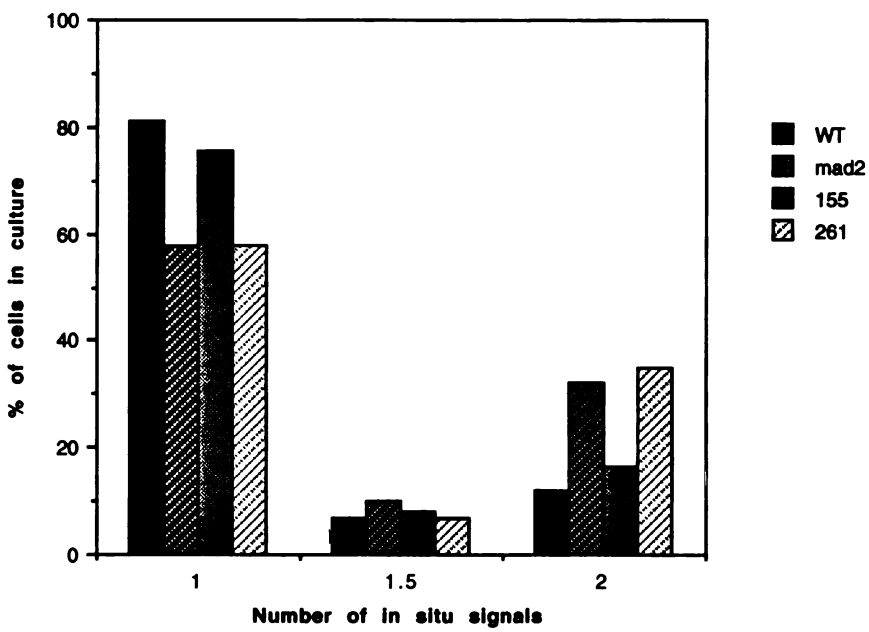

100
80
60
40
20
0
% of cells in culture
1
1.5
2
Number of in situ signals
WT
mad2
155
261

Figure 6. Short linear mini-chromosomes delay wild-type, 155 and *bub2*, but not *cdc55-215* or 261.

Exponentially growing cultures harboring either the linear or circular derivative of the mini-chromosome pVL106 were fixed and stained for tubulin immunofluorescence. Spindle morphology was scored as either a single dot (1), a short spindle, characteristic of G2/M (2), or a long, post anaphase spindle (3). The increased proportion of cells with short spindles in wild-type, 155 and *bub2* cells, compared with the corresponding cultures with circular mini-chromosomes, indicates that these strains can delay in response to linear mini-chromosomes.

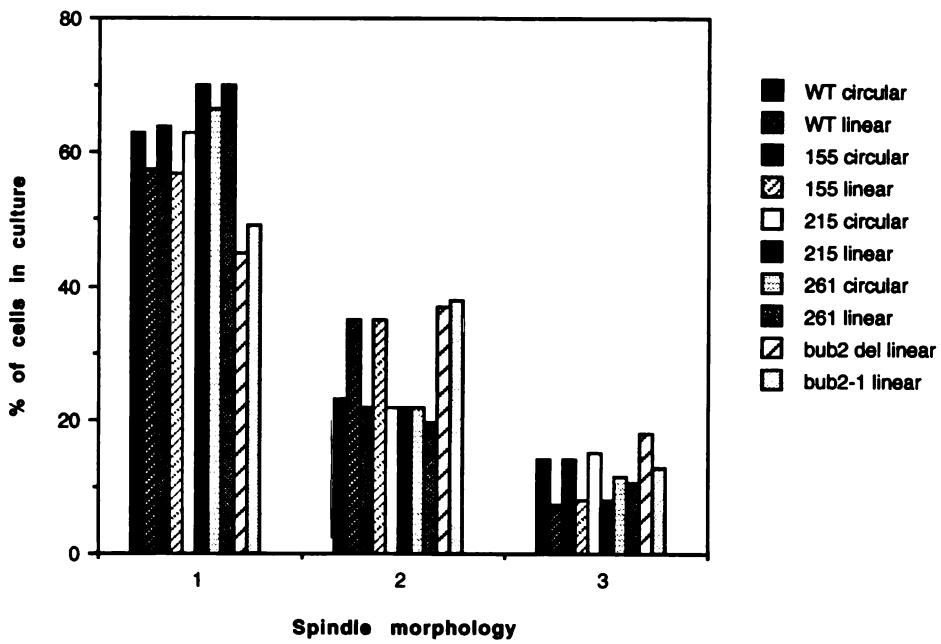
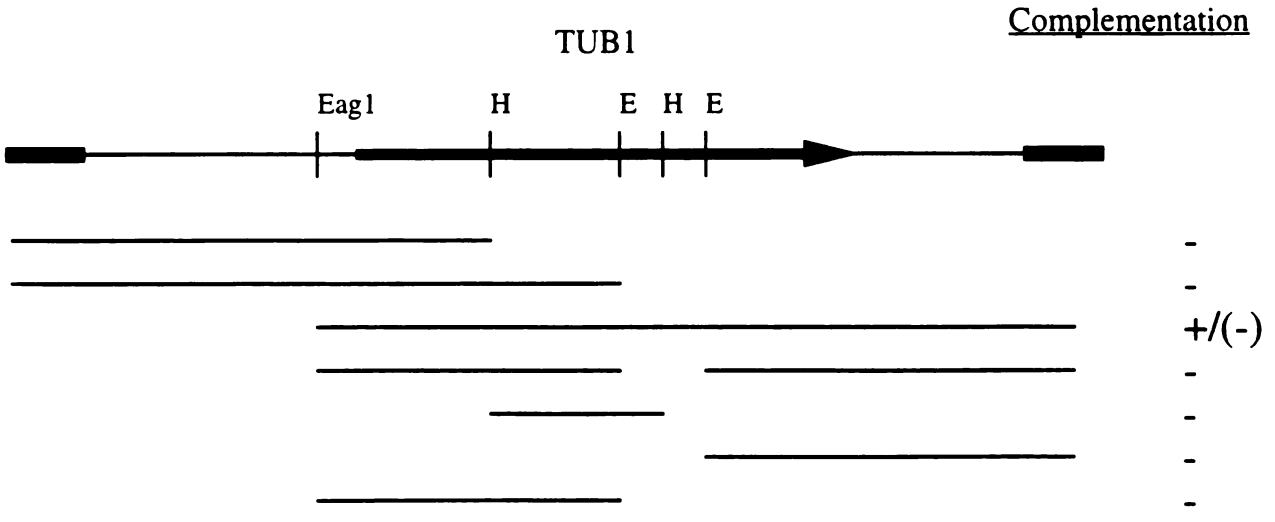

80
60
40
20
0
% of cells in culture
1
2
3
Spindle morphology
WT circular
WT linear
155 circular
155 linear
215 circular
215 linear
261 circular
261 linear
bub2 del linear
bub2-1 linear

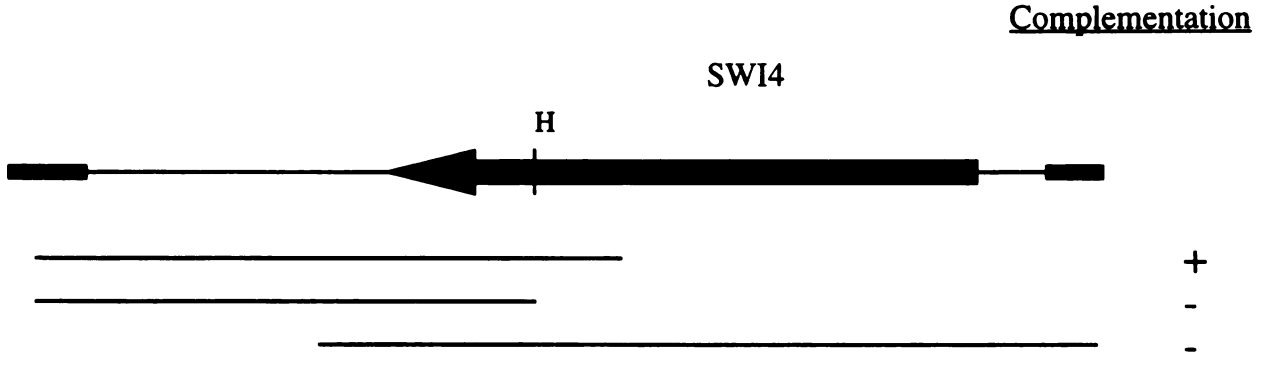
Figure 7. Rescue of mutants 155 and 261 by *TUB1* and truncated *SWI4* clones.

The figure illustrates the rescuing activities of various subclones of two positive clones obtained by screening a YCp50 based genomic library. (a) A fragment of *TUB1* that includes the entire gene and only a short promoter fragment has nearly full rescuing activity. (b) The rescuing activity of the other clone appears to reside in the 3' region, as when a random SauIIA fragment sub-library was prepared from this clone, all rescuing clones included the full 3' region. The only open reading frame in this region is the C-terminus of *SWI4*.

a) Rescue of 155 and 261 by *TUB1*



b) Rescue of 155 and 261 by a C-terminal portion of *SWI4* but not a complete *SWI4* clone



Chapter 3

An unusual mutant in alpha-tubulin demonstrates the existence of a spindle integrity checkpoint in budding yeast.

Abstract

The spindle assembly checkpoint delays the onset of anaphase in the presence of microtubule depolymerizing agents and unattached chromosomes. In a screen for more components of the spindle assembly checkpoint, we have isolated an allele of *TUB1*, the major α-tubulin of budding yeast. *tub1-171* continues cycling in nocodazole as evidenced by sister chromatid separation, rebudding, and rereplication, in contrast to other *TUB1* mutants. Clb2 cyclin levels remain elevated, but Clb2 associated H1 kinase activity decreases, suggesting an inhibitor of the kinase is produced. Our data may be consistent with a requirement for spindle microtubules to maintain mitotic kinase activity. A more likely explanation is that *tub1-171* is defective in a checkpoint that detects the integrity of the spindle. This proposed checkpoint is distinct from the spindle assembly checkpoint previously described, and may involve the *BUB2* gene.

Introduction

To ensure the fidelity of cell division, checkpoints can delay the progression of the cell cycle in response to failures in the completion of certain events (Hartwell and Weinert, 1989; Murray, 1992). The spindle assembly checkpoint detects problems in attaching chromosomes to the spindle (Rieder et al., 1994; Wells and Murray, 1995) and possibly in assembling the spindle, and delays the onset of anaphase. Studies in the budding yeast, *Saccharomyces cerevisiae*, have identified six genes involved in the spindle assembly checkpoint: *MAD1*, *MAD2* and *MAD3* (Li and Murray, 1991) and *BUB1*, *BUB2*, and *BUB3* (Hoyt et al., 1991). Mutants in the spindle assembly checkpoint fail to arrest in mitosis in response to microtubule depolymerizing drugs and therefore undergo a lethal mitosis (Hoyt et al., 1991; Li and Murray, 1991). Sister chromatids separate without segregation (Minshull et al., 1995) and levels of the B type cyclin, Clb2p, fall as if no drug was present (Hardwick and Murray, 1995; Minshull et al., 1995).

The six isolated mutants appear to have similar properties and a number of them can be placed in a single pathway (Hardwick and Murray, 1995). In the presence of nocodazole, Mad1p is hyperphosphorylated in wild type, *mad3* and *bub2* cells, but not in *mad2*, *bub1* or *bub3* cells. Therefore, *MAD2*, *BUB1* and *BUB3* are likely to lie upstream of *MAD1*. By this analysis, the essential protein kinase Mps1p, required for spindle pole body duplication (Winey et al., 1991) and the spindle assembly checkpoint (Weiss and Winey, 1995), also lies upstream of *MAD1*. *MAD3* and *BUB2* could lie downstream of *MAD1* or in a different pathway.

The three mutants which have been tested, *mad1*, *mad2*, and *mad3*, also fail to delay in mitosis in response to poorly segregating short linear mini-chromosomes, which are likely to have trouble attaching to the spindle (Wells and Murray, 1995). This agrees with studies in other organisms suggesting that chromosome attachment to the spindle is sensed by the checkpoint, and is sufficient by itself to cause mitotic delays (Rieder et al., 1994). Is this the only aspect of spindle structure that is sensed? This is difficult to assess,

as many other perurbations in spindle structure, such as microtubule depolymerization and the creation of monopolar spindles, would also generate problems in chromosome attachment. Sea urchin eggs, however, seem to lack the ability to detect unattached kinetochores, but still delay in mitosis unless a bipolar spindle is present (Sluder and Begg, 1983; Sluder et al., 1994). This suggests a positive requirement for a bipolar spindle for cell cycle progression to occur, in addition to the need for a lack of unattached chromosomes observed in other systems.

As few alleles were isolated in the original spindle assembly checkpoint screens (Hoyt et al., 1991; Li and Murray, 1991), we undertook a similar screen in an attempt to isolate further components of this sytem. One mutant that we isolated is an allele of *TUB1*, the major α-tubulin encoding gene in *S. cerevisiae*. Our results suggest that this mutant may be unable to detect the absence of a bipolar spindle, thus causing premature exit from mitosis.

