Roles for Mph1 kinase in the spindle checkpoint and chromosome segregation

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This thesis is presented for the degree of Doctor of Philosophy at the University of Edinburgh

DECLARATION OF AUTHENTICITY

I declare that this thesis was composed by myself ant that the research presented within is my own work, except where explicitly stated and acknowledgement is given.

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ABSTRACT

The spindle checkpoint monitors the metaphase-to-anaphase transition to ensure accurate segregation of sister chromatids during mitosis to prevent aneuploidy. Mph1, the fission yeast homologue of Mps1 is one of the kinases involved in the spindle checkpoint. Deletion of Mph1 results in cells with a compromised spindle checkpoint. They do not arrest in response to spindle damage by drugs such as benomyl or mutations such as the cold sensitive tubulin mutant *nda3*. Instead they attempt to continue through anaphase with the result being cells showing *cut* phenotypes and ultimately cell death due to chromosome loss.

Mph1 becomes phosphorylated during mitosis in an unperturbed mitosis and becomes hyperphosphorylated during spindle damage. The exact nature of this phosphorylation is unclear as no candidate kinase has been found that may be responsible for this modification, although autophosphorylation may play a role.

In addition to a role in the spindle checkpoint Mph1, (as well as Bub1, another spindle checkpoint kinase,) has a role in chromosome segregation. The Mph1 and Bub1 kinases appear to be the only spindle checkpoint components involved in chromosome segregation. In a deletion mutant a high percentage of lagging chromosomes and chromosome loss is observed, much the same as in a Bub1 deletion. When a double deletion of Mph1 and Bub1 is made an additive effect is seen with respect to chromosome segregation. The percentage of lagging chromosomes and chromosome loss almost doubles with respect to the single mutants. This suggests that these two kinases have distinct target required for effective chromosome segregation.

ABBREVIATIONS

Amp	Ampicillin
APC/C	Anaphase promoting complex/cyclosome
Bp	Base pairs
Bub	Budding uninhibited by benzimidazole
Cdc	Cell division cycle (mutant)
Cdk	Cyclin dependent kinase
CEN	Centromere
CENP	Centromere protein
Cnp	Centromere protein
Cut	Cell untimely torn
dH_2O	Distilled water
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra acetic acid
ECL	Enhanced chemiluminescence
EGTA	1,2,-Di (2-animoethoxy) ethane N,N,N,N'-tetra acetic acid
GFP	Green fluorescent protein
GST	Glutathione S-transferase
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
IF	Immunoflurescence
IP	Immunoprecipitation
Kt	Kinetochore
Mad	Mitotic arrest deficient
MT	Microtubule
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
PIPES	2-[4-(2-sulfoethyl)piperazin-1-yl]ethanesulfonic acid

rpm	Revolutions per minute
Slp	Sleepy
SPB	Spindle pole body
TEMED	N,N,N',N'-Tetramethylethylenediamine

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Chapter 1

Introduction

1.1 The Cell Cycle

For new cells to arise existing cells must divide. Cell division is very important for the growth and development of multicellular organisms. The cell cycle is a series of events that lead up to the division of the cell and is a highly regulated process. The cell cycle is divided into four main stages; G1, S-phase, G2 and mitosis (Figure 1.1). These stages vary in length between different organisms. G1 and G2 are the gap phases between S-phase and mitosis. During the cell cycle all the components of the cell must be replicated, for example cytoplasm, membranes and RNA and as such the cell size increases during the cell cycle. Chromosomes must also be duplicated and this happens during S-phase. Each chromosome must be duplicated to ensure that each daughter cell receives identical copies from the mother cell. The chromosomes must then be segregated to ensure that each daughter cell receives one copy of each chromosome. This segregation of chromosomes into the daughter cells must be carefully regulated to prevent the loss of genetic material from the daughter cells resulting in aneuploidy.

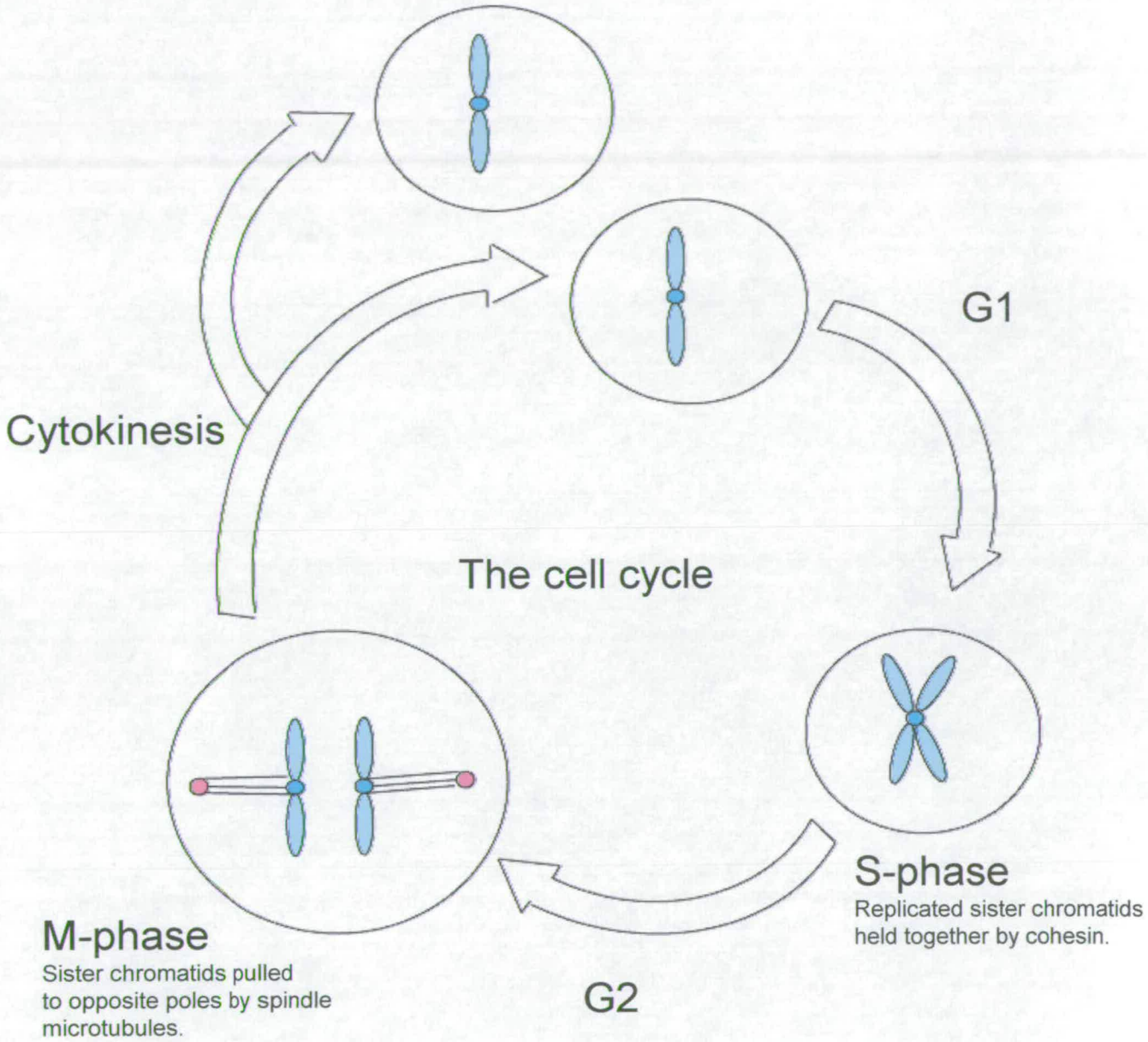


Figure 1.1: The cell cycle
The cell cycle consists of four phases: S-phase (DNA replication) and M-phase (mitosis) as well as two gap phases G1 and G2.

1.2 Cell Cycle Control

The cell cycle is driven by the activity of cyclin-dependent kinases (CDKs – there is only one CDK in *Schizosaccharomyces pombe*, Cdc2). CDKs must be bound to their activating molecules, cyclins, for them to be activated. Cyclins were discovered to be factors involved in cell cycle control as the levels fluctuated through the cell cycle. Cyclin-CDK activities rise and fall during the cell cycle and cause cyclical changes in the phosphorylation state of certain cell cycle components drive the cell through the specific phases of the cell cycle.

Phosphorylation also plays an important role in the control of CDK activity (figure 1.2). They are regulated by changes in phosphorylation state of specific tyrosine and threonine residues in the catalytic site of the kinase. In fission yeast the phosphorylation of Cdc2 is controlled by the Cdc25 phosphatase and the Wee1 kinase. These work to oppose each other by either inhibiting or activating the cyclin-CDK complex to allow entry into mitosis. Wee1 phosphorylates Cdc2 inactivating it to prevent entry into mitosis while Cdc25 dephosphorylates Cdc2 to allow irreversible entry into mitosis.

Different cyclins associate with CDKs depending on the phase of the cell cycle. Reviewed in (Nurse, 2000), (Murray, 2004) (Morgan, 2007)

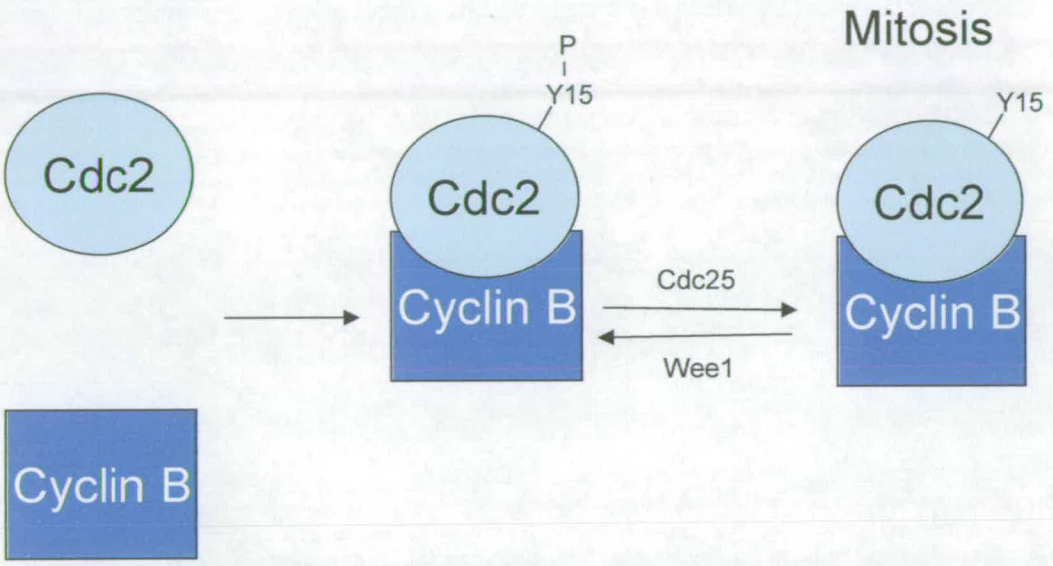


Figure 1.2: Cyclin-CDK regulation
The association of a cyclin with a CDK (Cdc2) is required for CDK activation. Two opposing enzymes control the activity of Cdc2 allowing entry into mitosis. These are the Cdc25 phosphatase and the Wee1 kinase. Dephosphorylation of tyrosine 15 by Cdc25 is required for entry in mitosis.

1.3 Fission Yeast as a Model Organism

The work detailed in this study was carried out in the fission yeast *Schizosaccharomyces pombe* which is an ideal model organism due to the ease at which genetic analysis can be carried out. They can be maintained as haploid organisms meaning that mutations can be made in a gene without the complication of a second copy of the gene being present allowing the study of recessive mutations. *S.pombe* has a relatively small genome that has been sequenced and they also control cell cycle in much the same way as higher eukaryotes. They also have a high rate of homologous recombination allowing mutations and deletions to be made using targeted recombination strategies and this can also be used to insert genes into the genome at specific sites. It is also possible to express genes in *S.pombe* under the control of regulatable promoters allowing the gene of interested to be switched off or up regulated at specific points in the cell cycle.

Another factor which makes fission yeast an attractive model organism is that it can proliferate rapidly in culture with a doubling time of around two to two and a half hours.

This is reviewed in (Morgan, 2007)

1.4 Mitosis

When a cell divides each daughter cell must receive an exact complement of the parental chromosomes. In order to ensure this, chromosomes must be segregated accurately and this happens during mitosis. To prevent loss of genetic material during this process which can be error prone, strict controls must be in place.

Mitosis occurs in several stages; prophase, when chromosomes condense; prometaphase, when the chromosomes become attached to the mitotic spindle;

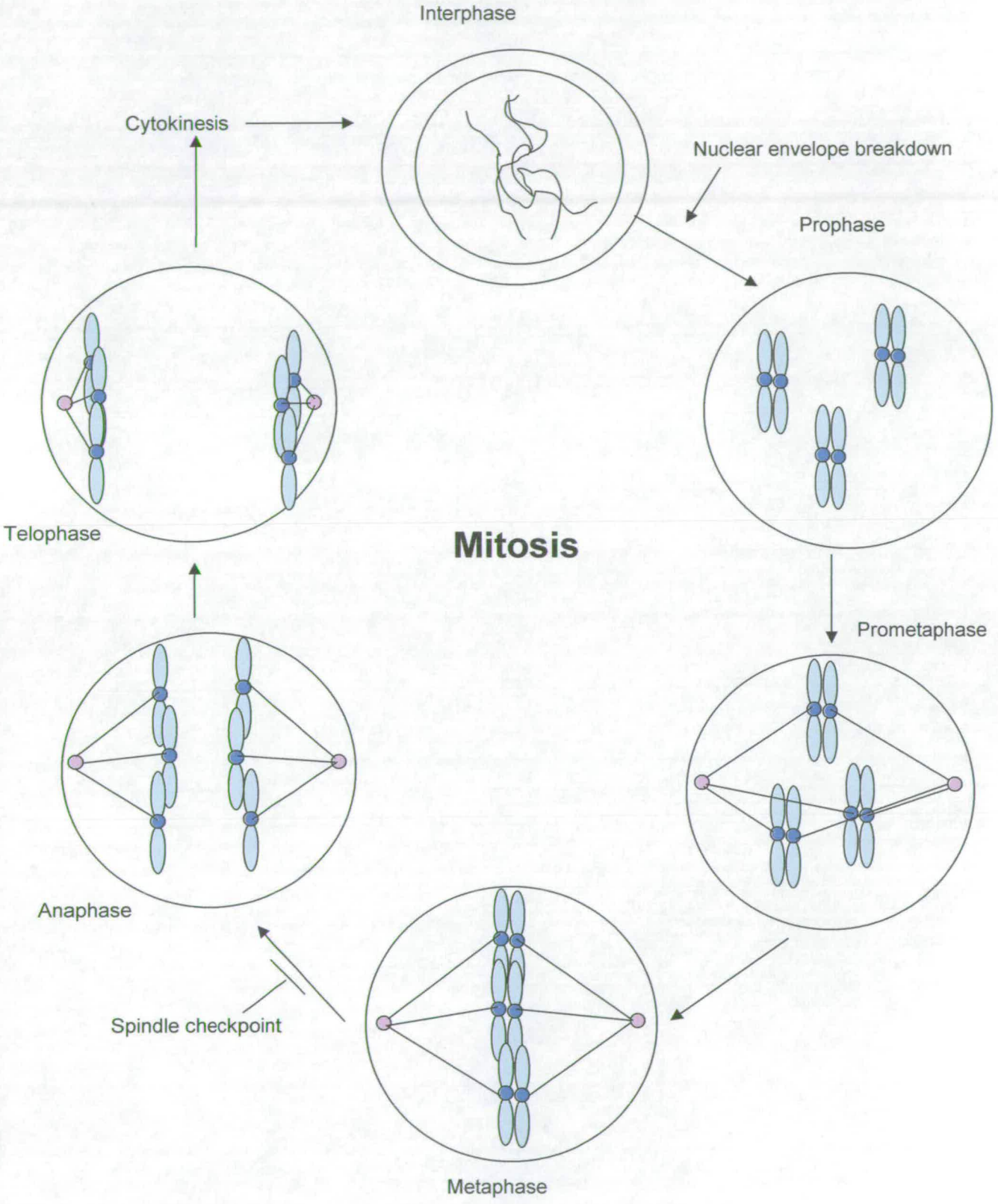
metaphase, when sister chromatids are aligned along the metaphase plate; anaphase, when the sister chromatids are separated to opposite poles and telophase when the segregated chromosomes and other nuclear components are packaged into the daughter nuclei (figure 1.3) This is not the case in yeast as they undergo a closed mitosis so the nuclear envelope never breaks down. The cell can then undergo cytokinesis.

1.4.1 Events in early mitosis

1.4.1.1 Chromosome cohesion

As cells progress into mitosis certain event must occur before they reach S-phase. These events are important to prevent the loss of genetic material during mitosis. Cytological studies identified the fact that during early mitosis the chromosomes appeared to condense and that the sister chromatids were held together until anaphase. The sister chromatids must be held together and properly bioriented during early mitosis to ensure correct attachment to the mitotic spindle. This ensures that each of the sisters is pulled to opposite poles during anaphase, ensuring that the resulting daughter cells receive the correct compliment of chromosomes. In order to achieve this, the replicating strands of DNA are held together during S-phase to prevent premature sister separation before anaphase onset (Watanabe, 2005) (Nasmyth, 2005), Chromosome cohesion is carried out by a protein complex that contains members of the structural maintenance of chromosomes (SMC) family of ATPases (Hagstrom and Meyer, 2003).

Figure 1.3: Mitosis
Mitosis is divided into several stages: prophase, when the chromosomes condense and individual sister chromatids can be seen; prometaphase, when the chromatids attach to the mitotic spindle; metaphase, when the chromatids align on the metaphase plate; anaphase, when the sister chromatids are separated to opposite poles and telophase, when the Segregated chromosomes are packaged in to daughter nuclei.
Interphase is what the cell is in before mitosis. The nuclear envelope breaks down as cells enter mitosis, however, this does not happen in yeast as they undergo a closed mitosis.


Interphase
Cytokinesis
Nuclear envelope breakdown
Prophase
Telophase
Mitosis
Prometaphase
Anaphase
Spindle checkpoint
Metaphase

The cohesin complex is composed of three subunits, two SMC subunits SMC1 and SMC3 (Psm1 and Psm3 in *S.pombe*) and a non-SMC subunit, Scc1 (Rad21 in *S.pombe*) (Nasmyth, 2005), (Tomonaga et al., 2000). The two SMC subunits are coiled coil proteins that each has a globular head domain at one end. The tails of the SMC molecules interact at one end of the complex and the head domains of these protein interact with Scc1 which acts as a linker between them forming a hinge-like structure (figure 1.4) (Lengronne et al., 2006). ATP molecules bind between the head domains of SMC1 and SMC3 and this promotes the association with Scc1 (Nasmyth, 2002). The cohesin complex is thought to form a ring like structure that encircles the DNA, holding the sisters together (Gruber et al., 2003).

Studies in budding yeast have shown cohesin to be loaded on to DNA during late G1 by a complex comprising of two more Scc proteins, Scc2 and Scc4. During S-phase the replicating DNA then passes through the cohesin ring resulting in the sister chromatids are held together as soon as the DNA is replicated (Lengronne et al., 2006). When cohesin is loaded on to DNA it is established along the chromosome arms and at the centromeres at Scc2/Scc4 binding sites (Watanabe, 2005). It is thought that pericentric heterochromatin also plays a role in cohesin recruitment as Swi6 (*S.pombe* homologue of the heterochromatin associating protein HP1) has been found to interact with SMC3 in yeast 2-hybrid studies (Bernard et al., 2001), (Nonaka et al., 2002). As cells reach the metaphase the cohesin at the arms has been removed and only the centromeric cohesion has to be removed to allow anaphase to occur.

Chromosome cohesion is an important factor in preventing loss of genetic material during anaphase. Chromatid cohesion may also be important for biorienting the sister chromatids to ensure that each kinetochore can be captured by microtubules coming from opposite poles.

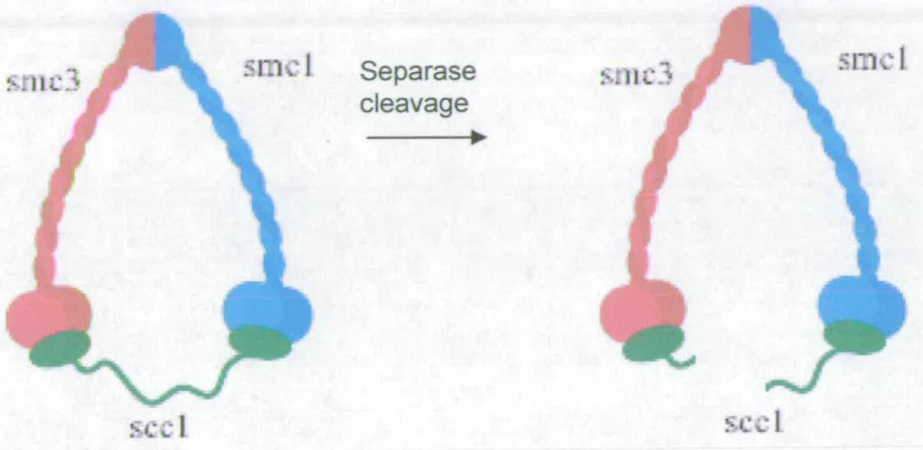
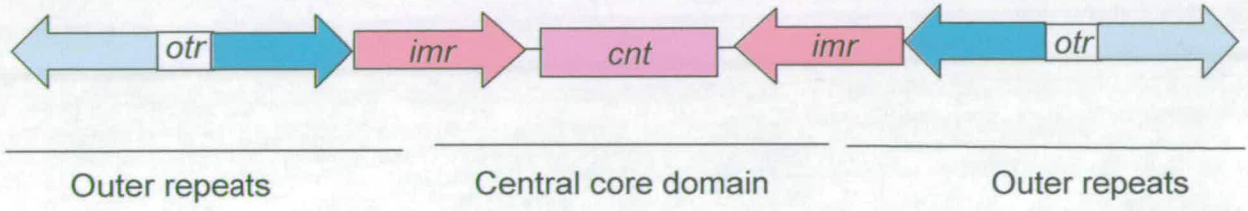


Figure 1.4: Structure of the cohesin complex
SMC1 and SMC3 ATPases are coiled coil proteins that interact with each other at on end and with Scc1 at the other end. The complex is thought to form a ring complex that encircles the sister chromatids holding them together until anaphase where Scc1 must be cleaved to allow anaphase movement of the chromosomes.
Diagram adapted from (Nasmyth 2005).

1.4.1.2 Centromeres and kinetochores

The centromere is the site on the chromosome where the kinetochore is established and where the chromosome becomes attached to spindle microtubules during mitosis. At this site the chromosomes also remain cohesed until anaphase onset. It was originally identified cytologically as the primary constriction (Vos et al., 2006). Centromeric regions contain a specialised type of chromatin called heterochromatin where the DNA is tightly packaged into nucleosomes. Genes present in heterochromatic regions are not expressed. This means that genes inserted into regions of heterochromatin become transcriptionally silenced (Allshire et al., 1995). Centromeres differ between different organisms; there is not a conserved sequence between organisms. Human and mouse centromeres are arranged as long arrays of satellite repeat DNA (alpha satellite repeats in human cells) (Cleveland et al., 2003), whereas the budding yeast centromeres are only 125bp (Cottarel et al., 1989). The centromeres of most organisms are made up of repeated sequences with budding yeast being the exception. Apart from budding yeast DNA sequence or length of the sequence is not important for establishing what defines a centromere and it is more likely that epigenetic regulation is important for defining centromeric regions (Karpen and Allshire, 1997, Sullivan et al., 2001). There are preferred sequences where centromeres are usually found, such as the alpha satellite repeats in human cells but these appear to be neither necessary, nor sufficient for establishing the site of a centromere. Insight into this has come from the study of a few special cases where dicentric chromosomes or neocentromere formation has arisen. A dicentric chromosome is one where there are two regions of centromeric DNA (alpha satellite repeats) but what was interesting when these were studied was that only one bound centromeric proteins. This means that only one site is active as a centromere.(Karpen and Allshire, 1997). The case of neocentromere formation has also been reported, where a centromere has arisen on a site that does not contain α-repeated elements (Karpen and Allshire, 1997).

A feature that is common among centromeres is that they all contain the histone H3 variant CENP A. The centromeres of the three chromosomes of *S.pombe* range from 40-120kb and are arranged in a similar manner (Karpen and Allshire, 1997) and (Cleveland et al., 2003), (Pidoux and Allshire, 2004). They are made up of three



Outer repeats | Central core domain | Outer repeats

Heterochromatin
H3K9 methylation
Clr4
Rik1
Swi6
cohesin

Unusual chromatin structure
Cnp1
Mis6, Mis12

Kinetochore complexes-
Ndc80
DASH

Figure 1.5: Fission yeast kinetochore structure
The centromeres of the chromosomes of *S.pombe* are arranged into two domains, the central core core domain flanked by the inverted repeats regions the inner most repeats (imr) and the outer repeats (otr). These domains differ with respect to the proteins that associate with them.

and Allshire, 2004) (figure 1.5). The central core DNA is non-repetitive and AT rich. The kinetochore is assembled on the region comprising of the central core and the *imr* regions. The *otr* regions around this are heterochromatic (Toyoda et al., 2002). The central core region is packaged into a unique chromatin structure as seen by the results of micrococcal nuclease digestion. Whereas most of the chromatin in a cell shows a ladder like pattern on a gel after micrococcal nuclease digestion, DNA from the central core region results in a smear on the gel (Polizzi and Clarke, 1991) and (Takahashi et al., 1992). The nucleosomes at the central core differ from those of euchromatic DNA as the central core region (*cnt* and *imr*) contain CENP-A (Cnp1 in fission yeast), a histone H3 variant, rather than histone H3 itself (Takahashi et al., 2000)and (Pidoux and Allshire, 2005).

The histone tails are subject to different modification, they can be acetylated, phosphorylated and methylated. These different types of modifications are important for determining whether the DNA is expressed or not in that region. The histone modifications help to define chromatin domains. The heterochromatic region of the *otr* tends to be hypoacetylated and methylation tends to play a role in heterochromatin formation (Ekwall et al., 1997), (Mellone et al., 2003). Methylation of histone H3 on lysine 9 (H3K9 methylation) is found in this region but not in regions of expressed DNA where it is usually histone H3 lysine 4 that is methylated (Hall et al., 2002). This methylation is caused by the actions of Clr4, a histone methyltransferase and the fission yeast homologue of Suvar39 (for suppressor of variegation as mutants were shown to alleviate silencing at the centromere in *Drosophila*)(Partridge et al., 2000). This modification allows Swi6, the fission yeast homologue of HP1, a chromodomain protein, to bind to these repeat regions (Ekwall et al., 1995). This binding also requires the presence of another protein Rik1 (Ekwall et al., 1996). Mutants of *swi6, clr4* and *rik1* show alleviation of silencing at the outer repeat regions of the fission yeast centromeres, shown by the expression of marker genes inserted into these regions. When the *swi6, clr4* and *rik1* genes are functional the marker genes are not expressed but this is not the case in *swi6, clr4* and *rik1* mutants (Allshire et al., 1995). Mutations in these genes also result in chromosome loss (Allshire et al., 1995). Mutants of the central core proteins Mis6 and Cnp1 also result in chromosome loss and alleviate silencing from the central core domain but not from the outer repeats.

The kinetochore is the site where microtubules attach during mitosis. They are proteinacious structures which assemble on the centromeric regions of chromosomes. Kinetochores are the sites of microtubules attachment during mitosis and as such are very important structures for maintaining genomic integrity into the next cell cycle. In terms of assembly and activity of kinetochores this is not conserved between organisms. The budding yeast kinetochore is attached to the spindle microtubules for most of the cell cycle and most of the components that make up the budding yeast kinetochore are essential indicating that the kinetochore is required throughout the cell cycle (Amor et al., 2004) and (McAinsh et al., 2003). This is not the case with vertebrate kinetochores which do not begin to assemble until late G2/S-phase, as seen in mammalian cells extracts and are seen as a mature laminar structure once the nuclear envelope has broken down which happens before mitosis takes place (nuclear envelope breakdown does not occur in fission yeast as yeast undergo a closed mitosis. (Maiato and Sunkel, 2004). Fission yeast kinetochores assemble on the central core region of the centromere and are present during interphase as they appear to be attached to SPBs during interphase.

A feature common to most kinetochores is that they are composed of constitutive and transiently associated proteins. In fission yeast the constitutive kinetochore components are bound to chromosomes throughout the cell cycle but the transient components only associate during mitosis (Liu et al., 2005). Constitutive components of the kinetochore include CENP-A (Cnp1), the centromere specific histone H3 variant, which is a found in the nucleosomes at this region, as described previously. Other constitutive components are Mis6 (homologue of human CENP-I) and Mis12 (homologue of budding yeast Mtw1), which were found in minichromosome instability screens along with Mal2 (Pidoux and Allshire, 2004), (Fleig et al., 1996), (Takahashi et al., 1994). Sim4 is another protein found constitutively at the kinetochore. It was found in a screen for fission yeast mutants which relieved silencing at the central core, hence silencing in the middle (Sim) and may form a complex with Mis6 as they have been shown to co-IP(Pidoux et al., 2003). The more transient components of the kinetochore tend to be those that interact with microtubules. Several complexes make up the kinetochore and many of these are conserved in budding yeast and fission yeast. These are the Ndc80, DASH and MIND complexes and Spc7 (Liu et al., 2005) and (Kerres et al., 2007). The Ndc80

complex comprising of Ndc80, Nuf2, Spc24 and Spc25, is conserved in higher eukaryotes but components of the DASH complex, such as Ask1 have not yet been found in higher eukaryotes (Li et al., 2002). The DASH complex was first identified in budding yeast and then homologues were identified in fission yeast (Liu et al., 2005). It is composed of the components Ask1, Dam1, Spc34, Duo1, Spc19, Dad2, Dad1, Dad5, Dad4 and Hsk3 and it is an essential complex in budding yeast, however this is not the case in fission yeast (Salmon, 2005). Although the DASH complex is a transient complex that only associates with the kinetochore during mitosis in fission yeast, one component, Dad1, was found to be constitutively associated with it(Liu et al., 2005).

1.4.1.3 Kinetochore-microtubule interactions

Microtubules are polymers of repeating α/β-tubulin heterodimers. Microtubules display dynamic instability as they switch between growth (rescue) and shrinkage (catastrophe) (Desai and Mitchison 1997). Spindle microtubules are nucleated from spindle pole bodies (SPBs, centrosomes in higher eukaryotes) with the minus ends found at the SPBs and the plus ends towards the kinetochores (Jasperson and Winey 2004).

The kinetochore is the site of microtubule attachment. Budding yeast kinetochores only make one connection with a microtubule whereas most other organisms make several, for example fission yeast kinetochores were found to have between 2 and 4 microtubule binding sites when studied by electron microscopy

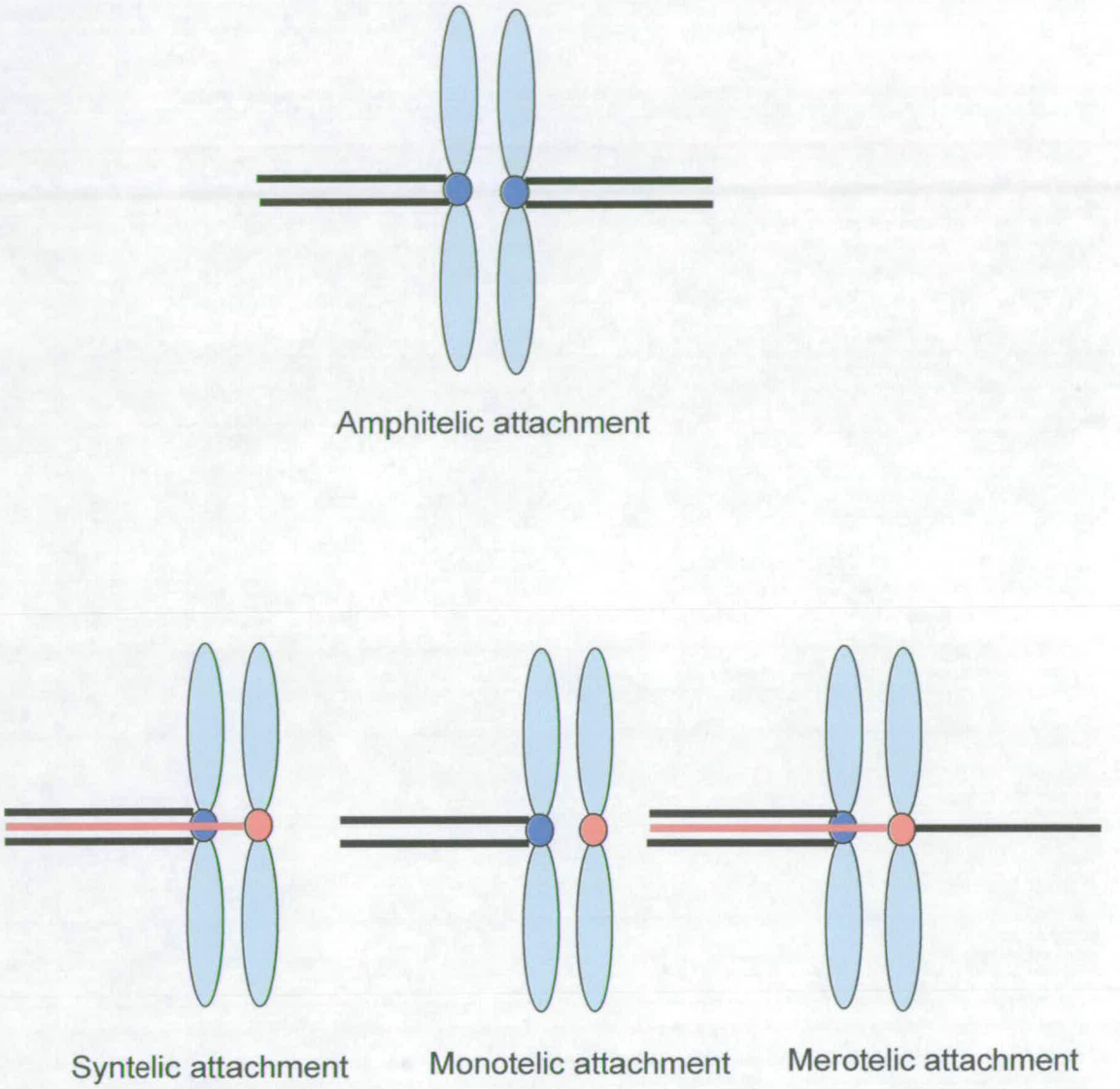


Figure 1.6: Types of attachment
Amphitelic is the correct, bipolar attachment required before anaphase onset to prevent chromosome loss. Errors in bipolar attachment occur resulting in several types of attachment defect. These are: syntelic attachment, where both sister chromatids are attached to microtubules from the same pole; monotelic attachment, where only one sister kinetochore is attached to spindle microtubules; and merotelic attachment, where one sister is attached to microtubules from both poles.

(Ding et al., 1993). For accurate chromosome segregation to occur the sister chromatids have to be properly bioriented and attached to microtubules coming from opposite poles. This is known as amphitelic attachment (figure 1.6) (Pidoux and Allshire, 2003), (Tanaka, 2005). When problems occur at the kinetochores attachment defects can occur. For example, both sister chromatids can become attached to microtubules coming from the same spindle pole, syntelic attachment, or only one sister can be properly attached, monotelic attachment (figure 1.6) (Pidoux and Allshire, 2003), (Tanaka, 2005). These faulty attachments can be recognised by the spindle checkpoint which delays anaphase onset to allow the defect to be corrected (discussed later). However there is one type of attachment defect that is not recognised by the spindle checkpoint and that is merotelic attachment. This is where one sister chromatid is attached to microtubules coming from both poles (Cimini et al., 2001), (Salmon et al., 2005). This type of defect is not seen in budding yeast as they only have one microtubule binding site per kinetochore and this defect is not recognised by the spindle checkpoint as there is both tension and attachment at the kinetochore (both requirements to prevent spindle checkpoint activation, discussed later). In order to prevent attachment problems the sister chromatids must be bioriented to ensure that the kinetochores face the correct poles (reviewed in (Pidoux and Allshire, 2003) and (Kotwaliwale and Biggins, 2006)).
Transient kinetochore components play a role in attaching chromosomes to microtubules. How the chromosomes then move towards the poles during anaphase differs between species. In higher eukaryotes microtubule motors play a role in moving the chromosomes to the spindle poles but in fission yeast it seems that microtubule depolymerisation is a more important force (Grishchuk and McIntosh 2006).

The exact way in which microtubules attach to the kinetochores of chromosomes is currently poorly understood although recent evidence from vertebrate cells (PtK1 cells) has suggested that Ndc80 might be important for this attachment (DeLuca et al., 2006). Depletion of Ndc80 lead to unstable kinetochore-microtubule attachments, however these attachments were not abolished altogether, so Ndc80 cannot be the only factor responsible for kinetochore-microtubule attachments (DeLuca et al., 2006). Work in *C.elegans* identified the KMN network which is important for kinetochore attachment to microtubules (Cheeseman et al., 2006). This network is

composed of KNL1, Mis12 and Ndc80 (Cheeseman et al., 2006). Mis12 did not appear to bind directly to microtubules but the other two did but with weak interactions that were strengthened when the whole complex was present. It has been shown in human cells that Mis12 is required for the localisation of Ndc80 (Kline et al., 2006). It was shown to be the Ndc80 and Nuf2 components of the Ndc80 complex that had the microtubule binding properties in *C.elegans,* human cells and PtK cells (Cheeseman et al., 2006)

The DASH complex, sometimes also called the Dam1 ring complex, binds to microtubules. It is thought to form rings around the microtubules and is linked to the kinetochore through binding to the Ndc80 complex (Salmon, 2005) (figure 1.7A). Other important factors in tethering microtubule plus ends to the kinetochore are microtubule associated proteins (MAPs) and motor proteins. In fission yeast these include Alp14 and Dis1, both members of the Dis1/Tog family of MAPs. These two proteins may form a bridge between the kinetochore and the plus ends of spindle microtubules and are thought to stabilise microtubules (Garcia et al., 2002). Klp5 and Klp6 share a similar function to Alp14 and Dis1 in fission yeast cells in helping to connect kinetochores and spindle microtubules. They are members of the Kin1 family of kinesins and form a heterodimer in cells. Although kinesins are motor proteins it is thought that Klp5 and Klp6 promote microtubule disassembly in order to drive chromosomes towards the poles (Garcia et al., 2002). It is thought that microtubule depolymerisation is a major force in driving chromosome separation at anaphase in fission yeast, unlike in other organisms where minus end directed motors such as dynein are thought to be important (Grishchuk and McIntosh 2006). Other factors also thought to be important for chromosome movement include EB1, which increases the dynamic instability of microtubules and CLIP-170 (both of which are plus end tracking proteins) (Schuyler and Pellman, 2001) and CENP-E (a vertebrate plus end directed kinesin).

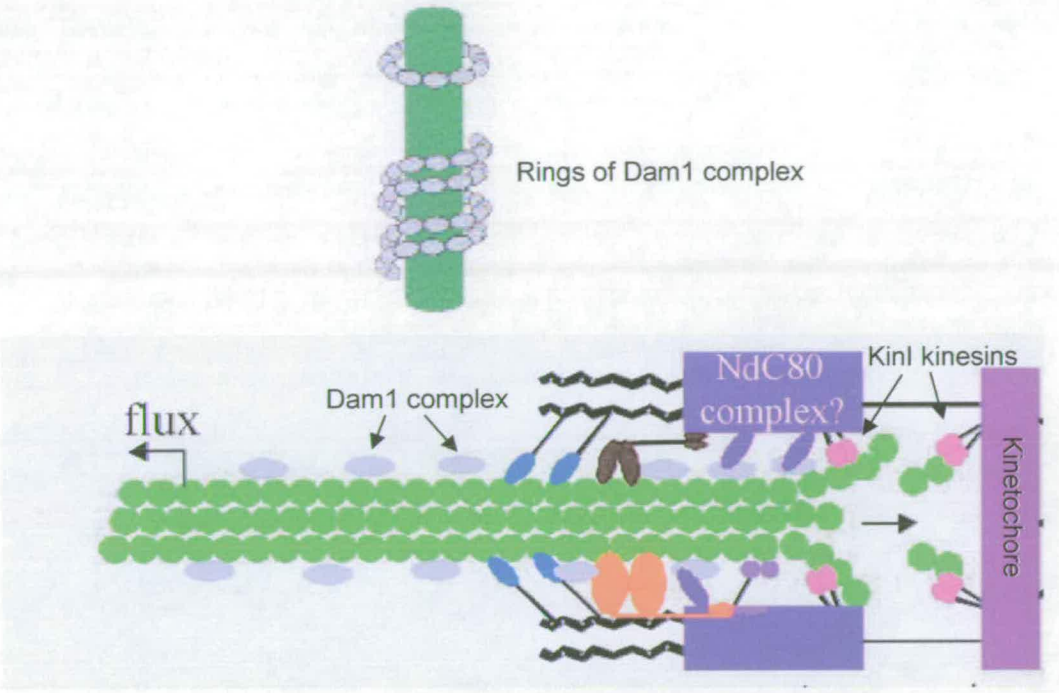
In terms of correct microtubule attachment the chromosomal passenger proteins are thought to play an important role (Maiato et al., 2004). Aurora B (fission yeast have only one Aurora protein called Ark1) promotes the dissociation of microtubules and kinetochores in response to inaccurate attachment. Aurora is part of the chromosomal passenger complex, first discovered in vertebrate cells (Earnshaw and Bernat, 1991) and found to be composed of Aurora B, Incenp, Survivin and

Figure 1.7: Kinetochore complexes and microtubule attachments

A. The Dam1 ring complex (DASH complex) is found in yeast and forms rings around the microtubules and is connected to the kinetochore through binding to the Ndc80 complex.

