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Analysis of Mph1 kinase and its substrates in spindle checkpoint signalling

Judith Zich

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Institute of Cell Biology

The University of Edinburgh

2009

Declaration:

I declare that this thesis was composed by myself and the research presented is my own except where otherwise stated.

Judith Zich

2009

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Abbreviations

ATP	Adenosine Triphosphate
bp	base pair
BSA	Bovine Serum Albumin
ChIP	Chromatin Immunoprecipitation
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediamine tetra acetic acid
EGTA	1,2-di (2-aminoethoxy) ethane-N, N, N, N' tetra acetic acid
FRAP	Fluorescence Recovery after Photobleaching
GFP	Green Fluorescent Protein
IgG	Immunoglobulin G
IP	Immunoprecipitation
Kb	kilo base pairs
kDa/Mda	kilo Dalton/mega Dalton
LB	Luria-Bertani
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PIPES	Piperazine-1,4-bis (2-ethanesulfonic acid)
PMG	Pombe Minimal Glutamate
PVDF	Polyvinylidene Fluoride
RNA	Ribonucleic acid
SDS	Sodium Dodecyl Sulphate
SPA	Synthetic Sporulation Agar
TAP	Tandem Affinity Purification
TE	Tris-EDTA
YES	Yeast Extract Supplemented

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Abstract

Accurate chromosome segregation is crucial as mis-segregation results in aneuploidy, which can lead to severe diseases such as cancer. The spindle checkpoint monitors sister-chromatid attachment and inhibits the onset of anaphase until all chromosomes are correctly bi-oriented on the mitotic spindle. The spindle checkpoint machinery of *S.pombe* is composed of many proteins, one of which is the kinase Mph1 (Mps1p-like pombe homolog). It previously has been shown that Mph1 is essential for the spindle checkpoint but not whether this is due to its kinase activity.

In this study we determined the role of Mph1 kinase activity in the spindle checkpoint. To do so a kinase-dead version of Mph1, which had no detectable kinase activity, was analysed. Using this kinase-dead allele we showed that lack of Mph1 kinase activity abolished the spindle checkpoint and led to chromosome mis-segregation. As a result of these two defects cell viability of cells lacking Mph1 kinase activity was severely impaired. These results led to the question of how Mph1 kinase activity regulates the spindle checkpoint.

Spindle checkpoint signalling is thought to mainly take place at two sites, at the kinetochore and at the anaphase promoting complex (APC). The APC is an E3 ubiquitin ligase that drives cells into anaphase by targeting the separase inhibitor securin and cyclin B for degradation by the 26 S proteasome. Upon activation of the spindle checkpoint the APC is inhibited by the mitotic checkpoint complex (MCC) composed of Slp1, Mad2 and Mad3. In this study we wanted to test whether the regulatory role of Mph1 kinase in the spindle checkpoint is via MCC binding to the APC. Using the kinase-dead version of Mph1 we showed that Mad2 and Mad3 binding to the APC is severely impaired in the absence of Mph1 kinase activity. This result led to the hypothesis that Mph1 might regulate Mad2 and Mad3 binding to the APC via phosphorylation.

Using kinase assays Mad2 and Mad3 were identified as *in vitro* substrates of Mph1 and phosphorylation sites in Mad2 and Mad3 were determined by mass spectrometry. Phosphorylation mutants of Mad2 and Mad3 showed spindle checkpoint defects, indicating that they are important Mph1 substrates.

Chapter 1

Introduction

Chapter 1: Introduction

1.1 General Introduction

Cells replicate by cell division and prior to each division the cell goes through four phases that form the cell cycle. The cell cycle is a very complicated sequence of events that have to be tightly controlled to ensure cell viability.

During M-phase the duplicated chromosomes are equally distributed to the two new daughter cells prior to cytokinesis and entry into the next cell cycle. All the events in a cell are directed by the genetic information encoded on the chromosomes. For that reason entry into mitosis and chromosome segregation has to be tightly controlled. Entry into mitosis without properly duplicated chromosomes or the next cell cycle with the wrong number of chromosomes would be devastating for a cell. Therefore, cells have evolved checkpoints that are present at certain critical stages of the cell cycle.

One of these checkpoints is the spindle checkpoint that ensures accurate chromosome segregation during mitosis. It monitors chromosome attachment to the mitotic spindle and delays anaphase until all chromosomes are correctly attached to the mitotic spindle. The spindle checkpoint prevents chromosome segregation and entry into anaphase by inhibiting the E3 ubiquitin ligase, the anaphase promoting complex (APC), which targets specific proteins for degradation by the proteasome and thus leads to entry into anaphase. Like other cell cycle checkpoints, the spindle checkpoint is regulated by post-translational modifications such as phosphorylation and ubiquitination that lead to activity changes and controlled proteolysis. This thesis will focus on regulation of the spindle checkpoint by Mph1 kinase in fission yeast.

1.2 Fission yeast

The organism used in this study is fission yeast. Fission yeast is a single celled free living archiascomycete fungal eukaryote. Its genome is fully sequenced and contains 13.8 Mb encoded on three chromosomes (I: 5.7 Mb, II:4.6 Mb, III:3.5 Mb) (Wood et al., 2002). Fission yeast is widely used as a research organism for several reasons. First, it is a stable haploid, which allows the isolation of recessive mutants. Second, cloning of conditional mutants is possible. Third, due to homologous recombination occurring frequently it is possible to replace genes and introduce mutated genes and gene deletions rather easily. Fourth, fission yeast only has a generation time of 2.5 hrs. Budding yeast also has the advantages listed above. However, fission yeast and budding yeast separated 330 - 420 million years ago and unlike budding yeast, fission yeast has no genome duplications and 145 genes within fission yeast genes are homologues to metazoan genes and are not found in budding yeast (Forsburg and Rhind, 2006; Wood et al., 2002). Furthermore, due to its size of 7 μm – 14 μm, microscopical analysis is easier in fission yeast than in budding yeast (3 - 5 μm), particularly due to the fact that the chromosomes can be seen individually during fission yeast mitosis.

1.3 The cell cycle

The time between two cell divisions is called the cell cycle (Hartwell and Weinert, 1989; Morgan, 2007). It can be divided into four phases. G1-phase, S-phase, G2-phase and M-phase. After cell division a cell enters G1 (gap1)-phase and only upon reaching a certain size and in the presence of nutrients, the cell commits to a new cell cycle and enters S-phase (Johnston, 1977). The transition from G1-phase to S-phase is controlled by the START (restriction point) checkpoint (Sherlock and Rosamond, 1993). In S-phase the chromosomes and the centrosome (spindle pole body in yeast) are duplicated (Adams and Kilmartin, 2000). Upon completion of DNA replication the cell enters G2 (gap2)-phase, which is followed by M-phase. Cells only enter mitosis once DNA replication is completed and this is controlled by the DNA

damage checkpoint and DNA replication checkpoint (Harrison and Haber, 2006). M-phase can be split into two sub-phases. Mitosis during which the sister-chromatids (duplicated chromosomes) are separated followed by cytokinesis (cell division) that leads to the formation of two daughter cells.

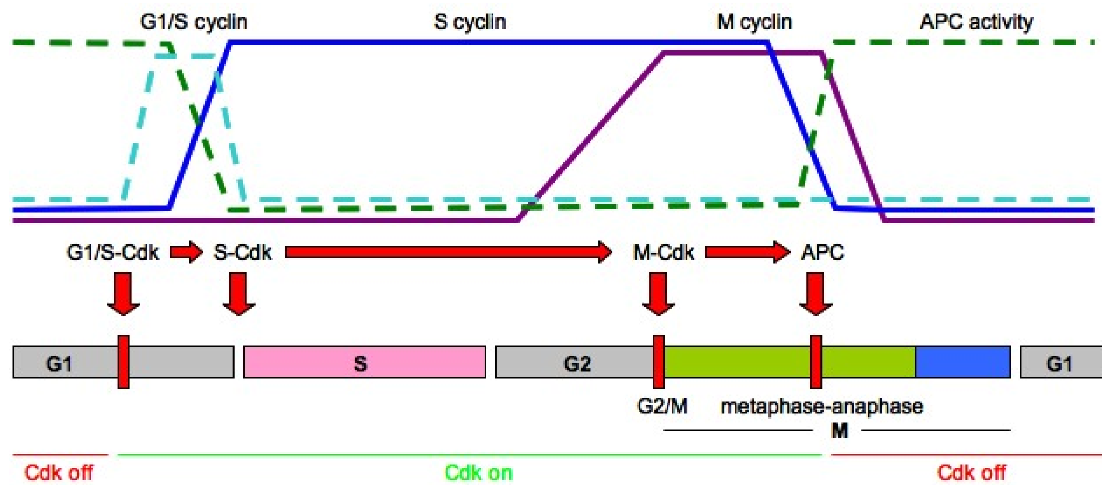


Figure 1.1: Model of the cell cycle

Picture adapted from D. Morgan, The cell cycle (Morgan, 2007)

Many of the genes encoding cell cycle regulatory proteins were first identified in budding yeast and called *CDC* (cell division cycle) genes (Hartwell et al., 1974). *cdc* mutants block the cell cycle at stages that only occur once, such as DNA replication or chromosome segregation. Some of the identified *CDC* genes encode kinases that were later renamed Cdk (cyclin dependent kinases). Cdks are the major kinases that control the cell cycle by modulating the activity of their substrates by phosphorylation (Morgan, 1997). All Cdks are conserved serine/threonine protein kinases. Their activities are partly regulated by the binding of activating co-factors, called cyclins. Most organisms have several different Cdks and cyclins. The combination of different Cdks with different cyclins regulates and controls certain cell cycle events such as entry into S-phase and M-phase as well as chromosome segregation. Cdks are present at constant levels throughout the cell cycle and to ensure that Cdks are only active at certain points in the cell cycle, cyclin protein levels are tightly controlled by gene expression and proteolysis.

