Studies on the interplay between the microtubule cytoskeleton and Cdc2-Cyclin B kinase at the onset of mitosis in fission yeast

Steven John Renwick

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Division of Yeast Genetics,
National Institute for Medical Research,
The Ridgeway,
Mill Hill,
London NW7 1AA.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

The aim of the work presented in this thesis was to investigate the roles of the microtubule cytoskeleton in the *S. pombe* cell cycle. I initially confirmed a previous observation by Hyams and colleagues that destruction of the microtubule cytoskeleton with the microtubule destabilising drug thiabendazole, inhibits Cdc2 kinase activation at the G2 to mitosis transition (Alfa et al., 1990). Importantly, this delay was not due to activation of the DNA damage or spindle assembly checkpoints and thus may represent a new pathway. Further investigation revealed that microtubule destruction also affected the re-localisation of the Cdc2 mitotic cyclin binding partner Cdc13 to the spindle pole bodies at the onset of mitosis, giving rise to a possible explanation for the inhibition of Cdc2 activity.

To investigate further the role of Cdc13 in the mitotic response to microtubule damage I characterised the thiabendazole sensitivity of a *cdc13-117* strain bearing a temperature sensitive mutant in Cdc13 (Booher and Beach, 1988). I found that *cdc13-117* cells were defective in the accuracy of chromosome segregation and performed a genetic screen for multicopy suppressors of the sensitivity of *cdc13-117* to thiabendazole. I identified several suppressors including a truncated allele of a gene encoding a protein with homology to the *S. cerevisiae* Dam1 microtubule binding protein that has a role in establishing chromosome bi-orientation (Cheeseman et al., 2002). Localisation studies established that Dam1 binds to the interface between mitotic spindle microtubules and kinetochores only in mitosis. To investigate the role of Dam1 in mitosis, the *dam1* gene was deleted. Cells lacking Dam1 showed frequent mitotic abnormalities and a high rate of chromosome mis-segregation. Initial studies suggest that Dam1 is a member of a 10 sub-unit complex that includes Duo1, Dad1, Spc34, and Ask1.

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List of Abbreviations

APC	Anaphase promoting Complex
BSA	Bovine serum albumin
CAK	Cdk-activating kinase
cdc	Cell division cycle
Cdk	Cyclin dependent kinase
cen1	centromere 1
CFP	Cyan fluorescent protein
CHIP	Chromatin immunoprecipitation
DAPI	4'6-Diamino-2-phenylindole
DMSO	Dimethyl sulphoxide
DNA	Deoxyribose nucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetra-acetic acid
EGTA	Ethylene glycol-bis(b-aminoethyl ether) N,N,N',N'-tetraacetic acid
EMM	Edinburgh minimal media
FHA	Forkhead associated
G2	Gap phase 2
G2-M	Gap phase 2 to mitosis
GAP	GTPase-activating protein
GFP	Green fluorescent protein
GST	Glutathione S-transferase
HA	Haemaglutanin
IPTG	Isopropyl-b-D-thiogalactopyranoside
kanR	Kanamycin resistance
Lat A	Latrunculin A
LB	Luria-Bertani
LiAc	Lithium Acetate
M	mitosis
MAPK	Mitogen-activated protein kinase

mcs	Mitotic catastrophe suppressor
MOPS	3-[N-Morpolino]propanesulphonic acid
OD	Optical density
O/N	Overnight
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PEM	Pipes EGTA magnesium sulphate
PEMBAL	Pipes EGTA magnesium sulphate BSA lysine
PEMS	Pipes EGTA magnesium sulphate sorbitol
PM	Potassium phosphate magnesium sulphate
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMSF	Phenylmethylsulphonyl fluoride
RT	Room temperature
SAK	Stress-activated kinase
SDS	Sodium dodecyl sulphate
SMC	Structural maintenance of chromosomes
SPB	Spindle pole body
TBZ	Thiabendazole
TAE	Tris acetate EDTA
TE	Tris EDTA
Tris	2-amino-2-(hydroxymathyl)propane 1-3-diol
ts	temperature sensitive
UBA	Ubiquitin associated
wt	wild type
YEP	Yeast extract plus peptone
YES	Yeast extract plus supplements

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Chapter 1

General introduction

The faithful transmission of genetic information from one generation of cells to the next is achieved by a mechanism termed the "cell cycle." The cell cycle is a tightly regulated series of events during which the cell's DNA is replicated and then segregated, so that the mother and daughter cell each receive one complete copy of the genome. Inaccuracies in these events are thought to be one of the prime causes of cancer or birth defects in humans. This has prompted our desire to develop a greater understanding of the cell cycle and the mechanisms that regulate it. Much of what we know so far has come from work carried out in the fission yeast *Schizosaccharomyces pombe* and the budding yeast *Saccharomyces cerevisiae*. Despite their evolutionary distance, the mechanisms that control the cell cycle have been conserved between humans and yeast. The ease with which yeast may be experimentally manipulated has made these cells an ideal model organism with which to study the eukaryotic cell cycle.

1.1 Fission yeast as a model organism

There are several features of the fission yeast *Schizosaccharomyces pombe* that make it an advantageous model organism. The speed of growth is one such advantage. The generation time of fission yeast varies between 2.5 and 4 hours depending on the incubation temperature and consequently, it is relatively easy to prepare large cultures of cells for protein preparation and biochemical study. In contrast to mammalian cells, fission yeast are easily genetically manipulated and the deletion of genes or their tagging with sequences encoding epitopes, such as green fluorescent protein, facilitates investigation of the roles of specific proteins. Fission yeast can grow as either haploid or diploid meaning that one copy of a gene, even if it is essential, can be deleted from diploid cells and the terminal phenotype observed by inducing sporulation and examining the germinating haploid cells. However, fission yeast grows best not as diploid but as haploid cells, hence screens to isolate genes by complementation of gene deletions or mutations are relatively straightforward to perform. Furthermore, due to the ease with which different strains containing gene deletions or mutations can be crossed

to each other, genetic interactions can be readily observed and the resulting progeny dissected. A further facility is that the entire genome sequence of the fission yeast has recently been published (Wood et al., 2002) and is accessible online (Hertz-Fowler et al., 2004).

1.1.1 The use of fission yeast in cell cycle studies

Fission yeast is of particular use for studies of the cell cycle. The high degree of evolutionary conservation within eukaryotes means that many of the genes encoding regulators of the cell cycle in fission yeast have homologues in humans. One example of this is the cyclin dependent kinase, Cdc2, which was first identified in fission yeast as a positive regulator of the cell cycle (Nurse and Thuriaux, 1980) and was subsequently found to have a homologue in humans that complements a fission yeast *cdc2* mutant (Lee and Nurse, 1987). This degree of conservation makes fission yeast a powerful tool with which to study molecular mechanisms that can then be applied to mammalian research.

S. pombe are rod shaped cells and, like mammalian cells, divide by medial fission. Their progression through the cell cycle can in part be determined by cell length, which is of particular use for synchronising cell cultures by elutriation. A wealth of *cdc* (cell division cycle) mutants facilitates the study of the cell cycle and also allows synchronisation by reversible cell cycle arrest of several of these mutants. Additionally, the cell's DNA and division septum can easily be stained with luminescent dyes, making nuclear division and cytokinesis easy to visualise by examining fixed cells at timepoints throughout the cell cycle.

1.2 The cell cycle

The eukaryotic cell cycle can be divided into 4 sequential stages: G1, S-phase, G2 and M-phase (Figure 1.1). G1 (gap phase 1) is the period between M-phase and S-phase during which cells are metabolically active and grow in size until they enter S-phase, where DNA replication takes place. Following S-phase, cells enter G2 (gap phase 2) in which the cells continue to grow until they are the requisite size to enter M-phase (Mitosis). During mitosis, the DNA replicated in S-phase is segregated by the mitotic spindle to either end of the cell so that following cytokinesis both mother and daughter cells each have one complete copy of the genome and can enter the subsequent G1 phase to continue the cell cycle. Fission yeast are unusual in that G1 and S-phase

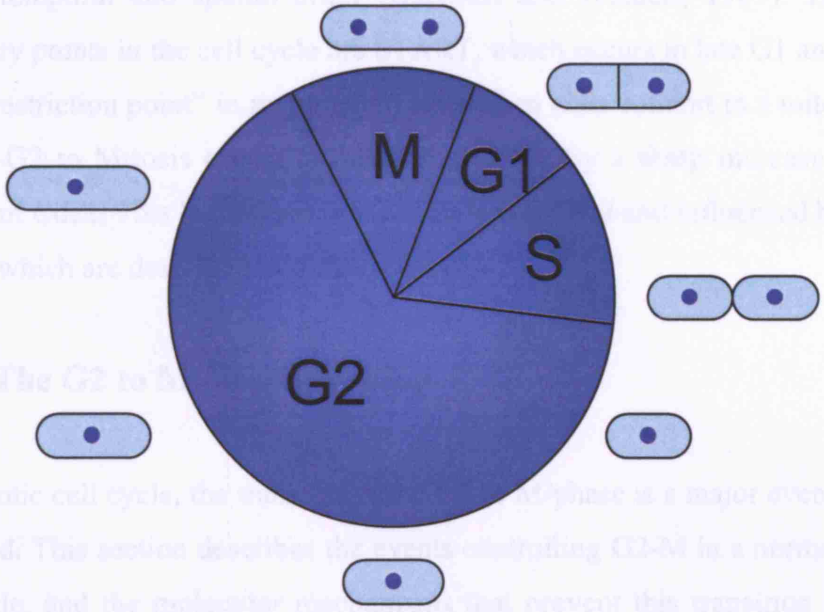


Figure 1.1 Model of the fission yeast cell cycle

Schematic diagram showing growth of the fission yeast during the cell cycle. Fission yeast grow by apical extension, meaning that cells of an equal size are at the same point within the cell cycle. Abbreviations: M, mitosis; S, DNA synthesis; G1 and G2, gap phases.

actually occur after the division site has been established but before cytokinesis occurs. This means that for most of the cell cycle, haploid cells actually have a 2C DNA content and prior to cytokinesis, have a 4C DNA content.

Progression through the cell cycle in eukaryotes is monitored by several checkpoints that ensure the fidelity of these cell cycle stages so that they occur in the correct temporal and spatial order (Hartwell and Weinert, 1989). The two major regulatory points in the cell cycle are START, which occurs in late G1 and is analogous to the "restriction point" in mammalian cells when cells commit to a mitotic cell cycle, and the G2 to Mitosis transition, which is driven by a sharp increase in the kinase activity of Cdc2. This latter transition is tightly regulated and influenced by a number of factors, which are described in the next section.

1.3 The G2 to Mitosis transition

In a mitotic cell cycle, the transition from G2 to M-phase is a major event and is tightly regulated. This section describes the events controlling G2-M in a normal fission yeast cell cycle, and the molecular mechanisms that prevent this transition in response to various signals.

1.3.1 Cdc2 kinase

Cdc2, the major fission yeast cyclin dependent kinase (Cdk), is the master regulator of the cell cycle and a sharp increase in its activity is the key event required to drive cells from G2 into mitosis. The activity of Cdc2 oscillates throughout the cell cycle due to a combination of its phosphorylation state and its periodic association with particular cyclins at specific cell cycle stages (Figure 1.2; (Simanis and Nurse, 1986; Moreno et al., 1989)). A prerequisite for *S. pombe* Cdc2 activity is the phosphorylation of Thr-167, located within the T-loop of the kinase, by the Mcs6 and Csk1 Cdk activating kinases (Gould et al., 1991; Lee et al., 1999). Comparison with the structure of human Cdk2 has suggested that Tyr-167 phosphorylation alters the substrate binding domain as well as promoting association with the cyclin (Jeffrey et al., 1995; Russo et al., 1996). Despite being essential for Cdc2 activation in *S. pombe* there is no evidence that phosphorylation of this residue is regulated by the cell cycle. However, in spite of this activating phosphorylation, the kinase is held in an inactive state during G2 due to phosphorylation of Tyr15 (Gould and Nurse, 1989), located within the ATP binding domain, by the kinases Wee 1 and, to a lesser extent, Mik1 (Lundgren et al., 1991). The

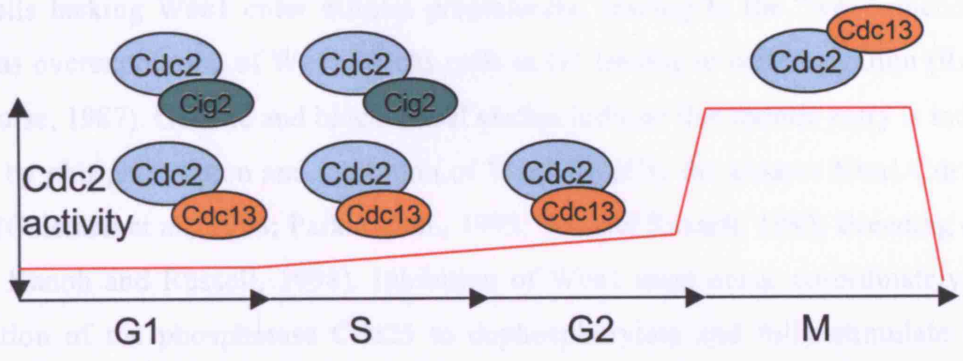


Figure 1.2 Cdc2 activity during the cell cycle

A schematic diagram showing Cdc2 kinase activity and association with Cig2 and Cdc13 cyclins during the cell cycle.

mechanism by which Tyr-15 phosphorylation prevents Cdc2 activation is unknown but could be involved in controlling the ability of the ATP-binding domain to bind nucleotide. However, it is clear that dephosphorylation of this residue by the phosphatase Cdc25 is the key event that triggers full activation of Cdc2 and entry into mitosis.

1.3.2 Wee1 and Cdc25

Wee1 is the main kinase that phosphorylates Cdc2 at Tyr15 to prevent its activity in G2 and cells lacking Wee1 enter mitosis prematurely, leading to the "wee" phenotype, whereas overexpression of Wee1 blocks cells in G2 leading to cell elongation (Russell and Nurse, 1987). Genetic and biochemical studies indicate that mitotic entry is induced partly by phosphorylation and inhibition of Wee1 itself by the kinases Nim1/Cdr1 and Cdr2 (Coleman et al., 1993; Parker et al., 1993; Wu and Russell, 1993; Breeding et al., 1998; Kanoh and Russell, 1998). Inhibition of Wee1 must occur co-ordinately with activation of the phosphatase Cdc25 to dephosphorylate and fully stimulate Cdc2 activity (Figure 1.3). Cdc25 is also regulated by phosphorylation and is subject to a variety of controls (Furnari et al., 1997; Zeng et al., 1998; Lopez-Aviles et al., 2005). Throughout G2, levels of Cdc25 accumulate until cells reach a critical size whereupon Cdc25 is activated to dephosphorylate and activate Cdc2 (Moreno et al., 1990; Creanor and Mitchison, 1996). The accumulation of Cdc25 may be under the translational control of an elongation factor, Tif1, and cells with mutations in Tif1 delay entry to mitosis (Daga and Jimenez, 1999). Interestingly, the mitotic cyclin Cdc13, whose levels similarly accumulate during G2, is also sensitive to translational activity consistent with the idea of a common mechanism regulating two key mitotic proteins (Daga and Jimenez, 1999). The steady accumulation of Cdc13 during G2 is also crucial to the Cdc2 kinase activity at mitosis.

1.3.3 Cdc13

The major and only essential mitotic cyclin in fission yeast is Cdc13, which is a homologue of human Cyclin B (Booher and Beach, 1988; Hagan et al., 1988). Protein levels of Cdc13 increase during G2, peak in metaphase and are sharply reduced in anaphase (Creanor and Mitchison, 1996). This reduction in protein levels is a major mechanism of inhibiting Cdc2 activity at the required time and is thought to be due to ubiquitin directed proteolysis. Consistent with this, Cdc13 contains an N-terminal "destruction box" motif common to other cyclin Bs and removal of this motif stabilises

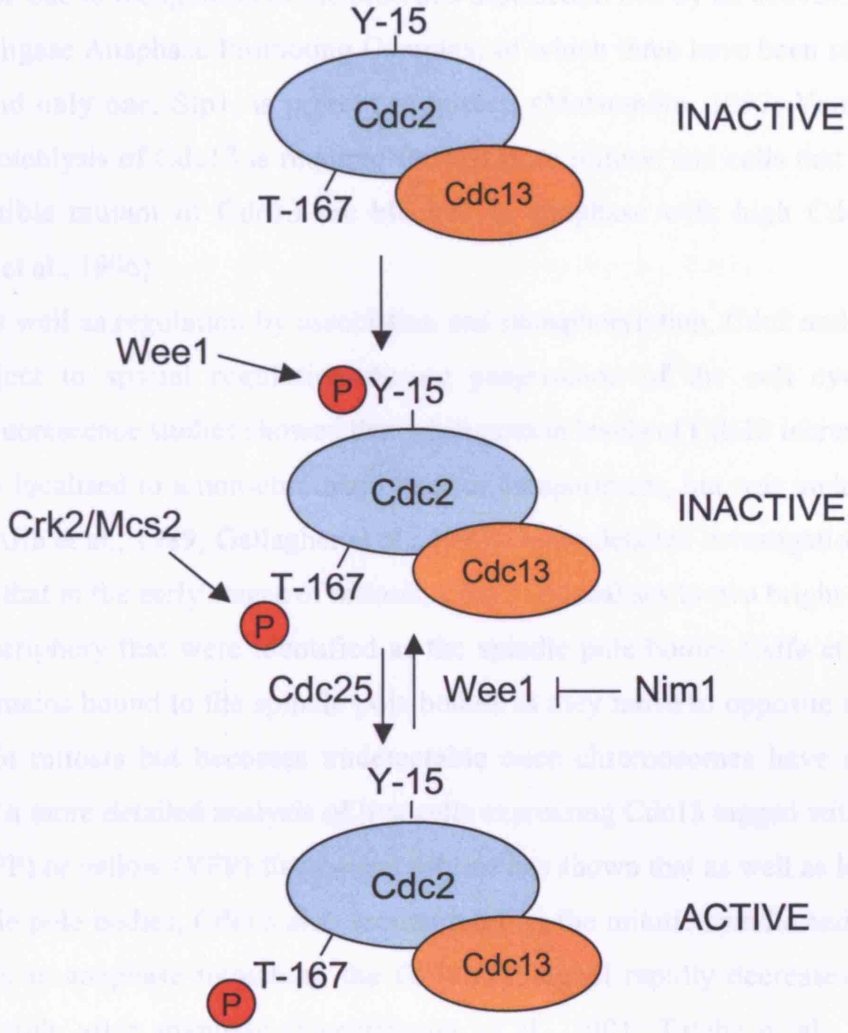


Figure 1.3 Regulation of the fission yeast Cdc2 protein kinase at the G2-M transition

Entry into mitosis requires activation of the Cdc2-Cdc13 complex. The Cdc2 protein kinase is maintained in an inactive state by the inhibitory phosphorylation on Tyr-15 via the Wee1 (and Mik1) kinase. The complex is primed for activation by phosphorylation of Thr-167 on Cdc2 by the cdk-activating kinase (CAK) composed of Crk2 and Mcs2, and by Cdc13 (Cyclin B) binding. Activation of Cdc2-Cdc13 kinase complex and the onset of mitosis require dephosphorylation of Tyr-15 on Cdc2, which is catalysed by Cdc25 phosphatase. Nim1 acts as a positive regulator of mitotic entry by inhibiting Wee1.

the protein during *in vitro* ubiquitination assays (Yamano et al., 1996). Ubiquitination is likely to be due to recognition of the protein's destruction box by an activator of the E3 ubiquitin ligase Anaphase Promoting Complex, of which three have been studied in *S. pombe* and only one, Slp1, is present in mitosis (Matsumoto, 1997; Yamada et al., 2000). Proteolysis of Cdc13 is required for exit from mitosis and cells that express an indestructible mutant of Cdc13 are blocked in anaphase with high Cdc2 activity (Yamano et al., 1996).

As well as regulation by association and phosphorylation, Cdc2 and Cdc13 are also subject to spatial regulation during progression of the cell cycle. Initial immunofluorescence studies showed that when protein levels of Cdc13 increased during G2 it was localised to a non-chromatin nuclear compartment, but was undetectable in mitosis (Alfa et al., 1989; Gallagher et al., 1993). More detailed investigation revealed, however, that in the early stages of mitosis, Cdc13 re-localises to two bright dots on the nuclear periphery that were identified as the spindle pole bodies (Alfa et al., 1990). Cdc13 remains bound to the spindle pole bodies as they move to opposite sides of the nucleus in mitosis but becomes undetectable once chromosomes have segregated. Recently, a more detailed analysis of live cells expressing Cdc13 tagged with either the green (GFP) or yellow (YFP) fluorescent protein has shown that as well as localising to the spindle pole bodies, Cdc13 also accumulates on the mitotic spindle and that at the metaphase to anaphase transition, the GFP/YFP signal rapidly decreases and is no longer visible after anaphase (Decottignies et al., 2001; Tatebe et al., 2001). An identical localisation pattern to this is seen using anti-Cdc2 antibodies (Alfa et al., 1990), although Cdc2-YFP is also found localised in both the cytoplasm and the nucleus in G2 cells (Decottignies et al., 2001).

1.3.4 Auto-amplification at the spindle pole bodies

Whilst the activation of Cdc2 is clearly required to drive cells into mitosis, it is unclear exactly how that signal is amplified so rapidly at G2-M. Studies in a number of organisms suggest that an auto-amplification loop involving the conserved polo-like kinase (Plk) family acts to rapidly activate Cdc25. Indeed, *Xenopus* Plk phosphorylates and activates Cdc25 *in vitro* (Kumagai and Dunphy, 1996) and depletion of Plk from egg extracts inhibits Cdc25 phosphorylation and prevents the timely activation of Cdc2 (Abrieu et al., 1998; Qian et al., 1998). As well as activating Cdc25, Plk may drive Cdc2 activation by inhibiting Wee1, since the budding yeast polo kinase Cdc5 binds to and phosphorylates the Wee1 kinase homologue Swe1 (Bartholomew et al., 2001).

Several lines of evidence suggest that entry into mitosis is controlled from the spindle pole bodies (SPBs) of fission yeast, which are homologous to the centrosomes of higher eukaryotes.

The *S. pombe* Polo kinase (Plo1) is also important for the entry to mitosis in this organism. Cells lacking Plo1 are unable to form a bipolar spindle whereas over-expression of the protein arrests cells with bipolar spindles and drives septation, in the absence of nuclear division, revealing a role in multiple stages of mitosis from G2-M to driving cytokinesis (Ohkura et al., 1995). Interestingly, Plo1 has a similar localisation pattern to Cdc2-Cdc13, associating only to the mitotic SPBs and disappearing in late anaphase B following spindle breakdown (Mulvihill et al., 1999), suggesting that mitotic commitment may be controlled from the SPBs. However, the SPB may be more than just a passive docking site for mitotic kinases and their regulators. A gain-of-function mutant in the SPB protein, Cut12, is able to promote premature association of Plo1 to the interphase SPBs and suppress the mitotic commitment defect of *cdc25-22* cells (Bridge et al., 1998; Mulvihill et al., 1999). Furthermore, association of Plo1 to the SPB may be regulated by the NIMA-related kinase Fin1, which is also found at the SPBs in late G2 and mitosis. NIMA kinases were first identified in the multicellular filamentous fungus *Aspergillus nidulans* in a screen for *nim* (never in mitosis) mutants (Morris, 1975), one of which, *nimA,* arrested cells in late G2 with duplicated SPBs and was found to encode a kinase designated NIMA (Oakley and Morris, 1983; Osmani et al., 1987; Osmani et al., 1988). Similar to the *cut12* gain of function mutant, over-expression of Fin1 drives the premature association of Plo1 to the SPBs and bypasses the requirement for Cdc25 (Grallert and Hagan, 2002).

These studies in fission yeast demonstrate that the SPB has a far greater role at G2-M than merely nucleating microtubules to form the mitotic spindle, serving to localise key mitotic proteins and potentially regulate some of their activity. The control of mitotic commitment from the SPB/centrosome appears to be a conserved feature. Indeed, the addition of *Xenopus* centrosomes to anucleate eggs has been found to trigger Cdc2 activation (Perez-Mongiovi et al., 2000) whilst in mammalian cells, active Cdk1-cyclin B has been shown to localise first to centrosomes (Jackman et al., 2003).

1.3.5 Checkpoints acting on Cdc2

Successful chromosome segregation is essential for the transmission of genetic material from one generation to the next and must only occur when the genome is undamaged and the cell is unstressed. To ensure this, fission yeast have checkpoint pathways to

prevent the activation of Cdc2 and the subsequent G2-M transition when cells are subject to genotoxic damage or environmental stresses. These checkpoint mechanisms target the regulators of Cdc2 activity and are conserved in higher eukaryotes, justifying the use of *S. pombe* as a model organism to study the G2-M transition.

1.3.5.1 DNA damage checkpoint

A particular checkpoint pathway is activated when the genome is either damaged or not completely replicated. Exactly how fission yeast cells sense DNA damage is unknown but seems to depend on Rad3 forming a complex with and phosphorylating Rad26 (Edwards et al., 1999). This step can occur independently of all other checkpoint proteins, suggesting that it may sense DNA damage directly and be upstream of the DNA damage response (Edwards et al., 1999). This checkpoint, by preventing activation of Cdc2, ultimately ensures that cells with unreplicated DNA or DNA damaged by ultraviolet light, gamma radiation or a DNA alkylating agent, do not enter mitosis. This checkpoint is largely conserved in eukaryotes and central to it are the PI3-like protein kinases. In fission yeast, the PI3-like protein kinase Rad3 is essential for this response and is required for phosphorylation and activation of the downstream effector kinase Chk1 (for the DNA-damage response) or Cds1(Chk2) (for the replication checkpoint) (Murakami and Okayama, 1995; Walworth and Bernards, 1996; Lindsay et al., 1998). Activation of Chk1 also depends on the protein Crb2, which binds to and regulates Chk1 in a complex with Cut5 (Saka et al., 1997; Esashi and Yanagida, 1999; Wilson et al., 1999). Chk1 and Cds1 do not inhibit Cdc2 directly but instead control the activity of the Cdc2 regulator Cdc25. Cdc25 is phosphorylated by Chk1 and Cds1 on at least nine sites in the N-terminus, which inhibits its ability to dephosphorylate and activate Cdc2 (Furnari et al., 1997; Zeng et al., 1998). Phosphorylation of Cdc25 also removes it from its substrate by promoting association with the 14-3-3 protein Rad24 leading to its nuclear exclusion (Zeng et al., 1998; Lopez-Girona et al., 1999).

1.3.5.2 Environmental stress

In mammalian cells, the G2-M transition may be inhibited by a variety of environmental stresses including osmotic stress, microtubule disassembly and inhibition of histone deacetylases. This checkpoint is distinct from the DNA damage checkpoint and requires activation of the p38 MAP kinase pathway (Wang et al., 2000; Bulavin et al., 2001; Matsusaka and Pines, 2004; Mikhailov et al., 2004). It was initially suggested

that, Cdc25 is phosphorylated by p38 to inhibit it and promote its association to 14-3-3 proteins (Bulavin et al., 2001). However, recent work has shown that the favoured phosphorylation site of p38 does not match the regulatory phosphorylation sites found on Cdc25 (Manke et al., 2005). In fact it was determined that p38 actually phosphorylates and activates an intermediate kinase, MAPKAP (mitogen-activated protein kinase-activated protein) Kinase-2, which then phosphorylates Cdc25 leading to its association to 14-3-3 proteins and subsequent inhibition (Manke et al., 2005).

In fission yeast, the p38 MAP kinase Sty1 has been found to link the cell cycle to environmental stress (Millar et al., 1995) and is known to bind to and phosphorylate the yeast MAPKAP kinase Srk1 (Sty1-regulated kinase 1) (Smith et al., 2002). Recently, López-Aviés and colleagues found that the yeast Srk1 acts in a similar fashion to its mammalian counterpart to phosphorylate Cdc25 following environmental stress leading to its association to 14-3-3 proteins and nuclear expulsion and inhibition (Lopez-Aviles et al., 2005).

1.4 Mitosis

Once G2 cells have reached the requisite size and are free from environmental and genotoxic stress, Cdc2 is activated as described in section 1.3 and the cells enter mitosis. Mitosis is arguably the most spectacular stage of the cell cycle during which replicated chromosomes are segregated into two daughter nuclei. This period of the cell cycle can itself be divided into four distinct stages that can be visualised in *S. pombe* by following a single GFP-marked chromosome and observation of cells expressing GFP-tubulin (Nabeshima et al., 1998; Tatebe et al., 2001). In pro-metaphase the three chromosomes of *S. pombe* undergo visible chromatin condensation. At the same time the spindle pole bodies (SPBs), homologous to the centrosomes of higher eukaryotes, move into the nuclear envelope and separate to opposite sides of the nucleus whilst nucleating microtubules within the nucleus to establish a short (~2μm) bipolar mitotic spindle. During metaphase, the spindle length is maintained whilst chromosomes become attached to it such that each sister chromatid of a chromosome is attached to microtubules nucleating from opposite SPBs. The next stage of *S. pombe* mitosis, anaphase, can be split into two parts. In anaphase A, sister chromatids separate and are segregated towards opposite SPBs then in anaphase B, the spindle elongates along the longitudinal axis of the cell to segregate chromosomes to either end of the cell. In the final stage of mitosis, telophase, chromosomes decondense and the spindle

disassembles. One complete copy of the genome has now been segregated to either end of the cell, which is now ready for cytokinesis to produce a mother and daughter cell. The molecular mechanisms controlling these events are described in more detail in the following sections.

1.4.1 Chromatin condensation

All eukaryotes must condense their chromatin prior to chromosome segregation in mitosis so that the DNA is compact enough to move along the mitotic spindle. Chromatin condensation requires recruitment of a condensin complex to the DNA (reviewed in (Strunnikov, 2003)). A five-subunit condensin complex, identified in frog extracts, has ATP-dependent DNA super-coiling activity *in vitro* that is thought to be important for chromatin condensation (Hirano and Mitchison, 1994; Hirano et al., 1997; Kimura and Hirano, 1997). An equivalent 5-subunit complex has been identified in *S. pombe* comprising Cut3 and Cut14 SMC (Structural Maintenance of Chromosomes; for review see (Hirano, 1999)), family members originally identified as mutants defective in chromosome condensation and segregation (Saka et al., 1994) together with Cnd1, Cnd2 and Cnd3 non-SMC proteins, mutants of which have the same phenotype as *cut3* mutants (Sutani et al., 1999). Notably, Cut3 is phosphorylated by Cdc2 in mitosis and this event is required for the re-localisation of this subunit from the cytoplasm to the nucleus, showing that Cdc2 directly promotes chromatin condensation (Sutani et al., 1999). Chromatin condensation in eukaryotes also correlates with the phosphorylation of Ser10 in the N-terminus of Histone H3, although how this modification contributes to condensation is unclear (reviewed in (Prigent and Dimitrov, 2003)). In *S. pombe,* Histone H3 is phosphorylated by the aurora-related kinase Ark1 and cells lacking this kinase activity display a stretched chromosome phenotype indicating that it is required for chromosome condensation (Petersen et al., 2001; Petersen and Hagan, 2003). Ablation of Ark1 prevents Histone H3 phosphorylation and the subsequent Cut3 re-localisation to the nucleus suggests that this phosphorylation controls the recruitment of condensin to chromatin in mitosis (Petersen and Hagan, 2003).

1.4.2 Forming the mitotic spindle

The increase in Cdc2 activity at the G2-M transition is concomitant with a rapid change in the microtubule cytoskeleton, which prepares the cell to assemble a bipolar mitotic spindle (Figure 1.4). These cytoskeletal re-arrangements are similar to those seen in

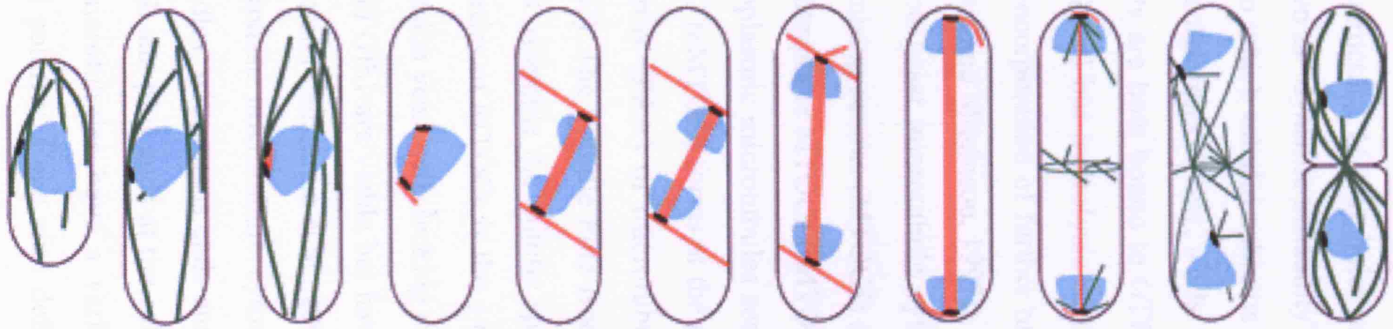


Figure 1.4 Changes in microtubule cytoskeleton during the fission yeast cell cycle

Interphase microtubules (green), mitotic microtubules (red), chromatin (blue) and SPBs (black) are all represented. The interphase microtubule cytoskeleton consists of 2-4 microtubule bundles spanning the longitudinal axis of the cell. In late G2 the SPBs duplicate. Upon commitment to mitosis the interphase microtubules disassemble and SPBs nucleate intranuclear microtubules. As SPBs move to opposite sides of the nucleus, intranuclear microtubules interdigitate to form the mitotic spindle. In anaphase A DNA separates and in anaphase B, spindle elongation occurs. In anaphase B astral microtubules nucleated from the SPBs into the cytoplasm are observed. Once nuclei reach the ends of the cells, the spindle disassembles and a post-anaphase array (PAA) of microtubules are nucleated from the eMTOC. Figure adapted from Hagan, 1998.

higher eukaryotes, making fission yeast a simple model in which to study spindle assembly.

1.4.2.1 The microtubule cytoskeleton

Microtubules form by the polymerisation of α and β tubulin heterodimers to assemble linear protofilaments that are arranged adjacent to each other, forming a hollow tube-like structure. Microtubules are inherently unstable and are in a constant state of flux known as "dynamic instability". This dynamic instability is most evident at the "plus end", to which tubulin subunits are added or lost during polymerisation or microtubule shrinkage respectively, whilst the "minus end" is less dynamic. The α and β tubulin subunits are both bound to GTP nucleotides. The GTP bound to α-tubulin cannot be hydrolysed but hydrolysis of the GTP bound to the β-tubulin subunit is required for the incorporation of further tubulin to the plus end during polymerisation (reviewed in (Desai and Mitchison, 1997)).

The fission yeast microtubule cytoskeleton is nucleated from γ-tubulin rich microtubule organising centres (MTOCS) of which there are three types in mitotic cells (Hagan, 1998). Interphase MTOCs (iMTOCS) associate with the nuclear envelope and nucleate the cytoplasmic microtubules seen during interphase (Tran et al., 2001). An equatorial MTOC (eMTOC) forms at the cell mid-zone in anaphase B and nucleates a post-anaphase array (PAA) of microtubules that disassembles following septation (Heitz et al., 2001). The Spindle Pole Bodies (SPBs) are the MTOCs from which the microtubules that assemble the mitotic spindle are nucleated. Key to the microtubule nucleating properties of MTOCs is the γ-tubulin ring complex (γ-TuRC) (Moritz and Agard, 2001). Fission yeast cells lacking components of the *S. pombe* γ-TuRC complex such as Mbo1 and Gfh1 are viable but have multiple defects associated with defective microtubule function (Venkatram et al., 2004).

The interphase microtubule cytoskeleton is arranged into 3-4 anti-parallel microtubule bundles, with minus ends anchored to the iMTOCs at the nucleus in the middle of the cell and plus ends at the cell tips (Hagan and Hyams, 1988; Tran et al., 2001). These microtubules have a variety of roles, from nuclear positioning to maintaining cell polarity. Cells with defective microtubules are unable to properly position the nucleus in the middle of the cell (Toda et al., 1983; Umesono et al., 1983) and it is thought that microtubules, tethered to the nucleus by MTOCS and extending to the cell tips, exert a balance of pushing forces to maintain the central position of the nucleus (Tran et al., 2001). Interphase microtubules are further required for the

establishment of polarised growth zones, since cells with defective microtubules can grow but have cell shape defects (Toda et al., 1983; Sawin and Nurse, 1998). Similar growth defects are found in *tea1* mutants (Snell and Nurse, 1994). Microtubules are thought to establish cell polarity in part, by localising the microtubule binding protein Tea1 onto the plus ends of microtubules and depositing it at cell ends to establish growth areas (Snell and Nurse, 1994; Verde et al., 1995; Mata and Nurse, 1997).

1.4.2.2 The mitotic spindle

At the G2-M transition, the interphase microtubule cytoskeleton disassembles and microtubules, nucleated within the nucleus from the SPBs, assemble to form the mitotic spindle. Studies of Xenopus egg extracts have shown that microtubule dynamics at G2-M are under the control of Cdc2-cyclin B (Verde et al., 1990; Verde et al., 1992), possibly through phosphorylation of microtubule associated proteins (MAPs) (Ookata et al., 1995). In fission yeast, the mitotic nucleating activity of SPBs can similarly be stimulated *in vitro* by *Xenopus* mitotic egg extract, however, this appears to be downstream of Cdc2 and only partially due to direct phosphorylation by the kinase (Masuda et al., 1992). Indeed, disruption of all kinases implicated in the G2-M transition in fission yeast – Plo1, Ark1 and Fin1 - perturb the assembly of the mitotic spindle (Ohkura et al., 1995; Petersen et al., 2001; Grallert and Hagan, 2002)

The fission yeast SPB spends most of the cell cycle located on the nuclear periphery (Ding et al., 1997). During late G2, the SPB duplicates and in prophase, invagination of the nuclear envelope followed by its localised breakdown allows entry of the duplicated SPBs (Ding et al., 1997). Each of the SPBs begin to nucleate intra-nuclear microtubules whilst moving through the nuclear envelope till they are situated opposite each other and the short (~2μm) bipolar spindle is formed (Ding et al., 1993; Ding et al., 1997). SPB movement and anchoring requires the protein Cut11, a protein with seven membrane-spanning domains, which is found in the nuclear envelope throughout the cell cycle and localises to the SPBs at the same time as they enter the envelope (West et al., 1998).

Electron microscopy of the fission yeast mitotic spindle has revealed that it consists of two arrangements of microtubules. During metaphase, microtubules extend from one pole to the other, whilst 2-4 microtubules associate with a region presumed to be the kinetochore of each sister chromatid. When the spindle elongates, the kinetochore-microtubules are no longer seen and instead of spanning from pole-to-pole, the non-kinetochore microtubules interdigitate at the spindle midzone (Ding et al.,

1993). Several kinesin-like proteins and non-motor MAPs localise to the spindle midzone and are proposed to generate the force necessary to elongate the spindle in anaphase B. For example, the kinesin related protein Cut7 localises to the midzone in anaphase B and *cut7* mutants fail to interdigitate midzone microtubules (Hagan and Yanagida, 1992). Another such protein, Ase1, also has a role in stabilising the region of minus-end microtubule overlap at the cytoplasmic MTOCs of interphase microtubules, as well as the plus end overlap at the spindle midzone (Loiodice et al., 2005). In addition, a group of proteins termed "chromosomal passengers" conserved from yeast to man, exhibit stage dependent re-localisation from kinetochores, where they are thought to be important in regulating microtubule-kinetochore attachments, to the spindle midzone, where they regulate interdigitating forces and are involved in determining the site of cytokinesis (reviewed in (Terada, 2001)). In fission yeast, these include the protein Bir1, which re-localises from kinetochores to the spindle midzone in anaphase A and is required for spindle elongation (Rajagopalan and Balasubramanian, 2002). Bir1 is required for the localisation of another chromosomal passenger, the Aurora kinase homologue Ark1 to kinetochores and then to the spindle midzone (Morishita et al., 2001). The elongation of the spindle in anaphase B is thought to be driven by sliding forces generated by the interdigitating spindle midzone and a change in microtubule dynamics (Ding et al., 1993; Mallavarapu et al., 1999). During mitosis, astral microtubules are nucleated from the SPBs and projected into the cytoplasm (Hagan and Hyams, 1988). These microtubules have been reported to orient the spindle along the longitudinal axis of the cell in metaphase (Gachet et al., 2001). However, recent work has shown that these astral microtubules are only nucleated in anaphase B (Zimmerman et al., 2004), suggesting that they act only to guide the elongating anaphase B spindle. In telophase, the spindle disassembles. The mechanisms controlling this process are unknown, although timely spindle disassembly seems to depend on the kinesin-like protein Klp2, since the anaphase spindle is hyper-elongated in cells lacking this protein (Troxell et al., 2001). The force generated by the mitotic spindle acts to separate sister chromatids to the mother and daughter cells. In metaphase, a protein complex called cohesin holds sister chromatids together and opposes this force.

1.4.3 Cohesion

Mitotic chromosomes consist of paired sister chromatids held together by a protein complex termed cohesin. Cohesion between sister chromatids is established in S phase but must be lost in anaphase to allow segregation of sisters to the mother and daughter

cells. In *S. cerevisiae,* the cohesin subunits consist of Smc1 and Smc3, (members of the SMC family also required for chromosome condensation) and two non-SMC proteins, Scc1 and Scc3, defects in any of which result in premature sister chromatid segregation (Guacci et al., 1997; Michaelis et al., 1997; Toth et al., 1999). Cohesin acts as a "glue" between sister chromatids and the key event marking the metaphase to anaphase transition in eukaryotes is the targeted proteolysis of a cohesin subunit allowing sisters to separate. Studies in budding yeast have established that the cohesin subunit Scc1 is cleaved by the protease Esp1 at the onset of anaphase (Ciosk et al., 1998; Uhlmann et al., 1999). Esp1 has been termed a "separase" and is inhibited prior to anaphase by association with the "securin" protein Pds1. The removal of cohesin is achieved by targeted ubiquitination of Pds1 by the E3 ubiquitin ligase Anaphase Promoting Complex, thus releasing Esp1 to degrade Scc1 (Uhlmann et al., 1999).

The fission yeast homologues of Smc1, Smc3 and Scc3 have been identified as Psm1, Psm3 and Psc3 respectively, all of which are essential for sister chromatid cohesion, whilst the homologue of Scc1 is Rad21, originally identified as a protein involved in DNA repair (Birkenbihl and Subramani, 1992; Birkenbihl and Subramani, 1995; Tatebayashi et al., 1998; Tomonaga et al., 2000). In addition to the cohesin complex, several other proteins are required to maintain cohesion of sister chromatids. Mis4, the homologue of budding yeast Scc2 (required for cohesion and essential during S-phase), is needed for cohesion and becomes essential during S-phase (Furuya et al., 1998), whilst fission yeast Eso4 is the homologue of budding yeast Eco1 and in both yeasts is similarly required for sister chromatid cohesion (Skibbens et al., 1999; Toth et al., 1999; Tanaka et al., 2000). In fission yeast, the homologues of budding yeast Esp1 and Pds1 are Cut1 and Cut2, which similarly form a complex (Kumada et al., 1998). Furthermore, Anaphase Promoting Complex dependant degradation of Cut2 is necessary for sister chromatid separation (Funabiki et al., 1996a; Kumada et al., 1998), suggesting that a common mechanism regulates separase activity in both yeasts. Indeed, the proteolysis of Rad21 is likely to be needed for the separation of sister chromatids as is its budding yeast homologue Scc1 since a non-cleavable Rad21 mutant blocks sister chromatid separation (Tomonaga et al., 2000).

1.4.4 Anaphase Promoting Complex (APC)

Once the appropriate spindle microtubule-kinetochore connections have been made, Cdc2 activity must be shut off and as described in section 1.4.3, separase must be released from the securin protein to allow sister chromatids to separate and anaphase to

proceed. To do this in fission yeast, Cdc13 and Cut2 (securin) are targeted for degradation by the covalent attachment of ubiquitin molecules by the Anaphase Promoting Complex (APC) (Funabiki et al., 1996b; Yamano et al., 1996; Funabiki et al., 1997; Yamano et al., 1998). The APC is a multi-subunit E3 ubiquitin ligase conserved throughout evolution (reviewed in (Peters, 2002)), which acts by transferring ubiquitin molecules to a lysine side chain of target proteins leading to their targeted degradation by the 26S proteosome (Wilkinson et al., 1999). To date, 13 APC sub-units have been identified in fission yeast by a combination of genetic analysis of anaphase defective mutant strains (reviewed in (Yanagida et al., 1999)), tandem affinity purification (TAP) and mass spectrometry (Yoon et al., 2002). Ubiquitination is a multi-step, multi-enzyme process in which the ubiquitin activating enzyme E1 activates ubiquitin, which is then transferred to the ubiqutin conjugating enzyme E2 that associates with the E3 ubiquitin ligase, thus allowing transfer of ubiquitin to the substrate (reviewed in (Fang and Weissman, 2004)). Most substrates of the APC contain a 9-residue conserved motif called the destruction box and are degraded during mitosis or G1. Overexpression of Cut2 or Cdc13 destruction boxes blocks cells in mitosis, whilst mutation of the lysine residues within the destruction boxes relieves this inhibition (Funabiki et al., 1996b; Yamano et al., 1996).

The substrate specificity of the APC requires the association of adaptor proteins containing WD (tryptophan, aspartic acid) repeats. In budding yeast, these are Cdc20 and its related protein Cdh1 (Hilioti et al., 2001; Pfleger et al., 2001; Schwab et al., 2001). It appears that in budding yeast, the association of Cdc20 or Cdh1 confers differing specificities because overexpression of Cdc20 leads to proteolysis of Pds1 but not mitotic cyclins, whereas overexpression of Cdh1 leads to mitotic cyclin proteolysis (Schwab et al., 1997; Visintin et al., 1997). Indeed, Cdc20 binds Pds1, whilst Cdh1 interacts with the mitotic cyclins Clb2 and Clb3 (Schwab et al., 2001). In fission yeast, WD-repeat containing proteins Slp1 and Ste9 may have a homologous role to Cdc20 and Cdh1, respectively. Slp1 appears to be important in controlling the APC and thus chromosome segregation but to date it has not been proven to specifically target Cut2 (Matsumoto, 1997; Kim et al., 1998). Ste9 mutants have higher levels of Cdc13 in G1 and its overexpression reduces cyclin levels (Kitamura et al., 1998; Kominami et al., 1998), suggesting that, as in budding yeast, this protein directs the APC to mitotic cyclins.

1.4.5 The Spindle Assembly Checkpoint

As mentioned previously, the WD-repeat proteins activate the APC to promote targeted degradation of Cut2 and Cdc13, thus promoting the metaphase to anaphase transition. Inhibition of these APC regulators delays the metaphase to anaphase transition and so they are targets of a checkpoint termed the Spindle Assembly Checkpoint (SAC) that operates in all eukaryotes. This checkpoint maintains the accuracy of chromosome segregation by ensuring that the metaphase to anaphase transition does not occur till every chromosome has established a bi-polar attachment to the metaphase spindle. Genetic screens in budding yeast for mutants that have died following treatment with a microtubule destabilising drug identified the key proteins involved in the SAC as Mad1, Mad2 and Mad3 (Li and Murray, 1991), and Bub1 and Bub3 (Hoyt et al., 1991). Another gene encoding a SAC protein, *MPS1,* was identified as a mutant that was also unable to duplicate SPBs (Weiss and Winey, 1996), suggesting a dual role. In *S. pombe,* homologues of Mad1 (Ikui et al., 2002), Mad2 (He et al., 1997), Mad3 (Millband and Hardwick, 2002), Bub1 (Bernard et al., 1998) and Bub3 (Bernard et al., 2001) have been identified. These checkpoint proteins have also been identified in humans showing that this checkpoint is evolutionarily conserved throughout eukaryotes (reviewed in (Millband et al., 2002)).

In vertebrates, the SAC is thought to monitor a combination of the microtubule occupancy at the kinetochore and the tension generated at kinetochores when they are correctly bi-oriented to these microtubules from opposite centrosomes (Li and Nicklas, 1995; Rieder et al., 1995). The requirement for balanced tension at the kinetochore as well as microtubule attachments was first shown in elegant experiments conducted in meiotic grasshopper spermatocytes, in which tension applied to an improperly attached chromosome with a micromanipulator was sufficient to overcome a metaphase to anaphase delay (Li and Nicklas, 1995). A single unattached kinetochore can delay anaphase (Rieder et al., 1994) and vertebrate Mad2 localises to empty kinetochores but not those attached to microtubules suggesting that this component of the SAC senses the microtubule occupancy of the kinetochore (Chen et al., 1996; Waters et al., 1998). Bub1 is a protein kinase that is also found to localise to kinetochores during early metaphase in vertebrate cells and is required to delay the cell cycle following spindle damage (Taylor and McKeon, 1997). Experiments in mammalian cells have shown that when kinetochores have microtubules attached but tension is abolished by treatment with the microtubule destabilising drug vinblastine, Bub1 is recruited to the kinetochore but not Mad2 (Skoufias et al., 2001). From these results, a model has been proposed,

whereby Mad2 senses the microtubule occupancy status of kinetochores, whilst Bub1 monitors the tension generated at the kinetochore by the attachment of the kinetochore to microtubules from opposite centrosomes. However, the situation seems more complex and work in both budding and fission yeast has revealed that several different complexes of SAC proteins are involved in activating this checkpoint.

The SAC inhibits the APC activator Cdc20 to delay chromosome segregation. In budding yeast Mad2 binds directly to and inhibits Cdc20 in a Mad1-dependent manner (Hwang et al., 1998). Mad1 becomes hyperphosphorylated when the SAC is activated and forms a complex with Mad2 that is required to maintain the checkpoint response (Hardwick and Murray, 1995). Work in *Xenopus* also suggests that the role of Mad1 is to recruit Mad2 to the kinetochore (Chen et al., 1998; Chen et al., 1999). In fission yeast, overexpression of Mad2 arrests cells in metaphase and has been shown to bind directly to the Cdc20 homologue Slp1, showing that it too delays cell cycle progression by preventing APC activation (He et al., 1997; Kim et al., 1998). As in vertebrates, fission yeast Mad2 has been found to localise to unattached kinetochores and in this situation is bound to Slp1 (Ikui et al., 2002), suggesting that this branch of the SAC is conserved in eukaryotes and assembles at kinetochores to sense their microtubule occupancy. Fission yeast Mad1 was isolated in a 2-hybrid screen using Mad2 as bait and the two found to interact directly. Furthermore, in cells lacking Mad1, the localisation of Mad2 is disrupted suggesting that, as in other eukaryotes, it has a role in recruiting Mad2 to the kinetochore (Ikui et al., 2002).

In human cells, a mitotic checkpoint complex (MCC) containing the APC activator Cdc20 together with the SAC proteins Mad2, BubR1(Mad3) and Bub3 has been identified. This complex inhibits the APC *in vitro* 3000-fold greater than Mad2 alone (Sudakin et al., 2001). Similar complexes have been found between Cdc20 and Mad2, Mad3 and Bub3 in budding yeast (Hardwick et al., 2000) and fission yeast (Millband and Hardwick, 2002).

Fission yeast Bub1 is also recruited to kinetochores in metaphase and is similarly maintained there and required to delay the metaphase to anaphase transition following spindle damage (Bernard et al., 1998). Bub1 is a substrate of Cdc2 and requires Cdc2-dependent phosphorylation for its full activity in the checkpoint response (Yamaguchi et al., 2003). Additionally, Bub1 has a non-checkpoint role since *bub1* mutants have a significantly increased chromosome loss rate during unperturbed growth (Bernard et al., 1998). This additional role in chromosome segregation may depend on the activity of the C-terminal kinase domain of Bub1. Kinase-dead mutants of *bub1* are

only partially checkpoint defective yet display increased levels of chromosome lagging in a normal cell cycle whilst the *bub1Δ28-160* mutant, which lacks the N-terminus of the protein, is checkpoint defective but displays few lagging chromosomes (Yamaguchi et al., 2003; Vanoosthuyse et al., 2004). Recent work has suggested the Bub1 in fission yeast may act with Bub3 as a scaffold on which to recruit further Bub3, Mad3 and Bub1 (Vanoosthuyse et al., 2004).

The SAC acts to delay the metaphase to anaphase transition to allow the cell to respond to a lack of microtubule attachment to the kinetochore or a lack of balanced tension at the kinetochore. If the checkpoint has been activated due to the lack of microtubules at the kinetochore, it is likely that the delay merely allows time for these attachments to be made. However, the checkpoint may still be activated by inappropriate microtubule-kinetochore connections that fail to generate tension, and the cell must exploit this delay. The following sections describe regulation of microtubule-kinetochore attachments.

1.4.6 Aurora kinases

1.4.6.1 Aurora kinases in humans

To segregate the genome successfully, sister chromatids must be attached to spindle microtubules from opposite poles. This arrangement is described as an "amphitelic" attachment and results in what is known as chromosome bi-orientation. However, several other kinetochore-microtubule arrangements are possible and must be avoided or corrected before anaphase can proceed. It is possible to have a "monotelic" attachment in which only one kinetochore is attached to microtubules from one SPB, a "syntelic" attachment where both kinetochores are attached to microtubules from only one SPB, or a "merotelic" attachment where one kinetochore is attached to microtubules from both SPBs (Figure 1.5). If any of these attachments remain uncorrected then chromosome mis-segregation may occur. Balanced tension is only established at the kinetochore when sister chromatids have an "amphitelic" attachment with equal numbers of microtubules from each SPB attaching to opposite kinetochores (Figure 1.5). To establish and maintain balanced tension at the kinetochore, a mechanism must exist to correct these improper microtubule-kinetochore attachments. Key players in the regulation of microtubule-kinetochore interactions have emerged as a family of proteins termed the "Aurora kinases."

Aurora kinase was first identified in *Drosophila* where *aurora* mutants failed to separate centrosomes and form a bipolar spindle in mitosis (Glover et al., 1995). In

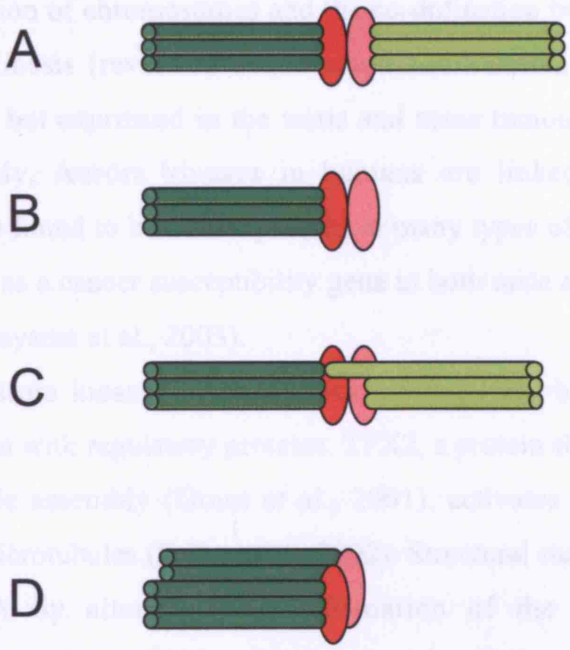


Figure 1.5 Microtubule-kinetochore attachments

Kinetochores that attach to multiple spindle microtubules can have several different attachment configurations. Bipolar chromosome attachment is achieved when kinetochores of replicated sister chromatids (red/pink) are attached to microtubules from opposite poles (green/light green). This is described as an (A) "amphitelic" attachment. When only one of the kinetochores of sister chromatids is attached to microtubules from one pole the attachment is described as (B) "monotelic". A (C) "merotelic" attachment occurs when a kinetochore is attached to microtubules from both poles. A (D) "syntelic" attachment is when both kinetochores of sister chromatids are attached to spindle microtubules from the same pole. Monotelic, syntelic and merotelic attachments must be corrected to achieve bipolar chromosome attachment.

mammals, the Aurora kinase family comprises three members: Aurora A, B and C. Aurora A is implicated in centrosome maturation and spindle assembly (reviewed in (Dutertre et al., 2002)) whilst Aurora B is thought to regulate kinetochore assembly, bipolar chromosome attachment and the spindle checkpoint, as well as the condensation and cohesion of chromosomes and the co-ordination between chromosome segregation and cytokinesis (reviewed in (Shannon and Salmon, 2002)). Aurora C is relatively unstudied but expressed in the testis and some tumour lines (Katayama et al., 2003). Importantly, Aurora kinases in humans are linked to carcinogenesis. They are frequently found to be overexpressed in many types of cancers and Aurora A has been identified as a cancer susceptibility gene in both mice and humans (Ewart-Toland et al., 2003; Katayama et al., 2003).