B. The chromosomal passenger proteins Survivin (Bir1- shown in blue) and INCENP (Sli15 – shown in pink) are thought
to be involved in sensing tension at the kinetochore. In response to weak tension they activate the Aurora B kinase (Ipl1) which phosphorylates certain kinetochore components (eg. Ndc80) and breaks these incorrect attachments

A



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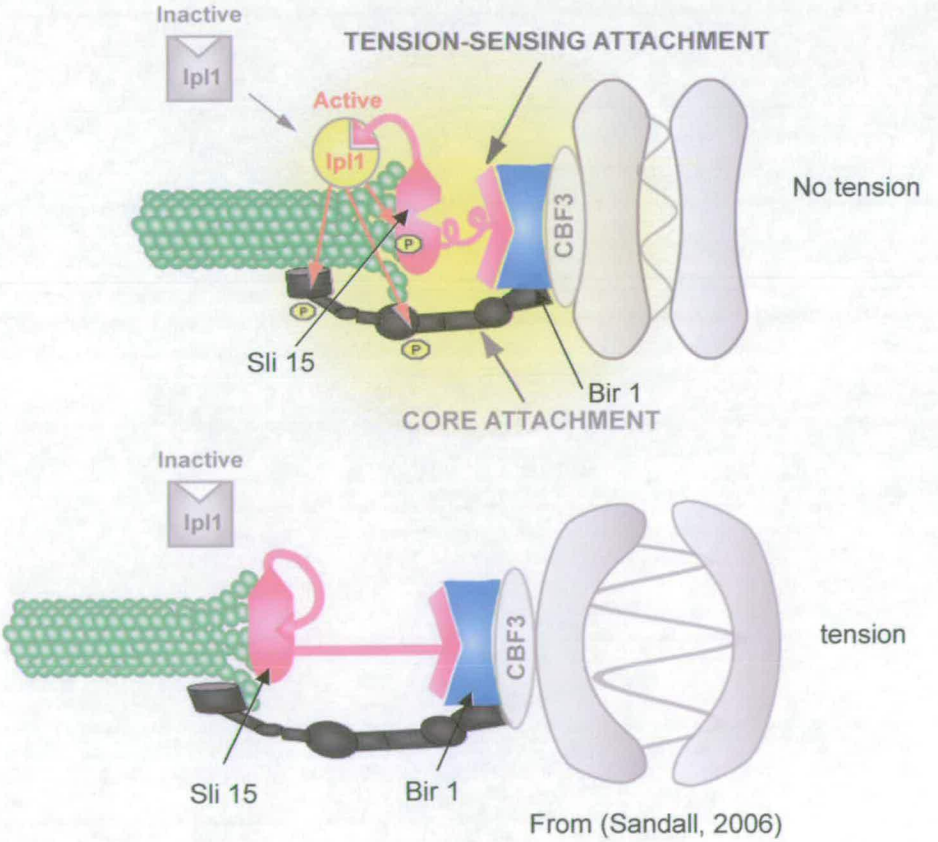
B



From (Sandall, 2006)

Borealin (Vagnarelli and Earnshaw, 2004) (Ark1, Pic1, and Cut17/Bir1 respectively in fission yeast) (Peterson 2001), (Leverson 2002), (Morishita et al., 2001). No fission yeast homologue of Borealin has as yet been found). A new model has emerged that suggests that the Aurora kinase may not be the only passenger involved in kt-mt attachments (Sandall et al., 2006). It has been suggested that in budding yeast not only are the Survivin and INCENP homologues (Bir1 and Sli15) important for the localisation of the Aurora homologue Ipl1, but they act as a tension sensor forming an attachment between the kinetochore and the microtubule and it is this that activates Ipl1 when there is no or only weak tension (Figure 1.7B) (Sandall et al., 2006)

Aurora B is thought to respond to weak attachments, such as those found in syntelically attached chromosomes that result in weak tension across the kinetochores and it facilitates the breakage of these weak attachments by phosphorylating kinetochore components, possibly Ndc80. This then allows the attachment defect to be corrected (Cheeseman et al., 2006). It is not only components of the Ndc80 complex that are phosphorylated by the Aurora B kinase, budding yeast Dam1 has also been shown to be a substrate (Shang et al., 2003) as well as the *Xenopus laevis* kinesin MCAK (Andrews 2004). XMCAK is involved in microtubule depolymerisation and phosphorylation by Aurora B appeared to inhibit this activity (Andrews et al., 2004).

1.4.2 Regulation by kinases/phosphatases

As previously discussed Aurora B kinase plays an important role in ensuring correct microtubule-kinetochore attachments during mitosis. It is not the only important kinase that acts to regulate mitosis; others include Polo-like kinase1, the spindle checkpoint kinases Mps1 and Bub1 (discussed later) and also the CDK Cdc2. Mammalian cells have at least 4 Polo-like kinases (Plks) whereas budding and fission yeast only have one (Plo1 in fission yeast). They have many functions in the cell and

it is thought to be important in the G2/M transition as it activates the phosphatase Cdc25 by phosphorylating it. At this point in the cell cycle Plo1 also phosphorylates the Wee1 kinase which down regulates it (Lee et al., 2005). Budding yeast and vertebrate Plks have also been found to be involved in the metaphase to anaphase transition by phosphorylating components of the cohesion complex on chromosome arms which reduces the affinity of cohesion for chromatin and so these molecules dissociate from the chromosome (Lee et al., 2005).

Phosphatases are other key regulators of the cell cycle. While kinases put phosphate groups on to proteins phosphatases remove them. There are several key phosphatases involved in mitosis. These are Cdc25, protein phosphatase 1 (PP1) and Cdc14. Cdc25, as discussed previously, is required to remove phosphorylation from Cdc2 to allow cells to progress in to mitosis. Protein phosphatase 1 (budding yeast Glc7) is antagonistic to Aurora B kinase (Pinsky et al., 2006). It does not dephosphorylated the Aurora kinase itself but acts on substrates of this kinase. This is required for cell cycle progression to occur (Pinsky et al., 2006). PP1 also has roles in nuclear envelope reassembly at the end of mitosis (not in yeast) (Trinkle-Mulcahy and Lamond, 2006).

Cdc14 is a regulator of late mitotic events and is important to allow cells to exit mitosis by dephosphorylating CDK targets (Trinkle-Mulcahy and Lamond, 2006).

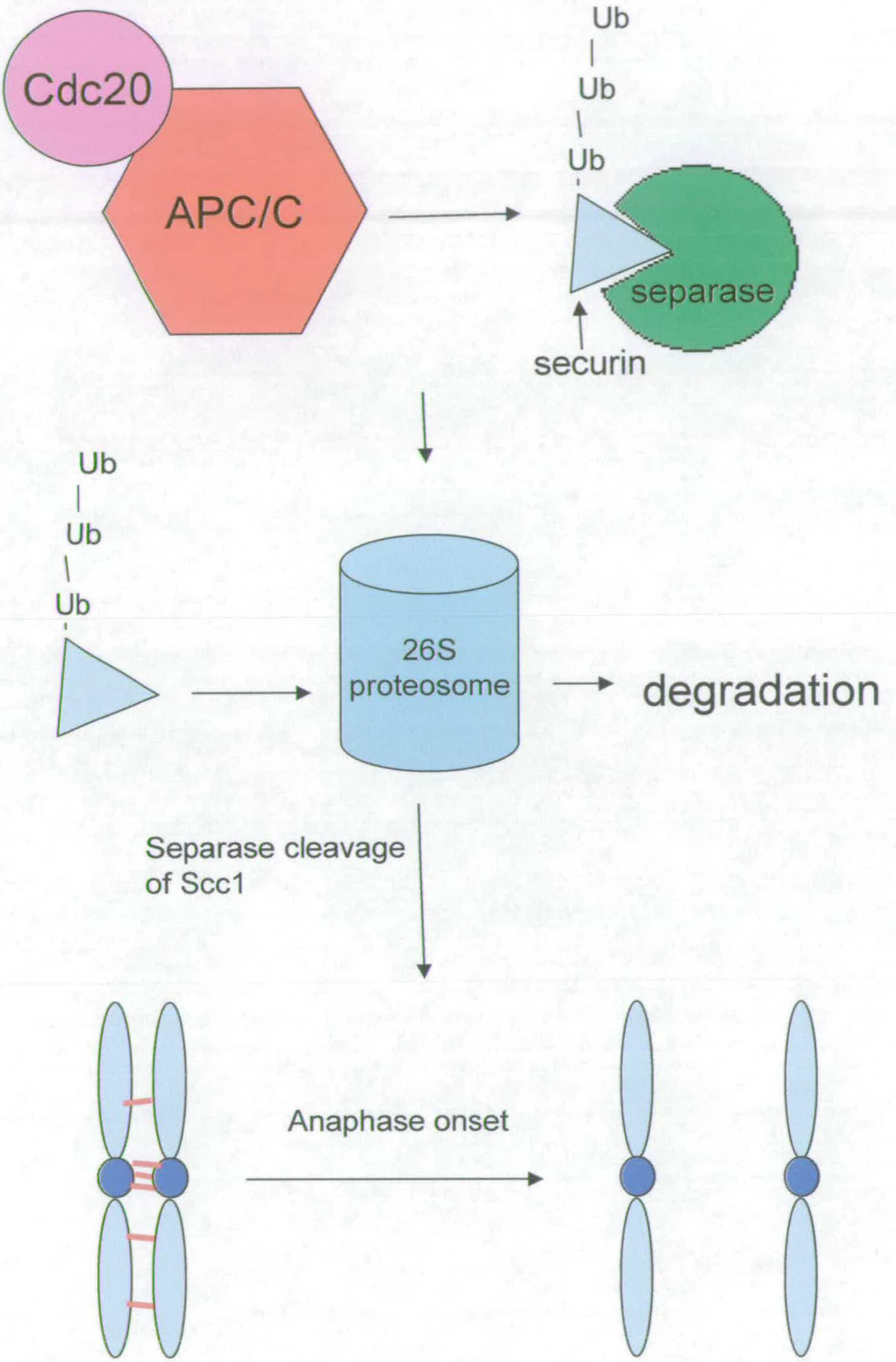
1.4.3 Mitotic progression by proteolysis

Proteolysis is required to allow progression from metaphase to anaphase as the cohesion at centromeres must be removed before the sister chromatids can be separated. The removal of cohesion and also the destruction of cyclin B is mediated by the anaphase promoting complex/cyclosome (APC/C) and its activator Cdc20 (Slp1 in fission yeast). The APC/C is a mutiprotein complex made up of about 13 subunits and has E3 ubiquitin ligase activity and so ubiquitinates substrate proteins to target them for destruction by the 26S proteosome (Peters, 2006). Ubiquitinatation requires not only an E3 enzyme but also an E1 enzyme which is an ATP-dependent

ubiquitin activating enzyme which then allows ubiquitin to be transferred to an E2 ubiquitin conjugating enzyme and the E3 enzyme (APC in this case) then promotes the binding of the ubiquitin chain to the substrate protein (Rape et al., 2006).
The APC/C does not actually ubiquitinate any of the proteins which make up the cohesin complex, instead it is the cysteine protease separase that is responsible for removing cohesin from the centromeres and allowing sister chromatid separation. This is achieved by the proteolytic degradation of the Scc1 subunit of the cohesion complex allowing the ring to be opened. To prevent premature separase activity before the onset of anaphase separase is inhibited by another protein, securin. This binds to separase preventing its activity (Peters, 2006). It is securin that is the target of the APC/C and the ubiquitination of this leads to its destruction by the 26S proteosome allowing separase to become active (see figure 1.8). The destruction of securin allows anaphase onset but cyclin B must also be targeted for destruction by the APC/C to allow mitotic exit.

It is thought that the activators of the APC/C, Cdc20 and Cdh1 are required for substrate recognition, although it seems that the APC/C is also able to recognise substrates itself. Cdc20 is the APC/C activator in mitosis whereas Cdh1 associates with the APC/C during other points during the cell cycle. Substrates of the APC/C contain specific sequence motifs such as destruction boxes (D-boxes) (Carroll et al., 2005).
Currently the evidence suggests that Cdc20 recognises and binds to D-boxes and Cdh1 can bind to both D-boxes and KEN boxes. Interestingly budding yeast Cdc20 itself contains a KEN box motif and gets degraded by APC/C^{Cdh1} at the end of mitosis. There is also evidence to suggest that the APC/C itself can bind to D-boxes through the Doc1 subunit (Carroll et al., 2005). It is, as yet, unclear whether this is also the case with KEN-box recognition.

Figure 1.8: APC/C mediated destruction of securin
The APC/C targets securin for destruction by polyubiquitinating it. This signals to the 26S proteosome to degrade it. The degradation of securin means that the protease separase becomes active and can cleave the Scc1 component of the cohesin complex. This removes cohesin from the centromeres of the sister chromatids and anaphase can occur.


Cdc20
APC/C
Ub
Ub
Ub
separase
securin
Ub
Ub
Ub
26S
proteosome
degradation
Separase cleavage
of Scc1
Anaphase onset

1.5 The Spindle Checkpoint

1.5.1 Checkpoints

Hartwell and Weinert first identified the concept of checkpoints when they noted that there was a dependence of a late cell cycle event on the completion of an earlier event. Using mutants in genes controlling certain cell cycle processes (e.g. *RAD9* gene involved in the DNA damage checkpoint) they showed that the mutations could alleviate this dependence and the cells could progress to the next stage of the cell cycle (Hartwell and Weinert, 1989).

To ensure all the events of the cell cycle occur accurately in the correct order and with the proper timing, control mechanisms must be in place within the cell. For example all the chromosomes must only be segregated and mitosis must only occur after DNA replication has taken place. This ensures that each daughter cell receives an entire copy of the genome no more, no less, to prevent aneuploidy. In order to achieve this cell cycle checkpoints regulate the transition between one phase and another. These are surveillance mechanisms that monitor cell cycle progression and prevent entry into the next phase of the cell cycle before all the requirements of the previous phase have been met (Hartwell and Weinert, 1989) and (Murray, 1994).

There are three main transitions in the cell cycle that require checkpoint activity. The first of these is at START, which is the point in mid G1 when cells become committed to the cell cycle. If conditions are not ideal and cell growth is not sufficient progression past this point is not permitted and cells exit the cell cycle and remain in G0, a non-dividing state, until sufficient conditions for growth are achieved (Morgan, 2007).

A second checkpoint exists to prevent entry into mitosis if DNA replication has not occurred correctly, or in the presence of DNA damage (Murray, 1994).

The main focus of this section will concern the third checkpoint found in cells. This controls the metaphase to anaphase transition. This checkpoint prevents premature sister chromatid separation by preventing cells from entering into anaphase before the sister chromosomes are properly attached to the mitotic spindle in order to prevent premature sister chromatid separation.

The control of cyclin-CDK activity is important for checkpoints to block cell cycle progression. The details of the cell cycle and cell cycle progression are reviewed in (Morgan, 2007), and (Murray, 2004).

1.5.2 Identification of components of the spindle checkpoint

The spindle checkpoint delays the metaphase to anaphase transition in response to unattached kinetochores and the lack of tension that results from this. A site of action for the checkpoint is at the kinetochores. This checkpoint is important to prevent chromosome loss when the chromosomes segregate caused by improper attachments to spindle microtubules. Chromosome loss results in aneuploidy which eventually leads to cell death in unicellular organisms and can result in diseases such as certain cancers in human cells and birth defects if this occurs during meiosis.

The MAD (mitotic arrest deficient) and BUB (budding uninhibited by benzimidazoles) proteins that are the main components of the spindle checkpoint were first identified in budding yeast by Li and Murray, (1991) and Hoyt et al., (1991) respectively.

The Mad and Bub proteins were found in separate screens but mutants in both sets of proteins failed to arrest mitosis in response to microtubule depolymerising drugs (e.g. benomyl). In both screens three genes were identified, which, when mutated prevented mitotic arrest in the presence of benomyl. These were *MAD1, MAD2, MAD3* and *BUB1, BUB2* and *BUB3*. The same genes were initially not found in both of the screens suggesting that the screens were not saturated. However, when the screens were repeated all of the Mad and Bub proteins were found showing that these were indeed the major components of the spindle checkpoint (Li and Murray, 1991), (Hoyt et al., 1991) The Mad and Bub proteins [apart from Bub2, a GTPase activating protein which is involved in a separate branch of the spindle checkpoint involved in exit from mitosis (Gardner and Burke, 2000)], are the core components of the spindle checkpoint. Another core component, found in a separate screen as it is involved in

spindle pole body duplication (detailed later), is Mps1 (Winey et al., 1991). Proteins with similar functions have been found in fission yeast and vertebrate cells (He et al., 1997, Bernard et al., 1998, He et al., 1998,.Li and Benezra, 1996 and Hardwick, 1998).

1.5.3 Spindle checkpoint monitoring

There is still some discussion as to what the spindle checkpoint actually monitors (figure 1.9.)

Attachment of the kinetochores to spindle microtubules from opposite poles (bipolar attachment) is important for cells to progress into anaphase. The spindle checkpoint monitors this bipolar attachment. The spindle checkpoint is a signal transduction pathway with sensors, signal transducing components and effectors (Wells, 1996). The sensors in this case monitor unattached kinetochores and in response to lack of bipolar attachment a signal transduction cascade is generated to inhibit anaphase onset. It was found in studies using meiotic cells by Li and Nicklas, (1995) that a "wait anaphase" signal was generated by lack of tension at the kinetochores. Using a glass needle to exert tension on a chromosome mimics the tension caused by bipolar spindle attachment and allows anaphase to occur. Rieder et al., (1995) demonstrated that and unattached kinetochore could generate a "wait anaphase" signal. Laser ablation of the last unattached kinetochore in Ptk cells prevented the delay into anaphase and allowed mitosis to continue.

The difference in the two factors activating the checkpoint may have been due to the cells used, meiotic cells in one case and mitotic cells in the other. However, in budding yeast cells, there is evidence for the spindle checkpoint monitoring tension. Experiments were carried out in *cdc6Δ* cells, which are unable to replicate their chromosomes. Lack of DNA replication led to cells entering mitosis with only single copies of each chromosome meaning that when attachment to microtubules occurred tension could not be established as there was no opposing force from a second sister

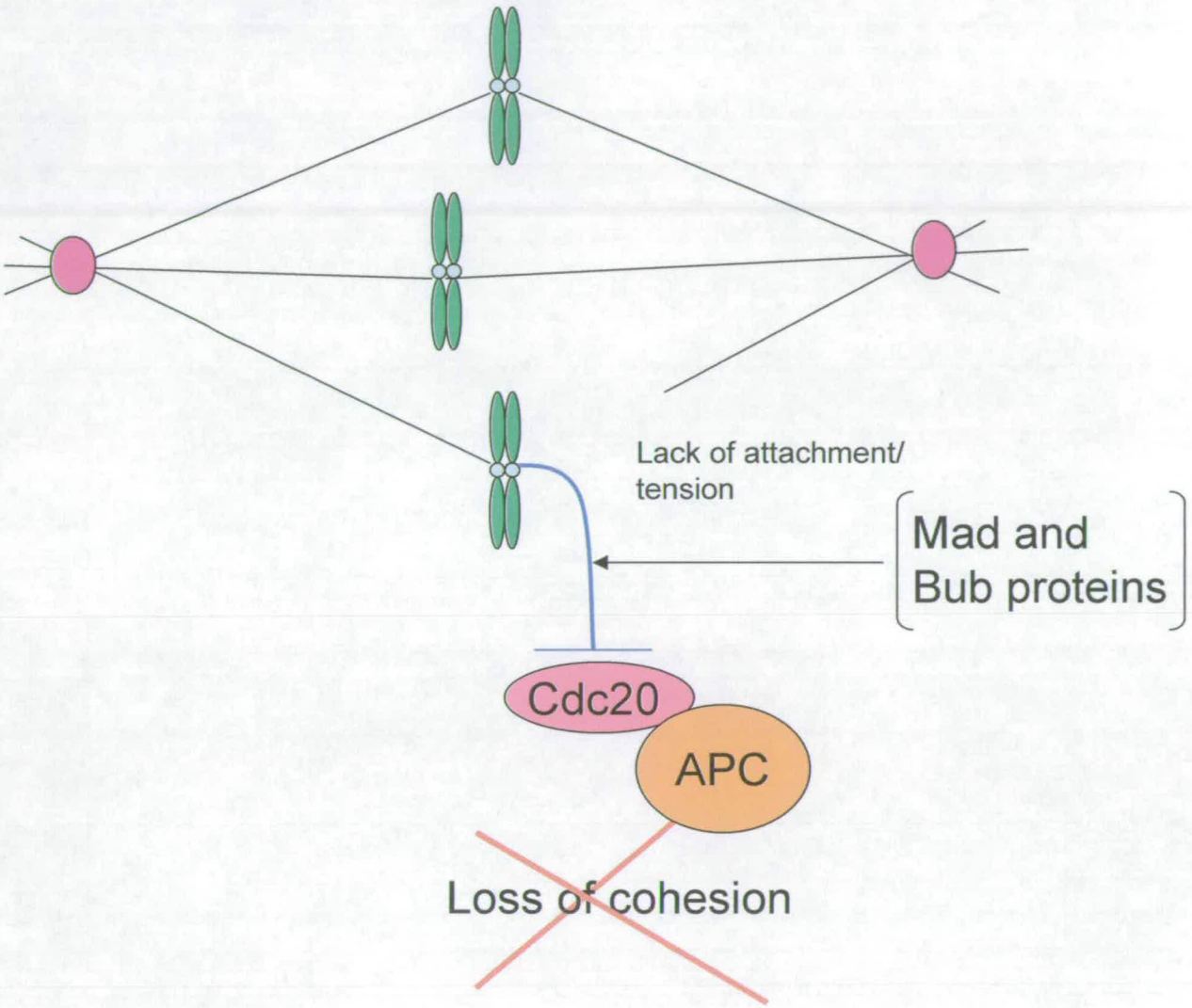


Figure 1.9: Schematic model of action of the spindle checkpoint

to pull against. It was shown that Pds1 (budding yeast securin) was not degraded in wild type cells so the checkpoint is activated, as in *cdc6Δmad2Δ* cells Pds1 was. degraded indicating that there was not a spindle checkpoint arrest and the APC was active. Budding yeast was utilised as it only has one microtubule binding site on each kinetochore so the results could not have been due to unoccupied microtubule binding sites (Stern and Murray, 2001).

1.5.4 Targets of the spindle checkpoint

It is now understood that the target of the checkpoint is the accessory subunit of the anaphase promoting complex/cyclosome (APC/C), Cdc20 (Slp1 in fission yeast). Cdc20 (Slp1) has been identified as the target for the spindle checkpoint in budding yeast (Hwang et al., 1998) and fission yeast (Kim et al., 1998). Yeast two-hybrid studies identified Mad2 in both systems, and Mad1 and Mad3 in budding yeast, as important regulators of Cdc20 function. Studies in budding yeast also showed Mad3 to interact with Cdc20, and both Mad2 and Mad3 require Mad1 for this function (Hwang et al., 1998). Mad2 has since been shown to exist in two conformations in cells, an open conformation (O-Mad2) and a closed conformation (C-mad2). The difference between these two conformations is in the "safety belt ", a 50 amino acid region in the C-terminus of Mad2 (De Antoni et al., 2005).

It is in this binding pocket that both Mad1 and Cdc20 can bind to Mad2. The interaction of Mad2 with Cdcd20 is important for inhibition of the APC/C by the spindle checkpoint (Figure 1.10). Since the energy needed to convert O-Mad2 to C-Mad2 is high it was initially though that Mad1 bound to Mad2 and facilitated the conversion of O-Mad2 to C-Mad2 and Mad1 could then be exchanged for Cdc20, which could occur more rapidly. However a new model has since been proposed, the "Mad2 template" model where the stable Mad1-C-Mad2 complex acts to recruit O-Mad2 to bind to Cdc20 (De Antoni et al., 2005).

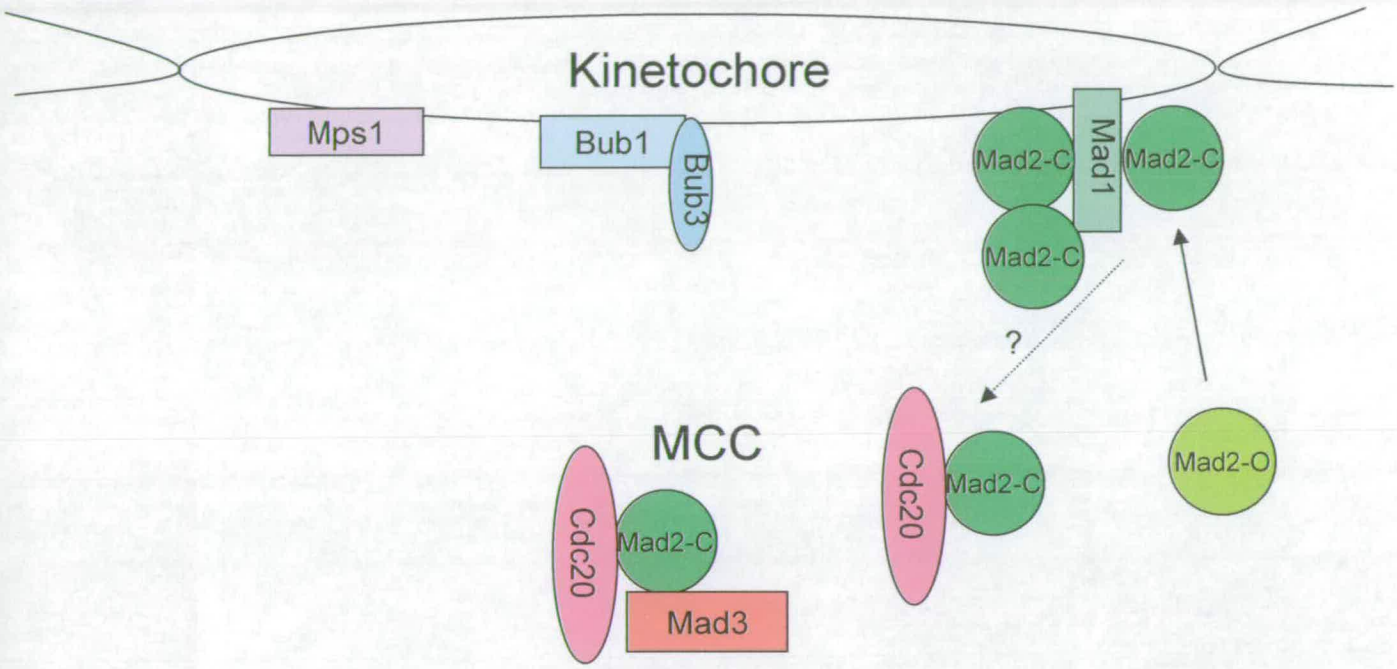


Figure 1.10: Checkpoint complexes
The sight of action for the spindle checkpoint is at kinetochores. Several complexes of spindle checkpoint components exist. Mad2 is thought to exist in 2 forms, open (Mad2-O) and Closed (Mad2-C). It adopts the closed conformation when bound to either Mad1 or Cdc20 and it is thought that the binding of Mad2 to Mad1 facilitates its binding to Cdc20.
The mitotic checkpoint complex (MCC) sequesters Cdc20 preventing it activating the APC/C so preventing anaphase onset.

It is now known that several complexes of checkpoint proteins exist and the actions of several of these proteins are dependent on other proteins (Musacchio and Hardwick, 2002) (Figure 1.10). It is currently thought that the Mad3, Bub3 and Mad2 exist as a complex which directly inhibits Cdc20 (reviewed in Musacchio and Hardwick, 2002 and Yu, 2002). It is likely that they form an inhibitory complex, termed the mitotic checkpoint complex (MCC), with Cdc20 and this sequesters Cdc20 preventing its interaction with the APC/C. In fission yeast there is currently no evidence to suggest that Bub3 is part of this complex (M Scznieckа, personal communication). Studies in *Xenopus* have shown Mad1 binds and recruits Mad2 to kinetochores (Chen et al., 1998). Other complexes that have so far been found are those involving Bub1-Bub3 and Bub3-Mad3. A Bub1-Bub3-Mad1 complex has also been found in yeast (Brady and Hardwick, 2000). High levels of this complex have been shown to be present in budding yeast cells in response to treatment of cells with a drug to disrupt microtubules The Bub1-Bub3-Mad1 appears to be cell cycle regulated, which does not seem to be the case for other complexes such as Bub1-Bub3. This suggests a potentially important signalling role for this complex in the spindle checkpoint (Brady and Hardwick, 2000).

1.5.5 The spindle checkpoint is a dynamic process

Many of the checkpoint components have been shown to be dynamic within the checkpoint, cycling on and off kinetochores. FRAP (fluorescence recovery after photobleaching) studies in mammalian cells have recently shown that only a small percentage of the total pool of spindle checkpoint proteins in a cell is actually bound to the unattached kinetochores (Howell et al., 2004) and (Shah et al., 2004). Mad1 and Bub1 are the least dynamic with relatively slow kinetics compared to many of the other checkpoint proteins and so are more likely to remain bound to the kinetochores. A portion of the cellular fraction of Mad2 is often found bound to Mad 1 and a

fraction of the BubR1 (mammalian Mad3) pool is found bound to Bub3 which leads to slower kinetics for these proteins. The unbound Mad2 and unbound BubR1, as well as Cdc20 and Mps1 are much more dynamic with faster kinetics suggesting that they shuttle on and off of kinetochores (Howell et al., 2004). This would indicate that there is likely to be a dynamic exchange of proteins at the kinetochore recruited by Mad1 and Bub1 and they may act as a scaffold (Howell et al., 2004).

1.6 Mph1

1.6.1 Original identification

MPS1 (Monopolar spindles) was originally identified in *S.cerevisiae*. It has since been shown that members of this family exist in almost all eukaryotes (with the exception of *C.elegans*. Mph1 is the fission yeast homologue of *MPS1*.
MPS1 was discovered in a screen for mutants that resulted in spindle pole duplication defects. A temperature sensitive mutation of *MPS1* (*mps1-1*) resulted in cells with monopolar spindles at the non-permissive temperature (Winey et al., 1991). These cells do not replicate their spindle pole bodies and this lead to the identification of Mps1 as being required for spindle pole body duplication. SPB duplication is required for proper bipolar spindle formation which is crucial for the segregation of chromosomes during anaphase. It was only later discovered that *MPS1* had a function in the spindle checkpoint (Weiss and Winey, 1996).

1.6.2 Mps1 as a component of the spindle checkpoint

The lack of mitotic arrest in monopolar cells with the *mps1-1* mutation suggested that this gene may have an additional role in cell cycle regulation.
In the same screen another gene involved in SPB duplication was identified, *MPS2*, and a mutation in it (*mps2-1*) also caused SPB duplication defects. In contrast to *mps1-1*, however, *mps2-1* cells do arrest and do not go through a lethal mitosis proving that it was not the absence of a duplicated SPB that was preventing the arrest. To determine if Mps1 had a role in the checkpoint cells were incubated with nocodozole at 25°C and 37°C. At 25°C cells arrested with G2 DNA content much the same as the wild type control. However at 37°C, the restrictive temperature for this mutation, cells did not arrest and continued with DNA synthesis and bud formation in the presence of disrupted spindle, much the same as the Mad1 mutant used as a control. To verify that this result was due to a failure in the spindle checkpoint and not because of an unreplicated SPB *mps1-1* cells were grown at 25°C to allow SPB formation and arrested in G2. Cells were then released in nocodozole at 37°C. They did not arrest in mitosis but again continued through bud formation and another round of DNA synthesis. This was conclusive evidence that Mps1 was a spindle checkpoint component (Weiss and Winey, 1996).

1.6.3 The Mps1 family are protein kinases

In an earlier study it had been shown that *MPS1* is a protein kinase. Sequence analysis of the C-terminus of *MPS1* from *S.cerevisiae* highlighted the fact that there was significant similarity to the consensus sequence eleven subdomains of a protein kinase domain (Lauze et al., 1995). Sequence analysis also showed significant homology to the kinase domains of human PYT/TTK and mouse *esk* genes, both now

shown to be the *MPS1* homologues in human and mouse cells respectively (Winey and Huneycutt, 2002). This highlighted the potential for *S.cerevisiae* Mps1 to be a dual specificity kinase phosphorylating serine and threonine residues as well as tyrosine residues (Lauze et al., 1995).

Weiss and Winey determined that since the *mps1-1* mutation is in the kinase domain this domain must be important for its SPB function as well as its checkpoint function (Weiss and Winey, 1996).

Studies in *Xenopus* egg extracts and budding yeast confirm that the kinase domain is required for spindle checkpoint function (Abrieu et al., 2001) and (Weiss and Winey, 1996). A kinase-dead version of Mps1 was made in *Xenopus* and was shown to still be able to localize to kinetochores but Mad1 and Mad2 were not recruited to kinetochores, so disrupting checkpoint signaling (Abrieu et al., 2001).

Mps1 in budding yeast has been shown to be required for Mad1 phosphorylation in response to checkpoint activation (Hardwick et al., 1996) again arguing that the kinase domain is important for the function of Mps1 in the spindle checkpoint.

1.6.4 Identification of an Mps1 homologue in *S.pombe*

A screen carried out in fission yeast by (He et al., 1998) was carried to find genes that caused metaphase arrest when overexpressed. A previous screen had found Mad2 in fission yeast (He et al., 1997). Whilst Mad2 overexpression does not cause metaphase arrest in budding yeast it does in fission yeast but Mps1 overexpression in budding yeast, however, does cause an arrest as does fission yeast Mph1 (Hardwick et al., 1996). It was shown that budding yeast cells overexpressing Mps1 arrested with G2 DNA content and short spindles, much the same as wild type cells at the same stage in mitosis. In cells that were mutant for the Mad and Bub checkpoint proteins Mps1 overexpression failed to cause an arrest (Hardwick et al., 1996).

Mph1 found in the fission yeast screen was also shown to arrest cells with the same spindle morphology and sequence alignment confirmed that this was the *S.pombe* homologue of Mps1 (He et al., 1998). Most of the sequence similarity occurs in the kinase domain, which is where most of the homology between members of this family of kinases is. There is usually very little homology in the N-terminus (Winey and Huneycutt, 2002).

The fact that Mph1 was the fission yeast homologue of Mps1 was also confirmed by the fact that Mph1 can not only partially rescue the checkpoint defects in an *mps1-1* mutant in *S.cerevisiae* but it also partially rescues the SPB defects. This is slightly surprising as Mph1 did not appear to be involved in SPB duplication in fission yeast. There is currently some controversy over whether this is the case for human Mps1. Research by Stucke et al., (2002) argued, by a combination of antibody staining and RNAi that Mps1 did not play a role in centrosome duplication in human cells. Studies by Liu et al., (2003) and Fisk et al., (2003) contradict these findings and claim that Mps1 is found at centrosomes and does play a role in their duplication.

Fission yeast Mph1 is not an essential gene unlike *S.cerevisiae MPS1* which meant that it was possible to construct a strain where the *mph1*$^{+}$ was deleted (*mph1Δ*) (He et al 1999). This mutant is alive and can grow until challenged with microtubule depolymerising drugs. When grown on media containing benomyl *mph1Δ* is one of the most benomyl sensitive of all the checkpoint proteins, along with Bub1 (Vanoosthuyse et al., 2004).

1.6.5 Dependencies of other checkpoint proteins on Mph1

It seems likely that Mph1 is an upstream component of the spindle checkpoint as it is required for the localisation of many of the other checkpoint proteins.

In the screen by (He et al., 1998) they also show that Mad2 is required for Mph1 overexpression to arrest cells in metaphase but Mph1 is not required for a Mad2 arrest. They conclude that this puts Mph1 upstream of Mad2 in the checkpoint pathway.

It has previously been shown that Mph1 is required for Mad3 localisation (Millband and Hardwick, 2002). In an *mph1Δ* mutant Mad3-GFP shows diffuse localisation rather that nuclear staining as in wild type suggesting that Mph1 (as well as Bub1 and Bub3) is required for the kinetochore localisation of Mad3.
It has also been suggested that Mph1 is also required for Bub1 and Bub3 localisation (Vanoosthuyse et al., 2004). This would indicate that Mph1 may be important for recruiting several of the checkpoint components to unattached kinetochores.
As has been previously described it is also known that Mps1 is required for the localization of Mad1 and Mad2 in vertebrate cells (Chen et al., 1999).

1.7 Aims of study

The main aims of this study are to investigate the Mph1 kinase in fission yeast, especially with respect to the function of the kinase domain in spindle checkpoint signalling. The regulation of Mph1 with respect to activation and inhibition during the cell cycle and other potential roles for Mph1, for example in chromosome segregation and the identification of any potential substrates or regulators of Mph1 are also important aims.

Chapter 2

Materials and Methods

2.1 Supplier Information.

Chemicals were purchases from the following sources, except where stated otherwise: BDH, Boerhinger Mannheim, Fisher, Gibco BRL and Sigma.

Restriction enzymes, DNA polymerase and other enzymes used in this work were obtained from the following sources, except where stated otherwise: New England Biolabs, Promega, Qiagen, Stratagen and Roche.

2.2 General information.

2.2.1 Sterilisation

Solutions were sterilised either by autoclaving or by filtration. Small volumes were filtered through acrodisc syringe filters (0.45μm). Large volumes were filtered through 250ml or 500ml filter units (0.45μm, Nalgene).

2.3 Bacterial manipulation

2.3.1 Bacterial growth and maintenance

2.3.1.1 Strains used in this study:

Table 2.1: Bacterial strains

Strain	Use in this study	Genotype	Origin
DH5α	General cloning and plasmid propagation	*F-φ80lacZΔM15(lacZYA-argF)U169 deo recA1 endA1 hsdR17(rk,mk) phoA supE44 thi-1 gyrA96 relA1 λ-*	Gibco BRL
XL1-Blue (electrocompetent)	cloning	F'::TN10 *proA*$^{+}$*B*$^{+}$ *lacI*^{q} *Δ(lacZ)M15/recA1 end A1 gyrA96(Nal*^{r}*) thi hsdR17 (*$r_k^- m_k^-$*) glnV44 relA1 lac*	Stratagene
BL21	Production of bacterially expressed GST fusion proteins	*E.coli* B F^{-} *dcm ompT hsdS(*$r_B^- m_B^-$*) gal*	Stratagene

Bacterial growth media

Table 2.2: Bacterial growth media

Bacterial media	Media components
LB*	1% (w/v) Bacto-tryptone 0.5% (w/v) Bacto-yeast extract 0.5% (w/v) NaCl pH adjusted to pH 7.2 with 5M NaOH
SOC	2% (w/v) Bacto-tryptone 0.5% (w/v) Bacto-yeast extract 20mM Glucose 10mM NaCl 10mM $MgCl_2$ 10mM $MgSO_4$ 2.5mM KCl

- For solid media, 2% (w/v) agar was added prior to autoclaving

2.3.1.2 Bacterial supplements

Table 2.3: bacterial supplements

Supplement	**Abbreviation**	**Stock solution Solvent and conc. (mg/ml)**	**Final conc.(µg/ml)**
Ampicillin (1000x)	Amp	Water 100mg/ml	100
Kanamycin (250x)	Kan	Water 10mg/ml	40
Isopropylthiogalactoside	IPTG	Water	20mg/ml
5-bromo-4-chloro-3-indolyl-B-D galactoside	X-gal	DMSO	20mg/ml

2.3.1.3 Bacterial growth conditions

Bacterial were typically grown at 37°C on solid LB media containing the appropriate antibiotic for selection of plasmid DNA or at 37°C with appropriate aeration and antibiotic in liquid media.

2.3.1.4 Storage of bacteria

Bacterial were stored indefinitely at -80°C in LB supplemented with 20% glycerol.

2.3.2 Bacterial transformations

2.3.2.1 Preparation of chemically competent *E.coli* cells

100ml DH5α (GIBCO, BRL) *E.coli* cells were grown in liquid LB at 37°C overnight to OD_{600}=0.5-0.7. Cells placed on ice for 10-15 minutes then centrifuged for 5 minutes at 5000rpm (4°C, Beckman JLA 10.500 rotor). From here cells were always kept on ice and all solutions used were prechilled. Cells were gently resuspended in 20ml of 0.1M $CaCl_2$ then collected by centrifugation for 5 minutes at 400 rpm. Cells

were then resuspended in 10ml 0.1 M $CaCl_2$ and glycerol was added to 20% w/v and cells measured into 100µl aliquots.

2.3.2.2 Transformation of chemically competent cells

Competent cells were thawed on ice. Typically up to 100ng of plasmid DNA was added to 50-100µl cells. The *E.coli*/DNA mixture was let to incubate on ice for 20 minutes then transferred to a 37°C water bath to heat shock the cells for 5 minutes. 900µl of LB was added to the cells and they were let to recover at 37°C, with shaking, for 1 hour after which time the cells were plated onto LB agar containing the appropriate antibiotic and incubated at 37°C.

2.3.2.3 Transformation of electo-competent XL1-Blue *E.coli*

XL1-Blue electro-competent *E.coli* (Stratagene) were purchased electro-competent. 40-50µl of cells were thawed on ice to which 100ng DNA for transformation was added. The cell/DNA mixture was transferred to a prechilled electroporation cuvette (0.2cm electrode gap). Cells were electroporated using a BioRad Gene PulserII at 200Ω resistance, 25mF capatcitance and 1.7kV voltage. Immediately after electroporation 1ml SOC medium was added and cells allowed to rest at 37°C with shaking for 1 hour. Cells then plated on LB containing the appropriate antibiotic for selection and grown at 37°C

Blue-white selection of clones was sometimes used where 0.1mM IPTG and 0.25% X-gal were spread on the plates to which the transformed bacteria would be plated. Colonies with the correct insert could be selected by picking white colonies as these could no longer express the β-galactosidase gene due to ligation of the DNA insert into the multiple cloning site of the vector within the *lacZ* gene.