In higher organisms the role of Cdks is not restricted to the regulation of the cell cycle. For example, human cells contain at least 9 Cdks and only Cdk1 - 4 are cell cycle regulators, whereas for example Cdk7, 8, 9 regulate gene transcription, while Cdk5 seems to regulate the cytoskeleton.

In addition to activation by binding of cylins, Cdk activity is also regulated by phosphorylation. Phosphorylation can both activate and inactivate Cdks depending on which residues are phosphorylated. Phosphorylation of a conserved threonine residue adjacent to the active site by Cdk activating kinases (CAK) activates Cdks (Fisher and Morgan, 1996). On the other hand phosphorylation of a conserved tyrosine residue, which is Tyr 15 in human, at the ATP-binding site by members of the Wee1 kinase family inactivates Cdks (Booher et al., 1997; Fattaey and Booher, 1997; Mueller et al., 1995b). This event can be reversed by Cdc25 phosphatases (Mueller et al., 1995a).

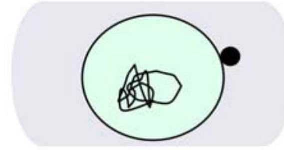
1.3.1 Fission yeast cell cycle

As described above, the fission yeast cell cycle can also be divided into four stages: G1-phase, S-phase, G2-phase and M-phase (mitosis and cytokinesis). However, two differences to other organisms are that G1-phase and S-phase can begin before cytokinesis is completed and that the G1-phase is very short (Nasmyth et al., 1979; Nurse, 1975). Furthermore, both fission and budding yeast differ from higher organisms as they perform a closed mitosis. The nuclear envelope is not broken down but remains intact throughout the cell cycle (Ding et al., 1997). As the G1-phase is very short, entry into the cell cycle, is not as tightly controlled as in other organisms. Instead, entry into mitosis is controlled and cells have to reach a certain size in addition to completing DNA replication, to be able to enter mitosis (Elledge, 1996; Fantes, 1977). If not enough nutrients are available, cells arrest in G1-phase or G2-phase and then have two options. They either enter stationary phase or if cells of the opposite mating type are present they form diploids and sporulate. Unlike in higher organisms, fission yeast only contains one Cdk, Cdc2/Cdk1 (Nurse, 1975;

Nurse and Bissett, 1981; Nurse et al., 1976). Cdc2/Cdk1 kinase activity is required for the G1/S and G2/M transition (Nurse and Bissett, 1981). Levels of Cdc2 are constant throughout the cell cycle. Its activity is controlled by binding of the cyclin B Cdc13, inhibitory phosphorylation by Wee1 and activating dephosphorylation by the phosphatase Cdc25. In G2-phase, Cdc2 is inactive as levels of Cdc13 are low, Wee1 activity is high and Cdc25 activity is low. At the end of G2-phase, Cdc13 levels rise and Cdc25 becomes more active. As a result of Cdc13 binding to Cdc2 and dephosphorylation of Cdc2 by Cdc25, Cdc2 becomes active and thus triggers entry of cells into mitosis. The Cdc2-Cdc13 complex itself then phosphorylates Wee1 and Cdc25, and inactivates and activates these enzymes respectively. Cdc2 activity then drops at the end of metaphase, when cyclin B is degraded by the proteasome (Morgan, 2007).

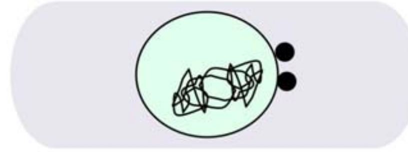
G1-phase

cell growth



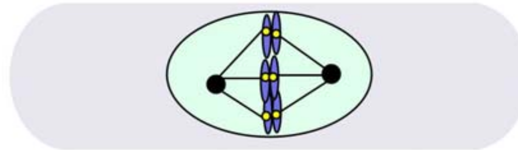
S-phase

DNA replication
SPB duplication



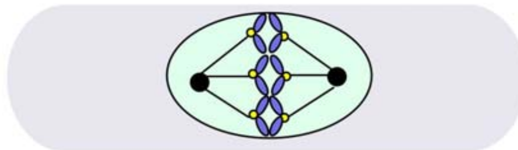
G2-phase
Mitosis
Prophase:

DNA condensation
DNA resolution
Spindle formation



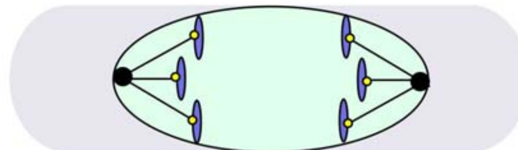
Metaphase

Bi-orientation of sister-chromatids



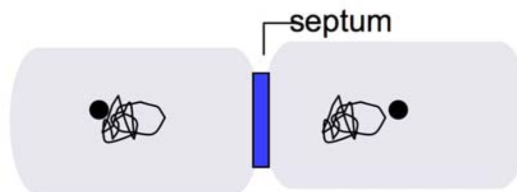
Anaphase A
Securin destruction

chromosome segregation
Sister-chromatids are pulled to opposite SPB



Anaphase B
Spindle elongation

Telophase



Spindle disassembly
Septation
Chromosome decondensation

Cytokinesis

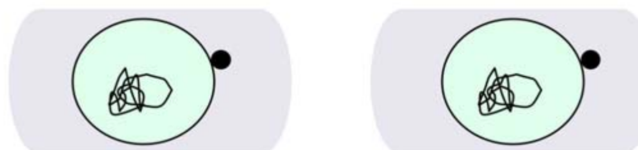


Figure 1.2: Model of the fission yeast cell cycle.

Picture adapted from M.Szczaniecka, Hardwick lab, PhD thesis, 2008.

1.4 Mitosis

Mitosis can be divided into five sub-phases in yeast and six sub-phases in higher organisms: prophase (not in yeast), prometaphase, metaphase, anaphase (anaphase A and anaphase B) and telophase.

After DNA replication, the sister-chromatids are tightly folded (catenated) and linked together by protein complexes called cohesin (sister-chromatid cohesion). Upon entry into prophase, two events take place. First, chromosomes condense, some of the cohesin that holds the sister-chromatids together is lost and DNA is decatenated. Second, the duplicated centrosomes/spindle pole bodies move apart and the mitotic spindle is formed. During prometaphase, the nuclear envelope breaks down (except in organisms like budding yeast and fission yeast that have a closed mitosis). The sister-chromatids become attached to the mitotic spindle and move to the centre of the spindle and during metaphase they align at the metaphase plate. Once all sister-chromatids are attached to opposite spindle poles (bi-oriented) the cell enters anaphase. During anaphase A the cohesin complex that holds the sister-chromatids together is disassembled and in anaphase B the centrosomes/spindle pole bodies move apart and the sister-chromatids are pulled to opposite spindle poles. During telophase the spindle is disassembled, the chromosomes start to decondense and the nuclear envelope starts to form. The final step of M-phase is cytokinesis, during which the cell is divided in two individual daughter cells (Morgan, 2007).

1.5 Regulation of the cell cycle by controlled proteolysis

1.5.1 The proteasome

As mentioned above proteolysis of certain proteins throughout the cell cycle is essential for cell viability. Most proteins are degraded by the multi-protein complex 26S proteasome, which is present in all eukaryotes (Baumeister et al., 1998). It is composed of the 20S core particle and two 19S regulatory particles. The 20S core

particle is a cylindrical barrel shaped 720 kDa complex composed of 28 subunits. On either side of the 20S core particle is an 890 kDa regulatory particle (19S complex) that can be divided into a lid and base complex. The 19S complex is composed of 19 subunits, of which six are ATPases. It is required for substrate recognition, binding, unfolding and translocation of the protein into the 20S core particle. The 20S core particle then cleaves proteins into small oligopeptides (Miller and Gordon, 2005; Wolf and Hilt, 2004).

With exceptions most proteins are recognised by the proteasome by attached ubiquitin chains. In most cases a chain of at least four ubiquitins has to be attached to the protein destined for degradation to be recognised by the proteasome (Pickart, 1997; Thrower et al., 2000).

Ubiquitin is a 76 amino acid polypeptide that is attached to proteins in an ATP dependent manner. This process requires three different enzymes: an ubiquitin-activating enzyme E1, an ubiquitin-conjugating enzyme E2 and an ubiquitin-protein ligase E3 (Pickart, 1997). The two major E3 protein ligases that control the cell cycle are the SCF and the anaphase promoting complex (APC).

1.5.2 E3 ubiquitin ligases

Controlled proteolysis of proteins throughout the cell cycle by the proteasome is catalysed by two E3 ubiquitin ligases, the SCF (Skp1, Cul1, Rbx1) complex and the APC (anaphase promoting complex).