Aurora kinases in vertebrates are regulated by their phosphorylation and by association with regulatory proteins. TPX2, a protein shown in *Xenopus* to be important for spindle assembly (Gruss et al., 2001), activates Aurora kinase and targets it to spindle microtubules (Kufer et al., 2002). Structural studies suggest that TPX2 activates Aurora A by altering the confirmation of the protein so that a regulatory phosphothreonine is hidden from dephosphorylation and deactivation (Bayliss et al., 2003). Aurora B is a chromosomal passenger protein that localises to centromeres from prometaphase to the metaphase to anaphase transition, when it re-localises to the spindle mid-zone and cell cortex (Murata-Hori et al., 2002). Aurora B is found in a complex with two other chromosomal passenger proteins, INCENP (inner centromere protein) and survivin, both of which are required for Aurora B re-localisation and full activity (Adams et al., 2000; Wheatley et al., 2001; Bolton et al., 2002).

Aurora B in vertebrates is implicated in the regulation of microtubule-kinetochore connections and thus chromosome segregation. Inhibition of Aurora B activity using antibodies, a specific kinase inhibitor, or RNA interference (RNAi) disrupts proper chromosome segregation and an increase in mono- and syntelically attached chromosomes is observed (Kallio et al., 2002; Hauf et al., 2003). This suggests a model in which the microtubule-kinetochore connections, found at mono- and syntelically attached chromosomes, are destabilised by the action of Aurora B kinase. A potential target for Aurora B kinase in the regulation of microtubule-kinetochore attachments has been identified as the mitotic centromere associated kinesin (MCAK). MCAK is a kinesin-like protein with microtubule depolymerisation activity that localises to centromeres and is required for chromosome segregation (Wordeman and Mitchison, 1995; Maney et al., 1998). Depletion of MCAK leads to multiple

segregation defects, with chromosomes displaying merotelic and syntelic attachments, indicating a role in correcting inappropriate microtubule-kinetochore connections (Kline-Smith et al., 2004). Two recent papers have shown that Aurora B phosphorylates MCAK *in vivo* and that this is required for localisation of MCAK to centromeres (Andrews et al., 2004; Lan et al., 2004). Furthermore, phosphorylation of MCAK by Aurora B inhibits the *in vitro* microtubule-destabilising activity of MCAK and mutation of the MCAK phosphorylation site increases the frequency of syntelic and monotelic microtubule-kinetochore attachments (Andrews et al., 2004). Taken together, these results suggest a model in which Aurora B directs MCAK to centromeres where it regulates its activity to correct improper microtubule-kinetochore attachments. Further insights into the mechanism by which Aurora kinases regulate microtubule-kinetochore attachments have come from work done in budding yeast.

1.4.6.2 Aurora kinases in budding and fission yeast

The *S. cerevisiae* Aurora kinase homologue, Ipl1, was first identified in a screen for mutants that increased in ploidy (Chan and Botstein, 1993). Budding yeast Ipl1 is essential for chromosome segregation and *ipl1* mutants die with high levels of mis-segregation (Francisco and Chan, 1994). The finding that the microtubule binding properties of kinetochores *in vitro,* in the presence of mutant Ipl1, were stabilised compared to the wild-type protein (Biggins et al., 1999) suggested that this chromosome segregation defect may be due to defective kinetochore regulation. Since Ipl1 localises to the mitotic spindle, this further suggested direct regulation of the kinetochore in mitosis (Biggins et al., 1999; Kim et al., 1999). Ipl1 binds to and phosphorylates the essential protein Sli15, the homologue of vertebrate INCENP, and this association is required to localise Ipl1 to the kinetochore and to stimulate its kinase activity (Kim et al., 1999; Kang et al., 2001). In an elegant set of experiments by Tanaka and colleagues, the kinetochores of wild-type cells were found to attach to old and new poles with equal frequency whereas in an *ipl1* mutant kinetochores always remained attached to the old pole indicating that Ipl1 controls chromosome bi-orientation (Tanaka et al., 2002). The outer kinetochore protein Dam1 is phosphorylated by Ipl1 kinase *in vitro* (Kang et al., 2001) and is likely to be a key target in regulating chromosome bi-orientation for the following reasons: firstly, cells expressing mutant Dam1, in which the proposed Ipl1 target residues were all mutated to non-phosphorylatable alanine, mimicked the phenotype of *ipl1* mutants (Cheeseman et al., 2002). Conversely, cells expressing a mutant Dam1 in which the target residues are mutated to negatively charged aspartic

acid, mimicking constitutive phosphorylation, suppress the phenotype of *ipl1* mutants but have a lagging chromosome phenotype on their own (Cheeseman et al., 2002). Importantly, Ipl1 itself appears to be involved in sensing the lack of tension at kinetochores as the SAC is unable to prevent chromosome segregation when Ipl1 is inactivated (Biggins and Murray, 2001). Genetic interactions have suggested that the phosphorylation of budding yeast kinetochore proteins by Ipl1 is counteracted by the essential type-1 phosphatase, Glc7 (Francisco et al., 1994; Tung et al., 1995). In *glc7* mutants, the kinetochore protein and Ipl1 target Ndc10 is hyperphosphorylated and these cells have a high rate of chromosome loss (Sassoon et al., 1999). Interestingly, a balance between Ipl1 and Glc7 activity has also been shown to control histone H3 phosphorylation (Hsu et al., 2000) suggesting that these proteins have a role in regulation chromosome condensation as well as segregation.

Together, these data suggest a model in which the lack of tension, due to the improper microtubule-kinetochore attachments, induces Ipl1 to both activate the SAC and phosphorylate kinetochore proteins including Dam1. This phosphorylation reduces the affinity of the kinetochore for the spindle microtubules, allowing any incorrect attachments to be disassociated. Subsequent dephosphorylation by Glc7 would allow the re-attachment of microtubules to the kinetochore and this process would continue until bi-orientation of sister chromatids was established. However, *S. cerevisiae* has one important difference from higher eukaryotes – each kinetochore binds only one microtubule (Winey et al., 1995), meaning that the only possible attachments are amphitelic or syntelic. Since fission yeast have 2-4 microtubules attached to each kinetochore (Ding et al., 1993), it can in theory have amphitelic, merotelic or syntelic attachments and thus is a better system from which to extrapolate to human cells where each kinetochore is attached to up to 20 microtubules (Cassimeris et al., 1990; Wendell et al., 1993).

S. pombe also contains an aurora kinase, Ark1, and although this has not been studied as much as its *S. cerevisiae* homologue, the data so far suggests that it has similar roles. For example, fission yeast cells lacking Ark1 display several distinctive phenotypes during mitosis. Firstly, Δ*ark1* cells cannot phosphorylate Histone H3 or recruit the condensin subunit, Cut3, resulting in a defect in chromosome condensation (Petersen et al., 2001; Petersen and Hagan, 2003). Next, many of these chromosomes were not associated with the mitotic spindle or were found only at one end of the spindle, suggesting a problem with kinetochore-microtubule attachments in the absence of Ark1 function (Petersen et al., 2001).

In other eukaryotes including budding yeast, Aurora kinase is found in a complex with INCENP and Survivin proteins, which are both required for full kinase activity (Bolton et al., 2002; Cheeseman et al., 2002). *S. pombe* contains homologues of INCENP and Survivin in Pic1 and Bir1 respectively (Rajagopalan and Balasubramanian, 1999; Morishita et al., 2001; Leverson et al., 2002). Similar phenotypes are observed in *bir1* and *ark1* mutants, such as a defect in chromosome condensation and Bir1 is required to recruit Ark1 to the spindle (Morishita et al., 2001) and for Ark1 kinase activity (Petersen and Hagan, 2003). When treated with microtubule destabilising drugs, *bir1* mutants or cells lacking Ark1 are unable to recruit Mad2 to the kinetochore to form a complex with Mad3 leading to a defective SAC although Bub1 and Mad3 localisation are not affected (Petersen and Hagan, 2003).

The substrates of Aurora kinases in humans and yeasts include proteins that are components of the kinetochore - a large multi-subunit proteinaceous structure that forms a bridge between mitotic spindle microtubules and chromosomes. Regulation of the kinetochore's phosphorylation state, as well as its assembly, is clearly important for accurate chromosome segregation. Kinetochores assemble in a hierarchical fashion at the centromeres of chromosomes – a specialised area of chromatin that is crucial for chromosome segregation in eukaryotes.

1.5 Centromeres and kinetochores

1.5.1 The centromere

The centromere is the region of chromatin at which the proteinaceous kinetochore assembles. Whilst serving the same purpose in all organisms, centromeres can have diverse structures. For example, the centromeres of budding yeast have been identified as comprising only a 125bp sequence, whilst the nematode *C. elegans* has holocentric kinetochores that assemble along the length of the chromosome arms. The centromeres of humans range from 0.3-5 Mb and consist of thousands of tandemly repeated arrays of a 171bp sequence element termed α-satellite (reviewed in (Murphy and Karpen, 1998)). The centromeres of fission yeast are larger than those found in budding yeast and, as in humans, also vary in size between chromosomes. Chromosome 1 of fission yeast, which is the largest chromosome at 5.6Mb has the smallest centromere (*cen1)* of ~35kb, whilst the centromere of the 4.4Mb chromosome 2 (*cen2*) is ~65kb and that of chromosome 3, the smallest at 2.5Mb, is the largest centromere (*cen3*) at ~110kb (Takahashi et al., 1992; Steiner et al., 1993; Wood et al., 2002). The fission yeast centromere is a modular

structure comprised of a central domain of non-repeating sequence that ranges in size from 4-7kb, flanked by inverted repeating domains termed the 'innermost' (*imr*) repeats that are unique to each centromere. Lying outside of the *imr* repeats are the 'outer' (*otr*) repeats composed of 'dg' and 'dh' elements, the number of which vary and confer the size difference between *cen1, cen2* and *cen3* (Figure 1.6; (Chikashige et al., 1989)).

The centromeres of fission yeast are transcriptionally silent areas and this feature has been exploited in screens for mutants in genes involved in centromere regulation that allow the transcription of reporter genes inserted into this region (Allshire et al., 1995). Mutations in *swi6, clr4* and *rik1* that alleviate the transcriptional silencing of this area also have a high incidence of chromosome segregation defects, showing that the structure of the centromeric chromatin is important for correct centromere function and chromosome segregation (Allshire et al., 1995; Ekwall et al., 1995; Ekwall et al., 1996). Swi6 is a chromodomain protein that is important in establishing heterochromatin at the outer repeats. Recruitment of Swi6 to the outer repeats requires deacetylation by the histone deacetylases (HDACs), Clr3 and Clr6, followed by methylation of histone H3 lysine 9 by the histone methyltransferase Clr4 (Nakayama et al., 2001).

Recent work has uncovered an unexpected role for non-coding RNA, transcribed from the outer repeats, in the establishment of functional centromeres in *S. pombe*. RNA interference (RNAi) is well documented in post-transcriptional gene silencing (reviewed in (Hutvagner and Zamore, 2002)). However, cells lacking genes encoding RNAi machinery proteins such as *ago1, dcr1, rdp1* and *rik1* lack histone H3 methylation. Consequently, they are unable to establish heterochromatin at the outer repeats and exhibit high levels of chromosome mis-segregation due to impaired centromere function (Volpe et al., 2002). It has been proposed that RNA transcribed towards the centromere from the *dh* elements is degraded by the RNAi machinery whilst low-level transcription from the forward strand allows the formation of dsRNA possibly involving amplification by the RNA polymerase Rdp1 (Volpe et al., 2002). Subsequent processing of the dsRNA by the RNAi machinery would produce RNA capable of recruiting the methyl-transferase Clr4 to the *dh* elements of the outer repeats, leading to histone H3 methylation and recruitment of Swi6 (Volpe et al., 2002). Methylation of histone H3 at the centromeres of mice chromosomes is directed by particular methyl-transferases and the discovery of RNA transcripts from the repeating satellite DNA of these regions (Rudert et al., 1995; Lehnertz et al., 2003), leads one to speculate whether the RNAi dependent assembly of heterochromatin at centromeres

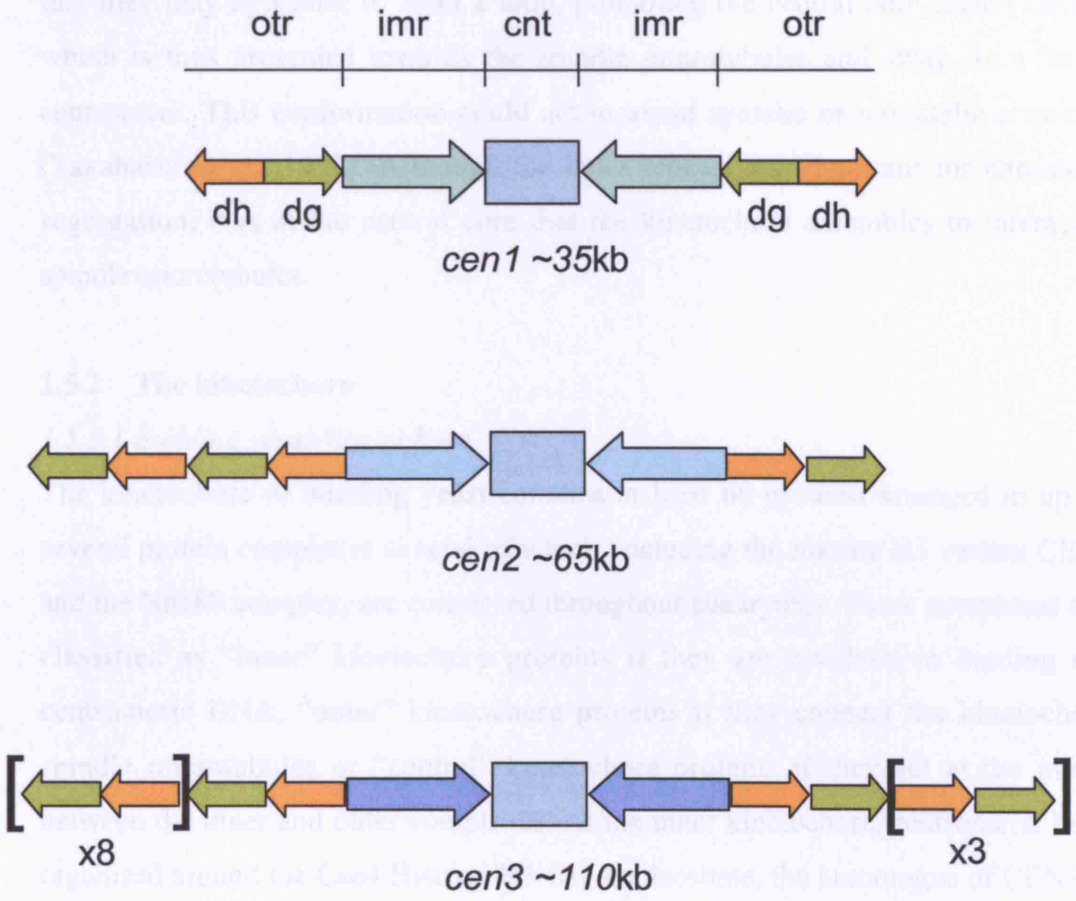


Figure 1.6 The centromeres of fission yeast

A schematic diagram showing the structure of fission yeast centromeres. A central domain (cnt) of non-repeating sequenece that ranges from 4-7kb is flanked in inverted innermost (imr) repeats. Outside the imr repeats lie the outer (otr) repeats composed of 'dh' and dg' repeats, the number of which varies between centromeres and confers their size difference.

might be a conserved feature amongst eukaryotes. Consistently, Dicer, one of the proteins involved in the RNAi machinery, is essential for the establishment of heterochromatin and proper chromosome segregation in vertebrates (Fukagawa et al., 2004). The sequence similarity of the outer repeat regions has lead to the suggestion that they may hybridise to form a loop, promoting the central core region outwards, which is thus presented towards the spindle microtubules and away from its sister centromere. This conformation could act to avoid syntelic or monotelic attachments (Takahashi et al., 1992). Although the outer repeats are important for chromosome segregation, it is at the central core that the kinetochore assembles to interact with spindle microtubules.

1.5.2 The kinetochore

1.5.2.1 Budding yeast kinetochore

The kinetochore of budding yeast contains at least 60 proteins arranged in up to 14 several protein complexes several of which, including the histone H3 variant CENP-A and the Ndc80 complex, are conserved throughout eukaryotes. These complexes can be classified as "inner" kinetochore proteins if they are involved in binding to the centromeric DNA, "outer" kinetochore proteins if they connect the kinetochore to spindle microtubules or "central" kinetochore proteins if they act at the interface between the inner and outer complexes. At the inner kinetochore, centromeric DNA is organised around the Cse4 Histone H3-like nucleosome, the homologue of CENP-A in humans (Meluh et al., 1998). The CBF3 complex, which contains the proteins Ndc10, Cep3, Ctf13 and Skp1 (Lechner and Carbon, 1991), is required for proper kinetochore function and to target Cse4 to the centromeric DNA (Goh and Kilmartin, 1993; Sorger et al., 1994; Ortiz et al., 1999). Also at the inner kinetochore are Cbf1 and Mif2, which have sequence similarity to human CENP-B and CENP-C respectively (Mellor et al., 1990). The central kinetochore includes the Ndc80 complex, which appears to be important in kinetochore-microtubule attachment regulation (Wigge and Kilmartin, 2001), and the large Ctf19 complex (Hyland et al., 1999; Ortiz et al., 1999). The outer kinetochore connects to the microtubules of the spindle. Proteins that localise here include microtubule-associated proteins, such as Stu2 and kinesin like proteins, such as Kip3 (Severin et al., 2001). Proteins like these are thought to provide the opposing forces at the kinetochore that are required for chromosome segregation. Recent work has identified a complex at the budding yeast outer kinetochore that appears to play a

key role in regulating microtubule-kinetochore connections. This complex, termed the DASH complex, is described in more detail in section 1.5.2.3.

1.5.2.2 Fission yeast kinetochore

In fission yeast, the central core region and the inner repeats are characterised by their unusual chromatin structure that can be detected by gel electrophoresis following partial micrococcal-nuclease digestion (Polizzi and Clarke, 1991; Takahashi et al., 1992; Marschall and Clarke, 1995). This feature was used in genetic screens to identify mutants that affect chromatin assembly in this region. In this way, Cnp1, the homologue of the centromere-specific histone H3 variant CENP-A found at mammalian centromeres was identified (Takahashi et al., 1992; Takahashi et al., 2000; Pidoux et al., 2003). Localisation of Cnp1 to the central core region is dependent on Mis6, a protein identified with Mis12 in a screen for '*mis*' mutants that have a high level of chromosome loss (Takahashi et al., 1994; Saitoh et al., 1997; Takahashi et al., 2000). A similar screen identified Mal2 (Fleig et al., 1996), which, with Mis6, has been localised to the central core region by chromatin-immunoprecipitation (ChIP) experiments (Saitoh et al., 1997; Goshima et al., 1999; Jin et al., 2002). Interestingly, *mis12* mutations do not activate the SAC in fission yeast whilst in budding yeast, a mutation in the *S. cerevisiae* homologue of Mis12, Mtw1, activates the SAC in an Ipl1-dependent manner, suggesting that Aurora kinase in budding yeast senses tension through Mtw1 (Goshima et al., 1999; Pinsky et al., 2003). However, whilst Mis12 localisation to centromeres in fission yeast is dependent on Cnp1, localisation of Mtw1 in budding yeast is independent of the Cnp1 homologue, Cse4, so it remains unclear whether these proteins have a common role and if the tension-monitoring feature is conserved between yeasts (Goshima et al., 1999; Pinsky et al., 2003).

Another protein found at the centromere of fission yeast is Sim4, identified in a further screen for mutants that allowed transcription from the central core region. Sim4 is in a complex with Mis6 and appears to co-operate in the loading of Cnp1 (Pidoux et al., 2003). Despite being in a complex with Mis6, there is evidence that Sim4 might have a different role. Mis6 mutants have a high rate of chromosome mis-segregation but display few lagging chromosomes suggesting an inability for one kinetochore to attach to microtubules (Saitoh et al., 1997). Meanwhile, Sim4 mutants have frequent lagging chromosomes suggesting that Sim4 is involved in the attachment-correction mechanism (Pidoux et al., 2003).

Further components of the fission yeast kinetochore, Ndc80, Nuf2 and Spc24 were identified by their homology to several proteins found in an analysis of highly enriched budding yeast SPBs by Matrix-assisted Laser Desorption/Ionisation (MALDI) and accordingly, these proteins localise to mitotic spindles and kinetochores (Nabetani et al., 2001; Wigge and Kilmartin, 2001). Electron microscopy analysis of fixed fission yeast cells has shown that during interphase, Ndc80 is located between Cnp1 in the central core region and the γ-tubulin of the SPB, suggesting that it may act as a bridge between the centromere and spindle microtubules (Kniola et al., 2001). Centromere localisation of Ndc80 is reduced in mutants that disrupt the central core region but not those affecting the heterochromatin of the outer repeats, strengthening the idea that these regions are regulated separately (Appelgren et al., 2003) but raising the question of what direct role the outer repeats have during chromosome segregation.

A possible answer may lie with two kinesin-like proteins Klp5 and Klp6, members of the Kinesin-8 family and homologous to vertebrate MCAK, found to destabilise microtubules rather than act as conventional motor proteins (Desai et al., 1999). Fission yeast null mutants in *klp5* or *klp6* exhibit altered microtubule dynamics, are resistant to microtubule destabilising drugs and have abnormal chromosome dynamics in mitosis resulting in a metaphase delay (West et al., 2001; West et al., 2002). These abnormal chromosome dynamics may indicate a problem in generating bipolar tension at the kinetochores as *klp5* or *klp6* mutants are synthetically lethal with cells lacking Bub1, which is implicated in tension monitoring, but not cells lacking Mad2, which is thought to respond to lack of attachment only (Skoufias et al., 2001; West et al., 2002). Importantly, Klp5 and Klp6 localise to the outer repeats of centromeres in metaphase, as determined by ChIP analysis (Garcia et al., 2002a), suggesting that controlled destabilisation of microtubules at this region may be important for generating tension in metaphase.

Localisation of Klp5 to centromeres is dependent on the microtubule binding protein Alp14 (Garcia et al., 2002b). Alp14 and Dis1, both members of the Dis1/TOG MAPs (microtubule associated protein) family, localise to centromeres in mitosis and are thought to stabilise microtubules to act in opposition to Klp5 and Klp6 (Garcia et al., 2001; Nakaseko et al., 2001; Garcia et al., 2002b). Dis1 had previously been found to be essential for sister chromatid separation and was found to bind to both interphase microtubules and the mitotic spindle (Ohkura et al., 1988; Nabeshima et al., 1995; Nakaseko et al., 1996; Nabeshima et al., 1998). Dis1 and Alp14 both bind to microtubules *in vitro* and whilst Dis1 localises to the central core region of the

centromere independently of microtubules, Alp14 localises to the inner and outer repeats for which it depends on microtubules (Garcia et al., 2001; Nakaseko et al., 2001). Recently work by T. Toda and colleagues has shown that localisation of Alp14 in mitosis is dependent on the TACC family protein Alp7 and that this is a partially mutual relationship because Alp14 is required to load Alp7 to the spindle but not SPBs (Sato et al., 2004). One can imagine a balance between microtubule destabilisation and stabilisation as being the force that establishes tension between sister chromatids. Indeed, the importance of this is evident in the observation that double mutants between Δ*alp14* or Δ*dis1* and either Δ*klp5* or Δ*klp6* are lethal (Garcia et al., 2002b), presumably due to an inability to establish bi-orientation.

1.5.2.2 The DASH complex

Balanced forces at the kinetochore may establish the tension required to allow anaphase to proceed but if an improper attachment occurs tension will not be balanced and a mechanism must exist to correct such an event. Although many proteins at the kinetochore known to be important for chromosome segregation have been described in fission yeast, how these kinetochore-microtubule attachments are actively regulated has not yet been determined. However, as described in section 1.4.6.2, recent studies in *S. cerevisiae* have suggested that phosphorylation of the outer kinetochore-associated microtubule binding protein Dam1, part of a protein complex termed the DASH complex, is important for correcting improper attachments (Cheeseman et al., 2002).

The first member of the DASH complex was identified in a screen for genes that when overexpressed, caused cell death and morphological abnormalities suggesting cytoskeletal defects. This was named Duo1 (Death Upon Overproduction) (Hofmann et al., 1998). The next member of the complex, Dam1 (Duo1 and Mps1 interacting factor), was captured in a 2-Hybrid screen using Duo1 as bait and independently isolated in a screen for mutants that were lethal in combination with a mutant of the SAC protein Mps1 (Hofmann et al., 1998; Jones et al., 1999). Dam1 and Duo1 localise to the mitotic spindle and kinetochore and cells expressing mutant proteins have spindle defects and a high rate of chromosome mis-segregation (Hofmann et al., 1998; Jones et al., 1999; Cheeseman et al., 2001b; Jones et al., 2001). These mis-segregation events are at a similar frequency to that of Ipl1 mutants (which fail to bi-orient sister chromatids) but contrasted with mutants in the kinetochore protein gene *ndc10* (which fail to segregate chromosomes at all) (Goh and Kilmartin, 1993; Biggins et al., 1999; Kim et al., 1999). This suggests that Dam1 mutants can establish microtubule-kinetochore attachments but

are unable to regulate them. Subsequently, more DASH complex proteins were identified, namely Dad1 (Duo1 and Dam1 interacting) (Enquist-Newman et al., 2001), Spc34 and Spc19 (Wigge and Kilmartin, 2001), Ask1, Dad2, Hsk1, Dad3, Dad4, Hsk2 and Hsk3 (Cheeseman et al., 2001a; Cheeseman et al., 2002; Janke et al., 2002; Li et al., 2002; Li et al., 2005). All these proteins are essential to budding yeast and mutants in any of the sub-units have similar mitotic spindle and chromosome segregation defects indicating an inability to generate bipolar microtubule-kinetochore attachments (Goh and Kilmartin, 1993; Biggins et al., 1999; Kim et al., 1999; Cheeseman et al., 2001a; Cheeseman et al., 2002; Janke et al., 2002; Li et al., 2002; Li et al., 2005). Importantly, phosphorylation of DASH complex subunits by the Aurora kinase Ipl1 appears to be important for regulating microtubule-kinetochore attachments and affects DASH complex interaction with the rest of the kinetochore, suggesting a mechanism for regulating chromosome bi-orientation (Cheeseman et al., 2002; Shang et al., 2003).

1.6 Aims of this thesis

The aim of this thesis was to characterise further the regulation of Cdc2/Cdc13 at the G2-M transition. As described in this introduction, much is known about the regulation of Cdc2/Cdc13 in response to cellular insults such as DNA damage and osmotic stress. It has, however, been demonstrated that the activity of Cdc2/Cdc13 is inhibited at the G2-M transition following microtubule damage (Alfa et al., 1990) but the mechanism by which this effect is imposed is unknown. This thesis begins by describing an approach to understand this effect and asks the following questions - is the inhibition of Cdc2 in response to microtubule damage due to a known checkpoint and if not then how might this effect be imposed?

Despite the requirement for Cdc2 to initiate mitosis, relatively little is known of its mitotic substrates. In this thesis, I investigate further the role of Cdc2/Cdc13 in mitosis, with particular emphasis on microtubule stability, by exploiting the TBZ sensitivity of a *cdc13-117* mutant strain to perform a screen designed to isolate other proteins involved in this process. One of these proteins is characterised to determine its role in microtubule dynamics and chromosome segregation in mitosis. The initial characterisation is presented of a protein complex previously unidentified in fission yeast.

Chapter 2

Materials and Methods

2.1 Yeast Techniques

2.1.1 Yeast Strains

A full list of *S. pombe* and *S. cerevisiae* strains used in this study is given in Table 2.1.

2.1.2 Yeast Media

All media were autoclaved at 120°C 15 p.s.i. for 10min.

Selective media – Edinburgh minimal media (EMM): 0.3% KH phthalate, 0.22% Na_2HPO_4, 0.5% NH_4Cl, 2% glucose, 20ml/l salts, 1ml/l vitamins, 0.1ml/l minerals (as detailed in Moreno *et al.*, 1991).

EMM agar: EMM plus 2% Bacto agar.
Glucose and EMM stocks were added separately after autoclaving. Additional nutritional supplements (histidine, uracil, adenine and leucine) were added to the media as required from sterile stocks of 10mg/ml to a final concentration of 100mg/l.

Rich media – Yeast extract plus supplements (YES): 0.5% Oxoid yeast extract, 2% glucose, 100mg/l adenine.

YES agar: YES plus 2% Bacto agar.
Glucose and adenine were added separately after autoclaving.

2.1.3 Yeast growth conditions

Liquid cultures were grown in a gyratory shaker at 200r.p.m. Standard growth temperature for wild type strains was 30°C. Temperature sensitive strains were grown at a permissive temperature of 25°C and arrested at a restrictive temperature of 36.5°C. Cold sensitive strains were grown at 28°C and arrested at a restrictive temperature of 18°C. Cells on agar plates were incubated in a constant temperature incubator. For

Table 2.1 Strains used in this study.

Strain	Genotype	Source
JM100	*leu1-32 ura4-D18 h+*	Lab stocks
JM109	*leu1-32 ura4-D18 h-*	Lab stocks
JM1590	*cdc25-22 leu1-32 ade6-216 h+*	Lab stocks
JM1591	*cdc25-22 leu1-32 ade6-210 h-*	Lab stocks
JM2602	*cdc25-22 cdc13-117 cdc13-gfp(LEU2) leu1.32 ura4-D18 h+*	Lab stocks
JM2580	*cdc13-117 cdc13-gfp(LEU2) leu1.32 ura4-D18 h-*	Lab stocks
JM2608	*cdc11-cfp:kanR leu1.32 ura4-D18 h+*	Lab stocks
JM2710	*cdc11-cfp:kanR cdc13-117 cdc13-gfp(LEU2) leu1.32 ura4-D18*	This study
JM1019	*nda3-KM311 leu1.32 h+*	
JM2706	*cdc13-117 cdc13-gfp(LEU2) nda3-KM311 leu1.32 h-*	This study
JM1696	*mad2::ura4 leu1.32 ura4-D18 h-*	T. Matsumoto
JM2465	*cdc25-22 mad2::ura4 leu1.32 ura4-D18*	Thus study
JM2323	*bub1::ura4 leu1.32 ura4-D18 h-*	K. Hardwick
JM2468	*cdc25-22 bub1::ura4 leu1.32 ura4-D18*	This study
JM1272	*rad3::ura4 leu1.32 ura4-D18 ade6-704 h-*	
JM1602	*cdc25-22 rad3::ura4 leu1.32 ura4-D18*	This study
JM2657	*dma1::kanR leu1.32 ura4-D18 h-*	This study
JM2687	*cdc25-22 dma1::kanR leu1.32 ura4-D18 h-*	This study
JM2976	*srk1::kanR leu1.32 ura4-D18 h-*	R. Aligue
JM2980	*cdc25-22 srk1::kanR leu1.32 ura4-D18 h-*	This study
JM2867	*cdc13-117 leu1.32 ura4-D18 ade6-210 h+*	Lab stocks
JM2868	*cdc13-117 leu1.32 ura4-D18 ade6-210 h-*	Lab stocks
JM2769	*cdc13-117 cdc11-gfp:kanR leu1.32 ura4-D18 h-*	This study
JM2591	*ndc80-gfp:kanR leu1.32 ura4-D18 h-*	Lab stocks
JM2878	*cdc13-117 ndc80-gfp:kanR cdc11-cfp:kanR leu1.32 ura4-D18*	This study
JM2763	*ndc80-gfp:kanR cdc11-cfp:kanR leu1.32 ura4-D18 h-*	Lab stocks
JM2800	*his7:gfp-lacI lys1:lacO leu1.32 ura4.D18 ade6-210 h-*	M. Yanagida
JM2900	*cdc13-117 his7:gfp-lacI lys1:lacO leu1.32 ura4-D18 cdc11-cfp:kanR h+*	This study

JM2790	*leu1.32 ura4-D18 ade6-210 h- Ch16 ade6-216 (bub1::ura4)*	J.P. Javerzat
JM2908	*cdc13-117 leu1.32 ura4-D18 ade6-210 h+ Ch16 ade6-216 (bub1::ura4)*	This study
JM2881	*dam1::kanR leu1.32 ura4-D18 h-*	This study
JM2899	*dam1(1-127):kanR leu1.32 ura4-D18*	This study
JM2959	*dam1-gfp:kanR leu1.32 ura4-D18 h+*	This study
JM2863	*dam1(1-127)-gfp:kanR leu1.32 ura4-D18 h+*	This study
JM2666	*ndc80-cfp:kanR leu1.32 ura4-D18 h-*	This study
JM2940	*ndc80-cfp:kanR dam1-gfp:kanR leu1.32 ura4-D18 h-*	This study
JM2880	*ndc80-cfp:kanR dam1(1-127)-gfp:kanR leu1.32 ura4-D18*	This study
JM3057	*dam1-gfp:kanR cdc11-cfp:kanR leu1.32 ura4-D18 h+*	This study
JM2879	*dam1(1-127)-gfp:kanR cdc11-cfp:kanR leu1.32 ura4-D18*	This study
JM2938	*dam1::kanR leu1.32 ura4-D18 ade6-210 h- Ch16 (ade6-216 bub1::ura4)*	This study
JM3029	*dam1::hygR cdc13-117 cdc13-gfp(LEU2) leu1.32 ura4-D18 h-*	This study
JM2748	*mad2-gfp(LEU2) leu1.32 ura4-D18 h+*	T. Toda
JM2758	*mad2-gfp(LEU2) cdc11-cgp:kanR leu1.32 ura4-D18 h-*	This study
JM2967	dam1::hygR mad2-gfp(LEU2) cdc11-cgp:kanR leu1.32 ura4-D18 h-	This study
JM2811	*bub1-gfp:kanR leu1.32 ura4-D18 h-*	Lab stocks
JM2760	*bub1-gfp:kanR cdc11-cfp:kanR leu1.32 ura4-D18 h+*	This study
JM3082	dam1::hygR bub1-gfp:kanR cdc11-cfp:kanR leu1.32 ura4-D18 h+	This study
JM2565	*lys1:nmt1-atb2-gfp leu1.32 ura4-D18 h-*	Lab stocks
JM2565	*mad2::ura4 lys1:nmt1-atb2-gfp leu1.32 ura4-D18 h-*	This study
JM2887	*dam1::kanR lys1:nmt1-atb2-gfp leu1.32 ura4-D18*	This study
JM3043	dam1::kanR mad2::ura4 lys1:nmt1-atb2-gfp leu1.32 ura4-D18 h+	This study
JM3053	*cdc25-22 dam1-3ha:kanR leu1.32 ura4-D18*	This study
JM3055	*cdc25-22 dam1(1-127)-3ha:kanR leu1.32 ura4-D18*	This study
JM2689	*cut11-cfp:kanR leu1.32 ura4-D18 h-*	J.R. McIntosh

	dam1-gfp:kanR cut11-cfp:kanR leu1.32 ura4-D18	This study
JM2971	*nmt1-gfp-dam1:kanR leu1.32 ura4-D18 h-*	U. Fleig
JM2949	*duo1::hygR leu1.32 ura4-D18 h-*	This study
JM3016	*spc34::hygR leu1.32 ura4-D18 h-*	This study
JM2948	*ask1::hygR leu1.32 ura4-D18 h-*	This study
JY741DH2	*hos2::ura4 leu1.32 ura4-D18 ade6-216 h-*	H. Aiba
JY741DH3	*hos3::ura4 leu1.32 ura4-D18 ade6-216 h-*	H. Aiba
JM3075	dam1-gfp:kanR duo1::hygR cdc11-cfp:kanR leu1.32 ura4-D18 h-	This study
JM3077	*dam1-gfp:kanR ask1::hygR cdc11-cfp:kanR leu1.32 ura4-D18 h-*	This study
JM3049	*dam1-gfp:kanR spc34::hygR cdc11-cfp:kanR leu1.32 ura4-D18 h+*	This study
JM3005	*ask1::hygR leu1.32 ura4-D18 h- ask:(leu1)*	This study
JM3031	*ask1::hygR leu1.32 ura4-D18 h- ask1(AAA):(leu1)*	This study

selective growth of antibiotic resistant strains, G418 (Invitrogen) or hygromycin (Sigma) was added at 100μg/ml, to YES agar plates.

2.1.4 Yeast storage conditions

Yeast strains were stored in 70% YES; 15% glycerol at –80°C.

2.1.5 Cell cycle synchronisation methods

2.1.1.1 cdc25-22 temperature block and release

The mutant strain *cdc25-22* was grown at 25°C to a density of 5×10^6 cells/ml in YES media, before being shifted to the restrictive temperature of 36.5°C for 4 hours, then cooled rapidly to the permissive temperature of 25°C.

Cell samples for biochemical analysis were collected at regular intervals by centrifugation (3200r.p.m. for 2min in a IEC Centra MP4 bench top centrifuge). 25ml samples were taken for each time-point, centrifuged, then washed in phosphate buffered saline (PBS: 140mM NaCl, 3.3 mM KCl, 10mM Na_2HPO_4 and 1.8mM KH_2PO_4), and the samples frozen on dry ice and stored at –80°C.

Cell samples for microscopic analysis were taken at regular time-points and processed as described in Section 2.2.

2.1.1.2 Hydroxyurea block and release

Cell cultures were grown to a density of 5×10^6 cells/ml in YES media. Hydroxurea (Sigma) was added at 11mM, and the culture incubated at the growth temperature for 4 hours. After 4 hours the culture was centrifuged (3200r.p.m. for 2min in a bench top centrifuge), washed once in fresh YES media, then resuspended in YES media at a density of 5×10^6 cells/ml.

Cell samples for biochemical or microscopic analysis were collected as described in section 2.1.5.1

2.1.1.3 Lactose gradient

A YES lactose gradient was made by loading a Fisherbrand gradient mixer with 10% lactose YES and 40% lactose YES to make a YES lactose gradient in a 50ml centrifuge tube with 10% lactose at the top, and 40% lactose at the bottom. Cell cultures were

grown to a density of 5×10^6 cells/ml in YES or EMM selective media as required. 50ml cell cultures were chilled on ice, and then collected by centrifugation (3200r.p.m. for 2min in a bench top centrifuge). Cell pellets were resuspended in 0.5ml YES and introduced carefully to the top of the YES lactose gradient, which was then centrifuged (1300r.p.m. at 4°C for 7min in a Sorvall bench top centrifuge). Using a syringe, 3ml samples were carefully extracted from the top of a broad band of cells that collected at the centre of the gradient during centrifugation. These cells were flushed into 40ml of ice-cold YES media before being harvested by centrifugation (3200r.p.m. for 2min in a bench top centrifuge) and resuspended in 3ml fresh YES media. Cell samples for microscopic analysis were collected at regular intervals and processed as described in Section 2.2.

2.1.6 Yeast transformations

2.1.6.1 Lithium Acetate (LiAc)

Cells were grown to a density of 5×10^6 cells/ml. 100ml of cells were harvested by centrifugation (3200r.p.m. for 2min in a bench top centrifuge), washed twice in distilled H_2O and resuspended in 500µl of TE/LiAc (0.1M LiAc/ 1×TE(10mM Tris/HCl pH8.0 and 1mM EDTA). To 100µl of LiAc cell suspension was added 10-20µg of transforming DNA and 100µg of sterile sonicated single stranded salmon sperm carrier DNA. 1200µl of 40% PEG_{3350}TE LiAc was added and the mix incubated at 30°C with agitation for 30min. Cells were then heat shocked at 42°C for 15min and harvested by centrifugation (13000r.p.m. for 10s in a bench top Heraeus Biofuge Pico microcentrifuge) before being resuspended in 200µl TE and spread onto the appropriate selective EMM agar plates. To select for antibiotic resistance, cells were initially spread onto YES agar plates and after 24h incubation at 30°C, replica plated to YES agar plates containing G418 or hygromycin.

2.1.6.2 Electroporation

Cells were grown to a density of 5×10^6 cells/ml. 50ml of cells were chilled for 15-30min then harvested by centrifugation at 4°C (3200r.p.m. for 2min in a bench top centrifuge), the washed twice in ice-cold distilled H_2O before being resuspended to a final concentration of $1\text{-}5\times10^9$ cells/ml in ice cold 1M sorbitol. 40µl of the cell suspension was added to 100ng of transforming DNA in chilled microcentrifuge tubes and incubated for 5min. The DNA and cells were transferred to chilled 0.2cm electrocuvettes (Biorad) and pulsed using a Bio-Rad Gene Pulser™ (Bio-Rad, UK)

(25μF, 200Ω, 2.5kV). 900μl of ice-cold 1M sorbitol was immediately added to the cells, which were then plated out onto the appropriate agar plates as described in section 2.1.6.1.

2.1.7 Isolation of yeast genomic DNA

A freshly growing yeast colony was resuspended in 200μl of STET buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 100mM Tris pH8.0, 1mM EDTA) in a microtube (Alpha, UK). 200μl of phenol-chloroform-isoamyl alcohol (25:24:1) was added, and glass beads (Biospec Products Inc, UK) added to the level of the meniscus. The cells were lysed in a Hybaid ribolyser (3x speed 4; 10s; chilled on ice between pulses). The lysate/phenol mix was collected by piercing the bottom of the sample tube with a needle, and collecting by into a fresh tube by centrifugation (2000r.p.m for 30s in a bench top centrifuge). The aqueous layer was then separated by centrifugation (13000r.p.m. for 10min in a benchtop microcentrifuge) and transferred to a fresh tube. To this was added ammonium acetate to a final concentration of 0.3M and 2.5 volumes of 100% Ethanol and the sample chilled on ice for 15min. The DNA was pelleted by centrifugation (13000r.p.m. for 10min in a benchtop microcentrifuge) and the pellet washed with 500μl of 70% ethanol. The tube was centrifuged again (13000r.p.m. for 5min in a benchtop microcentrifuge), and the DNA pellet dried in a 37°C incubator for 15min. The DNA pellet was resuspended in 50μl of water and 1μl used in a 50μl PCR reaction.

2.1.8 Mating

Equal quantities of freshly growing cells of opposite mating types were mixed with a toothpick on an EMM agar plate containing all nutrients and incubated at 28°C for 2-3 days. The formation of asci was monitored microscopically.

2.1.8.1 Random spore analysis

A small amount of the mating mix was resuspended in 1ml dH_2O, 0.5% Glucuronidase (Sigma), and incubated overnight at RT on a rotary wheel to digest asci. Spore numbers were estimated using a Thoma haemocytometer (Assistent, Germany) and approximately 500 spores were spread onto YES agar plates and incubated at 28°C. After 3-4 days, the colonies were replica plated to selective agar plates containing the appropriate nutritional supplements or antibiotics.

2.1.8.2 Tetrad dissection

Using a micromanipulator (Singer MSM), mature asci were placed in a line 3mm apart on a YES agar plate. The asci were left at 37°C for 3-4 hours or at 28°C overnight to allow the asci walls to break down. The 4 pores from each asci were then micromanipulated into a line 3mm apart. The spores were incubated at 28°C. After 3-4 days, the colonies were replica plated to selective agar plates containing the appropriate nutritional supplements or antibiotics.

2.2 Microscopy

Live analysis of cells was performed in an imaging chamber (CoverWell PCI-2.5, Grace Bio-labs) filled with 1ml of 1% agarose in minimal media and sealed with a 22×22mm glass coverslip. Constant temperature conditions were maintained using a full enclosure incubation chamber (Solent Scientific Ltd., UK). Fluorescent microscopy was performed on a Deltavision Spectris system containing a photometrics CH350L liquid cooled CCD camera and Olympus IX70 inverted microscope with a 100× objective. Stacks of images 0.3μm apart were acquired and processed using the deltavision data collection system (Applied Precision, Issaquah, WA, USA).

2.2.1 (4'6-diamino-2-phenylindole) DAPI and calcafluor staining

Cells were fixed in 70% ethanol and stored at 4°C until required. Fixed cells in ethanol were pelleted by centrifugation (3200r.p.m. for 2min in a bench top microcentrifuge) and rehydrated in dH_2O. 2μl of rehydrated cells were spread onto a microscope slide to form a monolayer of cells, and allowed to dry at room temperature. 1.5μl of DAPI plus calcafluor (1.5μg/ml DAPI in Vectashield© mounting medium (Vector laboratories, Burlingame, CA, USA) and 50μg/ml Calcafluor fluorescent brightener (Sigma)) was dotted onto the dried cells and gently sealed using a glass coverslip.

2.2.2 Rhodamine/phalloidin actin staining

Exponentially growing cells were fixed in an equal volume of formaldehyde-PM buffer (40% of 16% electronmicroscopy grade methanol free formaldehyde (TAAB Ltd., UK) and 60% Potassium phosphate magnesium sulphate buffer (PM: 35mM KPO_4 pH6.8 0.5mM $MgSO_4$)) for 30min at growth temperature. Cells were then pelleted by centrifugation (3200r.p.m. for 2min in a benchtop microcentrifuge) and washed 3 times in PM buffer, before being permeabilised in 1% Triton X-100 PM buffer for 1min, then

pelleted again by centrifugation (3200r.p.m. for 2min in a benchtop microcentrifuge) and washed a further 3 times in PM buffer. The cell pellet was finally resuspended in 20µl of rhodamine/phalloidin (Cambridge Bioscience, UK; stored in MeOH, reconstituted 1/100 in PM buffer) and incubated for 30min. 1.5µl of the suspension was spread on a microscope slide to form a monolayer of cells and allowed to dry at room temperature in the dark. 1.5µl of DAPI Vectashield© mounting medium was dotted on the surface of the cells and covered with a glass coverslip.

2.2.3 Antibody labelling of microtubules

10ml of 30% formaldehyde was made by adding 3g of paraformaldehyde to 10ml PEM Buffer (100mM Pipes, 1mM EGTA, 1mM $MgSO_4$ pH 6.9) and heating for 30min at 65°C. To this was added 120µl of 5M NaOH and the formaldehyde/buffer maintained at 65°C until almost all the formaldehyde had dissolved. The solution was cooled to RT and any undissolved formaldehyde pelleted by centrifugation (12,000 r.p.m. for 1min in a benchtop microcentrifuge). To fix cells, 0.125 volumes of 30% formaldehyde/PEM was quickly added to exponentially growing cells and mixed thoroughly. 30s to 1min later glutaraldehyde was added to a final concentration of 0.2% and the cells incubated at RT for 30-90min. To digest the cell walls, cells were pelleted by centrifugation (3200r.p.m. for 2min in a benchtop microcentrifuge) and washed three times in PEM buffer before being re-suspended in PEMS (PEM + 1.2M Sorbitol) + 1mg/ml Novozyme (Novo Biolabs, Denmark) + 0.3mg/ml 20T Zymolyase (ICN, UK) and incubated at 37°C for exactly 4min. Cells were again pelleted by centrifugation and washed three times in PEMS. Permeabilisation was done by incubating cells in PEMS + 1% Triton X 100 for 30s followed by 3 washes with PEMS buffer. Unreacted glutaraldehyde was quenched by incubating the cells 3 times in 500µl of sodium borohydride/PEM solution for 3-5min at RT. Cells were then washed twice in PEMS, resuspended in PEMBAL (PEM + 1% BSA, 0.1% NaN_3, 100mM lysine hydrochloride pH 6.9) and placed on a rotary wheel for 30min, then $1/10^{th}$ of the cell suspension in PEMBAL was transferred to a fresh tube and pelleted by centrifugation. The pellet was resuspended in 100µl of PEMBAL containing a 1:100 dilution of the tubulin antibody, tat1 (a gift from K. Gull), and cells incubated at RT overnight on a rotary wheel. Cells were again pelleted and washed three times in PEMS before being resuspended in 100µl of PEMBAL + 1% rhodamine conjugated anti-mouse antibody and incubated in the dark on a rotary wheel for 2-4h. Cells were pelleted by centrifugation, washed once in PEMBAL and once in PBS, before 1.5µl of the suspension was spread on a microscope

slide to form a monolayer of cells and allowed to dry at room temperature in the dark. 1.5µl of DAPI Vectashield© mounting medium was dotted onto the surface and covered with a glass coverslip.

2.2.4 Fixation of cells expressing green fluorescent protein (GFP) and cyan fluorescent protein (CFP)

Cells were fixed by addition of 0.1 volumes of 37% formaldehyde (Fisher Chemicals, UK) for 10min at growth temperature. Cells were pelleted by centrifugation (3200r.p.m. for 2min in a benchtop microcentrifuge) and washed 3 times in PBS. 1.5µl of cells were spread on a microscope slide form a monolayer of cells and dried at room temperature. 1.5µl of DAPI Vectashield© mounting medium was dotted onto the surface and covered with a glass coverslip.

2.2.5 Phase contrast microscopy

Cells were concentrated by centrifugation (3200r.p.m. for 2min in a benchtop microcentrifuge), a drop placed onto a microscope slide, and a coverslip placed on top. Cells were viewed on a Labphot-2 microscope (Nikon, UK) and images acquired with an Olympus DP12 (Olympus, UK) digital camera.

2.3 Nucleic acid manipulation

2.3.1 Polymerase chain reaction (PCR)

PCR was routinely carried out using either an OmnE or Techne thermal cycler in accordance with the manufacturers instructions. For amplification of DNA for cloning, gene disruptions and tagging, the Expand High Fidelity system was used in accordance with the manufacturers instructions. Diagnostic PCRs were carried out using *Taq* polymerase (AB Technologies) according to the manufacturer's instructions. A typical PCR mix contained 1×PCR buffer (Expand PCR buffer or *Taq* PCR buffer), 1.5mM $MgCl_2$, 200µM dNTPs, 300nM of each primer, 0.1-250ng template DNA, and the recommended units of enzyme. Oligonucleotides were manufactured by Genosys or Oswel (see Table 2.2 for a full list of oligonucleotides used in this study).

Table 2.2 Primers used in this study

Restriction sites are underlined with their corresponding enzyme in brackets.

Ref. no.	Sequence (5'-3')	Function
913	CGTTTTTGTCTCTTGTTGCT	dma1 deletion 'w'
915	GTTTAAACGAGCTCGAATTCATCGATACTGTT ATTTTCAGTATGGTTA	dma1 deletion 'y'
916	TAAAAGAGACCAAGTAGCAC	dma1 deletion 'z'
917	CAATTCAGTTAAGAGTTCTGT	Confirming dma1 deletion
1070	GGATGATAATGTGAGTGA	dam1(1-12&) truncation and tagging 'w'
1071	GGGGATCCGTCGACCTGCAGCGTACGAATCG TGAGAAATAACACCAC	dam1(1-127) tagging 'x'
1072	GGGGATCCGTCGACCTGCAGCGTACGACTAA TCGTGAGAAATAACACCAC	dam1(1-127) truncation 'x'
1073	GTTTAAACGAGCTCGAATTCATCGATTAAGC AAGGGAGACTGGTT	dam1(1-127) truncation and tagging 'y'
1074	TGAAGATTGGAGAAGCTATTA	dam1(1-127) truncation and tagging 'z'
1101	AAACCTTTACTAAGTCACTG	Confirming dam1(1-127) truncation and tagging
1089	CGTACTAAGATAGCGTCGG	dam1 deletion 'w'
1090	GGGGATCCGTCGACCTGCAGCGTACGAGTTT TCGCGACTTTGGGTGAG	dam1 deletion 'x'
1091	GTTTAAACGAGCTCGAATTCATCGATGCAAG GGAGACTGGTTGAAG	dam1 deletion 'y'
1092	GAAGATTGGAGAAGCTATTAC	dam1 deletion 'z'
1042	GCCACTGAAGTTGTAA	Confirming dam1 deletion
1093	GCAAATCAATGCTTTTTGCG	dam1 tagging 'w'
179	CGGATGTGATGTGAGAACTGTATCCTAGC	Confirming kanR integrations
1057	CGGCCTCCAGAAGAAGATGT	Confirming hygR integrations

1094	GGGGATCCGTCGACCTGCAGCGTACGATCTG GAAGCGGAATAGGTTT	dam1 tagging 'x'
1095	GTTTAAACGAGCTCGAATTCATCGATCAGCG AAGTGAACTGTAGAC	dam1 tagging 'y'
1096	CGCGGTACTTCCGAACAAAG	dam1 tagging 'z'
1081	GGTTGAGTGGTTTCACGAGTG	duo1 deletion 'w'
1082	GGGGATCCGTCGACCTGCAGCGTACGAGACT TCTAACCCAAACTCAC	duo1 deletion 'x'
1083	GTTTAAACGAGCTCGAATTCATCGATGTGTGC AATTTAAGCCTAAC	duo1 deletion 'y'
1084	GTAATGCACATAACTAACTCTTT	duo1 deletion 'y'
1047	GTATCAATGCATTTGCCAAATG	Confirming duo1 deletion
1077	GTATTGGGAGTAAGCCGATC	spc34 deletion 'w'
1078	GGGGATCCGTCGACCTGCAGCGTACGAGGAA GGCTATTTGCGTTGG	spc34 deletion 'x'
1079	GTTTAAACGAGCTCGAATTCATCGATCTGATG TAGCAGCAATAGAC	spc34 deletion 'y'
1080	GCAAGGAATGAACGCAATGC	spc34 deletion 'z'
1051	ACAAGCGACATTTCAATATGC	Confirming spc34 deletion
1085	GTTATTTGCTTTCTTAGTTT	ask1 deletion 'w'
1086	GGGGATCCGTCGACCTGCAGCGTACGAGCAC TGCTGAAGTTATCACC	ask1 deletion 'x'
1087	GTTTAAACGAGCTCGAATTCATCGATGGTGT AGGTATACTAGCGTCC	ask1 deletion 'y'
1088	TTATCAGGGTTCGTCAACTCC	ask1 deletion 'z'
1076	CAATGTTAGTTTCATAGATGGG	Confirming ask1 deletion
1255	GTACAGTCCGCTCCTAAGAA	ask1 mutagenesis forward T136A
1256	TTCTTAGGAGCGGACTGTAC	ask1 mutagenesis reverse T136A
1257	AGGATGCAGGCTCCGCTAAG	ask1 mutagenesis forward T163A
1258	CTTAGCGGAGCCTGCATCCT	ask1 mutagenesis reverse T163A

1259	GTGGAATGGCTCCACCTAA	ask1 mutagenesis forward S257A
1260	TTAGGTGGAGCCATTCCAC	ask1 mutagenesis reverse S257A
1261	AAAACTGCAGGAGGATGCTGGCAAAAGAC (PstI)	ask1 cloning into pJK148
1262	ACGCGTCGACCAAACTATTATTAACCAAGTC A (SalI)	ask1 cloning into pJK148
1271	ACTAGGATCCTTTTGGAAGCAGTTTTTTGAAG (BamHI)	ask1 cloning into pGEX
1268	TAGAAGATCTTCAGAGTTCCCAACTATCTT (BglII)	ask1 cloning into pGEX
387	GAATTCGAGCTCGGTACC	pUR19 sequencing
368	AAGCTTGCATGCCTGCAG	pUR19 sequencing
1263	TCCCGATGCAGCTGTACA	ask1 sequencing
1264	CGTGGAGTCACTTGTACTT	ask1 sequencing
1265	GCTTGCTGAGAAGAACCC	ask1 sequencing
1266	GAAACTCTTCGCATGCTCT	ask1 sequencing

2.3.2 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in 1% agarose (Invitrogen, UK) gels in 1xTAE (40mM Tris base, 1mM EDTA and 20mM glacial acetic acid, pH8.3) with TAE electrophoresis buffer. DNA was loaded with 1/6 volume DNA loading buffer (0.25% Bromophenol blue; 40% glycerol), and electrophoresed with a constant current of 60-100mA. DNA was stained by including ethidium bromide (Life Technologies, UK) in the gel at a final concentration of 0.5µg/ml, and visualised on a UV transilluminator, using 265nm wavelength light for analytical gels or 365nm wavelength light for preparative gels. The molecular size of DNA fragments was determined by comparison to DNA size markers (Invirtogen, UK).