Materials and Methods

Yeast strains and media

Table 2 lists the strains used in this work. *mad1* was backcrossed 3 times into the W303 background. All other strains are in the W303 background except BW224, which is S288c. Media were prepared and genetic manipulations were performed as described (Sherman et al., 1974).

Screen for checkpoint mutants, Death assays, Cloning, Rebudding, Western blotting, Immunoprecipitations, H1 kinase assays and FISH

See Chapter 2. Allelism of mutant 171 with *TUB1* was confirmed by crossing mutant 171 with *tub1-1* (6PD:0NPD:0T) and a marked integration of the *TUB1* rescuing clone (8PD:0NPD:0T).

Generation of other *tub1* mutants

Mutants generated in an alanine scanning mutagenesis were provided by K. Richards. The mutations introduced are as follows :

tub1-801 R2A, E3A

tub1-821 E161A, K164A

tub1-830 K279A, K281A

tub1-837 K339A, K340A

tub1-849 E430A, R431A, D432A

tub1-851 E443A, E444A, E445A, E446A

Linearised DNA encompassing the mutated TUB1 genes and an inserted LEU2 gene was transformed into wild-type W303 and LEU^+ transformants selected. Six transformants for each mutant were tested for benomyl sensitivity; most were benomyl sensitive. One of these was crossed to *tub1-1* to test for allelism (4PD:0NPD:0T for each transformant).

FACS Analysis

Cells were prepared for flow cytometry by the method of (Hutter and Eipel, 1979) using the DNA stain propidium iodide, and then analyzed on a Becton Dickinson FACSCAN flow cytometer using the LYSYS software package to obtain and analyze data.

Tubulin immunofluorescence

Exponentially growing cultures were adjusted to an OD_{600} of 0.3 and nocodazole added to final concentrations of 0, .5, 1, 2.5, 5, 7.5 and 10μg/ml. Cells were grown at 22°C for 30 minutes before processing for tubulin immunofluorescence as described in Chapter 1.

Results

Isolation of a mutant with defects in a cell cycle checkpoint

Previous screens for mutants in the spindle assembly checkpoint yielded 8 alleles defining 6 genes (Li and Murray, 1991; Hoyt et al., 1991). The low number of alleles suggests that more components of the checkpoint are yet to be discovered by this method. Therefore we performed a screen similar to one previously described (Li and Murray, 1991). The screen (Chapter 2) identified 10 alleles of the 6 *MAD* and *BUB* genes previously described and 7 alleles of 5 other genes which are under investigation (this Chapter, Chapter 2, Minshull et al., 1995, and J. Minshull and A. Straight, unpublished results). This chapter describes the characterisation of one mutant, identified by the isolation number 171. Mutant 171 is slightly less benomyl sensitive than *mad1* on plates (Figure 1a).

Mutants in the spindle assembly checkpoint fail to arrest the cell cycle in response to microtubule depolymerization (Li and Murray, 1991; Hoyt et al., 1991) and the failure of chromosomes to attach to the spindle (Wells and Murray, 1995). This results in rapid death of the mutants in the microtubule depolymerizing drug, nocodazole, as they go through an error-prone mitosis. (We used nocodazole in liquid cultures at 10-15mg/ml and a less potent microtubule depolymerizing drug, benomyl, at 7.5mg/ml in plates.) Structural mutants of the spindle are expected to arrest in mitosis and therefore recover from the nocodazole treatment. A collection of 250 benomyl sensitive mutants was screened by assaying for rapid death in nocodazole. The result of one such experiment involving *mad1* and mutant number 171 is shown in Figure 1. Both mutants die rapidly when exposed to nocodazole in both asynchronous (Figure 1b) and synchronous (Figure 1c) cultures, and this lethality no longer occurs in the presence of the DNA synthesis inhibitor, hydroxyurea, which prevents progression of the cell cycle. The reason for the rebound in viability at 150

minutes after release from α-factor in Figure 1b is not known, but a similar effect has been seen with the *bub2-1* mutant (Hoyt et al., 1991). Thus mutant 171 has the principal property of a checkpoint mutant: it loses viability due to cell cycle progression.

It remains possible that mutant 171 loses viability due to an irreversible error during arrest in mitosis, rather than incorrect progression through mitosis. We examined the progression of the cell cycle in mutant 171 in nocodazole by FACS analysis (Figure 2). Wild type cells arrest in mitosis with a 2N complement of DNA; eventually some cells leak through the arrest and a small peak of 4N cells appears. The peak appears at 4N because cytokinesis fails in the presence of high amounts of nocodazole. In mutant 171 the 4N peak appears earlier and is far more prominent as most cells enter the next cell cycle, having failed to arrest in the previous cell cycle.

Mutant 171 is an allele of *TUB1*, the major α-tubulin gene

In the course of attempting to clone another possible checkpoint mutant we isolated two genomic rescuing clones (Chapter 2). We tested the ability of these clones to rescue mutant 171. Both clones rescued mutant 171, and the rescuing regions were identified as the *TUB1* gene, the major a-tubulin in *Saccharomyces cerevisiae* (Schatz et al., 1986a; Schatz et al., 1986b) and a C-terminal portion of SWI4 (Figure 3). While the other mutant was not allelic to either *TUB1* or *SWI4*, mutant 171 was found to be allelic to *TUB1* (see Materials and Methods). It is therefore denoted from here on as *tub1-171*. The mechanism underlying rescue by the truncated SWI4 clone is not known, but notably it does not rescue either *tub1-1* or *tub3-1*, the first conditional mutants of the two *S. cerevisiae* a-tubulin genes to be characterised (Stearns and Botstein, 1988). Rescue of the *tub1-171* allele was performed by gap repair (Orr-Weaver et al., 1983). The mutant allele rescues *tub1-1*, a very benomyl sensitive mutant, to a level of benomyl resistance comparable to mutant 171, but lower than wild type levels (Figure 4). The rescued allele has not been sequenced as yet.

tub1-171 does not result in a visibly perturbed or nocodazole sensitive spindle

We were suprised that mutation in a structural gene such as *TUB1* could give even some of the phenotypes of a checkpoint mutant. Benomyl sensitive mutants defective in the spindle assembly checkpoint do not show a reduction of polymerized microtubules and spindle structures in low concentrations of microtubule depolymerizing drugs when compared to wild type cells, whereas such a reduction is seen in many mutants which are benomyl sensitive due to structural defects in the spindle (Li and Murray, 1991). Like *mad1*, but unlike *tub1-1*, *tub1-171* is not sensitive to microtubule depolymerizing agents in this manner (Figure 5). Staining of microtubules is essentially unaffected in both wild type and *tub1-171* cells in 2.5μg/ml nocodazole, a concentration at which nearly all microtubule staining is absent in *tub1-1* cells. At higher concentrations of nocodazole microtubule staining disappears coincidently in both wild type and *tub1-171* cells, indicating that microtubules are not visibly stabilised in *tub1-171*.

tub1-171 is unlike other mutants in *tub1*, which arrest in nocodazole

We wished to see if the checkpoint defects in *tub1-171* were a general property of mutants in *TUB1*. We therefore compared *tub1-171* to six other benomyl sensitive mutants in *TUB1* created by alanine scanning mutagenesis (alleles created by K. Richards and D. Botstein). Linear fragments containing the mutant *tub1* alleles were transformed into the W303 wild type strain. Benomyl sensitive transformants were isolated and tested for cosegregation of the benomyl sensitivity with the *TUB1* locus. All the mutants were considerably more benomyl sensitive than *tub1-171*.

When exponentially growing cultures of the seven *tub1* mutants were shifted to

media containing nocodazole, *tub1-171* lost viability rapidly (Figure 6), as seen before (Figure 1). Despite their benomyl sensitivity on plates, the other *tub1* mutants either did not lose viability or decreased in viability only slowly. The most severe mutant, *tub1-849*, still retains higher viability at 6 hours than *tub1-171* does at 90 minutes.

The lethal event for spindle assembly checkpoint mutants growing in nocodazole may be the entry into anaphase, measurable by observing the separation of sister chromatids (Guacci et al., 1994; A. Rudner, A. Dernburg, unpublished results). Using fluorescence *in situ* hybridization (FISH), the separation (although not segregation) of sister chromatids can be seen to occur as *mad* cells exit mitosis in the presence of nocodazole, giving rise to two signals per cell rather than one (J. Minshull, A. Dernburg and A. Rudner, unpublished results). We used this technique to ask if any of the *tub1* mutants separate their sister chromatids in nocodazole (Figure 7). *tub1-171* showed a decrease in the proportion of cells with a single *in situ* signal (Figure 7a), and an increase in the proportion of cells with two *in situ* signals (Figure 7b), indicating that sister chromatid separation is occuring. Essentially no sister chromatid separation occured for wild-type cells or most of the other *tub1* mutants. Some sister chromatid separation occured in the *tub1-849* and *tub1-851* mutants, but it was to a far lesser extent (11-16% of cells with two spots at 210 minutes, as compared to 44% for *tub1-171*).

A simple method by which cells in nocodazole can be scored for their entry into the next cell cycle is by observing the rate at which the large budded cells put out a new bud. This occurs without a preceeding cytokinesis due to the absence of a spindle. A small percentage of wild type cells rebud in nocodazole as they leak through the checkpoint (11% at 4.5 hours: Figure 8). The *tub1* mutants created by alanine scanning rebud to the same, or a lesser, extent to wild type. In contrast, *tub1-171* rebuds efficiently, with 71% of large budded cells rebudding after 4.5 hours in nocodazole. Thus *tub1-171* is unique amongst these *tub1* mutants; in the presence of nocodazole its cell cycle continues, as measured by loss of viability, sister chromatid separation, and rebudding.

tub1-171 and *mad1* detect different signals

The *mad1*, *mad2*, and *mad3* mutants fail to arrest in mitosis in response to poorly segregating, short linear mini-chromosomes, indicating that the *MAD* system usually detects the failure of chromosomes to attach to the spindle (Wells and Murray, 1995). The mini-chromosomes provide a means of testing whether the checkpoint detecting unattached kinetochores is intact, without disrupting the spindle. To test whether *tub1-171* can detect chromosome attachment failure, *tub1-171* was transformed with linear and circular derivatives of the plasmid pVL106 (V. Lundblad, pers. comm.) and exponentially growing cultures fixed and processed for anti-tubulin immunofluorescence. Wild type cells show an increased frequency of cells with short spindles in cells containing the linear form of pVL106 when compared to the circular form, indicating a delay in G2/M, while *mad1* cells do not show such a difference (Wells and Murray, 1995). Like wild type, but unlike *mad1*, *tub1-171* delays with linear pVL106 (Figure 9). This indicates that as long as the spindle remains intact, *tub1-171* is competent to delay the cell cycle in response to a failure to attach chromosomes to the spindle.

kar3 mutants were originally identified as defective both in karyogamy and an undefined step of mitosis due to mutation of a gene with homology to the microtubule motor, kinesin (Meluh and Rose, 1990). Kar3p is a minus end directed microtubule motor which may crosslink microtubules (Endow et al., 1994). Strains deleted for KAR3 are viable but delay in G2/M. This delay may be due to problems in chromosome attachment to the spindle, as Kar3p has been implicated in kinetochore function (Middleton and Carbon, 1994). If this delay is mediated by the *MAD* mitotic checkpoint it may be absent in *kar3Δ/mad* double mutants. If the delay is essential to allow the cell to overcome a deficiency in Kar3p, such double mutants may not be viable. The latter is true for *mad2-1* (R. Li, pers. comm.), *bub1-1* and *bub3Δ* (Meluh, 1992), which are all synthetically lethal

with *kar3Δ*, although not for *bub2-1* (Meluh, 1992). We wanted to see if the *kar3Δ* strain was synthetically lethal with *tub1-171*. The deletion plasmid pMR868 was cut with NruI, BamHI and NcoI and the large NruI/BamHI fragment isolated and transformed into *mad2-1* and *tub1-171*. The mutants were covered by their respective rescuing plasmids. Transformants obtained on double dropout plates were innoculated into non-selective media (2-3 transformants picked per transformation) and grown for four days with dilution into fresh non-selective media to allow loss of the rescuing plasmids. Cells were then plated out for single colonies and tested for loss of the rescuing plasmid. All colonies for all strains were LEU^+ due to the *kar3Δ*. For *mad2-1* all the colonies were also URA^+ due to the rescuing plasmid, demonstrating that *mad2-1* cannot coexist with the *kar3Δ* without the presence of a rescuing plasmid. In contrast, approximately one third of the *tub1-171* colonies were URA^-, indicating that they could lose the plasmid without becoming inviable. Thus while mutants in *MAD2*, *BUB1* and *BUB3* are synthetically lethal with *kar3Δ*, this is not the case for *tub1-171*. As with the short linear mini-chromosomes, *tub1-171* can still sense a defect and delay the cell cycle.

tub1-171 exits mitosis differently than *mad1*

Wild type cells arrested in nocodazole accumulate high levels of the B type cyclin, Clb2. *mad1* cells in nocodazole fail to accumulate Clb2p, destroying it as they exit mitosis (Hardwick and Murray, 1995). We tested *tub1-171* for the ability to accumulate Clb2p after release from an a-factor block into nocodazole. Nocodazole causes accumulation of Clb2p to high levels in *tub1-171*, although not as high as in wild type cells (Figure 10). Levels decline slightly by the last timepoint (210 minutes), but at 150 minutes there is no sign of Clb2p degradation, unlike in *mad1*. At 150 minutes many cells are already genetically dead and have shown signs of cell cycle progression as evidenced by rebudding (Figures 1 and 8).