1.5.2.1 SCF complex

The SCF complex consists of three invariable subunits Skp1, Cul1 and Rbx1 and a fourth variable subunit the F-box protein (Zheng et al., 2002), (for review (Nakayama and Nakayama, 2005)). The F-box protein regulates substrate specificity and to date over 70 different F-box proteins have been identified. The main role of the SCF complex was first thought to be the regulation of the transition from G1-phase to S-phase (Bai et al., 1996; Feldman et al., 1997; Skowyra et al., 1997).

Subsequently, it was discovered that the SCF complex regulates protein degradation throughout the whole cell cycle. For example it regulates the G2-M transition and the DNA damage checkpoint by targeting the kinase Wee1 and the phosphatase Cdc25 respectively for degradation by the proteasome (Bai et al., 1996; Busino et al., 2003; Jin et al., 2003; Watanabe et al., 2004). Furthermore, it also has a role in regulating APC activity by targeting the APC inhibitor Emi1 for degradation by the proteasome (Guardavaccaro et al., 2003; Margottin-Goguet et al., 2003; Reimann et al., 2001).

1.5.2.2 Anaphase promoting complex

1.5.2.2.1 Overview

The second E3 ubiquitin ligase catalysing controlled proteolysis during the cell cycle is the anaphase promoting complex (APC) (for review (Diaz-Martinez and Yu, 2007; Peters, 2006). It was first discovered for its essential role in cyclin degradation in mitosis (Irniger et al., 1995; King et al., 1995; Sudakin et al., 1995). Later on the critical mitotic substrates were identified as cyclin B and the separase inhibitor securin (Thornton and Toczyski, 2003). The APC is a multi-protein complex whose structure has been determined in several organisms (Dube et al., 2005; Gieffers et al., 2001; Herzog et al., 2009; Ohi et al., 2007; Passmore et al., 2005). Its activity has to be tightly regulated, as degradation of proteins at the wrong time can have severe consequences, such as aneuploidy and cell death. Therefore, APC activity is controlled by several mechanisms and requires the association of activating co-factors. Association of the two main co-factors Cdc20 or Cdh1 defines APC substrate specificity and depends on the phase of the cell cycle. Cdc20 activates the APC in mitosis, whereas Cdh1 activates the APC in late mitosis and G1-phase (Fang et al., 1998b; Zachariae et al., 1998). APC activity is also regulated by phosphorylation of APC subunits and its co-factors Cdc20 and Cdh1 by kinases such as polo kinase and Cdk. Furthermore, during the spindle checkpoint the APC is inhibited by the mitotic checkpoint complex (see section 1.6.2.1.5.3).

1.5.2.2.2 Composition of the APC

The APC is a multi-protein subunit complex composed of at least 13 subunits in budding and fission yeast (Yoon et al., 2002) and estimated masses for the APC vary between approximately 1 - 1.5 MDa (Dube et al., 2005; Ohi et al., 2007). The structure of fission yeast, human APC and *Xenopus* APC bound to its activators Cdc20 and Cdh1 respectively have recently been solved (Dube et al., 2005; Herzog et al., 2009) (Figure 1.3). They show that the overall basic structure of the APC is well conserved between species. Nevertheless, differences in the copy number of certain subunits have been reported between human, budding yeast and fission yeast (see Table 1.1).

Table 1.1: Identified APC subunits of fission yeast, budding yeast and human

Fission yeast	Budding yeast	Human	Domain
Cut4	Apc1	Apc1	
Apc5	Apc5*	Apc5	TPR Repeats
Apc2	Apc2*	Apc2	Cullin Repeats
Lid1	Apc4*	Apc4	
Nuc2*	Cdc27*	Apc3	TPR Repeats
Cut9*	Cdc16*	Apc6*	TPR Repeats
Cut23*	Cdc23*	Cdc23*	TPR Repeats
Apc10	Doc1*	Apc10	
Apc13	Swm1	Apc13	
Apc11	Apc11	Apc11	RING Domain
Apc14	------	------	
Apc15	Mnd2	------	
Hcn1	Cdc26	Cdc26	
------	------	Apc7	
------	Apc9	------	

* Subunits present in more than one copy. Adapted from (Ohi et al., 2007).

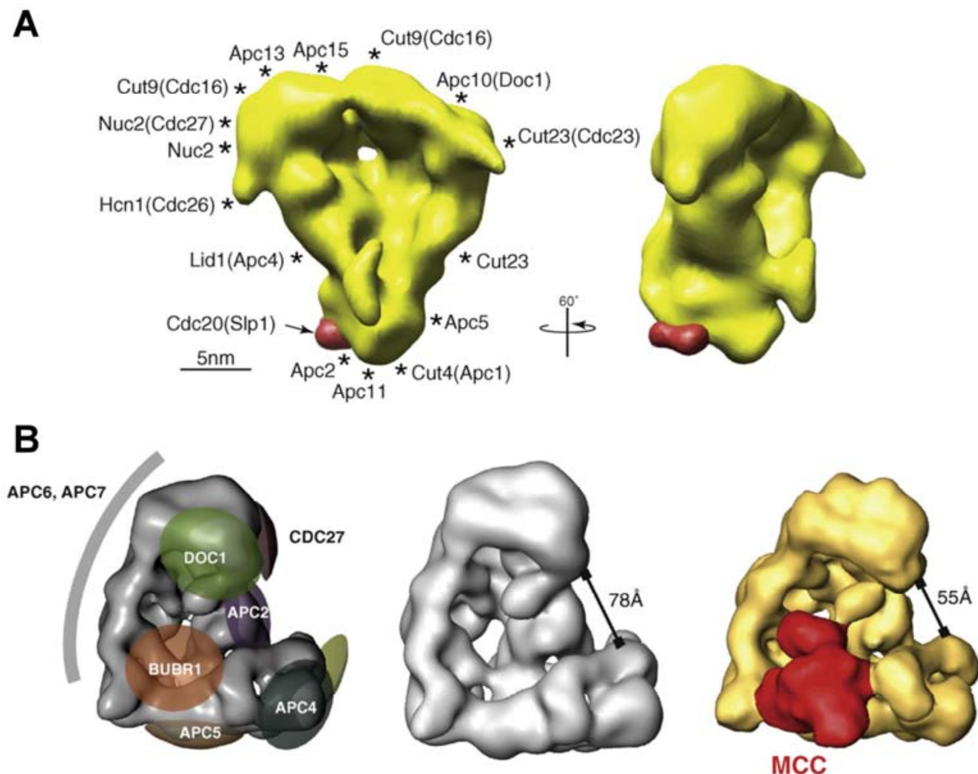


Figure 1.3: Model of fission yeast and human APC

(A) Fission yeast APC. The activating co-factor Cdc20/Slp1 is coloured in red. **(B)** Human APC. The mitotic checkpoint complex (MCC) is coloured in red. Picture taken from (Zich and Hardwick).

The role of many APC subunits in APC structure formation, activator binding and substrate binding has been studied. The APC is built of two subcomplexes that are bridged by the biggest subunit APC1. The catalytic center is formed by the cullin protein APC2 and the RING-H2 finger protein APC11. These two subunits are sufficient to catalyse protein ubiquitylation *in vitro*. However, they do not have substrate specificity (Tang et al., 2001b). APC11 can directly interact with an ubiquitin conjugating enzyme E2 and can even act as an E3 ubiquitin ligase *in vitro*, which is dependent on the RING-H2 Finger domain, in the absence of APC2 (Gmachl et al., 2000; Leverson et al., 2000).

Another well-studied subunit is **Doc1**, a regulatory subunit that is not required for APC structure (Passmore et al., 2003). It recognises substrates and promotes substrate binding to the APC and thus is required for full APC ligase activity and processivity. This is dependent on a destruction box in the substrate (Carroll et al., 2005). It is not clear whether Doc1 directly binds to substrates or indirectly promotes substrate binding to the APC by inducing conformational changes (Carroll and Morgan, 2002; Passmore et al., 2003).

1.5.2.2.3 APC activators

The two main activating co-factors of the APC are Cdc20/Slp1 and Cdh1/Hct1/Swr1 (Schwab et al., 1997; Visintin et al., 1997; Zachariae et al., 1998). Recently the structure of the APC in a complex with its activators Cdc20 and Cdh1 have been described (Dube et al., 2005; Herzog et al., 2009; Ohi et al., 2007).

Cdc20 and Cdh1 associate and activate the APC during different stages of the cell cycle and contribute to APC substrate specificity. APC-Cdc20 is active during mitosis, whereas APC-Cdh1 is active in late mitosis and G1-phase (Fang et al., 1998b; Zachariae et al., 1998).

One mechanism that regulates Cdc20 and Cdh1 association with the APC is phosphorylation by Cdk1/Cdc2. Cdh1 only activates the APC in G1-phase. Phosphorylation of Cdh1 in early mitosis prevents activation of the APC by Cdh1 and dephosphorylation of Cdh1 at the onset of anaphase enables it to activate the APC (Jaspersen et al., 1999; Kramer et al., 2000; Yamaguchi et al., 2000; Zachariae et al., 1998). Cdc20 on the other hand activates the APC during high Cdk1 activity and requires phosphorylation of the APC to be able to activate it (Kramer et al., 2000; Zachariae et al., 1998).