2.3.3 Recovery of DNA fragments from agarose gels

DNA was extracted from 1% low melting point agarose (Invitrogen, UK) gel using a Geneclean II glass milk extraction kit (BIO 101, UK) according to the manufacturer's instructions.

2.3.4 Phenol chloroform extraction of DNA

DNA preparations were mixed with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), and the layers separated by centrifugation (13000r.p.m. for 2min in a benchtop microcentrifuge). The DNA was recovered in the aqueous layer, and then concentrated by ethanol precipitation.

2.3.5 Ethanol precipitation of DNA

DNA was precipitated from an aqueous solution by the addition of 0.1 volumes of 3M sodium acetate pH 5.3 and 2.5 volumes of 100% ethanol. The sample was then chilled on ice for 15min and the DNA pelleted by centrifugation (13000r.p.m. for 10min in a benchtop microcentrifuge). The DNA pellet was washed with 70% ethanol, dried in a 37°C incubator for 15min and resuspended in dH_5O.

2.3.6 Restriction endonuclease digestion and DNA modification

DNA was incubated with restriction endonucleases (New England Biolabs) and the appropriate buffers for 4 hours at 37°C. T4 DNA ligase (New England Biolabs) and calf intestinal phosphatase (Boehringer Mannheim) were used according to the

manufacturers' instructions. Ligation of DNA fragments was carried out in a 10µl volume of 1× ligation buffer (50mM Tris/HCl, pH7.5, 10mM $MgCl_2$, 10mM dithiothreitol (DTT), 1mM ATP and 25µg/ml bovine serum albumin (BSA)) and 0.4 unit of T4 DNA ligase. Ligations were incubated at room temperature for 4 hours, or overnight at 16°C before transformation into *E. coli.*

2.3.7 DNA sequencing

DNA sequencing reactions were performed using the Big Dye DNA sequencing kit (Applied Biosystems, UK) according to the manufacturers instructions. DNA sequencing was carried out using an ABI PRISM 377 DNA Sequencer (Applied Biosystems, UK). DNA sequences were analysed using DNAStar (DNAStar, USA).

2.4 Bacterial techniques

2.4.1 Bacterial strains

For routine bacteriological work, *Escherichia coli* strain DH5α (suppE44 Δlac U169 (ϕ80 lacZΔM15) hsdR17 endA1$^-$ thi-1 gyrA96 relA1) was used. For electroporation, commercial strains Electroblue (Stratagene, UK), or Genehogs (Invitrogen, UK) were used.

2.4.2 Bacterial media

All media were autoclaved at 120°C 15p.s.i. for 10min. *E. coli* strains were grown in Luria-Bertani broth (LB (1% Bacto-tryptone, 0.5% Bacto yeast extract, 1% NaCl, pH7.5)) with the addition of 100µg/ml ampicillin for the selection of plasmids.

LB Agar: LB with 2% Bacto agar (Difco, UK).

2.4.3 Bacterial growth conditions

Cells in liquid culture were incubated at 37°C in a gyratory shaker (New Brunswick) at 300r.p.m. Cells on agar plates were incubated at 37°C in a constant temperature incubator.

2.4.4 Storage of bacterial strains

Strains were grown to stationary phase in LB with ampicillin if necessary and stored at –70°C in 20% glycerol.

2.4.5 *E. coli* transformation

2.4.5.1 Preparation of competent cells

A 200µl aliquot of an overnight culture of *E. coli* DH5α was inoculated into 50ml of fresh LB medium and grown to an OD_{600} of 0.3-0.4 then cooled on ice, and harvested (2500r.p.m. for 7min at 4°C in a Beckman centrifuge). Cells were washed twice in 10ml $CaCl_2$ solution (60mM CaCl2; 15% glycerol; 10mM PIPES; pH 7.0) before being resuspended in 2ml $CaCl_2$ solution and either used immediately or stored at –70°C in 100µl aliquots.

2.4.5.2 Transformation of E. coli

A 100µl aliquot of competent cells with 1-5µg transforming DNA was incubated on ice for 10min, then heatshocked at 42°C for 2min. 1ml of LB was added and the cells incubated at 37°C for 1h. An aliquot of cells was then plated onto selective LB agar plates.

2.4.5.3 Preparation of electrocompetent E. coli

A 5ml overnight culture of DH5α cells was inoculated into 500ml of fresh LB medium and grown to an OD_{600} of 0.4, then cooled on ice and harvested (2500r.p.m. for 5min at 4°C in a Beckman centrifuge), washed once with 0.5 volumes ice-cold water before being resuspended in 10% ice-cold glycerol. Electrocompetent cells were used immediately or stored at –70°C in 100µl aliquots.

2.4.5.4 Electrotransformation of E. coli

A 40µl aliquot of cells together with 1µg of transforming DNA was incubated on ice for 10min then transferred to a chilled 0.2cm electrocuvette (Bio-Rad, UK) and pulsed using a Bio-Rad Gene Pulser™ (25µF, 2.5kV, 200Ω). Immediately following the pulse, 900µl of fresh LB medium was added and the cells were incubated at 37°C for 1h before being plated onto LB agar containing 100µg/ml ampicillin.

2.5 Biochemical techniques

2.5.1 Preparation of crude yeast extract

Cell pellets were resuspended in 200µl Homogenisation Buffer (HB: 25mM MOPS pH7.2; 60mM β-glycerophosphate; 15mM EGTA; 15mM $MgCl_2$; 1mM DTT; 0.2mM Na-orthovanadate; 1% Triton X-100; 200mM NaCl; 15mM para-nitropheyl phosphate; 20µg/ml each of leupeptin, aprotonin, and pepstatin A; 0.5mM PMSF) and transferred to a chilled ribolyser tube on ice. Glass beads were added to just above the meniscus, and the cells lysed in a Hybaid ribolyser (3 x 10s; speed 10; kept on ice between pulses). The lysed cells were collected by piercing the bottom of the sample tube with a needle, and collecting by into a fresh tube by centrifugation (2000r.pm. for 30s at 4°C in a benchtop centrifuge), then the cell debris was pelleted by centrifugation (13000r.p.m. for 10min at 4°C in a benchtop microcentrifuge). The supernatant was taken and the protein content estimated using Bradford reagent (Bio-Rad) according to the manufacturer's instructions.

2.5.2 Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were denatured by boiling for 3min in an equal volume of 2× SDS gel-loading buffer (100mM Tris-HCl pH6.8, 200mM dithiothreitol, 4% SDS, 0.2% bromophenol blue and 20% glycerol) and run on 12% vertical SDS-PAGE gels using SDS-PAGE running buffer (25mM Tris/Hcl, pH8.3, 200mM glycine and 0.1% SDS). 16cm long gels were used and run at either 250V for 4h or at 35V overnight.

2.5.3 Coomassie-blue staining of proteins

SDS-PAGE gels were incubated in Coomassie-blue stain (46% methanol, 8% acetic acid and 0.25g/l Coomassie brilliant blue) for 1h at room temperature with constant agitation. Gels were destained in strong destain (50% methanol, 10% acetic acid) for 3-5h at room temperature with constant agitation. After destaining gels were either kept in low destain (5% methanol, 7% acetic acid) till further use, or dried using a Bio-Rad GelAir dryer system according to the manufacturer's instructions.

2.5.4 Western-blot analysis

Proteins were transferred from an SDS-PAGE gel to Immobilon™-P nitrocellulose membrane (Millipore, UK) using a semi-dry transfer cell (Bio-Rad) and transfer buffer

(39mM glycine, 48mM Tris/Hcl pH8.8, 20% methanol and 0.037% SDS) at 3mA/cm^2 for 1h. The membrane was blocked with PBS, 0.05% Tween, 5% Marvel dry milk powder for 1h at room temperature with constant agitation. After blocking the membrane was immersed in PBS with 0.05% Tween, 2% Marvel and the primary antibody at the appropriate dilution overnight at 4°C with constant agitation before being washed for 3×15min in PBS with 0.05% Tween at room temperature. The appropriate dilution of secondary antibody was then made in PBS, 0.05% Tween, 1% Marvel and then added to the membrane which was incubated in this solution for 2h at room temperature with constant agitation. The membrane was washed 3×15min in PBS with 0.05% Tween, and the proteins detected using an enhanced chemiluminescence detection (ECL) (Amersham Pharmacia Biotec, UK) system according to the manufacturer's instructions.

2.5.5 Immunoprecipitation

Proteins were immunoprecipitated from 1ml of crude cell extract in HB buffer by the addition of 1:1000 dilution of anti-Cdc13 (a gift from P. Nurse) and the mix incubated for 4h on a rotary wheel at 4°C. 40µl of Protein A slurry (Protein A sepharose beads (Amersham Pharmacia Biotech, UK), washed 3 times with HB and resuspended in HB buffer as a 50% slurry) was added and the incubation continued for 1h. The Protein A beads-antibody complex was then washed 3 times with HB, excess buffer removed, and then used in a kinase assay.

2.5.6 Kinase assays

Proteins were immunoprecipitated as described in section 2.4.5. The Protein A beads-antibody complex was washed 2 times with kinase assay buffer (KAB: 25mM MOPS pH7.2, 1mM DTT, 10mM $MgCl_2$). Excess KAB was replaced with 30µl of KAB containing 50µM ATP, 1:1000 dilution of 0.1µCi/µl γ^{32}P-ATP and 0.1mg/ml Histone H1 and the mix incubated at 30°C for exactly 10min per sample. The samples were then prepared as described in section 2.4.2 and electrophoresed, dried and autoradiographed. The kinase activity was quantified using a phosphorimager Storm80 (Amersham International) and Image Quant software.

2.5.7 GST-Ask1 fusion protein purification

5ml of an overnight culture of DH5α carrying the plasmid pGEX-Ask1 or pGEX-Ask1(AAA) was inoculated into 500ml of LB containing ampicillin and grown to an OD_{600} of 0.4. Isopropyl β-$_D$-thiogalacto pyranoside (IPTG) was added to a final concentration of 0.1mM and the culture incubated at 25°C for a further 2h. Cells were harvested by centrifugation (8000r.p.m. for 10min at 4°C in a Beckman JA20 rotor) and either used immediately or snap frozen on dry ice and stored at –80°C. Cells were resuspended in 5ml of EBC buffer (140mM NaCl, 0.5% Nonidet P-40, 100mM NaF, 200μM sodium orthovanadate, 50mM Tris-HCl pH8.0, Complete protease inhibitor cocktail (Roche), 1mM PMSF) and lysed by sonication (3×10s, 95% power (Novara Group Ltd., UK)). Cell debris was removed by centrifugation (16,000r.p.m. for 30min at 4°C in a Beckman JA20 rotor) and the supernatant transferred to a fresh tube. GST-fusion proteins from the supernatant were bound to glutathione-sepharose (Hagemeier et al., 1993) by incubating for 1h at 4°C with 250μl of glutathione sepharose equilibrated in EBC buffer (Glutathione Sepharose 4B (Amersham Pharmacia Biotech)), then washed 6 times with NETN buffer (100mM NaCl, 1mM EDTA, 0.5% Nonidet P-40, 20mM Tris-HCl pH8.0). Bound proteins were then eluted for 20min in 250μl of elution buffer (100mM NaCl, 50mM Tris-HCl pH7.5, 20mM reduced Glutathione and 0.1% Triton) and the GST-fusion proteins separated by centrifugation (13000r.p.m. for 2min at 4°C in a benchtop microcentrifuge). Protein content was estimated on a Coomassie blue stained SDS-PAGE gel and by using Bradford reagent (Bio-Rad) according to the manufacturer's instructions. GST-fusion proteins were used either immediately as a substrate in a kinase assay (see section 2.4.6) or resuspended in 20% glycerol and stored at –80°C for further use.

2.6 Strain construction

2.6.1 Deletion of genomic loci

2.6.1.1 Deletion of the dma1 locus

The *dma1* locus was deleted using a high efficiency PCR based approach (Krawchuk and Wahls, 1999). In the first stage, using a genomic library as a template (Barbet et al., 1992), primer pairs 913(w) and 914(x), and 915(y) and 916(z) (Table 2.2) were used in two separate PCRs to amplify 250bp regions 5' and 3' of the *dma1* locus, The oligos 914(x) and 915(y) contained sequence of the 5' and 3' of the *kan*^{r} cassette respectively. The products from the first stage were purified by extraction from an agarose gel (see

section 2.3.3) and used in a second PCR together with primers 913(w) and 916(z) in excess, to amplify the *kan*^{r} cassette, using pFA6a-kanMX6 as a template (Bahler et al., 1998). The product was purified by extraction from an agarose gel and transformed into wild type haploid strain JM100 or 109 (Table 2.1). Stable integrants were selected on YES plus G418 agar plates and confirmed by PCR of genomic DNA using oligonucleotides 917 and 179 (Table 2.2). A schematic diagram of the technique used to delete genomic loci in this study is shown in Figure 2.1.

2.6.1.2 Deletion of the dam1 locus

The *dam1* locus was deleted exactly as described above, except using the primer pairs 1089(w) and 1090(x), and 1091(y) and 1092(z) and tested using oligonucleotides 1042 and 179 (Table 2.2).

2.6.1.3 Deletion of the ask1, duo1 and spc34 loci

The *ask1* locus was deleted as described above using the primer pairs 1085(w) and 1086(x), and 1087(y) and 1088(z) (Table 2.2), except using a HygR cassette as a template in the second stage PCR (Sato et al., 2005). Integrants were selected on YES plus hygromycin agar plates and confirmed by PCR of genomic DNA using oligonucleotides 1076 and 1057 (Table 2.2). The *duo1* locus was deleted exactly as described above, using the primer pairs 1081(w) and 1082(x), and 1083(y) and 1084(z), and tested using oligonucleotides 1047 and 1057 (Table 2.2). The *spc34* locus was deleted exactly as described above, using the primer pairs 1077(w) and 1078(x), and 1079(y) and 1080(z) and tested using oligonucleotides 1051 and 1057 (Table 2.2).

2.6.1.4 Deletion of the C-terminus of the dam1 locus

Exactly the same approach as described in section 2.5.1.1 was used to make a truncation of the *dam1* coding sequence by introducing a stop codon into the gene. The primers pairs used were 1070(w) and 1072(x), and 1073(y) and 1074(z) and tested using oligonucleotides 1101 and 179 (Table 2.2).

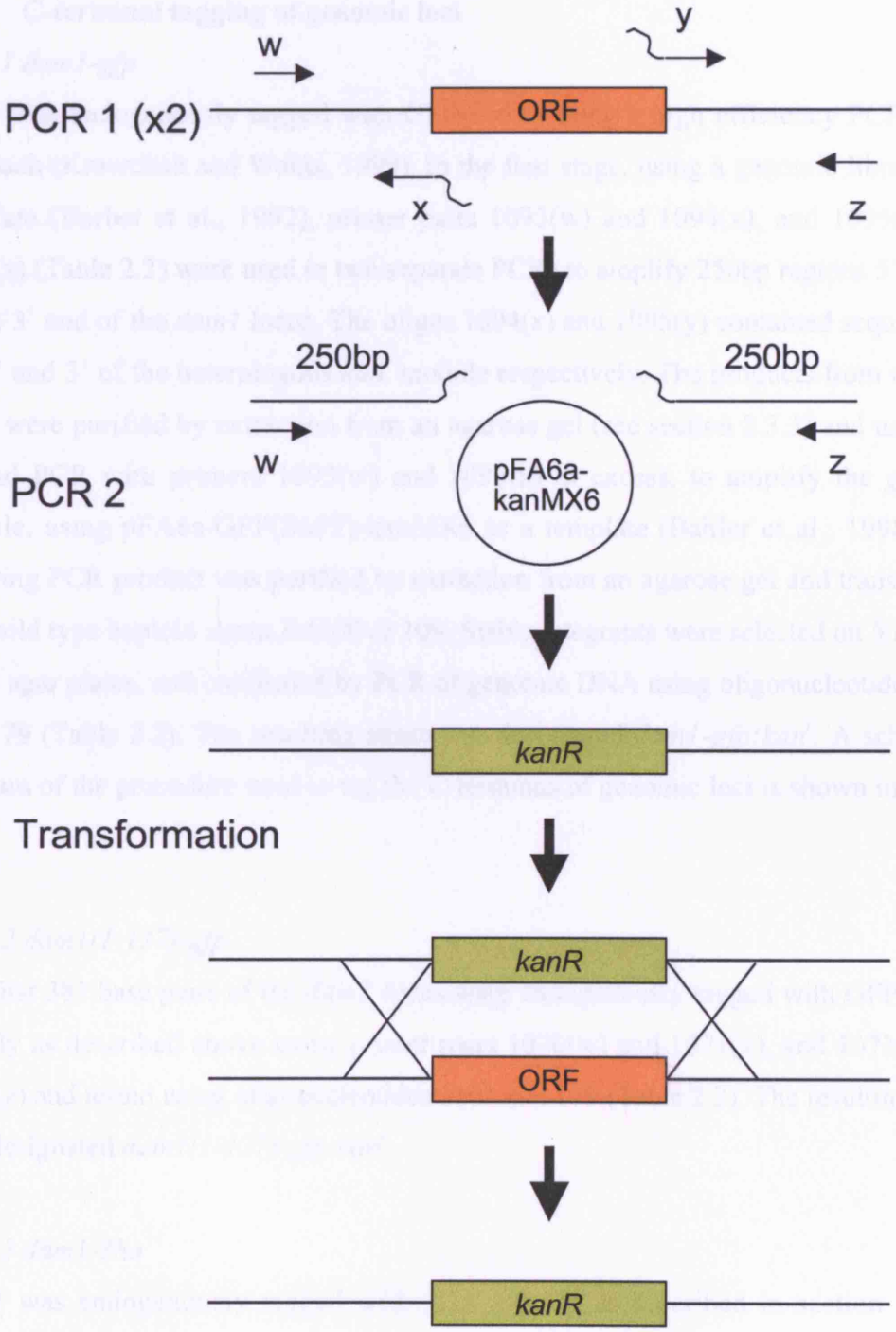


Figure 2.1 Deletion of genomic loci

Schematic diagram showing the PCR procedure used to delete genomic loci. See section 2.6.1 for full details.

2.6.2 C-terminal tagging of genomic loci

2.6.2.1 dam1-gfp

dam1 was endogenously tagged with GFP(S65T) using a high efficiency PCR based approach (Krawchuk and Wahls, 1999). In the first stage, using a genomic library as a template (Barbet et al., 1992), primer pairs 1093(w) and 1094(x), and 1095(y) and 1096(z) (Table 2.2) were used in two separate PCRs to amplify 250bp regions 5' and 3' of the 3' end of the *dam1* locus. The oligos 1094(x) and 1095(y) contained sequence of the 5' and 3' of the heterologous *kan*^{r} module respectively. The products from the first stage were purified by extraction from an agarose gel (see section 2.3.3) and used in a second PCR with primers 1093(w) and 1096(z) in excess, to amplify the *gfp-kan*^{r} module, using pFA6a-GFP(S65T)-kanMX6 as a template (Bahler et al., 1998). The resulting PCR product was purified by extraction from an agarose gel and transformed into wild type haploid strain JM100 or 109. Stable integrants were selected on YES plus G418 agar plates, and confirmed by PCR of genomic DNA using oligonucleotides 1101 and 179 (Table 2.2). The resulting strain was designated *dam1-gfp:kan*^{r}. A schematic diagram of the procedure used to tag the C-terminus of genomic loci is shown in Figure 2.2.

2.6.2.2 dam1(1-127)-gfp

The first 381 base pairs of the *dam1* locus were endogenously tagged with GFP(S65T) exactly as described above using primer pairs 1070(w) and 1071(x), and 1073(y) and 1074(z) and tested using oligonucleotides 1101 and 179 (Table 2.2). The resulting strain was designated *dam1(1-127)-gfp:kan*^{r}.

2.6.2.3 dam1-3ha

dam1 was endogenously tagged with 3HA exactly as described in section *2.5.2.1*, except that pFA6a-3HA-kanMX6 (Bahler et al., 1998) was used as a template to create the strain *dam1-3ha:kan*^{r}.

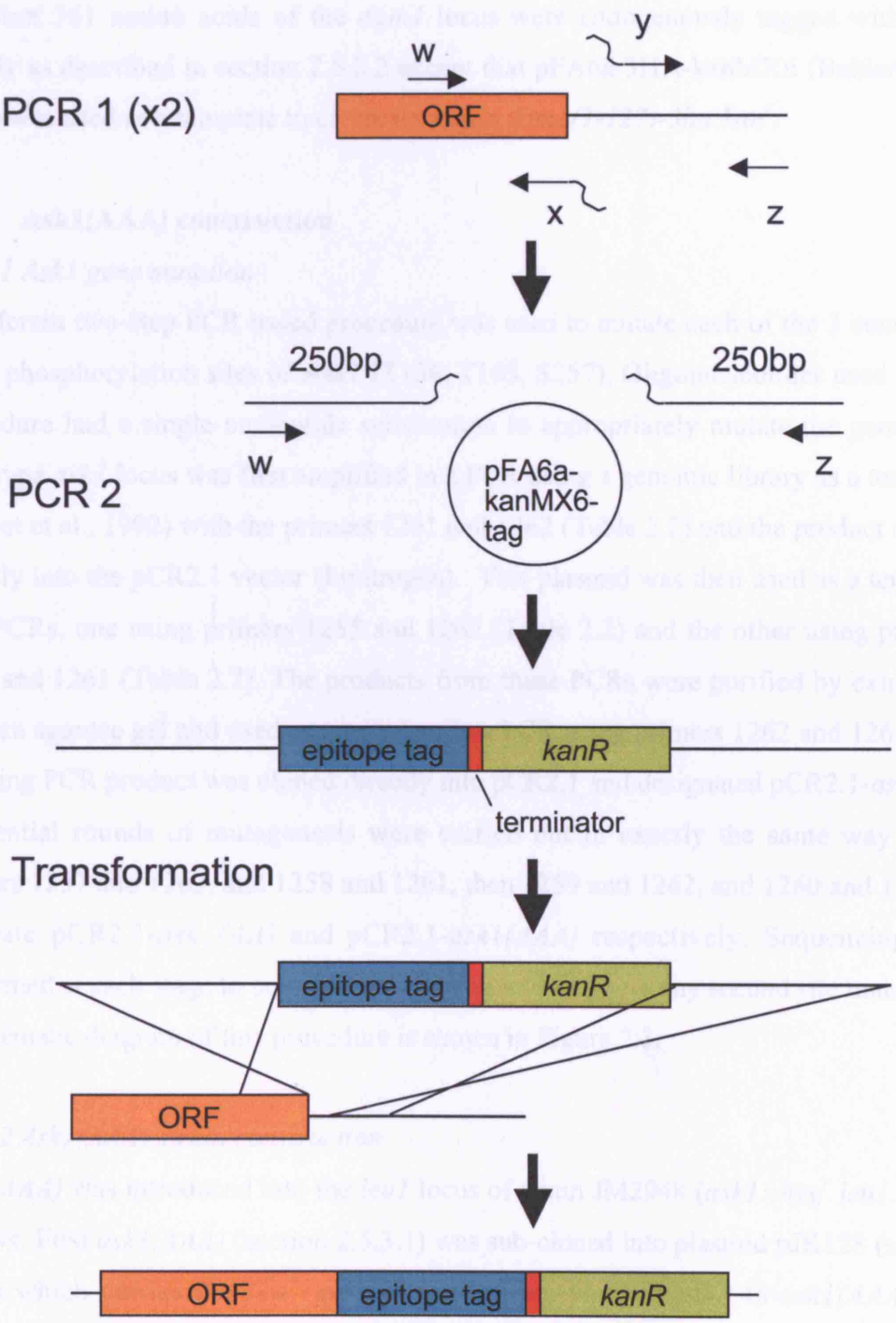


Figure 2.2 C-terminal tagging of genomic loci

Schematic diagram showing the PCR procedure used to enogenously place an epitope tag at the C-terminal codons of genomic loci. See section 2.6.2 for full details.

2.6.2.4 dam1(1-127)-3ha:kan^r

The first 381 amino acids of the *dam1* locus were endogenously tagged with 3HA exactly as described in section 2.5.2.2 except that pFA6a-3HA-kanMX6 (Bahler et al., 1998) was used as a template to create the strain *dam1(1-127)-3ha:kan^r*.

2.6.3 Ask1(AAA) construction

2.6.3.1 Ask1 gene mutation

A different two-step PCR based procedure was used to mutate each of the 3 consensus Cdc2 phosphorylation sites of Ask1 (T136, T163, S257). Oligonucleotides used in this procedure had a single nucleotide substitution to appropriately mutate the gene. The wild type *ask1* locus was first amplified in a PCR using a genomic library as a template (Barbet et al., 1992) with the primers 1261 and 1262 (Table 2.2) and the product cloned directly into the pCR2.1 vector (Invitrogen). This plasmid was then used as a template in 2 PCRs, one using primers 1255 and 1262 (Table 2.2) and the other using primers 1256 and 1261 (Table 2.2). The products from these PCRs were purified by extraction from an agarose gel and used as a template in a PCR using primers 1262 and 1261. The resulting PCR product was cloned directly into pCR2.1 and designated pCR2.1-*ask1(A)*. Sequential rounds of mutagenesis were carried out in exactly the same way using primers 1257 and 1262, and 1258 and 1261, then 1259 and 1262, and 1260 and 1261 to generate pCR2.1-*ask1(AA)* and pCR2.1-*ask1(AAA)* respectively. Sequencing was performed at each stage to confirm mutagenesis and to detect any second site mutations. A schematic diagram of this procedure is shown in Figure 2.3.

2.6.3.2 Ask1(AAA) strain construction

Ask1(AAA) was introduced into the *leu1* locus of strain JM2948 (*ask1::hyg^r leu1.32)* as follows: First *ask1(AAA)* (section 2.5.3.1) was sub-cloned into plasmid pJK128 (section 2.6.1) which carries the *leu$^+$* gene. The resulting plasmid pJK148-*ask1(AAA)* was linearised by cutting the unique *HindIII* site within the *leu$^+$* gene and the linear DNA transformed into JM2948 and *leu$^+$* transformants selected. This process was repeated with wild type *ask1*. A schematic diagram of this procedure is shown in Figure 2.4.

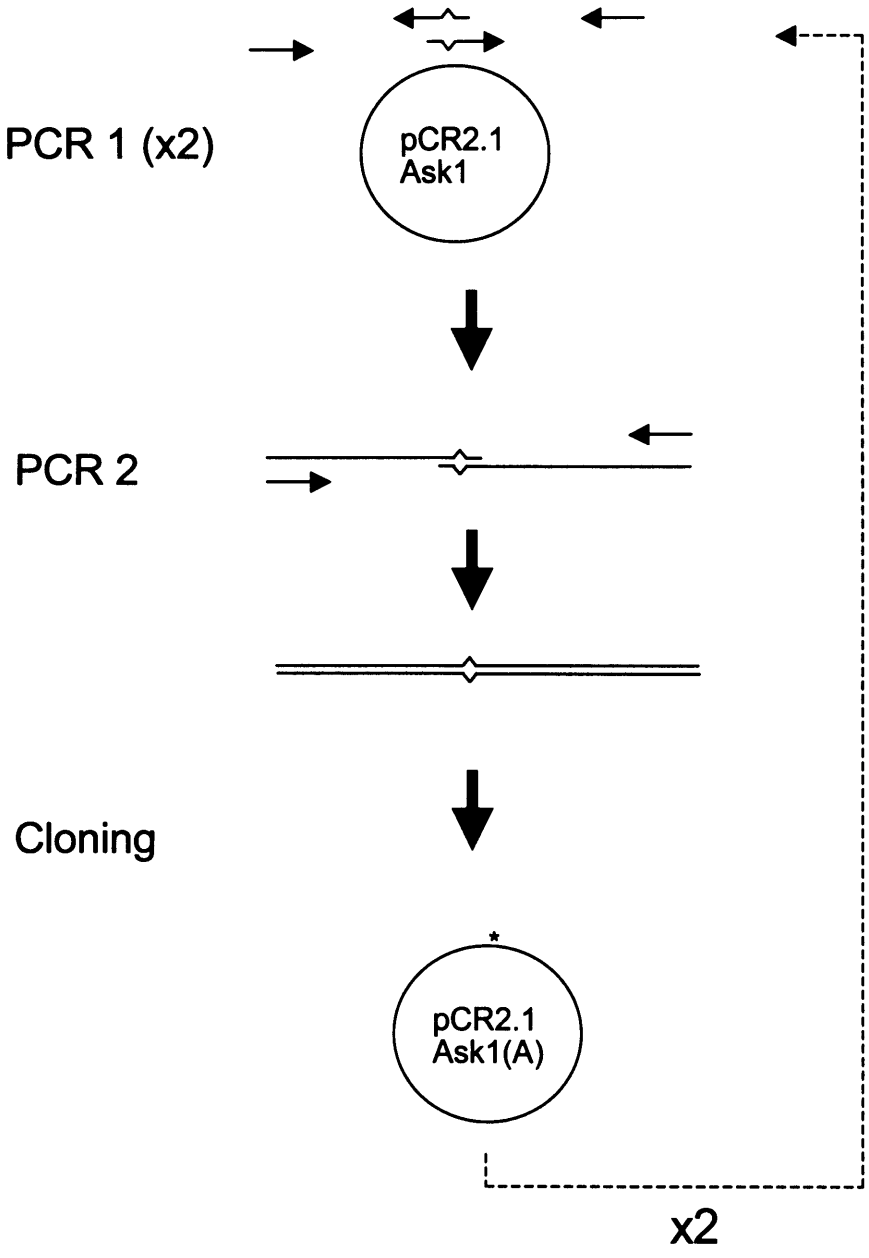


Figure 2.3 Ask1 mutagenesis

A schematic diagram showing the PCR based approach used to mutate the *ask1* gene. For details see section 2.6.3.1

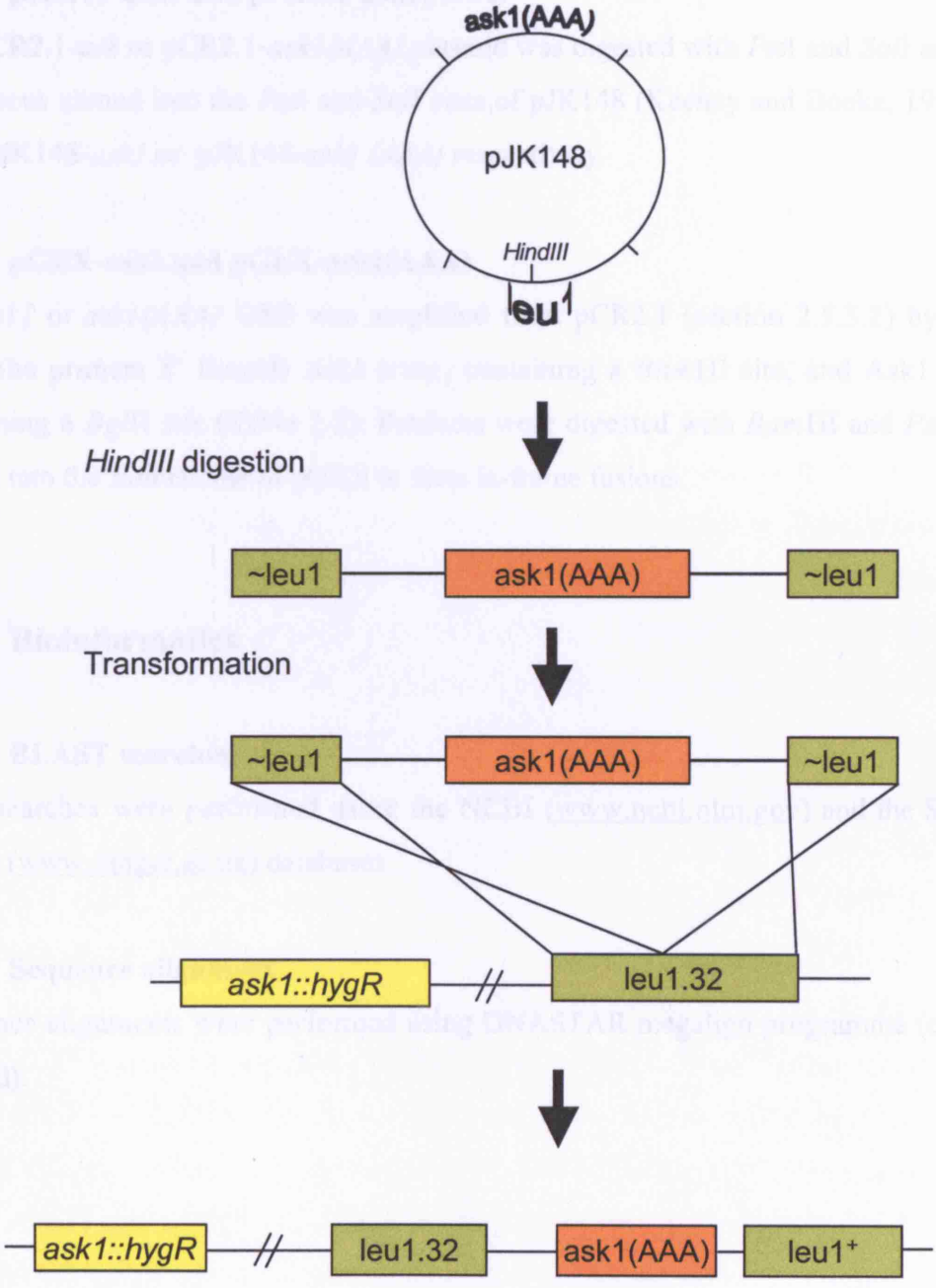


Figure 2.4 ask1(AAA) strain construction

A schematic diagram showing the integration of the linearised pCR2.1-ask1(AAA) at the leu1.32 locus of JM2948. For details see section 2.6.3.1

2.7 Plasmid construction

2.7.1 pJK128-ask1 and pJK148-ask1(AAA)

The pCR2.1-*ask* or pCR2.1-*ask1(AAA)* plasmid was digested with *Pst*I and *Sal*I and the *ask1* locus cloned into the *Pst*I and *Sal*I sites of pJK148 (Keeney and Boeke, 1994) to form pJK148-*ask1* or pJK148-*ask1 (AAA)* respectively.

2.7.2 pGEX-ask1 and pGEX-ask1(AAA)

The *ask1* or *ask1(AAA)* ORF was amplified from pCR2.1 (section 2.5.3.1) by PCR using the primers 5' BamHI Ask1 trunc, containing a *Bam*HI site, and Ask1 BglII containing a *Bgl*II site (Table 2.2). Products were digested with *Bam*HI and *Pst*I and cloned into the *Bam*HI site of pGEX to form in-frame fusions.

2.8 Bioinformatics

2.8.1 BLAST searches

Blast searches were performed using the NCBI (www.ncbi.nlm.gov) and the Sanger Centre (www.sanger.ac.uk) databases.

2.8.2 Sequence alignment

Sequence alignments were performed using DNASTAR megalign programme (cluster method).

Chapter 3

Inhibition of Cdc2 kinase activity at the G2 to M transition following microtubule damage.

3.1 Introduction

Cdc2 is required for the G1-S and G2-M transitions in fission yeast (Nurse et al., 1976; Nurse and Bissett, 1981). Protein levels of Cdc2 remain constant throughout the cell cycle whilst its protein kinase activity oscillates (Simanis and Nurse, 1986). Changes in Cdc2 activity are due to a combination of its periodic association with cyclin subunits and its phosphorylation state. The peak in Cdc2 activity at the G2 to M-phase transition drives cells into mitosis and is a result of association with Cdc13 (a homologue of Cyclin B) and its dephosphorylation (Gould and Nurse, 1989; Moreno et al., 1989). The activity of Cdc2 is kept low during G2 due to inhibitory phosphorylation of Tyr15 by the kinases Wee1 and Mik1 (Lundgren et al., 1991). Once cells reach a critical size the Cdc25 phosphatase dephosphorylates Tyr15 and activates Cdc2 to trigger mitotic entry (Millar et al., 1991). To ensure the correct timing of this event, the activities of Wee1, Mik1 and Cdc25 must themselves be co-ordinately regulated. Wee1 is itself phosphorylated and inhibited by the Nim1 and Cdr2 protein kinases (Coleman et al., 1993; Parker et al., 1993; Wu and Russell, 1993; Breeding et al., 1998; Kanoh and Russell, 1998), whilst regulation of Cdc25 activity appears to depend on regulation of both protein levels (Moreno et al., 1990) and phosphorylation by the Chk1, Cds1 (Chk2) and Srk1 kinases (Furnari et al., 1997; Sanchez et al., 1997; Zeng et al., 1998; Lopez-Aviles et al., 2005). In addition, Cdc13 levels are low during G1 and S phases, increase throughout G2 and peak in early mitosis when Cdc2 activity is high. An abrupt reduction in this activity is co-incident with the sudden disappearance of the Cdc13 protein at the metaphase to anaphase transition, which is due to proteolysis following its ubiquitination by the E3 ubiquitin ligase Anaphase Promoting Complex (APC) (Yamaguchi et al., 1997; Kitamura et al., 1998).

Cdc2 and Cdc13 are also subject to spatial regulation during progression of the cell cycle. Immunofluorescence studies showed that Cdc13 is localised to the nucleolus during G2 (Alfa et al., 1989; Gallagher et al., 1993) and re-localises to the spindle and

separated spindle pole bodies (SPBs) at the G2-M transition where it remains until it is degraded at the metaphase to anaphase transition (Decottignies et al., 2001; Tatebe et al., 2001). A similar localisation pattern is seen using anti-Cdc2 antibodies, (Alfa et al., 1990), although Cdc2 is localised in the cytoplasm as well as the nucleus in G2 cells (Decottignies et al., 2001).

Phosphorylation, cyclin binding and localisation, contribute to the regulation of Cdc2 activity. Importantly, these controls are themselves targets of checkpoints that delay Cdc2 activation and cell cycle progression in response to genotoxic or environmental stress. For example, following DNA damage with ultraviolet light, gamma radiation or a DNA alkylating agent in G2, the cell cycle is arrested. This checkpoint response requires Rad3 dependent phosphorylation of the protein kinases Chk1 (Walworth and Bernards, 1996) and Cds1 (Chk2) (Tanaka et al., 2001). The target of Chk1 and Cds1 kinases is Cdc25. Phosphorylation of Cdc25 by Chk1 and Cds1 inhibits its phosphatase activity and causes Cdc25 to be excluded from the nucleus, thus preventing Cdc2 activation and mitotic entry (Furnari et al., 1997).

In mammalian cells, Cdc2 activation at G2-M is delayed by a p38 MAP kinase pathway dependent "antephase checkpoint" following cellular insults including microtubule disassembly (Matsusaka and Pines, 2004; Mikhailov et al., 2004; Mikhailov et al., 2005). The following experiments were designed to elucidate whether a similar antephase checkpoint exists in fission yeast.

3.2 Results

3.2.1 Cdc2 kinase activation is inhibited by disruption of the microtubule cytoskeleton

Hyams and co-workers showed that the activation of Cdc2 kinase at the G2 to mitosis transition is inhibited upon treatment with the microtubule destabilising drug TBZ (Alfa et al., 1990). I reasoned that this might be due to activation of a novel cell cycle checkpoint. To confirm the effect of TBZ on Cdc2 activity, I first assayed Cdc2 kinase activity in *cdc25-22* cells arrested in late G2 and released to the permissive temperature (see Materials and Methods). Half and hour before release to the permissive temperature, the culture was split and TBZ added to one half at a final concentration of 100μg/ml. Samples were taken at regular time intervals, Cdc13 was immunoprecipitated and assayed for associated kinase activity using Histone H1 as a substrate (Figure

3.1.A). Quantification of the kinase activity showed that control cells had a marked increase from a basal level to 100% relative to Cdc2 kinase activity after release to the permissive temperature, whereas that of cells treated with TBZ only reached 20% of the maximum kinase activity (Figure 3.1.B).

To determine the IC50 of TBZ, the experiment was repeated using various concentrations of TBZ and samples were taken at the point of release and at a single time point 30min after release to permissive temperature (Figure 3.2.A). Quantification of the kinase activity showed that the Cdc2 kinase activity decreased progressively at increasing TBZ concentrations. The IC50 was calculated at 54.5μg/ml (Figure 3.2.B). To visualise the effect of TBZ on the microtubule cytoskeleton, immunofluorescence microscopy analysis was performed. *cdc25-22* cells arrested in late G2 at the restrictive temperature were treated with various concentrations of TBZ and samples were fixed and stained with anti-tubulin antibodies. Consistent with previous descriptions of the *S. pombe* microtubule cytoskeleton (Hagan and Hyams, 1988), control cells have 2-4 microtubules extending the length of the cell (Figure 3.3). At 50 and 75μg/ml TBZ, the microtubules became progressively shorter and were localised to the middle of the cell (Figure 3.3). At 100μg/ml TBZ, small dots of tubulin staining were present throughout the cytoplasm, and a few very short microtubules persisted at the middle of the cell (Figure 3.3). Similar experiments with the microtubule destabilising drug carbendazime showed identical results (data not shown). These results show that the inhibition of Cdc2 kinase activity by TBZ correlates with the destruction of the microtubule cytoskeleton.

To determine whether the inhibition of Cdc2 kinase activity by TBZ was a general response to cytoskeletal damage or specific to the disassembly of microtubules, Cdc2 kinase activity was examined following treatment with Latrunculin A (Lat A), a potent inhibitor of actin polymerisation (Spector et al., 1983; Ayscough et al., 1997). Temperature arrested *cdc25-22* cells were treated with Lat A to a final concentration of 30μM. After release to the permissive temperature, samples were taken and Cdc2 assayed for kinase activity (Figure 3.4.A). Quantification of the kinase activity showed that Lat A-treated cells increased Cdc2 activity to 95% of the maximum activity of the untreated cells (Figure 3.4.B). To examine the effect of LatA on the actin cytoskeleton, cells were fixed and stained for actin. Untreated cells showed actin patches at the tips of the cell and actin cables extending from the actin patches towards the middle of the cell (Figure 3.4.C), as previously described (Marks and Hyams, 1985). No actin structures

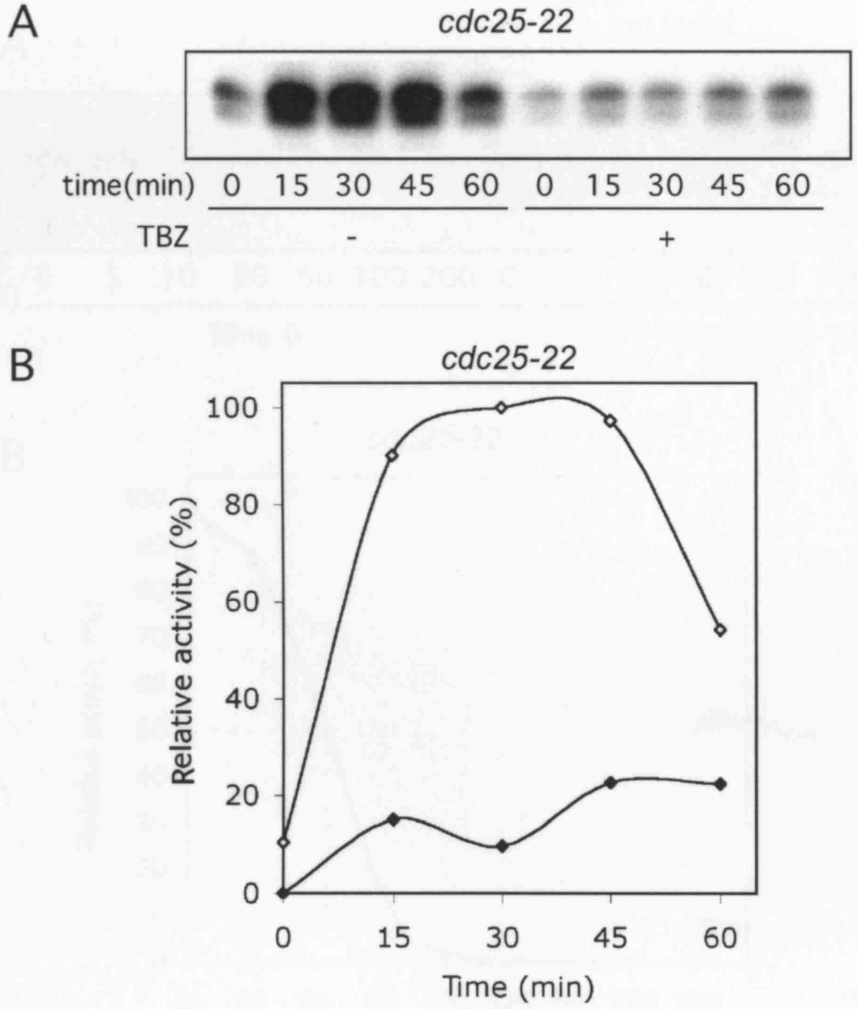


Figure 3.1. Cdc2 kinase activity at the G2/M transition is inhibited by thiabendazole

(A) *cdc25-22* cells were incubated at 36.6°C for 4h. 30min before rapid cooling to 25°C, the culture was split and thiabendazole (TBZ) to a final concentration of 100μg/ml was added to one culture and an equal volume of DMSO as a control to the other. Samples were taken every 15min. Autoradiograph shows the kinase activity of Cdc2 which was co-immunoprecipitated with an antibody to Cdc13 and protein A sepharose beads then assayed with [γ-P^{32}]ATP using Histone H1 as a substrate. (B) The relative kinase activity in (A) was quantified by measuring P^{32} incorporation with a phosphorimager screen. Open squares represent control cells, closed squares represent cells in the presence of 100μg/ml TBZ.

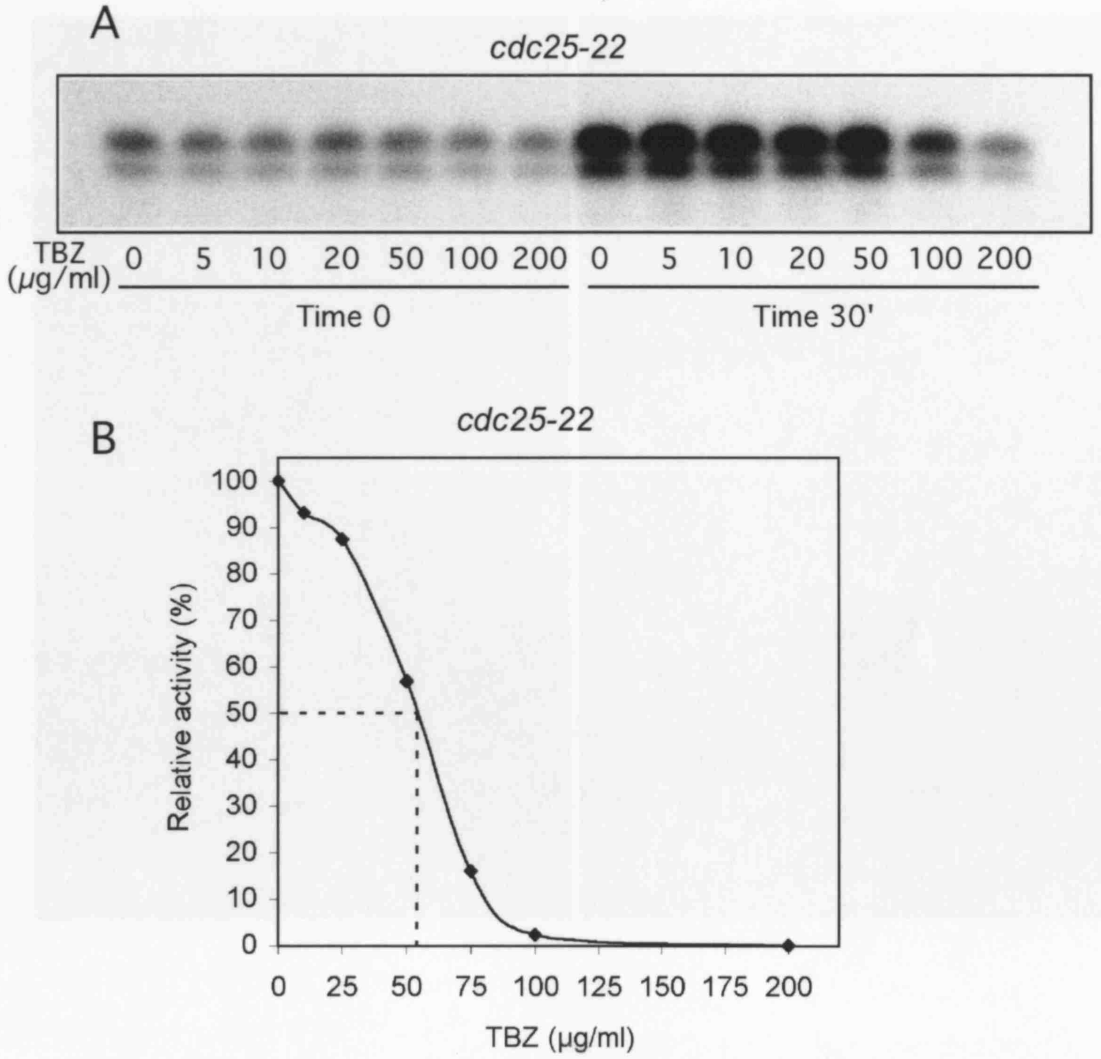


Figure 3.2. The IC50 of the TBZ inhibition of Cdc2

(A) *cdc25-22* cells were incubated at 36.5°C for 4h. 30min before rapid cooling to 25°C, the culture was split and TBZ to a final concentration of 5, 10, 20, 50, 100, 200μg/ml TBZ or an equal volume of DMSO, was added to the split cultures. Samples were taken immediately prior to and 30min after the shift to the permissive temperature. Autoradiograph shows the kinase activity of Cdc2 which was co-immunoprecipitated with an antibody to Cdc13 and protein A sepharose beads then assayed with [γ-P^{32}]ATP using Histone H1 as a substrate. (B) The relative kinase activity in (A) was quantified by measuring P^{32} incorporation with a phosphorimager screen. The dashed line shows the IC50, 54.5μg/ml.

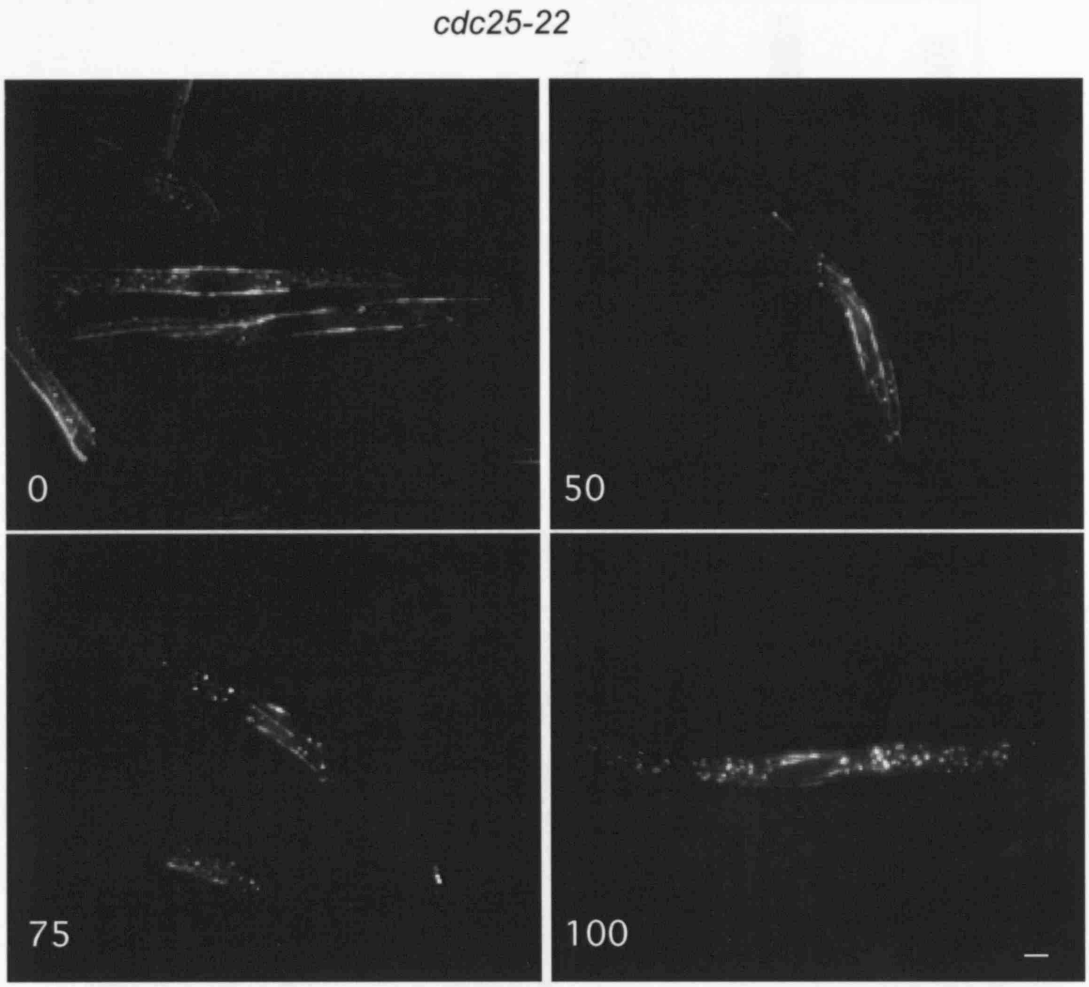


Figure 3.3. Immunofluorescence microscopy of the microtubule cytoskeleton

cdc25-22 cells incubated at 36.5°C for 3.5h were incubated for a further 30min in the presence of 50, 75, 100μg/ml TBZ or an equal volume of DMSO as a control. Samples were taken and the cells fixed in formaldehyde and stained for microtubules with the anti-tubulin antibody tat1. Scale bar is 2μm.