2.4 Growth and manipulation *S.pombe*

2.4.1 Fission yeast strains used in this study

Table 2.4: Strains used in this study

Strain	Genotype	Source
KP276	*wt ade6-210, leu1-32, ura4-D18, his3-D1, arg3-D4*	This Lab
KP175	*mph1Δ::ura4 leu1-32 ura4-D18 ade6-m216 h+*	S.Sazer
YLM16	*Ch16 minichromosome bub1Δ(ura) h+*	This lab
YLM22	*mph1Δ::ura4 ch16 leu1-32, ade6-210, ura4-D18*	This study
YLM36	*mad2Δ::ura4 leu1-32 ade6-210*	This lab
YLM39	*pREP41x Mph1 (LEU)*	S.Sazer
YLM42	*mph1Δ::NAT Cen2-GFP - lys1 ura4 cen2D107(::Kan-ura-lacO) his7::lacI-GFP leu1 ade6-210*	This study
YLM43	*bub1Δ::ura Cen2-GFP - lys1 ura4 cen2D107(::Kan-ura-lacO) his7::lacI-GFP leu1 ade6-210*	This study
YLM45	*Cen2-GFP h+ lys1 ura4 cen2D107(::Kan-ura-lacO) his7::lacI-GFP leu1 ade6-210*	Hiraoka
YLM46	*Cen2-GFP h- lys1 ura4 cen2D107(::Kan-ura-lacO) his7::lacI-GFP leu1 ade6-210*	Hiraoka
YLM48	*nda3 h+ leu1-32 ade6-210*	This lab
YLM60	*mph1Δ::NAT leu1-32, ade6-210, ura4-D18 h-*	This study
YLM61	*bub1Δ::ura4 h-*	This lab
YLM62	*mph1Δ::NAT bub1Δ::ura4 leu1-32, ade6-210, ura4-D18 h+*	This study
YLM63	*mph1Δ::NAT bub1Δ::ura4 cen2-GFP lys1 ura4 cen2D107(::Kan-ura-lacO) his7::lacI-GFP leu1 ade6-210 h+*	This study
YLM67	*bub1Δ::ura leu1-32 ura4DS/E ade6Đ216 his1Đ102 h+*	This lab
YLM70	*mph1Δ::NAT leu1-32, ade6-210, ura4-D18h+*	This study
YLM72	*mph1Δ::NAT bub1Δ::ura4 leu1-32, ade6-210, ura4-D18 h-*	This study
YLM77	*mph1Δ::NAT nda3 leu1-32, ade6-210, ura4-D18*	This study
YLM81	*bub1Δ::ura leu1Đ32 ura4DS/E ade6Đ216 his1Đ102 nda3*	This study
YLM109	*mph1-GFP (G418) ade6-210, leu1-32, ura4-D18, his3-D1, arg3-D4 h-*	This study
YLM111	*mph1k Δ-GFP ade6-210, leu1-32, ura4-D18, his3-D1, arg3-D4 (G418) h-*	This study
YLM112	*mph1-Tap (NAT) ade6-210, leu1-32, ura4-D18, his3-D1, arg3-D4h-*	This study
YLM129	*mph1-GFP mad2 Δ ade6-210, leu1-32, ura4-D18, his3-D1, arg3-D4*	This study
YLM131	*mph1k Δ-GFP mad2 Δ ade6-210, leu1-32, ura4-D18, his3-D1, arg3-D4*	This study
YLM133	*mph1-GFP cen2-GFP lys1 ura4*	This study

	cen2D107(::Kan-ura-lacO) his7::lacI-GFP leu1 ade6-210	
YLM135	*mph1k Δ-GFP cen2-GFP lys1 ura4 cen2D107(::Kan-ura-lacO) his7::lacI-GFP leu1 ade6-210*	This study
YLM137	*cdc25-22 ts*	
YLM138	*cdc25-22 mph1Δ::NAT ts*	This study
YLM139	*bub1 1-586-Tap (ura4)*	This lab
YLM141	*mph1-GFP nda3 ade6-210, leu1-32, ura4-D18, his3-D1, arg3-D4 h⁻*	This study
YLM143	*mph1k Δ-GFP nda3 ade6-210, leu1-32, ura4-D18, his3-D1, arg3-D4 h⁻*	This study
YLM152	*mad3-GFP (his⁺)*	This lab
YLM153	*cut7-24 leu1-32 ade6 210 h⁻*	R.Allshire
YLM164	*mph1k Δ-GFP bub1 1-586-Tap (ura4) (G418) h⁺*	This study
YLM171	*mph1Δ cut7-24*	This study
YLM174	*mph1k Δ-GFP cut7-24*	This study
YLM181	*cut7-24 mad2 Δ::ura4*	This lab
YLM182	*mph1Δ::NAT mad2 Δ::ura4*	This study
YLM185	*mph1kΔ-STAP ade6-210, leu1-32, ura4-D18, his3-D1, arg3-D4 h⁻*	This study
KP104	*bub3Δ::ura4 leu1-32 ade6 ura4-D18 h⁺*	This lab
YLM190	*mph1Δ::NAT bub3Δ::ura4*	This study
VV414	*nmt::GFP-plo1⁺ nda3*	H.Okhura
YLM201	*mph1Δ nmt::GFP-plo1⁺ nda3 leu1-32, ade6-210, ura4-D18*	This study
YLM207	*mph1KΔ-GFP nmt::GFP-plo1⁺ nda3*	This study
YLM217	*pyp3::ura4 leu1-32 ura4-D18 nda3-KM311*	This study
YLM218	*pyp3::ura4 leu1-32 ura4-D18 cdc25-22*	This study
YLM220	*bub1KΔ mph1::NAT nda3*	This study
YJZ4	*mph1-szz (G418)*	This lab
YLM222	*mph1-szz (G418) nda3-KM311*	This study

2.4.2 Fission yeast media

Table2.5: fission yeast media

Growth media	**Media components***
Yeast extract (YE)	0.5% (w/v) yeast extract 3% (w/v) glucose
Yeast extract supplemented (YE5A)	YE with 50x supplement cocktail$^{\Psi}$ and 25x uracil$^{\Psi}$
Yeast extract with Benomyl	YE with supplement$^{\Psi}$ and the appropriate concentration of benomyl$^{\Psi}$ (6,8,10μg/ml)
pombe minimal growth (PMG)	3g/l phtahlic acid, 2,2g/l Na_2HPO_4, 3.7 g/l L-

	glutamic acid, 20g/l D-glucose, 1x vitamins$^{\Psi}$, 1x minerals$^{\Psi}$, 1x salts$^{\Psi}$
Minimal supplemented	PMG supplemented with the appropriate 100x supplements and 25x uracil
SPA	10g/l Glucose 1g/l KH_2PO_4 1ml 1000x vitamins$^{\Psi}$ 2% w/v yeast agar

* For solid media add 2% w/v agar prior to autoclaving

$^{\Psi}$ added after autoclaving

2.4.3 Fission yeast nutrients and supplements

Table 2.6: *S.pombe* nutrients and supplements

Nutrients and supplements	**Components**
50x adenine	3.75g/l adenine
25x uracil	1.875g/l uracil
100x histidine	7.5g/l histidine
100x arginine	7.5g/l arginine
100x lysine	7.5g/l lysine
100x leucine	7.5g/l leucine
100x thiamine	4mg/ml
50x supplement cocktail	4g/l of each of: adenine, histidine, arginine, lysine, leucine
Benomyl	30mg/ml stock solution in DMSO added to boiling agar to appropriate concentration
1000x vitamins	1g/l pantothenic acid (4.2mM) 10g/l nicotinic acid (81.2mM) 10g/l inositol (55.5mM) 10mg/l biotin (40.8mM)
50x salts	53.5g/l $MgCl_2$ (0.26M) 1g/l $CaCl_2$ (4.99 mM) 50g/l KCl (0.67M) 2g/l Na_2SO_4
10 000x minerals	5g/l Boric acid (80.9mM) 4g/l Mn SO_4 (23.7mM) 4g/l Zn SO_4 (13.9mM) 2g/l $FeCl_3$ (7.4mM)

	0.4g/l molybdic acid (2.47mM) 1g/l KI (6.02mM) 0.4g/l Cu SO_4 (1.6mM) 10g/l citric acid (47.6mM)
G418 (kanamycin)	0.15mg/ml
clonNAT	Nouroseothricin (Werner Bioagents) 200mg/ml in dH_2O as a 200X stock

2.4.4 *S.pombe* growth conditions

Wild type *Schizosaccharomyces pombe* strains and strains that were not temperature sensitive were grown at 30°C. Temperature sensitive strains were typically grown at the permissive temperature 23-25°C and the restrictive temperature of 36°C. Cold sensitive strains were grown at the permissive temperature 30°C and the restrictive temperature 18°C. Strains grown in liquid media were incubated with shaking to provide appropriate aeration.

Strains grown on solid media were grown in the presence of appropriate supplements to select for plasmid DNA or mutations.

2.4.5 *S.pombe* cell cycle arrests

2.4.5.1 M-phase arrest using the *nda3-KM311* mutant

S.pombe cells containing the cold sensitive β-tubulin mutation *nda3-KM311* were grown in YE5A at 30°C to mid log phase then shifted to 18°C for 6-8 hours to activate the mutation and arrest cells in metaphase.

2.4.5.2 G2/M arrest using the *cdc25-22* mutant

S.pombe strains carrying the temperature sensitive *cdc25-22* mutation were grown at 25°C in YE5A to mid log phase. Cells were spun down by centrifugation and resuspended in prewarmed media at 36°C for 3 hours. The DNA of the cells was stained with DAPI to check for the arrest. Arrested cells should have elongated cells with only one DNA mass.

2.4.6 Strains containing the nmt promoter

The *nmt* promoter is an inducible promoter that is repressed in the presence of thiamine. Strains containing this promoter were grown in media containing 15μM thiamine then switched to media lacking thiamine to activate the promoter.

2.4.7 Benomyl sensitivity assay

Strains that had been freshly streaked out were used in this assay. Small amounts of cells were resuspended in 200μl dH_2O in the first row of a 96 well plate. Serial dilutions were then made by pipetting 20μl into 180μl dH_2O in series in the next 5 wells. The serially diluted cells were stamped onto a YE5A plates containing 0, 8, 10μg/ml Benomyl using a sterile stamper. The plates were incubated at 23°C for 2-3 days and growth analysed compared to relevant controls.

2.4.8 Half sectoring assay

Strains containing a short linear chromosome (530Kb) (Matsumoto et al., 1990) were used for a half sectoring assay to determine chromosome loss in those strains. The minichromosome contained the *ade6-216* mutation which complemented the *ade6-210* mutation contained in the parental strain. When grown on media containing minimal adenine colonies which have lost the minichromosome are red and those retaining it are which due to complementation of the 2 *ade* mutants. Strains were grown on media lacking uracil in order to prevent loss of the minicheomosome (which contained the *ura4-D18* mutation). Cells were resuspended in 1 ml dH_2O and

counted using a haemocytometer in order to get the correct volume to be plated to give approximately 250 cells per plate. These cells were plated on YE5A plates that contained one fifteenth the concentration of adenine than usual. The plates were incubated at 30°C for 3 days and colonies counted. Any colony that had at least half red sectoring was counted as chromosome loss as this indiacted that the chromosome was lost in the first division. Any completely red colonies were not counted.

2.4.9 Storage of *S.pombe* strains

For short term storage yeast strains were patched out on agar plates and kept at 4°C. For long term storage *S.pombe* strains were stored at -80°C in YE supplemented with 40% glycerol.

2.4.9 Transformation of *S.pombe*

2.4.9.1 Lithium acetate transformation

150ml culture grown to OD_{600}=600 in YE5A and cells were harvested by centrifugation at 3000rpm for 3 minutes.
Cells were washed in 50ml ddH_2O and spun down as before. Cells resuspended at 1.10^9cell/ml in 0.1M lithium acetate pH4.9 (pH adjusted with acetic acid). 100µl of this cell suspension was used for each transformation and were incubated at 30°C (25°C for ts mutants) for 60 minutes.
1µg of DNA was added and mixed by gentle vortexing to resuspend sedimented cells. 290µl 50% w/v PEG 4000 (prewarmed to 30°C, or 25°C for ts mutants) was added and cells incubated at 30°C for 60 minutes.
Cells were heat shocked at 42°C for 15 minutes then cooled to room temperature before being spun down by centrifugation at 13000rpm for 60 seconds. The supernatant was removed by aspiration and cells were resuspended in 10ml ½ YES (diluted with ddH_2O) and incubated with shaking at 30°C (25°C for ts mutants) for 60 minutes then plated onto YE5A plates and replica plated onto selective media.

2.4.9.2 Transformation of *S.pombe* by electroporation

Cells were grown at 30°C (or 25°C for ts mutants) in YE5A or appropriate selective media to an OD_{600}= 0.5 ($1x10^7$ cells/ml).

Cells were collected by centrifugation at 3000rpm for 3 minutes and washed once in ice cold ddH_2O and once in 1.2M sorbitol. Cells were spun down and resuspended in 1.2M sorbitol to a concentration of about $1x10^9$cells/ml. 100µl of this cell suspension was mixed with the DNA to be transformed and transferred to a pre-chilled 0.2cm electroporation cuvette.

Electroporation was carried out using a BioRad Gene Pulser II set at 200Ω, 25 mF and 1.5kV. Time constants between 4 and 5 milliseconds were aimed for.

Immediately after electroporation 900µl of 1.2M sorbitol was added to the cuvette and cell suspensions left on ice while other electroporations were carried out. 500µl cell suspension was plated out onto selective media and incubated at the appropriate temperature for 3-4 days.

2.5 Nucleic acid methods

2.5.1 Plasmids used in this study

Table 2.7: Plasmids used in this study

Plasmid	Source
pREP41x Mph1	S.Sazer
pREP41x EGFP	
pREP81x EGFP	K. Sawin
pGEX6P-1	Pharmacia Biotech

2.5.2 Phenol-chloroform extraction

Nucleic acids were purified away from proteins in an aqueous solution by adding an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) vortexing briefly to mix then centrifuging at 13000 rpm for 5 minutes. The upper aqueous phase that contained the nucleic acids was removed to a fresh tube.

2.5.3 Precipitation of nucleic acids

Nucleic acids were precipitated out of solution by adding 1/10th volume of 3M NaOAc and 2-3 volumes of ethanol and incubating on ice for 30 minutes before being centrifuged at 13000 rpm for 10 minutes at 4°C. The supernatant was removed and the pellet washed in 70% ethanol and centrifuged at 13000 rpm for 5 minutes at 4°C. The pellet was then air dried and resuspended in the appropriate volume of TE or dH_2O.

2.5.4 Plasmid mini-prep by rapid alkaline lysis

5ml of LB was supplemented with the appropriate antibiotic and inoculated with the *E.coli* strain containing the required plasmid and incubated overnight at 37°C with shaking.

The culture was spun down by centrifugation in a Falcon tube at 3000rpm for 3 minutes and the pellet was transferred to a 2.5ml Eppendorf tube. The cell suspension was resuspended in 100µl GTE (50mM glucose, 25 mM Tris-Cl pH 8.0, 10mM EDTA) and mixed by vortexing.

Cells were lysed by the addition of 200µl 1% w/v SDS/0.2M NaOH and gentle mixing until a clear, viscous state was achieved. To this 150µl 3MKOAc/2M HOAc was added and mixed well. The cell suspension was then spun down in a centrifuge at 1300rpm for 1 minute and the supernatant was decanted into a clean tube. The tube was filled with ethanol to precipitate the DNA and mixed well and chilled before being centrifuged for 5 minutes at 1300rpm. The supernatant was aspirated off and the DNA resuspended in 20µl TE (100mM Tris-HCl, 10mM EDTA, pH adjusted to pH8).

To precipitate the bulk of the RNA 40µl LiCl was added and chilled on ice for 10 minutes before being spun down at 1300 rpm for 3 minutes. The supernatant was transferred to a clean Eppendorf tube and ethanol added to precipitate the DNA. This was spun down at 1300rpm for 5 minutes and the DNA resuspended in 20µl TE.

2.5.5 Plasmid mini-prep by spin column.

Plasmid DNA was extracted from bacteria using a Qiagen mini-prep kit using the manufactures guidelines.

5ml LB was supplemented with the appropriate antibiotic and inoculated with bacteria containing the plasmid of interest and grown overnight at 36°C and plasmid DNA was extracted from this and resuspended in 50µl of elution buffer.

2.5.6 Yeast genomic DNA preparation

5ml YE5A or PMG with the appropriate supplements was inoculated with yeast and grown overnight at 30°C (25°C For ts strains). Cells were spun down at 3000rpm for 5 minutes and the supernatant discarded and cells resuspended in 200 µl "yeast crunch" lysis buffer (2% (v/v) Triton X-100, 1% (w/v) SDS, 100mM NaCl, 10mM Tris-HCl pH8.0, 1mM EDTA) and an equal volume phenol:chloroform:isoamyl alcohol (25:24:1) along with glass beads (150-212mm). Tubes were then subjected to bead beating (rapid mechanical vibration in a bead beater, Biospec prducts) for 1 minute to break open the cells before being centrifuged at 13000 rpm for 5 minutes. The upper phase was removed and the DNA was precipitated with ethanol (see 2.4.3) and the pellet dried then resuspended in TE or distilled water.

2.5.7 Agarose gel electrophoresis

Agarose gel electrophoresis of DNA fragments was carried out using 1% (w/v) agarose gels. To make the gels the appropriate amount of agarose was melted in 1x TBE buffer (Tris, boric acid, EDTA) with the addition of ethidium bromide after melting of the agarose. Samples to be loaded were mixed with "blue juice" loading buffer (0.25% (w/v) bromophenol blue, 30% (v/v) sucrose) which was approx. 5x concentration before being loaded in the wells. The gel was placed in a tank of TBE and run at 90-120V (depending on the size of gel). 1kb DNA ladder (Gibco BRL) was included as a size marker.

2.5.8 Extraction of DNA from agarose gels

DNA fragments for cloning were separated by agarose gel electrophoresis (section 2.4.6). Low intensity UV illumination of the gel was used to visualise the DNA bands and the band of interest was cut out of the gel with a scalpel and placed into a 1.5ml Eppendorf. The DNA was extracted from the agarose gel using a Qiagen gel extraction kit and following manufacturer's guidelines.

2.5.9 Restriction digestion of DNA

Restriction digestion of DNA was carried out in a volume of 50µl. This volume typically contained 5µl buffer (typically buffers 1-4 or unique buffers from New England Biolabs restriction enzymes), 10µl DNA, 1-5 units of enzyme and 0.5µl BSA depending on the requirements of the enzyme. This was made up to 50µl with distilled water. The digest was place in water bath at 37°C (or appropriate temperature for enzyme used) for approximately 2 hours.

2.5.10 Ligation of DNA molecules

Ligation reactions were carried out in a volume of 20µl containing:

- 100ng plus vector DNA
- At least 3 times this amount (with respect to the number of moles) insert DNA
- 1x ligation buffer (from a 10x stock)
- 2U T4 ligase
- distilled water to take mix to the correct volume

2.5.11 Amplification of DNA using the polymerase chain reaction

2.5.11.1 The polymerase chain reaction

Specific regions of DNA were amplified using the polymerase chain reaction (PCR). Reaction mix and conditions were dependent on the enzyme used. Reactions were carried out in a 0.5ml PCR tube

Typically Taq polymerase was used but for cloning and other applications where a high fidelity was required, such as cloning, Expand High Fidelity PCR system (Roche) was used following the manufacturer's guidelines. All reactions were carried out in either a PTC-150 minicycle or PTC-200 peltier thermal cycler (both MJ Research). A typical reaction is as follows:

Reaction mix

- 1x PCR buffer (from 10x stock)
- 1x dNTPs (dATP, dTTP, dGTP, dCTP)
- 0.5μM Forward primer
- 0.5μM Reverse primer
- 10-500ng template DNA (depending on source)
- DNA polymerase
- distilled water to 50μl

Reaction conditions

A typical 3 step reaction is as follows:

94°C for 1 minute (initial denaturation)

Followed by 30 cycles of:
94°C for 30 seconds
50°C* for 30 seconds
72°C for 30 seconds[φ]

Final step:
72°C for 5 minutes

* Usual T_d was 50°C but this was variable depending on primers and if product was obtained.
$^{\varphi}$ The extension time is dependent on the size of the product. For large products a longer extension time is needed.

2.5.11.2 PCR from bacterial colonies
A small amount of a single *E.coli* colony was picked with a sterile toothpick and added to the reaction mix. The reaction was carried as described previously (see 2.4.9.2).

2.5.11.3 PCR from yeast colonies
A small amount of cells picked from a single yeast colony with a sterile toothpick and put into an empty PCR tube. The cells were then boiled for 10 minutes at 94°C in the thermal cycler before the PCR reaction mix was added and the reaction carried out as described previously (see 2.4.9.2)
2.5.11.4 Purification of PCR products
DNA fragments obtained from PCR reactions (2.4.10.2) were purified away from excess oligonucleotides, unincorporated dNTPs, polymerase and buffer salts using a QIAquick PCR purification kit (Qiagen) and following the manufacturer's guidelines. The purified DNA products were usually eluted in 50µl elution buffer supplied and stored at -20°C.

2.5.12 PCR mediated cycle sequencing of DNA

Reactions were carried out using BigDye terminator v3.1 ready reaction cycle sequencing kit (Applied Biosystems). The reaction was set up as follows:

- Approx 0.5Ug double stranded template DNA or 180ng PCR product
- 1.6pmol primer
- 4µl terminator reaction mix
- distilled water to 10µl

The reaction conditions were as follows:

25 cycles of:

96°C for 30 seconds

50°C for 15 seconds

60°C for 4 minutes

Samples were run by the university sequencing service.

2.5.13 Primers used in this study

Table 2.8: Primers used in this study

Name	Sequence
Mph1BAHF	GAAAGGTCGGGTGAGTTGAGTAAGCACAAGCGATTAAATAAGGAACTTATTGATAGCATGGCTTATGATTG CGTTAGCAATTTACGAAAAATGCCAGAACGGATCCCCGGGTTAATTAA
Mph1BAHT	GGCTCCGTTATTTGATAACAGTCAAGCTACTCCCATACCCAAGCGTCAGCAGGACGTTGTTACTGTTGCCAA TCTACAATTTATCAAATTAGGAGTTGTTCGGATCCCCGGGTTAATTAA
Mph1BAHR	CTTTTAAAAGAGCGAAAAATTAAAGATTAAAAAAAAAAAGCCTTTAAGCTCCCTAAAAAAGTCGTTAACATT AAGAAACTCGTATGATGTTTGGGAAACTGAATTCGAGCTCGTATTAAAC
Mph1FORN	ATGCTAGTCGACAATGTCTAAGCGCAATCCTCC
Mph1REVC	GCTACCCGGGCTATTCTGGCATTTTTCGTAAATTG
Mph1REVKD	GCTACCCGGGCTAAACAACTCCTAATTTGATAAATTG
Mph1REV3	GTCACCCGGGCTATCGAGATTTCATTTCAATATCC
Mph1ABFOR	CCGGAATTCCGTGCTGAGTACACGTTGACG
Mph1ABREV	GCATGGTCGACCTAGGGAGTAGCTTGACTG

2.5.14 Determination of DNA content of cells by FACS analysis

Cells grown overnight in YE5A or the appropriate selective media and approximately $1x10^7$cells collected by centrifugation. Cells were then resuspended in 70% ethanol and vortexed (at this point cells can be stored at 4°C until needed.)
3ml of 50mM sodium citrate was added to 0.3ml of fixed cells and mixed. The cells were centrifuged and the supernatant removed and then they were resuspended in 0.5ml 50mM sodium citrate containing 0.4mg/ml RNase A and incubated overnight at 37°C.
The cells were then spun down and resuspended in 0.5ml 50mM sodium citrate containing 12μg/ml propidium iodide and left at 4°C until processed in the FACSCAN automated counter. Typically 20000 cells were counted for each sample.

2.6 Protein methods

2.6.1 Crude extraction of total cellular protein from yeast

Cells grown overnight in the appropriate media at the appropriate temperature. Cells were spun down by centrifugation at 3000rpm for 3 minutes. Pellet size was adjusted so each sample contained roughly the same volume. Pellets could then be snap frozen in liquid nitrogen or on dry ice and stored at -80°C. On ice 1x Sample buffer (2% w/v SDS, 80mM Tris-HCL pH6.8, 10% v/v glycerol, 10mM EDTA, Bromophenol blue to give desired colour, distilled water to appropriate volume) containing 1mM DTT, 1mM Pefabloc and LPC (10mg/ml leupeptin, pepstatin and chymostatin) was added to the cell pellet along with glass beads and subjected to ribolysing on setting 4 for 20 minutes (Nybaid Ribolyser). This was then spun down at 13000rpm in a microfuge for 1 minute at 4°C then the samples were boiled for 3 minutes at 96°C.

2.6.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels were poured between 2 glass plates with spacers between them held in a gel pouring rig. The bottom of the gel was sealed with 0.5% agarose in water then the resolving gel was poured on top of this. A thin layer of water saturated butan-1-ol was put on top pf the resolving gel while it polymerised. After polymerisation of the resolving gel the butan-1-ol was removed and a stacking gel layer was poured on top of the resolving gel and an appropriate comb inserted to give the correct number of wells.

The plates were fixed into the electrophoresis tank the chambers filled with running buffer (glycine, 60g Tris base, 0.1% w/v SDS, water to 10L)

Table 2.9 Resolving gel mixes

Reagent	**10%**	**12.5%**	**15%**
40% Acrylamide	3ml	3.75ml	4.5ml
2% Bis-acrylamide	789µl	624µl	516µl
1.5M Tris-HCl pH8.8	3ml	3ml	3ml
10% SDS	120µl	120µl	120µl
Water	5.1ml	4.5ml	3.86ml

Stacking gel recipe

5% w/v acrylamide

0.13% w/v bis acrylamide

125 mM Tris-Cl pH 6.8

0.1% w/v ammonium persulphate

0.01% TEMED

2.6.3 Western Blotting

2.6.3.1 Electrophoretic transfer of proteins from polyacrylamide gels onto nitrocellulose membrane.

Once protein gel was run it was transferred to either a wet or semi dry blotter. A sandwich was assembled with 2 pieces of 3MM filter paper, a piece of nitrocellulose, the gel and then another 2 pieces of 3MM filter paper. These were first soaked in transfer buffer.

Wet transfer – carried out in 1x Towbin buffer (25mM Tris Base, 192mM Glycine, 0.1% SDS, 20% methanol) run at 65V for 2 hours.

Semi dry transfer – run at 20V (180mA for one blot, 250mA for two) for 2 hours.

Transfer of proteins onto the membrane was confirmed by staining for proteins with Ponceau (12.5ml Acetic acid, 0.25g Ponceau stain, 237.5ml water) and the position of the molecular weight markers recorded with a pen mark on the membrane. The membrane was then cut to size and washed in 1x PBST (50ml 10x PBS (137mM NaCl, 2.7mM KCl, 10.1 mM Na_2PO_4, 1.76mM KH_2PO_4, pH adjusted to pH 7.2) 449.5ml water, 0.5ml 20% Tween-20) to remove the Ponceau stain and then the membrane was blocked in "blotto" (2.5g Marvel powdered milk, 45ml dH_2O, 5ml 10X PBS, 50µl 20% Tween-20) for 30 minutes with constant shaking to block unspecific interactions.

2.6.3.2 Antibody binding

The primary antibody was diluted as appropriate in fresh "blotto" and enough was added to cover the membrane. This was incubated at 4°C with constant shaking over night. The membrane was then washed 3 times for 5 minutes with either 1xPBST or

1x high salt PBST (50ml 10xPBS, 50ml 5M NaCl, 400ml dH_2O, 0.5ml Tween) depending on the antibody used. HRP conjugated secondary antibody was diluted into fresh "blotto" and incubated with the membrane at room temperature with shaking for 1 hour with constant shaking. The membrane was then washed in 1x PBST.

2.6.3.3 Immuno-detection with Enhanced Chemiluminescence (ECL)

Equal quantities of ECL reagents 1 and 2 (Amersham Biosciences) were mixed together and applied to the nitrocellulose membrane for one minute ensuring that the membrane was uniformly covered. Excess ECL solution was removed by blotting on 3MM paper and the membrane was placed on a glass plate and wrapped in cling film before being exposed to Kodak Bio-Max Light film. Exposure times varied depending on the antibody used and the strength of the signal.

2.6.4 Immuno-precipitation (IP)

Typically 50ml cultures were grown overnight and cell pellets collected by centrifugation before being lysed in 500µl lysis buffer (50mM HEPES (pH7.6), 75mM KCl, 1mM $MgCl_2$, 1mM EGTA, 0.1% triton X-100, 1mM Pefabloc and LPC) by bead beating for 1 minute. The lysed cells were spun down at 4°C at 13000 rpm for 3 minutes. The supernatant was transferred to a new 1.5ml eppendorf tube and 1mM DTT added. Another two clarifying spins were then carried out and after the final spin a 50µl sample was removed and frozen at -80°C to be used as whole cell extract. The affinity matrix (typically Protein A dynabeads) were washed three times in 1X PBS +) 0.1% Triton X-100 before being incubated in 100µl PBS + Triton and

the appropriate antibody (2.5µg antibody/10µl beads). This was incubated at 4°C with rotation for at least 1 hour. The beads were then washed twice in PBS + Triton and once in lysis buffer and the cell lysate added to them. The beads were incubated at 4°C with rotation for at least 2 hours. After incubation the supernatant was removed from the beads and they were washed three times in lysis buffer and twice in 1X PBS.

To remove the bound protein from the beads they were incubated in 1X sample buffer with 5% β-mercaptoethanol instead of DTT for 15 minutes at room temperature. The supernatant was then removed and the beads discarded.

λ-Phosphatase treating IPs

If the IP was to be phosphatase treated then after the last PBS wash step the beads were then washed in λ-phosphatase buffer (50mM Tris-HCl, 0.1mM Na_2EDTA, 5mM DTT, 0.01% Brij 35 and 2mM $MnCl_2$) and then incubated in 50µl λ-phosphatase buffer with 0.25 µl λ-phosphatase at 37°C for 30 minutes.

2.6.5 SZZ-tag pull down

3L cultures of yeast containing Mph1SZZ-tag were grown up overnight at 30°C in 4xYES to OD_{600}=12. If cells were to be arrested using the *nda3-KM311* mutation the temperature would be shifted to 18°C and cells incubated for a further 6 hours. Cells were pelleted in a centrifuge and the cell pellets resuspended in a minimal amount of dH_2O before being drop frozen in liquid nitrogen. The cells were lysed by grinding in an automatic grinder under liquid nitrogen.

60g of yeast powder was weighed out and resuspended in 60ml of 2x Hyman buffer (100mM bis-Tris propane pH7, 200mM KCl, 10mM EGTA, 10mM EDTA, 20% glycerol) including 1mM Pefabloc and LPC as well as phosphatase inhibitors (10mM

NaF, 0.4mM orthovanadate and 100nM microcystin). Once the powder was fully resuspended 12ml 10% Triton X-100 was added. The cell suspension was sonicated for 1 minute then pelleted in an ultracentrifuge at 45 000 rpm for 30 minutes at 4°C. 0.65g IgG sepharose slurry was washed with 10ml TST (50mM Tris-HCl pH7.4, 150mM NaCl, 0.1% Tween-20), 1ml 0.5M NH_4OAc pH3.4, 5ml TST, 1ml 0.5M NH_4OAc pH3.4, 5ml TST, 5ml 1X Hyman with 300mM KCl to remove unbound IgG.

The cell lysate was added to the IgG sepharose and incubated at 4°C with rotation for 4 hours. The supernatant was then removed from the IgG sepharose beads and they were washed with 1X Hyman buffer with 300mM KCl, then 1X Hyman buffer with 300mM KCl + 1mM DTT +0.1% Tween-20. The beads were resuspended in 1.5ml 1X Hyman buffer with 300mM KCl + 1mM DTT +0.1% Tween-20 and TEV protease and this was incubated over night at 4°C with rotation.

The supernatant was removed from the IgG beads and added to 85µl S-protein agarose slurry and incubated at 4°C with rotation for 4 hours. The supernatant was removed from the beads and they were washed with 10ml 1 Hyman buffer.

For mass spec analysis the beads were washed in a buffer of 50mM Tris pH8.8, 5mM EGTA, 1mM EDTA, 75mM KCl and the protein sample was eluted off the beads with 50µl urea buffer (50mM Tris pH8.8, 8M urea).

Mass spec analysis was carried out by Ali Sarkeshik at the Scripps Research Institute in California.

2.6.6 Creation of a polyclonal antibody

2.6.6.1 Expression of a GST-tagged fusion protein in bacteria

A 389bp section of Mph1 was transformed into the pGEX-6p vector (ref) was transformed into BL-21 chemical competent cells. 4L of transformed bacteria was

grown up in LB containing 75μg/ml ampicillin overnight at 37°C. When the OD_{600}=0.8-1 then IPTG was added to a concentration of 0.1 M and the cultures grown overnight at 18°C. This was to induce expression of the fusion protein. The cells were pelleted at room temperature in a centrifuge at max. speed for 10 minutes and the pellet resuspended in a minimal amount of dH_2O and drop frozen into liquid nitrogen. The frozen bacteria pellets were lysed by grinding in an automated grinder under liquid nitrogen. The cell powder was warmed to room temperature and when it had started to thaw it was resuspended in 5 volumes of lysis buffer (1X PBS containing 0.5% Tween-20, 1mM Pefabloc, 1M NaCl) and sonicated for 1 minute. After sonication DTT was added to 10mM. The lysates were spun at 35 000 rpm in an ultracentrifuge at 4°C. Glutathione agarose (Sigma) was rehydrated in PBS as per manufacturing instructions to a volume of 5-10 ml. This was packed into a column and the supernatant from the bacterial lysate was passed over it. The column was washed with 100ml wash buffer (1X PBS containing 0.05% Tween-20, 0.5mM DTT and 0.25M KCl) The flow through from this wash step was monitored by Bradford assay to make sure no protein was still coming off at the end of the wash step, if there was then washing was continued until no protein was detected. At the end of the wash step the column was then washed with 2 column volumes of wash buffer without Tween-20.

The column was eluted with elution buffer (50mM Tris pH8.1 containing 0.25M KCl and 5mM reduced glutathione). The elution was carried out by pipetting 1ml volumes of elution buffer directly onto the top of column bed. Each aliquot of flow through was collected in eppendorf tubes and assayed by Bradford assay to determine the peak fractions. These were then pooled and dialysed over night at 4°C in dialysis buffer (50mM HEPES pH7.6, 100mM KCl and 30% glycerol).

The dialysed sample was then checked on a polyacrylamide gel to ensure expression of the fusion protein had occurred. This was checked by staining the gel with Coomassie (500ml, 2.5g Coomassie blue R250, 225ml methanol, 50ml glacial acetic acid, 225ml dH_2O) to stain for protein. Excess Coomassie stain was removed by incubating the gel in desatin (125ml methanol, 35ml acetic acid, 340ml dH_2O).

The GST-$Mph1^{171-321}$ fusion protein was sent to Diagnostic Scotland to be injected into a rabbit and a sheep.

2.6.6.2 Coupling proteins to Affigel 10

In order to build a column to be used to purify the bleeds containing the polyclonal Mph1 antibody the GST-Mph1$^{171-321}$ had to be coupled to Affigel 10.
The desired amount of Affigel 10 was poured into a 15ml Falcon tube and washed into coupling buffer (50mM HEPES pH7.6 and 100mM KCl) by resuspending the beads in ice cold buffer and pelleting them in a bench top centrifuge at 4°C 3 times. After the final wash a 1:1 suspension of beads in coupling buffer was made the beads pelleted. The fusion protein was added to give a concentration of 1-5mg/ml. The beads were immediately pelleted and a 5µl sample taken and added to Bradford reagent to give a standard to compare the other samples against. The coupling reaction was incubated at 4°C with rotation and time points taken every 5 minutes by spinning down the beads and taking 5µl of supernatant. These time points were monitored by Bradford assay and when 25µl looked like the 5µl t=0 time point then the coupling reaction was complete.
The coupling reaction was stopped by adding 1M Tris pH7.5 to a final concentration of 50mM.

2.6.6.3 Affinity purification of polyclonal antibodies.

The Affigel column was washed with several column volumes of elution buffer (100mM triethylamine pH11.5 then 100mM glycine pH2.5). The column was then equilibrated with 1X PBS.
The crude serum was spun down for 10 minutes at 15000 rpm and sodium azide added to 0.04% to prevent bacterial growth.
The serum was passed over the column using a motorised pump at a flow rate of approximately 5 column volumes per hour. After all the serum had been passed over the column the column was washed with 2 column volumes of 0.2X PBS.
The column was first eluted with 100mM triethylamine pH 11.5. The elution was carried out by pipetting 1ml aliquots of elution buffer directly on to the column bed

and collecting each fraction in separate eppendorf tubes. The fractions were monitored by spotting a couple of µls on to nitrocellulose and staining with Ponceau to check for the presence of protein. Ten fractions were collected using this elution buffer then the elution was repeated using glycine. The fractions were neutralised by addition of the appropriate volume of 1M Tris-HCl (pH 6.8 to neutralise the triethylamine and pH7.4 to neutralise the glycine). The pooled fractions from each elution buffer were kept separate and dialysed into PBS containing 50% glycerol. The purified antibody was stored at -20°C.

2.7 Sera used in this study

Name	Concentration	Source
Anti-Mph1 Rb	1/1000	This study
Anti-Mph1 Sh	1/1000	This study
Tat1 (Ms monoclonal)	1/30(IF), 1/1000(Wb)	Keith Gull, University of Manchester
Anti rabbit HRP	1/3000	Amersham
Anti mouse HRP	1/3000	Amersham
Anti sheep HRP	1/10 000	Oxford Biotechnology

Table 2.10: Sera used in this study. Rb = rabbit, Sh = sheep, Ms = mouse, IF = immunofluorescence, Wb = western blotting

2.8 Microscopy

2.8.1 General microscopy

For live cell microscopy a 10µl sample of cell suspension was put onto a slide and covered with a cover slip.

For fixed cell microscopy cell were first fixed in ice cold methanol before being applied to a polylysine slide and a 10µl drop of Vectashield added before being

covered with a coverslip. If DNA was to be studied then Vectashield containing DAPI was used.

For imaging septa then cells were first fixed in ice cold methanol before being spun down and resuspended in PEM (100 mM Pipes pH6.9, 1 mM EGTA, 1 mM $MgSO_4$) and calcoflor added.

2.8.2 Immunofluorescence

Cell cultures grown overnight in the appropriate growth conditions. Filter cells and fix in ice cold methanol. The cells were spun out of methanol and resuspend in PEM (100 mM Pipes pH6.9, 1 mM EGTA, 1 mM $MgSO_4$), which was repeated twice. After the second washing in PEM the cells were incubated at 37°C for 30 minutes in PEMS (100 mM Pipes pH6.9, 1 mM EGTA, 1 mM $MgSO_4$, 1.2M Sucrose) containing 0.4mg/ml zymolyase. The cells were then washed twice in PEMS and resuspended in PEMS containing 1% Triton X-100 for 30 seconds. The cells were washed twice in PEM before being blocked in 5% milk in PEM (+ .1% azide to prevent bacterial growth) for 30 minutes. After blocking the appropriate amount of primary antibody in milk+PEM was added and the cells were incubated overnight at 4°C.

After the overnight incubation the primary antibody was removed but washing four time in milk+PEM and the secondary antibody in milk+PEM was added and incubated at room temperature for at lest 1 hour. The cells were then washed in PEM before being mounted on a slide for microscopy.

Chapter 3

Importance of the kinase domain for the checkpoint functions of Mph1.

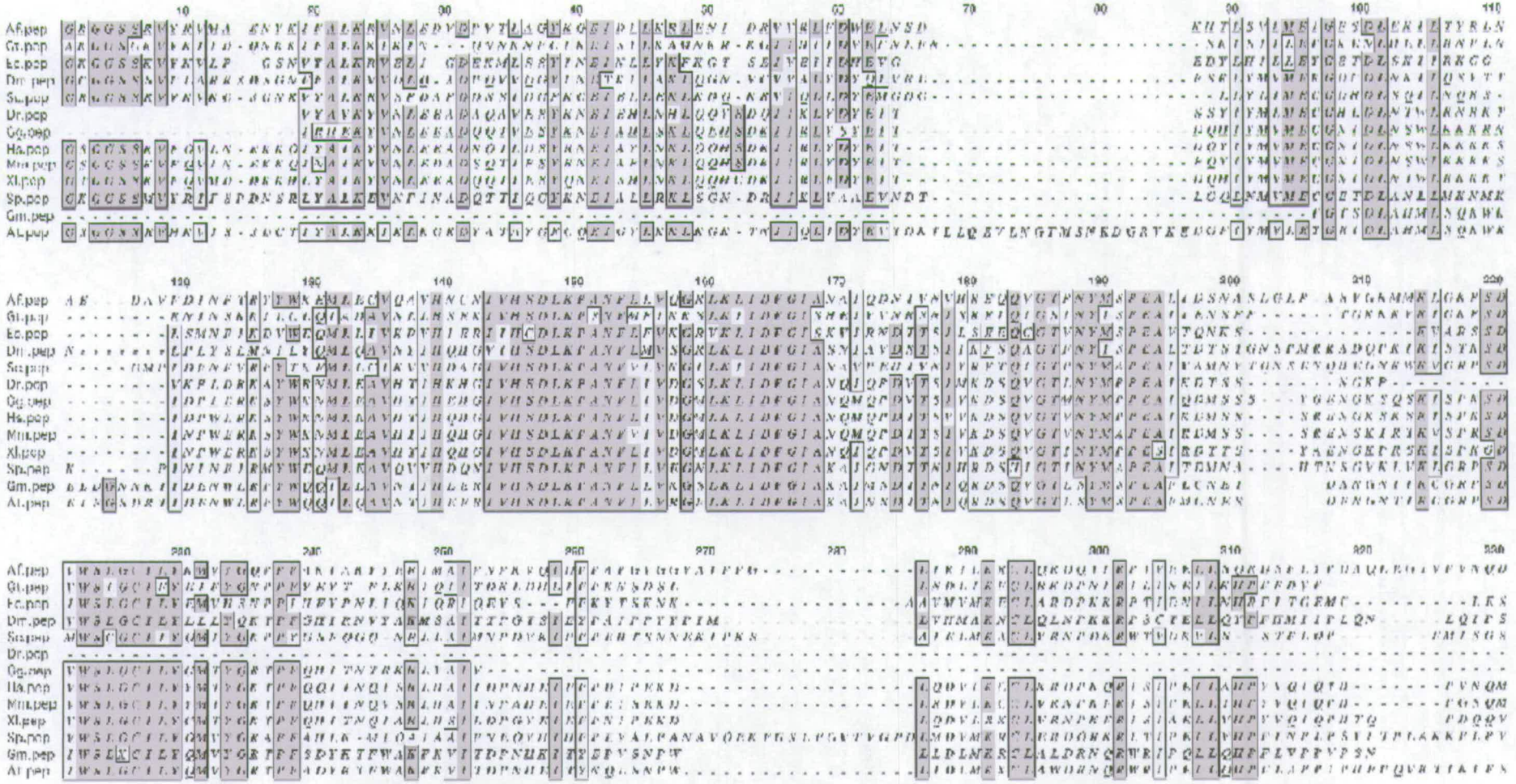
3.1 Introduction

The greatest sequence homology between members of the MPS1 family of protein kinases is found in the C-terminal kinase domain with most members having roughly 40% sequence identity with *S.cerevisiae* Mps1 (Figure 3.1). This is in comparison to the N-terminus which is very poorly conserved between the homologues in different organisms (Fisk and Winey, 2001). It is possible, then, that the kinase domain will have similar functions in members of the Mps1 family in different organisms.

In order to determine the importance of the kinase domain in budding yeast and vertebrate cells, kinase dead mutants have been made, as well as an analogue-sensitive version of *S.cerevisiae* Mps1. A typical kinase domain consists of 11 subdomains (Hanks et al., 1988). The kinase-dead allele of *S.cerevisiae* Mps1 was made by mutating D580 to A in a short sequence (DFG) which is in subdomain 7 (subdomains of the kinase domain illustrated in Figure 3.1) which is a highly conserved region in the kinase domains of all Mps1 homologues (Lauze et al., 1995). This allele was shown to no longer have the ability to phosphorylate MBP (maltose binding protein) *in vitro* and it exhibited greater mobility through a gel indicating that while Mps1 may phosphorylate itself this allele was not capable of this (Lauze et al., 1995).