A second mechanism of APC activation by Cdc20 and Cdh1 are protein levels. In budding yeast and human Cdh1 RNA levels and protein appear to be constant

throughout the cell cycle (Fang et al., 1998b; Prinz et al., 1998). This is in contrast to fission yeast Cdh1, that is phosphorylated by Cdk1/Cdc2 and thus destabilised (Yamaguchi et al., 2000).

Unlike Cdh1, Cdc20 protein levels change throughout the cell cycle. They are lowest in G1-phase when Cdh1 activates the APC. At the G1-phase to S-phase transition Cdc20 levels start to rise and are highest in metaphase and then drop again in G1-phase (Fang et al., 1998b; Prinz et al., 1998; Shirayama et al., 1998). Cdc20 degradation in G1-phase is mediated by APC-Cdh1 in a KEN-box and D-box dependent manner (Pfleger and Kirschner, 2000; Prinz et al., 1998). Stability of Cdc20 during metaphase and the spindle checkpoint is described in section 1.6.2.1.5.2.

1.5.2.2.4 Substrate recognition by the APC

The exact mechanism how the APC recognises its substrates still remains to be determined and appears to be complicated. Two consensus motifs have been identified that are recognised by APC-Cdc20 and APC-Cdh1: the D-box (R-X-X-X-L-X-X-X-X-N) and the KEN box (K-E-N-X-X-X-N) (Glotzer et al., 1991; Pfleger and Kirschner, 2000). It is not clear whether the APC itself recognises substrates or whether substrate recognition is mediated by Cdc20 and Cdh1. Most likely both contribute to substrate recognition. Studies in budding yeast and *Xenopus* reported that Cdc20 and Cdh1 can bind to substrates independently of the APC (Burton and Solomon, 2001; Burton et al., 2005; Pfleger et al., 2001; Schwab et al., 2001). However, Yamano et al. showed that the APC stably interacts with the D-box of cyclin B in *Xenopus* in the absence of Cdc20 (Yamano et al., 2004). It is also not clear how important the D-box and KEN-box are for substrate recognition by Cdh1. In budding yeast one study has been reported that binding to Cdh1 can be independent of the D-box (Schwab et al., 2001), whereas another study argued that substrate binding to Cdc20 and Cdh1 requires the KEN-box and D-box, respectively (Burton and Solomon, 2001). Furthermore, the APC subunit Doc1 is also able to

recognise substrates and promote substrate binding to the APC in a D-box dependent manner. However, it is not clear whether Doc1 directly binds to substrates (Carroll et al., 2005; Passmore et al., 2003).

1.5.2.2.5 Regulation of the APC

APC mis-regulation results in major cell cycle defects such as chromosome mis-segregation and DNA replication defects. Therefore, APC activity and substrate specificity is tightly regulated by the association of its co-factors Cdc20 and Cdh1 at different stages of the cell cycle. Furthermore, phosphorylation of APC subunits also appears to be important for APC activity throughout the cell cycle.

1.5.2.2.5.1 Phosphorylation of the APC

The APC is highly phosphorylated and many phosphorylation sites in APC subunits have been identified (Kraft et al., 2003; Steen et al., 2008). However, the physiological role for most of these sites still has to be determined. Unfortunately, a lot of identified phosphorylation sites are conserved between vertebrates but not with non-vertebrates, which makes the analysis even more complicated (Kraft et al., 2003).

Two kinases known to phosphorylate the APC are Cdk1 and Plk1 (Kraft et al., 2003; Rudner and Murray, 2000; Yoon et al., 2006). *In vitro* studies in HeLa cells show that Cdk1 and Plk1 phosphorylate different APC subunits and that kinase activity of both kinases is required to completely activate the APC (Golan et al., 2002). However, whereas Cdk1 seems to be sufficient to activate the APC, Plk1 only contributes to APC activity but is not sufficient or essential (Kraft et al., 2003). APC phosphorylation by Cdk1 increases Cdc20 binding (Kraft et al., 2003) and initiates anaphase (Rudner and Murray, 2000). Kraft et al. identified 34 phosphorylation sites but only 18 could be confirmed *in vitro* by Cdk1 and Plk1. Therefore, it is likely that

the APC is phosphorylated by other kinases in addition to Cdk1 and Plk1 (Kraft et al., 2003). Another kinase shown to phosphorylate human APC *in vitro* is protein kinase A (Kotani et al., 1998). Other candidates for APC phosphorylation are Mps1, Bub1, MAPK, Aurora kinase and in human also BubR1 kinase.

1.6 Cell cycle checkpoints

The cell cycle is a very complicated sequence of events that has to be tightly controlled to ensure cell viability. Therefore, cells have evolved checkpoints that are present at certain critical stages of the cell cycle. They ensure that important events are completed successfully before the initiation of the next event (Hartwell and Weinert, 1989). For example initiation of mitosis depends on successful DNA synthesis, which is controlled by the DNA damage checkpoint. During mitosis initiation of anaphase depends on successful bi-orientation of sister-chromatids to the mitotic spindle, which is controlled by the spindle checkpoint.

1.6.1 DNA damage checkpoint

The DNA damage checkpoint ensures that cells do not enter mitosis with damaged or unreplicated DNA (for review (Harrison and Haber, 2006)). A gene essential for the DNA damage checkpoint is RAD9, which is required for a cell cycle arrest due to DNA damage or incomplete DNA replication (Weinert and Hartwell, 1989; Weinert and Hartwell, 1988). RAD9 is not an essential gene. However, the chromosome loss rate in RAD9 deficient mutants is significantly elevated compared to wild-type cells, which ultimately leads to cell death (Weinert and Hartwell, 1990).

Another regulator of the DNA damage checkpoint is the tumor suppressor gene p53 (Meek, 2009). Mutations of p53 have been linked to many cancers (Nozaki et al., 1999). P53 activity is controlled in several ways, such as gene expression, post-translational modifications and controlled proteolysis by the proteasome. Binding of

Mdm2 to p53 catalyses its degradation by the proteasome (Prives and Hall, 1999; Zhang and Xiong, 2001). Upon DNA damage p53 gene expression is upregulated and phosphorylation of p53 stabilises and activates it (Prives and Hall, 1999). Importantly, a homologue of p53 has not been identified in yeast.

1.6.2 Mitotic Checkpoints

Mitosis is controlled by several checkpoints. Fission yeast was thought to have the **spindle orientation checkpoint** that monitors orientation of the mitotic spindle before the onset of anaphase (Gachet et al., 2001; Gachet et al., 2004). (for review see (Gachet et al., 2006). The proposed spindle orientation checkpoint (SOC) is dependent on the spindle checkpoint proteins Bub1, Bub3, Mps1 and Mad3 (see section 1.6.1.3) (Rajagopalan et al., 2004; Tournier et al., 2004). However, two recent studies argue that no spindle orientation checkpoint is present in fission yeast and that spindle orientation is determined by interphase microtubules (Meadows and Millar, 2008; Vogel et al., 2007).

Budding yeast has a checkpoint called the **spindle position checkpoint** that delays mitotic exit and cytokinesis until the spindle is correctly oriented (for review (Fraschini et al., 2008). The most conserved mitotic checkpoint is the **spindle checkpoint**, which ensures that cells only enter anaphase if the sister-chromatids are properly bi-oriented onto the mitotic spindle.

1.6.2.1 The spindle checkpoint

1.6.2.1.1 Overview

During mitosis the duplicated chromosomes have to be equally distributed between the mother and daughter cell. This process called chromosome segregation has to be tightly controlled as mis-segregation of chromosomes leads to aneuploidy. In order

to prevent aneuploidy cells have evolved a surveillance mechanism called the spindle checkpoint. It ensures that the chromosomes are properly bi-oriented on the mitotic spindle before cells enter anaphase. The spindle checkpoint is activated when an unattached kinetochore is present (Rieder et al., 1995) or if there is a lack of tension at the kinetochores (Li and Nicklas, 1995) (Figure 1.4). Upon activation of the spindle checkpoint the anaphase promoting complex (APC) is inhibited, which results in the stabilisation of cyclin B and securin and the onset of anaphase is delayed until all sister-chromatids are attached to opposite spindle poles (for review see (May and Hardwick, 2006).

The spindle checkpoint components were first identified in budding yeast in screens for mutants that did not delay mitosis upon treatment with a microtubule destabilising drug (Hoyt et al., 1991; Li and Murray, 1991). Later on these genes were also identified in other organisms and shown to be essential for the spindle checkpoint. The core components of the spindle checkpoint are the mad (mitotic arrest deficient) proteins: Mad1 (Hardwick and Murray, 1995), Mad2 (Chen et al., 1999; He et al., 1997), Mad3 (Hardwick et al., 2000; Millband and Hardwick, 2002) and the bud (budding uninhibited by benzimidazole) proteins: the kinase Bub1 (Bernard et al., 1998; Roberts et al., 1994) and Bub3 (Taylor et al., 1998; Vanoosthuyse et al., 2004). Another spindle checkpoint component is the kinase Mps1, which was first identified in budding yeast in a screen for mutants that had a spindle pole duplication defect and formed monopolar spindles (Winey et al., 1991). It later has been shown to also be essential for the spindle checkpoint (Weiss and Winey, 1996). The spindle checkpoint proteins, with the exception of Mps1 in budding yeast, are not essential in budding yeast. However, complete knockouts of spindle checkpoint proteins in mice are embryonic lethal (Dobles et al., 2000; Iwanaga et al., 2007; Kalitsis et al., 2000; Wang et al., 2004).