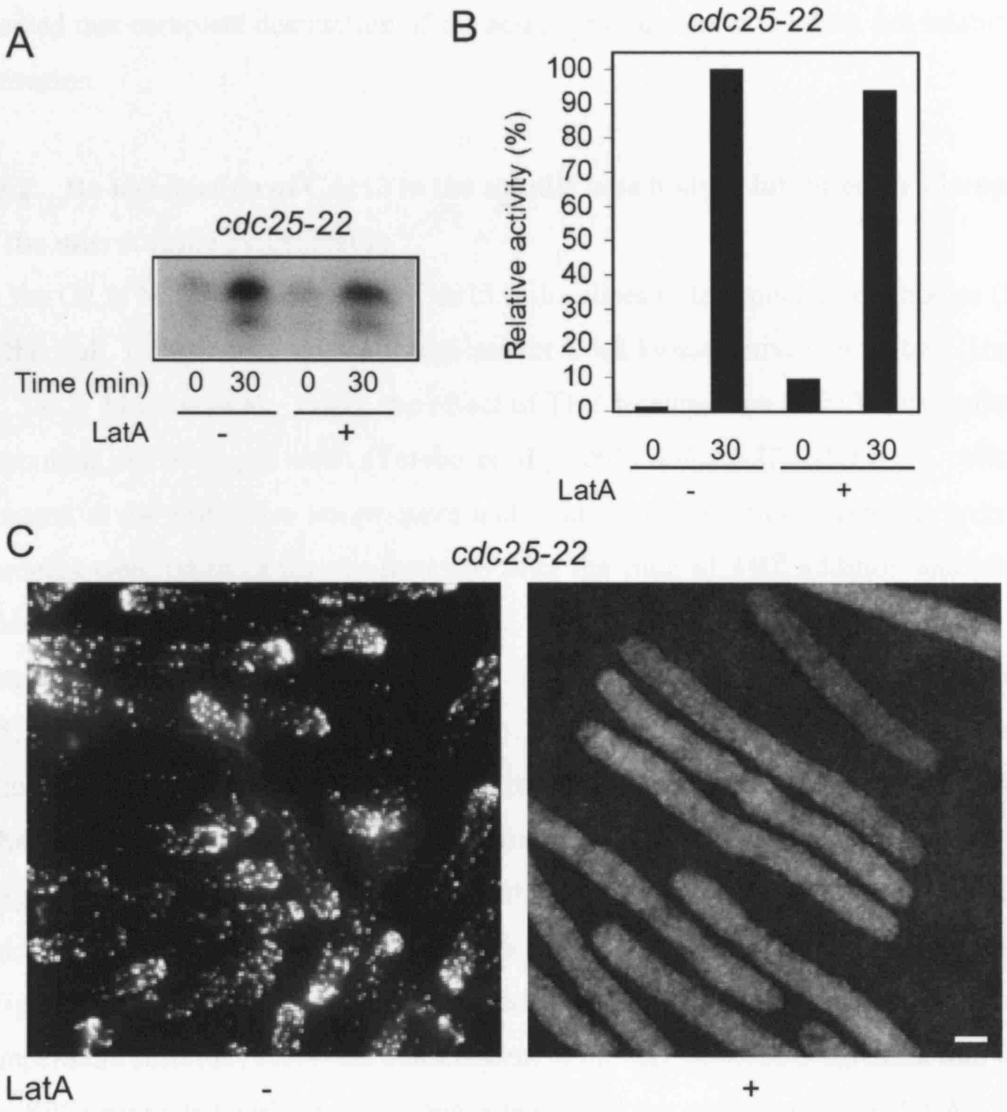


Figure 3.4. Cdc2 is not inhibited by actin cytoskeleton disruption

(A) *cdc25-22* cells were incubated at 36.5˚C for 4h. 30min before rapid cooling to 25˚C, the culture was split and LatA to a final concentration of 30μM or an equal volume of DMSO as a control, was added to the cells. Samples were taken at 0min and 30min after the shift to the permissive temperature. Autoradiograph shows the kinase activity of Cdc2 which was co-immunoprecipitated with an antibody to Cdc13 and protein A sepharose beads then assayed with [γ–P^{32}]ATP using Histone H1 as a substrate. (B) The relative kinase activity in (A) was quantified by measuring P^{32} incorporation with a phosphorimager screen. (C) Image of *cdc25-22* cells at the restrictive temperature which were incubated in the presence of 30μM LatA (+) or an equal volume of DMSO (-) for 30min. Samples were fixed with formaldehyde and stained for actin with Rhodamine-phalloidin. Scale bar is 2μm.

were visible in cells that had been treated with Lat A (Figure 3.4.C). These results showed that complete destruction of the actin cytoskeleton in G2 does not inhibit Cdc2 activation.

3.2.2 Re-localisation of Cdc13 to the spindle pole body is inhibited by disruption of the microtubule cytoskeleton

At the G2 to M transition, nuclear Cdc13 re-localises to the spindle pole bodies (SPBs) (Alfa et al., 1990). Since Cdc13 is required for Cdc2 kinase activity in mitosis (Hagan et al., 1988; Moreno et al., 1989), the effect of TBZ treatment on Cdc13 localisation was examined in *cdc13-gfp* cells (Tatebe et al., 2001). *cdc25-22 cdc13-gfp* cells were arrested at the restrictive temperature and treated, 30min prior to release, with TBZ. Samples were taken at regular intervals after the time of TBZ addition and after the release to the permissive temperature and fixed to visualise Cdc13-GFP. In *cdc25-22* arrested cells a 'faint' Cdc13 signal was observed on the unseparated SPBs (Figure 3.5.A) in 50-60% of cells (Figure 3.5.B). The percentage of control cells with faint Cdc13 at unseparated SPBs was constant till the temperature shift (Figure 3.5.B), whereupon the number of cells with faint Cdc13 decreased within 20min to 20% (Figure 3.5.B). Following the release to the permissive temperature "full" loading of Cdc13 to the SPBs was observed (Figure 3.5.A) increasing to 45% of cells after 30mins (Figure 3.5.B). The transition from faint to full Cdc13 localisation following temperature shift may represent commitment to mitosis. As cells progressed into mitosis the SPBs separated and moved to opposite sites of the nucleus (Figure 3.5.A). In cells treated with TBZ the faint Cdc13 signal on SPBs disappeared (Figure 3.5.A and 3.5.B). The effect of TBZ treatment on Cdc13 localisation was next examined in wild type *cdc11-cfp cdc13-gfp* cells, in which the SPB protein, Cdc11, is tagged with cyan fluorescent protein (CFP) (Krapp et al., 2001). *cdc11-cfp cdc13-gfp* cells were arrested in S phase by addition of hydroxyurea and then treated with 50µg/ml TBZ. Samples were taken before and after the release to fresh medium and fixed for visualisation at regular intervals. Control cells began re-localising Cdc13 to the SPB after 45min and after 75min 45% of all cells had Cdc13 associated to the SPBs (Figure 3.6). Cells treated with TBZ in this experiment were able to associate Cdc13 to the SPB. However, the re-localisation was delayed by 30min and after 105min only 27% of cells had SPB associated Cdc13 (Figure 3.6). These results suggest that microtubules are required at the G2-M transition for the re-localisation of Cdc13 to the SPB.

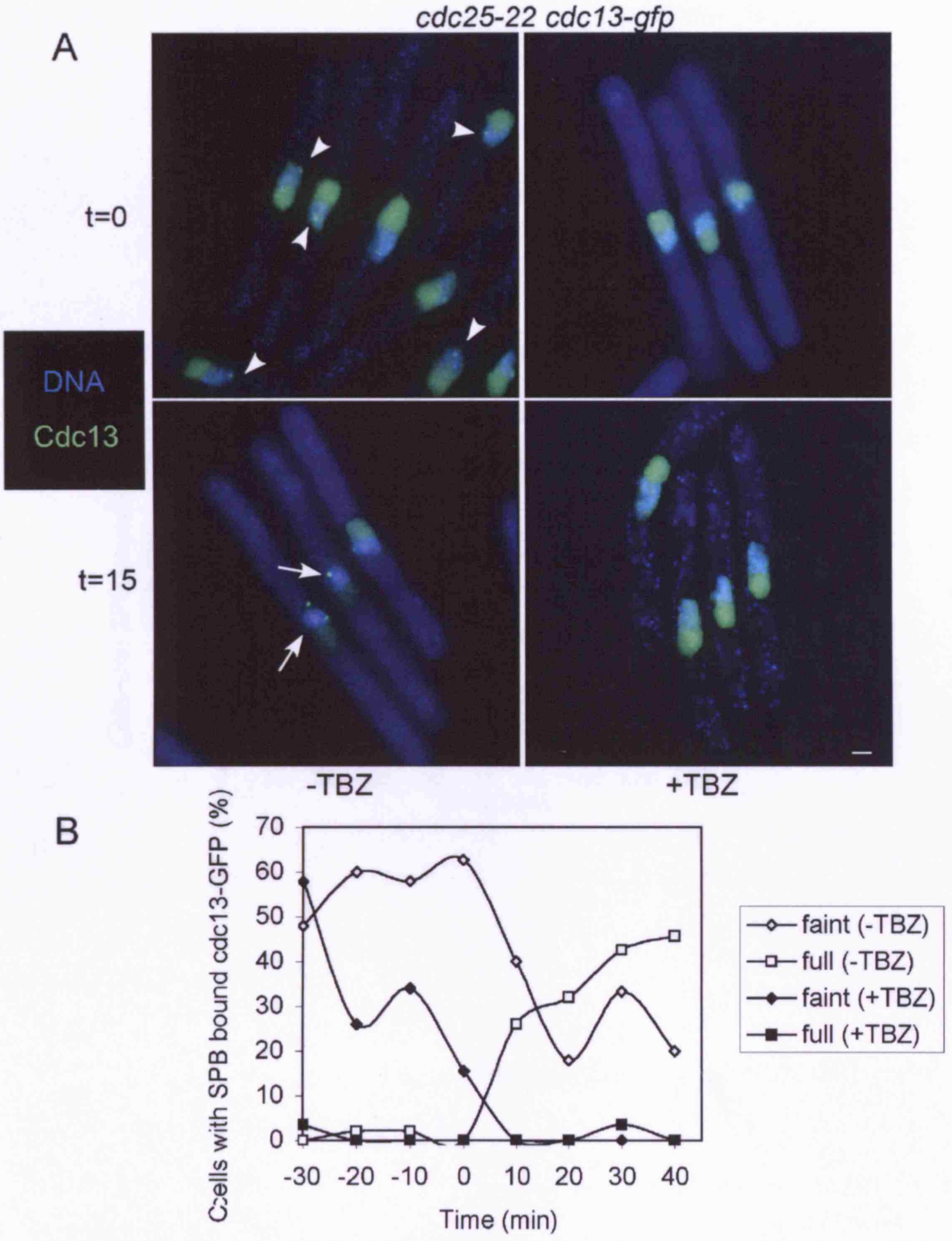


Figure 3.5. TBZ treatment disrupts the localisation of Cdc13

(A) *cdc25-22 cdc13-gfp* cells were incubated at 36.5˚C for 4h. 30min before rapid cooling to the permissive temperature, the culture was split and TBZ to a final concentration of 100µg/ml or an equal volume of DMSO was added. Cells were fixed in formaldehyde and scored for Cdc13-GFP localisation to the spindle pole body (SPB) as either "faint" (arrow heads) or "full" (arrows). Scale bar = 2µm. (B) Graph shows quantification of "faint" (diamonds) or "full" (squares) loading of Cdc13 to the SPBs in the presence of TBZ (filled symbols) or control cells (open symbols). n=250.

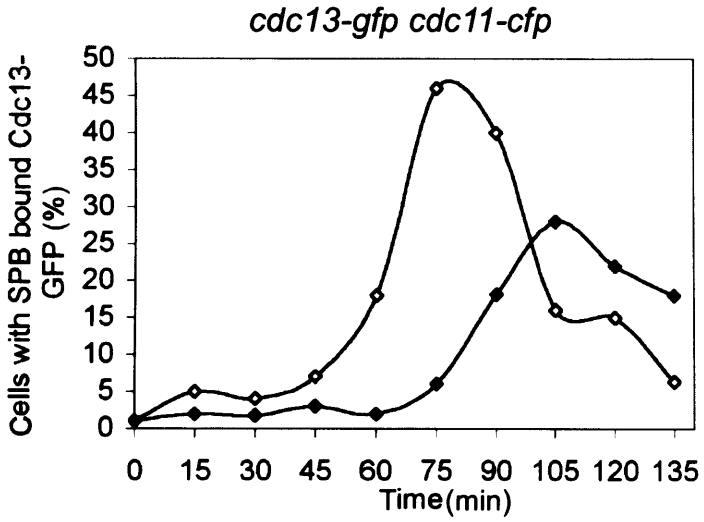


Figure 3.6 TBZ delays the localisation of Cdc13 in wild type cells

cdc13-gfp cdc11-cfp cells were incubated for 4h in hydroxyurea at a concentration of 11mM. 30 min before washing in fresh media, the culture was split, and TBZ to a final concentration of 50μg/ml or an equal volume of DMSO was added. Samples were taken every 15min and fixed in formaldehyde. Graph shows quantification of Cdc13 localisation to the SPBs. n=250.

To examine whether the effect of TBZ was due to a non-specific effect of the drug, Cdc13 localisation was examined in *nda3KM311 cdc13-cfp* cells. The mutant strain *nda3-KM311* was identified as a cold sensitive nuclear division arrest *(nda)* mutant that harbours a mutation in beta-tubulin (Toda et al., 1983; Umesono et al., 1983). At the restrictive temperature *nda3-KM311* cells arrest in a prophase like stage, with little or no microtubule structures (Hiraoka et al., 1984). To examine the effect of a mutant in tubulin on Cdc13 re-localisation, *cdc13-gfp* or *cdc13-gfp nda3-KM311* cells were arrested in S-phase with hydroxyurea and released to fresh medium at the restrictive temperature. Samples were taken at various time intervals and fixed for visualisation. The appearance of Cdc13-GFP at the spindle pole body in *nda3-KM311* cells was delayed by approximately 1h compared to wild type (Figure 3.7.A). Samples of cells at the restrictive temperature were also fixed and stained for tubulin. Control cells exhibited 2-4 microtubules extending the length of the cell (Figure 3.7.B), whilst *nda3-KM311* cells frequently had 3-4 short (approximately 1-3μm) microtubules, localised to the periphery of the nucleus (Figure 3.7.B). These results showed that although *nda-KM311* cells do not completely lack microtubules at the restrictive temperature, the re-localisation of Cdc13 to the SPBs is significantly delayed. Taken together these results confirm previous observations that microtubules are required at the G2-M transition and further indicate that this may be via affecting the re-localisation of Cdc13/Cdc2 to the SPB.

3.2.3 Inhibition of Cdc2 by disruption of the microtubule cytoskeleton is not due to activation of known checkpoints

The spindle assembly checkpoint (SAC) acts in mitosis to prevent the premature separation of sister chromatids (reviewed in (Lew and Burke, 2003)). Cells in which genes encoding the SAC components, Mad2 and Bub1, are deleted are unable to restrain mitotic progression when the mitotic spindle is damaged (He et al., 1997; Bernard et al., 1998). To investigate whether activation of the SAC might also inhibit Cdc2 at the G2-M transition in response to microtubule damage, cells lacking Mad2 and Bub1 were examined. *cdc25-22 mad2::ura4* cells arrested at the restrictive temperature were treated with TBZ as previously described in section 3.2.1. The kinase activity of Cdc2 at the G2-M transition was still inhibited following TBZ treatment in cells lacking Mad2 (Figure 3.8) or Bub1 (data not shown), suggesting that the SAC does not prevent activation of Cdc2 in late G2 following microtubule damage.

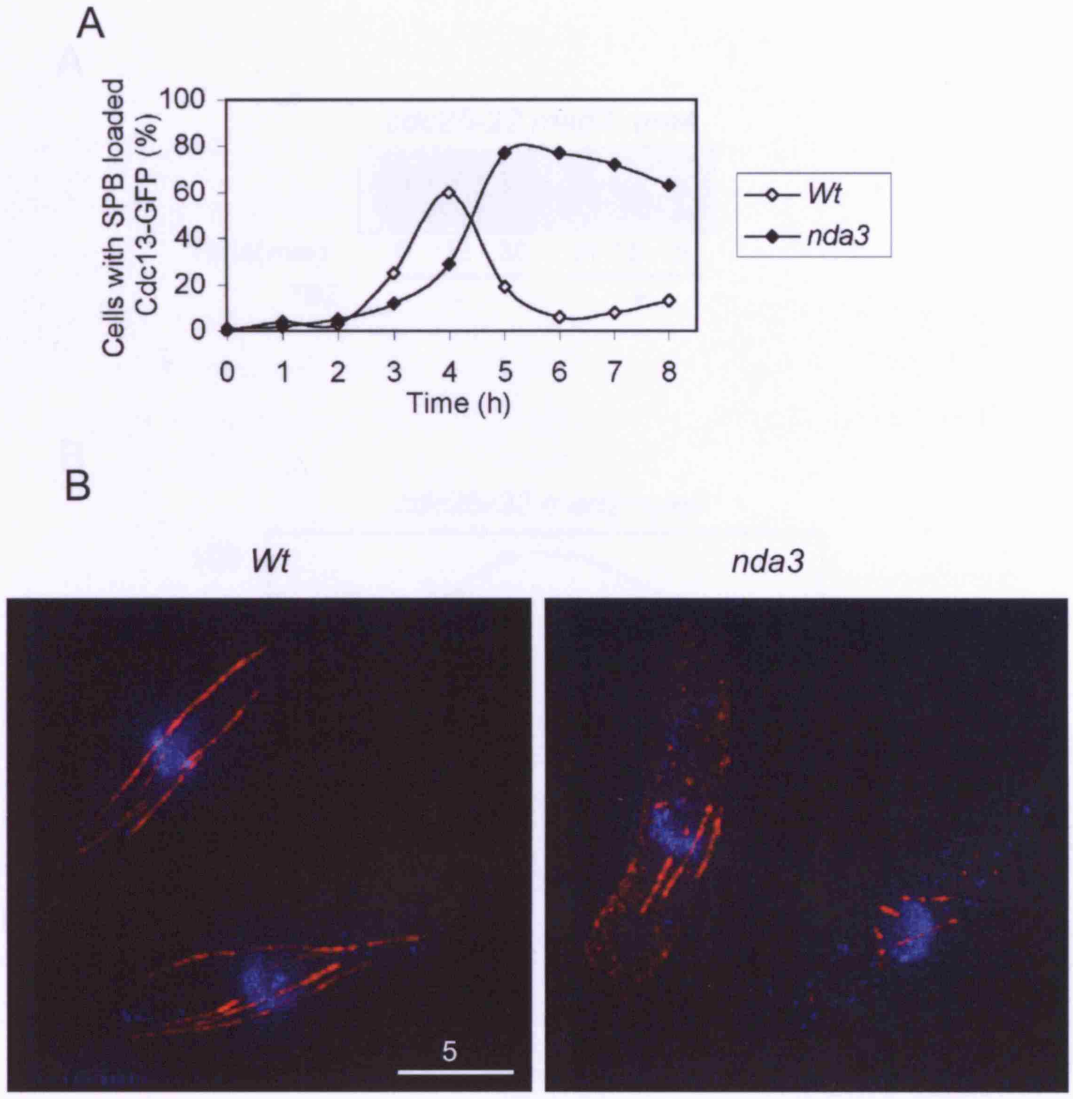


Figure 3.7. Cdc13-GFP loading is delayed in the tubulin mutant *nda3-KM311*

(A) *cdc13-gfp* (open squares; *Wt*) or *nda3-KM311 cdc13-gfp* (closed squares; *nda3*) cells were incubated at 28˚C for 4h in hydroxyurea at a concentration of 11mM. Cells were washed into fresh media, and incubated at 18˚C. Samples were taken every 60min after temperature shift and fixed in formaldehyde. Graph shows quantification of Cdc13 localisation to the SPBs. n=250. (B) Samples of cells from (A) were taken after 2h incubation at 18˚C, fixed in formaldehyde and stained for microtubules with the anti-tubulin antibody tat1. Scale bar = 5μm.

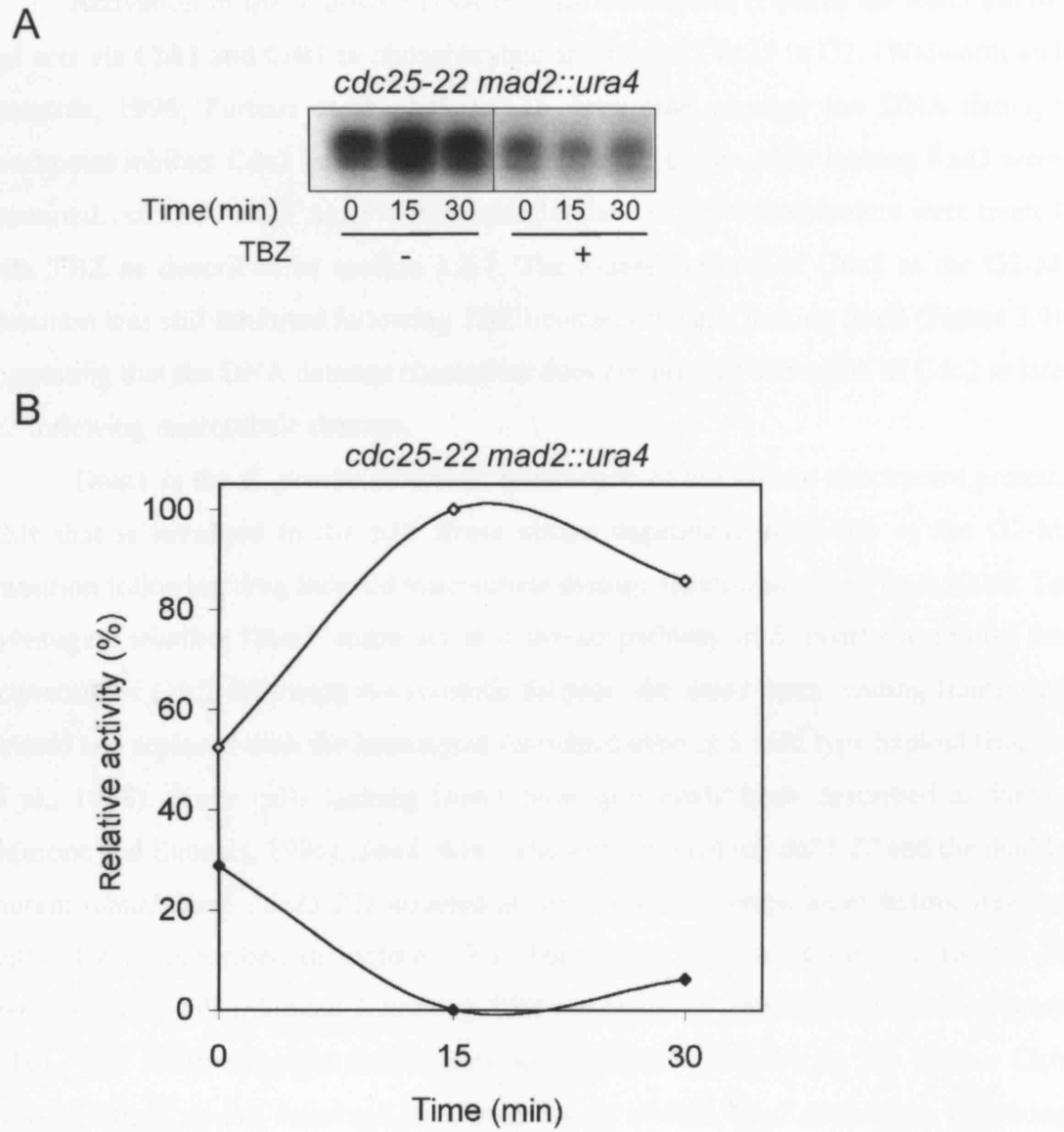


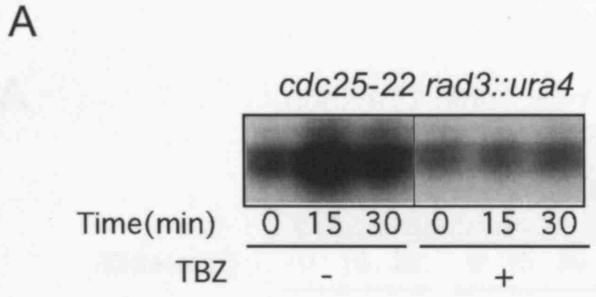
Figure 3.8. The inhibition of Cdc2 by TBZ is not due to the SAC

(A) *cdc25-22 mad2::ura4* cells were incubated at 36.5°C for 4h. 30min before rapid cooling to 25°C, the culture was split and TBZ to a final concentration of 100μg/ml or an equal volume of DMSO was added. Samples were taken at 0, 15 and 30min. Autoradiograph shows the kinase activity of Cdc2 which was co-immunoprecipitated with an antibody to Cdc13 and protein A sepharose beads then assayed with [γ-P^{32}]ATP using Histone H1 as a substrate. (B) The relative kinase activity in (A) was quantified by measuring P^{32} incorporation with a phosphorimager screen. Open squares represent control cells, closed squares represent cells in the presence of 100μg/ml TBZ.

Activation of the *S. pombe* DNA damage checkpoint requires the Rad3 kinase and acts via Chk1 and Cds1 to phosphorylate and inhibit Cdc25 in G2. (Walworth and Bernards, 1996; Furnari et al., 1997). To determine whether the DNA damage checkpoint inhibits Cdc2 in response to microtubule damage, cells lacking Rad3 were examined. *cdc25-22 rad3::ura4* cells arrested at the restrictive temperature were treated with TBZ as described in section 3.2.1. The kinase activity of Cdc2 at the G2-M transition was still inhibited following TBZ treatment in cells lacking Rad3 (Figure 3.9) suggesting that the DNA damage checkpoint does not prevent activation of Cdc2 in late G2 following microtubule damage.

Dma1 is the *S. pombe* structural homologue of the human checkpoint protein Chfr that is involved in the p38 stress kinase dependent inhibition of the G2-M transition following drug induced microtubule damage (Matsusaka and Pines, 2004). To investigate whether Dma1 might act in a similar pathway in *S. pombe* to inhibit the activation of Cdc2 following microtubule damage, the *dma1* open reading frame was deleted and replaced with the kanamycin resistance gene in a wild type haploid (Bahler et al., 1998). Since cells lacking Dma1 have previously been described as viable (Murone and Simanis, 1996), *dma1::kan^r* cells were crossed to *cdc25-22* and the double mutant *(dma1::kan^r cdc25-22)* arrested at the restrictive temperature before treating with TBZ as described in section 3.2.1. The kinase activity of Cdc2 at the G2-M transition was still inhibited following TBZ treatment in cells lacking Dma1 (Figure 3.10). This result suggests that despite its structural similarity to the human Chfr protein, Dma1 is not involved in a pathway to inhibit Cdc2 activation following microtubule damage.

Following exposure to osmotic stress, inhibition of *S. pombe* Cdc2 at G2-M may be dependent on Srk1 kinase (Lopez-Aviles et al., 2005). To determine whether Srk1 inhibits the activation of Cdc2 in response to microtubule damage, cells in which *srk1* was deleted were examined. *cdc25-22 srk1::kan^r* cells arrested at the restrictive temperature were treated with TBZ as described in section 3.2.1. The kinase activity of Cdc2 at the G2-M transition was still inhibited following TBZ treatment in cells lacking Srk1 (Figure 3.11) indicating that this response to microtubule damage in late G2 does not depend on Srk1. Taken together these results show that the inhibition of Cdc2 in fission yeast following microtubule damage is not due to activation of any known checkpoints that act on Cdc2 in G2 or respond to microtubule damage.



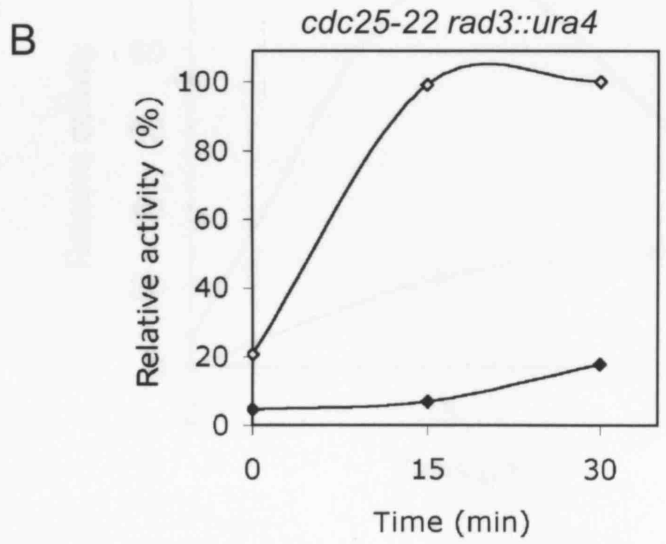


Figure 3.9. The inhibition of Cdc2 by TBZ is not due to the DNA damage checkpoint

(A) *cdc25-22 rad3::ura4* cells were incubated at 36.5°C for 4h. 30min before rapid cooling to 25°C, the culture was split and TBZ to a final concentration of 100μg/ml or an equal volume of DMSO was added. Samples were taken at 0, 15 and 30min. Autoradiograph shows the kinase activity of Cdc2 which was co-immunoprecipitated with an antibody to Cdc13 and protein A sepharose beads then assayed with [γ-P^{32}]ATP using Histone H1 as a substrate. (B) The relative kinase activity in (A) was quantified by measuring P^{32} incorporation with a phosphorimager screen . Open squares represent control cells, closed squares represent cells in the presence of 100μg/ml TBZ.

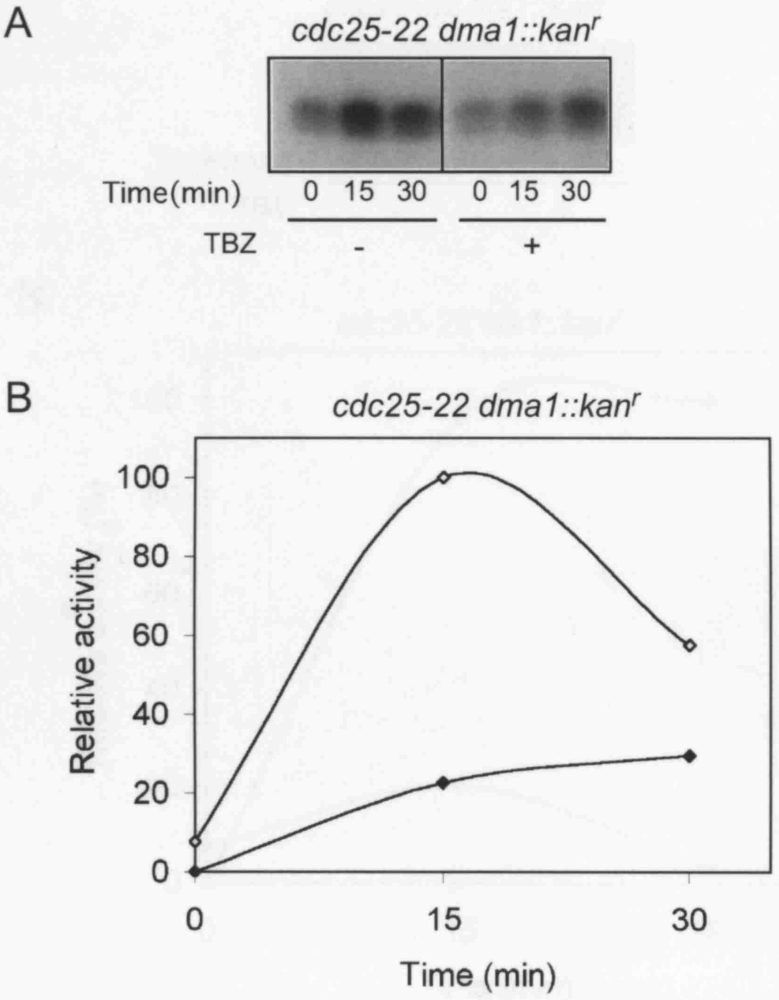


Figure 3.10. The inhibition of Cdc2 by TBZ is not due to the Chfr homologue Dma1

(A) *cdc25-22 dma1::kan*^{r} cells were incubated at 36.5°C for 4h. 30min before rapid cooling to 25°C, the culture was split and TBZ to a final concentration of 100μg/ml or an equal volume of DMSO was added. Samples were taken at 0, 15 and 30min. Autoradiograph shows the kinase activity of Cdc2 which was co-immunoprecipitated with an antibody to Cdc13 and protein A sepharose beads then assayed with [γ–P^{32}]ATP using Histone H1 as a substrate. (B) The relative kinase activity in (A) was quantified by measuring P^{32} incorporation with a phosphorimager screen. Open squares represent control cells, closed squares represent cells in the presence of 100μg/ml.

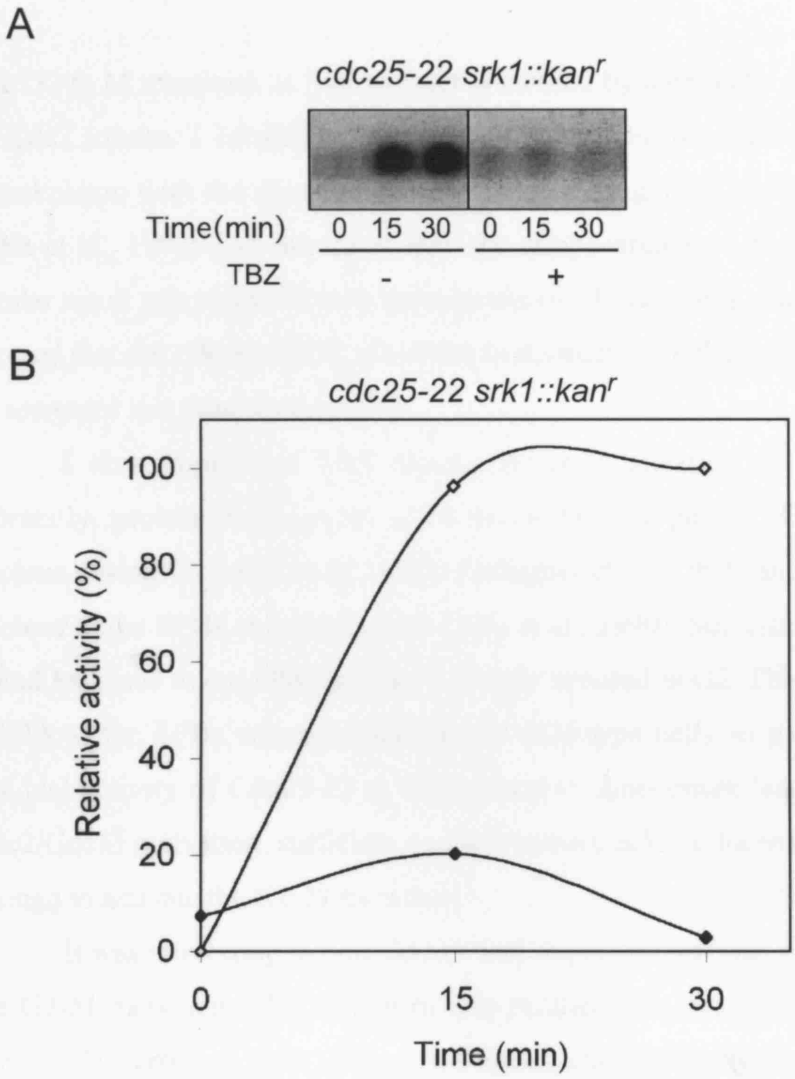


Figure 3.11. The inhibition of Cdc2 by TBZ is not due to the kinase Srk1

(A) *cdc25-22 srk1::kan*^{r} cells were incubated at 36°C for 4h. 30min before rapid cooling to 25°C, the culture was split and TBZ to a final concentration of 100μg/ml or an equal volume of DMSO was added. Samples were taken at 0, 15 and 30min. Autoradiograph shows the kinase activity of Cdc2 which was co-immunoprecipitated with an antibody to Cdc13 and protein A sepharose beads and then assayed with [γ-P^{32}]ATP using Histone H1 as a substrate. (B) The relative kinase activity in (A) was quantified by measuring P^{32} incorporation with a phosphorimager screen. Open squares represent control cells, closed squares represent cells in the presence of 100μg/ml TBZ.

3.3 Discussion

The G2 to M transition in fission yeast is marked by a dramatic increase in the activity of Cdc2 kinase. I confirmed previous observations that damaging the microtubule cytoskeleton with the microtubule destabilising drug TBZ inhibits activation of Cdc2 (Alfa et al., 1990) and correlates with the disappearance of microtubule structures. A similar result was observed with the microtubule destabilising drug carbendazime. I also showed that the effect of TBZ is not due to destruction of the actin cytoskeleton and can be recreated in a tubulin mutant.

I also found that TBZ blocks the re-localisation of Cdc13 to the SPBs. Normally, protein levels of the Cdc2 cyclin binding partner, Cdc13, increase in the nucleus during G2 (Alfa et al., 1989; Gallagher et al., 1993) and re-localises from the nucleus to the SPBs at mitotic onset (Alfa et al., 1990). Surprisingly, some Cdc13 was found localised to the SPBs of *cdc25-22* cells arrested in G2. The "faint" localisation of Cdc13 to the SPBs was not observed in wild type cells so probably represents the residual activity of Cdc25-22 at the restrictive temperature leading to a low level of Cdc2/Cdc13 activation, sufficient to allow some Cdc13 re-localisation to SPBs, but not enough to activate the G2-M transition.

It was interesting to note that as well as preventing Cdc13 loading to the SPBs at the G2-M transition, TBZ treatment also resulted in a loss of the faint Cdc13 loading seen in G2 arrested cells. This result indicates either that intact microtubules are required to maintain Cdc13 at the SPB, or that there is a constant cycle of Cdc13 loading on and off the SPB, in a process that requires microtubules. Microtubule dependent localisation of Cdc13 to the SPBs may explain why *cdc25-22* cells treated with TBZ are unable to activate Cdc2. If the SPBs are the site at which Cdc2 is activated, then perturbing the localisation of Cdc13 would affect mitotic entry. Whilst Cdc2 and Cdc13 both localise to the SPB in mitosis (Alfa et al., 1990; Decottignies et al., 2001), interdependence for their localisation has not yet been demonstrated so it is unclear whether Cdc2 localisation would also be affected following microtubule damage. Although Cdc25, the main activator of Cdc2, does not localise specifically to the SPB in mitosis (Lopez-Girona et al., 1999) several other proteins involved in regulating mitotic entry do, including the kinases Plo1 and Fin1 (Ohkura et al., 1995; Mulvihill et al., 1999; Grallert and Hagan, 2002). *stf1-1* mutants prematurely localise

Plo1 to the SPB, bypass the requirement for Cdc25 to activate Cdc2/Cdc13 and suppress the *cdc25-22* mutation (Hudson et al., 1990; Mulvihill et al., 1999) suggesting that this kinase activates Cdc2/Cdc13. Overexpression of Fin1 also drives premature Plo1 localisation and partially suppresses *cdc25-22* (Grallert and Hagan, 2002). Preventing loading of Cdc2/Cdc13 to the SPB may prevent its activation by these proteins and consequently mitotic entry.

The SAC is the only checkpoint in fission yeast that is known to sense microtubule status since it monitors the attachment of kinetochores to the mitotic spindle and acts to prevent the premature separation of sister chromatids in mitosis. However cells with a disabled SAC, lacking either Mad2 or Bub1, still failed to activate Cdc2 in the presence of TBZ, implying that the SAC is not involved. A checkpoint that responds to DNA damage and which is Rad3 dependent is known to regulate Cdc2 activity. Here I showed that cells lacking Rad3 also failed to activate Cdc2 in the presence of TBZ suggesting that this DNA damage pathway is not used in this response.

Treatment with microtubule destabilising drugs such as nocadazole and colcemid cause a delay in the G2-M transition in rat kangaroo kidney PtK_1 cells (Rieder and Cole, 2000). When PtK_1 cells already in prophase are treated with microtubule destabilising drugs, cells decondense their chromosomes and return to a pre-mitotic state (Rieder and Cole, 2000). This return to a pre-mitotic state is similar to that seen in vertebrate somatic cells following activation of the DNA damage checkpoint by irradiation in early prophase (Rieder and Cole, 1998). The response to irradiation appears to be distinct to that following microtubule damage, as inhibition of the DNA damage checkpoint by treatment with caffeine does not prevent the return of microtubule damaged cells to G2 as it does irradiated cells (Blasina et al., 1999; Rieder and Cole, 2000). Since chromosome condensation in PtK_1 cells is mediated by Cdk2/Cyclin A (Furuno et al., 1999), and commitment to mitosis, as in fission yeast, depends on a sharp increase in Cdk1/Cyclin B activity (Clute and Pines, 1999), it is likely that inhibition of Cdk activity by a checkpoint mechanism other than the DNA damage checkpoint, prevents the G2-M transition in response to microtubule disassembly. This checkpoint, termed the "antephase checkpoint" in reference to the period prior to mitosis when cells may be reversibly inhibited by cellular stress (Bullough and Johnson, 1951) responds to a variety of cellular insults, including hyperosmotic stress, microtubule disassembly, and inhibition of histone deacetylases (Matsusaka and Pines, 2004; Mikhailov et al., 2004). This checkpoint requires the activation of the p38 MAP kinase pathway but is independent of the ATM and ATR

checkpoint proteins (Wang et al., 2000; Bulavin et al., 2001; Matsusaka and Pines, 2004; Mikhailov et al., 2004). Recent work has shown that activation of p38 and the antephase checkpoint depends on the checkpoint protein Chfr (Matsusaka and Pines, 2004). Importantly, Chfr is absent or inactivated from several cell lines (Scolnick and Halazonetis, 2000) and tumours (Mizuno et al., 2002) demonstrating that overcoming the antephase checkpoint is a significant stage in the development of cancer.

In fission yeast, the structural homologue of Chfr is the Dma1 protein. I found that cells lacking Dma1 were unable to activate Cdc2/Cdc13 following microtubule damage suggesting that Dma1 does not play a similar role to Chfr in this response. The fission yeast p38 homologue Sty1 MAP kinase has been found to link the cell cycle to the extra-cellular environment (Millar et al., 1995; Shiozaki and Russell, 1995). In response to osmotic stress, Sty1 phosphorylates and activates Srk1 (Smith et al., 2002; Lopez-Aviles et al., 2005). Activated Srk1 prevents the G2-M transition by phosphorylating Cdc25 leading to its binding to the 14-3-3 protein Rad24, and accumulation of Cdc25 in the cytoplasm. I found that a strain lacking Srk1 was still unable to activate Cdc2 following microtubule damage. This suggests that the mechanism by which fission yeast inhibits Cdc2 following microtubule damage is distinct from that which senses environmental stress.

I conclude that none of the known checkpoints in fission yeast that respond to microtubule damage or regulate Cdc2 activity following cellular stress appear to be responsible for the inhibition of Cdc2 activity following microtubule damage. This suggests that either an unknown checkpoint responds to this stress, or that the inhibition of Cdc2 is an indirect affect due to it, or Cdc13's, inability to re-localise to the SPB following microtubule damage.

Chapter 4

A screen for multicopy suppressors of the sensitivity of *cdc13-117* to TBZ

4.1 Introduction

The Cdc2 mitotic binding partner, Cdc13, was identified as a multicopy suppressor of a cold sensitive *cdc2* mutant strain (Booher and Beach, 1987) and by complementation of a *cdc13-117* mutant strain at high temperature (Booher and Beach, 1988). As well as being temperature sensitive, *cdc13-117* cells are sensitive to the microtubule destabilising drug thiabendazole (TBZ). Notably, a multi-copy plasmid expressing *cdc2* rescues the sensitivity of *cdc13-117* cells to high temperature but not to TBZ (Booher and Beach, 1988). The sensitivity of *cdc13-117* to TBZ suggested an interaction between Cdc13 and microtubules and Cdc2/Cdc13 is found to bind to the mitotic spindle (Alfa et al., 1990; Decottignies et al., 2001). The precise role of Cdc2/Cdc13 on the mitotic spindle is, however, not well understood. Since one of the key events in G2-M is the re-organisation of the microtubule cytoskeleton to form a mitotic spindle, I reasoned that a genetic screen for multicopy suppressors of *cdc13-117* TBZ sensitivity may reveal proteins that either regulate microtubule stability, the association of Cdc13/Cdc2 to microtubules in mitosis or identify Cdc2 targets involved in mitotic spindle assembly. The results of the screen are presented here.

4.2 Results

4.2.1 *cdc13-117* cells are defective in the accuracy of chromosome segregation

At the permissive temperature, *cdc13-117* cells are unable to grow in the presence of 12.5μg/ml TBZ (Figure 4.1.A; (Booher and Beach, 1988)). Microscopic analysis showed that in the presence of TBZ *cdc13-117* cells die as micro-colonies after approximately 1-4 divisions (Figure 4.1.B). To analyse this phenotype further, wild type or *cdc13-117* cells that express the spindle pole body (SPB) protein Cdc11 tagged with GFP were synchronised in early G2 and released in the presence or absence of

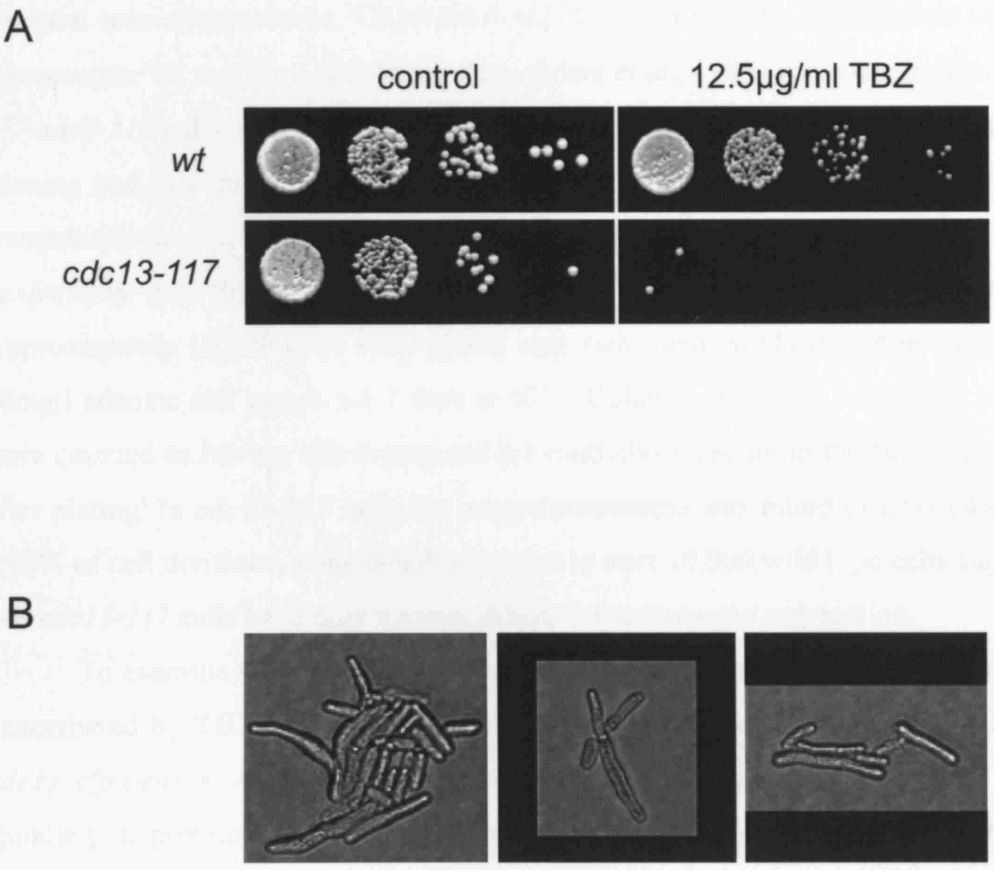


Figure 4.1. *cdc13-117* cells are sensitive to TBZ

(A) Wild type or *cdc13-117* cells were plated on rich medium in the presence of 12.5µg/ml TBZ or an equal volume of DMSO as a control and incubated for 2 days at 30°C. (B) Images of microcolonies of cdc13-117 growing on rich medium containing 12.5µg/ml TBZ for 2 days at 30°C were visualised by light microscopy.

12.5µg/ml TBZ. Entry into mitosis was judged by the appearance of separated SPBs (Figure 4.2.A). Wild type cells entered mitosis 60min after synchronisation in the presence or absence of TBZ (Figure 4.2.B). Addition of 12.5µg/ml TBZ also had little effect on the timing of mitotic entry in *cdc13-117* cells (Figure 4.2.B). To determine whether *cdc13-117* has a role in mitotic progression, the segregation of a linear non-essential mini-chromosome, Ch16(*ade6-M216)*, containing the centromeric region of chromosome III and the *ade6-M216* allele (Niwa et al., 1989) was examined in *cdc13-117 ade6-210* cells. *ade6-210* is an auxotrophic mutant that is unable to fully synthesise adenine and is complemented by *ade6-216.* When grown on medium with limiting amounts of adenine, loss of the mini-chromosome during cell division results in adenine auxotrophy and the growth of pink/white sectored colonies of *ade6-210* cells. Approximately 10,000 cells were plated onto rich medium plates supplemented with 10mg/l adenine and grown for 3 days at 30°C. Colonies that were more than half pink were counted as having mis-segregated the mini-chromosome in the first cell division after plating. In *cdc13-117* cells the mini-chromosome was found to mis-segregate in 0.02% of cell divisions, none could be scored in over 10,000 wild type cells suggesting that *cdc13-117* cells have only a minor defect in chromosome segregation.

To examine whether *cdc13-117* cells have a defect in mitotic progression that is exacerbated by TBZ, kinetochore behaviour was examined in *cdc13-117 ndc80-gfp cdc11-cfp* cells in which the kinetochore protein, Ndc80, is tagged with GFP and the spindle pole protein, Cdc11, is tagged with CFP. In the absence of drug, kinetochores segregated equally to the spindle poles (3:3) in wild type and *cdc13-117* cells (Figure 4.3.). However, in the presence of 12.5µg/ml TBZ, *cdc13-117 ndc80-gfp cdc11-cfp* cells showed an increase in the frequency of kinetochore mis-segregation: in 7/10 movies of *cdc13-117* cells kinetochores were mis-segregated (0:6, 1:5, or 2:4), whereas mis-segregated kinetochores were observed in only 1/10 movies of wild type cells. A cell in which 4 kinetochores segregate to one SPB and 2 to the other is shown in Figure 4.3. To quantify this phenotype more accurately, the segregation of the sister chromatids of chromosome 1 was examined in *lys1:lacO his7:GFP-LacI* cells (Nabeshima et al., 1998) in which the *lys1* locus on chromosome 1 is marked with GFP (called *cen1-gfp* hereafter). Wild type and *cdc13-117 cen1-gfp cdc11-cfp* cells were synchronised in early G2, released into rich medium in the presence or absence of 12.5µg/ml TBZ and the percentage of bi-nucleate cells assessed (Figure 4.4.A). After 140 minutes, the percentage of bi-nucleate cells in which both sister chromatids of

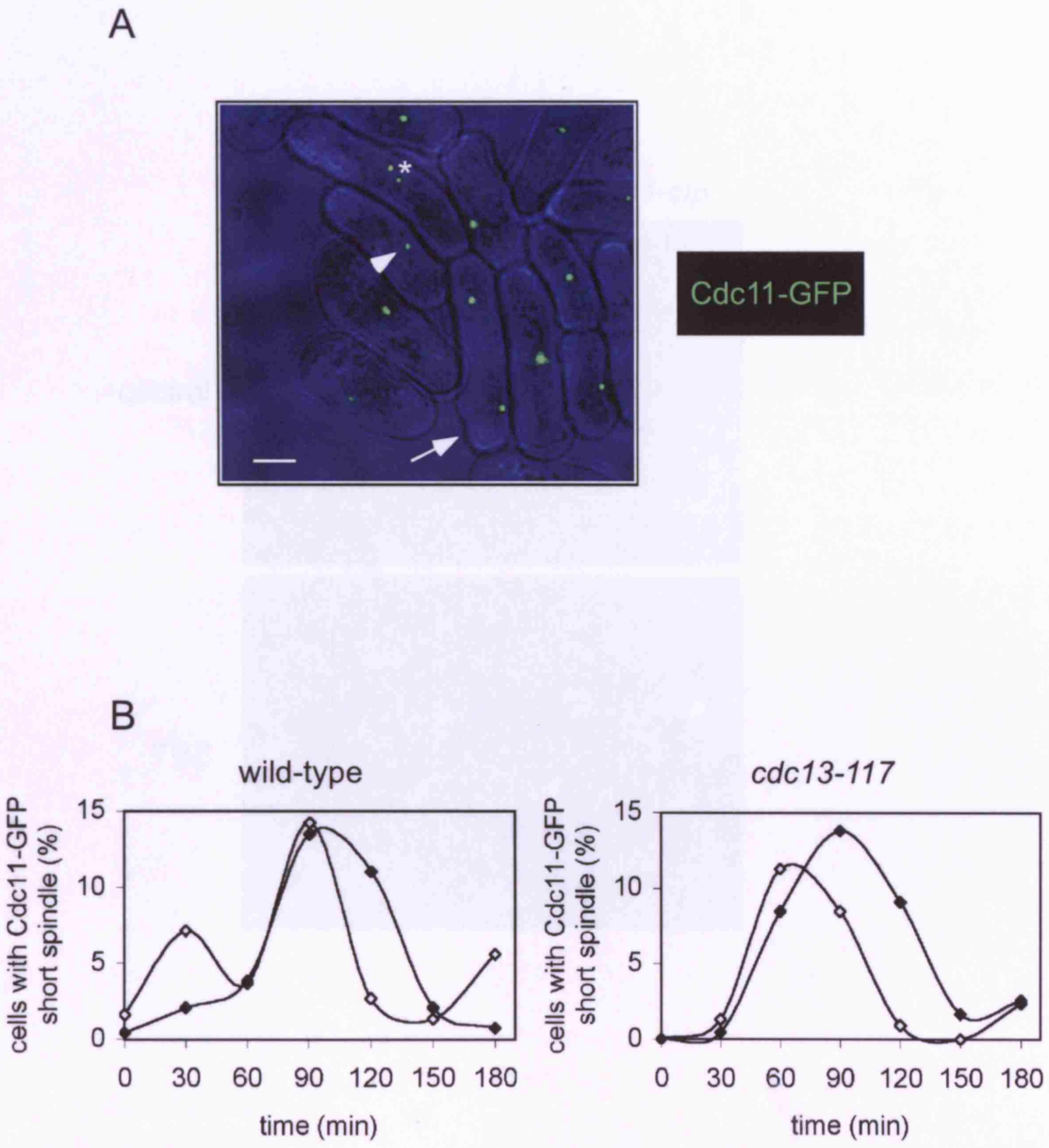


Figure 4.2 *cdc13-117* cells are not blocked in G2 in the presence of TBZ

(A) Image of log phase *cdc11-gfp* cells. Arrowhead indicates a cell in G2, asterix indicates a cell with a short (<2μm) mitotic spindle, arrow represents a cell in late anaphase. Scale bar = 2μm (B) *cdc11-gfp* or *cdc13-117 cdc11-gfp* cells were synchronised in early G2 and incubated in medium in the presence (closed symbols) or absence (open symbols) of 12.5μg/ml TBZ. Samples were taken every 30min, fixed with formaldehyde, and visualised by fluorescence microscopy. Cells were scored for Cdc11-GFP dots ≤2μm apart, indicating a short mitotic spindle.