How is *tub1-171* progressing in the cell cycle despite maintenance of Clb2 protein levels? Inactivation of $p34^{cdc2/CDC28}$ kinase activity provides a mechanism by which cells can escape mitosis without destroying B type cyclins (Minshull et al., 1995). We therefore tested whether the H1 kinase activity associated with Clb2p decreases when 171 mutant cells are grown in nocodazole. Figure 11 demonstrates that for *tub1-171* in nocodazole the kinase activity associated with $p34^{cdc2/CDC28}$/Clb2 complexes rises and then falls significantly, before the decrease in Clb2p levels occurs. One mechanism by which $p34^{cdc2/CDC28}$ can be inactivated is inhibitory phosphorylations on threonine 18 and tyrosine 19 (Coleman and Dunphy, 1994). A mutant version of $p34^{cdc2/CDC28}$ which cannot be phosphorylated due to mutation of these residues (to Val18 and Phe19 creating *cdc28-VF* (Booher et al., 1993)) prevents this means of inactivating kinase activity, and thus prevents the progression of the cell cycle of *cdc55* mutants grown in nocodazole (Minshull et al., 1995). Combination of the *cdc28-VF* mutant with *tub1-171* does not reduce the amount of rebudding in nocodazole (Figure 12), thus it seems unlikely that these phosphorylations are mediating the inapropriate inactivation of $p34^{cdc2/CDC28}$ kinase and subsequent exit of *tub1-171* from mitosis.

Discussion

In a screen for mutants in the spindle assembly checkpoint, we have isolated a mutant in the *TUB1* gene, the major a tubulin gene in *S. cerevisiae*. *tub1-171* has a spindle which is not visibly sensitive or resistant to nocodazole, but the cells fail to arrest in nocodazole. This leads to a rapid loss of viability as sister chromatids separate and the cells enter the next cell cycle, as evidenced by rebudding and rereplication. Other nocodazole sensitive mutants in *TUB1* do not show these phenotypes. Unlike *mad1*, *tub1-171* can still detect the defects due to short linear mini-chromosomes or *kar3Δ*, and maintains high Clb2p levels in nocodazole. Mitotic exit of *tub1-171* is associated with the inactivation of $p34^{cdc2/CDC28}$ kinase by some means other than the known inhibitory phosphorylations.

The isolation of a mutation in *TUB1* as a potential spindle assembly checkpoint mutant was surprising. A search for mutants in the replication checkpoint in *S. cerevisiae* has also yielded a mutant in a gene involved in the process being checked (Navas et al., 1995). In this case the *dun2* mutant fails to arrest the cell cycle in response to incomplete DNA replication. *DUN2* is identical to *POL2*, which encodes DNA polymerase epsilon, one of three DNA polymerases essential for DNA replication, and possibly a repair enzyme (Nishida et al., 1988; Wang et al., 1993). Its proximity to DNA metabolism suggests it may act as a sensor of unreplicated DNA. Likewise we consider the properties of *tub1-171* consistent with α-tubulin normally playing a role in sensing the integrity of the spindle and controlling cell cycle progression accordingly.

Defects in microtubule structure and dynamics have previously been associated with mitotic arrest rather than progression. Nocodazole treatment of wild type budding yeast cells causes microtubule disassembly and mitotic arrest (Jacobs et al., 1988) and, although tested mostly at the restrictive temperature rather than in nocodazole, many alleles of the tubulin genes are also observed to arrest in mitosis. Mutation of the *TUB2* gene in *S. cerevisiae*, encoding β-tubulin, causes some degree of mitotic arrest in all the mutants

examined, with the extent roughly proportional to the temperature sensitivity of the mutant (Huffaker et al., 1988; Reijo et al., 1994). Overexpression of *TUB2* causes a complete loss of microtubules and cell cycle arrest (Burke et al., 1989). Many mutations of the *TUB1* gene result in too few or too many microtubules at the restrictive temperature, and these mutants also arrest in mitosis (Schatz et al., 1988; Stearns and Botstein, 1988). Other mutants in *TUB1* are similar to *tub1-171*, however, in that little or no disruption of spindle structure is visible, and these mutants also generally show poor or no cell cycle arrest at the restrictive temperature (Schatz et al., 1988).

One possible explanation for the death of *tub1-171* in nocodazole is that nuclear division is occuring because the spindle microtubules are resistant to the drug and thus a complete mitotic spindle remains, but death ensues because astral microtubules are not drug resistant. Even with wild type tubulin, kinetochore microtubules have been reported as far more stable than other microtubules (Rieder, 1981). There are also precedents for mutants with differential stability of spindle and astral microtubules (Sullivan and Huffaker, 1992). In the absence of astral microtubules nuclear migration to the bud neck (Sullivan and Huffaker, 1992) and maintenance of spindle orientation at the bud neck (Palmer et al., 1992) both fail. Anaphase can occur, but usually takes place entirely within the mother cell, resulting in polyploid and aploid cells. These polyploid cells are viable, but if nuclear division also failed this may not be the case, as the single nucleus would contain multiple spindle poles. Mutants in BIN2 and BIN3, two genes implicated in actin and tubulin folding, form binucleate cells at low temperature by a similar process (Chen et al., 1994).

This explanation does not seem likely, however, as microtubule structures (nuclear or astral) are not visibly altered in *tub1-171*. In addition, *tub1-171* exits mitosis in an unusual manner, inactivating $p34^{cdc2/CDC28}$ kinase without destroying Clb2p. It seems likely that mutants in astral microtubule function would exit mitosis in the normal way, by destroying Clb2p.

tub1-171 clearly does exit mitosis in the presence of nocodazole, as evidenced by

its rapid death, sister chromatid separation, rebudding and rereplication. It does not, however, share all the properties of a conventional spindle assembly checkpoint mutant. Like wild type cells, *tub1-171* is able to delay passage through mitosis in response to poorly segregating, short linear mini-chromosomes. This suggests that, unlike *mad1*, *mad2*, and *mad3*, *tub1-171* can respond to a failure to attach chromosomes to the spindle (Wells and Murray, 1995). *tub1-171* is also viable in combination with a *kar3Δ*, which delays in G2/M (Meluh and Rose, 1990), possibly due to defects at the kinetochore (Middleton and Carbon, 1994). The inviability of other checkpoint mutants such as *mad2-1* with a *kar3Δ* may reflect their inability to detect this kinetochore related defect and delay the cell cycle, while again *tub1-171* and wild type cells can still delay.

The method by which *tub1-171* exits mitosis in the presence of nocodazole differs from *mad1*. In *mad1*, Clb2p is destroyed as if no drug was present, thus inactivating the $p34^{cdc2/CDC28}$/CLB2 kinase complex. In *tub1-171*, Clb2p levels remain high but $p34^{cdc2/CDC28}$ kinase is inactivated by some other means. This confirms the finding that the exit from mitosis can occur by more than one route (Minshull et al., 1995). Either means of inactivating $p34^{cdc2/CDC28}$ kinase appears to sufficient for mitotic exit; occurence of either one of the two events abrogates the need for the other.

How is $p34^{cdc2/CDC28}$ kinase activity inactivated in *tub1-171*? It is known that at least a subset of cyclin B (Alfa et al., 1990; Pines and Hunter, 1991; Jackman et al., 1995) and $p34^{cdc2/CDC28}$ (Bailly et al., 1989), as well as the majority of their associated kinase activity (Kubiak et al., 1993) is associated with the centrosome and spindle microtubules. Additionally, the activation of $p34^{cdc2/CDC28}$ kinase in *Schizosaccharomyces pombe* requires the presence of microtubules (Alfa et al., 1990). Therefore, one possibility is that the combination of nocodazole and the *tub1-171* mutation prevents any binding of $p34^{cdc2/CDC28}$ to the spindle or to smaller fragments of microtubules. Such binding may be necessary for the maintenance of the activity of $p34^{cdc2/CDC28}$ kinase, or its ability to keep the cell in mitosis. However, mutants in the TUB1 gene in budding yeast which lack

visible microtubules at the restrictive temperature invariably arrest in mitosis (Schatz et al., 1988). The specificity of the phenotype of *tub1-171* when compared to other mutants in *TUB1* in nocodazole (this study) also argues against such an explanation, and rather for one involving the disruption of a specific protein-protein interaction in *tub1-171*. In any case, in the high concentrations of nocodazole used in most of our experiments, it is unlikely that many spindle structures remain, even in wild type cells.

This raises the possibility that *tub1-171* identifies an active means by which the cell can exit mitosis when the spindle is normal, but in *tub1-171* exit occurs even when the spindle is disassembled. The ability of *tub1-171* to delay in response to short linear mini-chromosomes (and to a lesser extent its viability in combination with *kar3Δ*, since the mechanism of delay is more speculative in this case) suggests that the system defective in *tub1-171* does not respond to problems in chromosome attachment. An attractive possibility is that this sytem responds to the integrity of the spindle, perhaps detecting its bipolarity. Evidence from sea urchins implicates the necessity for a bipolar spindle for progression out of mitosis. In this system the creation of monopolar spindles results in a delay in mitosis, but this delay is prevented if a bipolar spindle is present in the same cytoplasm (Sluder and Begg, 1983; Sluder et al., 1994). This implies that in this organism the checkpoint governing chromosome attachment that has been defined in other systems (Rieder et al., 1994; Wells and Murray, 1995) is absent, but the cells still have a positive requirement for a bipolar spindle. Nocodazole is still competent to delay these cells in mitosis, presumably by destroying the bipolarity of the spindle (Sluder et al., 1994).

Other systems provide evidence for the existence of two mitotic checkpoints. A spindle assembly checkpoint can be detected in *Xenopus laevis* extracts by the addition of high concentrations of sperm nuclei and nocodazole (Minshull et al., 1994). This checkpoint responds to microtubule depolymerizing drugs, but not to the microtubule stabilizing drug, taxol (Minshull et al., 1994). In contrast, other cell types such as HeLa cells arrest in mitosis in response to both classes of drug (Jordan et al., 1992; Jordan et al.,

1993), suggesting that taxol may induce some other defect that only these latter systems can detect. It is difficult to determine which drug is causing which defect(s), as even at low concentrations of inhibitor, when spindle structure and chromosome attachment are not altered on a gross level, perturbations in kinetochore-microtubule interactions and centrosome organization can both be observed by electron microscopy (Wendell et al., 1993). The existence of two checkpoints could explain the contradictory phenotypes of *tub1-171*. In plate assays with low concentrations of benomyl *tub1-171* is slightly less sensitive than *mad1*, perhaps because a sensitive *MAD* checkpoint is still operating to some degree. In high concentrations of nocodazole, however, *tub1-171* exits mitosis and rebuds more rapidly than *mad1*, perhaps due to the failure of a second checkpoint when the spindle is completely destroyed.

A speculative model for the action of the *tub1-171* "spindle integrity" checkpoint is presented in Figure 13. An explanation of the phenotypes of various strains under various conditions using this model is presented in Table 1. In the model, a protein that binds to bipolar spindles (the 171-binding protein) is activated when it is displaced from the spindle by nocodazole. Perhaps there is a requirement for anti-parallel microtubules for binding of this protein to microtubules. Upon activation, the protein inhibits the production of an inhibitor of $p34^{cdc2/CDC28}$ kinase, thus preventing mitotic exit. The experiments of Sluder and Begg described above (Sluder and Begg, 1983), which provide evidence for the existence of a positive "exit mitosis" signal from a bipolar spindle, could be explained by the bipolar spindle sequestering the inhibitory 171-binding protein. In *tub1-171*, the 171-binding protein still binds to tubulin in the presence of nocodazole, and thus is not activated, allowing mitotic exit due to kinase inactivation.

To explain delays in response to short linear mini-chromosomes, the production of the inhibitor of $p34^{cdc2/CDC28}$ kinase must also be prevented in response to chromosome attachment defects, probably by the *MAD* checkpoint. The continued hyperphosphorylation of Mad1p in a *tub1-171* mutant in nocodazole (K. Hardwick, pers. comm.)

suggests that if the spindle assembly and spindle integrity checkpoints are linked in any way, the components associated with *tub1-171* are downstream of *MAD1*. In Figure 13, the *MAD* checkpoint is postulated to activate a protein X, which then inhibits $p34^{cdc2/CDC28}$ kinase inhibitor production. A new protein is invoked here, as in a *bub2Δ* strain, where the spindle integrity checkpoint is probably completely absent (see below), linear minichromosomes can still delay mitosis. In nocodazole, protein X no longer binds tubulin, and this mechanism is no longer operative. Thus for *tub1-171* in nocodazole, both protein X (no longer bound to tubulin) and 171-binding protein (incorrectly bound to tubulin) are inactive, and an aberrant exit from mitosis will occur.