In fission yeast it has been shown that the N-terminal region and the C-terminal kinase domain of Bub1 kinase have separate functions. The kinase domain is not required for a checkpoint arrest as strains with mutations in this region or with the kinase domain removed were still able to maintain an arrest in the presence of

Figure 3.1: Alignment of kinase domains of Mps1 homologues
A.f. Aspergilus fumigatis, A.t. Arabidopsis thaliana, D.m. Drosphila melanogaster, D.r. Danio rio (Zebra ®sh), E.c. Encephalitozoon cuniculi, G.g. Galus galus, G.m. Glycine max, H.s. Homo sapiens, M.m. Mus musculus, S.c. Saccharomyces cerevisiae, S.p. Schizosaccharomyces pombe, X.l. Xenopus laevis
The kinase domain is the most conserved region between members of the Mps1 family of kinases



Adapted from Winey and Huneycutt,. (2002)

microtubule drugs which destabilise microtubule and the *nda3-KM311* mutation which depolymerises microtubules (Vanoosthuyse et al., 2004). The N-terminus is required for targeting to the kinetochore and for Bub1 to function in the spindle checkpoint, whereas the kinase domain is required for the function of Bub1 in chromosome segregation. This study used a version of Mph1 where the kinase domain had been replaced by a marker gene (G418 resistance gene) to investigate the importance of the kinase domain in the functions of Mph1.

Overexpression of Mph1 and Mps1 in fission yeast and budding yeast respectively results in cells arresting with the checkpoint active (He et al., 1998), (Hardwick et al., 1996).
There is evidence from other organisms that the kinase domain of Mps1 is important for its role in the spindle checkpoint. This chapter will discuss whether or not this is the case with Mph1 and whether overexpression of Mph1-KΔ is able to establish a checkpoint arrest in fission yeast.

Nothing is known about the localisation of Mph1 in fission yeast at the current time and as such it is not known whether it has an involvement in spindle pole body (SPB) duplication like its budding yeast homologue or not (Winey et al., 1991). This is unlikely as if it was the case *mph1Δ* cells would probably be dead. It has already been shown is other organisms that Mps1 is generally localised to the kinetochore in mitosis, particularly during a mitotic arrest that activates the checkpoint, for example a nocodozole arrest (budding yeast , mouse (Fisk and Winey, 2001), human (Stucke et al., 2002), *Xenopus laevis* (Abrieu et al., 2001)). It was expected that this will be the case for Mph1 in fission yeast as it's involvement in the spindle checkpoint would suggest a localisation pattern similar to that the other components of the checkpoint in fission yeast.

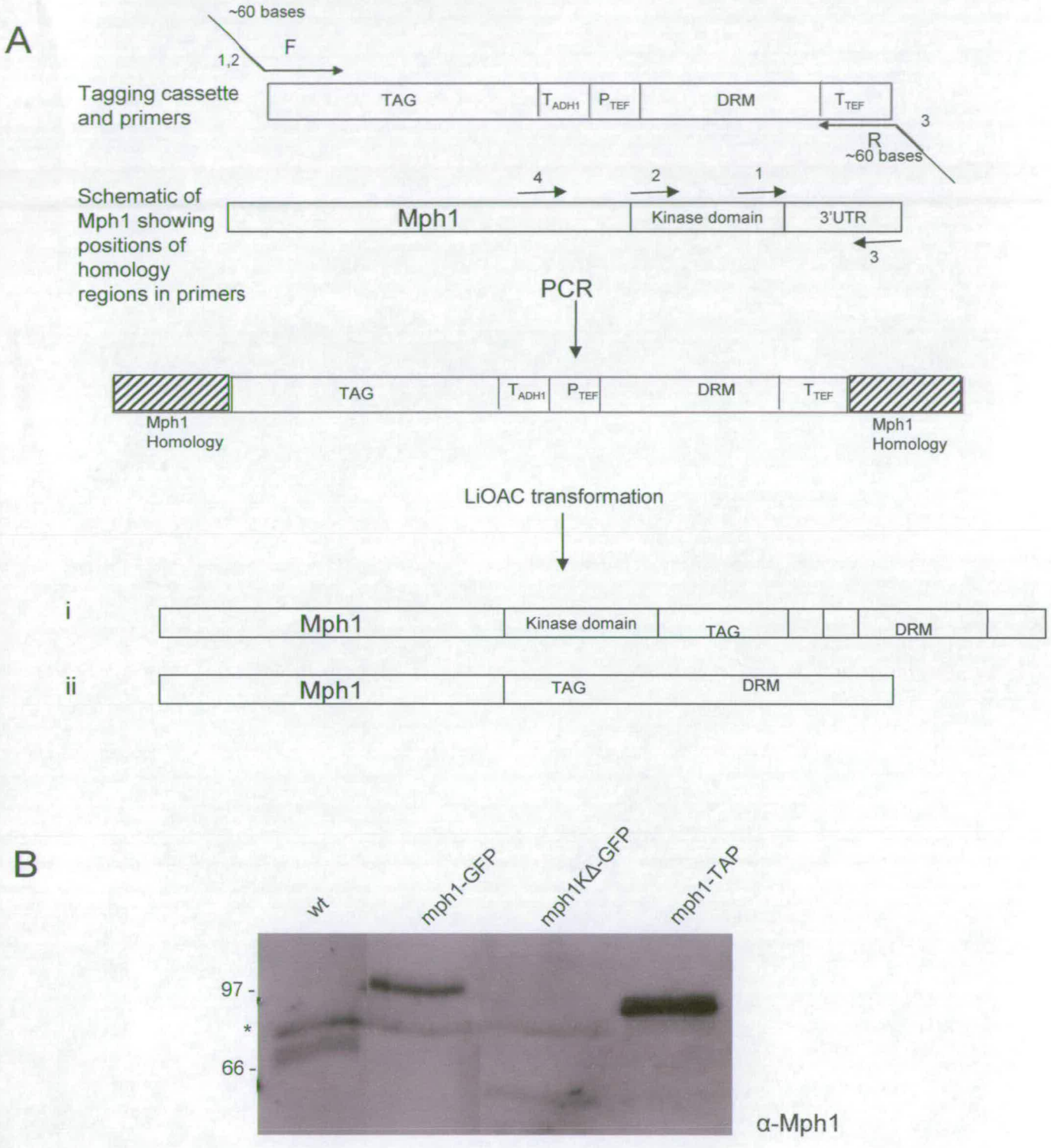
3.2 PCR-based tagging method

In order to study Mph1 it was beneficial to have it with a GFP or TAP tag on it. It was tagged with both green fluorescent protein (GFP) and the tandem affinity purification (TAP) tag. The GFP tagged strains were used in order to localise Mph1 by microscopy and the TAP tagged strains were used for biochemistry. This tagging method was also a convenient way of making a kinase-deleted version of Mph1. This was created by replacing the kinase domain of Mph1 with a GFP tag.

Tagged strains were made using a PCR-based strategy where primers were designed that contained homology to the tagging cassette of interest and to either the 3' untranslated region or just before the start of the kinase domain (See materials and methods primers MPH1BAHF, MPH1BAHT and MPH1BAHR) to give either a full length or kinase deleted tagged construct. The template used contained the tag of interest and a selectable marker (G418 resistance gene for the GFP tag and clonNAT resistance gene for the TAP tag) (Bahler et al., 1998). PCR generated a product that had the tag of interest, a selectable marker and homology to Mph1 at its 5' and 3' ends (figure 3.1). This was then integrated into the fission yeast genome by lithium acetate transformation and the transformants plated out onto YE5A media and incubated at 30°C. When colonies had formed these were replica plated on to selectable media in order to identify transformants. PCR of genomic DNA from these transformants using a primer inside the *Mph1* gene ((Figure 3.1, primer 4) was used to sequence the junction between Mph1 and the tag. Transformants were also subject to Western blot analysis to verify that the products were the correct size (Figure 3.1C). The Western blot was probed with rabbit α-Mph1 antibody (see Section 4.2 for details) to ensure any bands of different sizes seen were Mph1.

Figure 3.2: C-terminal PCR tagging of Mph1 using Bahler tagging cassettes (Bahler et al, 1998).
A.
The C-terminal tagging cassettes used were GFP and TAP. Both are C-terminal cassettes so don't contain a promoter . They have a drug resistance marker (DRM) downstream of the tag (G418 for GFP and NAT for TAP).
Forward and reverse primers (F and R on the schematic) were created to have homology to the tag and homology to regions of Mph1 (1 and 3 for full length, 2 and 3 for kinase deletion). PCR products generated were transformed by LiOAC transformation to give:
(i) full length tagged Mph1
(ii) Kinase deleted Mph1
B Western blot showing tagged strains. * indicates a non-specific band. Stains containing wt Mph1 and those clones thought to be positive for mph1-GFP, mph1KΔ-GFP and mph1-TAP were run out on the gel. The Western was probed with rabbit α-Mph1 primary antibody, see section 4.2 for details

Strains were checked using primer 4 to verify that they were tagged correctly by sequencing the junction between Mph1 and the tag.


A
~60 bases
F
1,2
Tagging cassette and primers
TAG
T_{ADH1}
P_{TEF}
DRM
T_{TEF}
3
R
~60 bases
Schematic of Mph1 showing positions of homology regions in primers
4
2
1
Mph1
Kinase domain
3'UTR
3
PCR
TAG
T_{ADH1}
P_{TEF}
DRM
T_{TEF}
Mph1 Homology
Mph1 Homology
LiOAC transformation
i
Mph1
Kinase domain
TAG
DRM
ii
Mph1
TAG
DRM
B
wt
mph1-GFP
mph1KΔ-GFP
mph1-TAP
97 -
*
66 -
α-Mph1

3.3 Benomyl sensitivity

In order to test at the importance of the kinase domain in the role of Mph1 in the spindle checkpoint a kinase deletion strain was made as described in section 3.1. The resulting *mph1KΔ-GFP* was viable in the absence of spindle damage. In order to study the affect of deleting the Mph1 kinase domain, *mph1KΔ-GFP* was tested for benomyl sensitivity. GFP tagged full length Mph1 was also tested to make sure that the tag did not impair the function of Mph1 and confer a phenotype on benomyl (figure 3.2).

Benomyl is a microtubule destabilising drug which perturbs kinetochore-microtubule interactions and as such most strains deleted for checkpoint components are at least mildly benomyl sensitive. This is because they do not arrest in response to impaired kinetochore-microtubule attachments due to the lack of a functional checkpoint. It has already been shown that Mph1 and Bub1 kinase deletions are among the most benomyl sensitive of the spindle checkpoint components (Vanoosthuyse et al., 2004) with Mph1 appearing even more sensitive than Bub1 (figure 3.2). To test how sensitive *mph1KΔ-GFP* was to benomyl serial dilutions of wt, *mph1Δ*, *bub1Δ* and *mph1KΔ-GFP* strains were plated out on to YE5A plates and YE5A plates containing 8μg/ml and 10μg/ml benomyl. This strain was compared to *mph1Δ* and *bub1Δ,* as well as *mad2Δ,* one of the less benomyl sensitive members of the spindle checkpoint mutants in fission yeast. Figure 3.2 shows that *mph1KΔ-GFP* is very benomyl sensitive, however it would appear that it is not as sensitive to this drug as the full Mph1 deletion but is still more sensitive than *mad2Δ.*

A

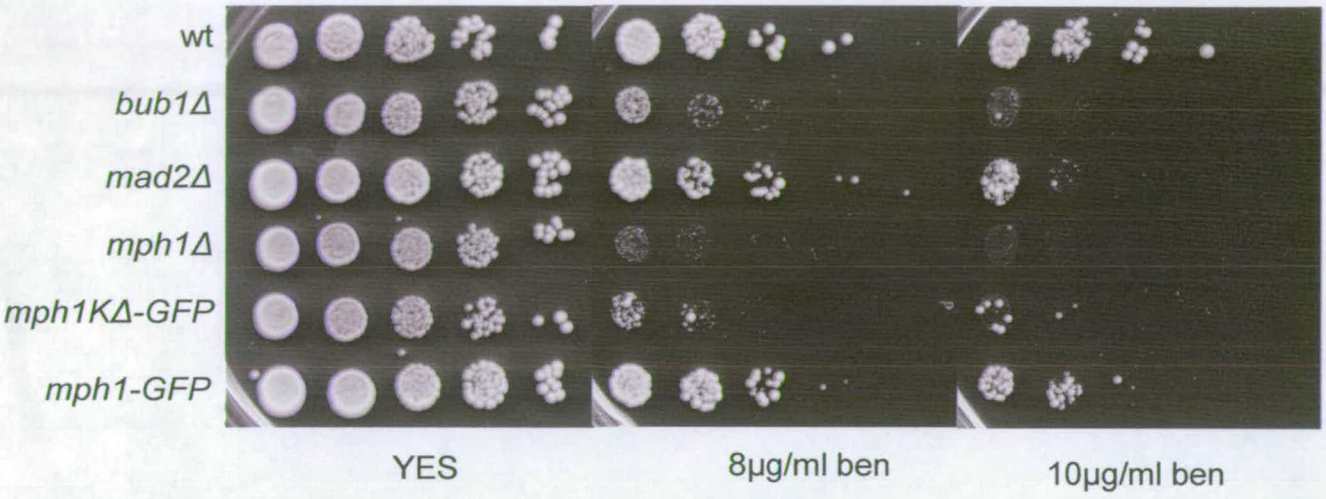


Figure 3.3: Benomyl sensitivity of checkpoint mutants

Serial dilutions (10-fold) were made with several checkpoint mutants and plated onto YE5A, 8µg/ml benomyl and 10µg/ml benomyl. Images were taken after 3 days at 30°C. On benomyl *mph1Δ* the most sensitive of the checkpoint mutants with *bub1Δ* slightly less sensitive but still more so that *mad2Δ*. The *mph1KΔ-GFP* does not appear to be as benomyl sensitive as *mph1Δ but* is more sensitive than *mad2Δ*. In terms of sensitivity to the drug it is comparable to *bub1Δ*.

3.4 The kinase domain of Mph1 is required for an arrest in response to microtubule depolymerisation.

The *nda3-KM311* mutation is a cold sensitive mutation in the *S.pombe* β-tubulin gene. Cells grown at 18°C containing the *nda3-KM311* mutant arrest in metaphase and this arrest is checkpoint dependent. This was due to an absence of both attachment and tension in this mutant (Hiraoka et al., 1984). When cells containing the *nda3-KM311* mutation were grown at 18°C they could not build a mitotic spindle meaning that cells with a functional spindle checkpoint should arrest. However, if the checkpoint is compromised they will not arrest but attempt to continue through mitosis in the absence of a mitotic spindle. This can be used as an assay to determine whether the spindle checkpoint is functional or not in combination with various other mutations. If the mutation of interest renders the checkpoint non-functional, cells will not arrest but attempt to go through anaphase in the absence of a spindle leading to aneuploidy and eventually cell death.

Using the *nda3-KM311* strain it was possible to score several factors which indicate a compromised spindle checkpoint such as loss of viability, septation and the *cut* phenotype which indicates chromosome mis-segregation.

3.4.1 *GFP-plo1*$^+$

One way of assessing checkpoint function in the *nda3-KM311* mutant was to use a strain that also contained expresses GFP tagged Polo. Polo is a kinase which has multiple roles in the cell. It is required for mitotic spindle formation, chromosome segregation and cytokinesis (Mulvihill et al., 1999). In fission yeast Polo kinase (Plo1) is recruited to SPBs during mitosis, and *GFP-plo1*$^+$ does not associate with

interphase SPBs, making this a useful indicator that cells are in mitosis (Mulvihill et al., 1999).

Strains containing *GFP-plo1*$^+$ under the control of the *nmt81* promoter and also containing *nda3-KM311* were used to look at *mph1Δ* and *mph1KΔ-GFP* over a 6 hour time course at 18°C with samples taken every hour. The number of cells showing *GFP-plo1*$^+$ at SPBs was counted as a percentage of total cells. During this time course it was possible to assess both whether the kinase domain of Mph1 was required to establish a checkpoint arrest and, if it could, whether it was required for the maintenance of the arrest. In an *nda3-KM311* strain with a functional spindle checkpoint the percentage of *GFP-plo1*$^+$ spots seen at SPBs increased steadily after 3 hours to give almost 90% of cells with *GFP-plo1*$^+$ at SPBs at 6 hours in the arrest. It was apparent from the results shown in figure 3.3 that this was not the case in an *mph1* mutant background. Neither *mph1Δ* nor *mph1KΔ-GFP* ever got above 5% of cells showing *GFP-plo1*$^+$ spots at their SPBs indicating that at no point during the time course does either strain delay or arrest in mitosis.

3.4.3 *mph1KΔ-GFP* dies rapidly in the presence of the *nda3-KM311* mutation

A second assay used to assess spindle checkpoint function in an *nda3-KM311* mutant is rate of death. If cells can not arrest in response to lack of a mitotic spindle they may attempt to go through an aberrant anaphase which will result in cells with a *cut (cells untimely torn)* phenotype, leading to aneuploidy and ultimately cell death.

For the rate of death assay cells were grown at 18°C for 6 hours and samples taken every 2 hours. At the first time point the number of cells per ml was counted using a haemocytometer in order to work out the volume need to plate a certain number of cells per plate (roughly 300 cells per plate). This volume was then plated out onto YE5A plates at every time point

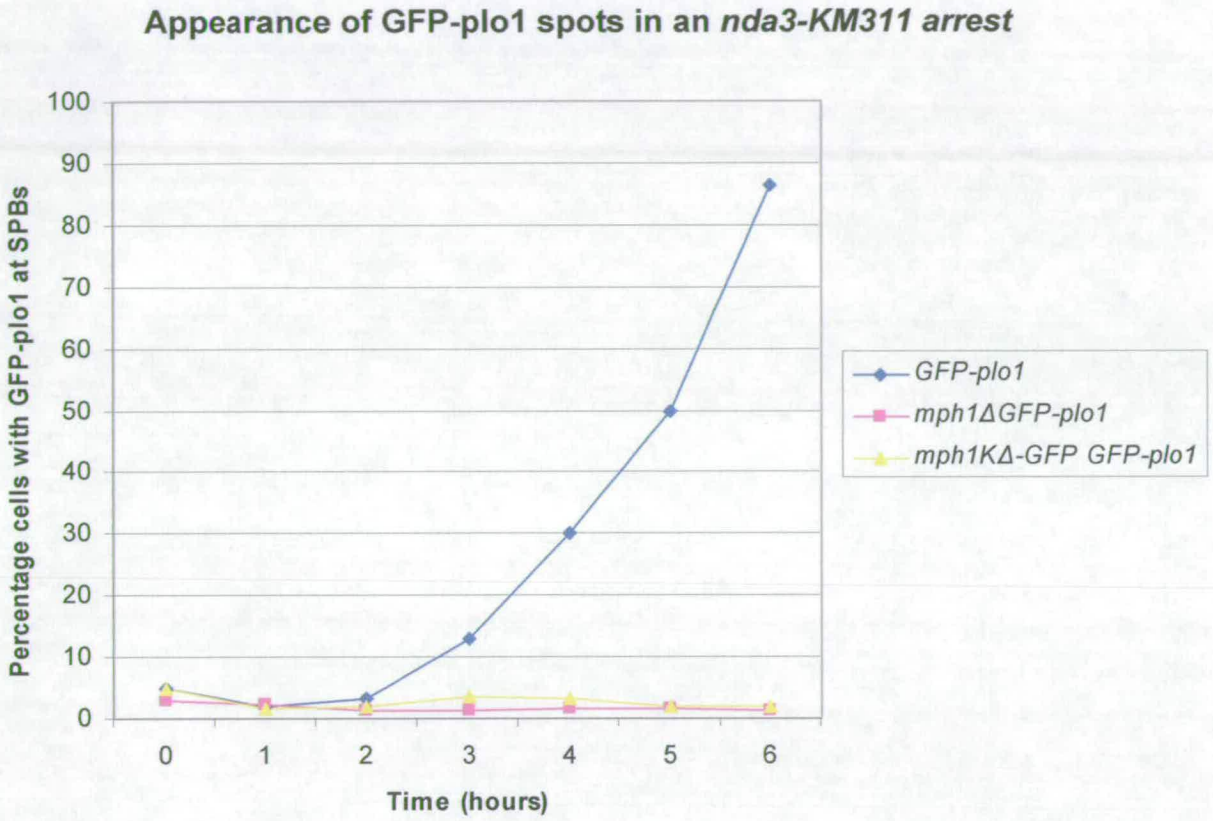


Figure 3.4: Mph1 mutants never show a high frequency of GFP-plo1 on SPBs in combination with the *nda3-KM31*1 mutation.

The *nda3-KM311* mutation was used to arrest cells containing *GFP-plo1,* under the control of the *nmt81* promoter. GFP-plo1 associates with SPBs during mitosis. *nda3-KM311* were grown at 18°C for 6 hours with samples taken every hour. Presence of GFP-plo1 at SPBs in methanol fixed cells was used as an indicator of whether the *mph1Δ* and *mph1KΔ-GFP* cells were able to arrest in response to the *nda3-KM311* mutation.
nda3-KM311 cells with a functional checkpoint arrest and *GFP-plo1* is enriched onto SPBs.
The *mph1Δ* and *mph1KΔ-GFP* do not arrest so this enrichment is never seen.

ensuring that roughly the same number of cells would be on each plate. If the *mph1KΔ-GFP* cells were dying in response to the *nda3-KM311* mutation the number of cells able to from colonies per plate would decrease as the time course continued. This was the case for both *mph1Δ* and *bub1Δ* as both begin to lose viability rapidly after 2 hours at 18°C (figure 3.4A). These both have a compromised spindle checkpoint and from figure 3.4A it can also be seen that *mph1KΔ-GFP* dies rapidly at 18°C, after only 2 hours. The likely reason for this inviability is that they attempt to continue on through anaphase in the absence of a spindle and lose chromosomes as a result.

3.4.3 *mph1KΔ-GFP* cells septate in the presence of the *nda3-KM311* mutation

Figure 3.4A has shown that *mph1Δ* and *mph1KΔ-GFP* die rapidly in combination with the *nda3-KM311* mutation. To investigate the reason for this an assay to quantitate septation in these cells was used. If cells arrest in response to the *nda3* mutation septa would not be seen as cells would remain in metaphase and not exit mitosis. If, however these cells do not arrest then they may attempt to continue on through anaphase and exit mitosis in the absence of a spindle. This would lead to aberrant chromosome segregation, aneuploidy, and cell death.

To investigate whether this was what was happening in the Mph1 mutants *mph1Δ nda3, mph1KΔ-GFP nda3* as well as *bub1Δ nda3* and *nda3* strains were grow at 30°C overnight to log phase and then shifted to 18°C and samples were taken every hour over a 6 hour time course. These samples were fixed with methanol before being resuspended in PEM and calcofluor (to visualize septa) and mounted with Vectashield containing DAPI.

Once again the *mph1Δ* and *bub1Δ* strains were used as positive controls for loss of checkpoint function as these are known to have a non-functional checkpoint and the *nda3* strain on its own was used as a positive control.

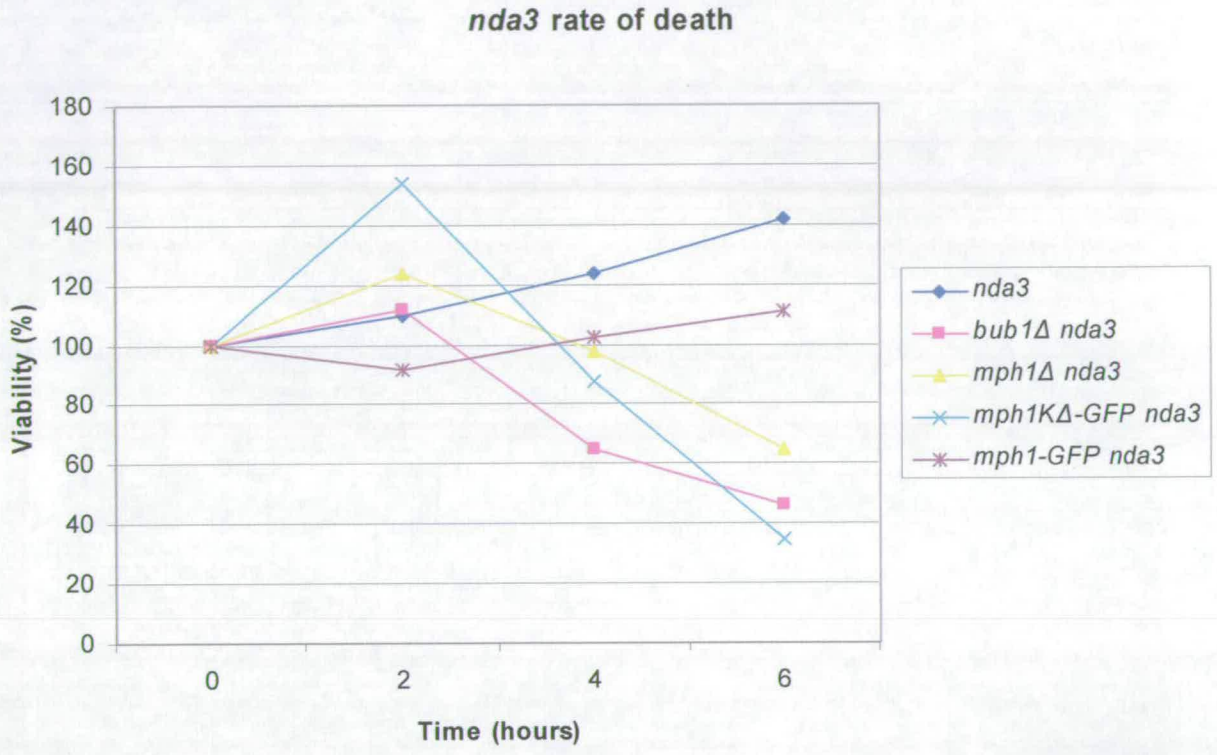
Figure 3.5: The kinase domain of Mph1 is required for it to sustain a mitotic arrest in the absence of a mitotic spindle

A. Cells were grown at 18°C for 6 hours and time points taken every 2 hours and plated out on YE5A plates.The *nda3-KM311* rate of death assay showed a rapid rate of death in combination with all checkpoint mutants tested, *mph1Δ, bub1Δ* and *mph1KΔ-GFP*, which is not seen in the *nda3* mutant alone.

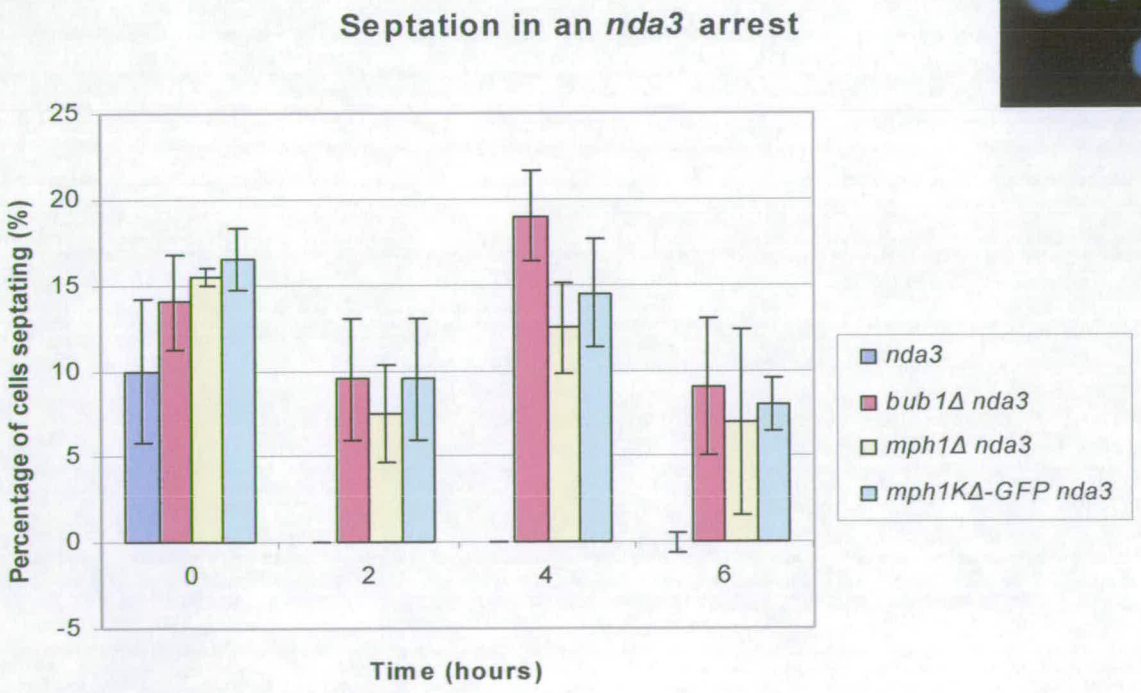
B. Cells were grown at 18°C for 6 hours with 2 hour time point. Calcofour was used to visualise septa. Taken as a percentage of total cells *nda3* on its own does not septate from 2 hours onwards whereas the checkpoint mutants continue to septate throughout the time course.

C. Inset shows a picture of *cut* cells from the *mph1Δ* mutant with the septa cutting thorough DNA that has not segregated properly. Scale bar shows 2μm.

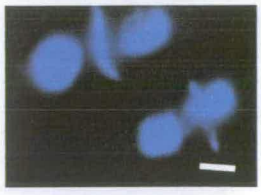
A


nda3 rate of death
Viability (%)
180
160
140
120
100
80
60
40
20
0
0
2
4
6
Time (hours)
nda3
bub1Δ nda3
mph1Δ nda3
mph1KΔ-GFP nda3
mph1-GFP nda3

B


Septation in an nda3 arrest
Percentage of cells septating (%)
25
20
15
10
5
0
-5
0
2
4
6
Time (hours)
nda3
bub1Δ nda3
mph1Δ nda3
mph1KΔ-GFP nda3

C



It could be seen when compared to a mutant carrying only the *nda3-KM311* mutation that the *mph1KΔ-GFP* mutant did not appear to arrest. Septa are seen throughout the time course at around the same percentage as at the beginning of the time course (figure 3.4B). This suggested that these cells attempted to continue cycling through the cell cycle in the absence of a mitotic spindle. The occurrence of cells showing a *cut* phenotype indicated that aneuploidy was occurring as a result. Therefore *mph1KΔ-GFP* could not arrest in the absence of a mitotic spindle and the reason for the rapid rate of death in combination with the *nda3-KM311* mutation seen in figure 3.4A is due to aneuploidy cause by aberrant chromosome segregation.

3.5 *mph1Δ* and *mph1KΔ-GFP* fail to arrest in response to spindle defects and continue to re-replicate their DNA.

Flow cytometry (FACS) analysis of *mph1Δ* and *mph1KΔ-GFP* was used as an assay to complement the *nda3* assays described previously. FACS is a method used to study the DNA content of a cell. Cells were fixed in ethanol and their DNA stained with propidium iodide which allowed DNA content to be measured by the FACScanner. FACS is a favourable method to used for this as it allows 20 000 cells to be counted very rapidly. A FACS profile was generated showing the percentage of cells with increasing amounts of DNA. Cells with unreplicated DNA show a peak at 1N to the left hand side of the graph. Cells with replicated DNA will then have a 2N peak to the right of this. If cells have become aneuploid and attempt to re-replicate their DNA a 4N peak will be seen as they will have a larger DNA content than cells with a single replicated genome.

FACS was used to study cell cycle progression in *mph1Δ, mph1KΔ-GFP* and *mad2Δ* mutants combined with *cut7-24,* a temperature sensitive mutant for a kinesin involved in spindle microtubule interdigitation during mitosis (Hagan and Yanagida, 1990;

Hagan and Yanagida, 1992). Cells arrest in response to this mutation with spindle defects (a V-shaped spindle is formed) and hypercondensed chromosomes indicating that they arrest in mitosis (Hagan and Yanagida, 1990). In a FACS profile, cells that arrest in response to this mutation will arrest with a large 2N peak as it is a mitotic arrest. If the strains do not arrest they will carry on into the next cell cycle and attempt to reduplicate their DNA and a 4N peak will be seen as many of these cells would be tetraploid. This is the case with *mad2Δ cut7-24* (Millband and Hardwick, 2002) which was used as a positive control for cells which don't arrest.

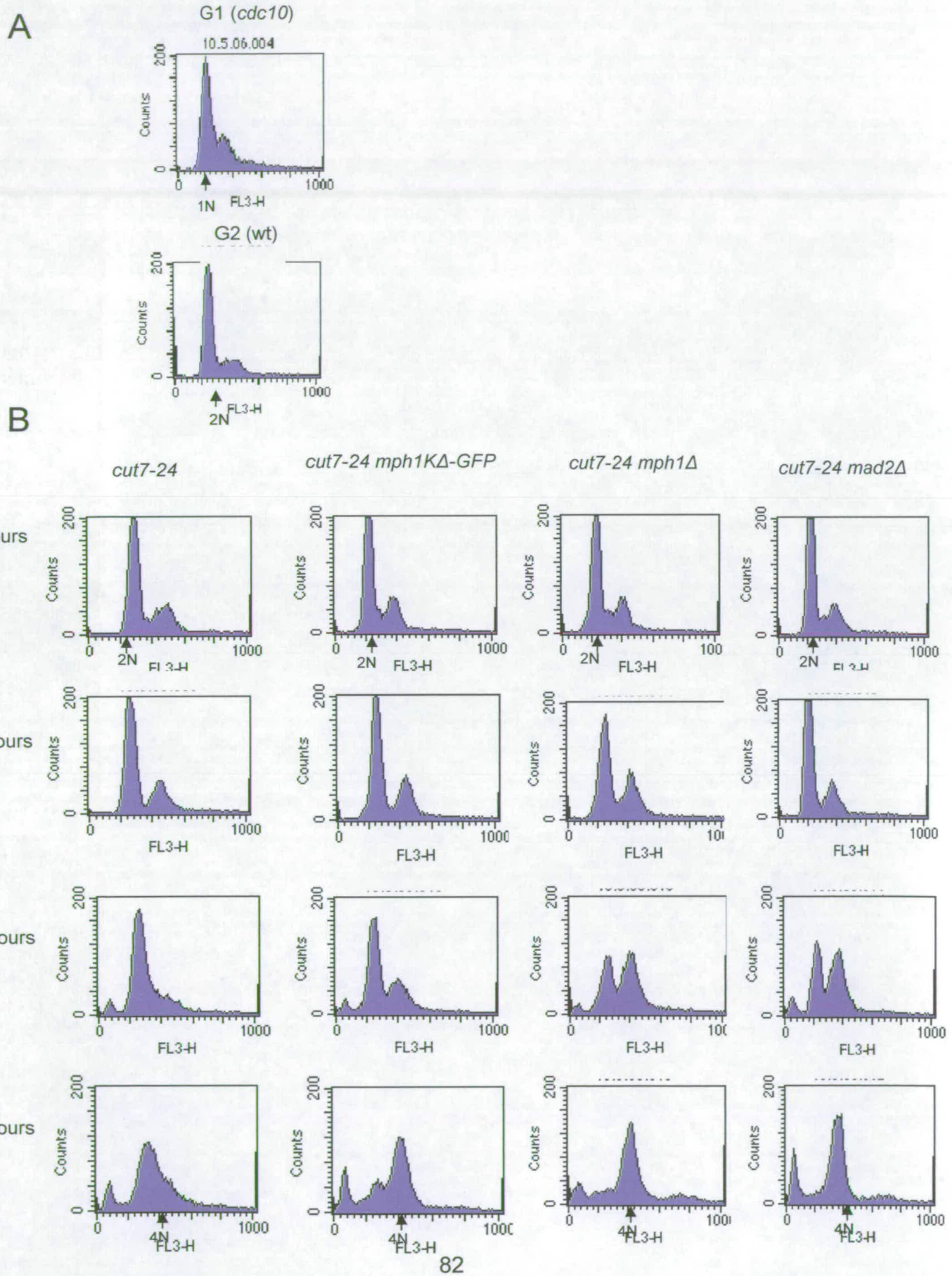
Log phase cultures were shifted to 36°C (the restrictive temperature for the *cut7-11* mutant) for 3 hours and samples were taken every 30 minutes during the arrest.

The FACS profiles shown in figure 3.5 show that *mph1Δ cut7-24* behaves like *mad2Δ cut7-24* meaning that this strain cannot establish an arrest. This was also true for the kinase deleted cells which look like the full deletion (figure 3.5). The FACS peaks moved to the right slightly during the time courses with all the mutants tested, even with the *cut7-24* which arrests. This is probably due to an increase in mitochondrial DNA during the time course as cells increase in size. The small peak to the far left of the profiles is probably due to aneucleate cells which result when the septa forms cutting off unsegregated cells so one daughter cells receives no DNA. The bulk of the DNA in all 3 checkpoint mutants moved significantly to the right and was seen as the 4N peak. This analysis gives a clear indication that the kinase of Mph1 is important for the establishment and/or maintenance of a checkpoint arrest.

Figure 3.6: FACS analysis of *mph1Δ* and *mph1KΔ-GFP* in a spindle checkpoint arrest.

A. *S.pombe* spends most of the cell cycle in G2 so wt cycling cells were used as a control for G2 cells showing a high 2N peak indicating replicated DNA.

B. Cells were arrested over 3 hours at 36°C using the *cut7-24* mutation which activates the spindle checkpoint. *mph1Δ* and *mph1KΔ-GFP* cells were studied to see if they could maintain a checkpoint arrest or attempt to carry on through to the next cell cycle like a *mad2Δ* which was used as a control. The *mph1Δ cut7-24* and *mph1KΔ-GFP cut7-24* mutants were grown at 36 °C and samples taken ever hour. The DNA content of these cells was analysed using a FACSCAN automated counter.


A
G1 (cdc10)
10.5.06.004
Counts
FL3-H
1N
G2 (wt)
2N
B
cut7-24
cut7-24 mph1KΔ-GFP
cut7-24 mph1Δ
cut7-24 mad2Δ
0 hours
1 hours
2 hours
3 hours
4N

3.6 Importance of the kinase domain for an arrest caused by Mph1 overexpression

3.6.1 Creation of an Mph1 overexpression cassette

A construct was made using a pREP plasmid that contained an *nmt* overexpression promoter. This is an inducible promoter with *nmt* standing for no message in thiamine. When cells containing this plasmid were grown in the presence of thiamine the promoter was not active and the gene that had been inserted into the plasmid was not expressed (sometimes low level of expression is seen when cells are grown in thiamine as leaky expression can occur). When cells were grown in the absence of thiamine the promoter was switched on and the gene of interest is expressed. In this study two versions of the pREP *nmt* plasmid were used, pREP 41x, a medium level overexpression plasmid and pREP 81x, a low level overexpression plasmid (Craven et al, 1998). Both of these contained an N-terminal GFP tag as well as a *S.cerevisiae LEU2* marker gene (figure 3.6A).
Primers Mph1FORN, Mph1REVC, Mph1REVKD and Mph1REV3 (see materials and methods) were used to amplify Mph1, or sections of Mph1, to be inserted into the plasmid. The primers were designed such that the resulting PCR products contained an N-terminal *SalI* site and a C-terminal *SmaI* to allow them to be ligated into the multiple cloning sites (MCS) of the pREP vector.

Three different Mph1 constructs were made using the pREP41x plasmid all containing different lengths of the *mph1* gene (see figure 3.6B). These comprised of the full length *mph1* gene, the gene up until the kinase domain (kinase deletion (KΔ) and the first third of the N-terminus only (1/3N). These constructs were important to assess the requirement for kinase domain in Mph1 localisation and also to attempt to map the minimal requirements for Mph1 to localise. This had been studied previously in human cells(Liu et al., 2003). By making constructs containing various regions of hMps1 it was found that only the first 300 amino acids were required to allow MPS1 to localise to the kinetochore (Liu et al., 2003). For this reason the

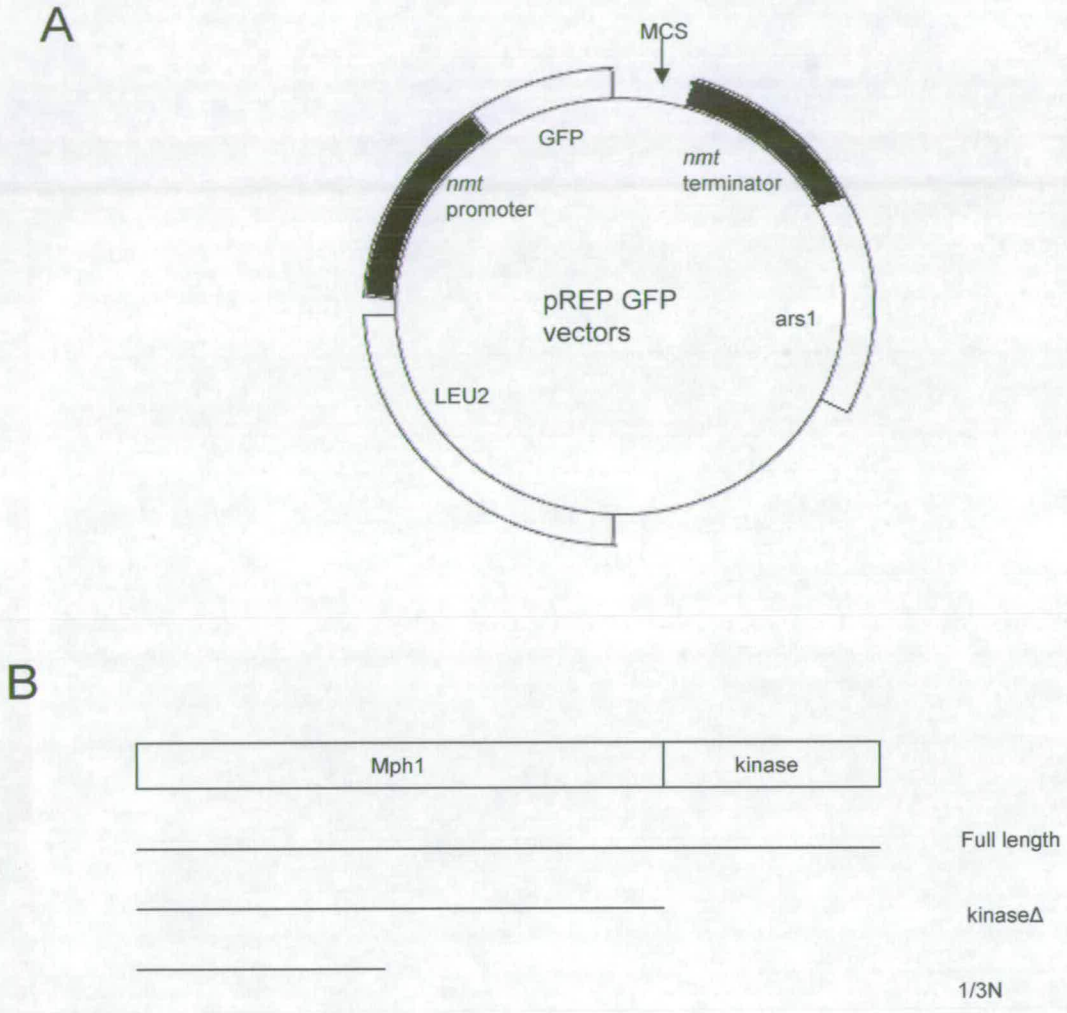


Figure 3.7: N-terminal tagging of Mph1 by insertion into overexpression plasmids

A. Schematic diagram of overexpression plasmids (pREP41 and pREP 81). Plasmid contains *nmt* inducible promoter, GFP tag next to the promoter before the Multiple cloning site (MCS) which contains the *SalI* and *SmaI* sites used to insert Mph1 into the plasmid. It also has an autonomous replication sequence and the *LEU2* gene as a selectable marker.