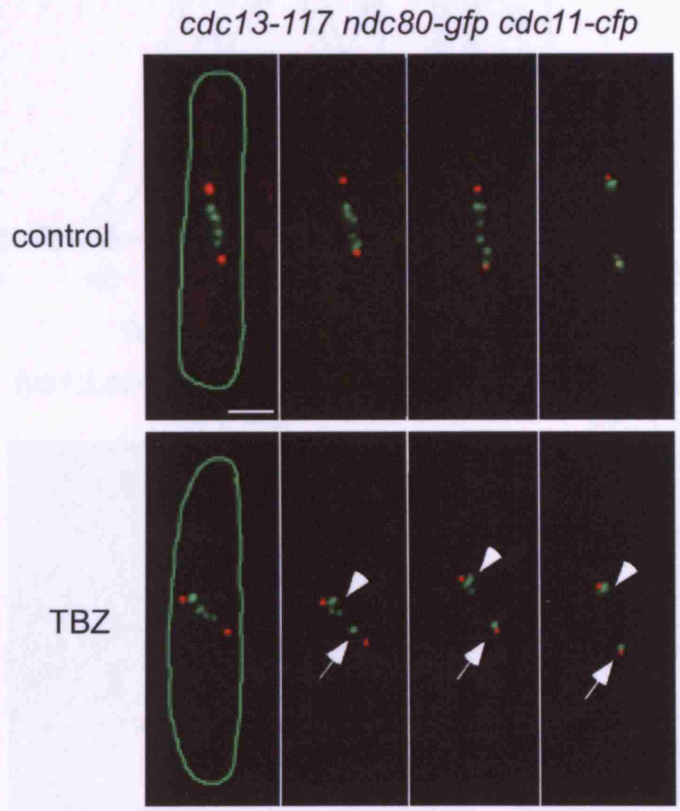


Figure 4.3 *cdc13-117* frequently mis-segregate chromosomes in the presence of TBZ

Log phase *cdc13-117 ndc80-gfp cdc11-cfp* cells were grown in the presence of 12.5μg/ml TBZ or an equal volume of DMSO as a control. Live cells were visualised by fluorescence microscopy. Arrow indicates 2 chromosomes segregating to one spindle pole, and arrowhead indicates 4 chromosomes segregating to the other spindle pole. Scale bar = 2μm.

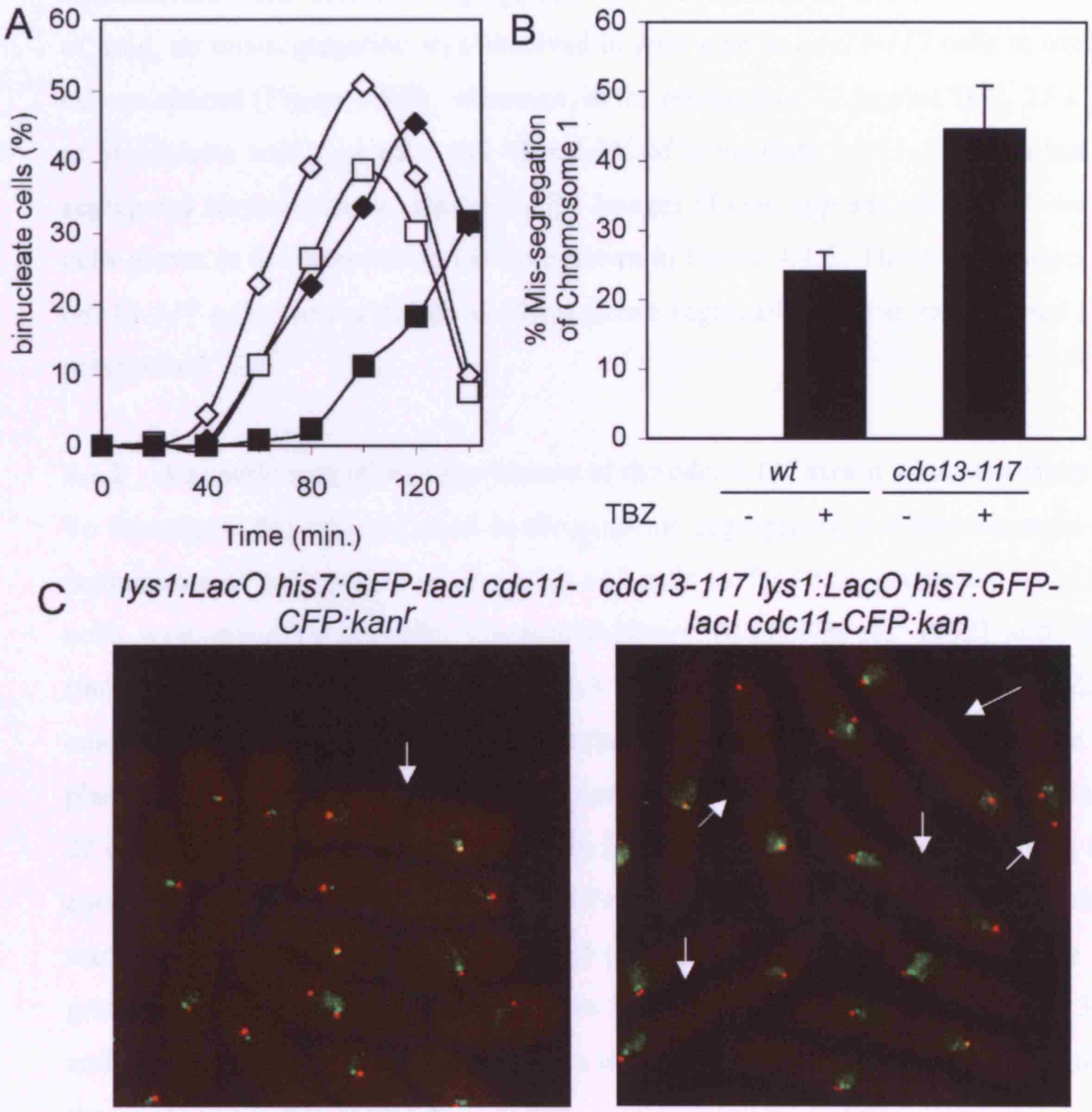


Figure 4.4 *cdc13-117* has an increased rate of chromosome mis-segregation in the presence of TBZ

(A) *lys1:LacO his7:GFP-lacI cdc11-CFP:kan*^{r} cells (diamonds) or *cdc13-117 lys1:LacO his7:GFP-lacI cdc11-CFP:kan*^{r} cells (squares), were synchronised in early G2 by lactose gradient and incubated in rich medium in the presence (closed symbols) or absence (open symbols) of 12.5μg/ml TBZ and the percentage of binucleate cells analysed at the times indicated. (B) From the experiment in (A) wild-type and *cdc13-117* cells incubated in the absence or presence of 12.5μg/ml TBZ were fixed in formaldehyde and visualised by fluorescence microscopy. The frequency of mis-segregation of chromosome 1 (2 dots in one nucleus) was expressed as a percentage of total binucleate cells. (C) Image of wild-type and *cdc13-117* cells incubated in TBZ from experiment in (B). Arrows indicate cells that have mis-segregated chromosome 1. Scale bar = 2μm.

chromosome 1 had been mis-segregated to the same pole was assessed. In the absence of drug, no mis-segregation was observed in wild type or *cdc13-117* cells in over 500 cells examined (Figure 4.4.B). However, in the presence of 12.5μg/ml TBZ, 25 ± 2.8% of bi-nucleate wild type cells and 43 ± 6.3% of bi-nucleate *cdc13-117* cells had mis-segregated chromosome 1 (Figure 4.4.B). Images of *cen1-gfp* and *cdc13-117 cen1-gfp* cells grown in the presence of TBZ are shown in Figure 4.4.C. These data suggest that *cdc13-117* cells have a defect in chromosome segregation that is exacerbated in the presence of TBZ.

4.2.2 A genetic screen for suppressors of the *cdc13-117* strain TBZ sensitivity

To investigate the role of Cdc13 in chromosome segregation, a screen for multi-copy suppressors of the sensitivity of *cdc13-117* cells to TBZ was performed. *cdc13-117* cells were transformed with a genomic library (Barbet et al., 1992) and 70,000 transformants were replica plated to rich medium containing 12.5μg/ml TBZ. 120 colonies were isolated after 3 days incubation. 86 of these were found to be non-plasmid dependent and were not studied further. Plasmid DNA was extracted from only 27 of the 34 suppressors and amplified in *E. coli.* For reasons that are not clear, DNA could not be isolated from the remaining 7 colonies. The recovered plasmids were then retransformed into *cdc13-117* cells and the strength of rescue classified into three groups; 'very strong' if colonies grew after 2 days, 'strong' if colonies grew after 3 days and 'weak rescue' if colonies took 4 days to grow (Table 4.1). A schematic diagram of the screen is given in Figure 4.5.

The plasmids were sequenced to identify the suppressors and a clone containing multicopy *cdc13* was isolated 5 times as a very strong suppressor and was the only suppressor that enabled *cdc13-117* to also grow at the restrictive temperature (Table 4.1). A clone containing Ucp3, a protein with a predicted GTPase and ubiquitin associated (UBA) domain was isolated 7 times. Another clone that was isolated 5 times contained a truncated allele of the SPAC589.08c ORF and another ORF (SPAC589.09). A clone containing a truncated allele of *dis2* type 1 phosphatase homologous to *S. cerevisiae GLC7* and another ORF (SPBC776.03) was isolated 9 times. A clone containing *inh2,* which encodes a small heat stable inhibitor of type 1 phosphatases, was isolated twice. Finally, a single clone containing a truncated allele of *pef1,* which encodes a cyclin dependent kinase homologous to *S. cerevisiae* Pho85, was isolated.

To confirm that the suppressors were rescuing the sensitivity of *cdc13-117* to TBZ by increasing the efficiency of chromosome segregation, three of the strongest

number of times isolated	ORF of interest in suppressor plasmid	strength of rescue
9	truncated *dis2*	++
7	*ucp3*	++
5	*cdc13*	+++
3	truncated SPAC589.08c	+++
2	*inh2*	++
1	truncated *pef1*	+

Table 4.1. Genomic clones isolated from the *cdc13-117* multicopy suppressor screen
The number of times each genomic clone was isolated, the ORF of interest expressed on each clone, and the relative strength of the rescue of *cdc13-117* TBZ sensitivity is shown.

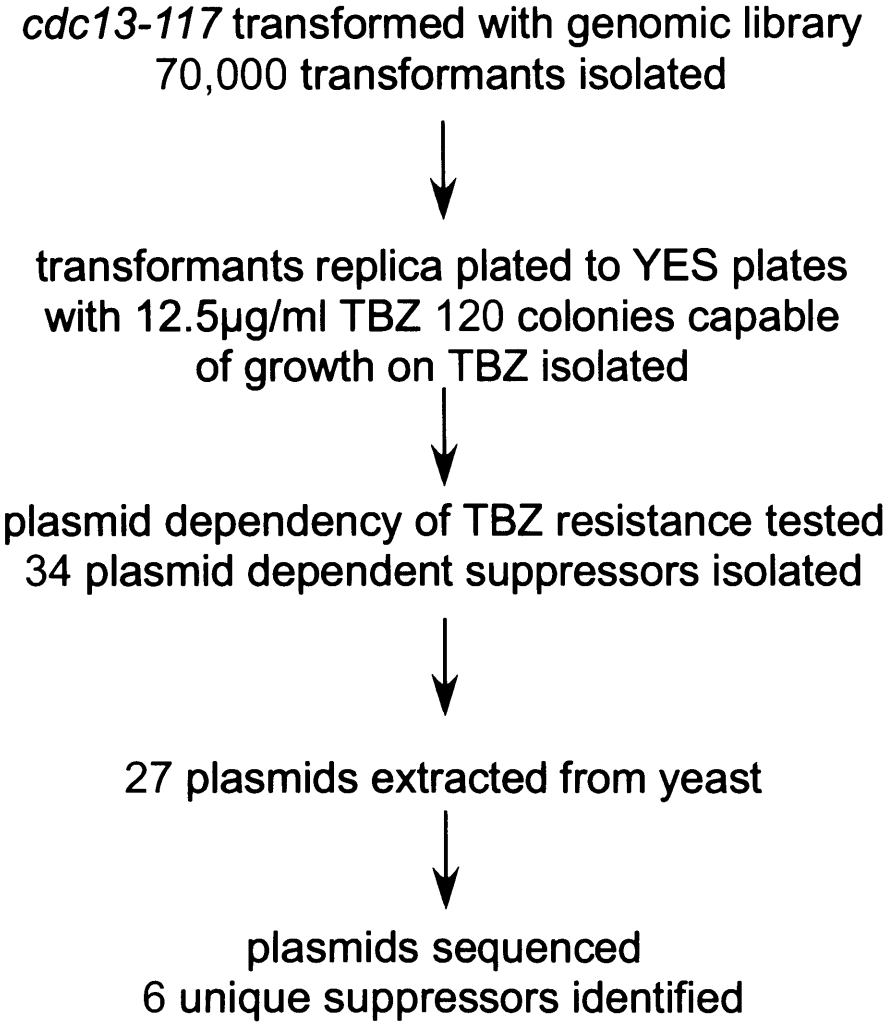


Figure 4.5 Screen for suppressors of the *cdc13-117* strain TBZ sensitivity

cdc13-117 cells were transformed with a genomic library, and 70,000 transformants were selected. The transformants were replica plated to rich medium plates containing 12.5µg/ml TBZ. 120 colonies capable of growth on TBZ plates were isolated and of these 34 were found to be plasmid dependent. Plasmid DNA was extracted from 27 of the colonies and amplified in E. coli. Multicopy suppressors were sequenced to identify the ORFs suppressing the TBZ sensitivity of *cdc13-117* cells.

suppressing clones and *cdc13* itself, were introduced by transformation into *cdc13-117 cen1-gfp cdc11-cfp* cells and segregation of chromosome 1 in the presence of 12.5μg/ml TBZ examined as described previously in this section. The clones suppressed the mis-segregation of chromosome 1 to varying degrees (Figure 4.6). These results suggest that the suppression of *cdc13-117* TBZ sensitivity by the clones isolated in the screen was due to an increase in the efficiency of chromosome segregation.

The clone containing a truncated allele of the SPAC589.08c ORF and the SPAC589.09 ORF was chosen for further study. To test which gene was responsible for the suppression sub-clones lacking truncated SPAC589.08c (KpnI SPAC589.08c and EcoRI SPAC589.08c respectively) were constructed. Additionally, a sub-clone containing truncated SPAC589.08c alone (EcoRI SPAC589.08c) was constructed. A diagram showing the genomic fragments in each sub-clone is shown in Figure 4.7.A. *cdc13-117* cells transformed with an empty plasmid, *cdc13*, the original suppressor, or one of each of the sub-clones were plated onto selective media plates containing 12.5μg/ml TBZ. Only cells transformed with plasmids containing either *cdc13* or the truncated allele of SPAC589.08c ORF were able to grow in the presence of TBZ (Figure 4.7), confirming that the protein encoded by truncated SPAC589.08c was responsible for suppressing *cdc13-117* TBZ sensitivity.

4.3 Discussion

The *cdc13-117* strain is sensitive to TBZ. The results in this chapter suggest that the *cdc13-117* strain is defective for the accurate segregation of sister chromatids during mitosis due to decreased stability of microtubules, a phenotype that is exacerbated in the presence of TBZ. Exactly why the mutant Cdc13-117 protein results in a defective mitosis is unclear, but is likely to involve de-regulation of the attachment of the mitotic spindle to the kinetochores of sister chromatids, or defective regulation of the SAC, since mutant proteins involved in these processes often have a very similar phenotype to *cdc13-117*. Alternatively, the Cdc13-117 protein may affect the Cdc2-dependent phosphorylation of one or more components of the mitotic machinery. One known target is the kinetochore protein Dis1 (Nabeshima et al., 1995), which is important in generating tension at the kinetochore (Garcia et al., 2002b). Although the significance of Dis1 phosphorylation *in vivo* has not been determined, its phosphorylation is required for full activity in mitosis.

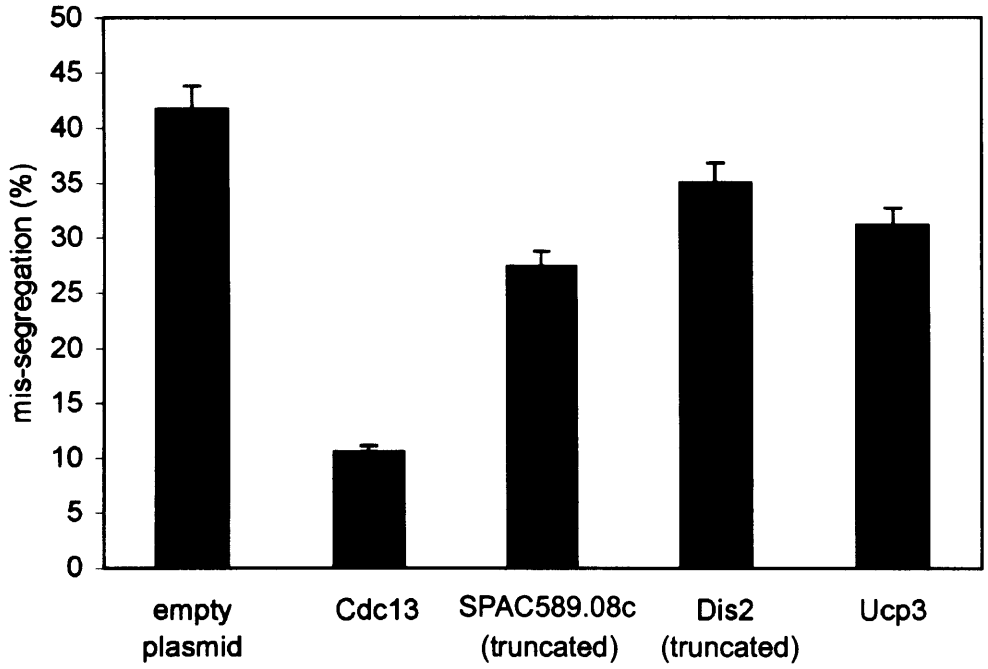


Figure 4.6 The suppressors reduce the chromsome segregation defect of *cdc13-117* cells. *cdc13-117 lys1:LacO his7:GFP-lacI cdc11-CFP:kan*^{r} cells were transformed with either empty plasmid, or plasmids expressing *cdc13*, SPAC589.08c, truncated *dis2*, or *ucp3* genes. Cells were synchronised in early G2 and incubated in fresh medium with 12.5μg/ml TBZ. After 140min samples were fixed and visualised by fluorescence microscopy. The frequency of mis-segregation of chromosome 1 (2 dots in one nucleus (Figure 4.4.C)) was expressed as a percentage of total binucleate cells. n=250.

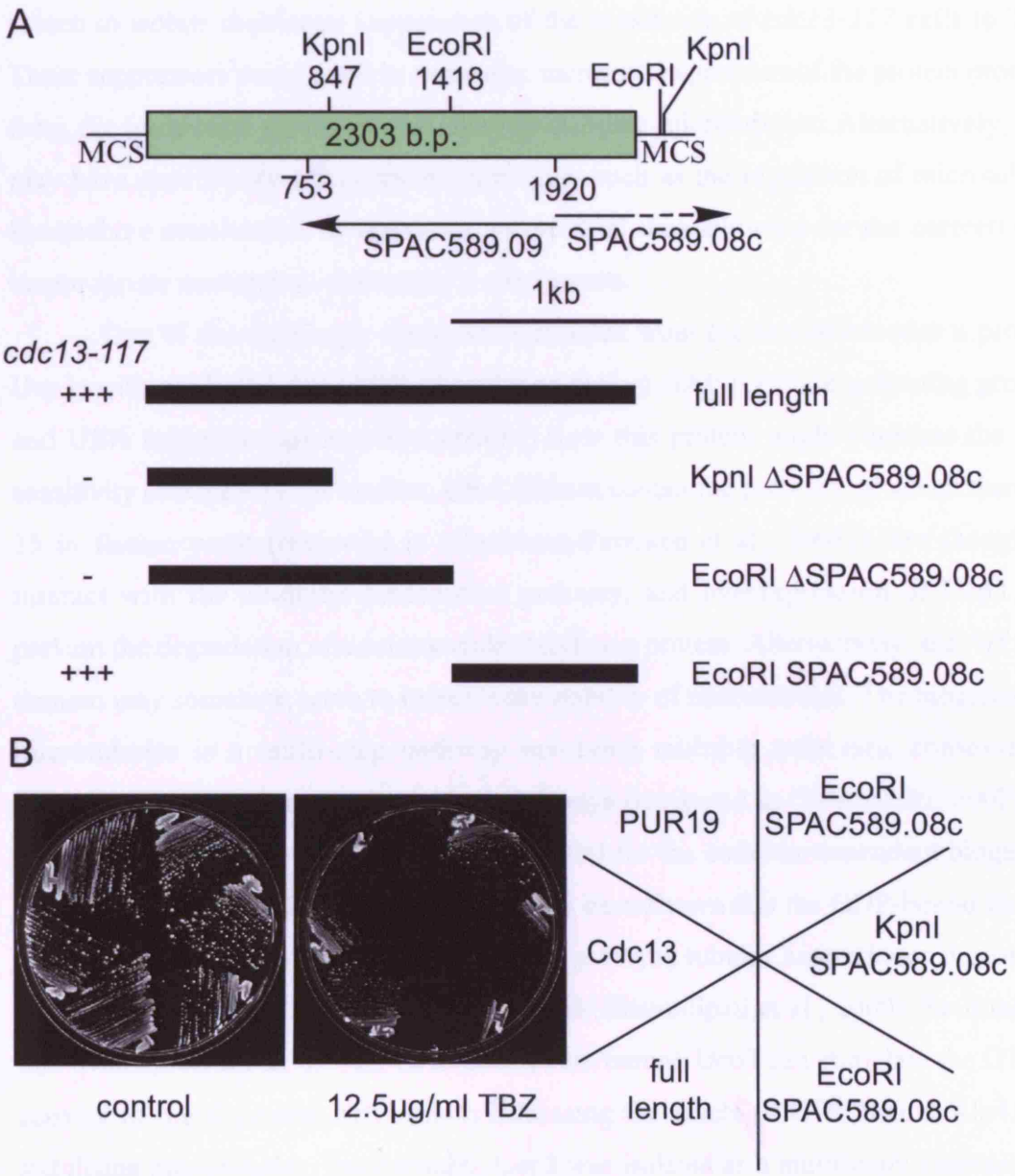


Figure 4.7 Truncated SPAC589.08c suppresses the TBZ sensitivity of *cdc13-117* cells.

(A) Restriction map of the genomic clone containing truncated *dam1*. Black arrows indicate the direction and size of the ORFs. Dashed line indicates the gene is truncated. The restriction enzyme sites in the clone and the plasmid multicloning site (MCS) used to create the sub-clones (thick black lines) are indicated. (B) cdc13-117 cells were transformed with the sub-clones, the full length clone, cdc13, or empty plamsid, and plated onto selective medium in the presence or absence of 12.5μg/ml TBZ and incubated for 3 days at 30°C.

To further investigate the defect of *cdc13-117* cells in mitosis, I performed a genetic screen to isolate multicopy suppressors of the sensitivity of *cdc13-117* cells to TBZ. These suppressors could work in two ways: increased expression of the protein products from the multicopy plasmids may directly stabilise microtubules. Alternatively, they may have specifically affect mitotic processes such as the regulation of microtubule-kinetochore attachments or activation of the SAC thus allowing for the correction of inappropriate microtubule-kinetochore attachments.

One of the multicopy suppressors isolated from the screen encodes a protein, Ucp3, with predicted Arf (ADP ribosylation factor) GAP (GTPase activating protein) and UBA (ubiquitin associated) domains. How this protein might suppress the TBZ sensitivity of *cdc13-117* is unclear. UBA domain containing proteins, of which there are 15 in fission yeast (reviewed in (Hartmann-Petersen et al., 2003)), are thought to interact with the ubiquitin-proteosome pathway, and overexpression of Ucp3 may prevent the degradation of a microtubule stabilising protein. Alternatively, the Arf GAP domain may somehow serve to increase the stability of microtubules. The biogenesis of microtubules is a multi-step pathway involving multiple cofactors, conserved in eukaryotes, acting in two symmetrical pathways (reviewed in (Szymanski, 2002)). In fission yeast the Arf protein, Alp41, is essential for the cofactor-dependent biogenesis of microtubules (Radcliffe et al., 2000). It has been shown that the GDP-bound form of the mammalian homologue of Alp41, Arl2, prevents tubulin heterodimer destruction when one of these co-factors is overexpressed (Bhamidipati et al., 2000). So it may be that overexpression of the Arf GAP domain containing Ucp3 can stimulate the GTPase activity of the *S. pombe* Arf, Alp41, increasing the levels of GDP-bound Alp41 and stabilising microtubules. Interestingly, Ucp3 was isolated as a multi-copy suppressor of a cold sensitive Cdc2 mutant (N. Walworth, personal communication). How Ucp3 interacts with Cdc2 has not been reported and whether this suppression is mechanistically similar to the suppression of *cdc13-117* TBZ sensitivity remains unclear.

One of the multicopy suppressors was a truncated allele of Dis2 type 1 phosphatase. Full length Dis2 was independently isolated as a mutant that blocked mitotic chromosome segregation (Ohkura et al., 1988; Ohkura et al., 1989) and also by its ability when overexpressed to prevent the mitotic entry of *cdc25-22 wee1-50* cells at high temperature (Booher and Beach, 1989). Furthermore, a truncated allele of *dis2* was also isolated in an independent screen to identify suppressors of the cell cycle arrest phenotype of the *mcs3-12 wee1.50 cdc25-22* strain (Samuel et al., 2000). Curiously, this

latter effect is a reversal of the inhibitory behaviour identified by Booher (Booher and Beach, 1989) and suggests that the truncated Dis2 may in fact act in a dominant negative manner. Intriguingly, Dis2 is homologous to the *S. cerevisiae* phosphatase Glc7, which has been shown genetically to act in opposition to the Aurora kinase Ipl1 (Francisco et al., 1994). Since Ipl1 is thought to phosphorylate the kinetochore protein Dam1 to promote the dissociation of incorrectly attached spindle microtubules (Kang et al., 2001; Cheeseman et al., 2002) it is likely that Glc7 dephosphorylates Dam1, thus allowing correct re-association. Interestingly, a truncated version of Glc7 lacking 126 amino acids from the C-terminus suppresses the chromosome segregation defect of an Ipl1 mutant (Francisco et al., 1994). If *S. pombe* Dis2 is a functional homologue of *S. cerevisiae* Glc7 then dominant negative truncation may be compensating for a lack of kinase activity suggesting that Aurora kinase activity is compromised in *cdc13-117* cells. In this respect, whether the *S. pombe* Aurora kinase Ark1 has a similar role to its *S. cerevisiae* homologue has not yet been proven, but cells lacking Ark1 mis-segregate chromosomes suggesting that kinetochore regulation is defective (Petersen et al., 2001). Intriguingly, a potential negative regulator of Dis2, Inh2, was isolated as a weak suppressor in the same screen, which encodes a small heat stable inhibitor of type 1 phosphatase and is homologous to the *S. cerevisiae* protein Glc8. Overexpression of Glc8 inhibits Glc7 (Tung et al., 1995). This suggests that if a similar scenario exists in *S. pombe* then Inh2, when overexpressed from a multicopy plasmid may serve to inhibit Dis2 and stabilise microtubule-kinetochore attachments. Another suppressor encodes a truncated version of a protein, Pef1, which may be in the same pathway as Dis2 and Inh2. Pef1 is a homologue of *S. cerevisiae* Pho85 kinase, and in fission yeast is implicated in the regulation of transcription at the G1-S transition (Tanaka and Okayama, 2000). In budding yeast, Pho85 phosphorylates and inhibits Glc8 (Tan et al., 2003) potentially activating Glc7 in mitosis. In fission yeast, Pef1 may have a similar role in which case the truncated protein may be a dominant negative version that prevents Inh2 inhibition, thus contributing to the inhibition of Dis2. The strongest suppressor isolated from the screen, the SPAC589.08c ORF, is analysed in detail in the next chapter.

Chapter 5

The function of *S. pombe* Dam1

5.1 Introduction

The SPAC589.08c ORF was isolated as a multi-copy suppressor of the TBZ sensitive *cdc13-117* mutant strain. In this chapter I have analysed the function of the protein encoded by the SPAC589.08c ORF.

5.2 Results

5.2.1 Dam1 regulates mitotic progression and resistance to environmental stress

The protein encoded by the SPAC589.08c ORF shares a short region of sequence homology to *S. cerevisiae* Dam1 (Figure 5.1), and was thus designated *S. pombe* Dam1. In *S. cerevisiae*, *dam1* is an essential gene and its protein product Dam1 is implicated in the regulation of chromosome bi-orientation (Jones et al., 1999; Cheeseman et al., 2002).

To investigate the function of *S. pombe* Dam1 a deletion was generated. First, a single allele of *dam1* was disrupted with the kanamycin resistance gene in a heterozygous h-/h+ strain (Bahler et al., 1998). Tetrad dissection of Δ*dam1/dam1+* heterozygous diploid gave rise to four viable spores that on germination showed a 2:2 segregation of kanamycin resistance. Thus unlike budding yeast Dam1 protein, its fission yeast homologue is non-essential for viability. Although loss of Dam1 only marginally affects the rate of cell proliferation, 8% of log phase Δ*dam1* cells display mitotic abnormalities that include hypercondensed or fragmented chromosomes, chromosome mis-segregation (unequal chromatin staining) or ectopic septation in the absence of chromosome segregation (cut phenotype) as judged by staining with DAPI and calcofluor (Figure 5.2.A). These phenotypes were fully suppressed by re-introduction of a plasmid expressing full length *dam1* gene (data not shown). To

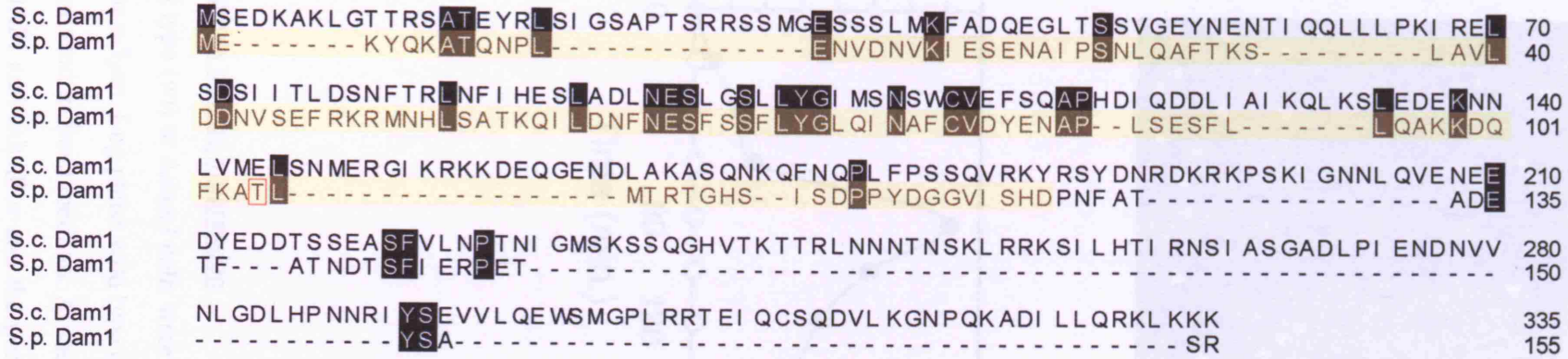


Figure 5.1 Dam1 in *S. cerevisiae* and *S. pombe*

Alignment of *S. cerevisiae* Dam1 and *S. pombe* Dam1 proteins. Black boxes denote the regions of homology. Red box indicates the putative Aurora kinase phophorylation site [(R/K)X(T/S)(I/L/V)] . The yellow highlighting indicates the truncated Dam1(1-127) protein.

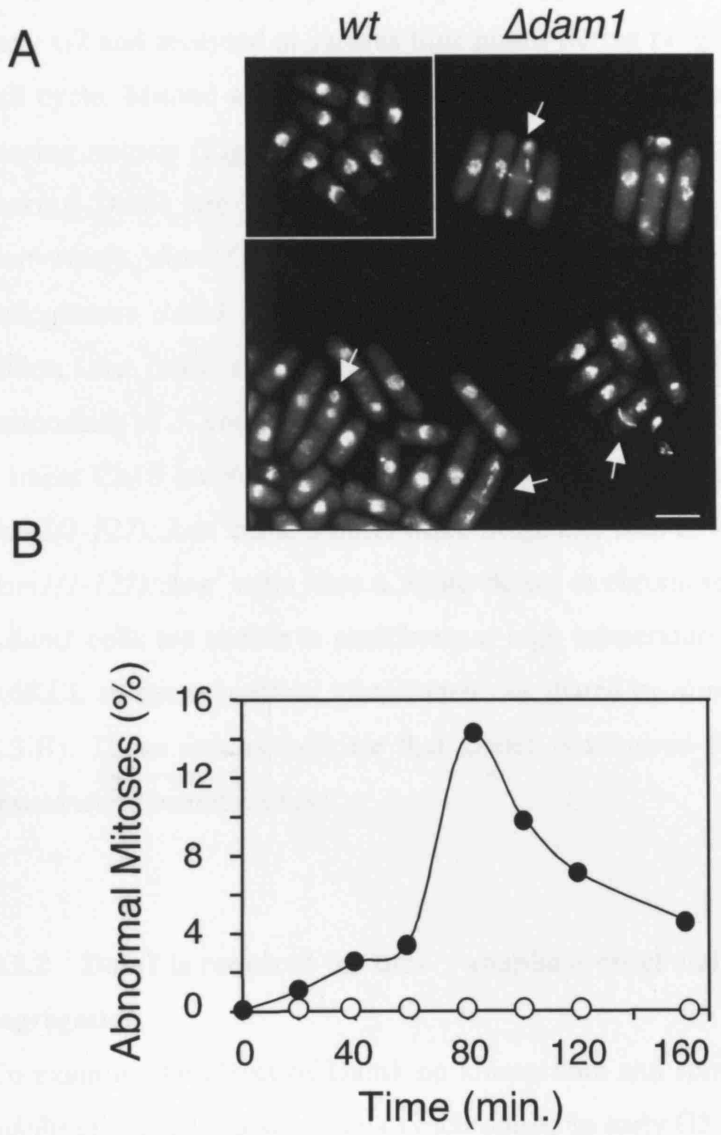


Figure 5.2 Dam controls mitotic progression

(A) Log phase wild type (wt) or Δ*dam1* cells were fixed and stained with DAPI and calcafluor. Scale bar = 5μm. Log phase wild type or Δ*dam1* cells were synchronised in early G2 and incubated in fresh medium. At the times indicated cells were fixed and stained with DAPi and calcuflor and the percentage of mitotic abnormalities expressed as a percentage of the total number of cells.

examine this phenotype more closely, wild type and Δ*dam1* cells were synchronised in early G2 and analysed at various time points during progression through the following cell cycle. Mitotic abnormalities were observed in only a proportion of Δ*dam1* cells passing mitosis (Figure 5.2.B). Consistent with a defect in mitotic progression, cells lacking Dam1 are more sensitive to TBZ than wild type cells (Figure 5.3.A). Conversely, *dam1(1-127):kanR* cells, in which the C-terminal 28 aminoacids of the endogenous *dam1* gene have been removed (see Figure 5.1) by insertion of a stop codon, are more resistant to thiabendazole, suggesting that the C-terminal 28 aminoacids of *S. pombe* Dam1 are important for this function (Figure 5.3.A). However, a linear Ch16 (ade6-M216) mini-chromosome was lost in 0.03% of cell divisions in *dam1(1-127)::kan*^{r} cells, 3 times more frequently than in wild type cells suggesting that *dam1(1-127)::kan*^{r} cells have a minor defect in chromosome segregation. In addition, Δ*dam1* cells are unable to proliferate at high temperature (37°C) or in the presence of 0.6KCl, an osmotic stress, phenotypes not shared by *dam1(1-127):kanR* cells (Figure 5.3.B). These results indicate that Dam1 is required for microtubule stability and resistance to osmotic stress.

5.2.2 Dam1 is required for timely anaphase onset and accurate chromosome segregation

To examine the effect of Dam1 on kinetochore and spindle pole dynamics a Δ*dam1 ndc80-gfp cdc11-cfp* strain was synchronised in early G2 and the percentage of cells in prometaphase or metaphase was analysed during the subsequent mitosis (Tournier et al., 2004). Consistent with a defect in mitotic progression, cells lacking Dam1 remain in prometaphase or metaphase on average twice as long as control cells (as judged by the area under the curve) (Figure 5.4.A). Filming of individual cells also revealed considerable cell to cell variation in both kinetochore behaviour and the overall time spent in prometaphase or metaphase. Images from movies of a representative wild type and Δ*dam1* cell undergoing sister chromosome segregation is shown in Figure 5.4.B. In the wild type cell, kinetochores oscillate between the SPBs and congress to the spindle mid-zone prior to anaphase onset, as previously observed (Figure 5.4.B; (Tournier et al., 2004)). In this and other movies kinetochores segregate equally to the SPBs (3:3). In the cells lacking Dam1 the mitotic spindle was found to be 3.6μm at anaphase onset as compared to 2.6μm in the wild type cell, because the onset of anaphase A occurs just after the onset of phase 3 (spindle elongation) (Figure 5.4.B). In the absence of Dam1,

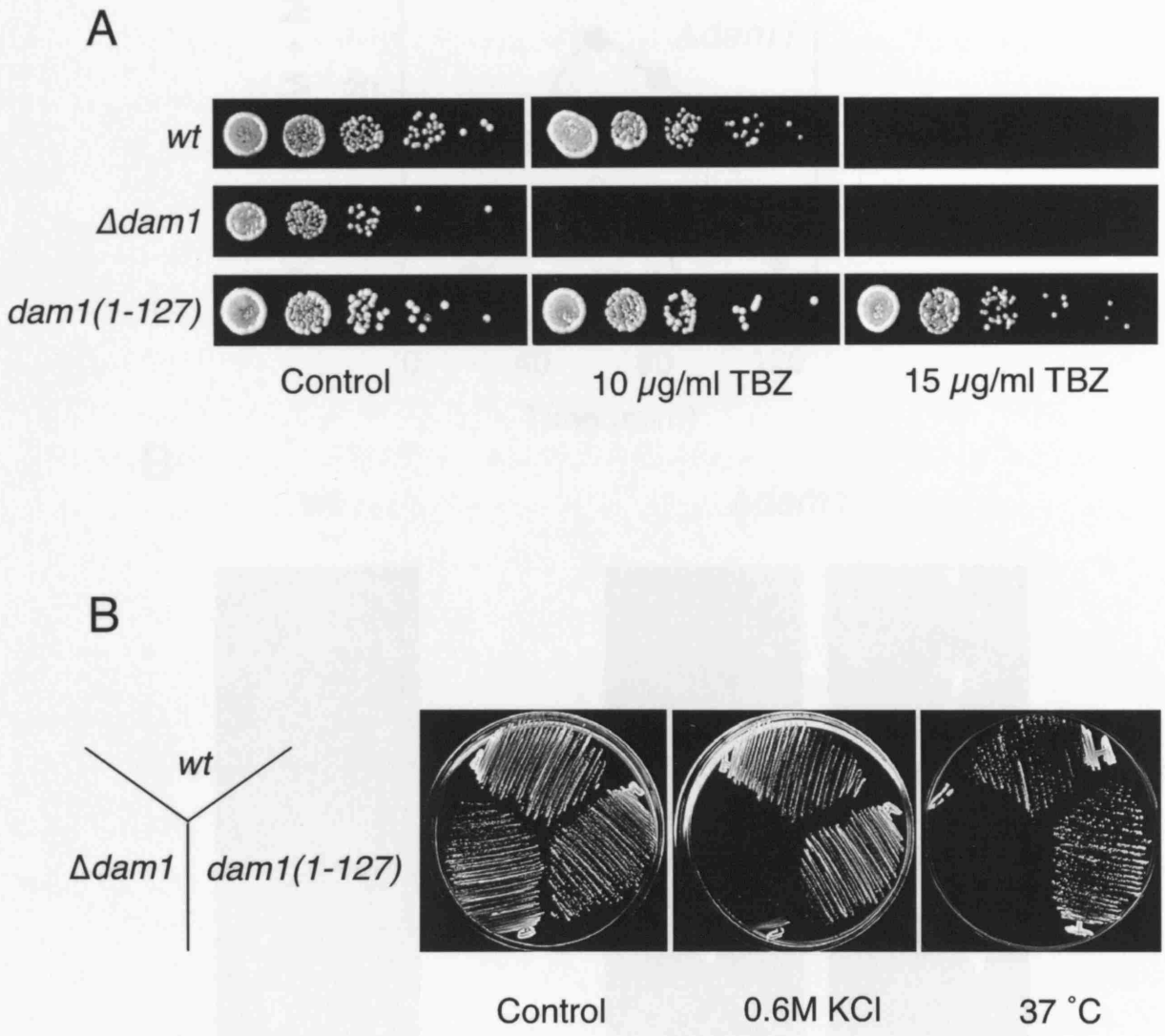


Figure 5.3 Cells lacking Dam1 are sensitive to TBZ and environmental stress.

(A) Ten fold serial dilutions of wild type, *Δdam1* or *dam1(1-127):kanR* cells were spotted onto rich medium either in the absence (control) or presence of either 10 μg/ml TBZ or 15 μg/ml TBZ and incubated for 3 days at 30°C. (B) Wild type (*wt*), *Δdam1* or *dam1(1-127):kanR* cells were streaked onto rich medium in the absence (control) or presence of 0.6M KCl (0.6M KCl) at 30°C or on rich medium at 37°C (37°C) for 3 days.

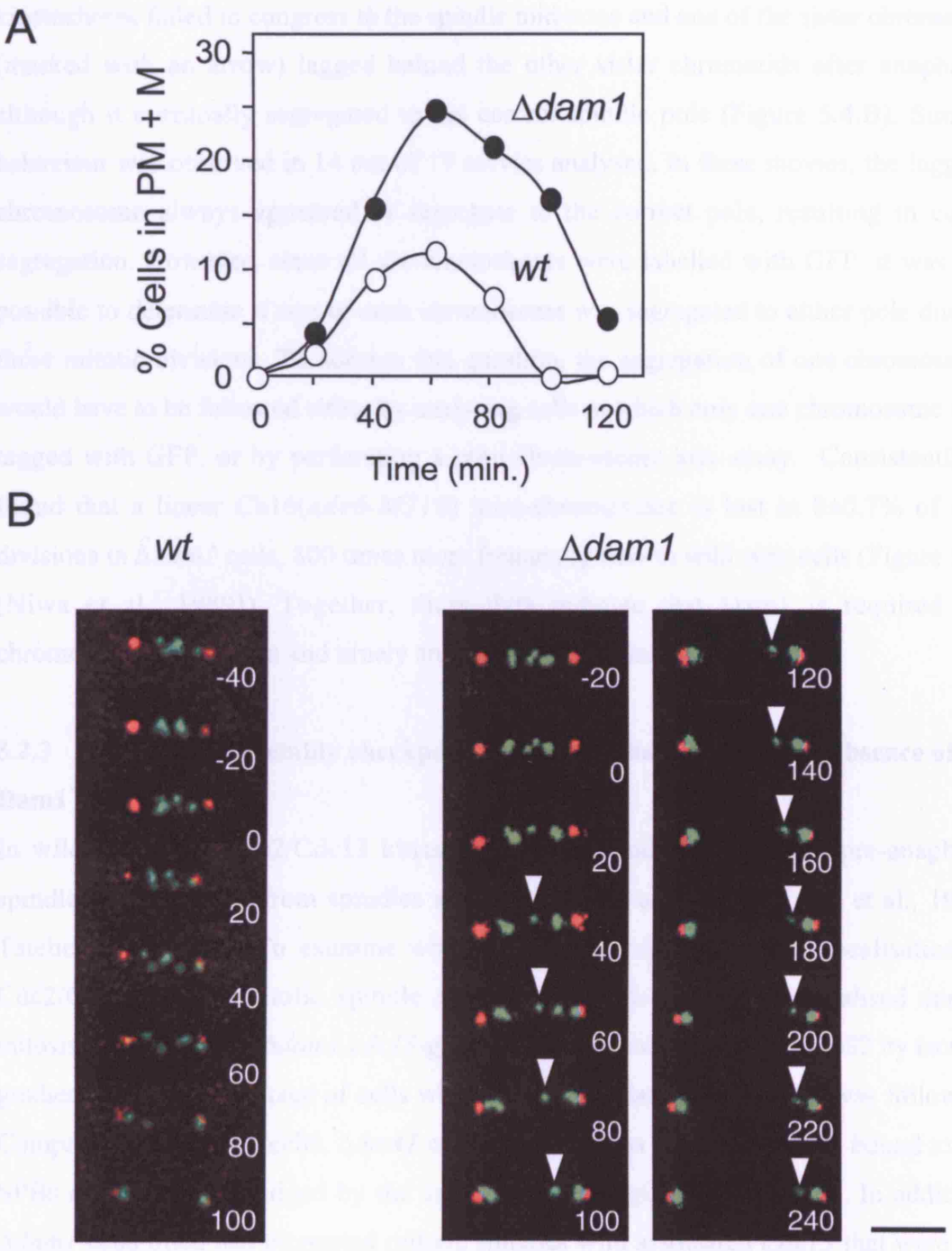


Figure 5.4 Dam1 is required for chromosome congression and timely anaphase onset.

(A) Log phase *ndc80-gfp cdc11-cfp* cells and *Δdam1 ndc80-gfp cdc11-cfp* cells were synchronised in early G2 and incubated in fresh medium. The percentage of cells in prometaphase and metaphase (PM+M) was calculated. (B) Images from movies of a *ndc80-gfp cdc11-cfp* cell (left panels) or a *Δdam1 ndc80-gfp cdc11-cfp* cell (right panel) in mitosis. Time zero is taken as the onset of anaphase A. Scale bar = 2 μm. Arrow indicate a lagging sister chromatid.

kinetochores failed to congress to the spindle mid-zone and one of the sister chromatids (marked with an arrow) lagged behind the other sister chromatids after anaphase, although it eventually segregated to the correct spindle pole (Figure 5.4.B). Similar behaviour was observed in 14 out of 19 movies analysed. In these movies, the lagging chromosome always appeared to segregate to the correct pole, resulting in equal segregation. However, since all six kinetochores were labelled with GFP, it was not possible to determine if one of each chromosome was segregated to either pole during these mitotic divisions. To address this question, the segregation of one chromosome would have to be followed either by analysing cells in which only one chromosome was tagged with GFP, or by performing a mini-chromosome loss assay. Consistently, I found that a linear Ch16(*ade6-M216*) mini-chromosome is lost in 8±0.7% of cell divisions in Δ*dam1* cells, 800 times more frequently than in wild type cells (Figure 5.5; (Niwa et al., 1989)). Together, these data indicate that Dam1 is required for chromosome segregation and timely anaphase onset in fission yeast.

5.2.3 The spindle assembly checkpoint delays anaphase onset in the absence of Dam1

In wild type cells Cdc2/Cdc13 kinase binds SPBs and short <2.5μm pre-anaphase spindles but is absent from spindles and SPBs during anaphase B (Alfa et al., 1990; Tatebe et al., 2001). To examine whether Dam1 is required for the localisation of Cdc2/Cdc13 to the mitotic spindle Δ*dam1 cdc13-gfp* cells were visualised during mitosis. *cdc13-gfp* and Δ*dam1 cdc13-gfp* cells were synchronised in early G2 by lactose gradient and the percentage of cells with spindle associated Cdc13-GFP was followed. Compared to wild type cells, Δ*dam1* cells spent twice as long with Cdc13 bound to the SPBs and spindle as judged by the area under the graph (Figure 5.6.A). In addition, Δ*dam1* cells often had elongated mitotic spindles with associated Cdc13 that were not seen in wild type cells (Figure 5.6.B). The prolonged association of Cdc13 to the spindle suggests that in the absence of Dam1 mitotic Cdc13 localisation is not prevented but that the activation of the anaphase promoting complex (APC) is delayed.

To examine whether the delay in anaphase onset was due to activation of the SAC, I monitored Mad2 and Bub1 localisation, components of the SAC that bind to the kinetochore either when spindle microtubules are not attached or the spindle is not under tension (Bernard et al., 1998; Ikui et al., 2002; Tournier et al., 2004). In wild type cells Mad2 re-localises to an area adjacent to one of the two SPBs during an unperturbed cell cycle (Tournier et al., 2004). I found that less than 2% of *mad2-gfp*

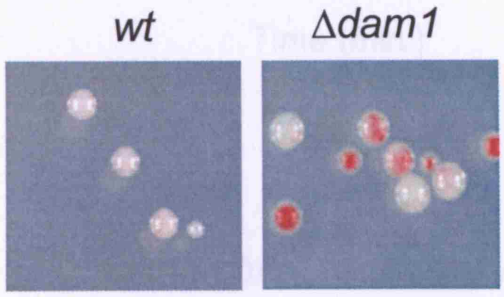
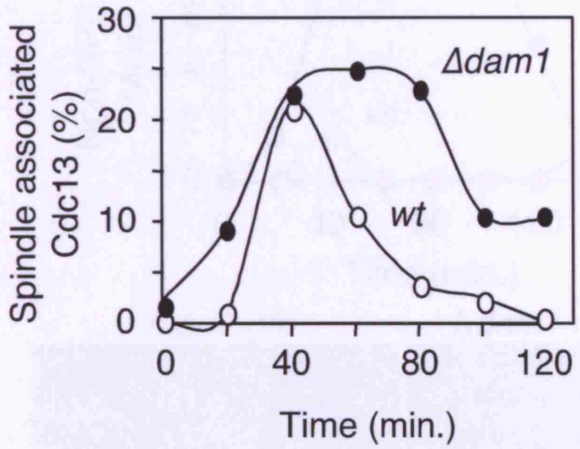


Figure 5.5 Mini-chromosome loss in cells lacking Dam1

Wild type or Δ*dam1* cells bearing a linear mini-chromosome Ch16 [ade6-M216] were plated on medium containing limiting adenine and the frequency of chromosome loss per division analysed. Approximately 10,000 colonies were counted and those that were more than half pink were counted as having mis-segregated the mini-chromosome in the first cell division after plating.

A



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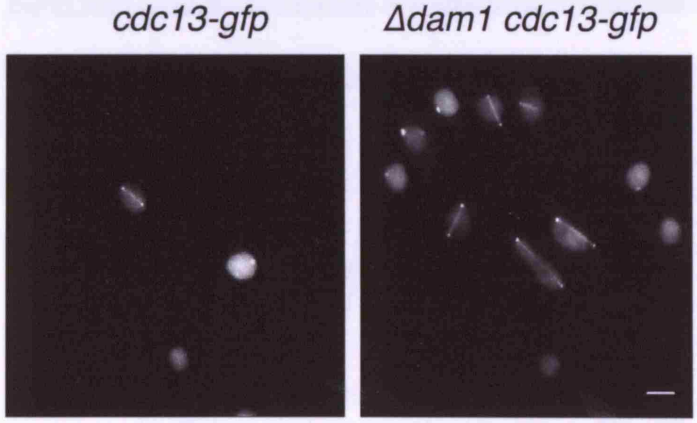


Figure 5.6 Cdc13 binds to mitotic spindles for longer in the absence of Dam1

(A) Log phase *cdc13-gfp* and *Δdam1 cdc13-gfp* cells were synchronised in early G2 and incubated into fresh medium. At the times indicated cells were fixed and visualised by fluorescence microscopy for the presence of spindle pole or spindle associated Cdc13. (B) Images of *cdc13-gfp* and *Δdam1 cdc13-gfp* cells are shown at the t = 40 minute time point. Scale bar = 2μm.

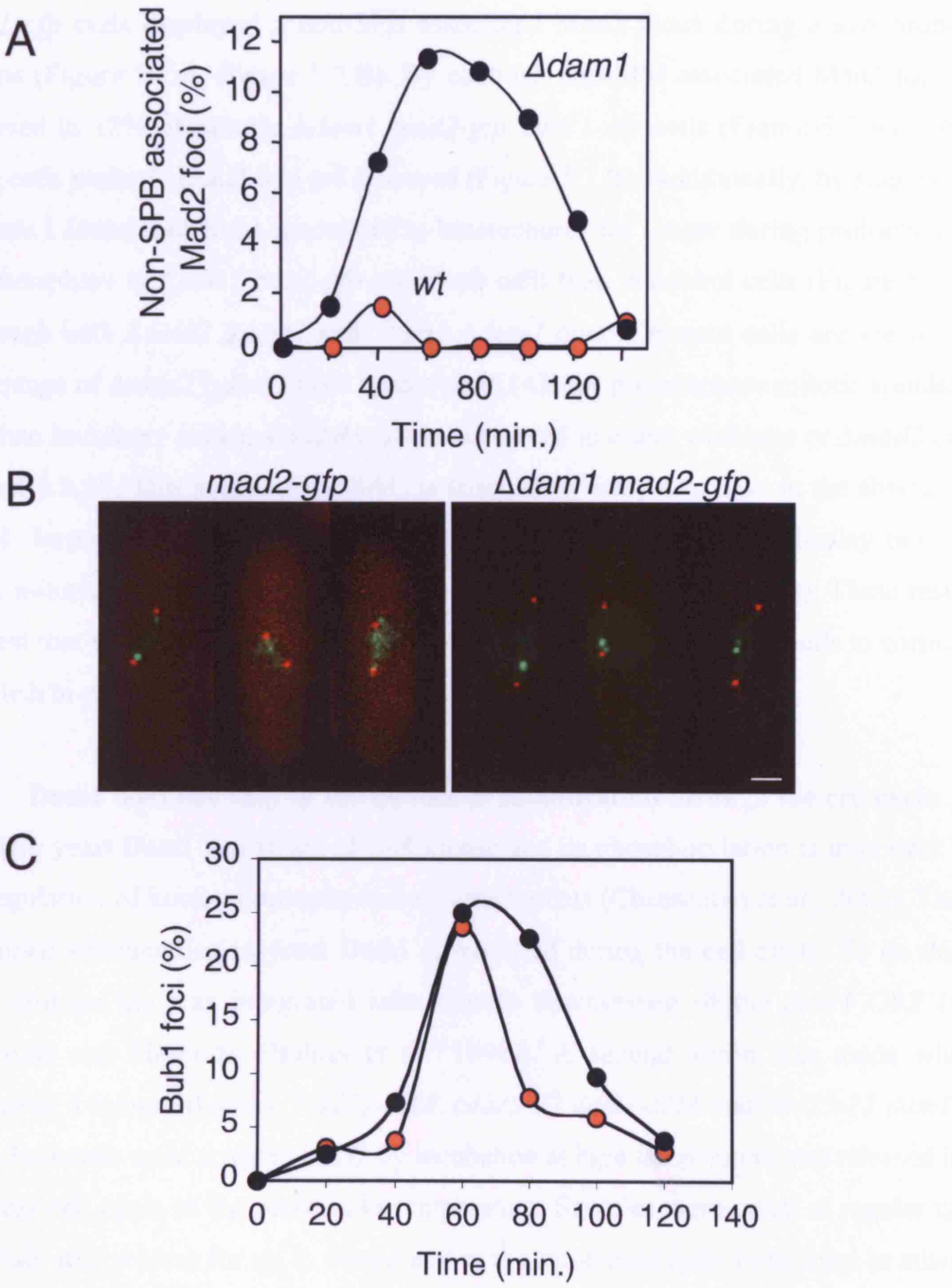


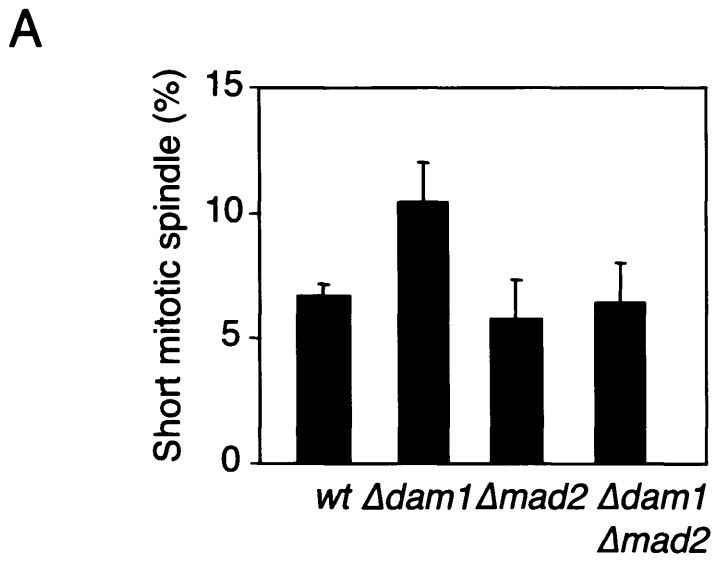
Figure 5.7 Mad2 and Bub bind to kinetochores for longer in the absence of Dam1.