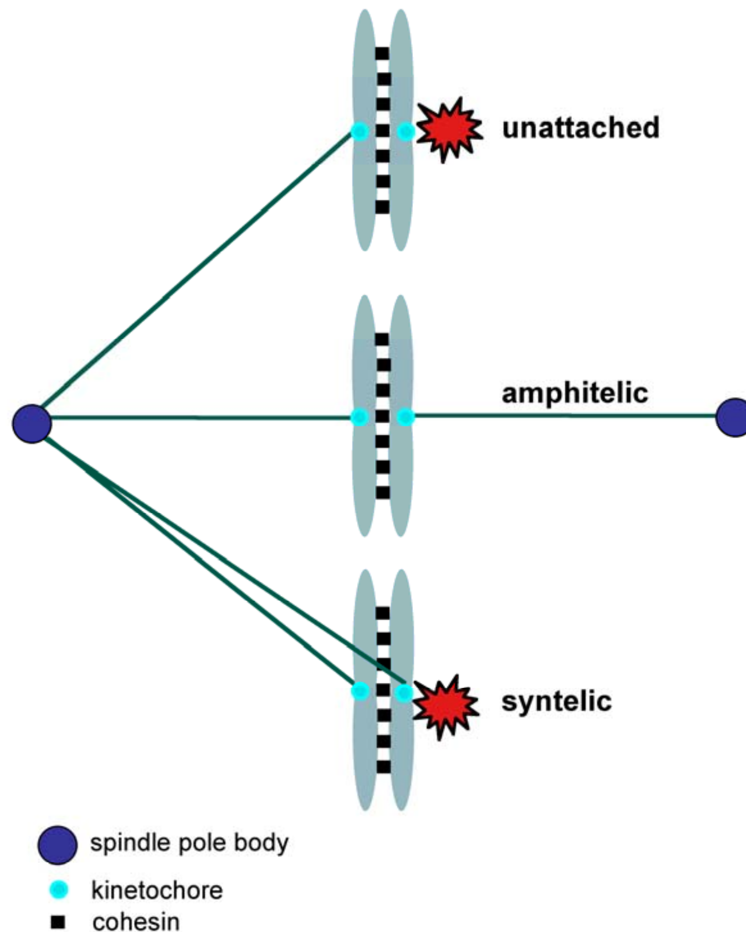


Figure 1.4: Model of defects in kinetochore-microtubule attachment Unattached kinetochores and syntelic attachments (both kinetochores are attached to one spindle pole) activate the spindle checkpoint.

1.6.2.1.2 The spindle checkpoint and disease

A functional spindle checkpoint is required for accurate chromosome segregation in mitosis and chromosome mis-segregation results in aneuploidy. Most solid tumors are aneuploid and a defective spindle checkpoint has been linked to tumorigenesis (for review (Holland and Cleveland, 2009; Kops et al., 2005b)). The spindle checkpoint has been found to be disrupted in many tumor cell lines (Cahill et al., 1998; Takahashi et al., 1999) and misexpression of spindle checkpoint proteins leads to an accelerated rate of tumor formation (Chi et al., 2009; Iwanaga et al., 2007; Li et

al., 2009; Schliekelman et al., 2009). Mad1 for example has been linked to tumorigenesis in human stomach and small lung cancer (Coe et al., 2006; Hanks et al., 2004; Osaki et al., 2007).

In addition to tumorigenesis, chromosome mis-segregation during meiosis is the most common genetic cause of spontaneous abortions and can result in birth defects, such as trisomy 21 (down syndrome) (for review (Hassold and Hunt, 2001).

1.6.2.1.3 The core spindle checkpoint components

1.6.2.1.3.1 The kinase Mps1

Mps1 (monopolar spindle 1) was first identified for its role in spindle pole duplication in budding yeast (Winey et al., 1991) and subsequently homologues of budding yeast Mps1 have been identified in fission yeast and vertebrates (Abrieu et al., 2001; Douville et al., 1992; Hogg et al., 1994; Lindberg et al., 1993; Mills et al., 1992; Schmandt et al., 1994). However, homologues have not been identified in *C.elegans*. With the exception of fission yeast, Mps1 kinases are essential for cell viability.

All identified Mps1 proteins belong to the MPS1 (MonoPolarSpindle 1) family of kinases and contain a conserved C-terminal kinase domain (Fisk and Winey, 2001; Lauze et al., 1995). They are dual-specificity kinases, which phosphorylate serine, threonine and tyrosine

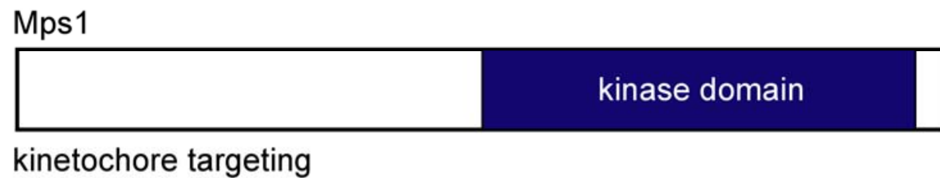


Figure 1.5: Schematic of Mps1. Mps1 kinases contain a conserved C-terminal kinase domain. The N-terminus is required for kinetochore targeting.

The structure of the catalytic domain of human Mps1 has been solved (Chu et al., 2008). It is composed of 11 subdomains. Subdomain VII is highly conserved and contains a consensus sequence DFG. Mutation of aspartic acid (D) to alanine (A) in this consensus sequence has been demonstrated to render Mps1 kinase-dead (Abrieu et al., 2001; Lauze et al., 1995). Another mechanism to "switch off" Mps1 kinase activity is the usage of an analogue-sensitive allele of *mps1* that can be inhibited using the ATP analogue 1NMPP1 (Jones et al., 2005; Tighe et al., 2008). Using these mutant alleles the roles of Mps1 in the spindle checkpoint and spindle pole duplication have been investigated.

In budding yeast, Mps1 is required for spindle pole/centrosome duplication (Jones et al., 2005; Winey et al., 1991) and the phosphorylation of spindle pole body subunits has been demonstrated to be dependent on Mps1 (Castillo et al., 2002; Friedman et al., 2001; Holinger et al., 2009). However, the role of Mps1 in centrosome duplication in vertebrates is controversial. Reports by Liu et al. and Fisk et al. show localisation of Mps1 to centrosomes throughout the cell cycle, which is independent of its kinase activity (Fisk and Winey, 2001). Furthermore, they suggest that Mps kinase activity is required for centrosome duplication (Fisk et al., 2003; Fisk and Winey, 2001). On the contrary, Stucke et al. argue that Mps1 does not localise to centrosomes and that it is not required for centrosome duplication (Stucke et al., 2004 Stucke et al., 2002). The exact role of Mps1 kinase activity in centrosome duplication remains to be determined.

There is no controversy in the question whether Mps1 kinases are required for the spindle checkpoint. Mps1 activity is elevated in mitosis and peaks during the spindle checkpoint. Mps1 kinases are essential for the spindle checkpoint and it has been shown that loss of Mps1 kinase activity abolishes the spindle checkpoint in several organisms (Abrieu et al., 2001; He et al., 1998; Jones et al., 2005; Kang et al., 2007; Tighe et al., 2008). Furthermore, using an analogue sensitive allele of Mps1 it has been demonstrated that Mps1 kinase activity is not only required for spindle checkpoint establishment but also for spindle checkpoint maintenance (Jones et al., 2005; Tighe et al., 2008).

Not many substrates of Mps1 in the context of the spindle checkpoint have been identified. Mps1 phosphorylates the spindle checkpoint protein Mad1 (Hardwick et al., 1996) and phosphorylation of human BubR1 is dependent on Mps1 (Huang et al., 2008). However, Mps1 phosphorylation sites in Mad1 and BubR1 have not been determined. Additional substrates of Mps1 could be APC subunits or nuclear pore proteins as human Mps1 interacts with the APC and localises to the nuclear pore complex (Liu et al., 2003).

Mps1 is a phosphoprotein with a peak of phosphorylation during spindle checkpoint activation (Liu et al., 2003; Stucke et al., 2002) and in fact it has been shown that Mps1 autophosphorylates. This autophosphorylation is required for full kinase activity (Jelluma et al., 2008a; Kang et al., 2007; Mattison et al., 2007). Reduced autophosphorylation has been shown to weaken the spindle checkpoint, lead to defects in centrosome duplication and to chromosome mis-alignment and thus chromosome mis-segregation and lagging chromosomes (Jelluma et al., 2008a; Kang et al., 2007; Mattison et al., 2007; Zhao and Chen, 2006).

Mps1 also has another mitotic role in addition to its role in the spindle checkpoint. It is required for efficient chromosome alignment and error correction. In an unperturbed mitosis lack of Mps1 leads to chromosome segregation defects, such as mis-aligned chromosomes and lagging chromosomes (Jelluma et al., 2008b; Jones et al., 2005; Liu et al., 2003; Tighe et al., 2008). Jelluma et al. propose that Mps1

regulates chromosome alignment and chromosome attachment by phosphorylating the chromosomal passenger complex component borealin. A phosphomutant of borealin showed chromosome alignment defects and reduced Aurora B activity, whereas a phosphomimic mutant restored chromosome alignment (Jelluma et al., 2008b). Mps1 has also been shown to be required for correct spindle formation (Jones et al., 2005) and phosphorylation of the kinetochore subunit Dam1 is required for the efficient connection between kinetochores and microtubules (Shimogawa et al., 2006).