B. Diagram showing the regions of Mph1 that were inserted into the plasmids. Three sections of varying length were used, full length Mph1, Mph1 minus the kinase domain (kinaseΔ) and the first third of the N-terminus of the protein only (1/3N) to assess the importance of the kinase domain and attempt to map the minimal region required for Mph1 localisation which was expected to be at the N-terminus.

The plasmids were transformed by electroporation (see materials and methods) into fission yeast strains containing either wt *mph1* or *mph1Δ* in order to see if it made a difference what background the plasmid was in. Transformed cells were plated onto PMG-*Leu +T* plates in order to prevent transformants from losing the plasmid and thiamine was added to prevent overexpression of Mph1 which would result in colonies not growing properly as overexpression of Mph1 causes a checkpoint arrest in fission yeast (He et al., 1998). For microscopy the transformants were grown in a starter culture of PMG *–Leu+T* for 8 hours at 30°C before being spun down and the thiamine washed out of the media. Cells were then grown overnight at 30°C for 16 hours in PMG-*LEU* to allow overexpression of the GFP-Mph1 constructs before being spun
down and fixed in methanol briefly before being mounted on a slide. Cultures were also set up and grown overnight in PMG-*Leu+T* to see if any leaky expression of Mph1 occurred and if this could be seen by fluorescence microscopy.

3.6.2 The metaphase arrest caused by overexpression of Mph1 requires the kinase domain.

It has previously been shown in both budding yeast and fission yeast that when Mps1 and Mph1 respectively are overexpressed they cause a metaphase arrest that is checkpoint dependent (budding yeast (Hardwick et al., 1996) fission yeast (He et al., 1998)). In fission yeast when Mph1 is overexpressed cells arrest with short metaphase spindles. In order to assess the importance of the kinase domain of Mph1 for this arrest the pREP constructs detailed in section 3.6.1 were used.

To investigate if the kinase domain of Mph1 was necessary for an arrest when the protein was overexpressed cultures of nmt41x EGFP-Mph1, nmt41x EGFP-mph1KΔ and nmt41x EGFP-1/3N were grown up in liquid PMG-Leu+T then the thiamine was washed out and the cells left to grow for 10 hours. Time points were taken every hour from 10 hours after induction until 16 hours after induction. The cells were spun down and fixed in methanol and stained with α-tat1 antibody (an antibody to α tubulin) to stain tubulin and the number of cells with short metaphase spindles was calculated as a percentage of total cell number (figure 3.7). At 10 hours only a small percentage of cells carrying the full length Mph1 overexpression construct had arrested in metaphase but as the time course progresses this eventually results in over 50% of the cells showing short metaphase spindle indicating that this construct could cause a metaphase arrest. This meant that Mph1 in this construct was functional. By comparison nmt41x GFP-mph1KΔ and nmt41x GFP-1/3N never even reach 10% arrested cells (figure 3.7). This suggests that the presence of the kinase domain is required to enable cells to arrest when Mph1 is overexpressed.

3.7 Mph1 localisation

3.7.1 Microscopy with PCR tagged *mph1-GFP*

Microscopy using the PCR tagged *mph1-GFP* was carried out by growing cells in a pre-culture of YE5A at 30°C then diluting this back this YE5A growing cells overnight at 30°C. These cells were spun down and either briefly fixed in methanol before mounting or mounted in YES low melting point agarose to look at live cells. It was not possible to see any GFP spots in either of the cell preparation. This may be due to Mph1 not being expressed at high levels in the cell.

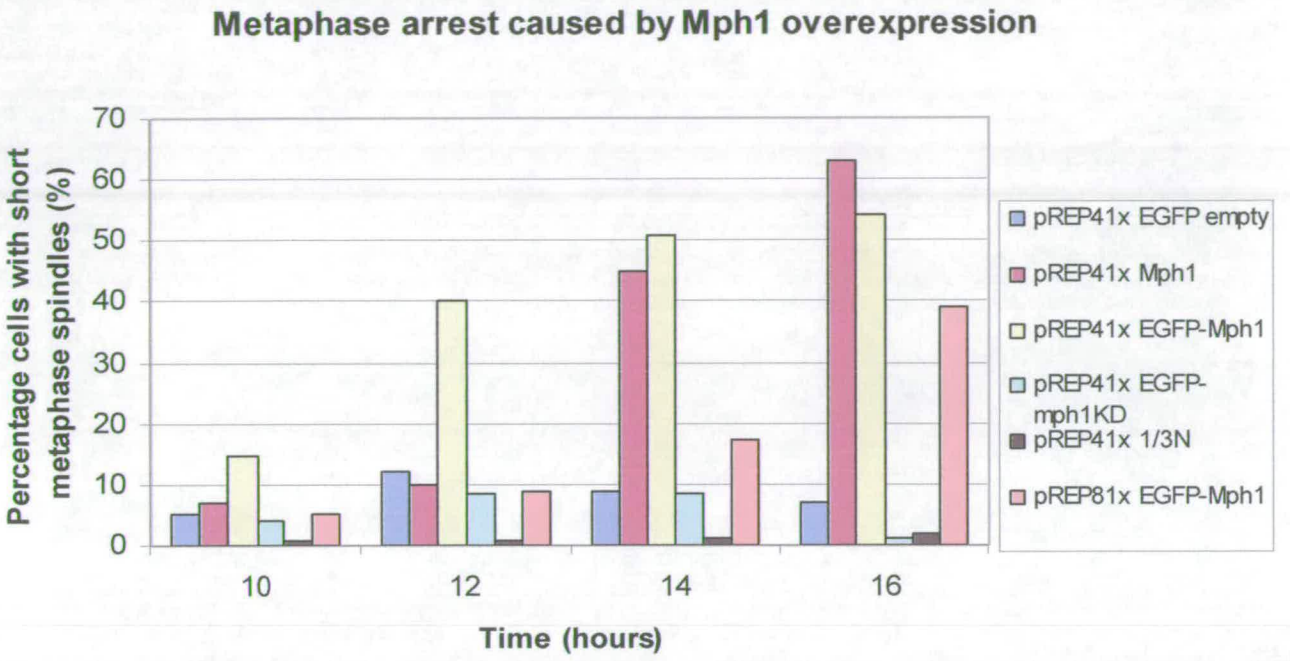


Figure 3.8: The kinase domain of Mph1 is required for a metaphase arrest cause when Mph1 is overexpressed.

Various lengths of Mph1 were inserted into an overexpression plasmid with an inducible promoter. The level of overexpression was sufficient to cause an arrest with full length Mph1, shown by an increase in short metaphase spindles over the course of the arrest. This was not the case when the kinase domain was removed and the phenotype appeared more severe when only the first third of the protein was present.

Since Mph1 acts during mitosis we considered that it may be localised more specifically at this point in the cell cycle making it easier to visualise. This was the case for *S.pombe* Mad3 (Millband and Hardwick, 2002). The pREP3x Mad2 plasmid was transformed into *mph1-GFP* cells as overexpression of Mad2 causes a metaphase arrest. *mph1-GFP* cells were also crossed to *nda3-KM311* as this mutation also causes a metaphase arrest when activated. Arresting these cells by overexpressing Mad2 or by growing them in the cold to activate the *nda3* mutation and studying both fixed and live cells under the microscope also did not allow localisation of Mph1 by microscopy. In an *nda3-KM311* arrest *mad3-GFP* was seen going on to kinetochores after 4 hours at 18°C and stays on kinetochores for the duration of the time course from this point on (figure 3.8A). This is not the case with *mph1-GFP* which is never seen to go on to kinetochore and is only ever seen very diffusely throughout the cell during the time course, even after 6 hours and this may just be background fluorescence (figure 3.8B). This is not because the tag renders Mph1 non-functional as *mph1-GFP* does not show benomyl sensitivity (figure 3.2) and is able to arrest in an *nda3* arrest. It seems likely that Mph1 is indeed expressed from its endogenous promoter at levels which are too low to be seen by fluorescence microscopy.

3.7.2 Microscopy with *GFP-mph1* on overexpression plasmids

3.7.2.1 pREP41x

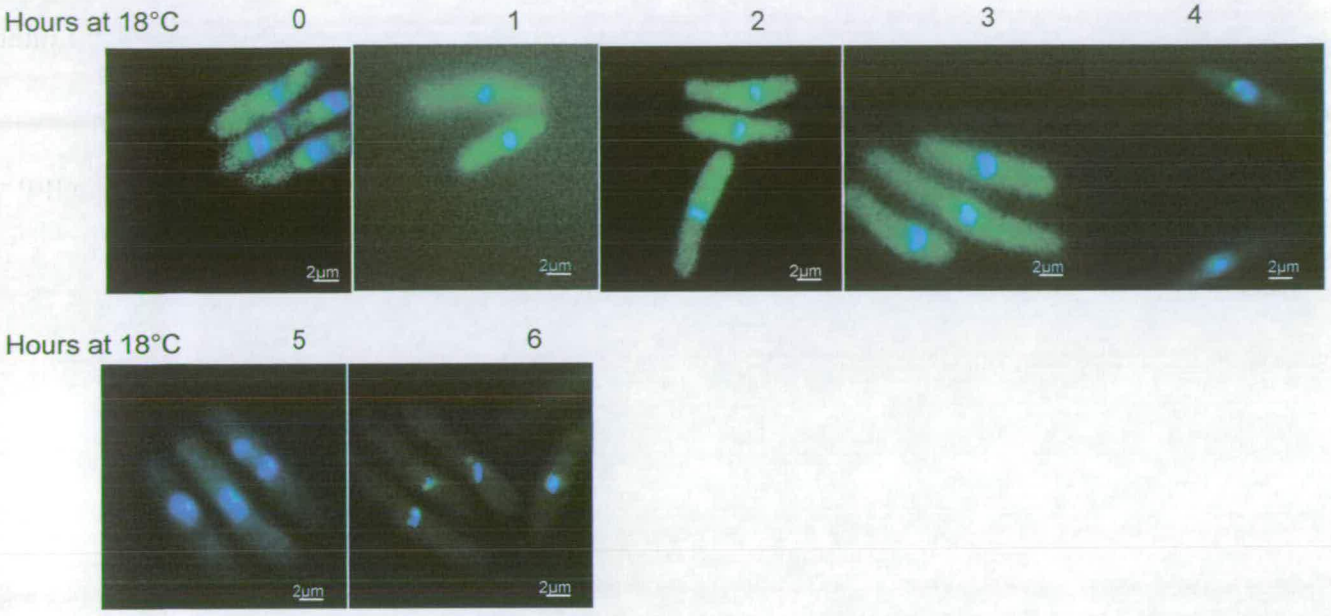
From the cultures grown in the presence of thiamine no GFP was seen at all and this correlates with the western blot of cells with the various Mph1 plasmids (Figure 3.9A). In the presence of thiamine no GFP-Mph1 is seen on the blot when probed with either α-Mph1 antibody or α-GFP antibody.

Figure 3.9: Comparison of *mad3-GFP* and *mph1-GFP* localisation in an *nda3-KM311* arrest

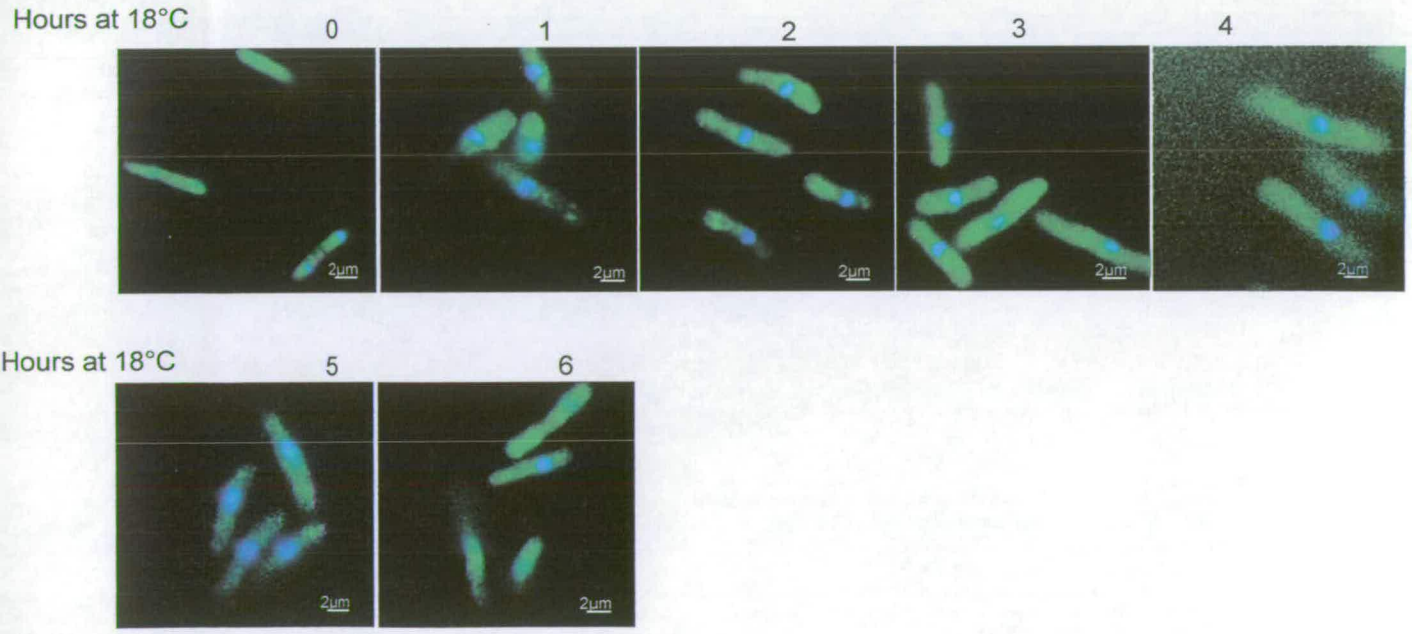
A. *mad3-GFP nda3-KM311* cells grown for 6 hours at 18°C to activate the *nda3-KM311* mutation Hourly time points were taken. Diffuse staining seen throughout the cell for the first 3 hours after which time distinct spots are seen as the arrest progresses.

B. *mph1-GFP nda3-KM311* grown as described above. Unlike *mad3-GFP* no change in GFP was seen as the time course progressed. Diffuse GFP staining was seen throughout the cells during the entire time course with discrete spot never being seen at the kinetochores.

A *mad3-GFP nda3*



B *mph1-GFP nda3*



Looking at pREP41x GFP-Mph1 grown in the absence of thiamine by fluorescence microscopy 12 hours after the promoter is switched on shows a diffuse nuclear signal. There are no defined spots that can be seen but it appears to be excluded from the nucleolus and cytoplasm which appear much less green than the nucleus (figure 3.9B).

After 16 hours in the absence of thiamine diffuse GFP staining was seen in the cytoplasm as well as the nucleus. This was probably due to the fact that by this time there will be a high concentration of Mph1 in the cell as it builds up as overexpression of Mph1 using this strength of promoter (*nmt41*) causes a metaphase arrest. Since cells were not dividing due to the arrest the level of Mph1 in the cell would continue to accumulate. This was similar to what was seen when mouse GFP-Mps1p is overexpressed as diffuse cytoplasmic staining was seen as well as being seen in the nucleus (Fisk and Winey, 2001).

When looking at the cells containing the truncated sections of Mph1 after 12 hours in the absence of thiamine (kinaseΔ and 1/3N) it was noted that the pattern of the fluorescence was different to that seen with full length Mph1. The bulk of the fluorescence was not seen in the nucleus but appears to be distributed evenly throughout the cell (figure 3.10B only kinaseΔ shown but results were the same for 1/3N). Again no defined spots of Mph1 are seen localised anywhere in the cell. This was the same after 16 hours, however with the 1/3N construct an even higher concentration of GFP is seen throughout the cell (figure 3.10C).

The reason for the diffuse GFP staining may be explained by studying the blot of cells expressing these constructs that had been in PMG-Leu-T for 16 hours (figure 3.9A). Instead of one large band of overexpressed Mph1 there was a ladder in all the lanes starting at a low MW and going up to the MW of that section of Mph1 plus GFP. This may be the result of the fusion protein being cleaved in the cell and so a lot of the diffuse staining seen was simply free GFP or GFP with very small portion of Mph1. However, another explanation for this ladder may be that it occurs when the protein extract is made. This was seen with all three of the constructs but the main band was still the full length band for each construct rather than a degradation product. Mph1 may be in a diffuse pool in the nucleus. This appears to be the case with the APC/C in *S.pombe* as studies using Cut9-GFP and Lid1-GFP (two components of the APC/C) have shown a pattern of diffuse nuclear staining rather than being seen as distinct spots of GFP (M. Sczaniecka, personal communication).

Figure 3.10: Analysis of overexpressed GFP-Mph1 from pREP41x and pREP81x plasmids

A. Western blot analysis of GFP-Mph1 pREP41x overexpression constructs containing varying lengths of Mph1 in the presence and absence of thiamine. Probed with anti-GFP antibody. Lack of thiamine switches on the *nmt* promotor driving the expression of the constructs. No protein is seen in the +T lanes. The smear of bands seen in the –T lanes is probably degradation products.

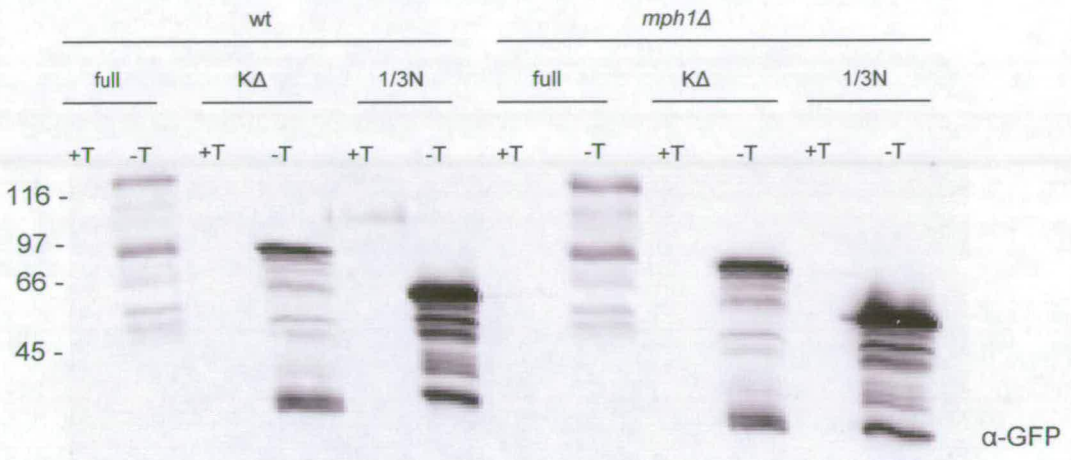
B. *pREP41x GFP-Mph1* and *pREP41x GFP-mph1kΔ* 12 hours after induction. Very little cytoplasmic GFP seen with full length GFP compared to the kinase deletion.

C. I6 hours after induction all constructs show both nuclear and cytoplasmic GFP staining.

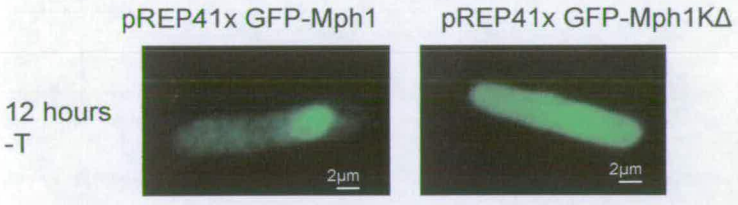
D. Western blot analysis of GFP-Mph1 pREP81x and pREP 41x overexpression constructs. Probed with rabbit anti-Mph1 antibody (see Section.4.2 for details)

pREP41x GFP-Mph1

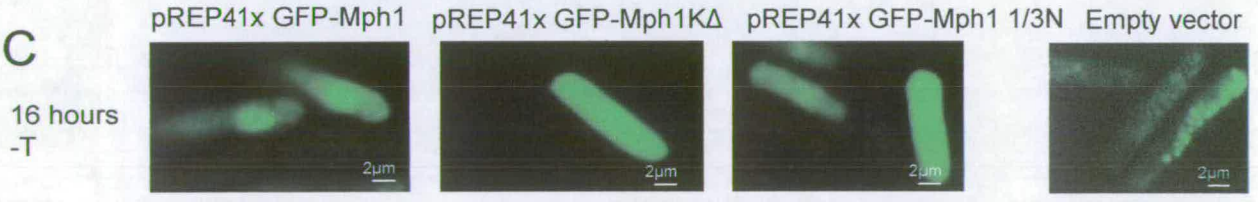
A



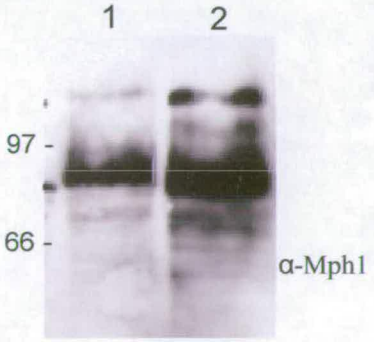
B



C



D



1. pREP81x GFP-Mph1

2. pREP41x GFP-Mph1

This was different from the Mad and Bub proteins which do localize to kinetochores as can be seen from mitotically arrested Mad3-GFP in figure 3.9A.

3.7.2.2 pREP81x

Putting Mph1 in a construct with a low level overexpression *nmt* promoter (pREP 81x GFP) showed a lower level of GFP-mph1 expressed when compared to that of pREP 41x GFP-mph1 as expected when analysed by Western blot analysis (Figure3.9D). At 12, 14 and 16 hours after induction in media lacking in thiamine GFP-mph1 was seen to stain the entire nucleus (data not shown) as was the case with the higher level overexpression promoter (figure 3.9B). At 6 and 8 hours after induction in PMG-LEU media no GFP is seen at all so it was likely that it was a case of "all-or-nothing" whereby either no GFP is seen or the whole nucleus is filled with GFP there does not appear to be a time where distinct spots of GFP-mph1 appear before the nucleus becomes saturated with GFP. As with the Western blot of the pREP 41x GFP-mph1 constructs, the western blot from extracts of cells expressing the pREP 81x GFP-mph1 construct (figure 3.9D, blot probed with α-Mph1 antibody see Section 4.2 for details) showed a ladder of degradation products, which is comparable with the pREP41x GFP-mph1 construct run on the same gel and it may be these that saturate the nucleus masking the full length construct. The fact that they only accumulate in the nucleus however was an argument against this as GFP does not have a nuclear localisation signal so should not be confined only to the nucleus but should be present in the cytoplasm as well (as is seen in the truncated GFP-mph1 constructs, kinaseΔ and 1/3N (figure 3.9C) where the cytoplasm was stained green as well as the nucleus).

3.8 Discussion

From the results detailed above it is apparent that the kinase domain of Mph1 is important for its role in the spindle checkpoint and its localisation. The results have shown that in the absence of the Mph1 kinase domain cells die in the presence of the microtubule drug benomyl and do not arrest in the absence of spindle microtubules which result when the cs mutant *nda3-KM311* is grown at its restrictive temperature. In the absence of a spindle the checkpoint should be active as there is no kinetochore-microtubule attachment or tension. In the *mph1KΔ* mutant there is no arrest in combination with this mutation as cells attempt to carry on through and exit mitosis resulting in a significant percentage of cells attempting to divide and as a result displaying a *cut* phenotype. It is the fact that chromosomes are lost when cells *cut* that leads to loss of viability in the presence of the *nda3-KM311* mutant. This loss of viability in not only observed in the *mph1Δ* mutant but also in the *mph1KΔ-GFP* mutant means that the kinase domain of Mph1 is required for it to function in the spindle checkpoint. This is unlike fission yeast Bub1 which does not require its kinase domain in order to function in the spindle checkpoint (Vanoosthuyse et al., 2004).

The kinase domain of Mph1 appears to be required for the establishment of a spindle checkpoint arrest. In order to determine whether the Mph1 kinase domain is also required for the maintenance of this arrest a conditional mutant would have to be made such as an analogue-sensitive allele similar to one that has been made with budding yeast Mps1 (Jones et al., 2005). This would allow a spindle checkpoint arrest to be established before the kinase function of Mph1 was inhibited.

The *GFP-plo1*$^{+}$ experiment confirms the requirement of the Mph1 kinase for establishment of an arrest, with the graphs of *GFP- plo1*$^{+}$ appearance in the *mph1Δ* and *mph1kΔ-GFP* showing a similar profile (figure 3.3). *GFP plo1*$^{+}$ in that experiment acts as a marker for mitosis so a population of cells which arrest at that point in response to the *nda3-KM311* mutation should an increase in *GFP- plo1*$^{+}$ whereas this is not the case for the Mph1 mutants. The inability to arrest in response to spindle damage is similar to what has been found in budding yeast when the kinase domain of Mps1 was perturbed (Jones et al 2005).

As detailed previously, in budding yeast an analogue-sensitive allele of Mps1 has been made (*mps1-as1*). This involved making a point mutation in the ATP binding pocket of the kinase domain. This mutant of Mps1 can be inhibited by the addition of the ATP-analogue 4-amino-1-(tert-butyl)-3-(1`-naphthylmethyl)pyrozolo[3,4-d]pyramidine (1NM-PP1) (Jones et al., 2005). This allowed the function of the kinase domain of Mps1 to be studied, specifically, as the protein was still present and the 1NM-PP1 only inhibited its enzymatic activity. As the protein was still present so any non-enzymatic function of Mps1, such as a possible function as a scaffold protein at the kinetochore to facilitate the recruitment of other proteins to the kinetochore, could still be carried out. In the presence of the inhibitor Mps1 autophosphorylation decreased and no arrest was seen when drugs which depolymerise microtubules were added (Jones et al., 2005). *Mps1-as1* cells are able to arrest in metaphase by checkpoint activation in the absence of the inhibitor. Once the arrest was established Jones et al then added the inhibitor and the arrest could not be maintained (Jones et al., 2005). This suggests a role for Mps1 kinase activity in both the establishment and maintenance of a checkpoint arrest.

In agreement with results seen for *S.cerevisiae* Mps1 the kinase domain is required for an arrest by Mph1 overexpression. This was seen by the absence of enrichment of short metaphase spindles when the kinase deletion mutant was overexpressed. This was the case when the full kinase domain is removed and when the kinase domain plus part of the N-terminus is removed (*mph1KΔ* and *1/3N* constructs). However to test this fully a kinase-dead version would have to be made to rule out the possibility that any effect on the ability of Mph1 to arrest is not due to the removal of the kinase domain rather than the absence of catalytic activity.

In Xenopus and human cells studies carried out to map the kinetochore targeting domain of Mps1 showed that only the N-terminus was required for this ((Abrieu et al., 2001) and (Liu et al., 2003) and (Stucke et al., 2004)). In the absence of the kinase domain Mps1 can still localise to the kinetochore however the results of Chapter 3 would suggest that this may not be the case in fission yeast as in the absence of the kinase domain the bulk of the GFP-Mph1 is lost from the nucleus and is seen diffusely in the cytoplasm.

At the present time it is still not possible to determine if Mph1 has a role in spindle pole body duplication in fission yeast. It was hoped that localisation studies would prove useful in answering this question but this has not been the case. It is unlikely that Mph1 plays an important role in SPB duplication as it is not an essential gene unlike in budding yeast (Winey et al., 1991).

As has been detailed in this chapter it was not possible to see GFP tagged Mph1 in cycling and arrested cells when under the control of its own promoter. The reason for this may be Mph1 is expressed at very low levels in the cell. Putting Mph1 under the control of a stronger promoter to overexpress it resulted in a diffuse nuclear staining of Mph1 compared to shorter sections of Mph1 which show cytoplasmic staining as well. This may mean that Mph1 is normally found diffusely in the nucleus and only gets recruited to the kinetochore during checkpoint activation, however, putting Mph1 under the control of the *nmt41x* has previously been shown to cause a metaphase arrest (He et al., 1998) so Mph1 should be recruited to kinetochores if this theory is true. Due to the overexpression of Mph1 and the fact that it may normally exist at very low levels in a cell very little of the bulk of the Mph1 present when it is being overexpressed may be required to be on kinetochores meaning that the remaining Mph1 in the nucleus masks the kinetochore staining.

In terms of localisation the question still to be answered is whether or not Mph1 is found at spindle pole bodies. This is something that it has as yet not been possible to determine using any of the GFP tagged constructs. Mps1 has been localised to SPBs in budding yeast (Winey et al., 1991) and mouse cells (Fisk and Winey, 2001) but is currently highly debated in human cells. Work by Stucke et al (Stucke et al., 2004; Stucke et al., 2002) argues that human Mps1 is not found on centrosomes while work by Fisk et al (Fisk et al., 2003) would suggest otherwise. Since *mph1Δ* cells are viable and appear able to complete a cell cycle with no defects in the absence of spindle damage would suggest that it does not have a role in SPB duplication unlike its budding yeast homologue. However, it is interesting that fission yeast Mph1 is able to partially complement the SPB duplication defects of a budding yeast *mps1* mutant. What is apparent from the localisation studies detailed in this chapter is that Mph1 is found in the nucleus and removing the kinase domain leads to much more diffuse localisation pattern. As yet a nuclear localisation signal has not been found in Mph1. Since it was not possible to tell from the studies overexpression GFP-Mph1

was whether or not Mph1 was at kinetochores. An alternative way of studying this would be to use chromatin immunoprecipitation (ChIP). I briefly attempted this but it has so far proved unsuccessful.

Chapter 4

Mph1 regulation

4.1 Introduction

Phosphorylation and degradation are two ways in which proteins can be regulated. Very little is currently known about the regulation of Mps1 kinases although previous studies in organisms such as *S.cerevisiae* and *Xenopus laevis* have shown that Mps1 is a phosphoprotein (Schutz et al., 1997), (Zhao and Chen, 2006). Phosphorylation would be a way of controlling the activity of the protein at different points in the cell cycle. The phosphorylation state of Mph1 is likely to be important for its function as it has been shown in human and budding yeast cells that Mps1 becomes hyperphosphorylated on checkpoint activation and is dephosphorylated on exit from mitosis (Liu et al., 2003), (Palframan et al., 2006). Degradation may also be important for the regulation of Mps1 activity. Since almost nothing is known about the regulation of Mph1 at the present time this study aims to address this and will show a potential candidate phosphatase involved in the regulation of Mph1 on exit from a mitotic arrest.

4.2 Mph1 polyclonal antibody

4.2.1 Making a polyclonal antibody to Mph1

Antibodies to proteins are incredibly useful reagents. They can be used for both immunofluorescence and for biochemistry and are particularly useful as tagging proteins can sometimes render them non-functional or only partially functional. An antibody to Mph1 was raised as the native protein could be studied in case tagging proved unsuccessful.

To make the antibody a small section of the *mph1* gene was amplified (389 bp directly upstream of the kinase domain (figure 4.1A). This section was chosen as it is in a similar region to the antibody created against human Mps1 (Stucke et al., 2004). The anti-Mph1 (α-Mph1) antibody was made using Mph1ABFOR and Mph1Y2HKREV primers (see materials and methods) giving a section of Mph1 from amino acids 171-321 when expressed. This section of DNA was flanked with *EcoRI* and *SalI* sites to allow insertion into the cloning site of the pGEX-6P-1 vector. The pGEX vector contained a glutathione S-transferase (GST) tag adjacent to the cloning site (see figure 4.1B for map of vector). It also contained the ampicillan resistance gene for selection in bacteria. The construct was transformed into bacteria (BL-21, see materials and methods) and grown on LB + ampicillin.

To purify the GST-Mph1$^{171-321}$ fusion protein transformants were grown in a preculture in LB + Amp liquid overnight which was then used to inoculate a larger culture to which IPTG was added to induce the production of the fusion protein. Induction was carried out at 18°C overnight. Cultures were then spun down and the cells drop frozen in liquid nitrogen as small pellets. These pellets were ground into a fine powder, under liquid nitrogen resuspended in lysis buffer and sonicated. The lysed cell suspension was subjected to ultracentrifugation and the supernatant saved as this contained the fusion protein. This was passed over a column of glutathione agarose beads which the GST bound to. The beads were washed and the fusion protein eluted and dialysed in a glycerol solution.

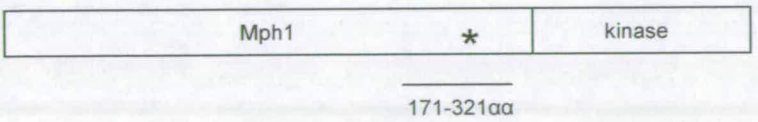
Figure 4.1: Making a polyclonal antibody to Mph1

A. Schematic representattion of Mph1 with * showing the region from 171-321 amino acids used to make a fusion protein with GST for use as an antigen to produce antibodies to Mph1.

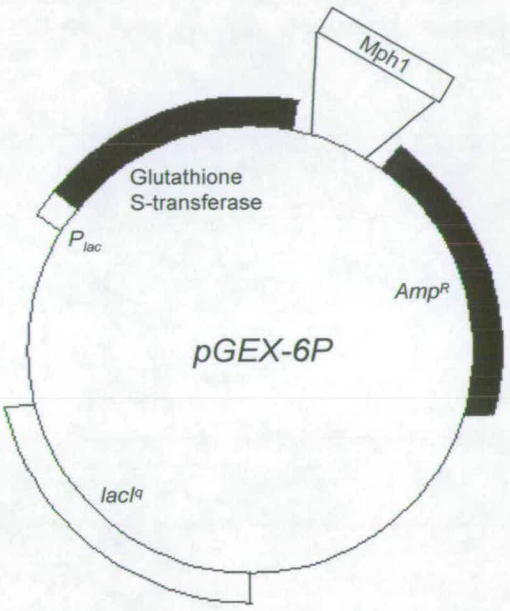
B. pGEX-6P vector containing a GST tag into which the section of Mph1 from A. was cloned then purified from bacteria.

C. Western blot of *wt*, pREP41x-Mph1 (grown for 16 hours in media lacking thiamine in order to overexpress and *mph1Δ* extracts to test rabbit α-Mph1antibody

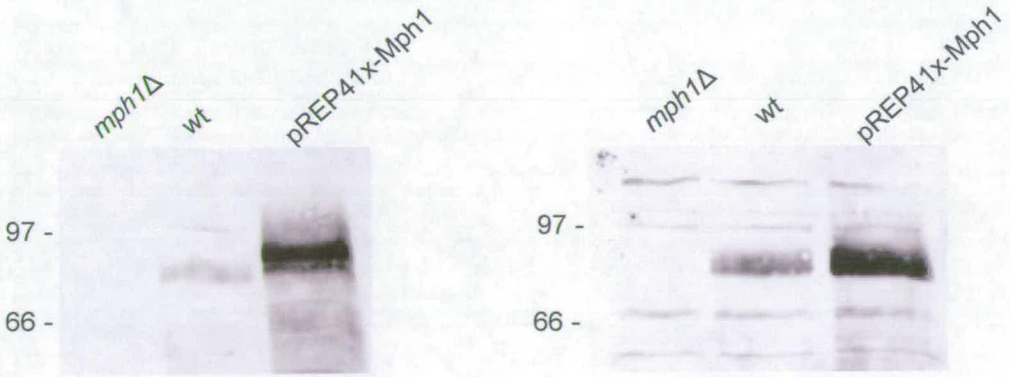
A


Mph1
*
kinase
171-321αα

B


Mph1
Glutathione
S-transferase
P lac
Amp R
pGEX-6P
lacI q

C


mph1Δ
wt
pREP41x-Mph1
97 -
66 -
Rabbit α-Mph1
mph1Δ
wt
pREP41x-Mph1
97 -
66 -
Sheep α-Mph1

To obtain antibodies the fusion protein was injected into both sheep and rabbit. Bleeds from injected animals were then purified by first passing it over a column of GST coupled to Affigel 10 to remove any antibodies specifically to GST from the serum. The flow through from this was then passed through a column of bacterial purified GST-Mph1$^{171-321}$. Mph1 antibodies were then eluted from this using high and low pH buffers (see materials and methods) and tested on a blot of *wt,* pREP41x-mph1 (grown in media lacking thiamine in order to overexpress Mph1)and *mph1Δ* cell extracts (figure 4.1C). No bands were seen in the *mph1Δ* lane, a band of the correct size was seen in the *wt* lane and a large hyperphosphorylated band was seen in the overexpressed lane proving that the antibody produced was specific for Mph1.

4.2.2 Fixed cell microscopy using α-Mph1 antibody

Since Mph1 could not be localised with the GFP tag (section 3.7.1) another way of localising it had to be found. There was a chance that the tag was affecting the localisation (even though *mph1-GFP* is not benomyl sensitive and cells arrest when combined with the *nda3-KM311* mutation, results not shown) fixed cell microscopy of endogenous Mph1 was carried out. Cells were fixed with formaldehyde (see materials and methods) and stained with rabbit α-GFP primary antibody (see section 3.2) and Alexa-coupled rabbit secondary antibody.
Microscopy using fixed and stained cells again failed to show localisation of Mph1 in either cycling cells or in Mad2 overexpressed or *nda3-KM311* arrested cells (data not shown). When Mad2 is overexpressed in fission yeast cells it cause a metaphase arrest that is dependent on the spindle checkpoint (He et al., 1998) , as is the arrest seen in the *nda3-KM311* mutant.

4.3 Mph1 phosphorylation throughout the cell cycle

4.3.1 Mph1 phosphorylation through a "normal" mitosis

Cdc25 is the phosphatase required to dephosphorylated Cdc2 to allow entry into mitosis. The *cdc25-22* mutant cells arrest in G2. Cells were arrested in G2 using the *cdc25*-22 temperature sensitive mutation by growing them for 3 hours at 36°C andf then released and allowed to continue through in to mitosis by shifting the temperature to 25°C. Samples were taken every 15 minutes. In a *cdc25-22* arrest the phosphorylation state of Mph1 does not differ from that of cycling cells (figure 4.2A). What is interesting that as you follow protein levels of Mph1 through release from this arrest, so the cells are synchronous, the phosphorylation state of Mph1 changed as the cells entered and exited mitosis. At 45 minutes when a large number of binucleate cells are present indicating that the cells are in mitosis a band is detected with a mobility shift. This suggests that Mph1 becoming hyperphosphorylated and as cells begin to exit mitosis (an abundance of septated cells are seen) Mph1 begins to become dephosphorylated, indicating that in a normal, unperturbed cell cycle Mph1 may get phosphorylated as it enters mitosis and this may be necessary to activated it.

4.3.2 Mph1 phosphorylation and cell cycle progression on release from a *cdc25-22* arrest in a *pyp3* mutant

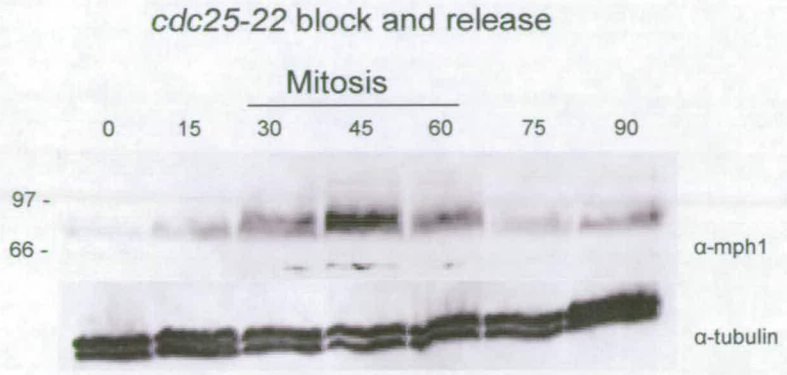
Since Mph1 appeared to be phosphorylated during mitosis but the level of phosphorylation fell as cells exited mitosis, this indicated that the protein was either being degraded, as is the case in budding yeast (Palframan et al., 2006) or that a phosphatase was acting on Mph1. In a yeast 2-hybrid screen carried out with only the kinase domain of Mph1 a phosphatase, the Pyp3 phosphatase, was identified as a possible interactor of Mph1 (P.Rengtved personal communication). *Pyp3* is a gene

Figure 4.2: Mph1 phosphorylation on release from cell cycle arrests

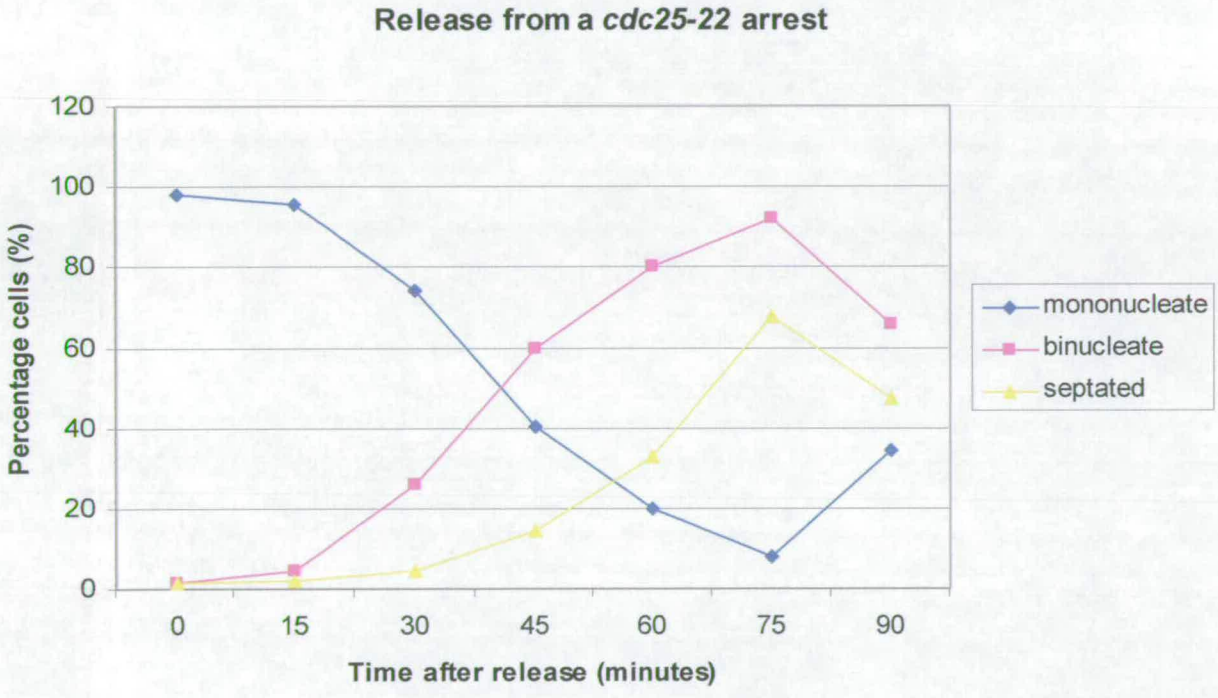
A. Release from a *cdc25-22* arrest. Cells containing the *cdc25-22* mutation were arrested in G2 for 3 hours by growing them at 36°C for 3 hours in order to synchronise them. After the 3 hour arrest the temperature was shifted to 25°C to release the cells and samples were taken every 15 minutes and proteins extracted and run out on an SDS-PAGE gel for Western blot analysis. The nitrocellulose membranes were probed with rabbit α-Mph1 antibody in order to observe levels of Mph1 through mitosis.
Levels of Mph1 and Mph1 phosphorylation increase as cells enter mitosis at 30 minutes after the release from the arrest.