(A) Log phase *mad2-gfp* and *Δdam1 mad2-gfp* cells were synchronised in early G2 and incubated into fresh medium. At the times indicated cells were fixed and visualised by fluorescence microscopy for the appearance of Mad2 foci. (B) Images of *mad2-gfp* and *Δdam1 mad2-gfp* cells are shown at the t = 40 minute time point. Scale bar = 2μm. (C) Log phase *bub1-gfp* and *Δdam1 bub1-gfp* cells were synchronised in early G2 and incubated into fresh medium. At the times indicated cells were fixed and visualised by fluorescence microscopy for the appearance of Bub1 foci.

cdc11-cfp cells displayed a non-SPB associated Mad2 focus during a synchronous mitosis (Figure 5.7.A; Figure 5.7.B). By contrast, non-SPB associated Mad2 foci are observed in 12% of mitotic Δ*dam1 mad2-gfp cdc11-cfp* cells (Figure 5.7.A) and in some cells multiple Mad2 foci are observed (Figure 5.7.B). Additionally, by single cells analysis I found that Bub1 associates to kinetochores for longer during prometaphase and metaphase in Δ*dam1 bub1-gfp cdc11-cfp* cells than in control cells (Figure 5.7.C). Although both Δ*mad2* Δ*dam1* and Δ*bub1* Δ*dam1* double mutant cells are viable, the percentage of Δ*mad2* Δ*dam1* cells with a short (<3μm) pre-anaphase mitotic spindle is less than in Δ*dam1* cells and similar to that observed in either wild type or Δ*mad2* cells (Figure 5.8.A). This suggests the SAC is required to delay anaphase in the absence of Dam1. Importantly, both Δ*mad2* Δ*dam1* and Δ*bub1* Δ*dam1* strains display twice as many mitotic abnormalities than observed in Δ*dam1* cells (Figure 5.8.B). These results suggest that the SAC delays anaphase to allow a proportion of Δ*dam1* cells to correctly establish bi-polarity.

5.2.4 Dam1 does not vary in abundance or modification through the cell cycle

Budding yeast Dam1 is a target of Ipl1 kinase and its phosphorylation is important for the regulation of kinetochore-microtubule attachments (Cheeseman et al., 2002). I next examined whether fission yeast Dam1 is modified during the cell cycle. To do this a *3HA* epitope tag was integrated immediately downstream of the *dam1* ORF (see Materials and Methods; (Bahler et al., 1998)). A second strain was made which expressed a truncated *dam1(1-127)-3HA*. *cdc25-22 dam1-3HA* and *cdc25-22 dam1(1-127)-3HA* cells were arrested in G2 by incubation at high temperature and released into the next cell cycle at the permissive temperature. Samples were taken at regular time intervals after release for up to 60min and at the same time cells were fixed in ethanol and scored for bi-nucleates to monitor cell cycle synchrony (Figure 5.9.A). Cell extracts were made and run on a 12% SDS-PAGE gel which was transferred to nitrocellulose and blotted with the mouse monoclonal anti-HA antibody 12CA5 (See Materials and Methods). Protein levels of both Dam1 and Dam1(1-127) were constant 60min into the cell cycle, and there was no visible mobility shift (Figure 5.9.B). Since changes in electrophoretic mobility normally indicate a change in phosphorylation, this result suggests that there is no change in phosphorylation status of either protein as cells enter mitosis or that such changes as there may be are not detectable by this method.



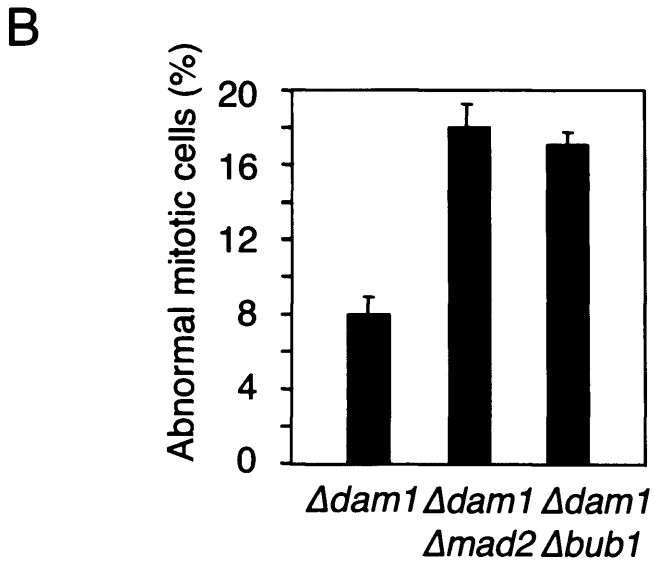
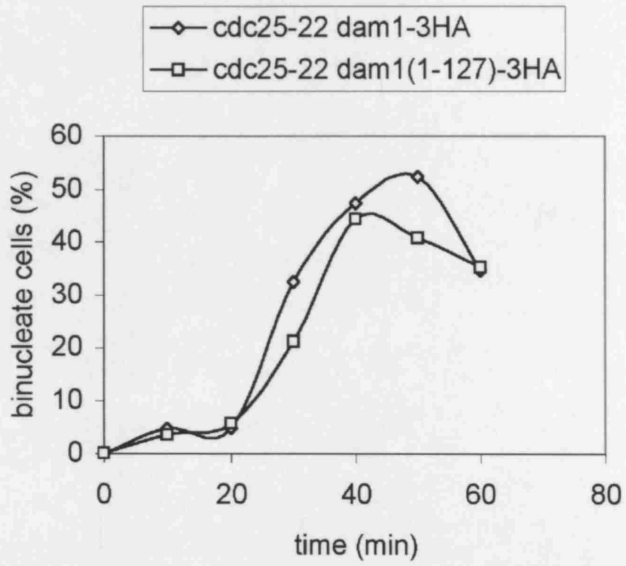


Figure 5.8 Loss of Dam1 causes activation of the spindle assembly checkpoint

(A) Log phase *nmt1-atb2-gfp*, *Δdam1 nmt1-atb2-gfp*, *Δmad2 nmt1-atb2-gfp* and *Δdam1Δmad2 nmt1-atb2-gfp* cells grown in the presence of thiamine were fixed and the percentage of cells with short (<3μm) pre-anaphase mitotic spindles determined. The results are the average of three independent experiments. (B) Log phase *Δdam1*, *Δdam1 Δmad2* or *Δdam1 Δbub1* cells were fixed and stained with DAPi and calcuflor. The percentage of mitotic abnormalities was expressed as a percentage of the total number of cells from three independent experiments.

A



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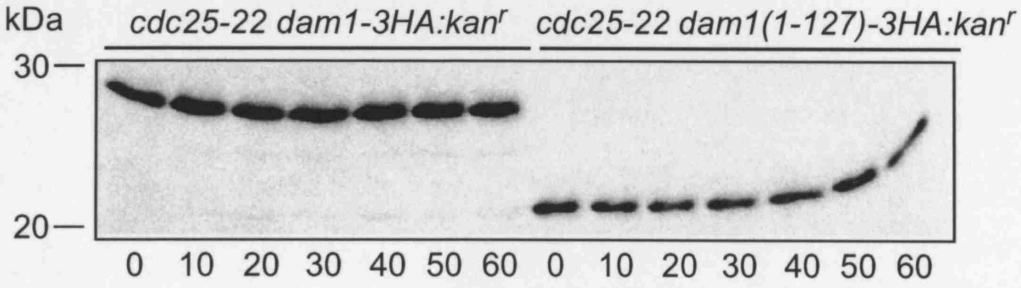


Figure 5.9 Dam1 and Dam1(1-127) are not phosphorylated in an unperturped cell cycle

(A) *cdc25-22 dam1-3HA:kan*^{r} or *cdc25-22 dam1(1-127)-3HA:kan*^{r} cells were incubated at the restrictive temperature for 4h then rapidly cooled to the permissive temperature. Samples were taken every 10min and the percentage of binucleate cells analysed. (B) From the experiment in (A) samples were taken and crude yeast extract was prepared as described in Materials and Methods. Crude extract was run on a 12% SDS-PAGE gel, transferred to Immobilon™-P nitrocellulose membrane, and probed with an anti-HA monoclonal antibody as described in Materials and Methods.

5.2.5 Dam1 associates to the plus end of spindle microtubules and kinetochores in mitosis

To determine the sub-cellular localisation of *S. pombe* Dam1, the *dam1* gene was tagged with the green fluorescent protein (GFP) (see Materials and Methods; (Bahler et al., 1998)) and examined *in vivo* in cells expressing the tagged kinetochore component Ndc80-CFP. Examination of fixed *dam1-gfp ndc80-cfp* cells showed that Dam1 does not associate with any identifiable cellular structure in interphase, but forms a single dot within the nucleus in early mitosis and multiple dots during pro-metaphase and metaphase, suggesting that it is recruited to kinetochores as cells enter mitosis (Figure 5.10.A). A high magnification image of a fixed *dam1-gfp ndc80-cfp* cell in metaphase shows that each of the Dam1 signals is associated closely, but not co-incidentally, with Ndc80 (Figure 5.10.B). A greater region of overlap between the Dam1 and Ndc80 signals is seen after anaphase A (Figure 5.10.B). Time-lapse analyses of live *dam1-gfp* cells show up to 6 dots of Dam1 that move rapidly in metaphase before separating to 2 dots that segregate to either end of the cell in anaphase (Figure 5.11.A). In movies of *dam1-gfp* cells, in addition to up to six stable Dam1 dots, I observed other Dam1 dots that rapidly appear and disappear during prometaphase and metaphase (see arrows Figure 5.11.A). By analysing fixed *dam1-gfp ndc80-cfp* cells I determined that these dots of Dam1 do not co-localise with Ndc80 (see arrow Figure 5.11.B). To determine if the non-Ndc80 associated dots of Dam were inside or outside the nucleus, *dam1-gfp cut11-cfp* cells were visualised in mitosis. Cut11 is associated with the nuclear envelope during interphase and with the nuclear envelope and SPBs during mitosis (West et al., 1998). The image in Figure 5.12.A shows seven Dam1 dots, six of which lie in a line between the SPBs whereas the seventh is located on the inner face of the nuclear envelope (see arrow Figure 5.12.A). During early mitosis, intranuclear spindle microtubules that are not part of the mitotic spindle are highly dynamic and can normally only be observed by live imaging (Zimmerman et al., 2004). However, I found that by overexpressing Dam1-GFP from the thiamine repressible *nmt1* promoter and staining of microtubules with a tubulin-specific antibody, I was able to observe Dam1 at the tips of intranuclear spindle microtubules (see arrow Figure 5.12.B). A truncated allele of *dam1,* Dam1(1-127) was tagged with GFP and its localisation examined in the same way to see if it was the same as the full length protein. Dam1(1-127) localised closely to, but not co-incidentally with Ndc80 in fixed mitotic cells (Figure 5.13.A). Time-lapse analysis of live cells showed fast moving dots of Dam1(1-127) (Figure 5.13.B), that were not associated with Ndc80 but were within the nucleus (data not

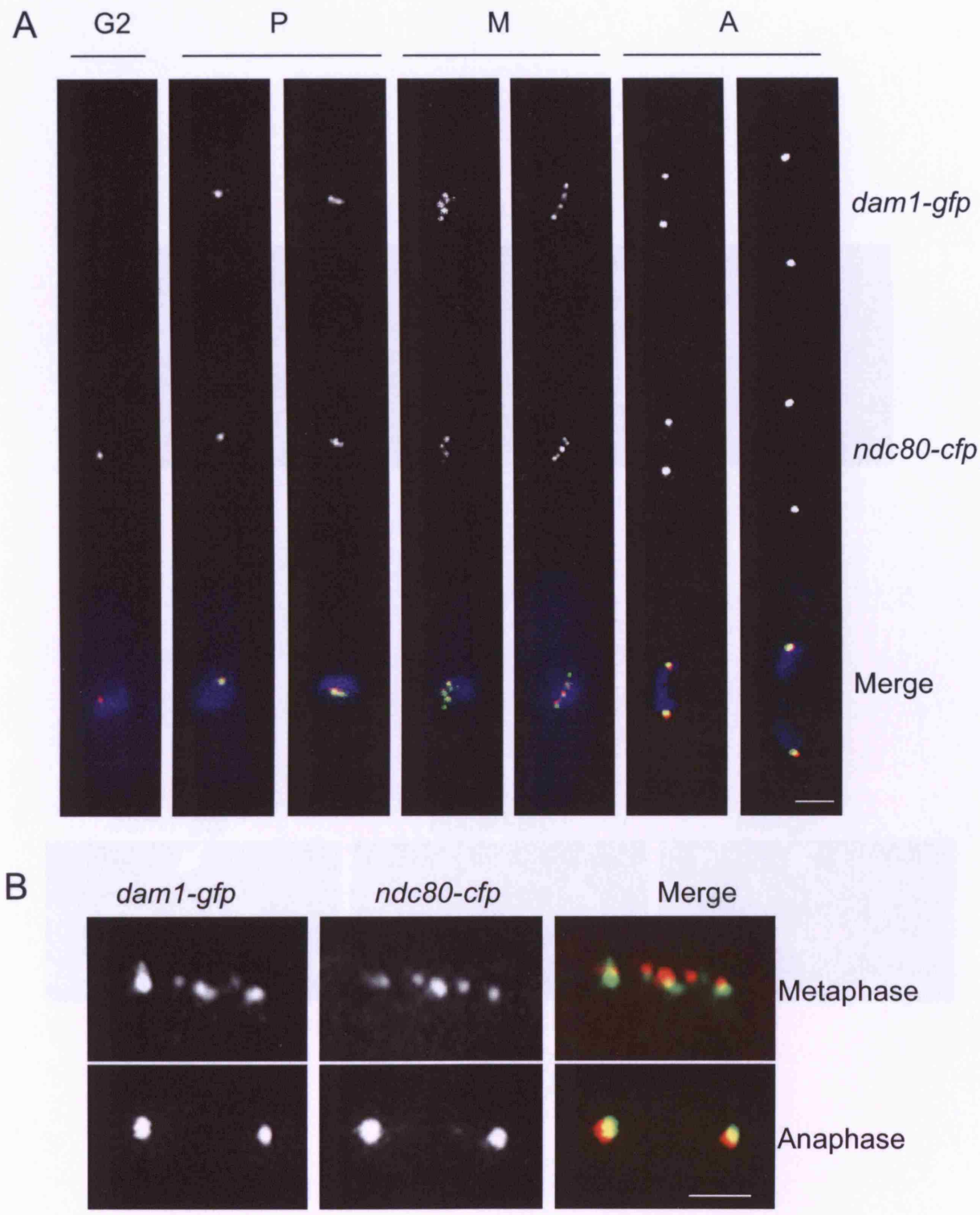


Figure 5.10. Dam1 associates with kinetochores during mitosis.

(A) Log phase *dam1-gfp ndc80-cfp* cells were fixed and analysed by fluorescence microscopy. Cells were catagorized for cell cycle distribution based on cell size and distribution of Ndc80 dots as either interphase (G2), prometaphase (P), metaphase (M) or anaphase (A). Scale bar = 2μm. (B) Higher magnification image of fixed *dam1-gfp ndc80-cfp* at metaphase or anaphase. Scale bar = 1μm.

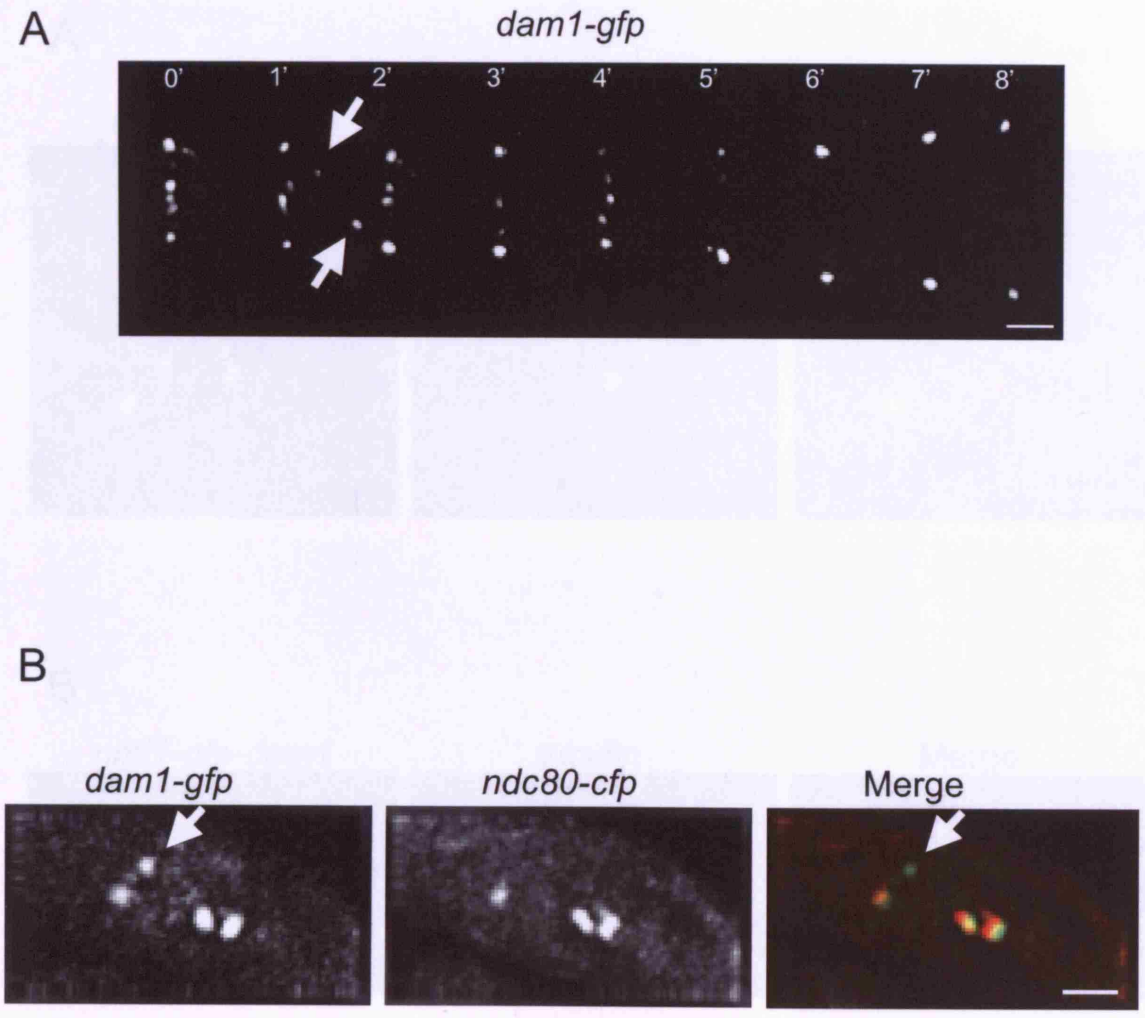


Figure 5.11 Dam1 associates to a non-kinetochore region in mitosis

(A) Images from a movie of *dam1-gfp* cells in mitosis. Arrow indicates association of Dam1 to a non-kinetochore region. Time in minutes. Scale bar = 1μm. (B) Image of fixed *dam1-gfp ndc80-cfp* cell in mitosis. Arrow indicates non-kinetochore associated Dam1. Scale bar = 1μm.

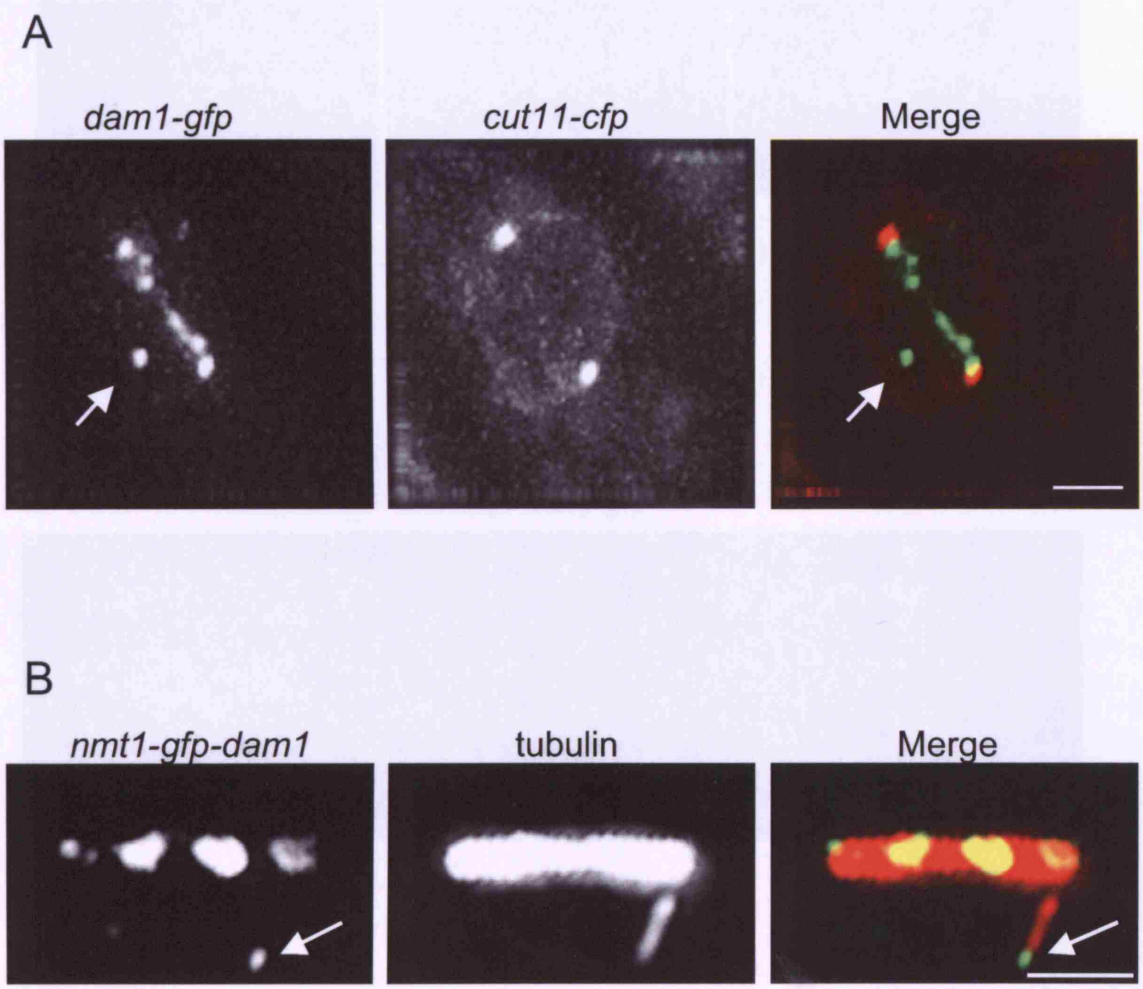


Figure 5.12 Dam1 associates with intranuclear microtubules

(A) Image of *dam1-gfp cut11-cfp* cell in mitosis. Six Dam1 dots are aligned between the spindle poles. The arrow indicates a seventh Dam1 dot which is inside the nucleus. (B) Image of fixed *nmt1-gfp*-dam1 cell in mitosis stained with a tubulin specific antibody. Arrow indicates Dam1 at the end of an intranuclear microtubule. Scale bars = 1μm.

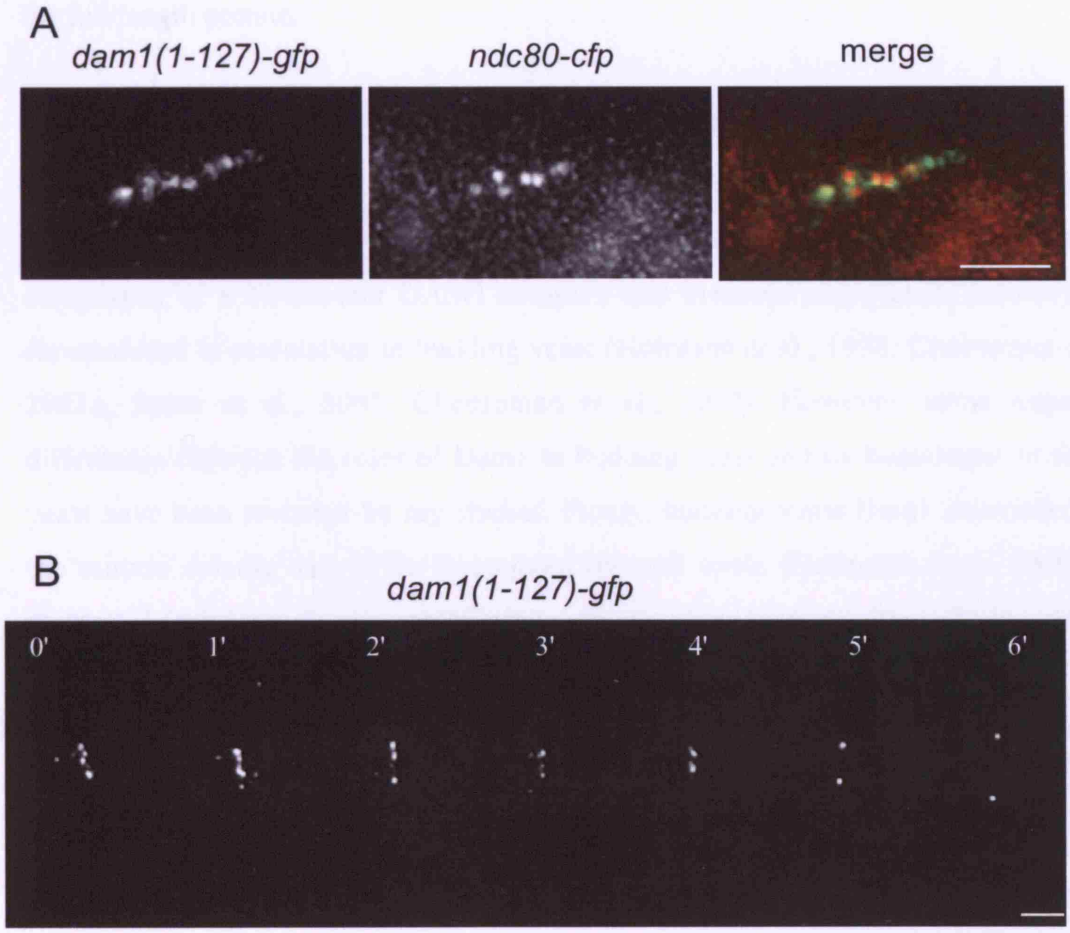


Figure 5.13. Dam1(1-127) displays an identical localisation to full length Dam1.

(A) Fixed *dam1(1-127)-gfp ndc80-cfp* cells in mitosis. Scale bar = 1μm. (B) Images from a movie of *dam1-gfp* cells in mitosis. Arrow indicates association of Dam1 to non-kinetochore associated microtubules. Time in minutes. Scale bar = 2μm.

shown). Taken together, these data strongly suggest that Dam1 binds to kinetochores and non-kinetochore associated spindle microtubules' tips during early mitosis and that the localisation of Dam1 protein lacking the C-terminal 28 amino acids is identical to the full length protein.

5.3 Discussion

A truncated allele of *dam1* was identified in a genetic screen for multicopy suppressors of the thiabendazole sensitivity of the *cdc13-117* mutant strain (Chapter 4). Dam1 is a component of a 10-subunit DASH complex that controls microtubule stability and chromosome bi-orientation in budding yeast (Hofmann et al., 1998; Cheeseman et al., 2001a; Jones et al., 2001; Cheeseman et al., 2002). However, some important differences between the roles of Dam1 in budding yeast and its homologue in fission yeast have been revealed by my studies. Firstly, budding yeast Dam1 associates with the mitotic spindle and SPBs throughout the cell cycle (Hofmann et al., 1998). In contrast, I found that fission yeast Dam1 only associates to kinetochores during mitosis. This finding is consistent with the observation that the kinetochores of budding yeast chromosomes are attached to microtubules from the SPB during interphase (Knop et al., 1999), whilst fission yeast chromosomes are directly attached to the SPB without any tubulin between the kinetochore and the SPB (Ding et al., 1993; Ding et al., 1997). In this respect, Dam1 shares a property with several other fission yeast proteins important in establishing chromosome bi-orientation including Dis1/Alp14 and Klp5/Klp6, which only associate to the kinetochore during mitosis (Nakaseko et al., 2001; West et al., 2002). Secondly, budding yeast Dam1 is essential (Jones et al., 1999). In contrast, I found that Dam1 is non-essential in fission yeast. This may be due to the difference in microtubule-kinetochore interactions between fission and budding yeast. During the establishment of chromosome bi-orientation in budding yeast, each kinetochore is attached to only one spindle microtubule (Winey et al., 1995). As chromosomes are replicated both sister chromatids are frequently captured by spindle microtubules from the same pole resulting in a syntelic attachment. In this situation tension cannot be applied across the mitotic spindle (Dewar et al., 2004). As cells enter mitosis the lack of spindle tension is sensed at the kinetochore by the Aurora-like kinase Ipl1, which phosphorylates a number of kinetochore associated proteins, including Dam1, to trigger microtubule detachment so that each sister chromatid can be attached to spindle microtubules from opposite poles (amphitelic attachment) (Cheeseman et al., 2002; Tanaka et al., 2002; Dewar et al., 2004). In the absence of Dam1, budding yeast cells

may be unable to resolve syntelic attachments resulting in lethal mis-segregation. In contrast, the kinetochores of replicated fission yeast sister chromatids are detached from microtubules till cells enter mitosis (Ding et al., 1993; Ding et al., 1997), reducing the likelihood of forming syntelic attachments. In budding yeast Dam1 may be the main protein that is involved in either microtubule-kinetochore attachment or correction, whilst in fission yeast other mechanisms are involved in these processes.

By careful time-lapse microscopy, I found that Dam1 binds not only to kinetochores, but also to non-kinetochore regions in early mitosis that have subsequently been shown to be the plus end of intra-nuclear spindle microtubules. These microtubules are highly dynamic and are thought to "search and capture" unattached kinetochores during pro-metaphase and metaphase and are not visible after anaphase onset (Sagolla et al., 2003; Zimmerman et al., 2004). Recently, purified budding yeast DASH complex has been found to preferentially bind GTP-tubulin *in vitro* (Westermann et al., 2005), providing an explanation for its affinity for the plus end of microtubules.

Cells lacking Dam1 are defective in the maintenance of a mini-chromosome. Since Dam1 only binds kinetochores in mitosis, this is likely to be due to inefficient mini-chromosome segregation rather than defective replication. This conclusion is borne out by live analysis of kinetochore dynamics. In wild type cells, kinetochores oscillate between the spindle poles during prometaphase and metaphase and congress to the spindle mid-zone just before anaphase onset. However in most cells lacking Dam1, chromosome congression does not take place and lagging sister chromatids are frequently observed during anaphase B. This may be due to persistent attachment of one of the two sister chromatids to microtubules from both poles. In a proportion of cells lacking Dam1, sister chromatid pairs segregate to the same pole, as judged by imaging of a single GFP-tagged chromosome. This suggests that the DASH complex is required for the efficient bi-orientation of chromosomes and that in its absence chromosomes become both merotelically and syntelically attached to spindle poles. Merotelic attachments most likely occur in wildtype cells due to the dynamic nature of 'search and capture' mitotic spindle microtubules, but in the presence of the DASH complex, these inappropriate attachments are resolved prior to anaphase. Studies of PtK1 cells have shown that 1.6% of unperturbed cells have a lagging chromosome in anaphase (Cimini et al., 2001). Observation of these lagging chromosomes by confocal microscopy shows that they are attached to spindle microtubules extending from either pole. Indeed, up to 30% of prometaphase cells have merotellic attachments that are are resolved before

cells enter anaphase (Cimini et al., 2003). This suggests that merotelic attachements are a consequence of having multiple spindle microtubules attaching to each kinetochore and that mechanisms have evolved to correct these inappropriate attachments. The mechanisms by which these attachments are corrected are of particular interest, since merotellic attachments are thought to lead to aneuploidy in mammalian cells (Cimini et al., 2001).

By analysis of kinetochore behaviour in both single cells and synchronised cell populations, I found that cells lacking Dam1 spend on average more than twice as long in pro-metaphase and metaphase than wild type cells. Moreover, degradation of Cdc13 (cyclin B) is delayed in cells lacking Dam1, indicating that activation of the anaphase promoting complex (APC) is inhibited. Secondly, in the absence of Dam1 the Mad2 spindle assembly checkpoint protein is recruited to kinetochores. Consistently I observed sustained recruitment of Mad2 and Bub1 spindle assembly checkpoint proteins to kinetochores in the absence of Dam1 and showed that the anaphase delay in Δ*dam1* cells in abolished by simultaneous inactivation of *mad2*. Importantly, Δ*dam1* Δ*mad2* and Δ*dam1* Δ*bub1* cells display a higher percentage of mitotic abnormalities than Δ*dam1* cells, suggesting that in the absence of Dam1 the spindle assembly checkpoint delays anaphase onset to allow chromosome bi-orientation to be established or corrected by another mechanism.

The allele of *dam1* isolated in the genetic screen encodes a protein lacking the C-terminal 28 amino acids that is expressed at the same level as the full length protein. Furthermore, localisation of the truncated protein was indistinguishable from full length protein, indicating that the C-terminus is not required for localisation of Dam1 to the kinetochore in mitosis. Importantly, I found the truncated *dam1(1-127)* allele confers TBZ resistance even when expressed as a single copy from its own chromosomal locus. This suggests that the C-terminal 28 amino acids of Dam1 are important for regulation of microtubule stability and explains why this allele, and not full length *dam1*, was isolated in the screen. However, *dam1(1-127)* cells have a elevated rate of chromosome mis-segregation, suggesting that the C-terminus of the protein is important for proper chromosome bi-orientation.

In budding yeast, phosphorylation of Dam1 by the Aurora kinase Ipl1 is thought to release inappropriately attached microtubules from kinetochores because a *dam1* allele that mimics its constitutive phosphorylation partially suppresses the lethality of a temperature sensitive *ipl1* mutant (Cheeseman et al., 2002). Budding yeast Dam1 can be detected as a doublet on SDS-PAGE/Western Blots during a normal cell cycle and the

slower migrating form is reduced in *ipl1* mutants (Kang et al., 2001; Li et al., 2002). Whilst budding yeast Dam1 has 3 Aurora kinase consensus phosphorylation sites [(R/K)X(T/S)(I/L/V)], fission yeast Dam1 has only one, in the C-terminus of the protein at aminoacid T105, an aminoacid which is still present in Dam1(1-127). Nevertheless it is possible that the C-terminus is important for association of the *S. pombe* Aurora kinase Ark1 to the DASH complex or its phosphorylation by Ark1. Surprisingly, examination of synchronous mitotic cells indicated that neither Dam1 or Dam1(1-127) are phosphorylated in a normal cell cycle. However, if Dam1 is phosphorylated in response to unbalanced tension, it may be undetectable in an unperturbed cell cycle.

Chapter 6

Identification of the S. pombe DASH complex

6.1 Introduction

The budding yeast Dam1 protein is a subunit of the DASH complex that binds the outer kinetochore and connects the centromere to the plus end of spindle microtubules throughout the cell cycle. This complex contains ten proteins, including Dam1, Duo1, Spc34 and Ask1 proteins, all of which are essential for viability (Hofmann et al., 1998; Cheeseman et al., 2001a; Cheeseman et al., 2001b; Enquist-Newman et al., 2001; Janke et al., 2002; Li et al., 2002). Budding yeast cells expressing mutants of these proteins have defects in mitotic spindle integrity and kinetochore function and have a high rate of chromosome mis-segregation due to a failure to bi-orient sister chromatids (Hofmann et al., 1998; Cheeseman et al., 2001a; Cheeseman et al., 2001b; Enquist-Newman et al., 2001; Janke et al., 2002; Li et al., 2002). In this chapter I have screened the *S. pombe* gene database for homologues of the *S. cerevisiae* DASH complex proteins and initiated analysis of their function.

6.2 Results

6.2.1 Dam1 requires DASH protein homologues to load in mitosis

A screen of the *S. pombe* protein database was carried out and *S. pombe* homologues of *S. cerevisiae* Duo1 (SPBC32F12.08c), Spc34 (SPAC8C9.17c), Ask1 (SPBC27.02c), Dad1 (SPAC16A10.05c) and Dad3 (SPAC14C4.16) were identified (Table 6.1). Sequence alignments of the *S. cerevisiae* and putative *S. pombe* Duo1, Spc34 and Ask1 proteins are presented in Figure 6.1. In addition, the previously described *S. pombe* gene products Hos2 and Hos3 were identified as structural homologues of *S. cerevisiae* Dad2 and Hsk3 respectively (Table 6.1). The *hos2* and *hos3* mutants were identified by their inability to proliferate under conditions of high osmotic stress, a phenotype associated with loss of Dam1 (Aoyama et al., 2000; Nakamichi et al., 2000). By analysing

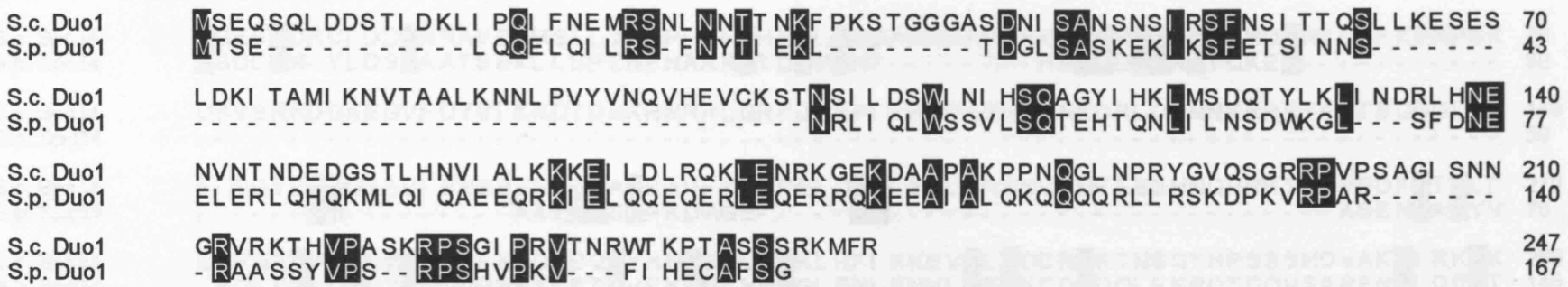
S.c. protein	*S.p.* gene/ chromosomal locus	BLAST Score	Proposed *S.p.* name	Protein Length (a.a.)
S.c. Dam1	SPAC589.08C	0.0008	*S.p.* Dam1	155
S.c. Duo1	SPBC32F12.08c	0.89*	*S.p.* Duo1	172
S.c. Ask1	SPBC27.02c	2.4 x e-09	*S.p.* Ask1	307
S.c. Spc34	SPAC8C9.17c	0.0073	*S.p.* Spc34	164
S.c. Spc19	SPCC1223	0.0098	*S.p.* Spc19	**
S.c. Dad1	SPAC16A10.05c	0.00049	*S.p.* Dad1	85
S.c. Dad2 (Hsk1)	*hos2*	5.4 x e-05	*S.p.* Dad2	94
S.c. Dad3	SPAC14C4.16	2.3 x e-06	*S.p.* Dad3	86
S.c. Dad4 (Hsk2)	SPBC3B9	0.00039	*S.p.* Dad4	**
S.c. Hsk3	*hos3*	0.012	*S.p.* Dad5	133

**S. pombe* Duo1 shows limited sequence homology to *S. cerevisiae* Duo1 but is required for binding of *S. pombe* Dam1 to kinetochores (see text).

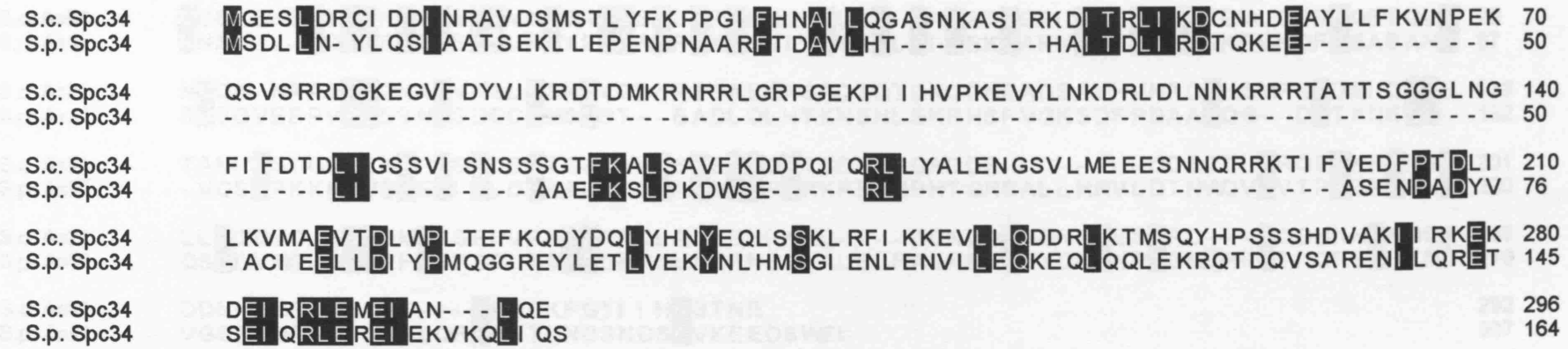
** *S. pombe* Spc19 and Dad4 have not been annotated as ORFs in the *S. pombe* GeneDB database.

Table 6.1 *S. pombe* orthologues of the *S. cerevisiae* DASH complex

A


S.c. Duo1 MSEQSQLDDSTIDKLIPQIFNEMRSNLNNTTNKFPKSTGGGASDNISANSNSIRSFNSITTQSLLKESES 70
S.p. Duo1 MTSE----------LQQELQILRS-FNYTIEKL---------TDGLSASKEKIKSFETSINNS------- 43
S.c. Duo1 LDKITAMIKNVTAALKNNLPVYVNQVHEVCKSTNSILDSWINIHSQAGYIHKLMSDQTYLKLINDRLHNE 140
S.p. Duo1 ----------NRLIQLWSSVLSQTEHTQNLILNSDWKGL---SFDNE 77
S.c. Duo1 NVNTNDEDGSTLHNVIALKKKEILDLRQKLENRKGEKDAAPAKPPNQGLNPRYGVQSGRRPVPSAGISNN 210
S.p. Duo1 ELERLQHQKMLQIQAEEQRKIELQQEQERLEQERRQKEEAIALQKQQQQRLLRSKDPKVRPAR------- 140
S.c. Duo1 GRVRKTHVPASKRPSGIPRVTNRWTKPTASSSRKMFR 247
S.p. Duo1 -RAASSYVPS--RPSHVPKV---FIHECAFSG 167

B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S.p. Spc34
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MSDLLN- YLQSIAATSEKLLEPENPNAARFTDAVLHT--------HAITDLIRDTQKEE---------- 50
S.c. Spc34
S.p. Spc34
QSVSRRDGKEGVFDYVI KRDTDMKRNRRLGRPGEKPI I HVPKEVYLNKDRLDLNNKRRRTATTSGGGLNG 140
---------- 50
S.c. Spc34
S.p. Spc34
FI FDTDLIGSSVI SNSSSGTFKALSAVFKDDPQI QRLLYALENGSVLMEEESNNQRRKTI FVEDFPTDLI 210
------LI---------AAEFKSLPKDWSE----RL----------ASENPADYV 76
S.c. Spc34
S.p. Spc34
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-ACI EELLDI YPMQGGREYLETLVEKYNLHMSGI ENLENVLLEQKEQLQQLEKRQTDQVSARENILQRET 145
S.c. Spc34
S.p. Spc34
DEIRRLEMEIAN--LQE 296
SEIQRLEREIEKVKQLI QS 164

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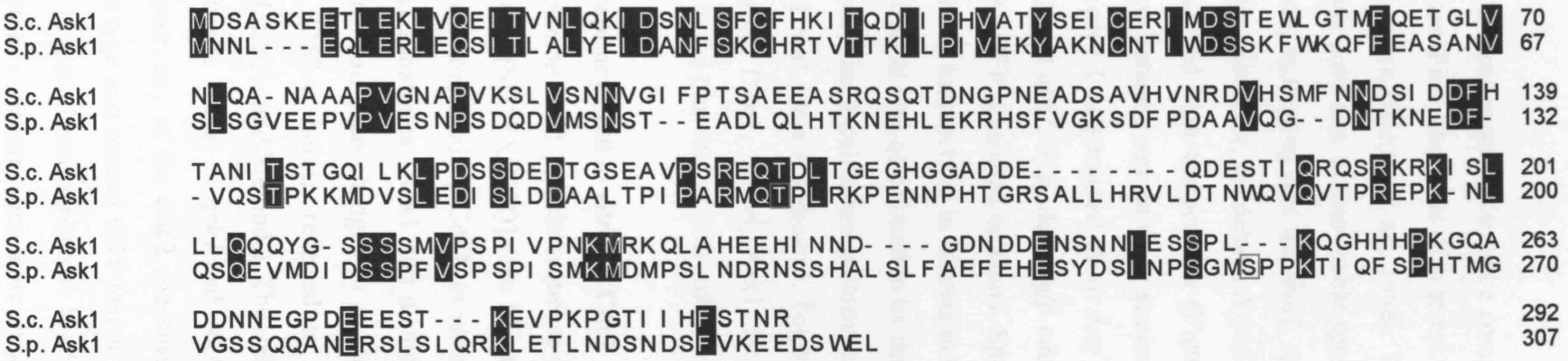

Figure 6.1 Duo1, Spc34 and Ask1 in *S. cerevisiae* and *S. pombe*

Alignments of *S. cerevisiae* and *S. pombe* (A) Duo1, (B) Spc34 and (C) Ask1 proteins. Black boxes denote the regions of homology. The red boxes indicate the Cdc2 consensus phosphorylation sites [(S/T)PX(K/R)] of *S. pombe* Ask1.

the *S. pombe* DNA sequence database, homologues of the *S. cerevisiae* proteins Spc19 and Dad4 were also identified on cosmids SPCC1223 and SPBC3B9 respectively although these had not been classified as ORFs in the fission yeast protein database (Table 6.1).

To examine if these proteins formed a complex with Dam1, I deleted the *duo1, spc34* and a*sk1* genes. First, the gene of interest was disrupted with kanamycin in a heterozygous h-/h+ strain (Bahler et al., 1998). Tetrad dissection of the each of the heterozygous diploids gave rise to four viable spores that showed a 2:2 segregation of kanamycin resistance, indicating that the *duo1, spc34* and *ask1* genes are each non-essential for viability. However, the Δ*duo1,* Δ*spc34* and Δ*ask1* strains were sensitive to TBZ, osmotic stress and high temperature (Figure 6.2.A; data not shown). Double mutant combinations between any of these strains showed no additive phenotype. To further test this theory I constructed *duo1::hyg*^{r} *dam1-gfp cdc11-cfp, spc34 ::hyg*^{r} *dam1-gfp cdc11-cfp* and *ask1::hyg*^{r} *dam1-gfp cdc11-cfp* strains to determine whether localisation of Dam1-GFP depended on Duo1, Spc34 or Ask1. I found that Dam1 was unable to localise to the kinetochore in mitosis in the absence of these proteins (Figure 6.2.B). Dam1 localisation was also found to be dependent on Hos2 and Hos3 (data not shown). Furthermore, the localisation patterns of Duo1-GFP and Spc34-GFP were identical to that of Dam1 (data not shown). Together, these results are consistent with the notion that Dam1, Duo1, Spc34, Ask1, Hos2 and Hos3 form a multi-subunit complex that binds to the plus end of microtubules and kinetochores.

6.2.2 Ask1 is phosphorylated *in vitro* by Cdc2

Analysis of the *S. pombe* Ask1 protein sequence showed that it has 3 consensus Cdc2 phosphorylation sites [(S/T)PX(K/R)] (see Figure 6.1.C). To investigate whether *S. pombe* Ask1 is phosphorylated by Cdc2, *in vitro* Cdc2 phosphorylation assays were performed using as substrates, Ask1 and a mutated Ask1 in which the consensus phosphorylation sites had been changed to an alanine that cannot be phosphorylated (T136A, T163A, S257A; herein referred to as Ask1(AAA)), by site directed mutagenesis (see Materials and Methods). The *ask1* coding sequence contains 2 introns at its N terminus, hence alleles of *ask1* and *ask1(AAA)* lacking the 5' 298 base pairs (which did not contain any of the Cdc2 consensus sites) were cloned into the pGEX vector and the wild type and mutant GST-fusion proteins expressed and purified from *E. coli* by binding to glutathione-sepharose beads followed by elution (see Materials and Methods). Protein concentrations were measured by Bradford assay and equal

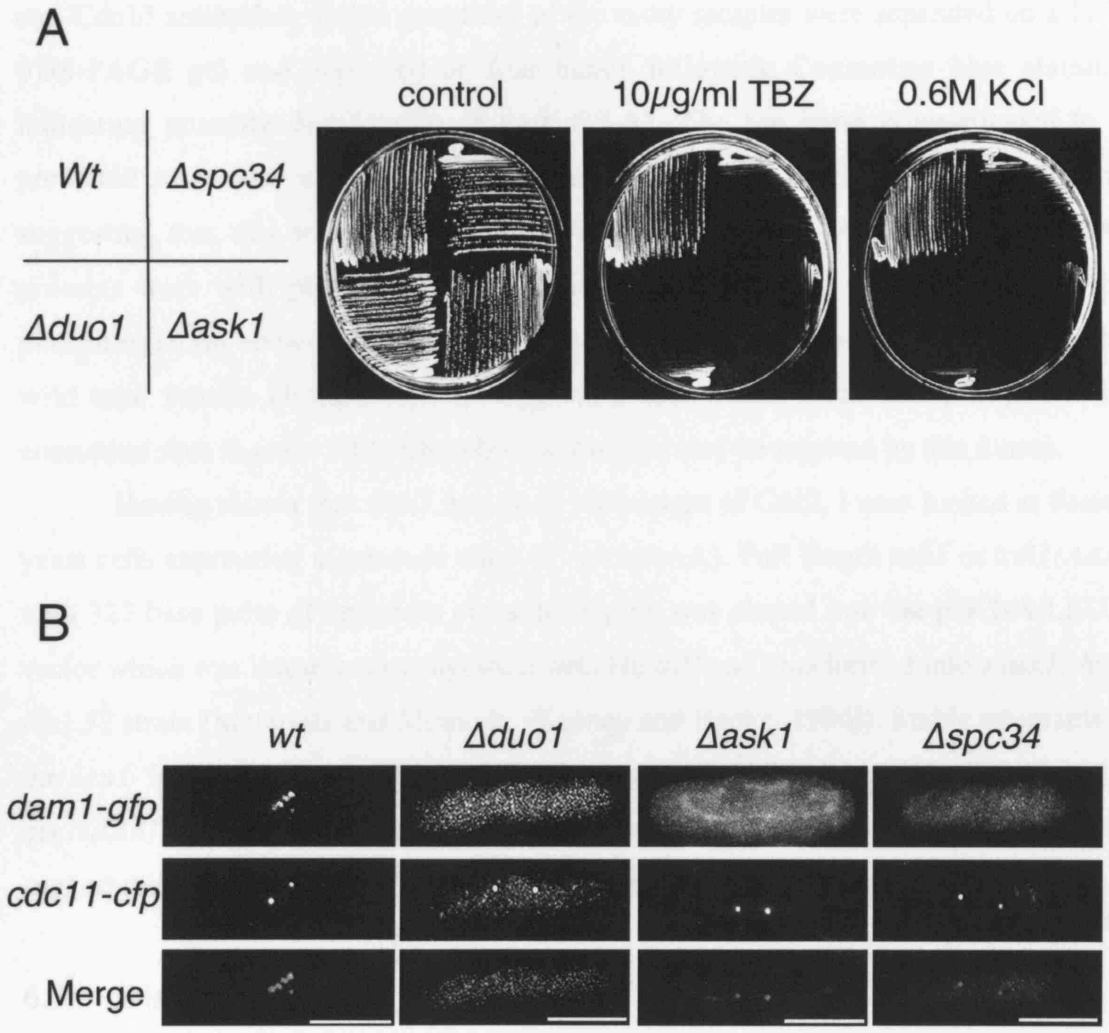

Figure 6.2 Duo1, Spc34 and Ask1 are required for association of Dam1 in mitosis.

(A) Wild type, *Δspc34*, *Δduo1* and *Δask1* cells were streaked either onto rich medium containing either no addition (control), 10 μg/ml TBZ (TBZ), 0.6M KCl (0.6M KCl) at 30°C or rich medium at 37°C and incubated for 3 days. (B) Log phase *dam1-gfp cdc11-cfp*, *Δduo1 dam1-gfp cdc11-cfp*, *Δspc34 dam1-gfp cdc11-cfp* and *Δask1 dam1-gfp cdc11-cfp* cells were fixed and stained with DAPi and calcuflor and visualised by fluorescence microscopy. The images show localisation of Dam1 in mitotic cells. Scale bar = 5 μm.

quantities of the wild type or mutant GST-fusion proteins were used as substrates for an *in vitro* phosphorylation assay using Cdc2 purified by co-immunoprecipitation with anti-Cdc13 antibodies. Equal quantities of the assay samples were separated on a 12% SDS-PAGE gel and appeared as four bands following Coomassie blue staining indicating possible degradation (Figure 6.3.A). The top band corresponded to a predicted molecular weight for the truncated Ask1-GST fusion protein of 50kDa, suggesting that this was a *bona fide* purified fusion protein. Ask1 and Ask1(AAA) proteins were both phosphorylated by Cdc2 (Figure 6.3.B). Quantification of the phosphorylation showed that the Ask1(AAA) was phosphorylated 30% less than the wild type protein (Figure 6.3.C), suggesting that whilst Cdc2 phosphorylates the consensus sites *in vitro,* other phosphorylation sites may be targeted by this kinase.

Having shown that Ask1 was an *in vitro* target of Cdc2, I next looked at fission yeast cells expressing a genomic copy of Ask1(AAA). Full length *ask1* or *ask1(AAA)* with 327 base pairs of upstream promoter region was cloned into the pJK148(LEU1) vector which was linearised by digestion with HindIII and transformed into a *ask1::hyg*^{r} *leu1.32* strain (Materials and Methods; (Keeney and Boeke, 1994)). Stable integrants at the *leu1* locus were selected by growth on plates lacking leucine. Surprisingly, *ask1(AAA)* cells were more resistant to TBZ than the control (Figure 6.4). These results suggest that phosphorylation of may be important for regulating microtubule stability.

6.3 Discussion

Structural homologues of all components of the budding yeast DASH complex were identified in the fission yeast database, including Duo1, Ask1 and Spc34, and I found that these proteins are required for Dam1 localisation in mitosis. Moreover, these proteins have similar localisation to Dam1 and cells lacking these proteins have identical phenotypes to Δ*dam1* cells, namely sensitivity to TBZ and osmotic stress. Together, these data indicate that, as in budding yeast, these proteins are part of a multi-subunit complex. Surprisingly, unlike in budding yeast, none of the components of the fission yeast DASH complex are essential for viability. As discussed in Chapter 5, this may represent the difference between microtubule-kinetochore attachments in budding yeast cells, which must resolve syntelic attachments in every cell cycle, and fission yeast cells which only become attached to microtubules from the SPB in mitosis or may be due to other mechanisms involved in microtubule-kinetochore attachment regulation existing in fission yeast but not in budding yeast.