In accordance with its role in the spindle checkpoint and chromosome segregation Mps1 localises transiently to kinetochores during mitosis, which is dependent on its N-terminus (Abrieu et al., 2001; Fisk and Winey, 2001; Howell et al., 2004; Liu et al., 2003; Stucke et al., 2004). A recent study showed that autophosphorylation of Mps1 in its N-terminus is required for kinetochore localisation (Xu et al., 2009). In addition, phosphorylation of *Xenopus* Mps1 by MAP kinase in the C-terminus is required for Mps1 kinetochore localisation and a functional spindle checkpoint (Zhao and Chen, 2006). Phosphorylation of the corresponding residue in human Mps1 also seems to contribute to Mps1 targeting to kinetochores (Xu et al., 2009).

In *Xenopus* Mps1 stays localised to kinetochores even after chromosome alignment, similar to BubR1 and Bub1 (Abrieu et al., 2001). Indeed in *Xenopus* Mps1 kinetochore localisation is dependent on Aurora B and Bub1 (Vigneron et al., 2004) and Mps1 is required for Bub1and BubR1 kinetochore recruitment (Kang et al., 2007; Vigneron et al., 2004). This appears not to be the case for human Mps1, which can localise to kinetochores independently of Bub1 and Aurora B (Stucke et al., 2004). However, dependency of human Mps1 on kinetochore localisation on the kinetochore components PRP4, Ndc80 and Nuf2 has been demonstrated (Martin-Lluesma et al., 2002; Montembault et al., 2007; Stucke et al., 2004). Mps1 also phosphorylates and interacts with the kinetochore subunit Ndc80, which is required for a fully functional spindle checkpoint (Kemmler et al., 2009). The exact role for Mps1 at the kinetochore has not been determined. It might act as a scaffold to recruit other checkpoint proteins as it is required for Mad1 and Mad2 recruitment to

kinetochores (Abrieu et al., 2001; Fisk et al., 2003; Liu et al., 2003; Martin-Lluesma et al., 2002; Tighe et al., 2008; Xu et al., 2009; Zhao and Chen, 2006). However, reports in human and *Xenopus* differ in whether Mps1 is required for Bub1 and BubR1 localisation to kinetochores (Kang et al., 2007; Liu et al., 2003; Tighe et al., 2008; Zhao and Chen, 2006).

Mps1 protein levels change throughout the cell cycle. Palframan et al. demonstrated degradation of budding yeast Mps1 during anaphase and G1-phase by the proteasome catalysed by APC-Cdc20 and APC-Cdh1 respectively, which has been suggested to be required for spindle checkpoint silencing (Palframan et al., 2006). Mps1 stability is also regulated by Cdk2 during S-phase. Inhibition of Cdk2 destabilises Mps1, which is then lost from centrosomes and thus centrosome duplication is inhibited (Fisk and Winey, 2001).

Fission yeast Mph1 (Mps1-like pombe homologue) has not been studied in depth. He et al. showed that Mph1 is a structural and functional homologue of budding yeast Mps1 regarding its role in the spindle checkpoint (He et al., 1998). Unlike budding yeast Mps1, Mph1 is not required for spindle pole body duplication and this is likely to be the reason why it is not essential (He et al., 1998). Nevertheless, Mph1 appears to have a role distinct from the spindle checkpoint as *mph1* null mutants have severe chromosome segregation defects (L.Milne, personal communication). Mph1 localisation to kinetochores has not been demonstrated in fission yeast. Several attempts in the Hardwick lab failed to show Mph1 localisation to kinetochores, such as CHIP analysis and Mph1 overexpression. In addition Mph1 appears to be relatively stable throughout the cell cycle (L.Milne, personal communication).

1.6.2.1.3.2 Mad1

Mad1 is an essential component of the spindle checkpoint (Chen et al., 1998; Hardwick and Murray, 1995).

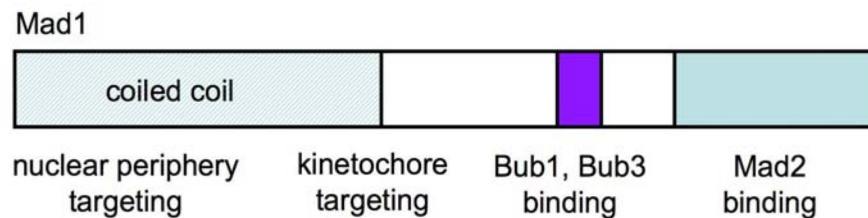


Figure 1.6: Schematic of Mad1. In budding yeast regions of the C-terminus of Mad1 have been demonstrated to be required for Mad2 binding (light green) (Chen et al., 1999) and Bub1 and Bub3 binding (purple) (Campbell et al., 2001), whereas the N-terminus is required for targeting of Mad1 to the nuclear periphery (Scott et al., 2005). Chung et al. proposed that the N-terminus is also required for kinetochore targeting of Mad1 (Chung and Chen, 2002).

During interphase Mad1 localises to the nuclear periphery (Campbell et al., 2001; Chen et al., 1998; Shah et al., 2004) and then localises to kinetochores early in mitosis (Campbell et al., 2001; Scott et al., 2005). Most of Mad1 is stably localised to unattached kinetochores (Shah et al., 2004). The remaining Mad1 only transiently localises to kinetochores (Shah et al., 2004) and travels between the kinetochores and the nuclear periphery during a spindle checkpoint arrest (Scott et al., 2005). Upon microtubule attachment Mad1 is released from the kinetochores and localises to the spindle poles (Shah et al., 2004). Mad1 kinetochore localisation in *Xenopus* is dependent on Bub1 (Sharp-Baker and Chen, 2001) and Mps1 (Abrieu et al., 2001). In human Mad1 localisation has been shown to be dependent on Mps1 (Liu et al., 2003), the kinetochore component Ndc80 (Martin-Lluesma et al., 2002) and PRP4 (Montembault et al., 2007).

Mad1 is a phosphoprotein that becomes hyperphosphorylated upon spindle checkpoint activation. Mps1 and Bub1 phosphorylate Mad1 *in vitro* (Hardwick et al., 1996; Seeley et al., 1999) and Mad1 phosphorylation *in vivo* is dependent on Bub1, Bub3 and Mad2 (Hardwick and Murray, 1995). However, specific phosphorylation sites in Mad1 and their physiological role in the spindle checkpoint have yet to be determined.

Mad1 has been demonstrated to form two complexes that are required for the spindle checkpoint (Brady and Hardwick, 2000; Chen et al., 1999). The Bub1-Bub3-Mad1 complex is formed in an unperturbed mitosis and at significantly elevated levels during an activated spindle checkpoint. It is dependent on Mps1 and Mad2 but not on Bub1 kinase activity (Brady and Hardwick, 2000). However, this complex was only detected in budding yeast and was reconstituted *in vitro* in human (Brady and Hardwick, 2000; Seeley et al., 1999).
The second complex is the Mad1-Mad2 complex that is easily detected in all organisms and was studied in more detail (see section 1.6.2.1.3.3).

1.6.2.1.3.3 Mad2

Mad2 is an essential component of the spindle checkpoint (Chen et al., 1999; Chen et al., 1996; He et al., 1997; Li and Benezra, 1996).

Figure 1.7: Schematic of Mad2. The C-terminus acts as a “seat-belt” to support different conformations of Mad2.

In interphase Mad2 is diffused in the cytoplasm and localises to the nuclear periphery (Campbell et al., 2001; Chen et al., 1998; Ikui et al., 2002), which is dependent on Mad1 (Ikui et al., 2002). After entry into mitosis Mad2 localises to unattached kinetochores, spindle fibres and spindle poles (Chen et al., 1998; Chen et al., 1996; Howell et al., 2000; Ikui et al., 2002). Importantly, Mad2 localisation to

kinetochores in vertebrate cells appears to be dependent on lack of kinetochore-microtubule attachment and not lack of tension at kinetochores (Shannon et al., 2002; Skoufias et al., 2001; Waters et al., 1998). As soon as all kinetochores are attached Mad2 is released from kinetochores and localises to the spindle (Ikui et al., 2002). Its localisation to kinetochores is transiently, with a pool stably associated with kinetochores (Howell et al., 2004; Shah et al., 2004; Vink et al., 2006). Shah et al. demonstrated that in vertebrate cells app. 50 % of Mad2 present are stably associated with kinetochores (Shah et al., 2004). Mad2 localisation to kinetochores depends on Mad1 (Chen et al., 1998; Luo et al., 2002; Sironi et al., 2001). In human it also depends on Mps1 (Liu et al., 2003), Ndc80 (Martin-Lluesma et al., 2002) and PRP4 (Montembault et al., 2007).

Mad1 and Mad2 form a complex (Campbell et al., 2001; Chen et al., 1998; Sironi et al., 2001), whose crystal structure has been solved (Sironi et al., 2002). It is present throughout the cell cycle independently of other checkpoint proteins and is required for checkpoint function (Chen et al., 1999).