If this model is correct, one candidate for the $p34^{cdc2/CDC28}$ kinase inhibitor would be p40/*SIC1*. This protein is an inhibitor of $p34^{cdc2/CDC28}$/CLB kinase complexes (Mendenhall, 1993) and is required to prevent the premature activation of $p34^{cdc2/CDC28}$ kinase complexed with the S phase cyclins Clb5p and Clb6p (Schwob et al., 1994). The protein and its activity are cell cycle regulated, appearing in a burst at around the M/G1 transition (Schwob et al., 1994; Donovan et al., 1994). Deletion of the *SIC1* gene causes slow growth associated with an increase in the fraction of cells in G2/M and increased frequencies of broken and lost chromosomes (Nugroho and Mendenhall, 1994; Donovan et al., 1994). These phenotypes are probably associated with the failure to regulate the $p34^{cdc2/CDC28}$/Clb5/6 complexes rather than a mitotic function of *SIC1*. A possible role for *SIC1* in this new checkpoint seems unlikely, however, as deletion of *SIC1* does not suppress the rebudding of *tub1-171* in nocodazole (not shown).

Most defects in spindle structure will necessarily result in problems in chromosome attachment. Why then would the cell need a second system to detect spindle integrity? One possibility is that this provides the cell with a rough indication of the likely fidelity of the following anaphase, whereas the chromosome attachment signal provides a more fine tuned measure. Even when a single chromosome is improperly attached to the spindle, the cell may choose to initiate anaphase if it has an indication that the spindle is largely intact, and

therefore few mistakes will occur. Evidence from other checkpoints indicates that checkpoint arrests can be "overruled" even in the presence of an irreparable mistake (Sandell and Zakian, 1993). In this case it is presumably more advantageous to undergo an error-prone division than no division at all. The method of mitotic exit in *tub1-171* meets these criteria - the inactivation of $p34^{cdc2/CDC28}$ kinase can override the attempt by the chromosome attachment checkpoint to keep the cell in mitosis by the maintenance of elevated Clb2p levels.

If *tub1-171* does represent the loss of a spindle integrity checkpoint, we can assess the source of the *MAD* checkpoint signal without inteference from a spindle integrity checkpoint. As *tub1-171* cells still maintain substantial Clb2p levels in the presence of the high concentrations of nocodazole we use in our assays, it seems likely that free kinetochores (Rieder et al., 1995) are the source of the *MAD* checkpoint signal, rather than, or as well as, monoattached chromosomes (Li and Nicklas, 1995; Nicklas et al., 1995).

Are there other proteins which may participate in the spindle integrity checkpoint? Bub2p is one possibility, as thus far its properties are identical to *tub1-171*. *bub2-1* mutants clearly progress out of mitosis in the presence of nocodazole, and $p34^{cdc2/CDC28}$ kinase activity drops, albeit slowly (Hoyt et al., 1991). The levels of Clb2p in nocodazole are not known. Like *tub1-171*, but unlike *mad1*, *bub2-1* is viable with *kar3Δ*, delays with short linear mini-chromosomes and is rescued by TUB1 and truncated SWI4 genomic clones (Chapter 2 and data not shown). Thus Bub2p is a candidate component of the spindle integrity checkpoint.

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Table 1. Rationalising the phenotypes of *mad1*, 261 and *tub1-171* in the context of a two checkpoint theory.

Clb2p levels are known, but the production (or not) of an inhibitor of $p34^{cdc2/CDC28}$/cyclin B complexes is only postulated to explain the known absence (or presence) of cell cycle delays. Either low Clb2p or high inhibitor (or both) can cause exit from mitosis/no delay in mitosis.

Explanation of phenotypes of mutants in two checkpoints

Strain	Condition	Clb2p		Is the CDC28 inhibitor inhibited?		Result
		Level	Explanation	Yes/No	Explanation	
WT	Normal exit from mitosis	Low	No unattached kinetochores	No	Bipolar spindle, 171-binding protein inactive	Exit
WT	nocodazole	High	MAD detects kinetochores	Yes	No bipolar spindle, 171-binding protein active	Delays
WT	linear CEN	High	MAD detects kinetochores	Yes	MAD activates "X"	Delays
mad1	nocodazole	Low	MAD fails	Yes	No bipolar spindle	No delay
mad1	linear CEN	Low	MAD fails	No	*mad* can't activate "X"	No delay
261	nocodazole	Low	MAD fails	Yes	No bipolar spindle	No delay
261	linear CEN	Low	MAD fails	Yes	MAD activates "X"	No delay
tub1-171	nocodazole	High	MAD detects kinetochores	No	171-binding protein not turned on by lack of bipolarity	No delay
tub1-171	linear CEN	High	MAD detects kinetochores	Yes	MAD activates "X"	Delays

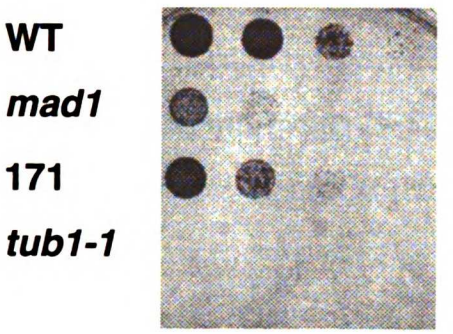
TABLE 2: Yeast Strains.

AFS34	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100*
BW128	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100, mad1Δ::HIS3*
BW182	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100, tub1-171*
BW278	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100, tub1-171, cdc28-VF*
JM82	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100, cdc55Δ::HIS3*
BW224	α	*ade2-1, ura3-52, tub1-1*
BW271	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100, tub1-801*
BW272	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100, tub1-821*
BW273	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100, tub1-830*
BW274	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100, tub1-837*
BW275	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100, tub1-849*
BW276	a	*ade2-1, ura3-1, trp1-1, leu2,3-112, his3-11, can1-100, tub1-851*
TBW136	as AFS34 but with linear pVL106	
TBW195	as AFS34 but with circular pVL106	
TBW194	as BW182 but with linear pVL106	
TBW209	as BW182 but with circular pVL106	

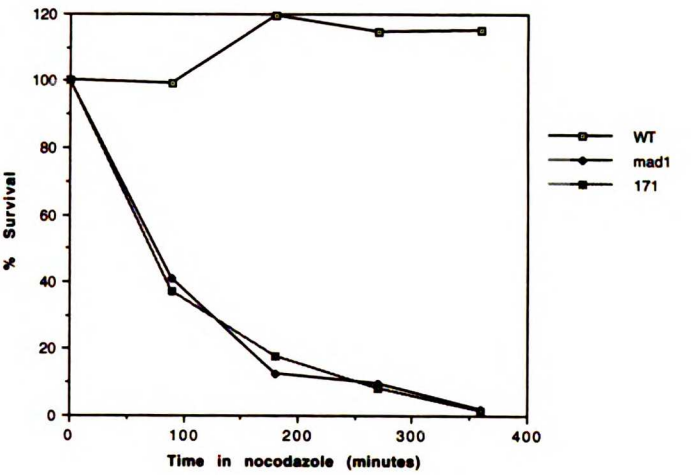
Figure 1. Rapid death of mutant 171 in nocodazole.

Mutant 171 is a benomyl sensitive mutant; slightly less benomyl sensitive on plates than *mad1*, as evidenced by spotting 4-fold dilutions of saturated cultures on a YPD plate with 7.5μg/ml benomyl (a). Exponentially growing (b) or α-factor arrested (c) cells were grown in YPD at 22°C with 15μg/ml nocodazole, and samples withdrawn at the indicated times and tested for viability by plating on YPD. Where indicated, the DNA synthesis inhibitor, hydroxyurea (HU), was added at the same time as the nocodazole at 10mg/ml. Viability can increase above 100% due to cell division.

a)


WT
mad1
171
tub1-1

b)


120
100
80
60
40
20
0
% Survival
0
100
200
300
400
Time in nocodazole (minutes)
WT
mad1
171

c)

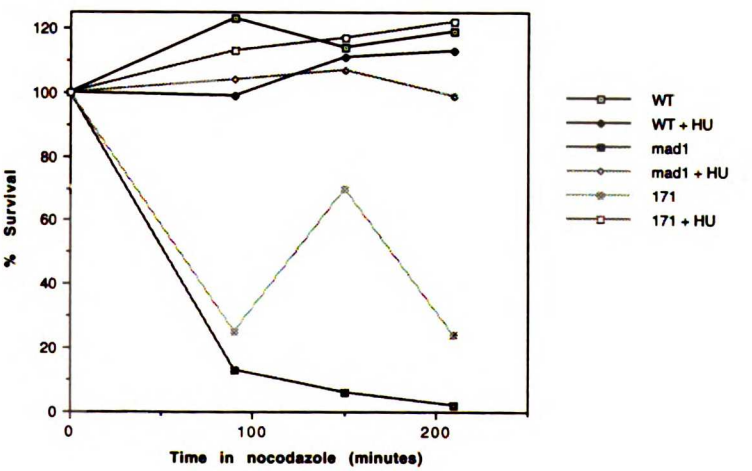
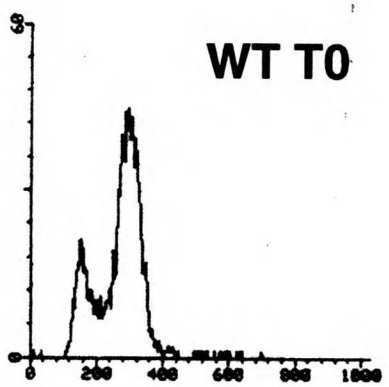
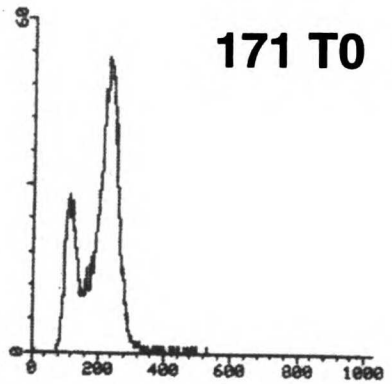
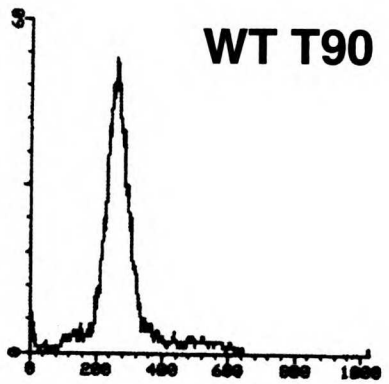

120
100
80
60
40
20
0
% Survival
0
100
200
Time in nocodazole (minutes)
WT
WT + HU
mad1
mad1 + HU
171
171 + HU

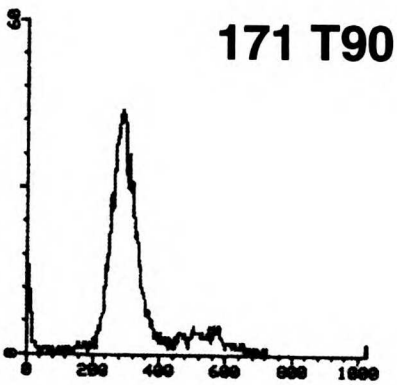
Figure 2. Rereplication of mutant 171 in nocodazole.

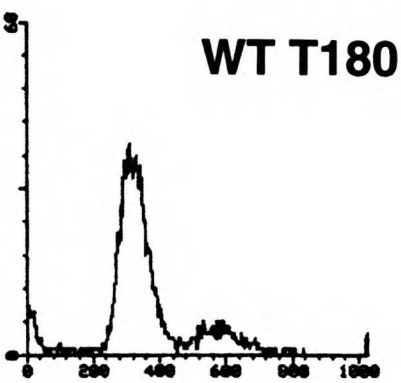
Exponentially growing cells were grown in YPD at 22°C with 15μg/ml nocodazole. Samples were withdrawn at the indicated times, fixed and processed for analysis by flow cytometry (FACS). Mutant 171 transiently arrests with mostly 2N DNA before entering the next cell cycle, as can be seen from the prominent 4N peak at 180 minutes.


WT T0


171 T0


WT T90


171 T90


WT T180

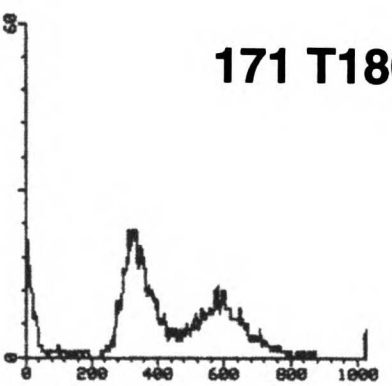
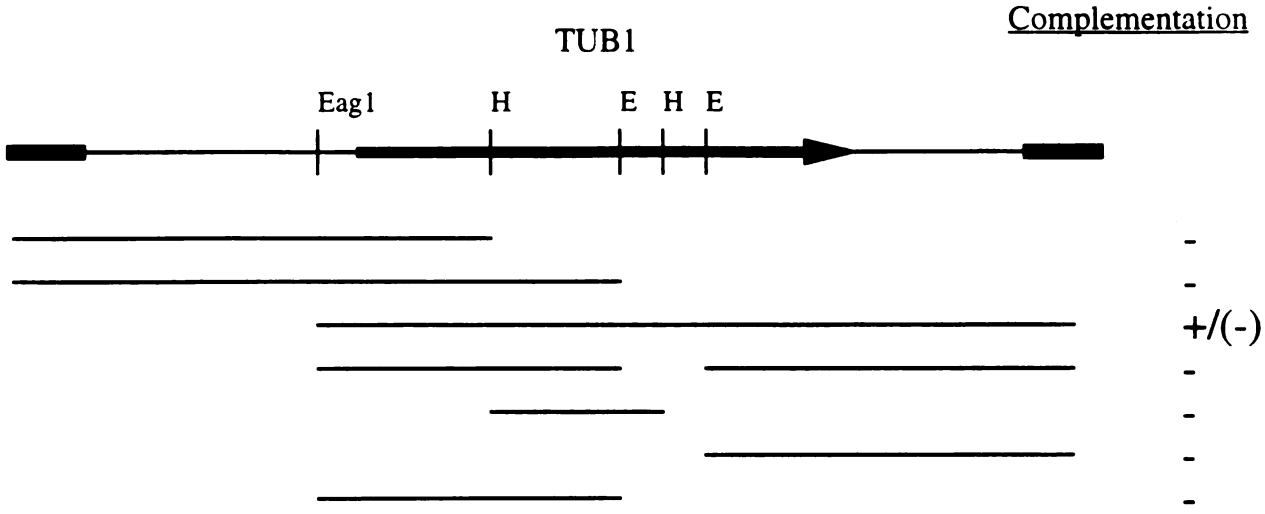

171 T180

Figure 3. Rescue of mutant 171 by *TUB1* and truncated *SWI4* clones.