B. Graph following the appearance of binucleate and septated cells showing progress through mitosis. Cells from each time point were fixed in methanol and stained with calcoflor and DAPI to visualise DNA and septa.

A


cdc25-22 block and release
Mitosis
0
15
30
45
60
75
90
97 -
66 -
α-mph1
α-tubulin

B


Release from a cdc25-22 arrest
120
100
80
60
40
20
0
Percentage cells (%)
0
15
30
45
60
75
90
Time after release (minutes)
mononucleate
binucleate
septated

that was first identified in a screen for genes which could rescue *cdc25* mutations (Millar et al., 1992). In this screen Pyp3 was found to be a phosphatase which could act to dephosphorylate Cdc2 to activate it in the absence of Cdc25 activity. It seemed likely that this maybe an auxiliary role for this phosphatase and it may have other substrates which it primarily dephosphorylates. It was possible that Mph1 is one of these substrates.

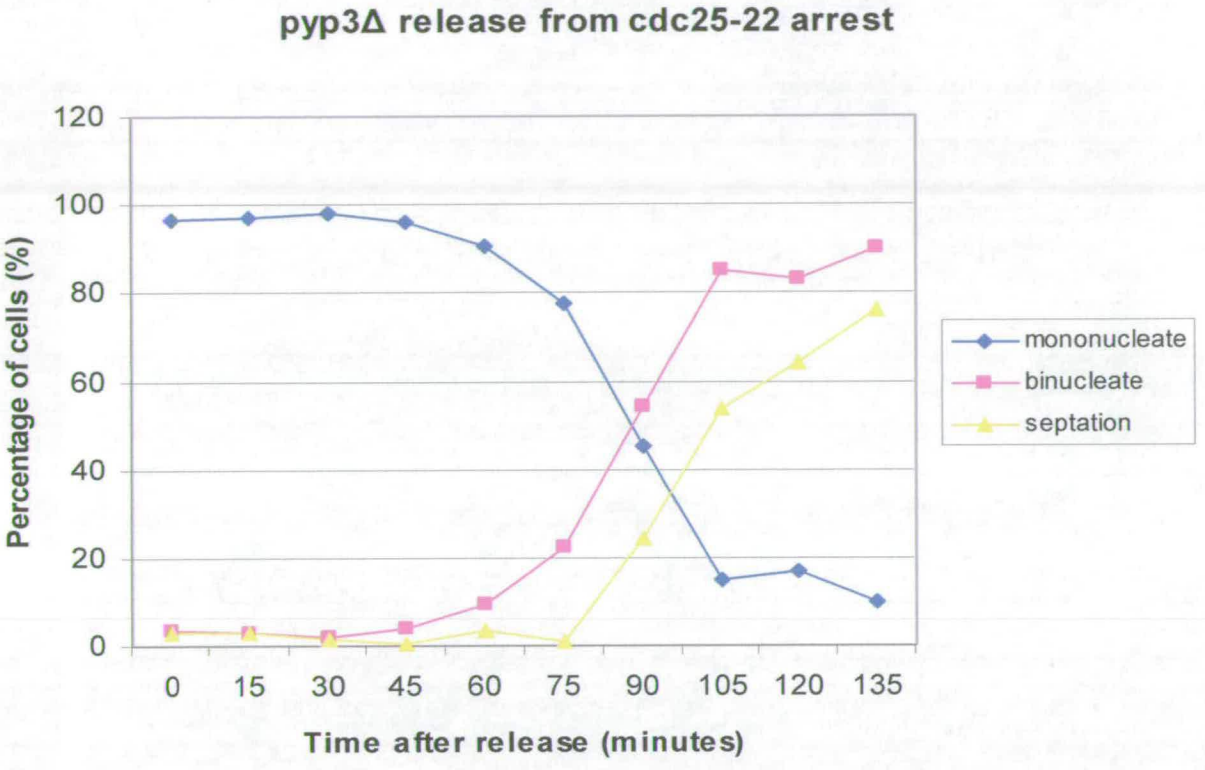
If Mph1 was dephosphorylated on exit from mitosis and Pyp3 was the phosphatase responsible then it was likely that Mph1 may be seen to be more phosphorylated in a normal unperturbed mitosis and that mitosis may be extended in a *pyp3Δ* strain. Pyp3 is not essential therefore it was possible for a deletion to be made (Millar et al., 1992). To study this *pyp3Δ* was crossed with *cdc25-22* to allow cells to be synchronised and released into a synchronous mitosis. This was achieved by growing the culture overnight at 25°C before shifting to 36°C for 3 hours to arrest the cells. They were then released by shifting the temperature back to 25°C and taking time point every 15 minutes for 165 minutes. Cells from these samples were either used for western blotting or fixed in methanol and stained with calcofluor and DAPI in order to visualise septa and DNA to plot the progress of the cells through mitosis. At each time point 200 cells were counted to get the percentage of mono and bi-nucleate cells and the number of cells septating. Plotting this on a graph showed that G2 in these cells appeared to be extended compared to G2 when cells were released from a *cdc25-22* arrest with a functional copy of Pyp3 (compare graphs in figure 4.3A and figure 4.2A). Whereas in the *cdc25-22* mutant alone cells entered mitosis after about 45 minutes it took double that time for cells to enter mitosis in the *pyp3Δ cdc25-22* double mutant. When the western blot of this time course was examined, however, it appeared that there was no change in the phosphorylation state of Mph1 in this double mutant when compared to the *cdc25-22* mutant alone (compare figures 4.3B and 4.2B). What is interesting when looking at the western blot is that Mph1 seems to become hyperphosphorylated at the same time in this mutant as in the single *cdc25-22* mutant. This suggests that Mph1 may get phosphorylated in G2 as the shift in phosphorylation state in both cases occurs just as the number of binucleate cells is starting to increase before the cells have entered mitosis.

Figure 4.3: *cdc25-22* arrest and release in a *pyp3Δ* mutant

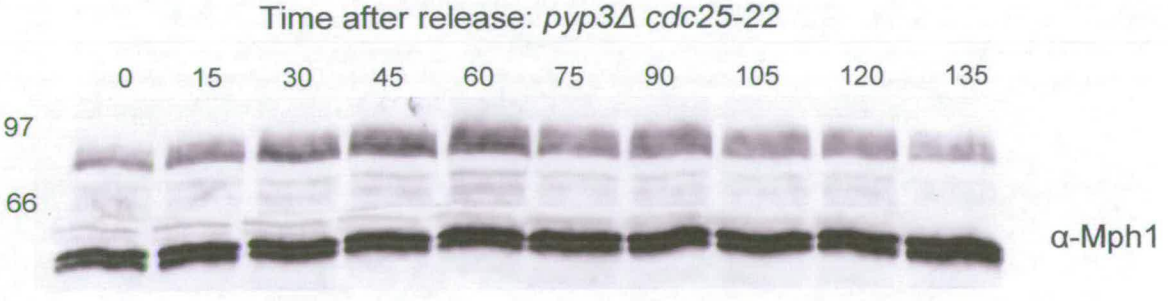
A. Graph following the appearance of binucleate and septated cells showing progress through mitosis following a G2 arrest using the *cdc25-22* mutation. Samples were taken every 15 minutes following the release from the arrest by shifting the temperature from 36°C to 25°C. Cells were fixed in methanol and stained with calcoflor and DAPI to stain septa and DNA. *pyp3Δ cdc25-22* cells show a delay entering mitosis when released from the arrest compared to *cdc25-22* single mutant.

B. Cells containing the *cdc25-22* mutation were arrested in G2 for 3 hours by growing them at 36°C for 3 hours in order to synchronise them. After the 3 hour arrest the temperature was shifted to 25°C to release the cells and samples were taken every 15 minutes and proteins extracted and run out on an SDS-PAGE gel for Western blot analysis. The nitrocellulose membranes were probed with rabbit α-Mph1 antibody in order to observe levels of Mph1 through mitosis.
Levels of Mph1 and Mph1 phosphorylation increases as cells enter mitosis at 30 minutes after the reslease from the arrest. This may mean that Mph1 is phosphorylated at the end of G2 prior to entering mitosis.

A


pyp3Δ release from cdc25-22 arrest
Percentage of cells (%)
120
100
80
60
40
20
0
0
15
30
45
60
75
90
105
120
135
Time after release (minutes)
mononucleate
binucleate
septation

B


Time after release: pyp3Δ cdc25-22
0
15
30
45
60
75
90
105
120
135
97
66
α-Mph1

4.3.3 Mph1 phosphorylation upon checkpoint activation

As was seen from the Western analysis presented in Figure 4.1C Mph1 was suspected to be a phosphoprotein. In cycling cells a doublet of bands is seen indicating that during a normal cell cycle Mph1 may phosphorylated. To confirm that Mph1 was a phosphoprotein an immunoprecipitation (IP) was carried out using cells arrested in mitosis using the pREP3x-Mad2 construct as overexpression of Mad2 causes a metaphase arrest. As a ladder of bands is seen in mitotically arrested cells it was likely that the bulk of the modification occurred in mitosis. The IP was carried out by extracting proteins from Mad2 overexpressed cells (grown in media lacking thiamine for 16 hours) and the extract was pulled down over proteinG Dynabeads bound with sheep α-Mph1 antibody. The beads were then split in half so half could be treated with lambda phosphatase. The IPs were run out on a gel for Western Blotting and the nitrocellulose was probed with rabbit α-Mph1. If Mph1 was phosphorylated a band shift would be seem as the ladder of bands would disappear and only one would be present. This was indeed the case (Figure 4.4A).

In order to investigate any potential changes in phosphorylation state as cells progress through mitosis a time course to follow Mph1 through an *nda3-KM311* arrest was used. A culture of *nda3* cells was grown up overnight at 30°Cin YE5A then shifted to 18°C for 6 hours. During this time samples were taken over 2 hour intervals to be used for Western analysis. The resulting nitrocellulose filter was probed with rabbit α-Mph1 (figure 4.4B). It can be seen that as the cells progress through the arrest the level of phosphorylation increases. As was seen with the *GFP-plo1*$^{+}$ time course (figure 3.2) most of the *nda3-KM311* cells have started to arrest by 4 hours and this correlates with the major band shift seen in Mph1 when a large ladder of bands is seen as the bulk of the protein shifts to the hyperphosphorylated form (figure 4.4A). Also the main bulk of Mph1 shifts up into the upper band with most of it seen roughly in the middle of the ladder of bands. By 6 hours at 18°C the bulk of the Mph1 has shifted to a highly phosphorylated form indicating that Mph1 is hyperphosphorylated in mitosis but as yet it is not clear is this is due to activation of the checkpoint or if this happens every time a cell enters mitosis.

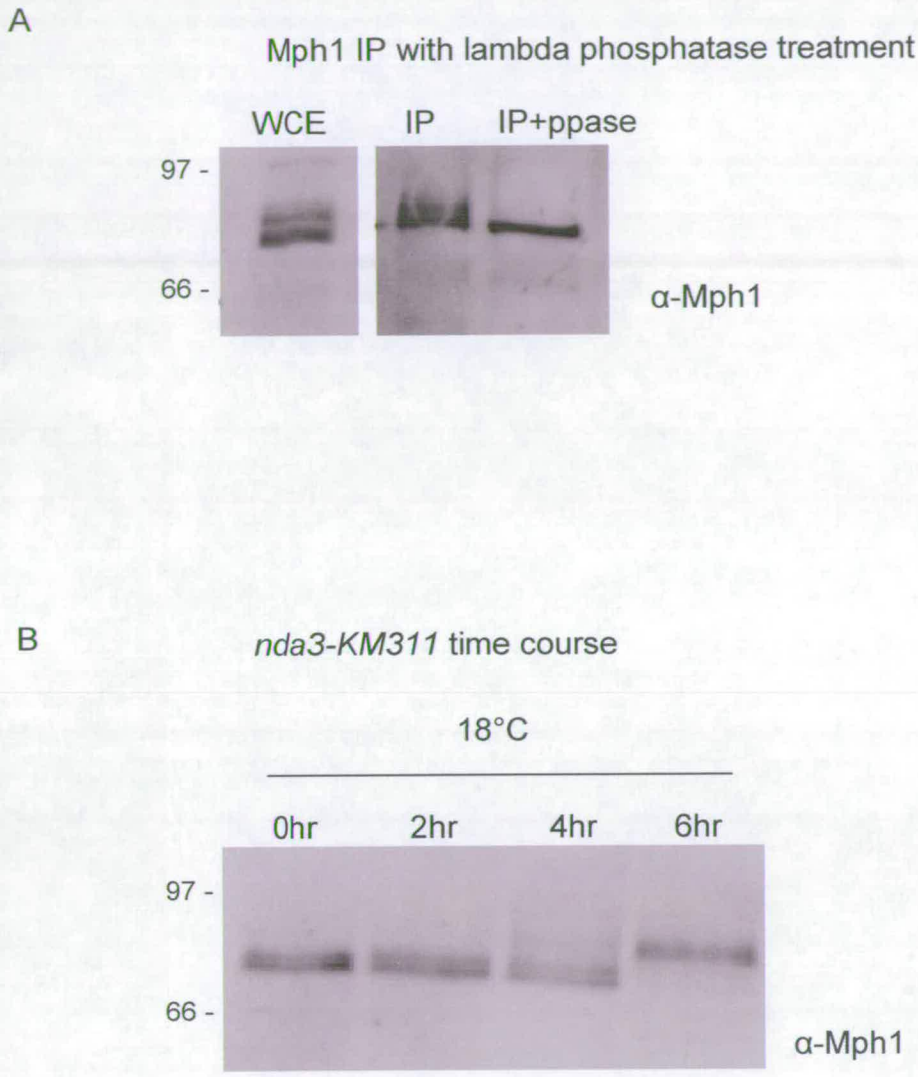


Figure 4.4: Mph1 phosphorylation

A. IP with λ-phosphatase treatment. Cells were arrested in mitosis by using the Mad2 overexpression cassette pREP3x-Mad2 by growing in media lacking thiamine for 16 hours. Whole cell extracts were prepared and Mph1 was immunoprecipitated (IP) using sheep α-Mph1bound to protein G Dynabeads. The Mph1 bound beads were split in half and one half was treated with λ-phosphatase (IP+ppase, lane 3). Western blot probed with rabbit α-Mph1 antibody.
The band shift indicates Mph1 is
a phosphoprotein

B. Mph1 phosphorylation during an *nda3-KM311* arrest. *Nda3-KM311* cells were grown at 18°C over 6 hours to arrest them. Samples were taken every 2 hours and protein extracts were run out on an SDS-PAGE gel for Western analysis. Western Blot probed with rabbit α-Mph1 antibody.

4.3.4 Checkpoint release

Since Mph1 is only seen to be hyperphosphorylated in mitosis, no hyperphosphorylation is seen in cycling cells; Mph1 must either be degraded or dephosphorylated as it exits mitosis. In budding yeast Mps1 gets degraded during anaphase so it was possible that the same could be true in fission yeast (Palframan et al., 2006). In order to study which of these is correct cells containing a wild type copy of *mph1* were arrested with the *nda3-KM311* mutation for 6 hours at 18°C as before (section 4.3.3). After the 6 hour arrest the culture was shifted back to 30°C to turn the mutation off and allow cells to begin to rebuild a spindle and continue through into anaphase.

Samples were taken every 5 minutes after the release to follow what happens to the Mph1 protein over time after release from the arrest.

From the western analysis of this time course (figure 4.5A) it seems that Mph1 is not degraded but instead is dephosphorylated following release from the arrest and this takes Mph1 back to the dephosphorylated form seen in cycling cells. This implied that Mph1 could be under the control of a phosphatase. The dephosphorylation is very rapid

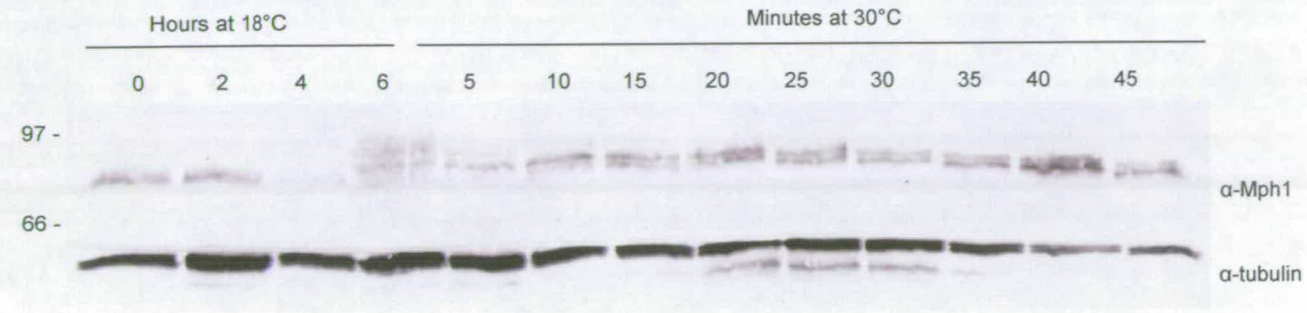
because from the blot it can be seen that after only 10/15 minutes the upper bands have started to disappear and the main bulk of Mph1 is beginning to move down into the lower bands again. The other possibility was that Mph1 was being degraded as cells exited mitosis.

4.3.5 Mph1 phosphorylation in a *pyp3* mutant on release from an *nda3* arrest

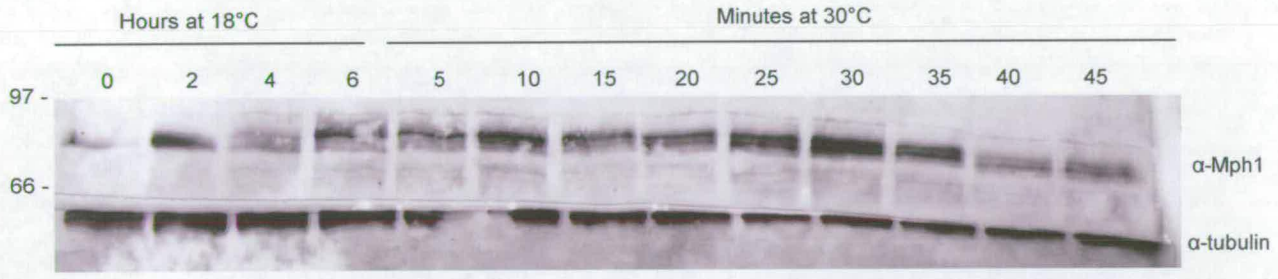
Since it was not clear from the *cdc25-22* block and release experiment in a *pyp3Δ* mutant if mitosis was extended as well as G2 an *nda3* block and release experiment was carried out in a *pyp3Δ* background. This was carried out as described previously

Figure 4.5: Mph1 phosphorylation in an *nda3-KM311* arrest
Cells were arrested at 18°C with the *nda3-KM311* mutation for 6 hour and then released at 30°C. Time points were taken every 2 hours during the arrest and every 15.minutes following the release. Western blots probed all probed with rabbit α-Mph1 antibody and tubulin was used as a loading control.
A Mph1 phosphorylation in an *nda3-KM311* mutant
B Mph1 phosphorylation in a *pyp3Δ nda3-KM311* mutant
C Mph1 phosphorylation in a *bub1KΔ nda3-KM311* mutant

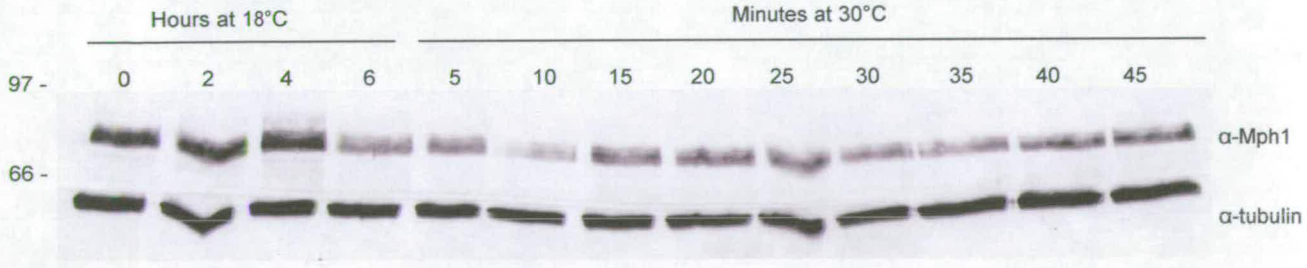
A *nda3-Km311*



B *pyp3Δ nda3-Km311*



C *bub1KΔ nda3-KM311*



(sections 4.3.3 and 4.3.4) with samples from each time point being run out on an SDS-PAGE gel for Western blotting.

Comparing the western blot of the same samples in figure 4.5B shows that the start of the dephosphorylation seen once the cells had been shifted back to 30°C correlated with that of the *nda3-KM311* single mutant (compare figures 4.5B and 4.5A). This would suggest that mitosis is not extended in this mutant. The fact that hyperphosphorylation of Mph1 was not seen for an extend period of time after the shift back to the permissive temperature meant that Pyp3 may not be the phosphatase responsible for the rapid dephosphorylation of Mph1 seen on exit from mitosis.

4.3.6 Mph1 phosphorylation on release from an *nda3-KM311* arrest in a *bub1KΔ* mutant

Since Bub1 is another kinase involved in the spindle checkpoint it seemed logical to investigate whether it was the kinase involved in phosphorylation of Mph1. To study this, a kinase-deleted version of Bub1 was used (Yamaguchi et al., 2003). This takes advantage of the fact that there are separation of function alleles of Bub1. Previous studies in fission yeast by (Vanoosthuyse et al., 2004) have shown that the N-terminus of Bub1 is sufficient for its role in the spindle checkpoint and that the kinase domain is required for its role in chromosome segregation. By using a Bub1 kinase deletion mutant (*bub1KΔ*) in combination with the *nda3-KM311* mutation it will still arrest in response to this mutation and Mph1 phosphorylation can be followed over the time course similar to that in figure 4.5A. Comparison of Figure 4.5A, showing Mph1 going into and out of a checkpoint arrest in the absence of any other mutations, with Figure 4.5C, showing the same arrest and release in the absence of Bub1 kinase activity it is clear that there is a difference between the two experiments. Mph1 still exhibits some hyperphosphorylation on checkpoint activation but not to the full extent as that seen when the kinase domain of Bub1 is present. This may indicate that Bub1 is involved in the regulation of Mph1 in a spindle checkpoint arrest. Another

possibility is that since this mutant did not arrest as well as a single *nda3-KM311* mutant at 18°C (data not shown, the single mutant displays 2% septation whereas the double displays 20%) the reason why there is less hyperphosphorylation seen could be because there are fewer cells in mitosis at any one time compared to the single mutant. This means that while the kinase domain of Bub1 is not thought to be required for the spindle checkpoint function of Bub1 this may not be entirely the case and the ability for the spindle checkpoint to allow cells to arrest efficiently in metaphase following tension and/or attachement defects is enhanced by the presence of the Bub1 kinase domain.

4.3.7 Mph1 phosphorylation in other kinase mutants

Since Mph1 is a phosphoprotein, it is phosphorylated in cycling cells and becomes hyperphosphorylated in mitosis, especially during a spindle checkpoint arrest, there must be a kinase responsible for Mph1 phosphorylation. There are several other kinases, other than Bub1 and Mph1, which act in mitosis and mutants of these (or mutants that affect their activity if the mutant of the actual kinase was not available) were used to test this. The mutants used were *plo1-ts19, plo1-ts2, cdc2-M26* and *cut17-275*. With the exception of *cut17-275* these are all mutants in kinases active in mitosis. Polo-like kinase 1 is found at SPBs in fission yeast during mitosis (discussed in section 3.3.1). In most eukaryotic cells it has been found to have a role in spindle pole maturation, regulation of a bipolar spindle and may help to regulate the activity of the APC/C. It may also have a role in commitment to exit from mitosis (Mulvihill et al., 1999). The spindle pole body association of Plo1 kinase requires Cdc2 and Cdc25 activity.

Cdc2 is required for entry into mitosis. It was the kinase found to be involved in the hyperphosphorylation of fission yeast Bub1 (Yamaguchi et al., 2003) so for this reason it was used in this assay to determine whether both of the spindle checkpoint

kinases were under the control of Cdc2 phosphorylation. The consensus sequence for this phosphorylation was found to be S/T-P-X-K/R. Four putative Cdk consensus sequences had been found in the sequence of *S.pombe* Bub1 and one had been identified as an actual site of this phosphorylation (Yamaguchi et al., 2003). Careful study of the protein sequence of Mph1 did not revealed any strong sequence similarity to this consensus sequence but there was a chance that it was a very weak match to this consensus sequence.

The Cut17 (also known as Bir1 in fission yeast) mutant is the only mutant used in this assay that was not a mitotic kinase. Cut17 is the fission yeast homologue of the passenger protein Survivin. It was found to be involved in chromosome condensation in mitosis anaphase spindle elongation. In the *cut17-275* mutant used in this assay it had been shown previously by (Morishita et al., 2001) that sister chromatid separation failed but securin was still degraded and the cell progressed through to cytokinesis. The reason this mutant was used in an assay looking at the effect of different kinase mutants on Mph1 phosphorylation was because it has been shown to be required for the localization of the fission yeast homologue of Aurora B, Ark1, which is a mitotic kinase (Morishita et al., 2001). It was possible that perturbing Ark1 localisation would affect its function and in the absence of a good mutant of Ark1 being readily available this was used instead in order to study if Ark1 was required for Mph1 phosphorylation.

Cultures of *plo1-ts2, plo1-ts19, cdc2-M26* and *cut17-275* were grown in YE5A overnight at 25°C and then shifted to the restrictive temperature, which was 36°C for all the mutants, for 3 hours. The cultures were then spun down and lysates made in SDS sample buffer. None of these mutants appeared to have an effect on Mph1 phosphorylation (data not shown) but this was difficult to study as the number of arrested cells for each mutant was variable and nowhere near as high as in the *nda3-KM311* mutant.

4.4 Mph1 degradation

From previous western analysis (Figure 4.4A) it was seen that the phosphorylation levels of Mph1 decreased as cells exited a mitotic arrest.This could be due to degradation of the protein, as is the case in budding yeast where Mps1 is degraded as cells progress through to anaphase (Palframan et al., 2006).

As has been seen previously Mph1 does not appear get degraded completely during release from G2 or mitotic arrests but it is possible that Mph1 is continually turned over during these arrests or on release from them. It was considered that if hyperphosphorylated Mph1 is required to maintain a spindle checkpoint dependent arrest then the release from this arrest may require Mph1 to be not only dephosphorylated but degraded as well.

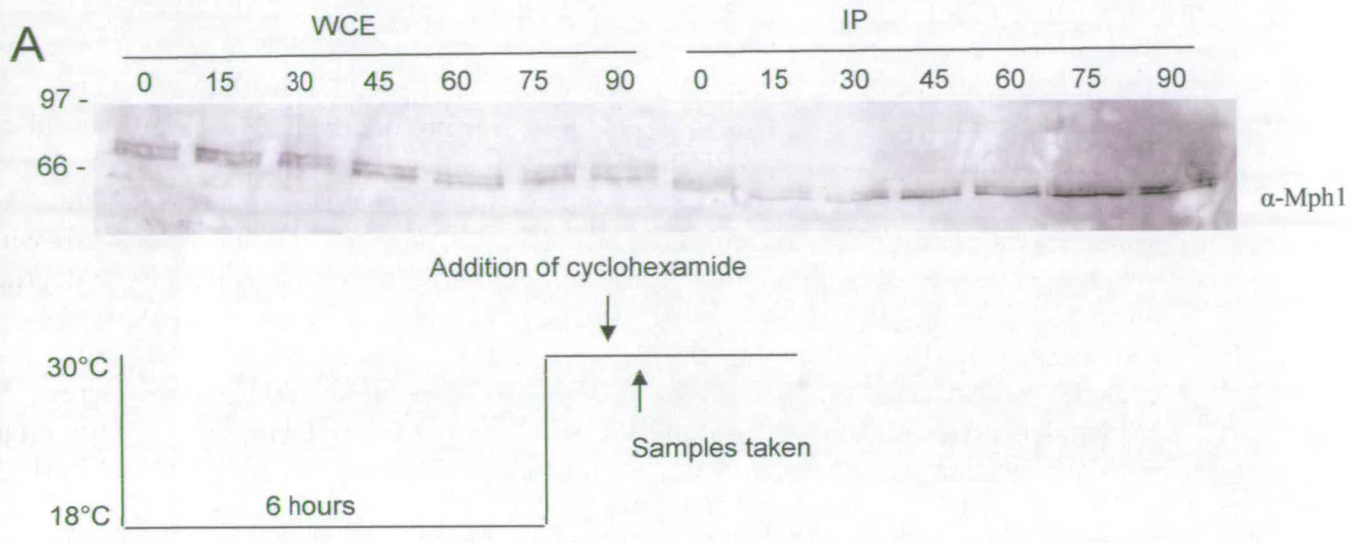
To assess this, cultures of *nda3-KM311* cells were grown up at 30°C and then shifted to 18°C (as detailed previously in this chapter) for 6 hours, to obtain a mitotically arrested population, after which time cycloheximide (from a 100x stock) was added to stop protein production. One of the cultures was shifted back to 30°C to investigate protein turnover on exit from a spindle checkpoint arrest (Figure 4.6A) and the other culture remained at 18°C to investigate protein turn over during the arrest (Figure 4.6B). In order to get a clear picture of this IPs were carried out with the samples and they were treated with λ-phosphatase to ensure that varying levels of phosphorylation did not prove confusing when looking at protein levels. If Mph1 was being turned over it would be seen by western blot as the levels of Mph1 protein would decrease over time. It has been shown for budding yeast that levels of Mps1p fall as cells enter anaphase (Palframan et al., 2006) (see inset in figure 4.6) so it was interesting to see if this was the case in fission yeast.

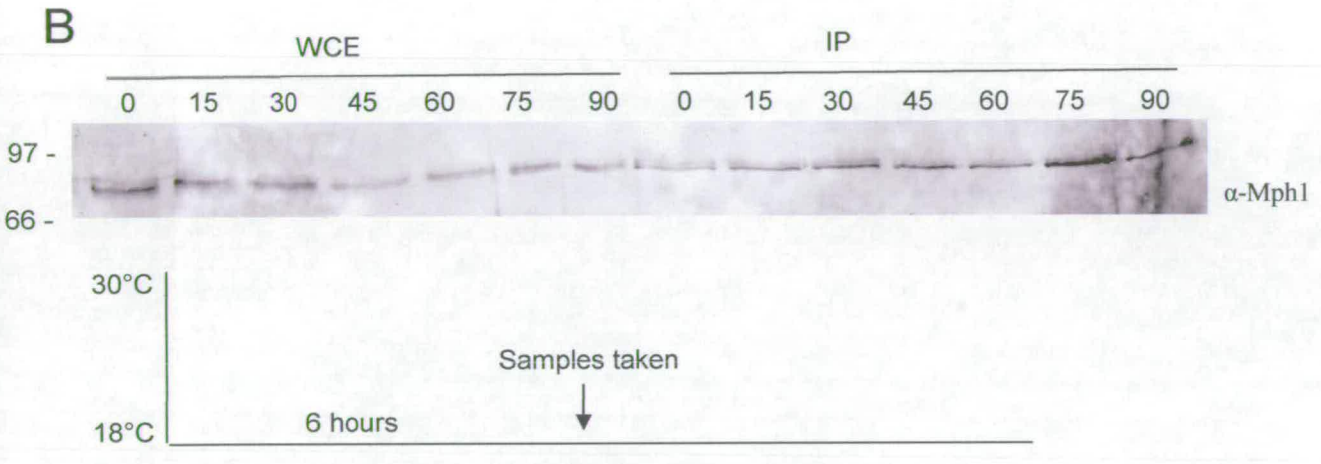
Figures 4.6A and B show that Mph1 does not appear to be degraded during a checkpoint arrest or on exit from mitosis. Levels of the protein remained fairly constant throughout the time courses unlike in budding yeast.

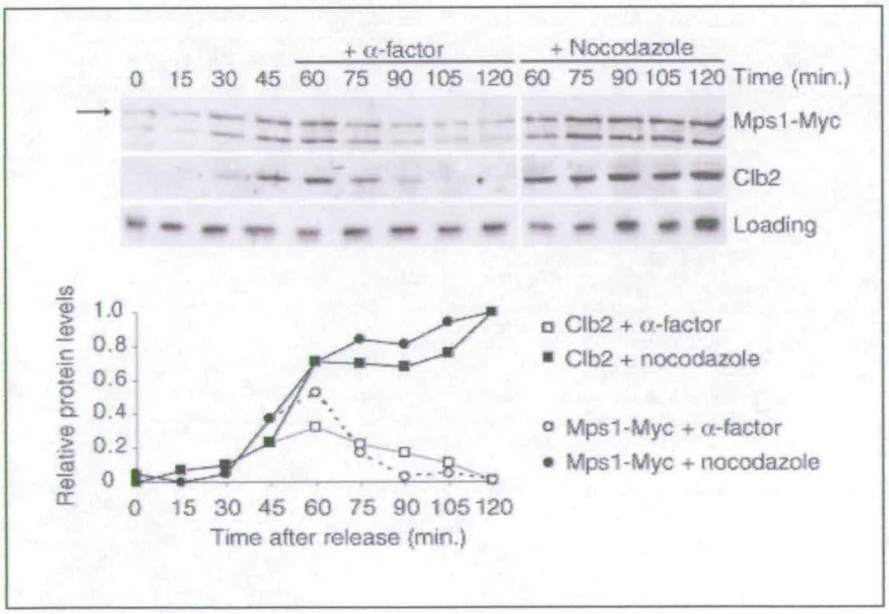
Figure 4.6: Turnover of Mph1

A. Turnover of Mph1 on release from an *nda3-KM311* arrest. *Nda3-KM311*cells were grown 30°C then shifted to 18°C for 6 hours to arrest cells. They were release at 30°C and cyclohexamide added to stop protein production. The culture remained at 30°C for 90 minutes with samples taken every 15 minutes.
IPs of 15 minute time pointes were carried out and λ- phosphatase treated to look at protein levels. The proteins were run out on an SDS-PAGE gel for Western analysis with the membrane probed with rabbit α-Mph1 antibody. Mph1 does not appear to be degraded on release from this arrest.

B. Turn over of Mph1 during an *nda3-KM311* arrest. Cells were arrested at 18°C and cyclohexamide was added after 6 hours as in A. IPs of 15 minute time pointes were carried out and phosphatase treated to look at protein levels. Again proteins run out on an SDS-Page gel and the membrane for Western analysis was probed with rabbit α-Mph1 antibody. Mph1 does not appear to be degraded during this arrest
Insert shows results from budding yeast showing Mph1 degradation during anaphase (Palframan 2006)







From
(Palframan 2006)

4.5 Discussion

It is apparent from the work detailed in this chapter that the phosphorylation state of Mph1 increases during mitosis so the creation of a polyclonal antibody to Mph1 has been invaluable to these studies. The most important experiment highlighting this is the *nda3-KM311* arrest and release experiment (Figure 4.2C), especially in comparison to the *cdc25-22* release experiment showing cells going through and unperturbed mitosis (Figure 4.2B). It shows that as cells arrest in response to the lack of a spindle by the action of the spindle checkpoint Mph1 becomes hyperphosphorylated. It is possible that this hyperphosphorylation is what activates Mph1 and is required for its role in a checkpoint arrest especially as no hyperphosphorylation is seen in a G2 arrest (Figure 4.2A).

It would be useful in the future to map the phosphorylation sites of Mph1 in order to study what effect mutating these would have on a cell's ability to activate the spindle checkpoint and arrest in metaphase. The prediction would be that if the correct phosphorylation sites are mutated then cells would not be able to arrest in response to the *nda3-KM311* mutation. However mutation the residues of the phosphorylation site to aspartic acid, which would mimic phosphorylation, may result in the spindle checkpoint of these cells being constitutively activated. For this to be possible the phosphorylation sites of Mph1 would first have to be identified and mapped. As yet no kinase has been identified as being involved in the regulation of Mph1 despite several mitotic kinases being investigated in this study (figure 4.4). It does not appear that the main mitotic kinases have a role in Mph1 phosphorylation as there is no decrease in the phosphorylated forms of Mph1 seen in these mutants.
At the present time it still remains unclear what kinase is responsible for the hyperphosphorylation of Mph1 in a spindle checkpoint arrest. The results of this study have shown that there may be some effect on hyperphosphorylation of Mph1 in a *bub1kΔ* mutant. Therefore it is possible that if Bub1 was further upstream than Mph1 it may activate Mph1 by phosphorylation which in turn could then phosphorylate other checkpoint components. For Bub1 to be identified as a regulator of Mph1 then it would be useful to investigate whether is can phosphorylate Mph1 in

a kinase assay. Unfortunately the consensus sequence for a Bub1 phosphorylation site is currently not known so it is not possible to determine if there are any in the Mph1 sequence.
It is entirely possible that autophosphorylation will be found to be important for Mph1 activity but as yet no consensus sequence has been identified for phosphorylation by Mps1 in any organisms. Mapping the phosphorylation sites of Mph1 will allow a phospho-mimic strain to be made in order to study the effect this would have on checkpoint activation and it may also give more of an insight into the kinase or kinases involved in the regulation of Mph1, especially during a spindle checkpoint arrest.

As yet the only candidates for the kinase responsible for Mps1 phosphorylation are Mps1 itself and MAP kinase in *Xenopus* cells (Zhao and Chen, 2006). It has been well documented in the past that it appears Mps1 is capable of autophosphorylation (Lauze et al., 1995), (Zhao and Chen, 2006) although it is likely that this is not the only kinase responsible for phosphorylation of Mph1. A recent study by Zhao and Chen (2006) have suggested MAP kinase as a potential regulator of *Xenopus* Mps1. By two-dimensional tryptic-phosphopeptide mapping they showed that there was a phosphopeptide present in metaphase that was not present when MAPK was inhibited. This modification appeared to be required for a functional spindle checkpoint but very little effect was seen on the kinase activity of *Xenopus* Mps1.

As cells are released from the *nda3-KM311* arrest Mph1 becomes dephosphorylated. This means that it must be under the control of a phosphatase. A yeast 2-hybrid study carried out recently in the Hardwick lab using the kinase domain of Mph1 as bait identified a potential candidate for this phosphatase, the Pyp3 phosphatase (P.Rengtved, personal communication). At present very little is known about the Pyp3 phosphatase except that it has a role in Cdc2 dephosphorylation in fission yeast. When overexpressed Pyp3 can compensate for a lack of Cdc25 in a cell. Cdc25 is the main phosphatase responsible for the dephosphorylation of Cdc2 to allow entry into mitosis (Millar et al., 1992). It did not, however, appear that Pyp3 was the phosphatase responsible for the hyperphosphorylation of Mph1 as dephosphorylation of Mph1 on release from an *nda3* arrest occurred at the same rate whether Pyp3 was

present in the cell or not. There is not a delay in mitosis in a *pyp3Δ* strain but G2 is extended when cells are released form a G2 arrest using the *cdc25-22* mutant.

Unlike in budding yeast cells (Palframan et al., 2006) it does not appear that protein degradation is a major factor in Mph1 regulation. It appears that the major regulation of Mph1 maybe through phosphorylation and dephosphorylation.

Chapter 5

A Role for Mph1 in Chromosome Segregation

5.1 Introduction

Studies by Bernard et al., (1998) and Vanoosthuyse et al, (2004) showed that in a *bub1Δ* mutant there are chromosome segregation defects. To date this has been the only mutant of the spindle checkpoint in fission yeast which showed these types of defects which were seen as a high rate of lagging chromosomes and chromosome loss.

Lagging chromosomes were typically seen in centromere mutants, such as mutants of *swi6* and *clr4* which show 34% and 46% (Ekwall et al., 1996). A lagging chromosome is seen as a mass of DNA left near the equator when the rest of the DNA has completed anaphase. They are thought to be the result of merotelically attached chromatids, where one sister chromatid is attached to spindle microtubules coming from both poles (Pidoux et al., 2000). These do not activate the spindle checkpoint as the requirement of tension and attachment are fulfilled. In mammalian cells the kinetochores of these merotelically attached chromatids were seen to be stretched, indicating tension, and electron microscopy in PtK cells have shown them to be attached to bundles of microtubules coming from both poles (Cimini et al., 2001). Lagging chromosomes are seen in fission yeast and higher eukaryotes as the kinetochores of these organisms contain more than one microtubule binding site. This is not the case with budding yeast which only has one microtubule binding site per kinetochore. Studies in budding yeast have revealed Mps1 to be involved in chromosome segregation (Jones et al., 2005) and this chapter investigates whether this is the case in fission yeast. In addition, since *mph1Δ* cells are found to display chromosome loss and lagging chromosomes were observed in *mph1Δ* it was of interest to study what happened in an *mph1Δbub1Δ* mutant since these are the only 2

checkpoint components that have been shown to have these chromosome segregation defects. The result of this was striking when compared to that of the single mutants and compared to *mph1Δ* combined with other checkpoint mutants.

In chapters 3 and 4 it has already been shown that the C-terminal kinase domain of Mph1 is required for its role in the spindle checkpoint (see chapter 3) so it was of interest to investigate if it was also required for proper chromosome segregation. It was also of interest to compare it to Bub1 and to investigate what the outcome would be if both Mph1 and Bub1 were deleted. Both of these proteins are important upstream components of the spindle checkpoint and are both implicated as having additional roles in the process of chromosome segregation.