The major role of Mad2 in the spindle checkpoint is the inhibition of Cdc20. Three different Mad2 complexes have been identified to be required for efficient APC inhibition. The Mad1-Mad2 complex described above, a Mad2-Cdc20 complex (Hwang et al., 1998; Ikui et al., 2002; Kallio et al., 1998; Kim et al., 1998; Wassmann and Benezra, 1998) that is required for the spindle checkpoint (Hwang et al., 1998; Kim et al., 1998) and the mitotic checkpoint complex composed of Mad2, Mad3, Bub3 (not detected in fission yeast) and Cdc20 (Fraschini et al., 2001; Sczaniecka et al., 2008; Sudakin et al., 2001).

Mad1 is required for Mad2-Cdc20 complex formation (Hwang et al., 1998) and the current model is that the Mad1-Mad2 complex catalyses Mad2-Cdc20 binding and thus APC inhibition during the spindle checkpoint (Chung and Chen, 2002; De Antoni et al., 2005). The proposed model for the activation of Mad2 at kinetochores to inhibit the APC is the Mad2 template model (De Antoni et al., 2005) (Figure 1.8). Mad2 exists in three conformations (Luo, 2008). An open conformation (O-Mad2),

a closed conformation (C-Mad2) and an intermediate conformation (I-Mad2). C-Mad2 is probably bound to kinetochores in a complex with Mad1. O-Mad2 is recruited to kinetochores by C-Mad2. At kinetochores O-Mad2 is "activated" and changes conformation to an intermediate state (I-Mad2) upon binding to Mad1 (De Antoni et al., 2005; Luo et al., 2002; Nezi et al., 2006; Sironi et al., 2002). I-Mad2 can bind Cdc20 and then change its conformation to C-Mad2 or I-Mad2 can be released from the kinetochores and change its conformation to C-Mad2. Cytosolic C-Mad2 can either form symmetric C-Mad2-C-Mad2 dimers that are active or asymmetric C-Mad2-O-Mad2 dimers that are inactive. Crystal structures of both dimers have been described (Mapelli et al., 2007; Yang et al., 2008)(for review (Yu, 2006)). This model is supported by the finding that the Mad1-Mad2 complex is required for the spindle checkpoint (Chen et al., 1999). What contradicts the template model is, that it has been demonstrated in yeasts that the MCC and the Mad2-Cdc20 complex can be formed independently of kinetochores (Fraschini et al., 2001; Poddar et al., 2005; Sczaniecka et al., 2008). However, MCC formed independently of kinetochores appears to be unable to inhibit the APC, which suggests that it lacks important post-translational modifications that it might receive at the kinetochores (Sczaniecka et al., 2008).

Post-translational modification of human Mad2 has been reported to regulate APC inhibition. Mad2 is a phosphoprotein with a peak in phosphorylation in mitosis (Wassmann et al., 2003). Wassmann et al. showed that a phosphomimic Mad2 mutant was unable to interact with either Mad1 or the APC and Cdc20 binding was perturbed (Wassmann et al., 2003). However, the kinase phosphorylating Mad2 has not been identified.

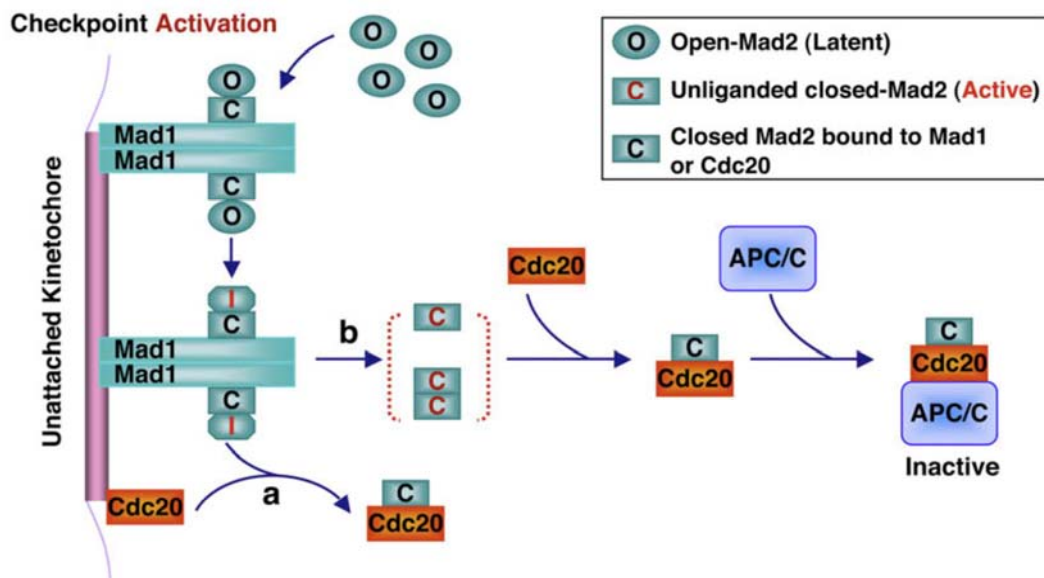


Figure 1.8: Mad2 template model. Closed-Mad2 is localised to kinetochores in a complex with Mad1. Open-Mad2 is recruited to kinetochores and changes its conformation to an intermediate form (I-Mad2) that is then released from the kinetochore. Upon release from the kinetochore I-Mad2 can immediately form a complex with Cdc20 or first form a dimer before binding to Cdc20. Picture was taken from (Luo, 2008).

1.6.2.1.3.4 Mad3/BubR1

Mad3/BubR1 is an essential component of the spindle checkpoint that is conserved between species (Chan et al., 1999; Chen, 2002; Hardwick et al., 2000; Millband and Hardwick, 2002). It has been suggested to be important for both the spindle checkpoint in response to unattached kinetochores and lack of tension at kinetochores. Importantly, unlike yeast, plant or *C.elegans* Mad3, BubR1 in higher organisms (human, *Xenopus*) contains a C-terminal kinase domain (Figure 1.9).

Mad3/BubR1 localises to unattached kinetochores early in metaphase until anaphase (Millband and Hardwick, 2002; Taylor et al., 2001). Localisation of Mad3 to kinetochores is dependent on Bub1, Bub3 and Mps1 in fission yeast (Millband and Hardwick, 2002). Mad3 is not stably associated with the kinetochore and is rapidly

exchanged (Howell et al., 2004; Rischitor et al., 2007). Unlike Mad2, vertebrate BubR1 localises to attached kinetochores upon loss of tension, which supports the idea that BubR1 has a role in the tension sensing checkpoint (Logarinho et al., 2004; Shannon et al., 2002; Skoufias et al., 2001). This relocalisation is dependent on Aurora B activity (Ditchfield et al., 2003).

Mad3/BubR1 has been shown to interact with other checkpoint proteins, such as Cdc20 and Bub3 (Davenport et al., 2006; Fang, 2002; Hardwick et al., 2000; Hwang et al., 1998; Millband and Hardwick, 2002). Mad3 also forms a complex with Bub3 that is present throughout the cell cycle (Hardwick et al., 2000; Millband and Hardwick, 2002) and has been suggested to have a role in APC inhibition as a pseudo-substrate (Burton and Solomon, 2007). Furthermore, Mad3 is a component of the mitotic checkpoint complex (MCC) that inhibits the APC upon spindle checkpoint activation (Fraschini et al., 2001; Sczaniecka et al., 2008; Sudakin et al., 2001) and it is possible that the interactions described above are subcomplexes of the MCC. Importantly, even though an interaction of Mad3 with Bub3 has been detected in fission yeast Bub3 has not been detected in the MCC (Millband and Hardwick, 2002; Sczaniecka et al., 2008).

Mad3 is a phosphoprotein that is hyperphosphorylated in mitosis and upon activation of the spindle checkpoint (Chan et al., 1999; Li et al., 1999; Rancati et al., 2005; Taylor et al., 2001). Phosphorylation of BubR1/Mad3 depends on several kinases including Polo kinase and Aurora B (Ditchfield et al., 2003; King et al., 2007a; Rancati et al., 2005). In budding yeast phosphorylation of Mad3 by Aurora is required for the spindle checkpoint in response to lack of tension at kinetochores (King et al., 2007a). BubR1 phosphorylation has also been shown to be dependent on Mps1 but specific sites have not been determined and analysis of phosphorylation mutants carried out yet (Huang et al., 2008).

In budding yeast protein levels of Mad3 are relatively constant throughout the cell cycle (Hardwick et al., 2000; Rancati et al., 2005) unlike in human in which BubR1 levels rise in late S-phase, G2-phase and upon spindle checkpoint activation (Chan et

al., 1999; Li et al., 1999). Interestingly in the study by Chan et al. analysis of BubR1 stability in two different cell lines revealed that BubR1 levels are stable in one cell line whereas they change in the other cell line throughout the cell cycle (Chan et al., 1999). Mad3/BubR1 contains two KEN boxes that are conserved between yeast and human (King et al., 2007b). In general the KEN box motif marks proteins for recognition by the APC and thus degradation by the proteasome. Indeed a study in budding yeast showed that disruption of the N-terminal KEN box leads to inhibition of Mad3 degradation in an APC-Cdh1 dependent manner in G1-phase (King et al., 2007b). However, most importantly both KEN boxes are essential for the spindle checkpoint in budding yeast and fission yeast (King et al., 2007b; Sczaniecka et al., 2008) (Figure 1.9). Mutation of the N-terminal KEN box disrupts MCC formation, Mad3 interaction with Cdc20 and Mad2 binding to the APC (King et al., 2007b; Sczaniecka et al., 2008).