The figure illustrates the rescuing activities of various subclones of two positive clones obtained by screening a YCp50 based genomic library. (a) A fragment of *TUB1* which includes the entire gene and only a short promoter fragment has nearly full rescuing activity. (b) The rescuing activity of the other clone appears to reside in the 3' region, as when a random SauIIA fragment sub-library was prepared from this clone, all rescuing clones included the full 3' region. The only open reading frame in this region is the C-terminus of *SWI4*.

a) Rescue of *tub1-171* by *TUB1*



b) Rescue of *tub1-171* by a C-terminal portion of *SWI4* but not a complete *SWI4* clone

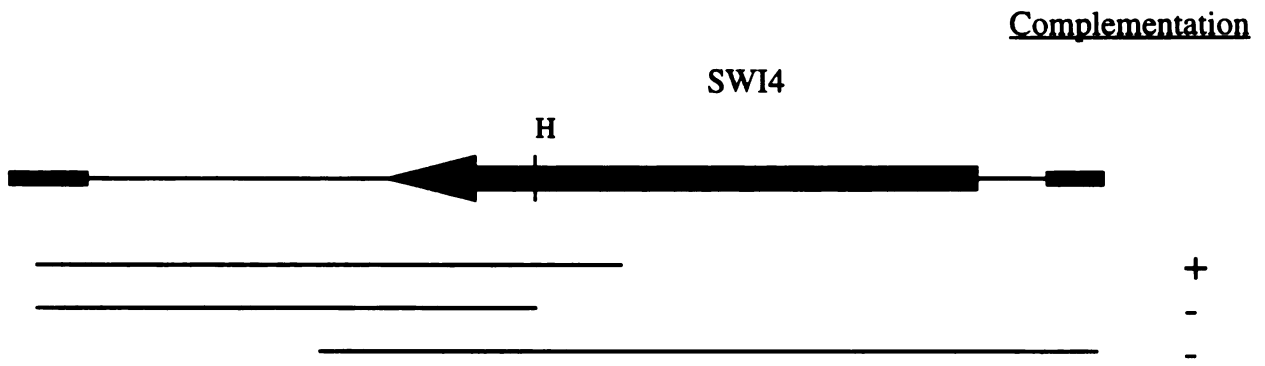


Figure 4. Rescue of the *tub1-171* allele.

The *tub1-171* allele was rescued by gap repair. When transformed into *tub1-1*, the rescued allele restores some benomyl resistance (as seen on a YPD plate with 7.5μg/ml benomyl), but *tub1-1* remains more benomyl sensitive than wild-type (represented here by *tub1-171* rescued by *TUB1*).

tub1-1 + YCp50

tub1-171 + TUB1

tub1-1 + YCptub1-171

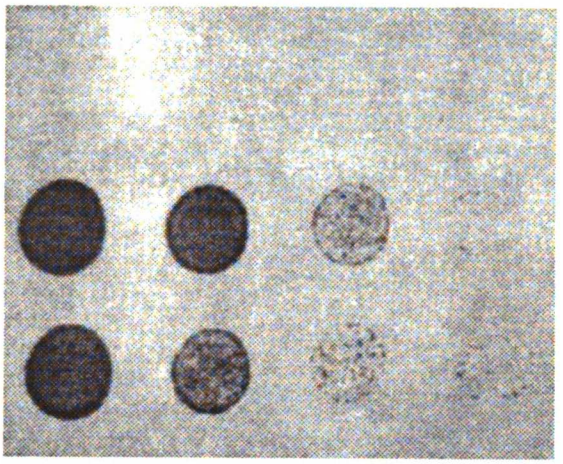
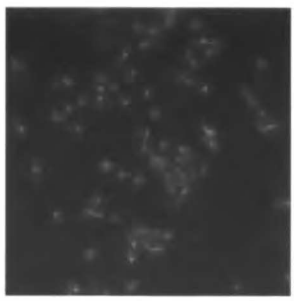
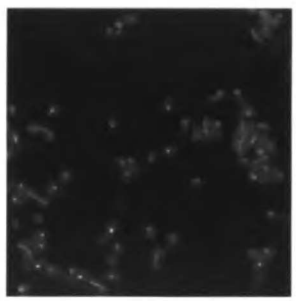


Figure 5. *tub1-171* does not lose microtubule structures in low concentrations of nocodazole.

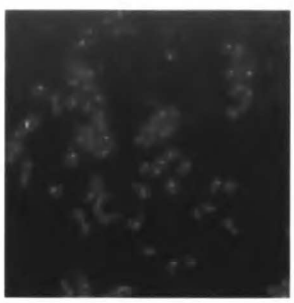
Exponentially growing cells were grown in YPD at 22°C with various concentrations of nocodazole for 30 minutes. Samples were then fixed and cells stained for anti-tubulin immunofluorescence. At 2.5μg/ml nocodazole, spindle structures appear normal in wild-type, *mad1* and*tub1-171* cells, but are absent in *tub1-1* cells (Figure panels). Spindle structures gradually decline in wild-type, *mad1* and *tub1-171* cells as the concentration of nocodazole is increased to 5, 7.5 and 10μg/ml (not shown). At 10μg/ml, almost no spindles remain in any strain.



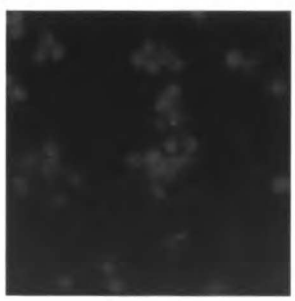
WT



mad1



tub1-171



tub1-1

Figure 6. Rapid death in nocodazole occurs for *tub1-171*, but not for other *tub1* alleles.

Exponentially growing cells were grown in YPD at 22°C with 15μg/ml nocodazole, and samples withdrawn at the indicated times and tested for viability by plating on YPD. Viability can increase above 100% due to cell division.

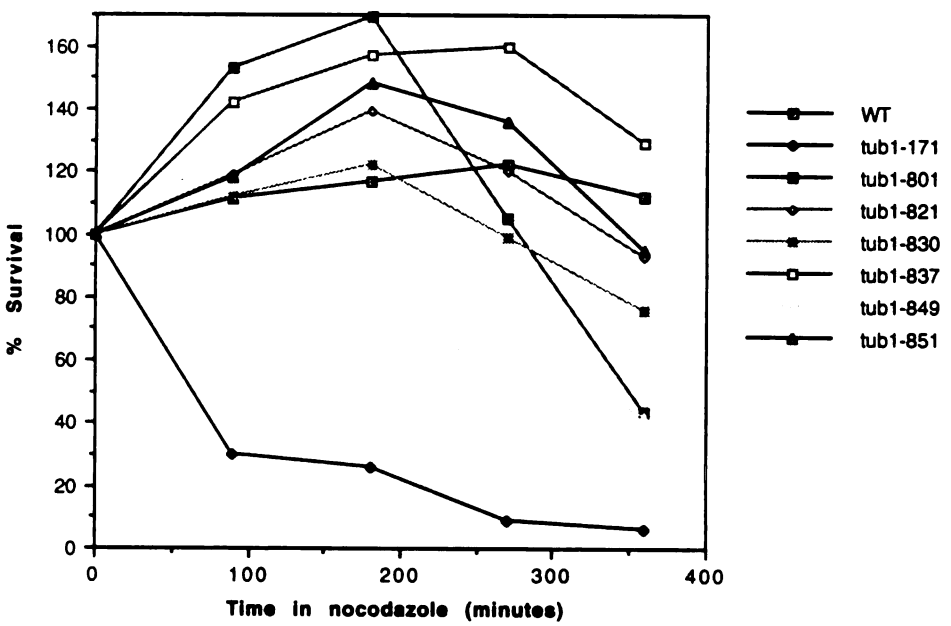
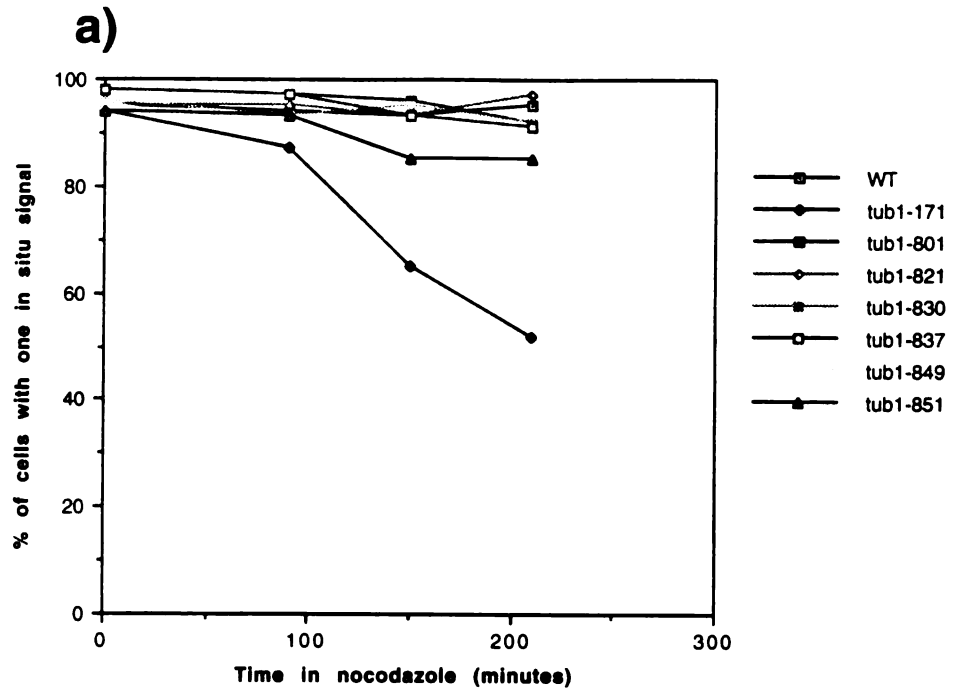

160
140
120
100
80
60
40
20
0
% Survival
0
100
200
300
400
Time in nocodazole (minutes)
WT
tub1-171
tub1-801
tub1-821
tub1-830
tub1-837
tub1-849
tub1-851

Figure 7. Sister chromatid separation in nocodazole in *tub1-171*, but not other *tub1* mutants.

Cells arrested in a-factor were released into YPD with 15μg/ml nocodazole at 22°C. Samples were withdrawn at the indicated times and fixed, spheroplasted and adhered to slides. The slides were probed with a fluorescent cosmid probe, which was visualized directly. Although proper segregation of chromosomes does not occur, sister chromatid separation can be easily visualized as the appearance of two dots in the cell where once there was one. *tub1-171* shows a decrease in the fraction of cells with one *in situ* signal, and an increase in the fraction with two signals, indicating that sister chromatid separation is occuring.


a)
100
80
60
40
20
0
% of cells with one in situ signal
0
100
200
300
Time in nocodazole (minutes)
WT
tub1-171
tub1-801
tub1-821
tub1-830
tub1-837
tub1-849
tub1-851

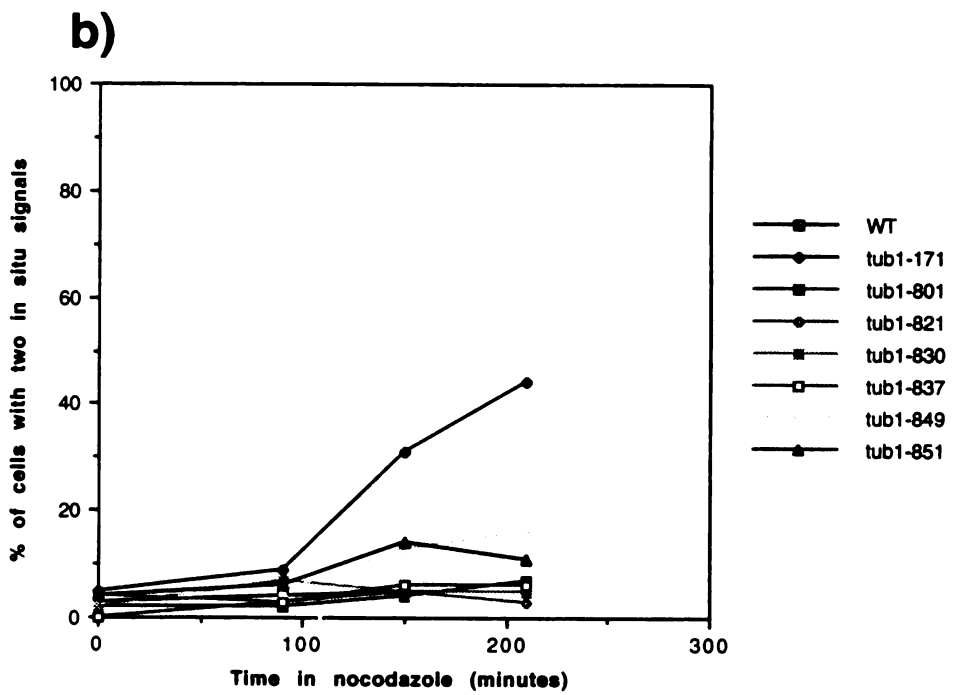

b)
100
80
60
40
20
0
% of cells with two in situ signals
0
100
200
300
Time in nocodazole (minutes)
WT
tub1-171
tub1-801
tub1-821
tub1-830
tub1-837
tub1-849
tub1-851

Figure 8. Rebudding in nocodazole in *tub1-171*, but not other *tub1* mutants.