5.2 Chromosome loss

Chromosome segregation defects are commonly seen as chromosome loss. When problems occur that prevent proper chromosome segregation there is a possibility that sister chromatids may be pulled to the same pole during anaphase leading to aneuploidy, chromosomes will be lost from one daughter and gained by the other. Yeast cannot tolerate chromosome loss as they are single cell organisms and loss of a chromosome would be potentially lethal. To investigate whether, as with *bub1Δ*, there was chromosome loss is observed in *mph1Δ*, two methods were employed.

5.2.1 Half-sectoring assay

The first method used a short (530Kb), linear minichromosome *Ch16* (Matsumoto et al, 1990) in a half sectoring assay (Allshire et al., 1995). The minichromosome contained the *ade6-216* mutation which complements the *ade6-210* mutation of the parent strains. When cells containing this minichromosome are grown on YES low ade plates the resulting colonies are white but if the minichromosome is lost then the colonies are red. When the minichromosome is lost in the first mitotic division the resulting colony will be half white and half red (figure 5.1A). This was the indicator used to score chromosome loss which was scored from the number of half sectored colonies out of total colonies, discounting any completely red colonies as these had lost the minichromosome before the first division.

Comparing chromosome loss in *mph1Δ* with that of the kinase deletion mutant, *mph1KΔ-GFP* shows both strains had a relatively high, but not identical, rate of chromosome loss. There is higher chromosome loss in the *mph1Δ* at 8% compared with 6% in the *mph1KΔ-GFP* (figure 5.1B). Chromosome loss in these mutants with the *Ch16* minichromosome is much higher than the published results for *bub1Δ* which only has a loss rate of 3.5% with the Ch16 minichromsome (Bernard et al., 1998).

5.2.2 *cen2-GFP*

The second method used for scoring chromosome loss used strains that contained GFP marked centromere 2 (*cen2-GFP*). These strains had a tandem array of *lac*O DNA (256 repeats) inserted 5kb from the centromere locus of centromere 2 and expressed GFP-tagged *lacI* which bound to the *lacO* showing centromere 2 as a green dot (Ding et al,. 2004). When cells undergo chromosome segregation without any problems, one dot is seen at each pole in a binucleate cell. However when problems have occurred during anaphase and chromosomes are lost

Figure 5.1: Chromosome loss is seen in mutants of Mph1

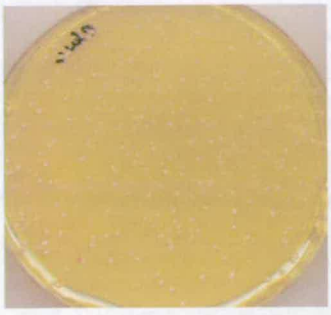
A. Minichromosome loss assay. Top panels show colonies from the half sectoring assay. 1. shows *Ch16* alone with predominately white colonies indicating no chromsome loss. 2. shows *mph1Δch16* colonies showing a high percentage of red secotorred colonies indicating a high rate chromsome loss. 3. shows a sectored colony along with white colonies that have retained the minichromosome and red colonies that did not contain the minichromosome when plated (these were not counted in the assay).
Graph shows that there is a higher percentage of chromosome loss in an *mph1Δ* than in the kinase deletion.

B. Cen2-GFP assay. Strains which had a tandem array of of *lac*O DNA (256 repeats) inserted 5kb from the centromere locus of centromere 2 and expressed GFP-tagged *lacI* which bound to th *lacO* showing centromere 2 as a green dot Cells which had a visible septa were counted in this assay (see table 5.2 for numbers counted). Where two green dots were seen on one side of the seprta this was scored as chromosme loss.Graph shows percentage chromosome loss in different strains especially comparing *mph1Δ* and *bub1Δ* and showing the effect on the double deletion.

C. Side panel shows Cen2-GFP spots properly segregated and a doublet at one pole indicating chromosome loss

A

1.

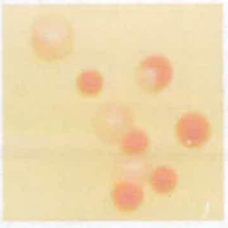


Ch16

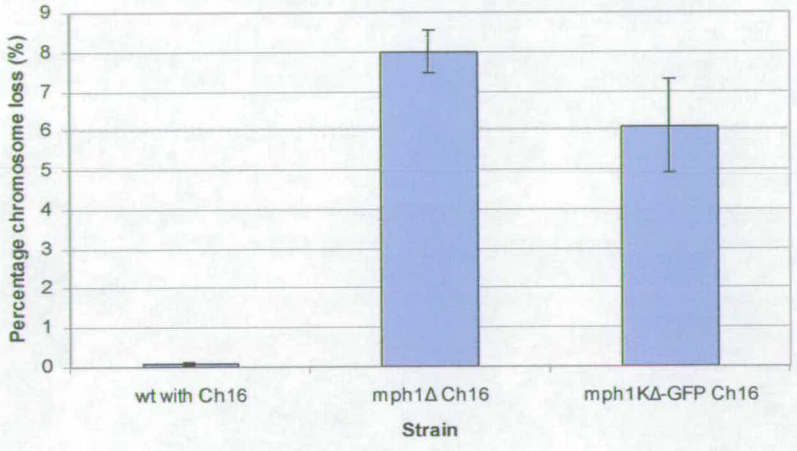
2.

mph1Δ Ch16

3.



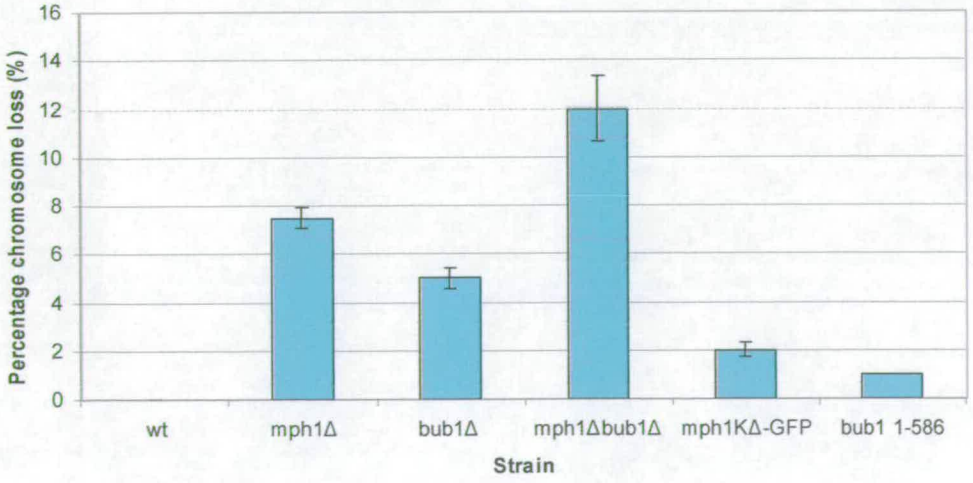
Ch16 minichromosome loss



B

C

Cen2-GFP chromsome loss



then two GFP spots will be seen at one pole and none at the other if it is chromosome 2 that had been lost (2:0 rather that 1:1 segregation) (Figure 5.1C). Cells that had a clear septa were counted to determine the rate of chromosome loss (for number of cells counted for each strain refer to Figure 5.6). Deletions of Mph1 and Bub1 were studied in order to compare them to see if they shared other functions apart from an involvement in the spindle checkpoint. The *mph1KΔ*-GFP was studied to investigate any potential role for the kinase domain of Mph1 in chromosome segregation and *mph1-GFP* was used as a control for this to ensure that the GFP tag was not having an effect on the function of the protein. As had been shown previously (Vanoosthuyse et al., 2004), *bub1Δ cen2-GFP* showed a high rate of chromosome loss (4.9%). Looking at a *mph1Δ cen2-GFP* mutant showed that there was also a high rate of chromosome loss in the absence of Mph1. The rate of chromosome loss in the absence of Mph1 is actually higher that in a *bub1Δ* at 8.1% (Figure 5.1B). The *mph1KΔ-GFP* mutant also showed chromosome loss in this assay, but again it was not to the extent seen in the full deletion of Mph1.

Since *mph1Δ* and *bub1Δ* both displayed chromosome segregation defects these mutants were crossed in order to see if a double mutant could be made or if this would be synthetic lethal. It was possible to generate an *mph1Δbub1Δ* double mutant from this cross so it was combined with *cen2-GFP* in this assay. This gave a striking result in that there was a greatly increased rate of chromosome loss at 12% in the double deletion compared to the single deletions (Figure 5.1B). This was interesting as it hinted that there may be an additive effect when these two mutations were combined.

5.3 Lagging chromosomes

Lagging chromosomes are currently thought to be the result of merotelically attached chromatids (Pidoux et al., 2000). This means that instead of the two sister chromatids being attached to spindle microtubules coming from opposite poles, one of the sisters is attached correctly and the other is attached to microtubules coming from both poles (Figure 5.2A). The result of this is that when anaphase occurs this chromatid cannot move to either pole as it is being pulled in both directions, so it gets stuck in the middle of the cell while the rest of the DNA segregates correctly to either pole. Another reason for lagging chromosomes could be that instead of being merotelic they are completely detached from the spindle and as such cannot move t either pole so remain where they are as anaphase progresses. When cells are stained for tubulin and DNA a lagging chromosome is seen at the centre of the spindle with two masses of DNA at either pole (Figure 5.2B). This segregation defect can occur in cells with a functional spindle checkpoint, unlike monotely and syntely where only one chromatid is attached or both sisters are attached to microtubules from the same pole. Syntelic and monotelic attachments are detected by the spindle checkpoint because both result in a lack of tension across the kinetochores and in the case of monotely there is a lack of attachment as well. This is not the case with merotely. Both kinetochores are attached to microtubules and tension still occurs across the kinetochores, so the spindle checkpoint does not recognise this as a defect and cells progress in to anaphase resulting in a lagging chromosome. This defective attachment will eventually be resolved and the chromatid will be able to move to one of the poles. However this is not necessarily the right pole. For this reason chromosome loss can occur as a result of merotelic attachment. This is not always the case and this is not the only reason for chromosome loss as it is possible for cells to exhibit chromosome loss but not merotely, for example chromosome loss can be seen in budding yeast but this cannot be due to merotelic attachments as budding yeast only have one microtubule binding site per kinetochore.

S.pombe and higher eukaryotes display lagging chromosome in certain kinetochore mutants but this is not the case with *S.cerevisiae* as kinetochores of this organism only contain one microtubule binding site so these kinetochores can only be attached to

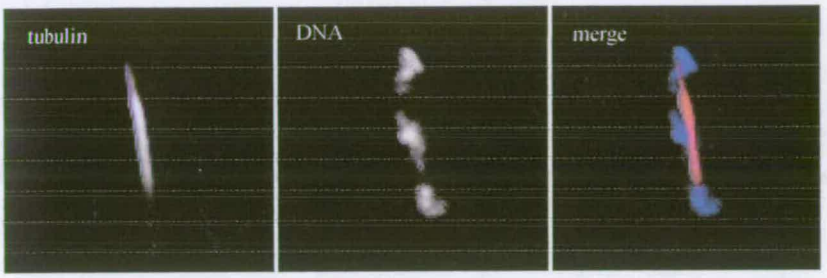
Figure 5.2: Deleting Mph1 results in lagging chromosomes

A. Lagging chromosomes are seen in an *mph1Δ* mutants two DNA masses moving towards the poles with a third mass seen in the middle of the spindle.

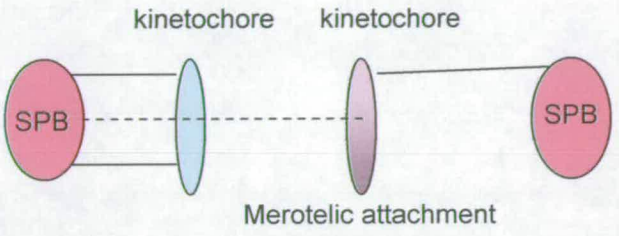
B. Schematic diagram showing a lagging chromosome caused by merotelic attachment.

C. *mph1Δ* and *bub1Δ* both have a high rate of lagging chromosomes. The *mph1KΔ-GFP* also shows lagging chromsomes but not to the extent seen in the full *mph1Δ* mutant. Table 5.2 details the number of cells counted for each strain.

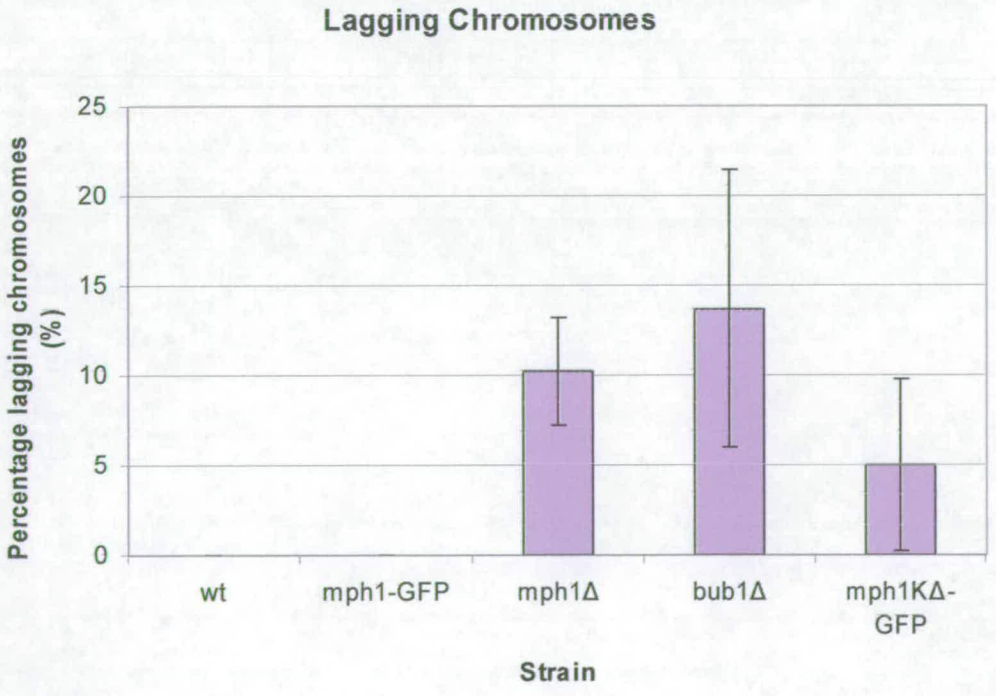
A


tubulin
DNA
merge

B


kinetochore
kinetochore
SPB
SPB
Merotelic attachment

C


Lagging Chromosomes
Percentage lagging chromosomes (%)
25
20
15
10
5
0
wt
mph1-GFP
mph1Δ
bub1Δ
mph1KΔ-GFP
Strain

one microtubule coming from one pole. *S.pombe*, however, contain 3-4 microtubule binding sites per kinetochore (Pidoux and Allshire, 2003).

5.3.1 *mph1Δ* lagging chromosomes

Even though chromosome loss is not always as a result of lagging chromosomes, the *mph1Δ* strain was studied for the presence of lagging chromosomes as they were seen in the *bub1Δ*.

To investigate lagging chromosomes in *mph1Δ,* cells were fixed with formaldehyde and stained with Tat 1 antibody (an antibody against α-tubulin) to stain microtubules and DAPI to stain the DNA. Under the fluorescence microscope cells in late anaphase had a long spindle and the DNA is seen as two masses of DAPI segregated at either pole. If a lagging chromosome was present in the cells they were seen as a small DNA mass near the middle of the spindle between the two main DNA masses (Figure 5.2B). Study of stained *mph1Δ* cells under the fluorescence microscope revealed that lagging chromosomes were found in *mph1Δ.* After counting approximately 1000 cells to determine the prevalence of lagging chromosomes in this strain it was found that there were 10% lagging chromosomes which was comparable to the 13% seen in *bub1Δ* cells (Figure 5.2C). This was a relatively high occurrence and is comparable to that found in deletions of known kinetochore components for example Swi6 and Clr4 (Ekwall et al., 1996). It was interesting that Mph1 and Bub1 were the only two checkpoint components found to have a role in chromosome segregation.

5.3.2 Additive effect of *mph1Δbub1Δ* on lagging chromosomes

Since *mph1Δ* and *bub1Δ* were the only spindle checkpoint mutants that showed lagging chromosomes it was interesting to see what happened when *mph1Δ* was combined with *bub1Δ,* especially considering the combined chromosome loss phenotype seen in this double mutant. Staining the double deletion in the same way as the single deletions and counting lagging chromosomes revealed that there was definitely a synergistic effect seen in the double mutation. There was a rate of 23% lagging chromosomes (Figure 5.3A) in the *mph1Δbub1Δ* mutant which is double that seen in the two single mutants. Importantly, a synthetic effect was not seen when *mph1Δ* was combined with other deletions of checkpoint components. The rate of lagging chromosomes seen in *mph1Δmad2Δ* and *mph1Δbub3Δ* are 9.8% and10.2% respectively and are comparable to an *mph1Δ* single mutant with 10.2% lagging chromosomes (Figure 5.3A). This means that the synergistic effect seen in the *mph1Δbub1Δ* is due to the specific combination of these two mutations and is not a common effect seen among the checkpoint mutants. This is also the case with benomyl sensitivity as *mph1Δmad2Δ* and *mph1Δbub3Δ* look like an *mph1Δ* single mutant on benomyl, whereas the *mph1Δbub1Δ* showed a more severe sensitivity to the drug than either of the single mutants (figure 5.3B)

5.3.3 Effect of removing the kinase domain of Mph1 on lagging chromosomes

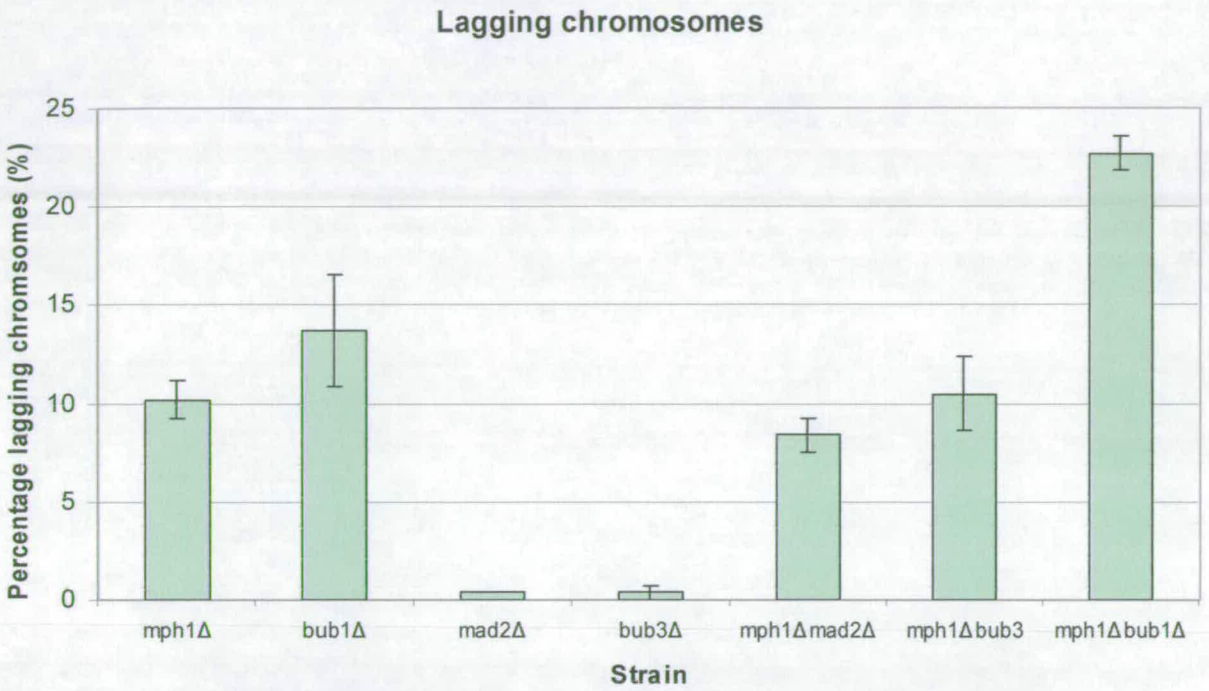
As was the case with chromosome loss in the *mph1kΔ* mutant which was less than that observed in the *mph1Δ* mutant this was also the case for lagging chromosomes which are not obseverd as frequently in this mutant as *mph1Δ,* as there was a high rate of lagging chromosomes in *mph1Δ* at 10% compared to 5% in the kinase deletion (Figure 5.4). This suggested that the kinase is important for the chromosome

Figure 5.3: Additivie effect of the *mph1Δbub1Δ* mutant

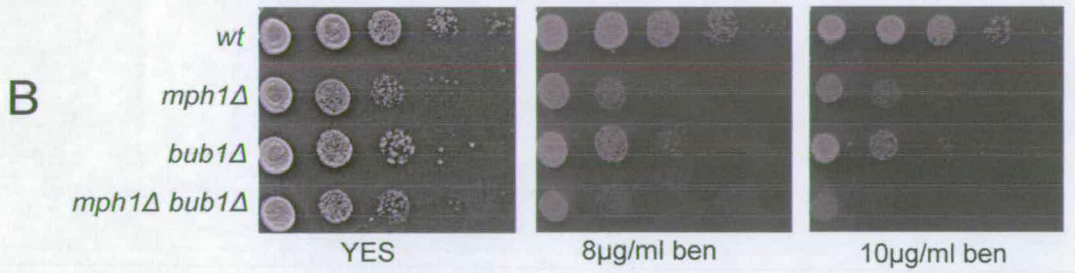
A. Graph showing lagging chromosomes in several mutants. The *mph1Δbub1Δ* has a significantly higher percentage of lagging chromosomes than the two single mutants or when *mph1Δ* is combined with *mad2Δ* or *bub3Δ*. For numbers of cells counted see Table 5.

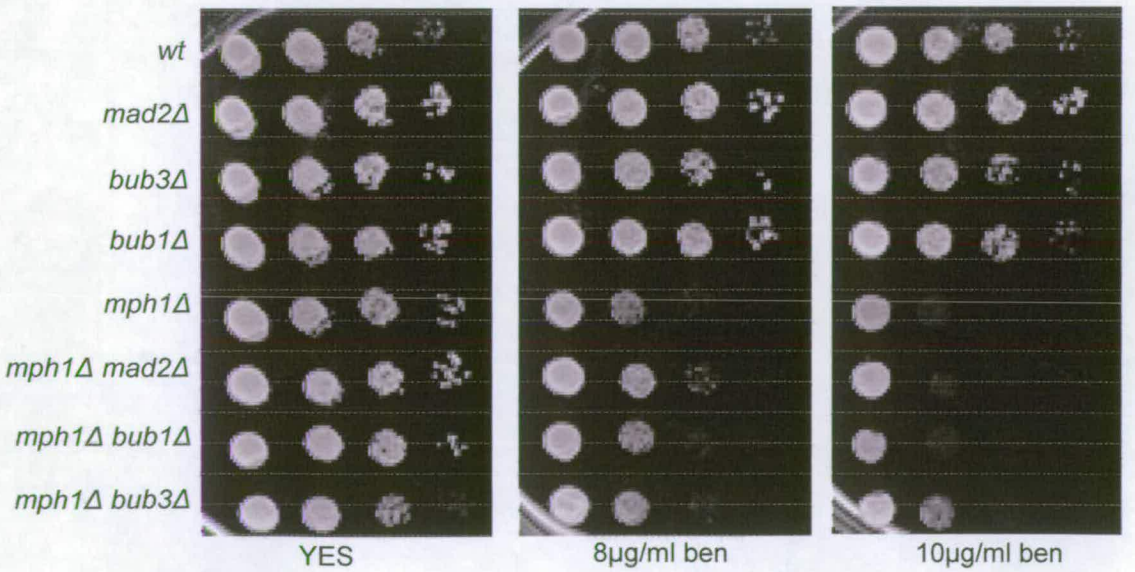
B. Single and double checkpoint mutants plated out on YE5A plates as 1:10 serial dilutions and YE5A containing 8 and 10μg/ml benomyl and incubated at 30°C for 3 days. The additive effect of the *mph1Δbub1Δ* mutant can be seen here as well.

A


Lagging chromosomes
Percentage lagging chromsomes (%)
25
20
15
10
5
0
mph1Δ
bub1Δ
mad2Δ
bub3Δ
mph1Δmad2Δ
mph1Δbub3
mph1Δbub1Δ
Strain

B


wt
mph1Δ
bub1Δ
mph1Δ bub1Δ
YES
8µg/ml ben
10µg/ml ben


wt
mad2Δ
bub3Δ
bub1Δ
mph1Δ
mph1Δ mad2Δ
mph1Δ bub1Δ
mph1Δ bub3Δ
YES
8µg/ml ben
10µg/ml ben

A

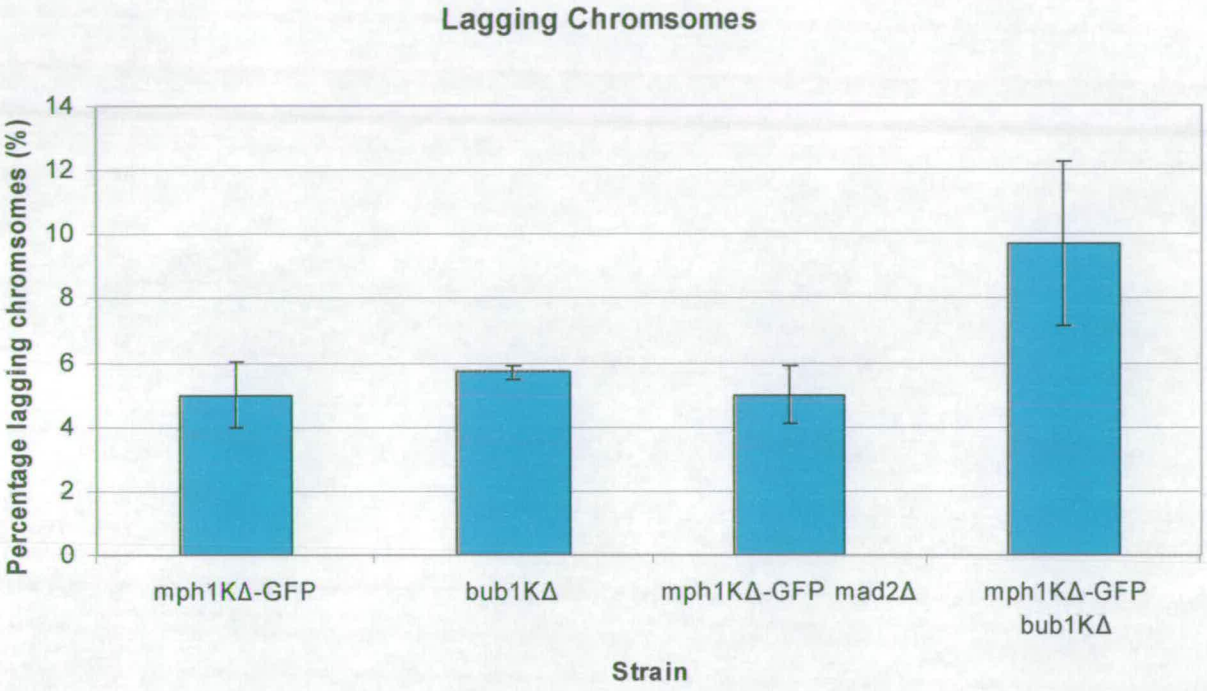


Figure 5.4: Affect of deleting the kinase domain of Mph1 on lagging chromosomes
Cells were grown in YE5A media overnight before being spun down and methanol fixed. They were stained with α-Tat 1 antibody and DAPI to allow visualisation of tubulin and DNA. Cells showing late anaphase spindles were counted. For number of cells counted see Table 5.2. Like the *bub1KΔ,* the *mph1KΔ* mutant has less lagging chromosomes that the full deletion. An additive effect is seen when both kinase deletions are combined.

segregation but the N-terminus may also be important. This is not the case with Bub1 which has a separation of function between the kinase domain and the N-terminus (Vanoosthuyse et al., 2004).

Interestingly, when a double mutant of the kinase deletions of both Mph1 and Bub1 (*mph1KΔ-GFP bub1kΔ*) was made there was also a slight additive effect seen with respect to lagging chromosomes similar to *mph1Δbub1Δ* but not to the same extent. The figure for lagging chromosomes in this mutant is 8.4% which is comparable to those of the two single mutants at 5% and 6% for *mph1KΔ-GFP and bub1kΔ* respectively (Figure 5.4). This suggests that the kinases domains of these proteins are important for the additive effect seen when the two full deletions are combined as there was also an additive effect seen when the kinase deletions were combined. As with *mph1Δmad2Δ* no additive effect was seen with the *mph1KΔ-GFPmad2Δ* double mutant.

5.4 Other synthetic interactions

It was interesting to note that while an additive effect was seen when *mph1Δ* and *bub1Δ* were combined, this was not the case when *mph1Δ* was combined with other spindle checkpoint mutants. *mph1Δmad2Δ* and *mph1Δbub3Δ* show the same percentage of lagging chromosomes as that seen in the single *mph1Δ* mutant. Our interpretation of this was that the defects are not made worse by disrupting the spindle checkpoint even more, but that it was the combination of two mutations (*mph1Δ* and *bub1Δ*) both showing chromosome segregation defects that causes this synthetic effect. An additive effect was also seen in the *mph1Δbub1Δ* double mutant in its sensitivity to benomyl.

In order to investigate further whether Mph1 and Bub1 are working in the same pathway or not, their genetic interactions were compared by reviewing what had been previously published in the literature. There have been several genetic interactions with *mph1Δ* and *bub1Δ* tested and reported in the literature for example with *rad21-K1* (Tomonaga et al., 2000), *clr4Δ*, *mal3Δ* (Asakawa et al., 2005), *atb2* (Asakawa et al., 2006), *klp5Δ* (West et al., 2002) and *taz1Δ*. This range of mutations included a subunit of the cohesion complex, a heterochromatin component, a kinetochore component, tubulin, a kinesin and a telomere protein. A summary of synthetic interactions between these mutants and mutants of Mph1 and Bub1 is shown in Table 5.1. From the table it can be seen that the genetic interactions between *mph1Δ* or *bub1Δ* and these mutations are the same in all cases except for *klp5Δ* which is synthetic lethal with *bub1Δ* but not *mph1Δ*. This was interesting as it suggested that Mph1 and Bub1 may not be working in the same pathway after all.

Another synthetic interaction noted with Mph1 and Bub1 also concerned *nda3-KM311*. When an attempt to make an *mph1Δbub1Δnda3-KM311* triple mutant was carried out by tetrad dissection of an *mph1Δnda3* x *bub1Δnda3-KM311* no viable triple mutant was found.

Strain	*mph1Δ*	*mph1KΔ-GFP*	*bub1Δ*	*bub1 1-586*
klp5Δ	Alive	N.D	Dead	N.D
rad21-K21ts	ts	ts	ts	ts
clr4Δ	Dead	Dead	N.D	N.D
atb2-983	Dead	N.D	Dead	N.D
mal3	ts	N.D	ts	N.D
taz1Δ	ts	N.D	ts	N.D

Table 5.1: Synthetic interactions
Results of a literature search to compare synthetic interactions of an *mph1Δ* mutant and a *bubΔ* mutant to try and identify differences in interactions.

5.5 Discussion

The results of this study demonstrate that along with Bub1, Mph1 has a role within chromosome segregation and are the only spindle components in fission yeast for which this has been found to be the case in. Since the common feature of both of these proteins was that they were kinases and since the kinase domain of Bub1 was shown to be required for proper chromosome segregation in *S.pombe* (Vanoosthuyse et al., 2004), the effect of deleting the kinase domain of Mph1 was studied to determine whether this was the case for Mph1 as well (a summary of all the chromosome mis-segregation results is shown in Table 5.2). It was interesting to note that while lagging chromosomes and chromosome loss were seen in the *mph1KΔ-GFP,* it was not to the extent seen in the full deletion, which means that there may be a part of the N-terminus that is important for this function as well. This means that the N-terminus must be important for carrying out some of the functions of Mph1, for example it may be important for the localisation of Mph1. If Mph1 acts as a scaffold at the kinetochore for other proteins to bind to, then the N-terminus may have a role in getting it there while the C-terminus may then be important for the phosphorylation of interacting proteins. Since *mph1Δ* and *bub1Δ* both resulted in a compromised spindle checkpoint and chromosome segregation defects, they were crossed to determine whether a double mutant would be viable. This was the case, so the double mutant was used in the chromosome segregation assays detailed in this chapter. The outcome of these assays was somewhat surprising as there appeared to be a synthetic effect of combining these mutants. It was originally thought that the phenotype seen would be similar to that seen in *bub1Δ* with respect to chromosome segregation defects as it is the one that shows the highest percentage of these defects. This is because compared to the single mutants the double *mph1Δbub1Δ* strain showed a much higher rate of both chromosome loss and lagging chromosomes. It is not surprising that there is an additive effect on benomyl with the double deletion considering the chromosome loss and lagging chromosome results. Benomyl is a drug that destabilises microtubules which can lead to improper mt-kt attachments that would not be corrected in cells lacking a functional checkpoint such as the *mph1Δbub1Δ* mutant. This combined with the high frequency of chromosome segregation defects seen in this mutant meant that the combination of them was seen as a very low tolerance to the drug.

Strain	Lagging chromosomes	Mini chromosome loss	Cen2-GFP chromosome loss
wt	0% (0/517)	wt+Ch16 0.092% (1/1086)	0% (0/636)
mph1Δ	10.2% (128/1246)	282/3560 (79/1000)	7.4% (74/992)
bub1 Δ	13.7% (131/955)	-	4.4% (32/732)
mph1 Δ bub1 Δ	22.6% (238/1050)	-	12% (95/791)
mph1-GFP	0.2% (1/461)	-	0% (0/670)
mph1K Δ-GFP	5% (50/1008)	235/3872 (61/1000)	1.7% (14/807)
bub1K Δ	5.7% (28/487)	-	1% (7/730)
mph1K Δ-GFP bub1K Δ	9.7% (84/863)	-	-
mad2 Δ	0.4% (2/500)	-	-
bub3 Δ	0.4% (3/736)	-	-
mph1 Δmad2 Δ	10% (46/466)	-	-
mph1 Δbub3 Δ	10.5% (75/713)	-	-
mph1K Δ-GFP mad2 Δ	5.7% (48/844)	-	-

Table 5.2: Summary of chromosome segregation defect results

The fact that an *mph1Δbub1Δnda3-KM311* triple mutant could not be obtained was most likely due to the fact that all three of these mutants show chromosome mis-segregation and the combination of all of them means that the cells have such a high rate of chromosome loss that they are not viable.

There could be several explanations for the synthetic effects seen when *mph1Δ* and *bub1Δ* were combined. Either they could both be part of different pathways that both have similar, over-lapping functions, or they could be part of the same pathway. If they were part of two separate pathways then it may be the case that when one pathway is defective chromosome segregation defects occur but when both pathways are defective the phenotype becomes even more severe as there is no compensation for lack of function. This could also be the case if they are part of the same pathway. Loss of either Mph1 or Bub1 may not completely disable it but loss of both would and so the chromosome mis-segregation phenotype seen would be more severe in the double mutant. The model shown in Figure 5.5 illustrates these two possible explanations. Rad21 is the fission yeast homologue of the cohesin subunit Scc1. It was first identified as a protein involved in DNA repair (Tatebayashi et al., 1998). Rad21 is essential for mitotic progression; it must be degraded for cells to progress through to anaphase (Toyoda et al., 2002).

The Rad21 mutant used in this study was the temperature sensitive (ts) mutant *rad21-K1*. At the permissive temperature cells with this mutation showed a range of abnormalities such as unevenly segregated chromosomes, aberrant early to mid-anaphase chromosomes, a displaced nucleus and sometimes showed a *cut* phenotype (Tatebayashi et al., 1998). During interphase this mutant showed 2 GFP spots when GFP labelled chromosome I was used to look at sister separation. The presence of 2 spots at this time indicated that premature sister separation had occurred (Tomonaga et al., 2000). At the permissive temperature these arrest in mitosis and this arrest is dependent on the spindle checkpoint as *rad21-K1 mad2Δ* failed to arrest at the restrictive temperature (Tomonaga et al., 2000). It was thought that problems with kt-mt interactions were responsible for causing spindle checkpoint activation in this mutant (Toyoda et al., 2002). Since this mutation on its own displayed chromosome segregation defects and died at the restrictive temperature

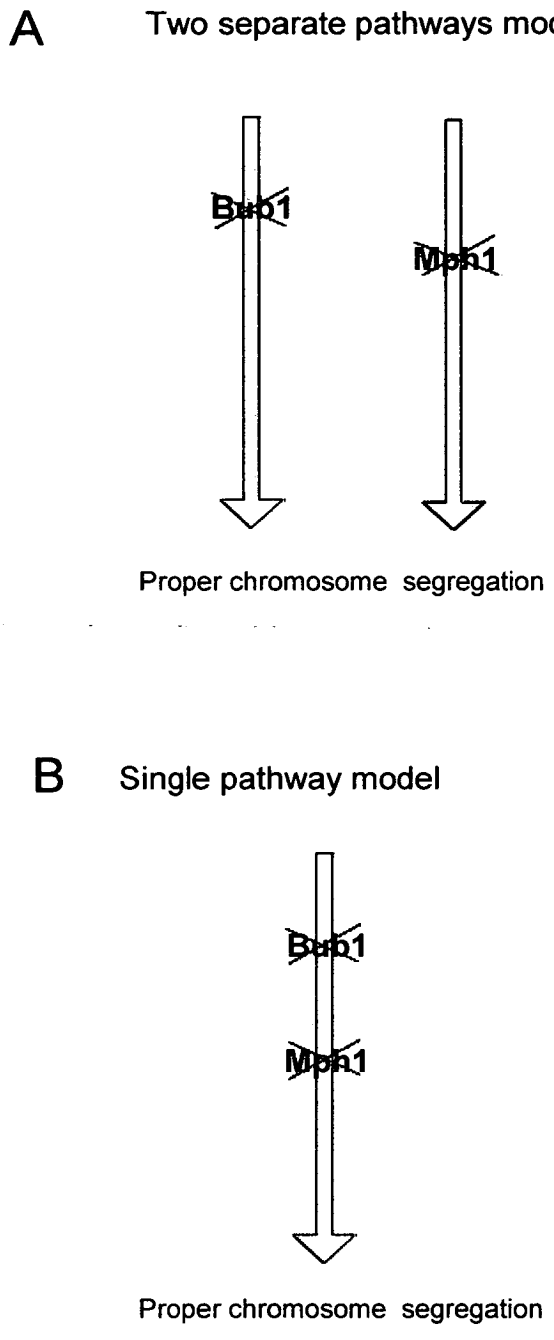


Figure 5.5: Mph1 and Bub1 pathway models

A. Two separate pathway model where Mph1 and Bub1 both act in separate pathways that are both involved in chromosome segregation.

B Single pathway model where Mph1 and Bub1 would both at in the same pathway to control proper chromosome segregation.

when in combination with *mad2Δ*, it was not surprising that this was also the case with *mph1Δ* and *bub1Δ* making the *rad21-K1 mph1Δ* and *rad21-K1 bub1Δ* mutants ts.

Clr4 in fission yeast is a histone methyl transferase that is responsible for the methylation of histone H3 on lysine 9 (Nakayama et al., 2001). This is required to establish regions of transcriptionally inactive DNA to set up regions of heterochromatin at the silent mating type locus, the telomeres and the centromeres. This lysine 9 methylation recruits another protein called Swi6 (the fission yeast HP1 homologue) which binds to these regions and this is required for a functional centromere. In the absence of Clr4 (and Swi6) silencing was lost from the centromere; reporter genes inserted into this region could be expressed (Nakayama et al., 2001). These mutants also showed a high rate of chromosome loss and also lagging chromosomes similar to those described previously in this chapter (Ekwall et al., 1996). It is likely that the combination of two mutations, both with chromosome segregation defects, is lethal and this is why double mutants between *mph1Δ* and *clr4Δ* (and *bub1Δ* and *clr4Δ*) are not viable.

Mal3 is the fission yeast homologue of the microtubule associated protein EB1. It has been shown to be a plus-end tracking protein involved in the regulation of microtubule dynamics and possibly also chromosome stability during mitosis. Mal3 has also been shown to be involved in establishment of bipolar attachment of chromosomes to the mitotic spindle (Asakawa et al., 2005). This is achieved in cooperation with the spindle checkpoint. Double mutants of *mal3Δ* and spindle checkpoint mutants such as *bub1Δ bub3Δ, mad3Δ* and *mph1Δ* were found to have growth defects; they are viable but temperature sensitive. These double mutants showed a high rate of chromosome missegregation at the restrictive temperature (36°C) (Asakawa et al., 2005).

Taz1 is a telomere protein. It is involved in regulating telomere function in fission yeast cells in mitosis and meiosis. In the absence of Taz1 cells show aberrant chromatin structure at telomeres which include highly elongated telomeres and loss of repression of genes adjacent to telomeres. When *taz1Δ* was crossed to *mph1Δ* and *bub1Δ* cells showed a greater loss in viability at 20°C than either of the single mutants.

Atb2 is the fission yeast α-tubulin. The *atb2-983* mutant of this gene is a ts mutant that showed defects in microtubule dynamics during interphase and a high incidence of chromosome segregation defects, such as minichromosome loss and unequal chromosome segregation (Asakawa et al., 2006). The Bub1 branch of the spindle checkpoint was activated in these mutants as Bub1, but not Mad2, was shown to be recruited to the kinetochores. For this reason it was not surprising that when *atb2-983* was crossed to *bub1Δ* the progeny were not viable as the checkpoint response is required to detect the kt-mt defects and delay anaphase onset in this mutant(Asakawa et al., 2006). If this did not happen then the chromosome segregation defects were not rectified and a very high rate of chromosome missegregation was seen. This was also the case when *atb2-983* was crossed to *mph1Δ* in this study; viable spores of the double mutant were not obtained.

The study by (Asakawa et al., 2006) showed that it was *mph1Δ* and *bub1Δ* in combination with *atb2-983* that were synthetic lethal but not *mad2Δ* in combination with *atb2-983.* They suggested that this meant it was the Bub1 branch of the spindle checkpoint was responsible for the arrest in response to the defects in MT-KT interactions that occurred in the *atb2-983* mutant. Since removing the kinase domain of Bub1 had no effect on *atb2-983* viability, it was concluded that it was the spindle checkpoint function, rather than the chromosome segregation function of Bub1 that was important for maintaining viability (Asakawa et al., 2006). Since Mad2 is important for a checkpoint arrest in response to unattached kts it has been suggested that the defect in the *atb2-983* cells may be due the fact that microtubules are attached to kts but not in a bipolar way. This may mean that Bub1 and Mph1 are responding to a lack of tension in this mutant.