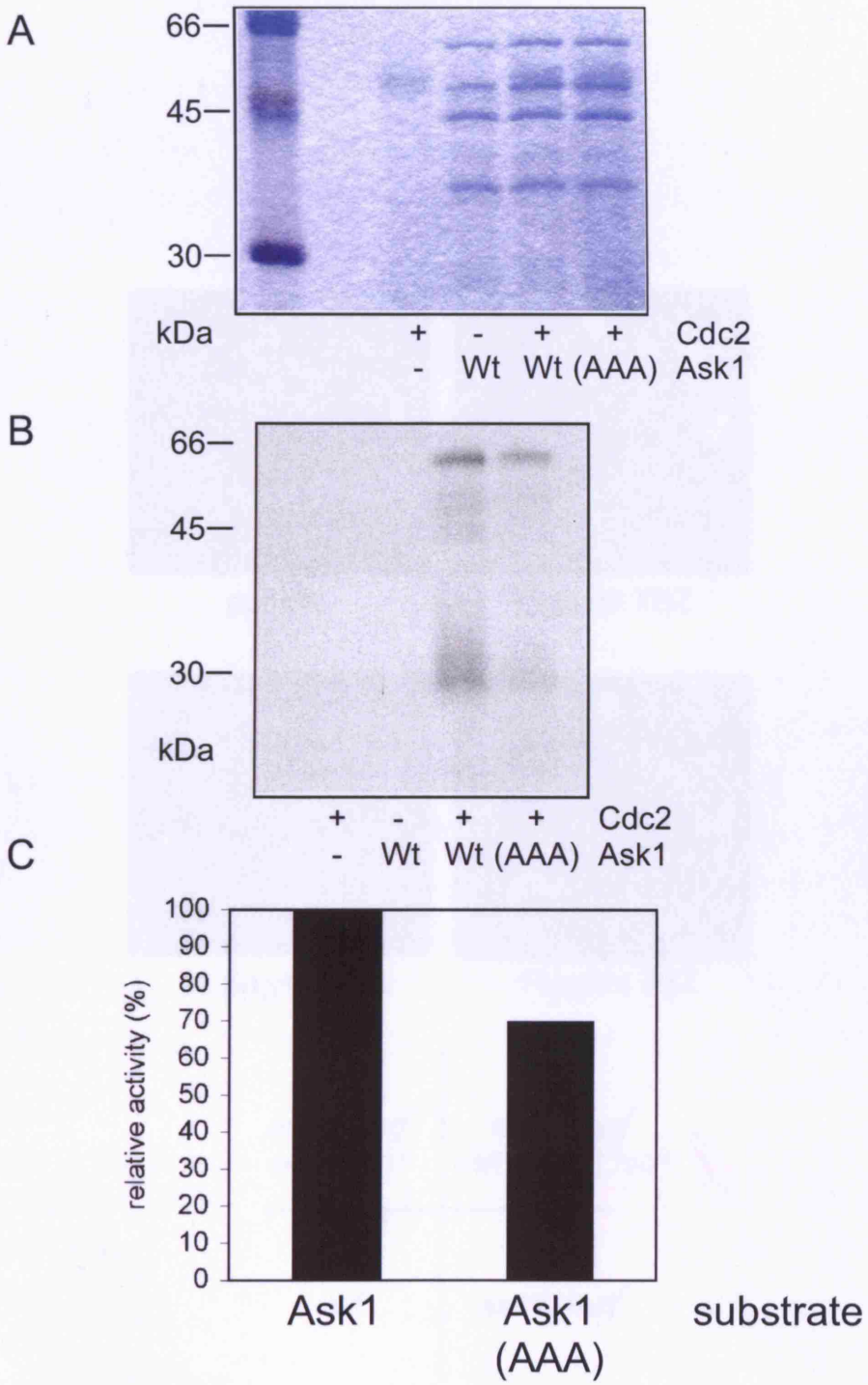

Figure 6.3. Ask1 is phosphorylated *in vitro* by Cdc2

(A) Picture of Coomasie stained 12% SDS-PAGE gel loaded with equal amounts of wildtype (Wt), mutant (AAA) Ask1, or no (-) protein following an *in vitro* Cdc2 kinase assay. (B) Autoradiograph showing incorportation of P^{32} from the kinase assay in (A). (C) Graph showing quantification of kinase activity (%) plotted relative to the maximum activity in the experiment.

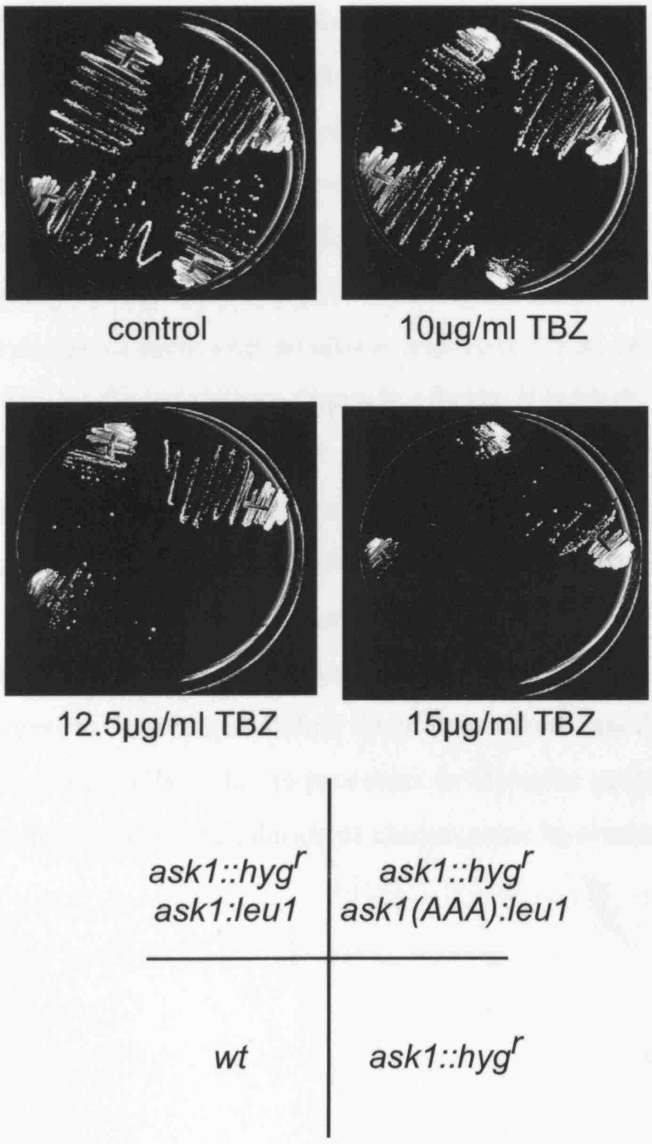

Figure 6.4. *ask1::hyg^r ask1(AAA):leu1* cells are resistant to TBZ

Wildtype, *ask1::hyg^r*, *ask1::hyg^r ask1:leu1*, or *ask1::hyg^r ask1(AAA):leu1* cells were streaked onto rich medium plates containing either no addition (control) or 10, 12.5 or 15µg/ml TBZ and incubated at 30°C for 3 days.

The fission yeast DASH complex localises to kinetochores only in mitosis and therefore requires Cdc2/Cdc13 activation. This may be an indirect requirement, depending on the nucleation of microtubule within the nucleus that is stimulated by Cdc2/Cdc13 activity, or a direct requirement due to phosphorylation of DASH complex sub-units by Cdc2/Cdc13. Fission yeast Ask1 has 3 consensus Cdc2 phosphorylation sites [(S/T)PX(K/R)] and I found that it is a *in vitro* substrate of Cdc2. Notably, an Ask1 protein in which these 3 sites are replaced with non-phosphorylatable alanine residues (Ask1(AAA)), is still an *in vitro* substrate of Cdc2, albeit to a lesser degree. Ask1 has several other SPs and TPs which are partial consensus sites and which may be phosphorylated by Cdc2 *in vitro,* but whether these sites are biologically significant and phosphorylated *in vivo* remains to be determined.

Intriguingly, cells expressing Ask1(AAA) were resistant to TBZ suggesting that the phosphorylation of these sites *in vivo* is important for its cellular function. Since the DASH complex localises to kinetochores in mitosis, it is likely that this effect is due to regulation of microtubule-kinetochore attachments. Phosphorylation of Ask1 by Cdc2 may prime the complex for regulation during mitosis, perhaps allowing association of the Aurora kinase Ark1, so that syntelic and merotelic connections can be corrected. In the absence of phosphorylation, the microtubule-kinetochore interaction may lose its capacity to be regulated by Ark1, preventing the turnover of microtubule connections. It will be of interest to determine whether the increased resistance to TBZ seen in *dam1(1-127)* and *ask1(AAA)* cells is due to processes in the same pathway ultimately affecting the DASH complex and the regulation of chromosome bi-orientation.

Chapter 7

Summary

7.1 The aims of the thesis.

The aim of this thesis was to investigate further the regulation of Cdc2/Cdc13 at the onset of mitosis in fission yeast. In particular, I examined the interplay between the microtubule cytoskeleton and Cdc2/Cdc13. Firstly, I investigated the influence of the microtubule destabilising drug TBZ on Cdc2/Cdc13 activation at the G2-M transition. Secondly, I performed a genetic screen to isolate multi-copy suppressors of a TBZ sensitive *cdc13-117* mutant strain. The function of one of these suppressors, Dam1, was chosen for further study.

7.2 Regulation of Cdc2 kinase activity at the G2-M transition.

7.2.1 A novel microtubule dependent G2-M checkpoint.

Hyams and co-workers had previously shown that activation of Cdc2 kinase at the G2-M transition in fission yeast is inhibited following treatment with the microtubule destabilising drug TBZ (Alfa et al., 1990). I confirmed this finding and showed that the inhibition of Cdc2 correlated with the destruction of the microtubule cytoskeleton.

The cold-sensitive tubulin mutant *nda3-KM311* showed a delay in Cdc13 re-localisation to the SPB, but tubulin polymers were still visible in these cells at the restrictive temperature. A tighter tubulin mutant is required to determine whether the inhibition of Cdc2 activity is due to the inability to re-localise Cdc13 to the SPB. Other *nda* mutants such as *nda3-1828* could be tested (Toda et al., 1983; Umesono et al., 1983). The γ-tubulin complex-associated protein Mbo1 is required for the nucleation of cytoplasmic microtubules from the non-SPB MTOCs (Sawin et al., 2004; Venkatram et al., 2004). Cells lacking Mbo1 could be used as a genetic model to further investigate the role of microtubules in the G2-M transition, although it must be noted that these cells still nucleate a single cytoplasmic microtubule bundle from the SPB (Sawin et al.,

2004; Venkatram et al., 2004). The identification of a conditional mutant that is unable to nucleate cytoplasmic microtubules may be required to further these studies.

I found that inhibition of Cdc2 activity following microtubule damage did not depend on either DNA structure checkpoints or the spindle assembly checkpoint. Hyams and co-workers showed that the inhibitory Tyr-15 phosphorylation of Cdc2 is maintained when cells are treated with TBZ suggesting that Cdc25 phosphatase is inhibited (Alfa et al., 1990). The DNA structure checkpoint causes phosphorylation of Cdc25, which causes its inactivation and nuclear exclusion (Furnari et al., 1997; Zeng et al., 1998; Lopez-Aviles et al., 2005). A mutant Cdc25 has been constructed in which nine phosphorylation sites for Chk1 and Cds1 are mutated to alanine (Zeng and Piwnica-Worms, 1999). The *cdc25-9A* allele is unable to delay the cell cycle in response to unreplicated or damaged DNA (Zeng and Piwnica-Worms, 1999). Furthermore, overexpression of Srk1, a p38/Sty1 activated kinase, fails to block the cell cycle in *cdc25-9A* cells suggesting that the environmental stress checkpoint also depends on phosphorylation of Cdc25 at some of these sites (Lopez-Aviles et al., 2005). It would be interesting to determine whether the inhibition of Cdc2 activity following microtubule damage depends on phosphorylation and inhibition of Cdc25. This could be examined by following Cdc2 kinase activity in *cdc25-9A* cells treated with TBZ or in a *nda* mutant. It is possible that additional as yet unidentified kinases, other than Chk1, Cds1 and Srk1, phosphorylates Cdc25 on these sites in response to microtubule disruption. In mammals the antephase checkpoint is mediated by the p38 stress-activated kinase and requires the Chfr checkpoint protein (Matsusaka and Pines, 2004). I found that fission yeast cells lacking the structural homologue of Chfr, Dma1, were unable to activate Cdc2/Cdc13 following TBZ treatment. Although this shows that Dma1 is not part of this response in fission yeast, it is still unclear exactly how Chfr in human cells may sense microtubule damage and transmit this signal to p38.

7.2.2 Microtubules are required for the re-localisation of Cdc13 to the SPBs.

In fission yeast the Cdc2/Cdc13 re-localises from the nucleolus to the SPB at the G2/M transition, although it is not known whether this is a consequence or a cause of Cdc2 activation. Furthermore it is not known to what protein(s) Cdc2/Cdc13 binds on the SPB or how this re-localisation occurs. Immunogold labelling and electron microscopy have been used in fission yeast to determine the localisation of Cut12, a protein important in spindle formation, to the nuclear face of the SPB (Bridge et al., 1998) and this technique could similarly be used to examine the precise localisation of

Cdc2/Cdc13. At the onset of mitosis, the SPB enters the nuclear envelope (Ding et al., 1997) and it would be interesting to investigate by electron microscopy whether this event also depends on intact microtubules and the re-localisation of Cdc2/Cdc13. Regardless, I found that treatment with TBZ blocks association of the cyclin Cdc13 (Cyclin B) to the SPB in mitosis, suggesting that the inhibition of Cdc2 activity may be due to disrupted Cdc13 localisation. Work in other organisms suggests that Polo kinases, which associate to the centrosome, are involved in an auto-amplification loop at the G2-M transition (Kumagai and Dunphy, 1996; Abrieu et al., 1998; Qian et al., 1998; Bartholomew et al., 2001) and in fission yeast the activity of several SPB associated kinases, including Fin1 and Plo1, are required at the onset of mitosis (Ohkura et al., 1995; Krien et al., 1998; Petersen et al., 2001). In particular, premature association of Plo1 to the SPB in *stf1-1 (cut12-sf1)* mutant cells suppresses the mitotic commitment defect of *cdc25-22* cells suggesting that an auto-amplification loop also exists at the G2/M transition in fission yeast (Bridge et al., 1998; Mulvihill et al., 1999). Microtubule damage may prevent the activation of one of these kinases, thus preventing auto-amplification of Cdc2/Cdc13 activation. To investigate whether these kinases are affected by microtubule damage, the localisation of plo1-GFP and fin1-GFP could be examined following TBZ treatment. Secondly, the localisation of Cdc13 and the activity of Cdc2 could be examined in *stf1-1* cells after TBZ treatment to determine whether premature localisation on Plo1 to the SPB can bypass this inhibition. In mammalian cells the Aurora A kinase is implicated in centrosomes maturation and spindle assembly (Dutertre et al., 2002). The fission yeast Aurora kinase, Ark1, is thought to perform the same role as both Aurora A and B in humans (Petersen et al., 2001). Ark1 activity may be inhibited by TBZ treatment, which might prevent Cdc13 localisation to the SPB and Cdc2/Cdc13 activation. Cdc13 localisation and Cdc2 activity could be examined following TBZ treatment in cells overexpressing Ark1 to investigate if this can overcome the effect of microtubule damage.

Another possibility is that TBZ activates an inhibitor of the G2/M transition. A candidate for this might be the Cdc14-like phosphatase, Clp1, which displays similar cell cycle dependent localisation to Cdc13 (Trautmann et al., 2001). In budding yeast Cdc14 is involved in the mitotic exit network (Shou et al., 1999). In fission yeast Clp1, in addition to a role in the septum initiation network, is thought to have a role at the G2-M transition. Cells lacking Clp1 have a semi-*wee* phenotype and overexpression of Clp1 delays the G2-M transition while Cdc2 activity is kept low and its inhibitory phosphorylation of Tyr-15 maintained at interphase levels (Trautmann et al., 2001). It

would be of interest to investigate whether Clp1 is required for inhibition of Cdc2/Cdc13 activation following TBZ treatment.

7.3 The *S. pombe* DASH complex and the establishment of chromosome bi-orientation.

7.3.1 Identification and function of the *S. pombe* DASH complex

In a genetic screen for multi-copy suppressors of a TBZ sensitive *cdc13-117* mutant strain (Booher and Beach, 1988) I isolated a truncated allele of *dam1*. Analysis of the full length Dam1 protein showed that the protein localises to kinetochores and intra-nuclear spindle microtubules in mitosis and that in the absence of Dam1, cells have a chromosome mis-segregation defect. Cells lacking Dam1 spend more than twice as long in pro-metaphase and metaphase than wild type cells and recruit Mad2 and Bub1 to kinetochores suggesting that this delay is due to activation of the SAC. Consistently, this delay was reduced in cells lacking SAC proteins, but these cells had an increase in mitotic abnormalities suggesting that the SAC delays anaphase to allow chromosome bi-orientation to be established or corrected by another mechanism.

In budding yeast, Dam1 is part of a complex containing 10-subunits, all of which are essential for viability (Hofmann et al., 1998; Cheeseman et al., 2001a; Cheeseman et al., 2001b; Enquist-Newman et al., 2001; Janke et al., 2002; Li et al., 2002). *S. pombe* homologues of these sub-units were identified in the *S. pombe* protein database. Unlike in budding yeast, fission yeast cells lacking Duo1, Spc34 or Ask1 were viable. Furthermore, localisation of Dam1 to the kinetochores in mitosis depends on other DASH complex proteins. These findings are consistent with the notion that the DASH proteins form a multi-subunit complex in fission yeast. Interestingly, Dam1 did not appear to completely co-localise with the kinetochore protein Ndc80 in mitosis. During metaphase, this probably represents DASH complex on the plus ends of intra-nuclear microtubules that are searching for the Ndc80 containing kinetochores. Although the DASH complex must associate with the kinetochore in metaphase, it may not form a secure attachment until balanced tension has been established. During anaphase, although the DASH complex and Ndc80 may be more closely associated, Ndc80 may act as a 'bridge' between the outer most complex, DASH, and the centromere contacting Cnp1. The limitations of fluorescence microscopy, however, restrict the interpretations that can be made on such small differences in localisation. In order to accurately determine the localisation of the DASH complex in relation to other

kinetochore proteins, such as Ndc80, it will be necessary to perform electron microscopy studies. Although Dam1 was seen to associate with kinetochores in mitosis, it was unclear whether the DASH complex was also found at SPBs Dam1 was often observed adjacent to the the SPB and at the outer-most ends of the mitotic spindle. This could have been DASH complex bound to the SPB itself, or DASH complex bound to short, mitoic spindle microtubules emanating from the SPB. Again, the exact loaclisation of Dam1 near the SPB could be examined by electron microscopy.

7.3.2. Regulation of the DASH complex by Aurora kinase

In budding yeast, phosphorylation of Dam1 by the Aurora kinase Ipl1 is important for correcting improper microtubule-kinetochore attachments (Cheeseman et al., 2002). My analysis of synchronized *dam1-3ha* fission yeast cells did not suggest that Dam1 is phosphorylated during the cell cycle. However, Dam1 phosphorylation may be undetectable in an unperturbed cell cycle and this experiment could be repeated in the cold sensitive tubulin mutant *nda3-KM311* where chromosomes would lack balanced tension in mitosis due microtubule disruption. It is important to determine whether Dam1 in fission yeast is a target of the Aurora kinase Ark1. This would be done firstly by purifying epitope-tagged Dam1 and using it as a substrate in an *in vitro* kinase assay with purified epitope-tagged Ark1. Secondly, it will be of interest to examine whether any phosphorylation of Dam1 is cell cycle regulated. This could be done by raising an antibody to phosphorylated Thr-105 (at the Aurora kinase consensus phosphorylation site KATL) of Dam1 and following the phosphorylation of Dam1 in synchronous cells by Western blot analysis. Dam1 in fission yeast is indeed phosphorylated during the cell cycle then it is possible that mutant alleles of *dam1,* in which Thr-105 is changed to a non-phosphorylatable alanine or a glutamic acid to mimic constitutive phosphorylation, would have chromosome segregation defects. Interestingly, cells expressing a truncated allele of *dam1* at the genomic locus were TBZ resistant but had a slightly elevated chromosome mis-segregation suggesting that the C-terminus of the protein is important for the regulation of microtubule stability, perhaps in the establishment of chromosome bi-orientation. The truncated Dam1, lacking the C-terminal 28 amino acids, still contains the Aurora kinase consensus site at Thr-105. However, the C-terminus of the protein may still be important for the association of Ark1 to Dam1 or for the phosphorylation of Dam1. Again, this could be tested by a combination of experiments looking at the phosphorylation of Dam1(1-127) by Ark1 *in vitro* and examining whether phosphorylation of Dam1(1-127) is affected *in vivo.*

7.3.3 Regulation of the DASH complex by Cdc2/Cdc13 kinase.

Unlike in budding yeast, the fission yeast DASH complex proteins localise to the kinetochores and plus end of spindle microtubules only in mitosis. Therefore, their localisation is dependent of the activity of Cdc2/Cdc13 in mitosis. This is likely to be due to the increased dynamics of microtubules forming the spindle within the nucleus stimulated by Cdc2/Cdc13 activity, and due to phosphorylation of DASH complex components. Consistently, I found that Ask1 is an *in vitro* substrate of Cdc2/Cdc13. A mutant of Ask1 (denoted Ask1(AAA)), which contains alanines in place of either serine or threonine at the three Cdc2 consensus phosphorylation sites, was phosphorylated significantly less *in vitro* than the wild type protein. Surprisingly, *ask1(AAA)* cells were more resistant to TBZ than wild type cells. This suggests that phosphorylation of Ask1 is not required for the localisation of the DASH complex to kinetochores in mitosis but is important for regulating microtubule stability at the kinetochore. Dam1-GFP localisation should be observed in *ask1(AAA)* cells to confirm that DASH complex localisation is unaffected in the absence of phosphorylation by Cdc2/Cdc13. A possible model is that phosphorylation of the Ask1 by Cdc2 in mitosis, "primes" the DASH complex to define a window during which it may be regulated (Figure 7.1). While Ask1 is phosphorylated, the Aurora kinase Ark1 may be able to phosphorylated Dam1 to correct improper microtubule-kinetochore attachments (Figure 7.1). If so, then in *ask1(AAA)* cells, Ark1 may be unable to destabilise microtubule-kinetochore attachments, leading to an increased stability in spindle microtubules, but an elevated chromosome loss rate due to the inability to resolve improper attachments. Conversely, an Ask1 mutant containing glutamic acid residues at the Cdc2 consensus phosphorylation sites mimicking constitutive phosphorylation might be unable to switch off Ark1 leading to unstable microtubule-kinetochore attachments and an increase in chromosome mis-segregation. This model could be tested by a number of experiments. Firstly, the localisation of Ark1 could be examined in *ask1(AAA)* cells by observing both a GFP epitope tagged Ark1 in these cells, and by ChIP experiments. Secondly, the phosphorylation of Dam1 could be followed by Western blot of epitope tagged Dam1, or using a phospho-specific Dam1 antibody in *ask1(AAA)* cells to examine whether Dam1 is phosphorylated in the absence of Ask1 phosphorylation. Interestingly, recent experiments in *Drosophila* have shown that the expression of a non-degradable Cyclin B mutant prevents the Aurora B release from anaphase kinetochores, resulting in anaphase chromosomes which exhibit cycles of re-orientation from one pole to the other

with an accumulation of merotelic attachments (Parry et al., 2003). This data supports my hypothesis that the degradation of Cyclin B at the metaphase to anaphase transition is important to "seal" microtubule-kinetochore attachments by shutting off Cdc2 phosphorylation of Ask1, thus preventing Aurora kinase phosphorylating the DASH complex.

7.3.4 A special role for Dad1

Further work in this lab has revealed that one of the DASH complex proteins, Dad1, is localised to the kinetochores throughout the cell cycle (see Appendix). This observation, and further genetic interactions, indicates that Dad1 is both a component of the DASH complex, and a component of the inner kinetochore. Intriguingly, the *S. cerevisiae* DASH complex has recently been shown to form both rings and paired helical structures on microtubules *in vitro* (Figure 7.2; (Miranda et al., 2005; Westermann et al., 2005)). It will be of great interest for future studies to investigate exactly how the ring complex interacts with the kinetochore and is regulated during the establishment of bi-orientation, particularly by Aurora kinase. The DASH ring complex could form a "collar" at the plus end of spindle microtubules, which then docks at the kinetochore to form a stable attachment. Further work has shown that Dad1 does not localise to kinetochores in *mis6* or *mal2* mutants, whereas its localisation is unaffected in *mis12, nuf2 or cnp1* mutants, suggesting that the Mis6 complex provides the docking site for the DASH complex at kinetochores (see Appendix). One exciting possibility is that Dad1 at the kinetochore nucleates a change in DASH structure from ring to double helix configuration thus stabilising amphitelic attachment of spindle microtubules to the kinetochore. To test this hypothesis it will be necessary to construct Dad1 mutants that bind only the DASH complex but not the Mis6 complex.

7.3.5 Other pathways controlling bipolar chromosome attachment in *S. pombe*

Components of the DASH complex (except Dad1) share a property of the Alp14, Dis1, Klp5 and Klp6 proteins, namely associating to the kinetochores exclusively in mitosis (Nabeshima et al., 1998; Garcia et al., 2001; Nakaseko et al., 2001; Garcia et al., 2002a; Garcia et al., 2002b; West et al., 2002). Importantly, further studies in this lab found that cells lacking both Dam1 and Klp5 are inviable due to chromosome mis-segregation during the first mitosis even though bipolar spindle assembly is not affected (see Appendix). In most *Δdam1Δklp5* cells, the sister chromatids of chromosome 1 fail to segregate or both segregate to the same pole. This indicates that the DASH complex and

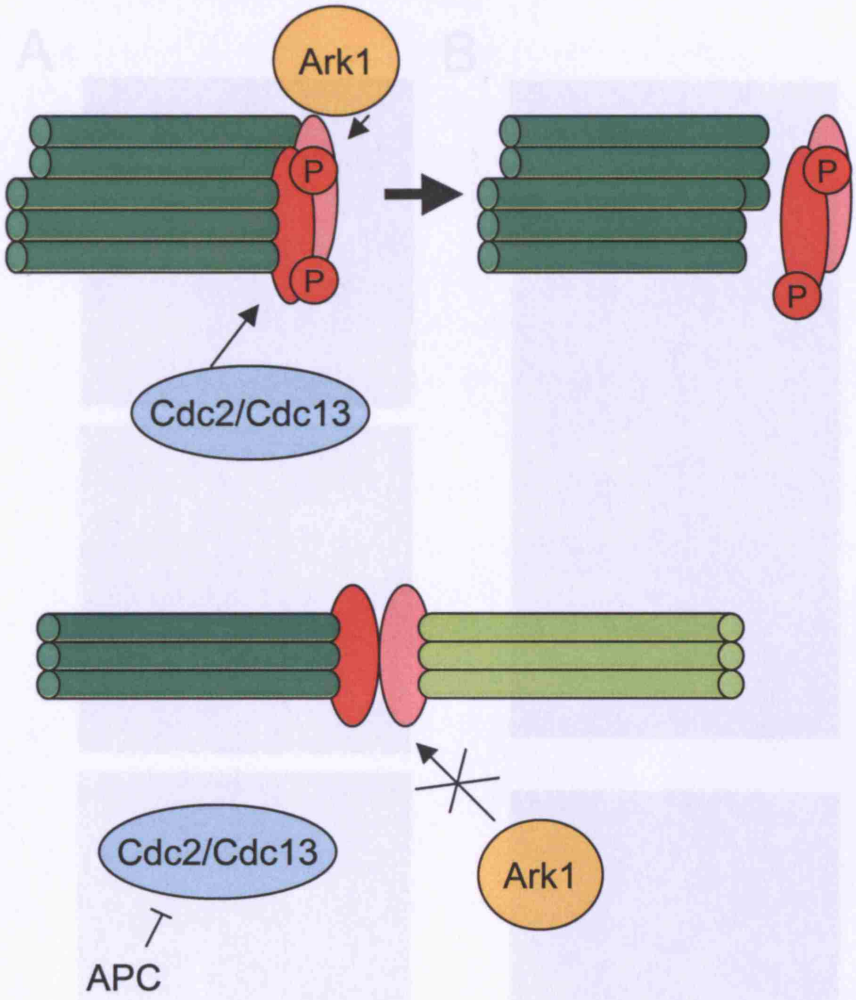

Figure 7.1 Model for the co-ordinate regulation of the DASH complex by Cdc2 and Ark

Schematic diagram showing a model for how Cdc2/Cdc13 and the Aurora kinase, Ark1, might regulate DASH complex to correct improper microtubule(green)-kinetochore(red/pink) attachments in mitosis. In mitosis, Ask1 is phosphorylated by Cdc2/Cdc13. When Ask1 is phosphorylated, Ark1 is able to phosphorylate Dam1 to correct microtubule-kinetochore attachments. Once balanced tension is established at the kinetochores by an amphitelic attachment, Cdc2/Cdc13 activity is switched off by the APC. Ask1 is no longer phosphorylated and Ark1 cannot phosphorylate Dam1 so the microtubule-kinetochore attachment is "sealed".

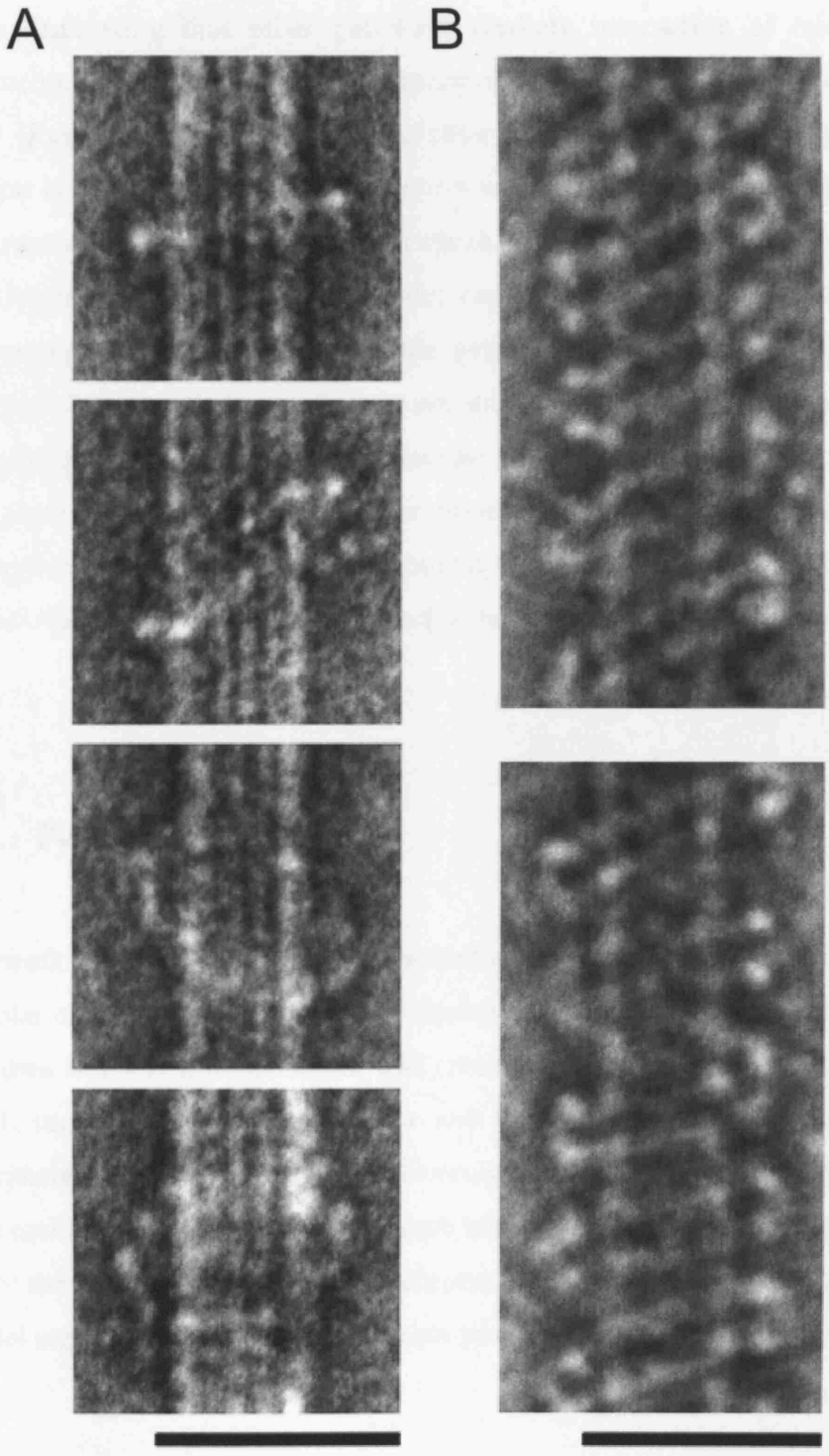

Figure 7.2 Different modes of microtubule decoration by DASH complex

Gallery of negatively stained microtubules in the presence of DASH complex showing (A) single rings and (B) paired helices. Scale bar = 500 Å. Adapted from Miranda et al, 2005.

Klp5/6 co-ordinately control bipolar chromosome attachment in fission yeast. In some cells lacking Dam1 and Klp5, chromosome 1 is segregated properly to opposite spindle poles, indicating that other pathways mediate interaction of microtubules with kinetochores. This may be through interaction of Mal3 (EB-1) with kinetochore bound Spc7 (Kerres et al., 2004), or some other microtubule attachment process, but this process is either inefficient or not subject to a correction process. This could be tested by crossing *Δmal3 Δdam1* cells (which are viable; our unpublished results) to *Δklp5Δmal3* cells (which are viable; our unpublished results) and looking at the segregation of chromosome 1 in the germinating cells to determine whether any microtubule-kinetochore attachments are still made. Together, this data suggests that the pathways that mediate bipolar chromosome attachment in fission yeast differ from those that operate in budding yeast. One reason for this may be because fission yeast kinetochores are bound to 2-4 microtubules, whereas budding yeast kinetochores are bound only to a single microtubule and do not encounter merotelic attachments.

7.4 Perspective

The work presented in this thesis has shed new light on a protein complex that controls bi-polar chromosome attachment in fission yeast. There is much to be investigated in this area and future experiments will reveal how Cdc2/Cdc13 and the Aurora kinase, Ark1, regulate the DASH complex and how Klp5/6 and the DASH complex co-ordinately establish and regulate microtubule-kinetochore attachments. Fission yeast, with multiple microtubules binding each kinetochore, is an excellent system in which to study the mechanisms controlling chromosome bi-orientation and could become the model organism of choice to study these processes.

References

Abrieu, A., Brassac, T., Galas, S., Fisher, D., Labbe, J. and Doree, M. (1998) The Polo-like kinase Plx1 is a component of the MPF amplification loop at the G2/M-phase transition of the cell cycle in Xenopus eggs. *J Cell Sci*, **111**, 1751-1757.

Adams, R.R., Wheatleya, S.P., Gouldsworthy, A.M., Kandels-Lewis, S.E., Carmena, M., Smythe, C., Gerloff, D.L. and Earnshaw, W.C. (2000) INCENP binds the Aurora-related kinase AIRK2 and is required to target it to chromosomes, the central spindle and cleavage furrow. *Current Biology*, **10**, 1075-1078.

Alfa, C.E., Booher, R., Beach, D. and Hyams, J.S. (1989) Fission yeast cyclin: subcellular localisation and cell cycle regulation. *J Cell Sci Suppl*, **12**, 9-19.

Alfa, C.E., Ducommun, B., Beach, D. and Hyams, J.S. (1990) Distinct nuclear and spindle pole body population of cyclin-cdc2 in fission yeast. *Nature*, **347**, 680-682.

Allshire, R.C., Nimmo, E.R., Ekwall, K., Javerzat, J.P. and Cranston, G. (1995) Mutations derepressing silent centromeric domains in fission yeast disrupt chromosome segregation. *Genes Dev*, **9**, 218-233.

Andrews, P.D., Ovechkina, Y., Morrice, N., Wagenbach, M., Duncan, K., Wordeman, L. and Swedlow, J.R. (2004) Aurora B Regulates MCAK at the Mitotic Centromere. *Developmental Cell*, **6**, 253-268.

Aoyama, K., Kawaura, R., Yamada, H., Aiba, H. and Mizuno, T. (2000) Identification and characterization of a novel gene, hos3+, the function of which is necessary for growth under high osmotic stress in fission yeast. *Biosci Biotechnol Biochem*, **64**, 1099-1102.

Appelgren, H., Kniola, B. and Ekwall, K. (2003) Distinct centromere domain structures with separate functions demonstrated in live fission yeast cells. *J Cell Sci*, **116**, 4035-4042.

Ayscough, K.R., Stryker, J., Pokala, N., Sanders, M., Crews, P. and Drubin, D.G. (1997) High rates of actin filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed using the actin inhibitor latrunculin-A. *J Cell Biol*, **137**, 399-416.

Bahler, J., Wu, J.Q., Longtine, M.S., Shah, N.G., McKenzie, A., 3rd, Steever, A.B., Wach, A., Philippsen, P. and Pringle, J.R. (1998) Heterologous modules for efficient and versatile PCR-based gene targeting in Schizosaccharomyces pombe. *Yeast*, **14**, 943-951.

Barbet, N., Muriel, W.J. and Carr, A.M. (1992) Versatile shuttle vectors and genomic libraries for use with Schizosaccharomyces pombe. *Gene*, **114**, 59-66.

Bartholomew, C.R., Woo, S.H., Chung, Y.S., Jones, C. and Hardy, C.F.J. (2001) Cdc5 Interacts with the Wee1 Kinase in Budding Yeast. *Mol. Cell. Biol.*, **21**, 4949-4959.

Bayliss, R., Sardon, T., Vernos, I. and Conti, E. (2003) Structural Basis of Aurora-A Activation by TPX2 at the Mitotic Spindle. *Molecular Cell*, **12**, 851-862.

Bernard, P., Hardwick, K. and Javerzat, J.-P. (1998) Fission Yeast Bub1 Is a Mitotic Centromere Protein Essential for the Spindle Checkpoint and the Preservation of Correct Ploidy through Mitosis. *J. Cell Biol.*, **143**, 1775-1787.

Bernard, P., Maure, J.F. and Javerzat, J.P. (2001) Fission yeast Bub1 is essential in setting up the meiotic pattern of chromosome segregation. *Nat Cell Biol*, **3**, 522-526.

Bhamidipati, A., Lewis, S.A. and Cowan, N.J. (2000) ADP ribosylation factor-like protein 2 (Arl2) regulates the interaction of tubulin-folding cofactor D with native tubulin. *J Cell Biol*, **149**, 1087-1096.

Biggins, S. and Murray, A.W. (2001) The budding yeast protein kinase Ipl1/Aurora allows the absence of tension to activate the spindle checkpoint. *Genes Dev*, **15**, 3118-3129.

Biggins, S., Severin, F.F., Bhalla, N., Sassoon, I., Hyman, A.A. and Murray, A.W. (1999) The conserved protein kinase Ipl1 regulates microtubule binding to kinetochores in budding yeast. *Genes Dev*, **13**, 532-544.

Birkenbihl, R.P. and Subramani, S. (1995) The rad21 gene product of Schizosaccharomyces pombe is a nuclear, cell cycle-regulated phosphoprotein. *J Biol Chem*, **270**, 7703-7711.

Birkenbihl, R.P. and Subramani, S. (1992) Cloning and characterization of rad21 an essential gene of Schizosaccharomyces pombe involved in DNA double-strand-break repair. *Nucleic Acids Res*, **20**, 6605-6611.

Blasina, A., Price, B.D., Turenne, G.A. and McGowan, C.H. (1999) Caffeine inhibits the checkpoint kinase ATM. *Current Biology*, **9**, 1135-1138.

Bolton, M.A., Lan, W., Powers, S.E., McCleland, M.L., Kuang, J. and Stukenberg, P.T. (2002) Aurora B Kinase Exists in a Complex with Survivin and INCENP and Its Kinase Activity Is Stimulated by Survivin Binding and Phosphorylation. *Mol. Biol. Cell*, **13**, 3064-3077.

Booher, R. and Beach, D. (1989) Involvement of a type 1 protein phosphatase encoded by bws1+ in fission yeast mitotic control. *Cell*, **57**, 1009-1016.

Booher, R. and Beach, D. (1988) Involvement of cdc13+ in mitotic control in Schizosaccharomyces pombe: possible interaction of the gene product with microtubules. *Embo J*, **7**, 2321-2327.

Booher, R. and Beach, D. (1987) Interaction between cdc13+ and cdc2+ in the control of mitosis in fission yeast; dissociation of the G1 and G2 roles of the cdc2+ protein kinase. *Embo J*, **6**, 3441-3447.

Breeding, C.S., Hudson, J., Balasubramanian, M.K., Hemmingsen, S.M., Young, P.G. and Gould, K.L. (1998) The cdr2+ Gene Encodes a Regulator of G2/M Progression and Cytokinesis in Schizosaccharomyces pombe. *Mol. Biol. Cell*, **9**, 3399-3415.

Bridge, A.J., Morphew, M., Bartlett, R. and Hagan, I.M. (1998) The fission yeast SPB component Cut12 links bipolar spindle formation to mitotic†control. *Genes Dev.*, **12**, 927-942.

Bulavin, D.V., Higashimoto, Y., Popoff, I.J., Gaarde, W.A., Basrur, V., Potapova, O., Appella, E. and Fornace, A.J., Jr. (2001) Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase. *Nature*, **411**, 102-107.

Bullough, W.S. and Johnson, M. (1951) The energy relations of mitotic activity in adult mouse epidermis. *Proc R Soc Lond B Biol Sci*, **138**, 562-575.

Cassimeris, L., Rieder, C.L., Rupp, G. and Salmon, E.D. (1990) Stability of microtubule attachment to metaphase kinetochores in PtK1 cells. *J Cell Sci*, **96 (Pt 1)**, 9-15.

Chan, C. and Botstein, D. (1993) Isolation and Characterization of Chromosome-Gain and Increase-in-Ploidy Mutants in Yeast. *Genetics*, **135**, 677-691.

Cheeseman, I.M., Anderson, S., Jwa, M., Green, E.M., Kang, J.-s., Yates, I., John R., Chan, C.S.M., Drubin, D.G. and Barnes, G. (2002) Phospho-Regulation of Kinetochore-Microtubule Attachments by the Aurora Kinase Ipl1p. *Cell*, **111**, 163-172.

Cheeseman, I.M., Brew, C., Wolyniak, M., Desai, A., Anderson, S., Muster, N., Yates, J.R., Huffaker, T.C., Drubin, D.G. and Barnes, G. (2001a) Implication of a novel multiprotein Dam1p complex in outer kinetochore function. *J. Cell Biol.*, **155**, 1137-1146.

Cheeseman, I.M., Enquist-Newman, M., Muller-Reichert, T., Drubin, D.G. and Barnes, G. (2001b) Mitotic Spindle Integrity and Kinetochore Function Linked by the Duo1p/Dam1p Complex. *J. Cell Biol.*, **152**, 197-212.

Chen, R.-H., Brady, D.M., Smith, D., Murray, A.W. and Hardwick, K.G. (1999) The Spindle Checkpoint of Budding Yeast Depends on a Tight Complex between the Mad1 and Mad2 Proteins. *Mol. Biol. Cell*, **10**, 2607-2618.

Chen, R.-H., Shevchenko, A., Mann, M. and Murray, A.W. (1998) Spindle Checkpoint Protein Xmad1 Recruits Xmad2 to Unattached Kinetochores. *J. Cell Biol.*, **143**, 283-295.

Chen, R.-H., Waters, J.C., Salmon, E.D. and Murray, A.W. (1996) Association of Spindle Assembly Checkpoint Component XMAD2 with Unattached Kinetochores. *Science*, **274**, 242-246.

Chikashige, Y., Kinoshita, N., Nakaseko, Y., Matsumoto, T., Murakami, S., Niwa, O. and Yanagida, M. (1989) Composite motifs and repeat symmetry in S. pombe centromeres: direct analysis by integration of NotI restriction sites. *Cell*, **57**, 739-751.

Cimini, D., Howell, B., Maddox, P., Khodjakov, A., Degrassi, F., Salmon, E.D. (2001) Merotelic kinetochore orientation is a major mechanism of aneuploidy in mitotic mammalian tissue cells. *J. Cell Biol.*, **153**, 517-527.

Cimini, D., Moree, B., Canman, J.C., Salmon, E.D., (2003) Mertotelic kinetochore orientation occurs frequently during early mitosis in mammalian tissue cells and error correction is achieved by two different mechanisms. *J Cell Sci.*, **116**, 4213-4225.

Ciosk, R., Zachariae, W., Michaelis, C., Shevchenko, A., Mann, M. and Nasmyth, K. (1998) An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. *Cell*, **93**, 1067-1076.

Clute, P. and Pines, J. (1999) Temporal and spatial control of cyclin B1 destruction in metaphase. *Nature Cell Biology*, **1**, 82-87.

Coleman, T.R., Tang, Z. and Dunphy, W.G. (1993) Negative regulation of the wee1 protein kinase by direct action of the nim1/cdr1 mitotic inducer. *Cell*, **72**, 919-929.

Creanor, J. and Mitchison, J. (1996) The kinetics of the B cyclin p56cdc13 and the phosphatase p80cdc25 during the cell cycle of the fission yeast Schizosaccharomyces pombe. *J Cell Sci*, **109**, 1647-1653.

Daga, R. and Jimenez, J. (1999) Translational control of the cdc25 cell cycle phosphatase: a molecular mechanism coupling mitosis to cell growth. *J Cell Sci*, **112**, 3137-3146.

Decottignies, A., Zarzov, P. and Nurse, P. (2001) In vivo localisation of fission yeast cyclin-dependent kinase cdc2p and cyclin B cdc13p during mitosis and meiosis. *J Cell Sci*, **114**, 2627-2640.

Desai, A. and Mitchison, T.J. (1997) MICROTUBULE POLYMERIZATION DYNAMICS. *Annual Review of Cell and Developmental Biology*, **13**, 83-117.

Desai, A., Verma, S., Mitchison, T.J. and Walczak, C.E. (1999) Kin I kinesins are microtubule-destabilizing enzymes. *Cell*, **96**, 69-78.

Dewar, H., Tanaka, K., Nasmyth, K. and Tanaka, T.U. (2004) Tension between two kinetochores suffices for their bi-orientation on the mitotic spindle. *Nature*, **428**, 93-97.

Ding, R., McDonald, K. and McIntosh, J. (1993) Three-dimensional reconstruction and analysis of mitotic spindles from the yeast, Schizosaccharomyces pombe. *J. Cell Biol.*, **120**, 141-151.

Ding, R., West, R.R., Morphew, D.M., Oakley, B.R. and McIntosh, J.R. (1997) The spindle pole body of Schizosaccharomyces pombe enters and leaves the nuclear envelope as the cell cycle proceeds. *Mol Biol Cell*, **8**, 1461-1479.

Dutertre, S., Descamps, S. and Prigent, C. (2002) On the role of aurora-A in centrosome function. *Oncogene*, **21**, 6175-6183.

Edwards, R.J., Bentley, N.J. and Carr, A.M. (1999) A Rad3-Rad26 complex responds to DNA damage independently of other checkpoint proteins. *Nat Cell Biol*, **1**, 393-398.

Ekwall, K., Javerzat, J.P., Lorentz, A., Schmidt, H., Cranston, G. and Allshire, R. (1995) The chromodomain protein Swi6: a key component at fission yeast centromeres. *Science*, **269**, 1429-1431.

Ekwall, K., Nimmo, E., Javerzat, J., Borgstrom, B., Egel, R., Cranston, G. and Allshire, R. (1996) Mutations in the fission yeast silencing factors clr4+ and rik1+ disrupt the localisation of the chromo domain protein Swi6p and impair centromere function. *J Cell Sci*, **109**, 2637-2648.

Enquist-Newman, M., Cheeseman, I.M., Van Goor, D., Drubin, D.G., Meluh, P.B. and Barnes, G. (2001) Dad1p, Third Component of the Duo1p/Dam1p Complex Involved in Kinetochore Function and Mitotic Spindle Integrity. *Mol. Biol. Cell*, **12**, 2601-2613.

Esashi, F. and Yanagida, M. (1999) Cdc2 phosphorylation of Crb2 is required for reestablishing cell cycle progression after the damage checkpoint. *Mol Cell*, **4**, 167-174.

Ewart-Toland, A., Briassouli, P., de Koning, J.P., Mao, J.-H., Yuan, J., Chan, F., MacCarthy-Morrogh, L., Ponder, B.A.J., Nagase, H. and Burn et, a. (2003) Identification of Stk6/STK15 as a candidate low-penetrance tumor-susceptibility gene in mouse and human. *Nature Genetics*, **34**, 403-412.

Fang, S. and Weissman, A.M. (2004) A field guide to ubiquitylation. *Cell Mol Life Sci*, **61**, 1546-1561.

Fleig, U., Sen-Gupta, M. and Hegemann, J. (1996) Fission yeast mal2+ is required for chromosome segregation. *Mol. Cell. Biol.*, **16**, 6169-6177.

Francisco, L. and Chan, C.S. (1994) Regulation of yeast chromosome segregation by Ipl1 protein kinase and type 1 protein phosphatase. *Cell Mol Biol Res*, **40**, 207-213.

Francisco, L., Wang, W. and Chan, C.S. (1994) Type 1 protein phosphatase acts in opposition to IpL1 protein kinase in regulating yeast chromosome segregation. *Mol Cell Biol*, **14**, 4731-4740.

Fukagawa, T., Nogami, M., Yoshikawa, M., Ikeno, M., Okazaki, T., Takami, Y., Nakayama, T. and Oshimura, M. (2004) Dicer is essential for formation of the heterochromatin structure in vertebrate cells. *Nat Cell Biol*, **6**, 784-791.

Funabiki, H., Kumada, K. and Yanagida, M. (1996a) Fission yeast Cut1 and Cut2 are essential for sister chromatid separation, concentrate along the metaphase spindle and form large complexes. *Embo J*, **15**, 6617-6628.

Funabiki, H., Yamano, H., Kumada, K., Nagao, K., Hunt, T. and Yanagida, M. (1996b) Cut2 proteolysis required for sister-chromatid seperation in fission yeast. *Nature*, **381**, 438-441.

Funabiki, H., Yamano, H., Nagao, K., Tanaka, H., Yasuda, H., Hunt, T. and Yanagida, M. (1997) Fission yeast Cut2 required for anaphase has two destruction boxes. *Embo J*, **16**, 5977-5987.

Furnari, B., Rhind, N. and Russell, P. (1997) Cdc25 Mitotic Inducer Targeted by Chk1 DNA Damage Checkpoint Kinase. *Science*, **277**, 1495-1497.

Furuno, N., den Elzen, N. and Pines, J. (1999) Human cyclin A is required for mitosis until mid prophase. *The Journal Of Cell Biology*, **147**, 295-306.

Furuya, K., Takahashi, K. and Yanagida, M. (1998) Faithful anaphase is ensured by Mis4, a sister chromatid cohesion molecule required in S phase and not destroyed in G1 phase. *Genes Dev.*, **12**, 3408-3418.

Gachet, Y., Tournier, S., Millar, J.B. and Hyams, J.S. (2001) A MAP kinase-dependent actin checkpoint ensures proper spindle orientation in fission yeast. *Nature*, **412**, 352-355.

Gallagher, I.M., Alfa, C.E. and Hyams, J.S. (1993) p63cdc13, a B-type cyclin, is associated with both the nucleolar and chromatin domains of the fission yeast nucleus. *Mol Biol Cell*, **4**, 1087-1096.

Garcia, M.A., Koonrugsa, N. and Toda, T. (2002a) Two Kinesin-like Kin I Family Proteins in Fission Yeast Regulate the Establishment of Metaphase and the Onset of Anaphase A. *Current Biology*, **12**, 610-621.

Garcia, M.A., Koonrugsa, N. and Toda, T. (2002b) Spindle-kinetochore attachment requires the combined action of Kin I-like Klp5/6 and Alp14/Dis1-MAPs in fission yeast. *EMBO J.*, **21**, 6015-6024.

Garcia, M.A., Vardy, L., Koonrugsa, N. and Toda, T. (2001) Fission yeast ch-TOG/XMAP215 homologue Alp14 connects mitotic spindles with the kinetochore and is a component of the Mad2-dependent spindle checkpoint. *Embo J*, **20**, 3389-3401.

Glover, D.M., Leibowitz, M.H., McLean, D.A. and Parry, H. (1995) Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles. *Cell*, **81**, 95-105.

Goh, P.Y. and Kilmartin, J.V. (1993) NDC10: a gene involved in chromosome segregation in Saccharomyces cerevisiae. *J Cell Biol*, **121**, 503-512.

Goshima, G., Saitoh, S. and Yanagida, M. (1999) Proper metaphase spindle length is determined by centromere proteins Mis12 and Mis6 required for faithful chromosome segregation. *Genes Dev.*, **13**, 1664-1677.

Gould, K.L., Moreno, S., Owen, D.J., Sazer, S. and Nurse, P. (1991) Phosphorylation at Thr167 is required for Schizosaccharomyces pombe p34cdc2 function. *Embo J*, **10**, 3297-3309.

Gould, K.L. and Nurse, P. (1989) Tyrosine phosphorylation of the fission yeast cdc2+ protein kinase regulates entry into mitosis. *Nature*, **342**, 39-45.

Grallert, A. and Hagan, I.M. (2002) Schizosaccharomyces pombe NIMA-related kinase, Fin1, regulates spindle formation and an affinity of Polo for the SPB. *Embo J*, **21**, 3096-3107.

Gruss, O.J., Carazo-Salas, R.E., Schatz, C.A., Guarguaglini, G., Kast, J., Wilm, M., Le Bot, N., Vernos, I., Karsenti, E. and Mattaj, I.W. (2001) Ran Induces Spindle Assembly by Reversing the Inhibitory Effect of Importin [alpha] on TPX2 Activity. *Cell*, **104**, 83-93.

Guacci, V., Koshland, D. and Strunnikov, A. (1997) A direct link between sister chromatid cohesion and chromosome condensation revealed through the analysis of MCD1 in S. cerevisiae. *Cell*, **91**, 47-57.

Hagan, I., Hayles, J. and Nurse, P. (1988) Cloning and sequencing of the cyclin-related cdc13+ gene and a cytological study of its role in fission yeast mitosis. *J Cell Sci*, **91 (Pt 4)**, 587-595.

Hagan, I. and Yanagida, M. (1992) Kinesin-related cut7 protein associates with mitotic and meiotic spindles in fission yeast. *Nature*, **356**, 74-76.

Hagan, I.M. (1998) The fission yeast microtubule cytoskeleton. *J Cell Sci*, **111 (Pt 12)**, 1603-1612.

Hagan, I.M. and Hyams, J.S. (1988) The use of cell division cycle mutants to investigate the control of microtubule distribution in the fission yeast Schizosaccharomyces pombe. *J Cell Sci*, **89 (Pt 3)**, 343-357.

Hagemeier, C., Bannister, A.J., Cook, A. and Kouzarides, T. (1993) The activation domain of transcription factor PU.1 binds the retinoblastoma (RB) protein and the transcription factor TFIID in vitro: RB shows sequence similarity to TFIID and TFIIB. *Proc Natl Acad Sci U S A*, **90**, 1580-1584.

Hardwick, K. and Murray, A. (1995) Mad1p, a phosphoprotein component of the spindle assembly checkpoint in budding yeast. *J. Cell Biol.*, **131**, 709-720.