Exponentially growing cells were grown in YPD at 22°C with 15µg/ml nocodazole, and samples withdrawn at the indicated times, fixed and the cell morphology determined. A minimum of 200 cells were counted for each strain and timepoint. At the first timepoint >95% of cells were unbudded, and at later timepoints >90% of cells were large or triply budded cells. The few unbudded or small budded cells are not represented in the figure, which shows the proportion of large budded cells which rebudded.

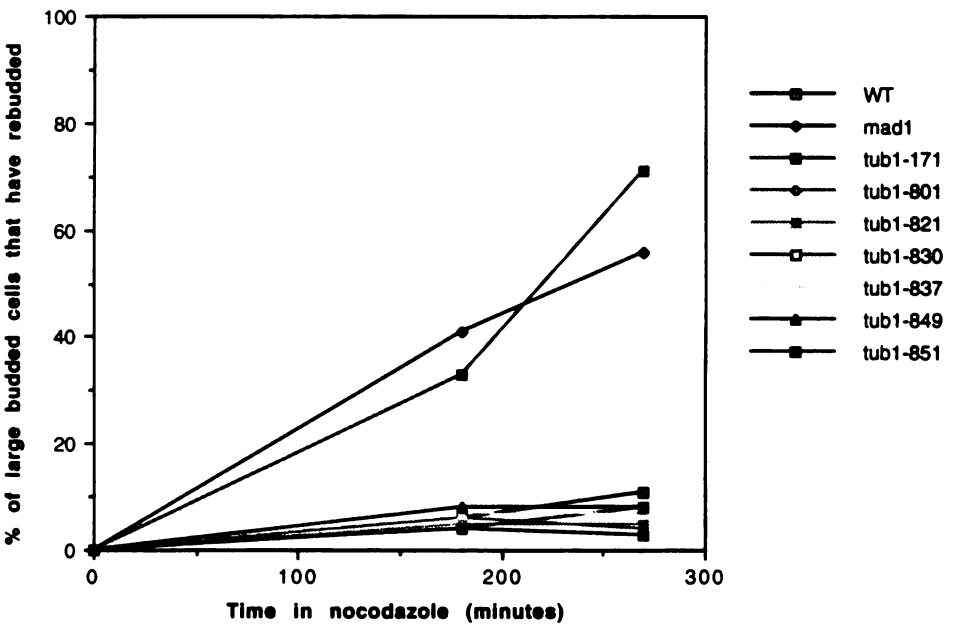

% of large budded cells that have rebudded
100
80
60
40
20
0
0
100
200
300
Time in nocodazole (minutes)
WT
mad1
tub1-171
tub1-801
tub1-821
tub1-830
tub1-837
tub1-849
tub1-851

Figure 9. Short linear mini-chromosomes delay wild-type and *tub1-171*.

Exponentially growing cultures harboring either the linear or circular derivative of the mini-chromosome pVL106 were fixed and stained for tubulin immunofluorescence. Spindle morphology was scored as either a single dot (1), a short spindle, characteristic of G2/M (2), or a long, post anaphase spindle (3). The increased proportion of cells with short spindles in wild-type and *tub1-171* cells, compared with the corresponding cultures with circular mini-chromosomes, indicates that these strains can delay in response to linear mini-chromosomes.

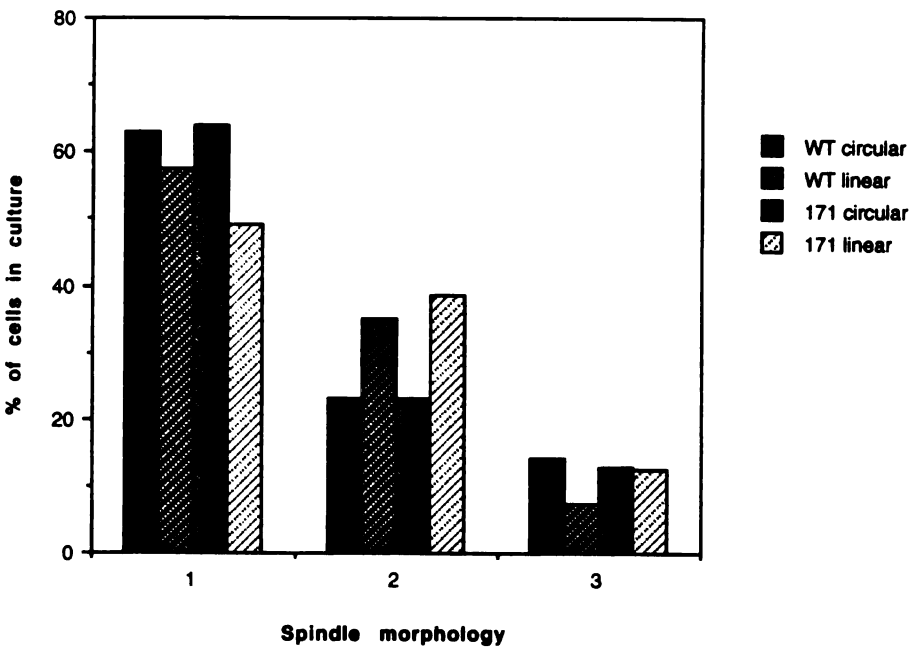

80
60
40
20
0
% of cells in culture
1
2
3
Spindle morphology
WT circular
WT linear
171 circular
171 linear

Figure 10. Clb2p levels of *tub1-171* in nocodazole.

Cells arrested in α-factor were released into YPD with 15μg/ml nocodazole at 22°C. Samples were withdrawn at the indicated times and protein extracts made. The extracts were run on a gel and probed for Clb2p. Clb2p levels rise and stay high in wild-type and rise and fall in *mad1*. In *tub1-171*, Clb2p levels rise to levels close to wild-type and decline only partially by the last timepoint.

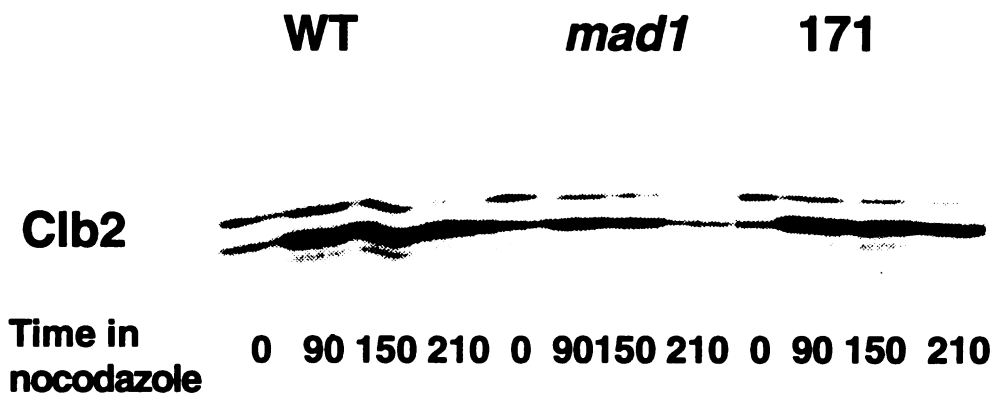

WT
mad1
171
Clb2
Time in nocodazole
0 90 150 210 0 90150 210 0 90 150 210

Figure 11. Clb2p-associated H1 kinase levels in *tub1-171* in nocodazole.

Cells arrested in α-factor were released into YPD with 15μg/ml nocodazole at 22°C. Samples were withdrawn at the indicated times and protein extracts made. The extracts were run on a gel and probed for Clb2p and immunoprecipitated with anti-Clb2p antibody before assaying for H1 kinase activity. (a) Clb2p levels rise and stay high in wild-type, *cdc55*, and mutant 155. (b) H1 kinase activity stays high in wild-type, but drops significantly in *cdc55* and *tub1-171*. The drop in H1 kinase levels in *tub1-171* is significant by 150 minutes, when Clb2p levels have not begun to fall.

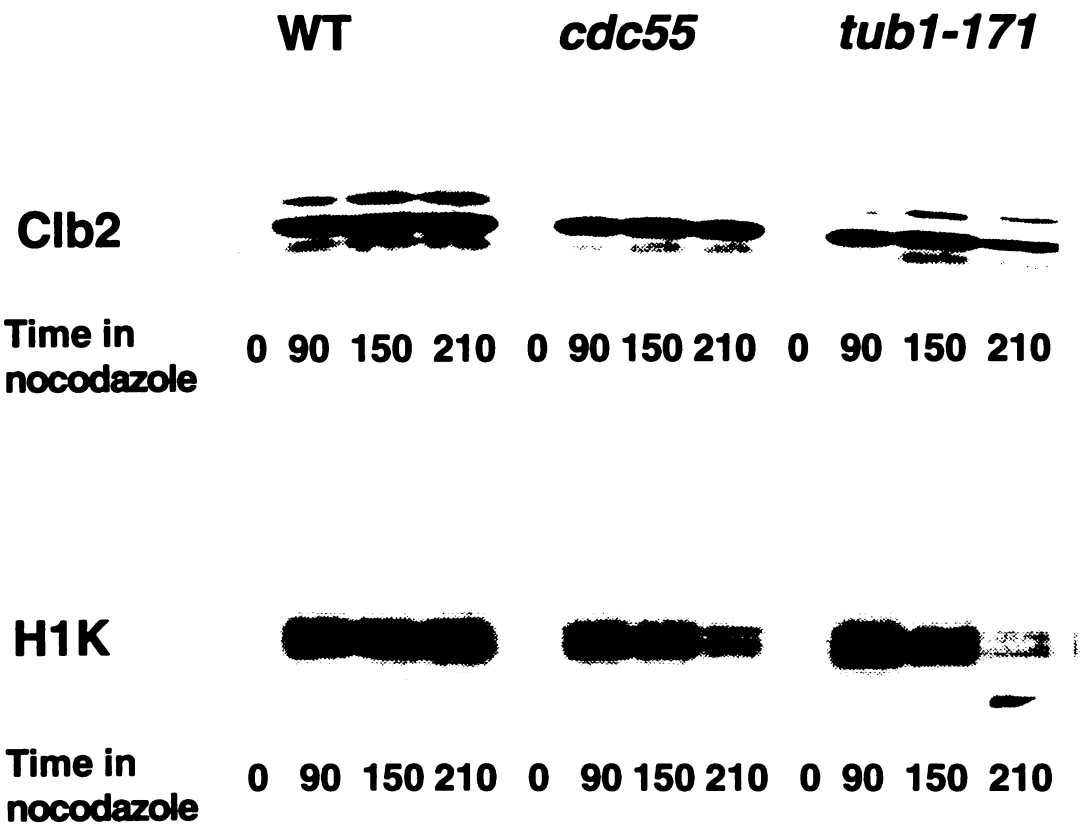

WT
cdc55
tub1-171
Clb2
Time in nocodazole
0 90 150 210 0 90 150 210 0 90 150 210
H1K
Time in nocodazole
0 90 150 210 0 90 150 210 0 90 150 210

Figure 12. The *cdc28-VF* mutant does not rescue the rebudding phenotype of *tub1-171*.

Exponentially growing cells were grown in YPD at 22°C with 15μg/ml nocodazole, and samples withdrawn at the indicated times, fixed and the cell morphology determined. A minimum of 200 cells were counted for each strain and timepoint. At the first timepoint >95% of cells were unbudded, and at later timepoints >90% of cells were large or triply budded cells. The few unbudded or small budded cells are not represented in the figure, which shows the proportion of large budded cells which rebudded. The extent of rebudding is identical for *tub1-171* alone or in combination with *cdc28-VF*, which cannot be phosphorylated on two inhibitory residues.