Klp5 is a motor protein which belongs to the Kin1 family of kinesins in fission yeast (West et al., 2002). In fission yeast there are two such proteins, Klp5 and Klp6. They have similar functions and a *klp5ΔklpΔ* double mutant was found to have a similar phenotype to that of the *klp5Δ* single mutant. It has since been found that these two proteins exist as a heterodimer (Garcia et al., 2002). For this reason only Klp5 was used in this study as it was assumed that the same result would be obtained from Klp6 as well.

Deletion of Kpl5 was found to result in stabilised microtubules and cells can withstand a high concentration of TBZ (West et al., 2002). For this reason it was

inferred that Klp5 may be involved in microtubule disassembly. The deletion strain also showed abnormal chromosome distribution along the spindle, for example two or three uneven DNA masses were often seen as cells entered mitosis. As cells elongated, however, only two segregating DNA masses were seen, much like in a wild type cell meaning that there was a problem between prophase chromosome condensation and anaphase A but this was rectified by the time cells entered anaphase B (West et al., 2002). The *klp5Δ* cells showed a delay in mitotic progression which may have been a result of spindle checkpoint activation which would have been necessary to allow time to rectify the problems seen in early mitosis. The defect at this point was thought to be in the capture of the kinetochore by the microtubules of the mitotic spindle (Garcia et al., 2002). Since the spindle checkpoint was thought to play a role in this mutant the interaction between Klp5 and members of the checkpoint was investigated. It was found that *klp5Δ* was synthetic lethal with *bub1Δ* and mutants of the APC/C (West et al., 2002). At high temperatures the *klp5Δ bub1Δ* was dead although neither of the single mutants were ts. The reason for this was likely to be because a delay could not be established so the chromosome segregation defects could not be rectified. In the case of the *bub1Δ* it has its own segregation defects as shown in this study and the combination of the two would lead to a high frequency aneuploidy and cell death. What is interesting then was that while a synthetic interaction is seen between Klp5 and Bub1 this is not the case with Mph1.
It was interesting to note that Mph1 and Bub1 display very similar interactions with respect to the mutants detailed above. However a major difference is in their interaction with the motor protein Klp5, as a double mutant with *bub1Δ* had a much reduced viability compared to a double mutant with *mph1Δ*. This may suggest that while they both have functions at the kinetochore, possibly with respect to kinetochoret-microtubule interactions, they are not working in the same pathway. Mph1 may have a related function to Klp5 at the kinetochore which may indicate that they both lie on the same pathway.

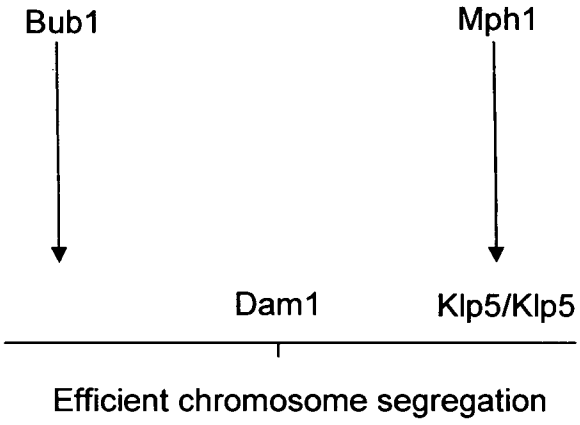
To better understand the synthetic interactions detailed in this chapter and to investigate further whether Mph1 and Bub1 are working in the same pathway or not direct interactors and substrates of these proteins must be found.

Chapter 6

Discussion

This study has identified several roles for the protein Mph1 in the fission yeast *S.pombe*. As it was known to be the homologue of the budding yeast spindle checkpoint component Mps1 it was first tested to ensure it functioned in the spindle checkpoint in fission yeast as well. This study has shown that Mph1 is required for cells to arrest in response to the lack of a mitotic spindle and to prevent cells attempting to re-replicate their DNA after a mitosis in which the spindle structure was disrupted (in a *cut7-24* mutant). This was a good indication that Mph1 was indeed a checkpoint component in fission yeast. Deletion of the kinase domain of Mph1 in fission yeasts results in cells unable to arrest in response to lack of a spindle, in a cold sensitive β-tubulin mutant *nda3-KM311* or a disrupted spindle in a *cut7-24* mutant. The results of this study also show that Mph1 has an additional role in fission yeast apart from in the spindle checkpoint and this is in chromosome segregation. The kinase domain has also been shown to be important for this as lagging chromosomes and chromosome loss are seen in both a full deletion and a kinase deletion mutant. From the work on chromosome segregation it has become likely that Mph1 and Bub have distinct targets and are possibly not working in the same pathway even though they have similar functions (figure 6.1A). This was surprising as it was assumed that the opposite would be the case, but they do not appear to regulate each other and genetic analysis has revealed differing synthetic interactions leading to the assumption that they are in separate pathways that carry out similar functions. With regards to the spindle checkpoint both Mph1 and Bub1 regulate different components as discussed in the introduction and they do not appear to phosphorylate each other (Figure 6.1B).

A Chromosome segregation



B Spindle Checkpoint

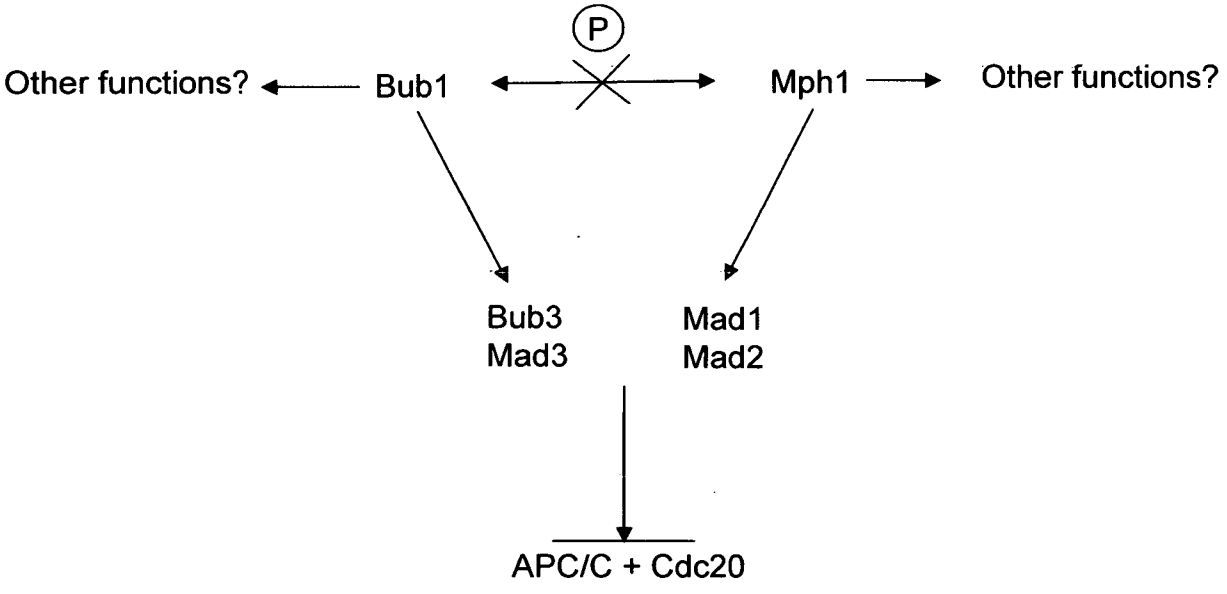


Figure 6.1: Models for Mph1 and Bub1 activity

A. Mph1 and Bub1 may be in separate pathways that are both involved in ensuring correct chromosome segregation.

B. Mph1 and Bub1 regulated different components of the spindle checkpoint and do not appear to phosphorylate each other.

6.1 The kinase domain of Mph1 is important for its role in the spindle checkpoint

Since Mph1 was a protein kinase it was of interest to determine the importance of the kinase domain for the function of Mph1. By making a construct that had the kinase domain of Mph1 replaced with a GFP tag or TAP tag (Figure 3.1) it was possible to assess the function of a kinase deleted Mph1. Using the cold-sensitive β-tubulin mutant *nda3-KM311* mutant in combination with *mph1Δ* or *mph1KΔ-GFP* in a series of experiments it was apparent that the kinase deletion behaved in a similar manner to that of the full deletion. The spindle checkpoint appeared compromised as the cells could not arrest in response to the lack of attachment and tension indicating that kinase domain was involved in the function of Mph1 in the spindle checkpoint.
It was not possible to determine whether the Mph1 kinase domain was required for establishment and maintenance of a checkpoint response or whether it was only necessary for the initial establishment. To determine this is a conditional mutant could have to be used similar to one that has been made in budding yeast. This could be an analogue sensitive allele or perhaps a temperature/cold sensitive mutant. This type of mutation has been used successfully to study other protein kinases such as budding yeast Cdc28 (Bishop et al., 2000). In budding yeast this allele of Mps1 has a mutation in the ATP-binding region of the kinase domain as it removes a bulky hydrophobic side chain. (Jones et al., 2005). This allele can still hydrolyse ATP but when a non-hydrolysable analogue of ATP (1NMPP1) was introduced it was able to bind into the mutated ATP binding pocket of the kinase domain (but not in binding pocket of the wild type kinase domain of other protein kinases present in the cell) and would prevent kinase activity. If this allele could be made in fission yeast then the *plo1*$^{+}$*-GFP nda3-KM311* experiment detailed in section 3.4.1 could be used to investigate whether the kinase domain of Mph1 was important for maintenance of a checkpoint response. By growing cells at 18°C to inactivate the Nda3 protein and in the absence of 1NMPP1 the cells would be able to establish an arrest. If the ATP analogue were then to be introduced then the disappearance of GFP spots at the SPBs would be an indication that the kinase domain was required to maintain a checkpoint arrest.

The use of this analogue sensitive allele in budding yeast demonstrated that that kinase domain was important for the function of Mps1 as cells containing this mutation did not arrest in the presence of mt-depolymerising drugs when 1NMPP1 was added and that it was required for maintenance of an arrest (Jones et al., 2005). Since Mph1 is an upstream component of the spindle checkpoint it is likely that it gets recruited to the kinetochore and acts as a scaffold there to recruit other proteins to the kinetochore. This means that it may have a structural role at the kinetochore as well as an enzymatic function. The implications of this for the role of Mph1 at the kinetochore, as well as in spindle checkpoint signaling could be to help maintain the structural integrity of the kinetochore either by binding to kinetochore components and helping to stabilize the structure or by phosphorylating kinetochore and/or spindle checkpoint components. So far in fission yeast Mph1 has not been shown to phosphorylate spindle checkpoint proteins, however *S.cerevisiae* Mps1 phosphorylated Mad1.

If Mph1 is recruited to kinetochores in order to carry out its function it would be of interest to investigate how functional the kinase domain on its own would be. This would indicate whether the N-terminus is required for a structural role, and assuming this was the case and assuming that it is important to get the protein onto kinetochores (more detailed localisation studies would be required to confirm this) then it would also indicate whether the Mph1 kinase could carry out its function away from the kinetochore.

6.2 Mph1 has a role in chromosome segregation

The work detailed in chapter 5 suggested a role for Mph1 in chromosome segregation. This has also been found to be the case with budding yeast Mps1 (Jones et al., 2005). When Mph1 is deleted then chromosome segregation defects such as chromosome

loss and lagging chromosomes are seen. This suggests a role for Mph1 at the kinetochore, although what this may be is, as yet, unclear. The budding yeast *mps1-as* mutant shows chromosome loss in anaphase in the presence of the inhibitor (Jones et al., 2005). No lagging chromosomes are seen as budding yeast kinetochores only contain one microtubule binding site so merotelic attachments are not possible in budding yeast and that is what is thought to be the cause of the lagging chromosomes in fission yeast. The fact that the *mph1KΔ-GFP* shows a reduced rate for both chromosome loss and lagging chromosomes (figures 5.1 and 5.2 respectively) compared with the full Mph1 deletion suggests that even though the kinase domain is required for efficient chromosome segregation the N-terminus is also required for this. As has been mentioned previously Mph1 may play a structural role at the kinetochore and act as a scaffold, not only for spindle checkpoint components but also for kinetochore proteins.

A surprising result that came out of the chromosome segregation study was the effect of making an *mph1Δbub1Δ* double mutant. This cross was initially made to investigate any possible synthetic interactions between these two kinases. As it was possible to obtain a double mutant from the cross they were not synthetic lethal. What was interesting was the fact that the chromosome missegregation phenotypes (chromosome loss and lagging chromosomes) seen in this double mutant were more severe than that seen in either of the two single mutants (figures 5.1 and 5.2). Bub1 has been shown to be involved in chromosome segregation in fission yeast (Vanoosthuyse et al., 2004) showing both chromosome loss and lagging chromosomes. There was a clear additive effect seen in the *mph1Δbub1Δ* mutant when compared to the single mutants. We propose that even though Bub1 and Mph1 appear to have similar functions with respect to chromosome segregation it is possible that they work in separate, but overlapping pathways.
So far any synthetic interactions between Mps1 and Bub1 in other organisms have not been shown and would be difficult to test due to the fact that Mph1 and Bub1 are essential proteins in many organisms so it is not clear if this is exclusive to fission yeast or if it is universal.

6.3 Mph1 localisation

Due to the evidence from budding yeast, human and mouse cells showing differing results with respect to the localization of Mps1 it of interest to study this in fission yeast. In budding yeast Mps1 is localized not only to the kinetochores but also the SPBs (Winey et al., 1991) which is also the case in mouse cells (Fisk and Winey, 2001). The work on human Mps1 has resulted in conflicting evidence for whether or not Mps1 is at centrosomes. Evidence from one group suggests that it is (Fisk et al., 2003) whereas work from a second group suggests that it is not at ccentrosomes and is restricted to kinetochores (Stucke et al., 2004; Stucke et al., 2002). It was not possible to determine from the work detailed in this study if fission yeast Mph1 was found at SPBs or not as Mph1 was found to diffusely stain the nucleus. It is likely that there is no important role at the SPBs for Mph1 as Mph1 is not essential, unlike budding yeast Mps1. There is possibly another protein which carries out the spindle pole duplication function of Mps1 in fission yeast. However, Mph1 may have partially retained some of this function as it is partially able to rescue the SPB duplication function of a budding yeast ts mutant of Mps1.

The diffuse localization pattern of Mph1 possibly reflects the fact that it has several roles within the nucleus such as the spindle checkpoint function and the chromosome segregation function described in this study. It may also have other functions, for example it may interact with the proteosome, which localises to the nuclear periphery (Wilkinson et al., 1998), as several proteosome components were pulled out of a TAP tag pull down with full length Mph1 (see Appendix 1). It has also been described that human Mps1 co-IPs with two subunits of the APC/C Cdc27 and Cdc16 (fission yeast Nuc2 and Cut9 respectively). This is interesting because Cut9 came out of a yeast 2-hybrid screen in the lab using the kinase domain of Mph1 (P. Rengtved personal communication). This may help to explain the diffuse localisation of Mph1 as the APC/C has been seen to have a diffuse localisation pattern throughout the nucleus (M. Sczaniecka, personal communication) and if Mph1 associates with it then it would be likely that would have a similar localisation pattern.

6.4 Mph1 regulation

The results shown in Chapter 4 of this study indicate that Mph1 is primarily subject to post-translational modifications in mitosis (Figures 4.2 and 4.4). As cells enter a normal, unperturbed mitosis Mph1 is phosphorylated and when the spindle checkpoint is activated Mph1 becomes hyperphosphorylated. It is likely that it is this phosphorylation that is responsible for the activity of Mph1 in the spindle checkpoint. Unlike budding yeast, where Mps1 is a target for APC mediated degradation (Palframan et al., 2006) it does not appear that proteolysis plays a significant role in the regulation of fission yeast Mph1. After release from an α-factor arrest *S.cerevisiae* Mps1 protein level are seen to increase as the cell enters mitosis, then as the cell progresses in to anaphase the protein levels decrease(Palframan et al., 2006). This was not the case for nocodozole arrested cells (metaphase arrest) as the levels of Mps1 remained constant throughout the arrest (Palframan et al., 2006). This is in contrast to the situation with *S.pombe* Mph1. Although levels of Mph1 remain constant during an *nda3-KM311* arrest (cells also arrested in metaphase), the levels of Mph1 did not decrease once Mph1 was released from this arrest and could progress through anaphase (Figure 4.8). This is consistent with the fact that no KEN box or D-box motifs have been found in the protein sequence of Mph1. What was noticeable about the release from this arrest was that while Mph1 was hyperphosphorylated during the arrest it became gradually dephosphorylated as the cells entered anaphase and exited mitosis. This implies that Mph1 is not regulated in the same manner as budding yeast Mps1, and that instead of degradation, a phosphatase may be involved in controlling the activity of Mph1. It has been suggested that budding yeast Mps1 gets degraded at anaphase in order to disable the checkpoint during anaphase in order to prevent segregating sister chromatids activating the checkpoint. Palframan et al (2006) have shown that if Mps1 is expressed during anaphase, even in the absence of spindle damage, then high levels of Pds1 (securin) are seen, indicating that the checkpoint has been activated. It is likely that this occurs because the checkpoint is responding to lack of tension created when the sister chromatids separate (Palframan et al., 2006). This meant that regulating the activity of Mps1 (and it is probably also the case for Mph1) appeared to be important to allow anaphase to progress correctly.

As for what activates Mph1, that is still very unclear. Several candidate kinases were tested but they did not appear to have any effect on Mph1 phosphorylation. Unlike fission yeast Bub1, Mph1 does not appear to be phosphorylated by Cdc2 (figure 4.6).

6.5 Future work

Over all it appears that fission yeast Mph1 shares many of the functions as Mps1 in other organisms, particularly budding yeast. There are, however some striking differences, particularly in terms of its regulation.

In order to fully understand the many roles that Mph1 may have in *S.pombe* it will be important in the future to identify substrates for the kinase. This will be instrumental in defining what processes Mph1 is involved with in the cell and for determining how it functions. It was hoped that kinetochore proteins would be pulled down in the TAP tag pull down but unfortunately this was not the case. Several kinetochore complexes have been purified by other labs, particularly budding yeast complexes and these may prove useful as an indicator of potential targets of Mph1 kinase activity if they are used in a kinase assay with Mph1. This approach is currently being undertaken by a new PhD student in the lab and so far preliminary results using budding yeast kinetochore complexes in a kinase assay with fission yeast Mph1 has identified Ndc80 and Dsn1, a component of the Mtw1/Mis12 complex, as potential substrates (J. Zich personal communication). Studies in budding yeast previously identified Dam1 as a substrate for the Mps1 kinase (Shimogawa et al., 2006). These finding may indicate that Mph1 and Mps1 share a similar, overlapping role to the Aurora kinase at kinetochores.

Determining potential kinases and phosphatases involved in controlling the activity of Mph1 will also be important. It was hoped that potential candidates would have been found through the TAP pulldown but unfortunately this was not the case. This may be because these would be very transient interactions. Unfortunately the one candidate for a phosphatase (Pyp3) turned out to have no clear effect on Mph1 phosphorylation. Using mass spec to map phosphorylation sites on Mph1 may help in identifying candidate kinases if any of these sites match known consensus sites for kinases.

Mutating any phosphorylation sites will give an insight into how important phosphorylation is for Mph1 activity and whether the same sites are important for allowing it to function in the spindle checkpoint and in chromosome segregation.

Bibliography

Abrieu, A., Magnaghi-Jaulin, L., Kahana, J. A., Peter, M., Castro, A., Vigneron, S., Lorca, T., Cleveland, D. W., and Labbe, J. C. (2001). Mps1 is a kinetochore-associated kinase essential for the vertebrate mitotic checkpoint. Cell *106*, 83-93.

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.

Amor, D. J., Kalitsis, P., Sumer, H., and Choo, K. H. (2004). Building the centromere: from foundation proteins to 3D organization. Trends Cell Biol *14*, 359-368.

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. Dev Cell *6*, 253-268.

Asakawa, K., Kume, K., Kanai, M., Goshima, T., Miyahara, K., Dhut, S., Tee, W. W., Hirata, D., and Toda, T. (2006). The V260I mutation in fission yeast alpha-tubulin Atb2 affects microtubule dynamics and EB1-Mal3 localization and activates the Bub1 branch of the spindle checkpoint. Mol Biol Cell *17*, 1421-1435.

Asakawa, K., Toya, M., Sato, M., Kanai, M., Kume, K., Goshima, T., Garcia, M. A., Hirata, D., and Toda, T. (2005). Mal3, the fission yeast EB1 homologue, cooperates with Bub1 spindle checkpoint to prevent monopolar attachment. EMBO Rep *6*, 1194-1200.

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.

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., Partridge, J. F., Genier, S., Javerzat, J. P., and Allshire, R. C. (2001). Requirement of heterochromatin for cohesion at centromeres. Science *294*, 2539-2542.

Bishop, A. C., Ubersax, J. A., Petsch, D. T., Matheos, D. P., Gray, N. S., Blethrow, J., Shimizu, E., Tsien, J. Z., Schultz, P. G., Rose, M. D., *et al.* (2000). A chemical switch for inhibitor-sensitive alleles of any protein kinase. Nature *407*, 395-401.

Brady, D. M., and Hardwick, K. G. (2000). Complex formation between Mad1p, Bub1p and Bub3p is crucial for spindle checkpoint function. Curr Biol *10*, 675-678.

Carroll, C. W., Enquist-Newman, M., and Morgan, D. O. (2005). The APC subunit Doc1 promotes recognition of the substrate destruction box. Curr Biol *15*, 11-18.

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. (2001). Implication of a novel multiprotein Dam1p complex in outer kinetochore function. J Cell Biol *155*, 1137-1145.

Cheeseman, I. M., Chappie, J. S., Wilson-Kubalek, E. M., and Desai, A. (2006). The conserved KMN network constitutes the core microtubule-binding site of the kinetochore. Cell *127*, 983-997.

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.
Cimini, D., Howell, B., Maddox, P., Khodjakov, A., Degrassi, F., and Salmon, E. D. (2001). Merotelic kinetochore orientation is a major mechanism of aneuploidy in mitotic mammalian tissue cells. J Cell Biol *153*, 517-527.
Cleveland, D. W., Mao, Y., and Sullivan, K. F. (2003). Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. Cell *112*, 407-421.
Cottarel, G., Shero, J. H., Hieter, P., and Hegemann, J. H. (1989). A 125-base-pair CEN6 DNA fragment is sufficient for complete meiotic and mitotic centromere functions in Saccharomyces cerevisiae. Mol Cell Biol *9*, 3342-3349.
De Antoni, A., Pearson, C. G., Cimini, D., Canman, J. C., Sala, V., Nezi, L., Mapelli, M., Sironi, L., Faretta, M., Salmon, E. D., and Musacchio, A. (2005). The Mad1/Mad2 complex as a template for Mad2 activation in the spindle assembly checkpoint. Curr Biol *15*, 214-225.
DeLuca, J. G., Gall, W. E., Ciferri, C., Cimini, D., Musacchio, A., and Salmon, E. D. (2006). Kinetochore microtubule dynamics and attachment stability are regulated by Hec1. Cell *127*, 969-982.
Ding, R., McDonald, K. L., and McIntosh, J. R. (1993). Three-dimensional reconstruction and analysis of mitotic spindles from the yeast, Schizosaccharomyces pombe. J Cell Biol *120*, 141-151.
Earnshaw, W. C., and Bernat, R. L. (1991). Chromosomal passengers: toward an integrated view of mitosis. Chromosoma *100*, 139-146.
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. R., Javerzat, J. P., 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 (Pt 11)*, 2637-2648.
Ekwall, K., Olsson, T., Turner, B. M., Cranston, G., and Allshire, R. C. (1997). Transient inhibition of histone deacetylation alters the structural and functional imprint at fission yeast centromeres. Cell *91*, 1021-1032.
Fisk, H. A., Mattison, C. P., and Winey, M. (2003). Human Mps1 protein kinase is required for centrosome duplication and normal mitotic progression. Proc Natl Acad Sci U S A *100*, 14875-14880.
Fisk, H. A., and Winey, M. (2001). The mouse Mps1p-like kinase regulates centrosome duplication. Cell *106*, 95-104.
Fleig, U., Sen-Gupta, M., and Hegemann, J. H. (1996). Fission yeast mal2+ is required for chromosome segregation. Mol Cell Biol *16*, 6169-6177.
Friedman, D. B., Kern, J. W., Huneycutt, B. J., Vinh, D. B., Crawford, D. K., Steiner, E., Scheiltz, D., Yates, J., 3rd, Resing, K. A., Ahn, N. G., *et al.* (2001). Yeast Mps1p phosphorylates the spindle pole component Spc110p in the N-terminal domain. J Biol Chem *276*, 17958-17967.
Garcia, M. A., Koonrugsa, N., and Toda, T. (2002). 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.

Gardner, R. D., and Burke, D. J. (2000). The spindle checkpoint: two transitions, two pathways. Trends Cell Biol *10*, 154-158.
Gruber, S., Haering, C. H., and Nasmyth, K. (2003). Chromosomal cohesin forms a ring. Cell *112*, 765-777.
Hagan, I., and Yanagida, M. (1990). Novel potential mitotic motor protein encoded by the fission yeast cut7+ gene. Nature *347*, 563-566.
Hagan, I., and Yanagida, M. (1992). Kinesin-related cut7 protein associates with mitotic and meiotic spindles in fission yeast. Nature *356*, 74-76.
Hagstrom, K. A., and Meyer, B. J. (2003). Condensin and cohesin: more than chromosome compactor and glue. Nat Rev Genet *4*, 520-534.
Hall, I. M., Shankaranarayana, G. D., Noma, K., Ayoub, N., Cohen, A., and Grewal, S. I. (2002). Establishment and maintenance of a heterochromatin domain. Science *297*, 2232-2237.
Hanks, S. K., Quinn, A. M., and Hunter, T. (1988). The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science *241*, 42-52.
Hardwick, K. G. (1998). The spindle checkpoint. Trends Genet *14*, 1-4.
Hardwick, K. G., Weiss, E., Luca, F. C., Winey, M., and Murray, A. W. (1996). Activation of the budding yeast spindle assembly checkpoint without mitotic spindle disruption. Science *273*, 953-956.
Hartwell, L. H., and Weinert, T. A. (1989). Checkpoints: controls that ensure the order of cell cycle events. Science *246*, 629-634.
He, X., Jones, M. H., Winey, M., and Sazer, S. (1998). Mph1, a member of the Mps1-like family of dual specificity protein kinases, is required for the spindle checkpoint in S. pombe. J Cell Sci *111 (Pt 12)*, 1635-1647.
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.
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.
Howell, B. J., Moree, B., Farrar, E. M., Stewart, S., Fang, G., and Salmon, E. D. (2004). Spindle checkpoint protein dynamics at kinetochores in living cells. Curr Biol *14*, 953-964.
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.
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.
Jones, M. H., Huneycutt, B. J., Pearson, C. G., Zhang, C., Morgan, G., Shokat, K., Bloom, K., and Winey, M. (2005). Chemical genetics reveals a role for Mps1 kinase in kinetochore attachment during mitosis. Curr Biol *15*, 160-165.
Karpen, G. H., and Allshire, R. C. (1997). The case for epigenetic effects on centromere identity and function. Trends Genet *13*, 489-496.
Kerres, A., Jakopec, V., and Fleig, U. (2007). The Conserved Spc7 Protein Is Required for Spindle Integrity and Links Kinetochore Complexes in Fission Yeast. Mol Biol Cell.
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.

Kline, S. L., Cheeseman, I. M., Hori, T., Fukagawa, T., and Desai, A. (2006). The human Mis12 complex is required for kinetochore assembly and proper chromosome segregation. J Cell Biol *173*, 9-17.
Kotwaliwale, C., and Biggins, S. (2006). Microtubule capture: a concerted effort. Cell *127*, 1105-1108.
Lauze, E., Stoelcker, B., Luca, F. C., Weiss, E., Schutz, A. R., and Winey, M. (1995). Yeast spindle pole body duplication gene MPS1 encodes an essential dual specificity protein kinase. Embo J *14*, 1655-1663.
Lee, K. S., Park, J. E., Asano, S., and Park, C. J. (2005). Yeast polo-like kinases: functionally conserved multitask mitotic regulators. Oncogene *24*, 217-229.
Lengronne, A., McIntyre, J., Katou, Y., Kanoh, Y., Hopfner, K. P., Shirahige, K., and Uhlmann, F. (2006). Establishment of sister chromatid cohesion at the S. cerevisiae replication fork. Mol Cell *23*, 787-799.
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.
Li, Y., and Benezra, R. (1996). Identification of a human mitotic checkpoint gene: hsMAD2. Science *274*, 246-248.
Liu, S. T., Chan, G. K., Hittle, J. C., Fujii, G., Lees, E., and Yen, T. J. (2003). Human MPS1 kinase is required for mitotic arrest induced by the loss of CENP-E from kinetochores. Mol Biol Cell *14*, 1638-1651.
Liu, X., McLeod, I., Anderson, S., Yates, J. R., 3rd, and He, X. (2005). Molecular analysis of kinetochore architecture in fission yeast. Embo J *24*, 2919-2930.
McAinsh, A.D., Tytell, J.D., and Sorger, P.K. (2003) Structure, function and regulation of budding yeast kinetochores. Annu Rev Cell Dev Biol *19*, 519-539.
Maiato, H., DeLuca, J., Salmon, E. D., and Earnshaw, W. C. (2004). The dynamic kinetochore-microtubule interface. J Cell Sci *117*, 5461-5477.
Maiato, H., and Sunkel, C. E. (2004). Kinetochore-microtubule interactions during cell division. Chromosome Res *12*, 585-597.
Mellone, B. G., Ball, L., Suka, N., Grunstein, M. R., Partridge, J. F., and Allshire, R. C. (2003). Centromere silencing and function in fission yeast is governed by the amino terminus of histone H3. Curr Biol *13*, 1748-1757.
Millar, J. B., Lenaers, G., and Russell, P. (1992). Pyp3 PTPase acts as a mitotic inducer in fission yeast. Embo J *11*, 4933-4941.
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.
Morgan, D. O. (2007). The cell cycle : principles of control (Northants: Oxford University Press).
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.
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.
Murray, A. (1994). Cell cycle checkpoints. Curr Opin Cell Biol *6*, 872-876.

Murray, A. W. (2004). Recycling the cell cycle: cyclins revisited. Cell *116*, 221-234.
Musacchio, A., and Hardwick, K. G. (2002). The spindle checkpoint: structural insights into dynamic signalling. Nat Rev Mol Cell Biol *3*, 731-741.
Nakayama, J., Rice, J. C., Strahl, B. D., Allis, C. D., and Grewal, S. I. (2001). Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science *292*, 110-113.
Nasmyth, K. (2002). Segregating sister genomes: the molecular biology of chromosome separation. Science *297*, 559-565.
Nasmyth, K. (2005). How might cohesin hold sister chromatids together? Philos Trans R Soc Lond B Biol Sci *360*, 483-496.
Nonaka, N., Kitajima, T., Yokobayashi, S., Xiao, G., Yamamoto, M., Grewal, S. I., and Watanabe, Y. (2002). Recruitment of cohesin to heterochromatic regions by Swi6/HP1 in fission yeast. Nat Cell Biol *4*, 89-93.
Nurse, P. (2000). A long twentieth century of the cell cycle and beyond. Cell *100*, 71-78.
Palframan, W. J., Meehl, J. B., Jaspersen, S. L., Winey, M., and Murray, A. W. (2006). Anaphase inactivation of the spindle checkpoint. Science *313*, 680-684.
Partridge, J. F., Borgstrom, B., and Allshire, R. C. (2000). Distinct protein interaction domains and protein spreading in a complex centromere. Genes Dev *14*, 783-791.
Peters, J. M. (2006). The anaphase promoting complex/cyclosome: a machine designed to destroy. Nat Rev Mol Cell Biol *7*, 644-656.
Pidoux, A., and Allshire, R. (2003). Chromosome segregation: clamping down on deviant orientations. Curr Biol *13*, R385-387.
Pidoux, A. L., and Allshire, R. C. (2004). Kinetochore and heterochromatin domains of the fission yeast centromere. Chromosome Res *12*, 521-534.
Pidoux, A. L., and Allshire, R. C. (2005). The role of heterochromatin in centromere function. Philos Trans R Soc Lond B Biol Sci *360*, 569-579.
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., Kotwaliwale, C. V., Tatsutani, S. Y., Breed, C. A., and Biggins, S. (2006). Glc7/protein phosphatase 1 regulatory subunits can oppose the Ipl1/aurora protein kinase by redistributing Glc7. Mol Cell Biol *26*, 2648-2660.
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.
Rape, M., Reddy, S. K., and Kirschner, M. W. (2006). The processivity of multiubiquitination by the APC determines the order of substrate degradation. Cell *124*, 89-103.
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.
Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. (1999). A generic protein purification method for protein complex characterization and proteome exploration. Nat Biotechnol *17*, 1030-1032.
Salmon, E. D. (2005). Microtubules: a ring for the depolymerization motor. Curr Biol *15*, R299-302.
Salmon, E. D., Cimini, D., Cameron, L. A., and DeLuca, J. G. (2005). Merotelic kinetochores in mammalian tissue cells. Philos Trans R Soc Lond B Biol Sci *360*, 553-568.

Sandall, S., Severin, F., McLeod, I. X., Yates, J. R., 3rd, Oegema, K., Hyman, A., and Desai, A. (2006). A Bir1-Sli15 complex connects centromeres to microtubules and is required to sense kinetochore tension. Cell *127*, 1179-1191.
Schutz, A. R., Giddings, T. H., Jr., Steiner, E., and Winey, M. (1997). The yeast CDC37 gene interacts with MPS1 and is required for proper execution of spindle pole body duplication. J Cell Biol *136*, 969-982.
Schuyler, S. C., and Pellman, D. (2001). Microtubule "plus-end-tracking proteins": The end is just the beginning. Cell *105*, 421-424.
Shah, J. V., Botvinick, E., Bonday, Z., Furnari, F., Berns, M., and Cleveland, D. W. (2004). Dynamics of centromere and kinetochore proteins; implications for checkpoint signaling and silencing. Curr Biol *14*, 942-952.
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.
Shimogawa, M. M., Graczyk, B., Gardner, M. K., Francis, S. E., White, E. A., Ess, M., Molk, J. N., Ruse, C., Niessen, S., Yates, J. R., 3rd, *et al.* (2006). Mps1 phosphorylation of dam1 couples kinetochores to microtubule plus ends at metaphase. Curr Biol *16*, 1489-1501.
Stern, B. M., and Murray, A. W. (2001). Lack of tension at kinetochores activates the spindle checkpoint in budding yeast. Curr Biol *11*, 1462-1467.
Stucke, V. M., Baumann, C., and Nigg, E. A. (2004). Kinetochore localization and microtubule interaction of the human spindle checkpoint kinase Mps1. Chromosoma *113*, 1-15.
Stucke, V. M., Sillje, H. H., Arnaud, L., and Nigg, E. A. (2002). Human Mps1 kinase is required for the spindle assembly checkpoint but not for centrosome duplication. Embo J *21*, 1723-1732.
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.
Tanaka, T. U. (2005). Chromosome bi-orientation on the mitotic spindle. Philos Trans R Soc Lond B Biol Sci *360*, 581-589.
Tatebayashi, K., Kato, J., 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*, 49-57.
Tomonaga, T., Nagao, K., Kawasaki, Y., Furuya, K., Murakami, A., Morishita, J., Yuasa, T., Sutani, T., Kearsey, S. E., Uhlmann, F., *et al.* (2000). Characterization of fission yeast cohesin: essential anaphase proteolysis of Rad21 phosphorylated in the S phase. Genes Dev *14*, 2757-2770.
Toyoda, Y., Furuya, K., Goshima, G., Nagao, K., Takahashi, K., and Yanagida, M. (2002). Requirement of chromatid cohesion proteins rad21/scc1 and mis4/scc2 for normal spindle-kinetochore interaction in fission yeast. Curr Biol *12*, 347-358.
Trinkle-Mulcahy, L., and Lamond, A. I. (2006). Mitotic phosphatases: no longer silent partners. Curr Opin Cell Biol *18*, 623-631.

Vagnarelli, P., and Earnshaw, W. C. (2004). Chromosomal passengers: the four-dimensional regulation of mitotic events. Chromosoma *113*, 211-222.
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.
Vos, L. J., Famulski, J. K., and Chan, G. K. (2006). How to build a centromere: from centromeric and pericentromeric chromatin to kinetochore assembly. Biochem Cell Biol *84*, 619-639.
Watanabe, Y. (2005). Sister chromatid cohesion along arms and at centromeres. Trends Genet *21*, 405-412.
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.
Wells, W. A. (1996). The spindle-assembly checkpoint: aiming for a perfect mitosis, every time. Trends Cell Biol *6*, 228-234.
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.
Wilkinson, C. R., Wallace, M., Morphew, M., Perry, P., Allshire, R., Javerzat, J. P., McIntosh, J. R., and Gordon, C. (1998). Localization of the 26S proteasome during mitosis and meiosis in fission yeast. Embo J *17*, 6465-6476.
Winey, M., Goetsch, L., Baum, P., and Byers, B. (1991). MPS1 and MPS2: novel yeast genes defining distinct steps of spindle pole body duplication. J Cell Biol *114*, 745-754.
Winey, M., and Huneycutt, B. J. (2002). Centrosomes and checkpoints: the MPS1 family of kinases. Oncogene *21*, 6161-6169.
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.
Yu, H. (2002). Regulation of APC-Cdc20 by the spindle checkpoint. Curr Opin Cell Biol *14*, 706-714.
Zhao, Y., and Chen, R. H. (2006). Mps1 phosphorylation by MAP kinase is required for kinetochore localization of spindle-checkpoint proteins. Curr Biol *16*, 1764-1769.

Appendix

TAP-tag pull down of Mph1

A.1 SZZ-tag

Currently very little is known about interactors and substrates of Mps1 homologues in any organism. The only interactions that have been shown were in budding yeast where yeast 2-hybrid analysis showed it to interact with Mob1, a component of the mitotic exit network (MEN) and Spc110, a spindle pole body component (Winey and Huneycutt, 2002). The only substrate known for Mps1 has also only been found in budding yeast these are Spc110 (Friedman et al., 2001) and Dam1 (Shimogawa et al., 2006) and it phosphorylates Mad1. This is not the case in fission yeast where there is no clear evidence that Mad1 is phosphorylated.
For this reason a TAP-tag pull down was carried out using full length Mph1 fused to an SZZ-tag. The tagging was carried out using a pFA6 tagging vector containing the SZZ-tag construct. The tag was transformed into yeast as described previously (Section 3.2). The SZZ-tag is a modified version of the tandem affinity purification (TAP) tag (Rigaut et al., 1999) with an S-tag in place of the calmodulin binding domain and the ZZ tag (minimal protein A binding domain) after the TEV cleavage site (Cheeseman et al., 2001).

Two Mph1 SZZ-tag pull downs were carried out, one using cycling cells and one using *nda3* arrested cells. This was in order to identify and substrates that are specifically phosphorylated when the spindle checkpoint is activated and potential regulators of Mph1 activity. It was also hoped that some kinetochore components may be pulled down as it seems that Mph1 has a role at the kinetochore.

A.2 Protein pull down

The pull down was carried out by growing up 4L of yeast, either *mph1-SZZ* or mph1-*SZZ nda3*. The *nda3* strain was arrested by shifting temperature to 18°C for 6 hours. The cells were then spun down and ground to a powder under liquid nitrogen. The powder was resuspended in buffer and sonicated. The extract was then passed over S-agarose beads to allow the tag to bind. The bound protein was subjected to TEV cleavage overnight to remove it from the beads and was then passed over protein A beads. Since the tag binds with a high affinity to these beads it had to be eluted off in denaturing conditions using 6M urea. These samples were sent off to a lab in the Scripps Research Institute in California for mass spec analysis.

A.3 Mass spec results

A.3.1 Results from cycling cells

The mass spec analysis of the extract from cycling cells produce very few hits (figures A1.A and A2). This could be because Mph1 is only fully active during mitosis (as seen with the phosphorylation of Mph1 in chapter 4). The few potential significant hits from this extract had a slightly lower than desirable sequence coverage but what was interesting was that Klp6 appear to be pulled down from cycling cells. This correlates with the genetic interactions data shown in chapter 5 where Klp5/6 are synthetic lethal with Bub1 but not Mph1 possibly putting Mph1 and Klp5/6 in the same pathway. This suggests that Mph1 has a function at the kinetochore. Unfortunately no other kinetochore components were pulled down.

A.3.2 Results from *nda3* arrested cells

The mass spec analysis on the extract from *nda3* arrested cells produced many more hits than the cycling cell extract (tables A1 and A2). This could indicate that Mph1 is significantly more active when the spindle checkpoint is active than at any other point in the cell cycle.

The most significant hits from this set of mass spec are proteosome components with 7 components appearing in this data. If Mph1 interacts with the 26S proteosome this may partially explain the Mph1 localisation pattern seen (Chapter 3). The 26S proteosome has been shown to localise to the nuclear periphery in both mitosis and interphase (Wilkinson et al., 1998) so Mph1 would not only be found at the kinetochore but also at this region of the cell. Again the sequence coverage is fairly low with many of these hits but in the case of both samples it may be that if the proteins interacting with Mph1 are highly phosphorylated then it may be difficult to detect them by mass spec. This may also be the case for Mph1 itself as in both extracts it appears with low sequence coverage. This may mean that several important interactors of Mph1 have not appeared in this data set as they may be highly phosphorylated.

Mph1-Tap from cycling cells

Protein	Sequence coverage	Number of peptides
Sam1 (S-adenosylmethionine synthetase	18.1%	3
Hsp60	13.6%	3
Mph1	13.1%	8
Sum3	11.3%	4
Pyk1	8.8%	2
Sad1 interacting factor	8.5%	3
Klp6	4%	3

Table A1: Mass spec results from *mph1-SZZ* in cycling cells
Potential significant results in red

Mph1-Tap from *nda3* arrested cells

Protein	Sequence coverage	Number of peptides
Arg5	61.2%	25
40S ribosomal protein S17	56.1%	9
40S ribosomal protein S2	51%	16
Act1	48.8%	20
Gpd3	46.6%	21
Rpt3 (proteasome component)	35.7%	13
Rpt2 (proteasome component)	24.6%	9
Rpt6 (proteasome component)	20.8%	6
Mts4 (proteasome component)	19.1%	10
Rpt5 (proteasome component)	13.9%	6
20S proteasome component	12.7%	3
Rpt4 (proteasome component)	8%	2
Mph1	7.1%	2
Tea3	6.9%	6

Table A2: Mass spec results from *mph1-SZZ nda3-KM311* arrested cells
Potential significant results in red