Hardwick, K.G., Johnston, R.C., Smith, D.L. and Murray, A.W. (2000) MAD3 Encodes a Novel Component of the Spindle Checkpoint which Interacts with Bub3p, Cdc20p, and Mad2p. *J. Cell Biol.*, **148**, 871-882.

Hartmann-Petersen, R., Semple, C.A.M., Ponting, C.P., Hendil, K.B. and Gordon, C. (2003) UBA domain containing proteins in fission yeast. *The International Journal of Biochemistry & Cell Biology*, **35**, 629-636.

Hartwell, L.H. and Weinert, T.A. (1989) Checkpoints: controls that ensure the order of cell cycle events. *Science*, **246**, 629-634.

Hauf, S., Cole, R.W., LaTerra, S., Zimmer, C., Schnapp, G., Walter, R., Heckel, A., van Meel, J., Rieder, C.L. and Peters, J.-M. (2003) The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. *J. Cell Biol.*, **161**, 281-294.

He, X., Patterson, T.E. and Sazer, S. (1997) The Schizosaccharomyces pombe spindle checkpoint protein mad2p blocks anaphase and genetically interacts with the anaphase-promoting complex. *Proc Natl Acad Sci U S A*, **94**, 7965-7970.

Heitz, M.J., Petersen, J., Valovin, S. and Hagan, I.M. (2001) MTOC formation during mitotic exit in fission yeast. *J Cell Sci*, **114**, 4521-4532.

Hertz-Fowler, C., Peacock, C.S., Wood, V., Aslett, M., Kerhornou, A., Mooney, P., Tivey, A., Berriman, M., Hall, N., Rutherford, K., Parkhill, J., Ivens, A.C., Rajandream, M.-A. and Barrell, B. (2004) GeneDB: a resource for prokaryotic and eukaryotic organisms. *Nucl. Acids Res.*, **32**, D339-343.

Hilioti, Z., Chung, Y.-S., Mochizuki, Y., Hardy, C.F.J. and Cohen-Fix, O. (2001) The anaphase inhibitor Pds1 binds to the APC/C-associated protein Cdc20 in a destruction box-dependent manner. *Current Biology*, **11**, 1347-1352.

Hirano, T. (1999) SMC-mediated chromosome mechanics: a conserved scheme from bacteria to vertebrates? *Genes Dev.*, **13**, 11-19.

Hirano, T., Kobayashi, R. and Hirano, M. (1997) Condensins, chromosome condensation protein complexes containing XCAP-C, XCAP-E and a Xenopus homolog of the Drosophila Barren protein. *Cell*, **89**, 511-521.

Hirano, T. and Mitchison, T.J. (1994) A heterodimeric coiled-coil protein required for mitotic chromosome condensation in vitro. *Cell*, **79**, 449-458.

Hiraoka, Y., Toda, T. and Yanagida, M. (1984) The NDA3 gene of fission yeast encodes beta-tubulin: a cold-sensitive nda3 mutation reversibly blocks spindle formation and chromosome movement in mitosis. *Cell*, **39**, 349-358.

Hofmann, C., Cheeseman, I.M., Goode, B.L., McDonald, K.L., Barnes, G. and Drubin, D.G. (1998) Saccharomyces cerevisiae Duo1p and Dam1p, Novel Proteins Involved in Mitotic Spindle Function. *J. Cell Biol.*, **143**, 1029-1040.

Hoyt, M.A., Totis, L. and Roberts, B.T. (1991) S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function. *Cell*, **66**, 507-517.

Hsu, J.Y., Sun, Z.W., Li, X., Reuben, M., Tatchell, K., Bishop, D.K., Grushcow, J.M., Brame, C.J., Caldwell, J.A., Hunt, D.F., Lin, R., Smith, M.M. and Allis, C.D. (2000) Mitotic phosphorylation of histone H3 is governed by Ipl1/aurora kinase and Glc7/PP1 phosphatase in budding yeast and nematodes. *Cell*, **102**, 279-291.

Hudson, J.D., Feilotter, H. and Young, P.G. (1990) stf1: Non-Wee Mutations Epistatic to cdc25 in the Fission Yeast Schizosaccharomyces pombe. *Genetics*, **126**, 309-315.

Hutvagner, G. and Zamore, P.D. (2002) RNAi: nature abhors a double-strand. *Current Opinion in Genetics & Development*, **12**, 225-232.

Hwang, L.H., Lau, L.F., Smith, D.L., Mistrot, C.A., Hardwick, K.G., Hwang, E.S., Amon, A. and Murray, A.W. (1998) Budding Yeast Cdc20: A Target of the Spindle Checkpoint. *Science*, **279**, 1041-1044.

Hyland, K.M., Kingsbury, J., Koshland, D. and Hieter, P. (1999) Ctf19p: A Novel Kinetochore Protein in Saccharomyces cerevisiae and a Potential Link between the Kinetochore and Mitotic Spindle. *J. Cell Biol.*, **145**, 15-28.

Ikui, A.E., Furuya, K., Yanagida, M. and Matsumoto, T. (2002) Control of localization of a spindle checkpoint protein, Mad2, in fission yeast. *J Cell Sci*, **115**, 1603-1610.

Jackman, M., Lindon, C., Nigg, E.A. and Pines, J. (2003) Active cyclin B1-Cdk1 first appears on centrosomes in prophase. *Nat Cell Biol*, **5**, 143-148.

Janke, C., Ortiz, J., Tanaka, T.U., Lechner, J. and Schiebel, E. (2002) Four new subunits of the Dam1-Duo1 complex reveal novel functions in sister kinetochore biorientation. *Embo J*, **21**, 181-193.

Jeffrey, P.D., Russo, A.A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J. and Pavletich, N.P. (1995) Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex. *Nature*, **376**, 313-320.

Jin, Q.-W., Pidoux, A.L., Decker, C., Allshire, R.C. and Fleig, U. (2002) The Mal2p Protein Is an Essential Component of the Fission Yeast Centromere. *Mol. Cell. Biol.*, **22**, 7168-7183.

Jones, M.H., Bachant, J.B., Castillo, A.R., Giddings, T.H., Jr. and Winey, M. (1999) Yeast Dam1p Is Required to Maintain Spindle Integrity during Mitosis and Interacts with the Mps1p Kinase. *Mol. Biol. Cell*, **10**, 2377-2391.

Jones, M.H., He, X., Giddings, T.H. and Winey, M. (2001) Yeast Dam1p has a role at the kinetochore in assembly of the mitotic spindle. *PNAS*, **98**, 13675-13680.

Kallio, M.J., McCleland, M.L., Stukenberg, P.T. and Gorbsky, G.J. (2002) Inhibition of Aurora B Kinase Blocks Chromosome Segregation, Overrides the Spindle Checkpoint, and Perturbs Microtubule Dynamics in Mitosis. *Current Biology*, **12**, 900-905.

Kang, J.-s., Cheeseman, I.M., Kallstrom, G., Velmurugan, S., Barnes, G. and Chan, C.S.M. (2001) Functional cooperation of Dam1, Ipl1, and the inner centromere protein (INCENP)-related protein Sli15 during chromosome segregation. *J. Cell Biol.*, **155**, 763-774.

Kanoh, J. and Russell, P. (1998) The Protein Kinase Cdr2, Related to Nim1/Cdr1 Mitotic Inducer, Regulates the Onset of Mitosis in Fission Yeast. *Mol. Biol. Cell*, **9**, 3321-3334.

Katayama, H., Brinkley, W.R. and Sen, S. (2003) The Aurora kinases: Role in cell transformation and tumorigenesis. *Cancer and Metastasis Reviews*, **22**, 451-464.

Keeney, J.B. and Boeke, J.D. (1994) Efficient Targeted Integration at leu1-32 and ura4-294 in Schizosaccharomyces pombe. *Genetics*, **136**, 849-856.

Kerres, A., Vietmeier-Decker, C., Ortiz, J., Karig, I., Beuter, C., Hegemann, J., Lechner, J. and Fleig, U. (2004) The Fission Yeast Kinetochore Component Spc7 Associates with the EB1 Family Member Mal3 and Is Required for Kinetochore-Spindle Association. *Mol. Biol. Cell*, **15**, 5255-5267.

Kim, J.H., Kang, J.S. and Chan, C.S. (1999) Sli15 associates with the ipl1 protein kinase to promote proper chromosome segregation in Saccharomyces cerevisiae. *J Cell Biol*, **145**, 1381-1394.

Kim, S.H., Lin, D.P., Matsumoto, S., Kitazono, A. and Matsumoto, T. (1998) Fission Yeast Slp1: An Effector of the Mad2-Dependent Spindle Checkpoint. *Science*, **279**, 1045-1047.

Kimura, K. and Hirano, T. (1997) ATP-dependent positive supercoiling of DNA by 13S condensin: a biochemical implication for chromosome condensation. *Cell*, **90**, 625-634.

Kitamura, K., Maekawa, H. and Shimoda, C. (1998) Fission Yeast Ste9, a Homolog of Hct1/Cdh1 and Fizzy-related, Is a Novel Negative Regulator of Cell Cycle Progression during G1-Phase. *Mol. Biol. Cell*, **9**, 1065-1080.

Kline-Smith, S.L., Khodjakov, A., Hergert, P. and Walczak, C.E. (2004) Depletion of Centromeric MCAK Leads to Chromosome Congression and Segregation Defects Due to Improper Kinetochore Attachments. *Mol. Biol. Cell*, **15**, 1146-1159.

Kniola, B., O'Toole, E., McIntosh, J.R., Mellone, B., Allshire, R., Mengarelli, S., Hultenby, K. and Ekwall, K. (2001) The Domain Structure of Centromeres Is Conserved from Fission Yeast to Humans. *Mol. Biol. Cell*, **12**, 2767-2775.

Knop, M., Pereira, G. and Schiebel, E. (1999) Microtubule organization by the budding yeast spindle pole body. *Biol Cell*, **91**, 291-304.

Kominami, K., Seth-Smith, H. and Toda, T. (1998) Apc10 and Ste9/Srw1, two regulators of the APC-cyclosome, as well as the CDK inhibitor Rum1 are required for G1 cell-cycle arrest in fission yeast. *Embo J*, **17**, 5388-5399.

Krapp, A., Schmidt, S., Cano, E. and Simanis, V. (2001) S. pombe cdc11p, together with sid4p, provides an anchor for septation initiation network proteins on the spindle pole body. *Curr Biol*, **11**, 1559-1568.

Krawchuk, M.D. and Wahls, W.P. (1999) High-efficiency gene targeting in Schizosaccharomyces pombe using a modular, PCR-based approach with long tracts of flanking homology. *Yeast*, **15**, 1419-1427.

Krien, M., Bugg, S., Palatsides, M., Asouline, G., Morimyo, M. and O'Connell, M. (1998) A NIMA homologue promotes chromatin condensation in fission yeast. *J Cell Sci*, **111**, 967-976.

Kufer, T.A., Sillje, H.H.W., Korner, R., Gruss, O.J., Meraldi, P. and Nigg, E.A. (2002) Human TPX2 is required for targeting Aurora-A kinase to the spindle. *J. Cell Biol.*, **158**, 617-623.

Kumada, K., Nakamura, T., Nagao, K., Funabiki, H., Nakagawa, T. and Yanagida, M. (1998) Cut1 is loaded onto the spindle by binding to Cut2 and promotes anaphase spindle movement upon Cut2 proteolysis. *Curr Biol*, **8**, 633-641.

Kumagai, A. and Dunphy, W.G. (1996) Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from Xenopus egg extracts. *Science*, **273**, 1377-1380.

Lan, W., Zhang, X., Kline-Smith, S.L., Rosasco, S.E., Barrett-Wilt, G.A., Shabanowitz, J., Hunt, D.F., Walczak, C.E. and Stukenberg, P.T. (2004) Aurora B Phosphorylates Centromeric MCAK and Regulates Its Localization and Microtubule Depolymerization Activity. *Current Biology*, **14**, 273-286.

Lechner, J. and Carbon, J. (1991) A 240 kd multisubunit protein complex, CBF3, is a major component of the budding yeast centromere. *Cell*, **64**, 717-725.

Lee, K.M., Saiz, J.E., Barton, W.A. and Fisher, R.P. (1999) Cdc2 activation in fission yeast depends on Mcs6 and Csk1, two partially redundant Cdk-activating kinases (CAKs). *Current Biology*, **9**, 441-444.

Lee, M.G. and Nurse, P. (1987) Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2. *Nature*, **327**, 31-35.

Lehnertz, B., Ueda, Y., Derijck, A.A.H.A., Braunschweig, U., Perez-Burgos, L., Kubicek, S., Chen, T., Li, E., Jenuwein, T. and Peters, A.H.F.M. (2003) Suv39h-Mediated Histone H3 Lysine 9 Methylation Directs DNA Methylation to Major Satellite Repeats at Pericentric Heterochromatin. *Current Biology*, **13**, 1192-1200.

Leverson, J.D., Huang, H.-k., Forsburg, S.L. and Hunter, T. (2002) The Schizosaccharomyces pombe Aurora-related Kinase Ark1 Interacts with the Inner Centromere Protein Pic1 and Mediates Chromosome Segregation and Cytokinesis. *Mol. Biol. Cell*, **13**, 1132-1143.

Lew, D.J. and Burke, D.J. (2003) THE SPINDLE ASSEMBLY AND SPINDLE POSITION CHECKPOINTS. *Annual Review of Genetics*, **37**, 251-282.

Li, J.-m., Li, Y. and Elledge, S.J. (2005) Genetic Analysis of the Kinetochore DASH Complex Reveals an Antagonistic Relationship with the Ras/Protein Kinase A Pathway and a Novel Subunit Required for Ask1 Association. *Mol. Cell. Biol.*, **25**, 767-778.

Li, R. and Murray, A.W. (1991) Feedback control of mitosis in budding yeast. *Cell*, **66**, 519-531.

Li, X. and Nicklas, R.B. (1995) Mitotic forces control a cell-cycle checkpoint. *Nature*, **373**, 630-632.

Li, Y., Bachant, J., Alcasabas, A.A., Wang, Y., Qin, J. and Elledge, S.J. (2002) The mitotic spindle is required for loading of the DASH complex onto the kinetochore. *Genes Dev.*, **16**, 183-197.

Lindsay, H.D., Griffiths, D.J.F., Edwards, R.J., Christensen, P.U., Murray, J.M., Osman, F., Walworth, N. and Carr, A.M. (1998) S-phase-specific activation of Cds1 kinase defines a subpathway of the checkpoint response in Schizosaccharomyces†pombe. *Genes Dev.*, **12**, 382-395.

Loiodice, I., Staub, J., Setty, T.G., Nguyen, N.-P.T., Paoletti, A. and Tran, P.T. (2005) Ase1p Organizes Antiparallel Microtubule Arrays during Interphase and Mitosis in Fission Yeast. *Mol. Biol. Cell*, **16**, 1756-1768.

Lopez-Aviles, S., Grande, M., Gonzalez, M., Helgesen, A.-L., Alemany, V., Sanchez-Piris, M., Bachs, O., Millar, J.B.A. and Aligue, R. (2005) Inactivation of the Cdc25 Phosphatase by the Stress-Activated Srk1 Kinase in Fission Yeast. *Molecular Cell*, **17**, 49-59.

Lopez-Girona, A., Furnari, B., Mondesert, O. and Russell, P. (1999) Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. *Nature*, **397**, 172-175.

Lundgren, K., Walworth, N., Booher, R., Dembski, M., Kirschner, M. and Beach, D. (1991) mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. *Cell*, **64**, 1111-1122.

Mallavarapu, A., Sawin, K. and Mitchison, T. (1999) A switch in microtubule dynamics at the onset of anaphase B in the mitotic spindle of Schizosaccharomyces pombe. *Curr Biol*, **9**, 1423-1426.

Maney, T., Hunter, A.W., Wagenbach, M. and Wordeman, L. (1998) Mitotic Centromere-associated Kinesin Is Important for Anaphase Chromosome Segregation. *J. Cell Biol.*, **142**, 787-801.

Manke, I.A., Nguyen, A., Lim, D., Stewart, M.Q., Elia, A.E. and Yaffe, M.B. (2005) MAPKAP kinase-2 is a cell cycle checkpoint kinase that regulates the G2/M transition and S phase progression in response to UV irradiation. *Mol Cell*, **17**, 37-48.

Marks, J. and Hyams, J.S. (1985) Localization of F-actin through the cell division cycle of Schizosaccharomyces pombe. *EUR. J. CELL BIOL.*, **39**, 27-32.

Marschall, L. and Clarke, L. (1995) A novel cis-acting centromeric DNA element affects S. pombe centromeric chromatin structure at a distance. *J. Cell Biol.*, **128**, 445-454.

Masuda, H., Sevik, M. and Cande, W. (1992) In vitro microtubule-nucleating activity of spindle pole bodies in fission yeast Schizosaccharomyces pombe: cell cycle-dependent activation in xenopus cell-free extracts. *J. Cell Biol.*, **117**, 1055-1066.

Mata, J. and Nurse, P. (1997) tea1 and the microtubular cytoskeleton are important for generating global spatial order within the fission yeast cell. *Cell*, **89**, 939-949.

Matsumoto, T. (1997) A fission yeast homolog of CDC20/p55CDC/Fizzy is required for recovery from DNA damage and genetically interacts with p34cdc2. *Mol. Cell. Biol.*, **17**, 742-750.

Matsusaka, T. and Pines, J. (2004) Chfr acts with the p38 stress kinases to block entry to mitosis in mammalian cells. *J. Cell Biol.*, **166**, 507-516.

Mellor, J., Jiang, W., Funk, M., Rathjen, J., Barnes, C.A., Hinz, T., Hegemann, J.H. and Philippsen, P. (1990) CPF1, a yeast protein which functions in centromeres and promoters. *Embo J*, **9**, 4017-4026.

Meluh, P.B., Yang, P., Glowczewski, L., Koshland, D. and Smith, M.M. (1998) Cse4p is a component of the core centromere of Saccharomyces cerevisiae. *Cell*, **94**, 607-613.

Michaelis, C., Ciosk, R. and Nasmyth, K. (1997) Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. *Cell*, **91**, 35-45.

Mikhailov, A., Shinohara, M. and Rieder, C.L. (2005) The p38-mediated Stress-activated Checkpoint: A Rapid Response System for Delaying Progression through Antephase and Entry into Mitosis. *Cell Cycle*, **4**.

Mikhailov, A., Shinohara, M. and Rieder, C.L. (2004) Topoisomerase II and histone deacetylase inhibitors delay the G2/M transition by triggering the p38 MAPK checkpoint pathway. *J. Cell Biol.*, **166**, 517-526.

Millar, J.B., Buck, V. and Wilkinson, M.G. (1995) Pyp1 and Pyp2 PTPases dephosphorylate an osmosensing MAP kinase controlling cell size at division in fission yeast. *Genes Dev*, **9**, 2117-2130.

Millar, J.B., McGowan, C.H., Lenaers, G., Jones, R. and Russell, P. (1991) p80cdc25 mitotic inducer is the tyrosine phosphatase that activates p34cdc2 kinase in fission yeast. *Embo J*, **10**, 4301-4309.

Millband, D.N., Campbell, L. and Hardwick, K.G. (2002) The awesome power of multiple model systems: interpreting the complex nature of spindle checkpoint signaling. *Trends Cell Biol*, **12**, 205-209.

Millband, D.N. and Hardwick, K.G. (2002) Fission yeast Mad3p is required for Mad2p to inhibit the anaphase-promoting complex and localizes to kinetochores in a Bub1p-, Bub3p-, and Mph1p-dependent manner. *Mol Cell Biol*, **22**, 2728-2742.

Miranda, J.L., Wulf, P.D., Sorger, P.K. and Harrison, S.C. (2005) The yeast DASH complex forms closed rings on microtubules. **12**, 138-143.

Mizuno, K., Osada, H., Konishi, H., Tatematsu, Y., Yatabe, Y., Mitsudomi, T., Fujii, Y. and Takahashi, T. (2002) Aberrant hypermethylation of the CHFR prophase checkpoint gene in human lung cancers. *Oncogene*, **21**, 2328-2333.

Moreno, S., Hayles, J. and Nurse, P. (1989) Regulation of p34cdc2 protein kinase during mitosis. *Cell*, **58**, 361-372.

Moreno, S., Nurse, P. and Russell, P. (1990) Regulation of mitosis by cyclic accumulation of p80cdc25 mitotic inducer in fission yeast. *Nature*, **344**, 549-552.

Morishita, J., Matsusaka, T., Goshima, G., Nakamura, T., Tatebe, H. and Yanagida, M. (2001) Bir1/Cut17 moving from chromosome to spindle upon the loss of cohesion is required for condensation, spindle elongation and repair. *Genes Cells*, **6**, 743-763.

Moritz, M. and Agard, D.A. (2001) [gamma]-Tubulin complexes and microtubule nucleation. *Current Opinion in Structural Biology*, **11**, 174-181.

Morris, N.R. (1975) Mitotic mutants of Aspergillus nidulans. *Genet Res*, **26**, 237-254.

Mulvihill, D.P., Petersen, J., Ohkura, H., Glover, D.M. and Hagan, I.M. (1999) Plo1 Kinase Recruitment to the Spindle Pole Body and Its Role in Cell Division in Schizosaccharomyces pombe. *Mol. Biol. Cell*, **10**, 2771-2785.

Murakami, H. and Okayama, H. (1995) A kinase from fission yeast responsible for blocking mitosis in S phase. *Nature*, **374**, 817-819.

Murata-Hori, M., Tatsuka, M. and Wang, Y.-L. (2002) Probing the Dynamics and Functions of Aurora B Kinase in Living Cells during Mitosis and Cytokinesis. *Mol. Biol. Cell*, **13**, 1099-1108.

Murone, M. and Simanis, V. (1996) The fission yeast dma1 gene is a component of the spindle assembly checkpoint, required to prevent septum formation and premature exit from mitosis if spindle function is compromised. *Embo J*, **15**, 6605-6616.

Murphy, T.D. and Karpen, G.H. (1998) Centromeres Take Flight: Alpha Satellite and the Quest for the Human Centromere. *Cell*, **93**, 317-320.

Nabeshima, K., Kurooka, H., Takeuchi, M., Kinoshita, K., Nakaseko, Y. and Yanagida, M. (1995) p93dis1, which is required for sister chromatid separation, is a novel microtubule and spindle pole body-associating protein phosphorylated at the Cdc2 target sites. *Genes Dev*, **9**, 1572-1585.

Nabeshima, K., Nakagawa, T., Straight, A.F., Murray, A., Chikashige, Y., Yamashita, Y.M., Hiraoka, Y. and Yanagida, M. (1998) Dynamics of centromeres during metaphase-anaphase transition in fission yeast: Dis1 is implicated in force balance in metaphase bipolar spindle. *Molecular Biology Of The Cell*, **9**, 3211-3225.

Nabetani, A., Koujin, T., Tsutsumi, C., Haraguchi, T. and Hiraoka, Y. (2001) A conserved protein, Nuf2, is implicated in connecting the centromere to the spindle during chromosome segregation: a link between the kinetochore function and the spindle checkpoint. *Chromosoma*, **110**, 322-334.

Nakamichi, N., Yamamoto, E., Yamada, H., Aiba, H. and Mizuno, T. (2000) Identification and characterization of a novel gene, hos2+, the function of which is necessary for growth under high osmotic stress in fission yeast. *Biosci Biotechnol Biochem*, **64**, 2493-2496.

Nakaseko, Y., Goshima, G., Morishita, J. and Yanagida, M. (2001) M phase-specific kinetochore proteins in fission yeast: Microtubule-associating Dis1 and Mtc1 display rapid separation and segregation during anaphase. *Current Biology*, **11**, 537-549.

Nakaseko, Y., Nabeshima, K., Kinoshita, K. and Yanagida, M. (1996) Dissection of fission yeast microtubule associating protein p93Dis1: regions implicated in regulated localization and microtubule interaction. *Genes Cells*, **1**, 633-644.

Nakayama, J.-i., Rice, J.C., Strahl, B.D., Allis, C.D. and Grewal, S.I.S. (2001) Role of Histone H3 Lysine 9 Methylation in Epigenetic Control of Heterochromatin Assembly. *Science*, **292**, 110-113.

Niwa, O., Matsumoto, T., Chikashige, Y. and Yanagida, M. (1989) Characterization of Schizosaccharomyces pombe minichromosome deletion derivatives and a functional allocation of their centromere. *Embo J*, **8**, 3045-3052.

Nurse, P. and Bissett, Y. (1981) Gene required in G1 for commitment to cell cycle and in G2 for control of mitosis in fission yeast. *Nature*, **292**, 558-560.

Nurse, P. and Thuriaux, P. (1980) REGULATORY GENES CONTROLLING MITOSIS IN THE FISSION YEAST SCHIZOSACCHAROMYCES POMBE. *Genetics*, **96**, 627-637.

Nurse, P., Thuriaux, P. and Nasmyth, K. (1976) Genetic control of the cell division cycle in the fission yeast Schizosaccharomyces pombe. *Mol Gen Genet*, **146**, 167-178.

Oakley, B.R. and Morris, N.R. (1983) A mutation in Aspergillus nidulans that blocks the transition from interphase to prophase. *J Cell Biol*, **96**, 1155-1158.

Ohkura, H., Adachi, Y., Kinoshita, N., Niwa, O., Toda, T. and Yanagida, M. (1988) Cold-sensitive and caffeine-supersensitive mutants of the Schizosaccharomyces pombe dis genes implicated in sister chromatid separation during mitosis. *Embo J*, **7**, 1465-1473.

Ohkura, H., Hagan, I.M. and Glover, D.M. (1995) The conserved Schizosaccharomyces pombe kinase plo1, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G1 and G2 cells. *Genes Dev*, **9**, 1059-1073.

Ohkura, H., Kinoshita, N., Miyatani, S., Toda, T. and Yanagida, M. (1989) The fission yeast dis2+ gene required for chromosome disjoining encodes one of two putative type 1 protein phosphatases. *Cell*, **57**, 997-1007.

Ookata, K., Hisanaga, S., Bulinski, J., Murofushi, H., Aizawa, H., Itoh, T., Hotani, H., Okumura, E., Tachibana, K. and Kishimoto, T. (1995) Cyclin B interaction with microtubule-associated protein 4 (MAP4) targets p34cdc2 kinase to microtubules and is a potential regulator of M-phase microtubule dynamics. *J. Cell Biol.*, **128**, 849-862.

Ortiz, J., Stemmann, O., Rank, S. and Lechner, J. (1999) A putative protein complex consisting of Ctf19, Mcm21, and Okp1 represents a missing link in the budding yeast kinetochore. *Genes Dev.*, **13**, 1140-1155.

Osmani, S.A., May, G.S. and Morris, N.R. (1987) Regulation of the mRNA levels of nimA, a gene required for the G2-M transition in Aspergillus nidulans. *J Cell Biol*, **104**, 1495-1504.

Osmani, S.A., Pu, R.T. and Morris, N.R. (1988) Mitotic induction and maintenance by overexpression of a G2-specific gene that encodes a potential protein kinase. *Cell*, **53**, 237-244.

Parker, L.L., Walter, S.A., Young, P.G. and Piwnica-Worms, H. (1993) Phosphorylation and inactivation of the mitotic inhibitor Wee1 by the nim1/cdr1 kinase. *Nature*, **363**, 736-738.

Parry, D.H., Hickson, G.R. and O'Farrell, P.H. (2003) Cyclin B destruction triggers changes in kinetochore behavior essential for successful anaphase. *Curr Biol*, **13**, 647-653.

Perez-Mongiovi, D., Beckhelling, C., Chang, P., Ford, C.C. and Houliston, E. (2000) Nuclei and Microtubule Asters Stimulate Maturation/M Phase Promoting Factor (MPF) Activation in Xenopus Eggs and Egg Cytoplasmic Extracts. *J. Cell Biol.*, **150**, 963-974.

Peters, J.M. (2002) The anaphase-promoting complex: proteolysis in mitosis and beyond. *Mol Cell*, **9**, 931-943.

Petersen, J. and Hagan, I.M. (2003) S. pombe Aurora Kinase/Survivin Is Required for Chromosome Condensation and the Spindle Checkpoint Attachment Response. *Current Biology*, **13**, 590-597.

Petersen, J., Paris, J., Willer, M., Philippe, M. and Hagan, I.M. (2001) The S. pombe aurora-related kinase Ark1 associates with mitotic structures in a stage dependent manner and is required for chromosome segregation. *J Cell Sci*, **114**, 4371-4384.

Pfleger, C.M., Lee, E. and Kirschner, M.W. (2001) Substrate recognition by the Cdc20 and Cdh1 components of the anaphase-promoting complex. *Genes Dev.*, **15**, 2396-2407.

Pidoux, A.L., Richardson, W. and Allshire, R.C. (2003) Sim4: a novel fission yeast kinetochore protein required for centromeric silencing and chromosome segregation. *J. Cell Biol.*, **161**, 295-307.

Pinsky, B.A., Tatsutani, S.Y., Collins, K.A. and Biggins, S. (2003) An Mtw1 Complex Promotes Kinetochore Biorientation that Is Monitored by the Ipl1/Aurora Protein Kinase. *Developmental Cell*, **5**, 735-745.

Polizzi, C. and Clarke, L. (1991) The chromatin structure of centromeres from fission yeast: differentiation of the central core that correlates with function. *J. Cell Biol.*, **112**, 191-201.

Prigent, C. and Dimitrov, S. (2003) Phosphorylation of serine 10 in histone H3, what for? *J Cell Sci*, **116**, 3677-3685.

Qian, Y.-W., Erikson, E., Li, C. and Maller, J.L. (1998) Activated Polo-Like Kinase Plx1 Is Required at Multiple Points during Mitosis in Xenopus laevis. *Mol. Cell. Biol.*, **18**, 4262-4271.

Radcliffe, P.A., Vardy, L. and Toda, T. (2000) A conserved small GTP-binding protein Alp41 is essential for the cofactor-dependent biogenesis of microtubules in fission yeast. *FEBS Letters*, **468**, 84-88.

Rajagopalan, S. and Balasubramanian, M.K. (2002) Schizosaccharomyces pombe Bir1p, a Nuclear Protein That Localizes to Kinetochores and the Spindle Midzone, Is Essential for Chromosome Condensation and Spindle Elongation During Mitosis. *Genetics*, **160**, 445-456.

Rajagopalan, S. and Balasubramanian, M.K. (1999) S. pombe Pbh1p: an inhibitor of apoptosis domain containing protein is essential for chromosome segregation. *FEBS Letters*, **460**, 187-190.

Rieder, C., Schultz, A., Cole, R. and Sluder, G. (1994) Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors sister kinetochore attachment to the spindle. *J. Cell Biol.*, **127**, 1301-1310.

Rieder, C.L. and Cole, R. (2000) Microtubule disassembly delays the G2-M transition in vertebrates. *Current Biology*, **10**, 1067-1070.

Rieder, C.L. and Cole, R.W. (1998) Entry into mitosis in vertebrate somatic cells is guarded by a chromosome damage checkpoint that reverses the cell cycle when triggered during early but not late prophase. *The Journal Of Cell Biology*, **142**, 1013-1022.

Rieder, C.L., Cole, R.W., Khodjakov, A. and Sluder, G. (1995) The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. *J Cell Biol*, **130**, 941-948.

Rudert, F., Bronner, S., Garnier, J.M. and Dolle, P. (1995) Transcripts from opposite strands of gamma satellite DNA are differentially expressed during mouse development. *Mamm Genome*, **6**, 76-83.

Russell, P. and Nurse, P. (1987) Negative regulation of mitosis by wee1+, a gene encoding a protein kinase homolog. *Cell*, **49**, 559-567.

Russo, A.A., Jeffrey, P.D. and Pavletich, N.P. (1996) Structural basis of cyclin-dependent kinase activation by phosphorylation. *Nat Struct Biol*, **3**, 696-700.

Sagolla, M.J., Uzawa, S. and Cande, W.Z. (2003) Individual microtubule dynamics contribute to the function of mitotic and cytoplasmic arrays in fission yeast. *J Cell Sci*, **116**, 4891-4903.

Saitoh, S., Takahashi, K. and Yanagida, M. (1997) Mis6, a fission yeast inner centromere protein, acts during G1/S and forms specialized chromatin required for equal segregation. *Cell*, **90**, 131-143.

Saka, Y., Esashi, F., Matsusaka, T., Mochida, S. and Yanagida, M. (1997) Damage and replication checkpoint control in fission yeast is ensured by interactions of Crb2, a protein with BRCT motif, with Cut5 and†Chk1. *Genes Dev.*, **11**, 3387-3400.

Saka, Y., Sutani, T., Yamashita, Y., Saitoh, S., Takeuchi, M., Nakaseko, Y. and Yanagida, M. (1994) Fission yeast cut3 and cut14, members of a ubiquitous protein family, are required for chromosome condensation and segregation in mitosis. *Embo J*, **13**, 4938-4952.

Samuel, J.M., Fournier, N., Simanis, V. and Millar, J.B. (2000) spo12 is a multicopy suppressor of mcs3 that is periodically expressed in fission yeast mitosis. *Mol Gen Genet*, **264**, 306-316.

Sanchez, Y., Wong, C., Thoma, R.S., Richman, R., Wu, Z., Piwnica-Worms, H. and Elledge, S.J. (1997) Conservation of the Chk1 Checkpoint Pathway in Mammals: Linkage of DNA Damage to Cdk Regulation Through Cdc25. *Science*, **277**, 1497-1501.

Sassoon, I., Severin, F.F., Andrews, P.D., Taba, M.-R., Kaplan, K.B., Ashford, A.J., Stark, M.J.R., Sorger, P.K. and Hyman, A.A. (1999) Regulation of Saccharomyces cerevisiae kinetochores by the type 1†phosphatase Glc7p. *Genes Dev.*, **13**, 545-555.

Sato, M., Dhut, S. and Toda, T. (2005) New drug-resistant cassettes for gene disruption and epitope tagging in Schizosaccharomyces pombe. *Yeast*, **22**, 583-591.

Sato, M., Vardy, L., Angel Garcia, M., Koonrugsa, N. and Toda, T. (2004) Interdependency of Fission Yeast Alp14/TOG and Coiled Coil Protein Alp7 in Microtubule Localization and Bipolar Spindle Formation. *Mol. Biol. Cell*, **15**, 1609-1622.

Sawin, K.E., Lourenco, P.C.C. and Snaith, H.A. (2004) Microtubule Nucleation at Non-Spindle Pole Body Microtubule-Organizing Centers Requires Fission Yeast Centrosomin-Related Protein mod20p. *Current Biology*, **14**, 763-775.

Sawin, K.E. and Nurse, P. (1998) Regulation of Cell Polarity by Microtubules in Fission Yeast. *J. Cell Biol.*, **142**, 457-471.

Schwab, M., Lutum, A.S. and Seufert, W. (1997) Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. *Cell*, **90**, 683-693.

Schwab, M., Neutzner, M., Mocker, D. and Seufert, W. (2001) Yeast Hct1 recognizes the mitotic cyclin Clb2 and other substrates of the ubiquitin ligase APC. *Embo J*, **20**, 5165-5175.

Scolnick, D.M. and Halazonetis, T.D. (2000) Chfr defines a mitotic stress checkpoint that delays entry into metaphase. *Nature*, **406**, 430-435.

Severin, F., Habermann, B., Huffaker, T. and Hyman, T. (2001) Stu2 Promotes Mitotic Spindle Elongation in Anaphase. *J. Cell Biol.*, **153**, 435-442.

Shang, C., Hazbun, T.R., Cheeseman, I.M., Aranda, J., Fields, S., Drubin, D.G. and Barnes, G. (2003) Kinetochore Protein Interactions and their Regulation by the Aurora Kinase Ipl1p. *Mol. Biol. Cell*, **14**, 3342-3355.

Shannon, K.B. and Salmon, E.D. (2002) Chromosome Dynamics: New Light on Aurora B Kinase Function. *Current Biology*, **12**, R458-R460.

Shiozaki, K. and Russell, P. (1995) Cell-cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. *Nature*, **378**, 739-743.

Shou, W., Seol, J.H., Shevchenko, A., Baskerville, C., Moazed, D., Chen, Z.W.S., Jang, J., Shevchenko, A., Charbonneau, H. and Deshaies, R.J. (1999) Exit from Mitosis Is Triggered by Tem1-Dependent Release of the Protein Phosphatase Cdc14 from Nucleolar RENT Complex. *Cell*, **97**, 233-244.

Simanis, V. and Nurse, P. (1986) The cell cycle control gene cdc2+ of fission yeast encodes a protein kinase potentially regulated by phosphorylation. *Cell*, **45**, 261-268.

Skibbens, R.V., Corson, L.B., Koshland, D. and Hieter, P. (1999) Ctf7p is essential for sister chromatid cohesion and links mitotic chromosome structure to the DNA replication machinery. *Genes Dev.*, **13**, 307-319.

Skoufias, D.A., Andreassen, P.R., Lacroix, F.B., Wilson, L. and Margolis, R.L. (2001) Mammalian mad2 and bub1/bubR1 recognize distinct spindle-attachment and kinetochore-tension checkpoints. *PNAS*, **98**, 4492-4497.

Smith, D.A., Toone, W.M., Chen, D., Bahler, J., Jones, N., Morgan, B.A. and Quinn, J. (2002) The Srk1 Protein Kinase Is a Target for the Sty1 Stress-activated MAPK in Fission Yeast. *J. Biol. Chem.*, **277**, 33411-33421.

Snell, V. and Nurse, P. (1994) Genetic analysis of cell morphogenesis in fission yeast--a role for casein kinase II in the establishment of polarized growth. *Embo J*, **13**, 2066-2074.

Sorger, P.K., Severin, F.F. and Hyman, A.A. (1994) Factors required for the binding of reassembled yeast kinetochores to microtubules in vitro. *J Cell Biol*, **127**, 995-1008.

Spector, I., Shochet, N.R., Kashman, Y. and Groweiss, A. (1983) Latrunculins: novel marine toxins that disrupt microfilament organization in cultured cells. *Science*, **219**, 493-495.

Steiner, N.C., Hahnenberger, K.M. and Clarke, L. (1993) Centromeres of the fission yeast Schizosaccharomyces pombe are highly variable genetic loci. *Mol Cell Biol*, **13**, 4578-4587.

Strunnikov, A.V. (2003) Condensin and biological role of chromosome condensation. *Prog Cell Cycle Res*, **5**, 361-367.

Sudakin, V., Chan, G.K.T. and Yen, T.J. (2001) Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. *J. Cell Biol.*, **154**, 925-936.

Sutani, T., Yuasa, T., Tomonaga, T., Dohmae, N., Takio, K. and Yanagida, M. (1999) Fission yeast condensin complex: essential roles of non-SMC subunits for condensation and Cdc2 phosphorylation of Cut3/SMC4. *Genes Dev*, **13**, 2271-2283.

Szymanski, D. (2002) Tubulin Folding Cofactors: Half a Dozen for a Dimer. *Current Biology*, **12**, R767-R769.

Takahashi, K., Chen, E.S. and Yanagida, M. (2000) Requirement of Mis6 Centromere Connector for Localizing a CENP-A-Like Protein in Fission Yeast. *Science*, **288**, 2215-2219.

Takahashi, K., Murakami, S., Chikashige, Y., Funabiki, H., Niwa, O. and Yanagida, M. (1992) A low copy number central sequence with strict symmetry and unusual chromatin structure in fission yeast centromere. *Mol Biol Cell*, **3**, 819-835.

Takahashi, K., Yamada, H. and Yanagida, M. (1994) Fission yeast minichromosome loss mutants mis cause lethal aneuploidy and replication abnormality. *Mol Biol Cell*, **5**, 1145-1158.

Tan, Y.S.H., Morcos, P.A. and Cannon, J.F. (2003) Pho85 Phosphorylates the Glc7 Protein Phosphatase Regulator Glc8 in Vivo. *J. Biol. Chem.*, **278**, 147-153.

Tanaka, K., Boddy, M.N., Chen, X.-B., McGowan, C.H. and Russell, P. (2001) Threonine-11, Phosphorylated by Rad3 and ATM In Vitro, Is Required for Activation of Fission Yeast Checkpoint Kinase Cds1. *Mol. Cell. Biol.*, **21**, 3398-3404.

Tanaka, K. and Okayama, H. (2000) A Pcl-like Cyclin Activates the Res2p-Cdc10p Cell Cycle "Start" Transcriptional Factor Complex in Fission Yeast. *Mol. Biol. Cell*, **11**, 2845-2862.

Tanaka, K., Yonekawa, T., Kawasaki, Y., Kai, M., Furuya, K., Iwasaki, M., Murakami, H., Yanagida, M. and Okayama, H. (2000) Fission Yeast Eso1p Is Required for Establishing Sister Chromatid Cohesion during S Phase. *Mol. Cell. Biol.*, **20**, 3459-3469.

Tanaka, T.U., Rachidi, N., Janke, C., Pereira, G., Galova, M., Schiebel, E., Stark, M.J. and Nasmyth, K. (2002) Evidence that the Ipl1-Sli15 (Aurora kinase-INCENP) complex promotes chromosome bi-orientation by altering kinetochore-spindle pole connections. *Cell*, **108**, 317-329.

Tatebayashi, K., Kato, J.-i. and Ikeda, H. (1998) Isolation of a Schizosaccharomyces pombe rad21ts Mutant That Is Aberrant in Chromosome Segregation, Microtubule Function, DNA Repair and Sensitive to Hydroxyurea: Possible Involvement of Rad21 in Ubiquitin-Mediated Proteolysis. *Genetics*, **148**, 49-58.

Tatebe, H., Goshima, G., Takeda, K., Nakagawa, T., Kinoshita, K. and Yanagida, M. (2001) Fission yeast living mitosis visualized by GFP-tagged gene products. *Micron*, **32**, 67-74.

Taylor, S.S. and McKeon, F. (1997) Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. *Cell*, **89**, 727-735.

Terada, Y. (2001) Role of chromosomal passenger complex in chromosome segregation and cytokinesis. *Cell Struct Funct*, **26**, 653-657.

Toda, T., Umesono, K., Hirata, A. and Yanagida, M. (1983) Cold-sensitive nuclear division arrest mutants of the fission yeast Schizosaccharomyces pombe. *J Mol Biol*, **168**, 251-270.

Tomonaga, T., Nagao, K., Kawasaki, Y., Furuya, K., Murakami, A., Morishita, J., Yuasa, T., Sutani, T., Kearsey, S.E., Uhlmann, F., Nasmyth, K. and Yanagida, M. (2000) Characterization of fission yeast cohesin: essential anaphase proteolysis of Rad21 phosphorylated in the S phase. *Genes Dev*, **14**, 2757-2770.

Toth, A., Ciosk, R., Uhlmann, F., Galova, M., Schleiffer, A. and Nasmyth, K. (1999) Yeast cohesin complex requires a conserved protein, Eco1p(Ctf7), to establish cohesion between sister chromatids during DNA replication. *Genes Dev*, **13**, 320-333.

Tournier, S., Gachet, Y., Buck, V., Hyams, J.S. and Millar, J.B.A. (2004) Disruption of Astral Microtubule Contact with the Cell Cortex Activates a Bub1, Bub3, and Mad3-dependent Checkpoint in Fission Yeast. *Mol. Biol. Cell*, **15**, 3345-3356.

Tran, P.T., Marsh, L., Doye, V., Inoue, S. and Chang, F. (2001) A Mechanism for Nuclear Positioning in Fission Yeast Based on Microtubule Pushing. *J. Cell Biol.*, **153**, 397-412.

Trautmann, S., Wolfe, B.A., Jorgensen, P., Tyers, M., Gould, K.L. and McCollum, D. (2001) Fission yeast Clp1p phosphatase regulates G2/M transition and coordination of cytokinesis with cell cycle progression. *Current Biology*, **11**, 931-940.

Troxell, C.L., Sweezy, M.A., West, R.R., Reed, K.D., Carson, B.D., Pidoux, A.L., Cande, W.Z. and McIntosh, J.R. (2001) pkl1+and klp2+: Two Kinesins of the Kar3 Subfamily in Fission Yeast Perform Different Functions in Both Mitosis and Meiosis. *Mol. Biol. Cell*, **12**, 3476-3488.

Tung, H., Wang, W. and Chan, C. (1995) Regulation of chromosome segregation by Glc8p, a structural homolog of mammalian inhibitor 2 that functions as both an activator and an inhibitor of yeast protein phosphatase 1. *Mol. Cell. Biol.*, **15**, 6064-6074.

Uhlmann, F., Lottspeich, F. and Nasmyth, K. (1999) Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1. *Nature*, **400**, 37-42.

Umesono, K., Toda, T., Hayashi, S. and Yanagida, M. (1983) Cell division cycle genes nda2 and nda3 of the fission yeast Schizosaccharomyces pombe control microtubular organization and sensitivity to anti-mitotic benzimidazole compounds. *J Mol Biol*, **168**, 271-284.

Vanoosthuyse, V., Valsdottir, R., Javerzat, J.P. and Hardwick, K.G. (2004) Kinetochore targeting of fission yeast Mad and Bub proteins is essential for spindle checkpoint function but not for all chromosome segregation roles of Bub1p. *Mol Cell Biol*, **24**, 9786-9801.

Venkatram, S., Tasto, J.J., Feoktistova, A., Jennings, J.L., Link, A.J. and Gould, K.L. (2004) Identification and Characterization of Two Novel Proteins Affecting Fission Yeast {gamma}-tubulin Complex Function. *Mol. Biol. Cell*, **15**, 2287-2301.

Verde, F., Dogterom, M., Stelzer, E., Karsenti, E. and Leibler, S. (1992) Control of microtubule dynamics and length by cyclin A- and cyclin B-dependent kinases in Xenopus egg extracts. *J Cell Biol*, **118**, 1097-1108.

Verde, F., Labbe, J.C., Doree, M. and Karsenti, E. (1990) Regulation of microtubule dynamics by cdc2 protein kinase in cell-free extracts of Xenopus eggs. *Nature*, **343**, 233-238.

Verde, F., Mata, J. and Nurse, P. (1995) Fission yeast cell morphogenesis: identification of new genes and analysis of their role during the cell cycle. *J. Cell Biol.*, **131**, 1529-1538.

Visintin, R., Prinz, S. and Amon, A. (1997) CDC20 and CDH1: A Family of Substrate-Specific Activators of APC-Dependent Proteolysis. *Science*, **278**, 460-463.

Volpe, T.A., Kidner, C., Hall, I.M., Teng, G., Grewal, S.I.S. and Martienssen, R.A. (2002) Regulation of Heterochromatic Silencing and Histone H3 Lysine-9 Methylation by RNAi. *Science*, **297**, 1833-1837.

Walworth, N.C. and Bernards, R. (1996) rad-Dependent Response of the chk1-Encoded Protein Kinase at the DNA Damage Checkpoint. *Science*, **271**, 353-356.

Wang, X., McGowan, C.H., Zhao, M., He, L., Downey, J.S., Fearns, C., Wang, Y., Huang, S. and Han, J. (2000) Involvement of the MKK6-p38gamma cascade in gamma-radiation-induced cell cycle arrest. *Molecular And Cellular Biology*, **20**, 4543-4552.

Waters, J.C., Chen, R.-H., Murray, A.W. and Salmon, E.D. (1998) Localization of Mad2 to Kinetochores Depends on Microtubule Attachment, Not Tension. *J. Cell Biol.*, **141**, 1181-1191.

Weiss, E. and Winey, M. (1996) The Saccharomyces cerevisiae spindle pole body duplication gene MPS1 is part of a mitotic checkpoint. *J Cell Biol*, **132**, 111-123.

Wendell, K., Wilson, L. and Jordan, M. (1993) Mitotic block in HeLa cells by vinblastine: ultrastructural changes in kinetochore-microtubule attachment and in centrosomes. *J Cell Sci*, **104**, 261-274.

West, R.R., Malmstrom, T. and McIntosh, J.R. (2002) Kinesins klp5(+) and klp6(+) are required for normal chromosome movement in mitosis. *J Cell Sci*, **115**, 931-940.

West, R.R., Malmstrom, T., Troxell, C.L. and McIntosh, J.R. (2001) Two related kinesins, klp5+ and klp6+, foster microtubule disassembly and are required for meiosis in fission yeast. *Mol Biol Cell*, **12**, 3919-3932.

West, R.R., Vaisberg, E.V., Ding, R., Nurse, P. and McIntosh, J.R. (1998) cut11+: A Gene Required for Cell Cycle-dependent Spindle Pole Body Anchoring in the Nuclear Envelope and Bipolar Spindle Formation in Schizosaccharomyces pombe. *Mol. Biol. Cell*, **9**, 2839-2855.

Westermann, S., Avila-Sakar, A., Wang, H.-W., Niederstrasser, H., Wong, J., Drubin, D.G., Nogales, E. and Barnes, G. (2005) Formation of a Dynamic Kinetochore-Microtubule Interface through Assembly of the Dam1 Ring Complex. *Molecular Cell*, **17**, 277-290.

Wheatley, S.P., Carvalho, A., Vagnarelli, P. and Earnshaw, W.C. (2001) INCENP is required for proper targeting of Survivin to the centromeres and the anaphase spindle during mitosis. *Current Biology*, **11**, 886-890.

Wigge, P.A. and Kilmartin, J.V. (2001) The Ndc80p Complex from Saccharomyces cerevisiae Contains Conserved Centromere Components and Has a Function in Chromosome Segregation. *J. Cell Biol.*, **152**, 349-360.

Wilkinson, C.R., Penney, M., McGurk, G., Wallace, M. and Gordon, C. (1999) The 26S proteasome of the fission yeast Schizosaccharomyces pombe. *Philos Trans R Soc Lond B Biol Sci*, **354**, 1523-1532.

Wilson, S., Warr, N., Taylor, D. and Watts, F. (1999) The role of Schizosaccharomyces pombe Rad32, the Mre11 homologue, and other DNA damage response proteins in non-homologous end joining and telomere length maintenance. *Nucl. Acids Res.*, **27**, 2655-2661.

Winey, M., Mamay, C., O'Toole, E., Mastronarde, D., Giddings, T., Jr, McDonald, K. and McIntosh, J. (1995) Three-dimensional ultrastructural analysis of the Saccharomyces cerevisiae mitotic spindle. *J. Cell Biol.*, **129**, 1601-1615.

Wood, V., Gwilliam, R., Rajandream, M.-A., Lyne, M., Lyne, R., Stewart, A., Sgouros, J., Peat, N., Hayles, J., Baker, S., Basham, D., Bowman, S., Brooks, K., Brown, D., Brown, S., Chillingworth, T., Churcher, C., Collins, M., Connor, R., Cronin, A., Davis, P., Feltwell, T., Fraser, A., Gentles, S., Goble, A., Hamlin, N., Harris, D., Hidalgo, J., Hodgson, G., Holroyd, S., Hornsby, T., Howarth, S., Huckle, E.J., Hunt, S., Jagels, K., James, K., Jones, L., Jones, M., Leather, S., McDonald, S., McLean, J., Mooney, P., Moule, S., Mungall, K., Murphy, L., Niblett, D., Odell, C., Oliver, K., O'Neil, S., Pearson, D., Quail, M.A., Rabbinowitsch, E., Rutherford, K., Rutter, S., Saunders, D., Seeger, K., Sharp, S., Skelton, J., Simmonds, M., Squares, R., Squares, S., Stevens, K., Taylor, K., Taylor, R.G., Tivey, A., Walsh, S., Warren, T., Whitehead, S., Woodward, J., Volckaert, G., Aert, R., Robben, J., Grymonprez, B., Weltjens, I., Vanstreels, E., Rieger, M., Schafer, M., Muller-Auer, S., Gabel, C., Fuchs, M., Fritzc, C., Holzer, E., Moestl, D., Hilbert, H., Borzym, K., Langer, I., Beck, A., Lehrach, H., Reinhardt, R., Pohl, T.M., Eger, P., Zimmermann, W., Wedler, H., Wambutt, R., Purnelle, B., Goffeau, A., Cadieu, E., Dreano, S., Gloux, S., Lelaure, V., Mottier, S., Galibert, F., Aves, S.J., Xiang, Z., Hunt, C., Moore, K., Hurst, S.M., Lucas, M., Rochet, M., Gaillardin, C., Tallada, V.A., Garzon, A., Thode, G., Daga, R.R., Cruzado, L., Jimenez, J., Sanchez, M., del Rey, F., Benito, J., Dominguez, A., Revuelta, J.L., Moreno, S., Armstrong, J., Forsburg, S.L., Cerrutti, L., Lowe, T., McCombie, W.R., Paulsen, I., Potashkin, J., Shpakovski, G.V., Ussery, D., Barrell, B.G. and Nurse, P. (2002) The genome sequence of Schizosaccharomyces pombe. *Nature*, **415**, 871-880.

Wordeman, L. and Mitchison, T. (1995) Identification and partial characterization of mitotic centromere- associated kinesin, a kinesin-related protein that associates with centromeres during mitosis. *J. Cell Biol.*, **128**, 95-104.

Wu, L. and Russell, P. (1993) Nim1 kinase promotes mitosis by inactivating Wee1 tyrosine kinase. *Nature*, **363**, 738-741.

Yamada, H., Matsumoto, S. and Matsumoto, T. (2000) High dosage expression of a zinc finger protein, Grt1, suppresses a mutant of fission yeast slp1(+), a homolog of CDC20/p55CDC/Fizzy. *J Cell Sci*, **113**, 3989-3999.

Yamaguchi, S., Decottignies, A. and Nurse, P. (2003) Function of Cdc2p-dependent Bub1p phosphorylation and Bub1p kinase activity in the mitotic and meiotic spindle checkpoint. *Embo J*, **22**, 1075-1087.

Yamaguchi, S., Murakami, H. and Okayama, H. (1997) A WD Repeat Protein Controls the Cell Cycle and Differentiation by Negatively Regulating Cdc2/B-Type Cyclin Complexes. *Mol. Biol. Cell*, **8**, 2475-2486.

Yamano, H., Gannon, J. and Hunt, T. (1996) The role of proteolysis in cell cycle progression in Schizosaccharomyces pombe. *Embo J*, **15**, 5268-5279.

Yamano, H., Tsurumi, C., Gannon, J. and Hunt, T. (1998) The role of the destruction box and its neighbouring lysine residues in cyclin B for anaphase ubiquitin-dependent proteolysis in fission yeast: defining the D-box receptor. *Embo J*, **17**, 5670-5678.

Yanagida, M., Yamashita, Y.M., Tatebe, H., Ishii, K., Kumada, K. and Nakaseko, Y. (1999) Control of metaphase-anaphase progression by proteolysis: cyclosome function regulated by the protein kinase A pathway, ubiquitination and localization. *Philos Trans R Soc Lond B Biol Sci*, **354**, 1559-1569; discussion 1569-1570.

Yoon, H.-J., Feoktistova, A., Wolfe, B.A., Jennings, J.L., Link, A.J. and Gould, K.L. (2002) Proteomics Analysis Identifies New Components of the Fission and Budding Yeast Anaphase-Promoting Complexes. *Current Biology*, **12**, 2048-2054.

Zeng, Y., Forbes, K.C., Wu, Z., Moreno, S., Piwnica-Worms, H. and Enoch, T. (1998) Replication checkpoint requires phosphorylation of the phosphatase Cdc25 by Cds1 or Chk1. *Nature*, **395**, 507-510.

Zeng, Y. and Piwnica-Worms, H. (1999) DNA Damage and Replication Checkpoints in Fission Yeast Require Nuclear Exclusion of the Cdc25 Phosphatase via 14-3-3 Binding. *Mol. Cell. Biol.*, **19**, 7410-7419.

Zimmerman, S., Daga, R.R. and Chang, F. (2004) Intra-nuclear microtubules and a mitotic spindle orientation checkpoint. **6**, 1245-1246.

Appendix - Publication