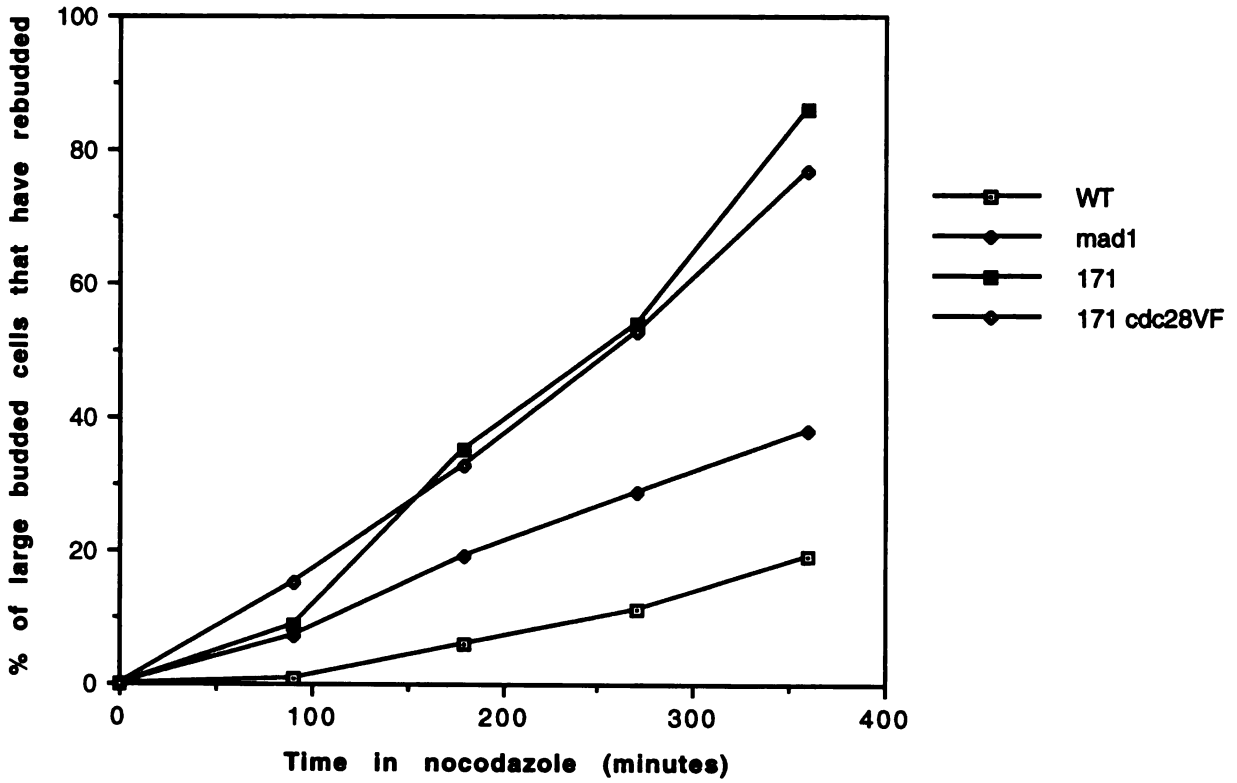
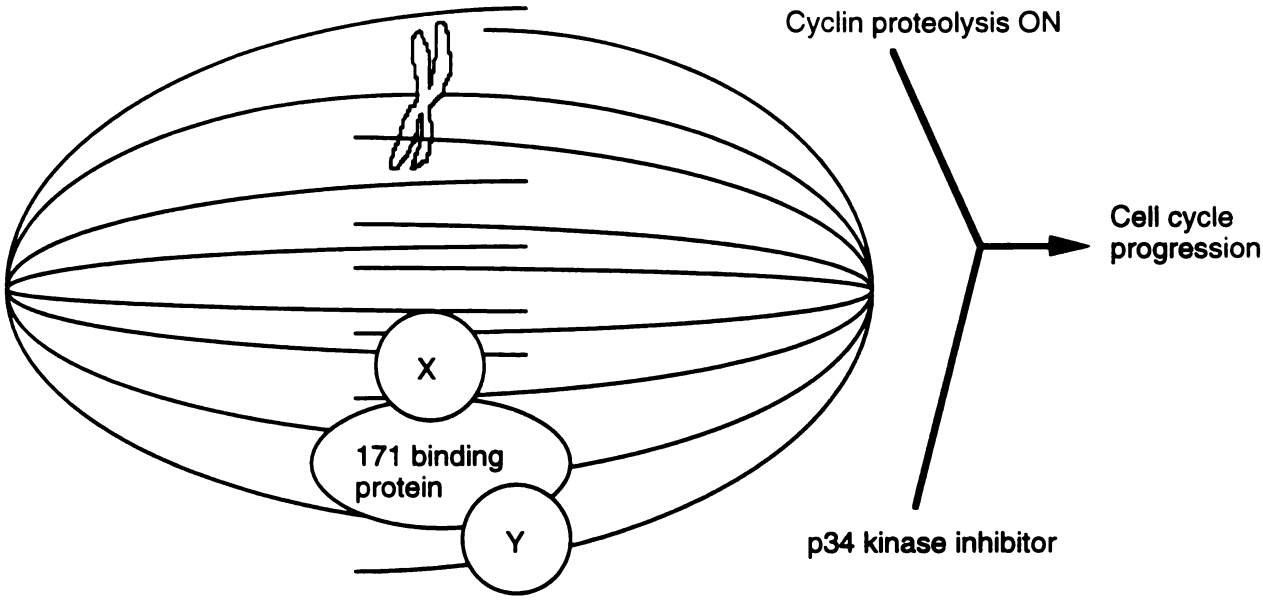

% of large budded cells that have rebudded
100
80
60
40
20
0
0
100
200
300
400
Time in nocodazole (minutes)
WT
mad1
171
171 cdc28VF

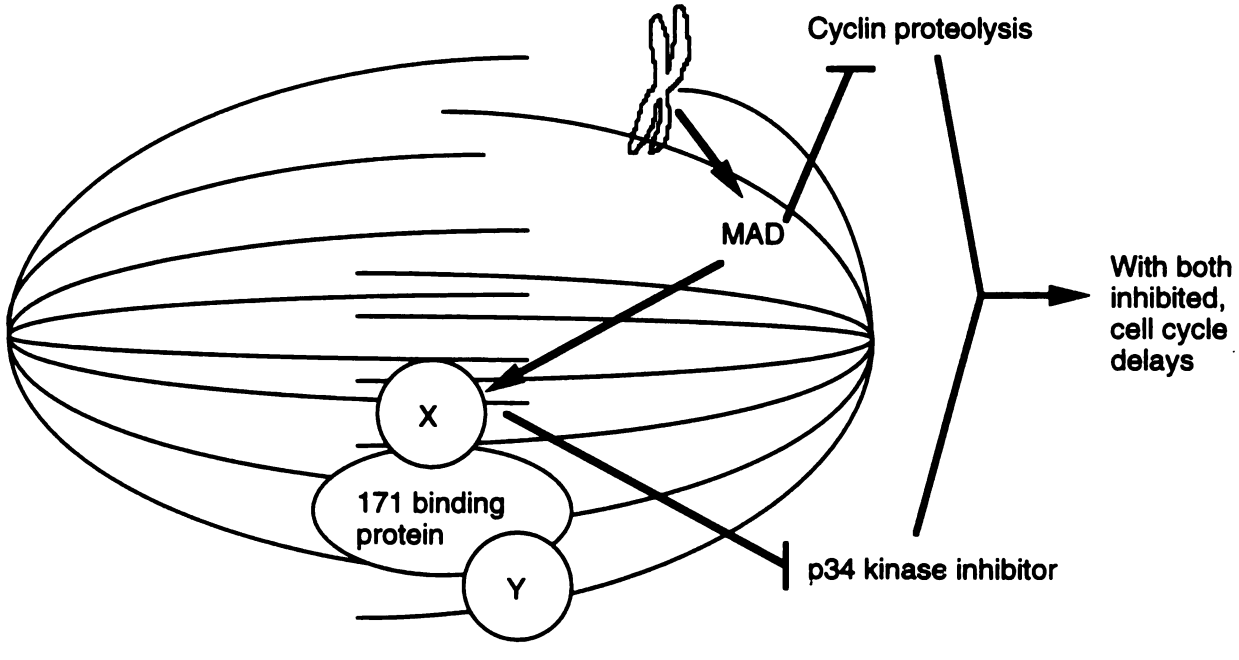
Figure 13. A speculative model for the "spindle integrity" checkpoint.

(a) In a regular cell cycle in either wild type or *tub1-171*, cyclin proteolysis and an inhibitor of $p34^{cdc2/CDC28}$/cyclin B complexes ensure exit from mitosis. The spindle integrity checkpoint protein Y (e.g. Bub2p) does not interfere with the action of the kinase inhibitor because protein Y is bound to the bipolar spindle. (b) Unattached or monoattached chromosomes activate the spindle assembly checkpoint, which prevents cyclin proteolysis and, through protein X, inhibits the kinase inhibitor. The end result is a mitotic delay. (c) Wild type cells in nocodazole also activate the spindle assembly checkpoint and prevent cyclin proteolysis. With protein X no longer bound to the spindle, however, the kinase inhibitor cannot be shut off by this means. Instead the newly released 171-binding protein–protein Y complex performs this task. The cell cycle delays. (d) The *tub1-171* mutant does not delay in mitosis in nocodazole because the 171-binding protein maintains an association with tubulin, and protein Y is therefore unable to signal to inactivate the kinase inhibitor. The cells exit mitosis prematurely due to inhibition of $p34^{cdc2/CDC28}$/cyclin B.

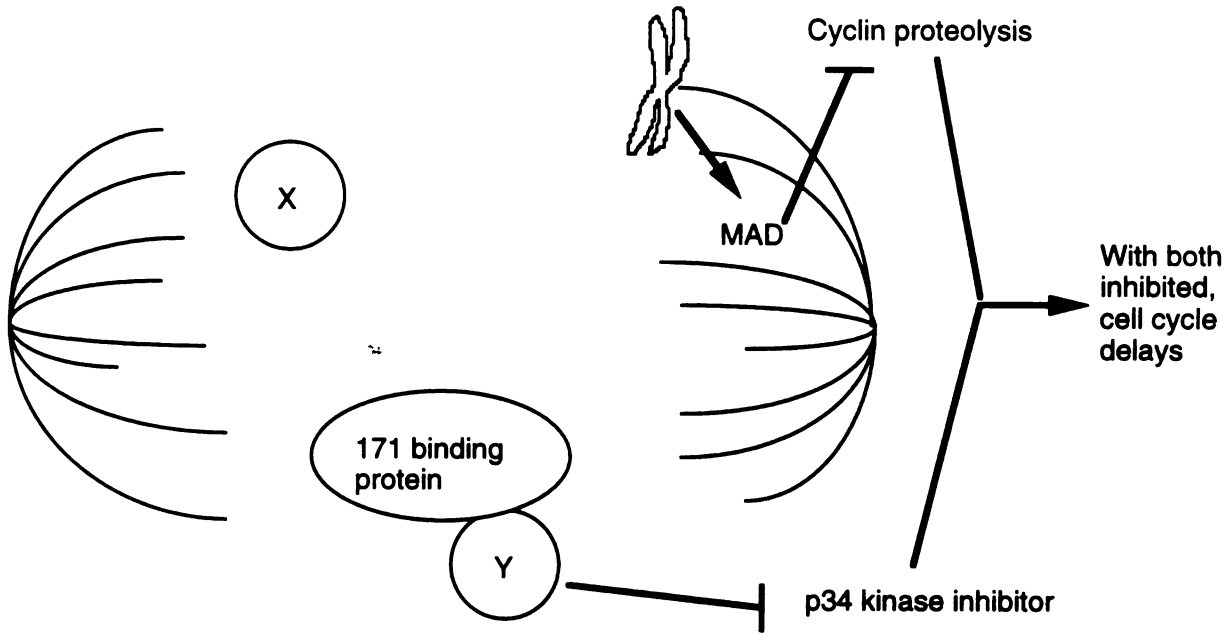
(a) Regular cell cycle:



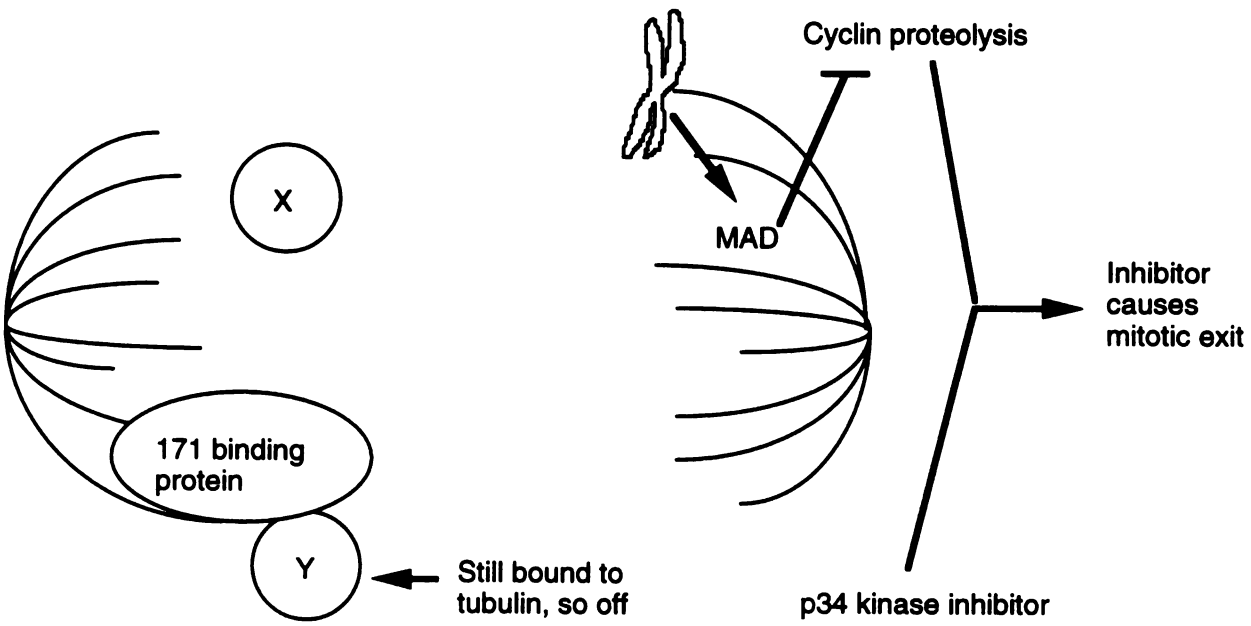
(b) With short linear mini-chromosome:



(c) Wild type in nocodazole:



(d) *tub1-171* in nocodazole:



Discussion

Discussion

One difficulty in analyzing the spindle assembly checkpoint has been the lack of specificity of the agents used to perturb mutants; microtubule depolymerizing drugs. These drugs cause disruption of kinetochore-microtubule interactions, spindle structure, and, in other systems, centrosome structure (Wendell et al., 1993). This makes it difficult to determine which defect activates the checkpoint. Using mutants in other mitotic genes in yeast is one solution, but many of these mutations also disrupt all microtubule based structures and interactions (e.g. *cin1*, *mps2*) or it is unknown or uncertain which structures are disrupted (e.g. *kar3*). A primary contribution of this work is the dissection of the signals which activate the spindle assembly checkpoint pathway.

While this work was in progress, workers in another system established that the presence of monoattached chromosomes was sufficient to activate a checkpoint delaying anaphase onset (Rieder et al., 1994). However, the demonstration in Chapter 1 that various mini-chromosomes cause *mad*-dependent mitotic delays shows clearly for the first time that this applies to *Saccharomyces cerevisiae*, and that the *MAD* checkpoint is the system responsible. The correlation of errors in segregation with rarer delays is further evidence that the *MAD* checkpoint plays a surveillance role in the absence of drugs (Li and Murray, 1991).

Our data does not reveal the underlying problems which cause mini-chromosomes to segregate with low fidelity and activate the checkpoint. The maintenance of checkpoint delays upon mutating the *SIR2* gene provides further evidence that proximity of telomeres is unlikely to be the problem causing poorer segregation for linear mini-chromosomes when compared to their circular counterparts (Murray and Szostak, 1986). Precocious separation of sister chromatids is the most likely problem for linear mini-chromosomes, and both circular and linear mini-chromosomes are likely to have trouble acheiving correct alignment on the metaphase plate due to their small size (see Chapter 1 discussion).

Without cytological information, it is not possible to tell whether the delays we see are due to mini-chromosomes attaching to only one pole or not attaching to the spindle at all. The visualization of mini-chromosomes bearing multiple copies of the *lac* operator in conjunction with a GFP-Lac repressor reporter protein may at least show if the mini-chromosomes are within the normal confines of the spindle. Direct evidence for the mechanism of generation of the checkpoint signal is more easily obtained in more cytologically tractable systems (Li and Nicklas, 1995; Nicklas et al., 1995; Rieder et al., 1995). These studies have established evidence for free kinetochores (Rieder et al., 1995) and attached kinetochores lacking tension (Li and Nicklas, 1995; Nicklas et al., 1995) as the source of the checkpoint signal. Indirect evidence from our studies supports the idea that free kinetochores can signal. Wild-type cells maintain an arrest in very high concentrations of nocodazole, and it seems unlikely that microtubule-kinetochore interactions remain in these conditions. Cell cycle progression is not prevented by some other toxic effect of the nocodazole, as *mad* mutants still cycle.

The screen for more components of the spindle assembly checkpoint, described in Chapter 2, more than doubled the number of alleles of the known checkpoint genes, and has led to the analysis of five other genes. The two described in Chapter 2 remain to be cloned; use of an alternative library may help in this endeavor, and for 261 a candidate gene homologous to an isolated frog gene may be revealed by the sequencing of *S. cerevisiae* chromosome VII. It will be interesting to determine if the 261 gene product interacts with Tub1p or Bub2p, which are implicated in the same part of the checkpoint.

The only defect identified in nocodazole arrested 155 mutant cells is rebudding. Analysing whether G1 cyclin transcription and rereplication are also occuring will allow determination of the level at which 155 mutant cells are deregulated. If many G1/S events are occuring, it is more likely that 155 represents a downstream effector of the spindle assembly checkpoint, responsible for repressing G1 events while cells are arrested in mitosis. 155 may be necessary for the cell to recognize that it is in mitosis and not G1; for

example it may play a role in the repression of G1 cyclin transcription by $p34^{cdc2/CDC28}$/CLB complexes (Amon et al., 1993). It will also be important to determine if the genes normally needed for budding and G1/S progression, such as *SWI4*, *SWI6* and the G1 cyclins, are required for the rebudding of 155.

The expression of the *MPS1* kinase under the control of the galactose promoter may help define if 155 is part of the checkpoint pathway. Overexpression of *MPS1* in wild-type cells leads to inapropriate activation of the spindle assembly checkpoint and a transient cell cycle arrest with elevated levels of Clb2p. Mutants defective in components of the checkpoint downstream from *MPS1* do not arrest. *MPS1* overexpression would likely cause elevated Clb2p levels in 155, as 155 maintains high Clb2p levels in nocodazole. If rebudding in 155 is a result of some unknown effect of nocodazole arrest, but not of mitotic arrest in general, *MPS1* overexpression will prevent the entry of 155 into the next budding cycle. The opposite result would be more difficult to interpret; rebudding by 155 with *GAL-MPS1* may signify that it lies downstream in the checkpoint pathway, or it may reflect the failure of a separate pathway leading from active $p34^{cdc2/CDC28}$/CLB complexes to the control of budding.

The work in Chapter 1 yielded an important tool which was used to analyse the checkpoint mutants isolated in the screen described in Chapter 2, notably the *tub1-171* mutant discussed in Chapter 3. The frequency of checkpoint delays observed with short linear mini-chromosomes is large enough to allow the detection of the delay by cell morphology rather than the more time-consuming pedigree analysis. This provides the basis for asserting in Chapter 3 that if proteins associated with TUB1 are part of a checkpoint that normally detects mitotic defects, chromosome attachment is not one of these defects. Such a dissection of the checkpoint signals is not possible with microtubule depolymerizing drugs.

We established that *tub1-171* fails to arrest in nocodazole, undergoing a lethal division in which it separates its chromosomes, rebuds and rereplicates. Clb2p levels

remain high, but associated kinase activity falls. None of these events occur in other *tub1* mutants analysed. Thus the combination of nocodazole and *tub1-171* leads to inactivation of $p34^{cdc2/CDC28}$ kinase, either due to the dissociation of the kinase from the spindle (unlikely: see Chapter 3) or due to an active mechanism of inhibition induced by faulty regulation of *tub1-171* or associated proteins, which fail to sense that the spindle is disassembled. The identification of the putative kinase inhibitor would help prove this latter theory; mutation of such an inhibitor should suppress the rapid death and rebudding of *tub1-171* in nocodazole. As *tub1-171* is not especially sensitive to low concentrations of benomyl, the screen would be best performed using the reverse of the *BUB* screen, in which cells are analysed after a brief pulse of a high concentration of drug. Backing for this theory may also come from further analysis of the *bub2* mutant, which appears to have the same phenotype as *tub1-171*.

As discussed in the Introduction, cyclin destruction may be dependent on an interaction with the spindle, and it can be imagined that the operation of the *MAD* checkpoint could also require this interaction. This does not, however, explain the phenotype of *tub1-171*, as the exit from mitosis in this mutant is not associated with cyclin destruction, but inactivation of $p34^{cdc2/CDC28}$ by some other means.

The *tub1-171* "spindle integrity" checkpoint may explain the behaviour of *mad* cells that we have observed in the microcolony and rebudding assays. The mutant cells do divide or rebud more rapidly than wild-type, but they do not behave exactly as though no drug was present. *mad* cells will eventually slow down due to chromosome loss and damage, but the residual slowing of the cell cycle observed in early cycles may be due to the spindle integrity checkpoint. In a normal mitosis in the absence of drugs, the synchrony and speed of the exit from mitosis may be enhanced by the putative inhibitor produced by an intact spindle. When the spindle is destroyed by nocodazole treatment, this inhibitor is no longer made in either wild-type or *mad* cells. *mad* cells still exit mitosis due to destruction of B type cyclins, but the exit is slower than in the absence of the drug.

If the spindle integrity checkpoint proves to be a reality, we now have the ability to activate and inactivate only the chromosome attachment checkpoint (with mini-chromosomes and the *mad* mutants, respectively) or to inactivate only the spindle integrity checkpoint (with *tub1-171* and possibly *bub2-1*). The selectivity of these effects will aid in dissecting the cell's ability to delay in mitosis and prevent chromosome loss and non-disjunction.

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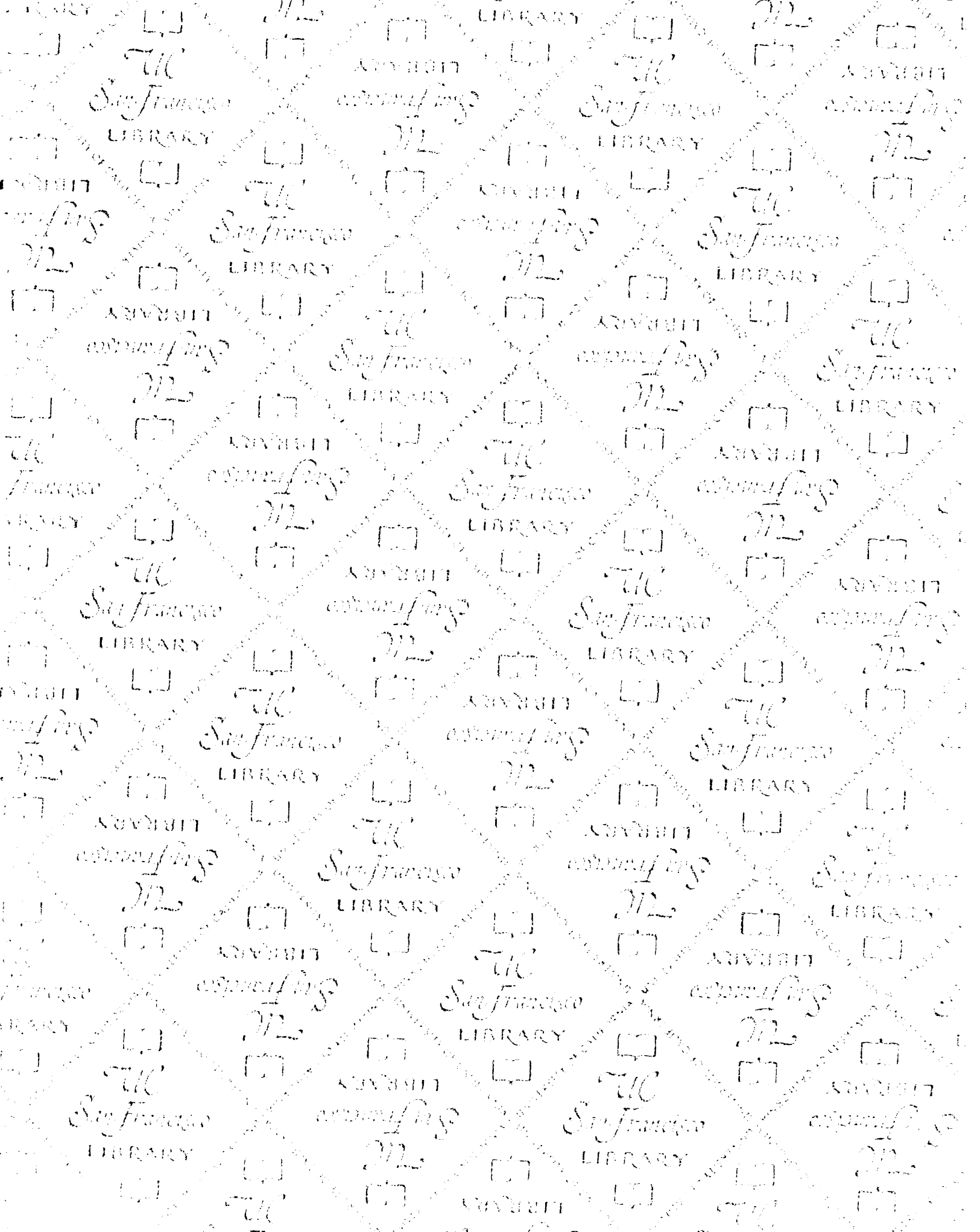
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