As mentioned above Mad3/BubR1 in higher organisms contains a C-terminal kinase domain. BubR1 autophosphorylates *in vitro* (Taylor et al., 1998), but the reports of whether BubR1 kinase activity is required for a functional spindle checkpoint differ (Chen, 2002; Harris et al., 2005; Mao et al., 2003). These contradicting results could be due to experimental differences.

In human additional roles for BubR1 apart from its role in the spindle checkpoint have been suggested. BubR1 has been shown to regulate microtubule-kinetochore attachment (Lampson and Kapoor, 2005). Furthermore BubR1 deficient cells have chromosome alignment defects (Chan et al., 1999; Ditchfield et al., 2003; Lampson and Kapoor, 2005). It is noteworthy that *mad3* null mutants do not have chromosome segregation defects in fission yeast and a very low rate in budding yeast (Vanoosthuyse et al., 2004; Warren et al., 2002). Harris et al. proposed two distinct roles for BubR1: spindle checkpoint signalling is dependent on the N-terminus that is conserved between species and chromosome movement is dependent on the C-terminal kinase domain (Harris et al., 2005). This model could explain the differences between yeast and higher organisms.

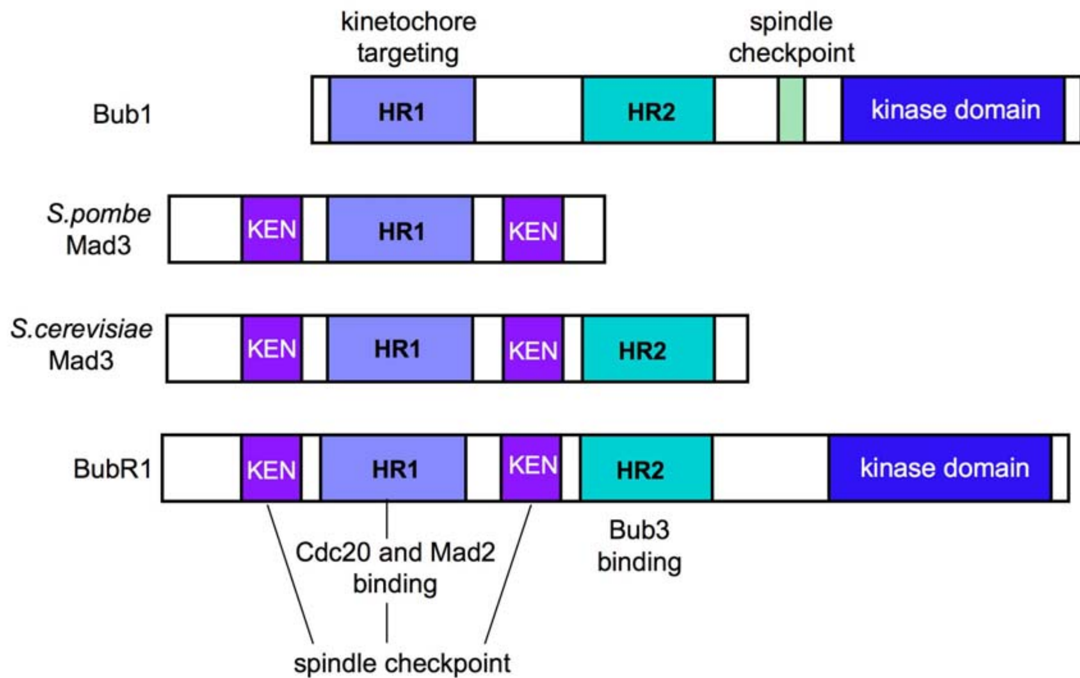


Figure 1.9: Schematic of Bub1. *S.pombe* Mad3, *S.cerevisiae* Mad3 and BubR1. Bub1 contains two homology regions to *S.cerevisiae* Mad3 and BubR1 (HR1: light blue; HR2: green). HR2 is not present in *S.pombe* Mad3. Bub1 and BubR1 contain a C-terminal kinase domain that is not present in yeast Mad3. All Mad3/BubR1 homologues contain a conserved N-terminal and C-terminal KEN box (purple). The KEN boxes are required for a functional spindle checkpoint in yeasts (King et al., 2007b; Sczaniecka et al., 2008). HR1 is required for Cdc20/Mad2 binding and HR2 for Bub3 binding in *S.cerevisiae* (Hardwick et al., 2000). The N-terminus of Bub1 is required for kinetochore targeting in *S.pombe* (Vanoosthuyse et al., 2004). A region in the N-terminus of human Bub1 is required for the spindle checkpoint (Klebig et al., 2009).

1.6.2.1.3.5 The kinase Bub1

Bub1 kinase is an essential component of the spindle checkpoint (Bernard et al., 1998; Sharp-Baker and Chen, 2001; Taylor and McKeon, 1997). It has been proposed to be important for the spindle checkpoint response to unattached

kinetochores and lack of tension at kinetochores (Fernius and Hardwick, 2007; Skoufias et al., 2001; Taylor et al., 2001).

Bub1 localises to kinetochores during an unperturbed mitosis (Bernard et al., 1998; Gillett et al., 2004) and upon spindle checkpoint activation (Bernard et al., 1998) and stays attached to kinetochores until anaphase (Jablonski et al., 1998; Sharp-Baker and Chen, 2001; Taylor et al., 2001; Taylor and McKeon, 1997). Unlike Mad2, that is released from kinetochores once the sister-chromatids are attached to the mitotic-spindle, Bub1 is recruited to kinetochores if tension is not strong enough (Shannon et al., 2002; Skoufias et al., 2001).

Bub1 is stably bound to kinetochores during mitosis (Shah et al., 2004) and is thought to act as a scaffold at kinetochores. Whether this scaffolding role of Bub1 depends on its kinase activity is not clear. However, a study in fission yeast showed that the N-terminus of Bub1 is sufficient to recruit Mad3 and Bub3 to telomeres (Rischitor et al., 2007). Bub1 is required for the localisation of Mad3/BubR1 and Bub3 to kinetochores (Johnson et al., 2004; Sharp-Baker and Chen, 2001). Localisation of Bub1, Bub3 and Mad3 is dependent on the N-terminus of Bub1 in fission yeast (Kadura et al., 2005; Vanoosthuyse et al., 2004). The importance of Bub1 in Mad1 and Mad2 localisation to kinetochores varies between organisms (Chen, 2004; Johnson et al., 2004; Kadura et al., 2005; Sharp-Baker and Chen, 2001).

Two Bub1 complexes have been identified. Bub1 interacts with Bub3 (Roberts et al., 1994; Seeley et al., 1999; Taylor et al., 1998) and in budding yeast Bub1 forms a complex with Mad1 and Bub3 (Brady and Hardwick, 2000).

Bub1 is a kinase that is capable of autophosphorylation (Roberts et al., 1994; Seeley et al., 1999; Taylor et al., 1998). It is phosphorylated upon spindle checkpoint activation (Chen, 2004; Sharp-Baker and Chen, 2001; Taylor et al., 2001) and Bub1 phosphorylation by Cdc2 (Cdk1) in fission yeast (Yamaguchi et al., 2003) and MAPK in *Xenopus* (Chen, 2004) is required for a fully functional spindle checkpoint.

The role of Bub1 autophosphorylation has not been investigated in great depth. However, several Bub1 substrates have been identified. Bub1 phosphorylates Bub3 (Roberts et al., 1994) and Mad1 *in vitro* (Seeley et al., 1999). The physiological role of this phosphorylation has still to be determined. Furthermore, human Bub1 phosphorylates Cdc20 *in vitro* and this phosphorylation is needed for *in vitro* inhibition of the APC and a fully functional spindle checkpoint (Tang et al., 2004). Even though Bub1 phosphorylates spindle checkpoint proteins the role of Bub1 kinase activity for the spindle checkpoint is controversial. Reports vary between kinase activity not being required for the spindle checkpoint (Sharp-Baker and Chen, 2001; Vanoosthuyse et al., 2004; Warren et al., 2002) or slight checkpoint defects in the absence of Bub1 kinase activity (Chen, 2004; Klebig et al., 2009; Yamaguchi et al., 2003). A recent study in budding yeast demonstrates that Bub1 kinase activity is not required for the spindle checkpoint response due to unattached kinetochores but is for the response to lack of tension at kinetochores (Fernius and Hardwick, 2007). These different observations even in the same organisms could be due to different assays and different kinase-deficient alleles of Bub1.

Nevertheless, Bub1 has a second role apart from its role in the spindle checkpoint. *bub1* null mutants in yeast and human display chromosome segregation defects (Bernard et al., 1998; Johnson et al., 2004; Morrow et al., 2005; Vanoosthuyse et al., 2004; Warren et al., 2002) and accurate chromsome segregation in yeast has been shown to be dependent on Bub1 kinase activity (Fernius and Hardwick, 2007; Vanoosthuyse et al., 2004). It has been proposed that Bub1 regulates proper bi-orientation through shugoshin 1 and 2 (Sgo1 and Sgo2). Localisation of Sgo1 and Sgo2 to centromeres is dependent on Bub1 (Fernius and Hardwick, 2007; Kitajima et al., 2004). In budding yeast Sgo1 localisation is dependent on Bub1 kinase activity and *bub1* null mutants and *sgo1* null mutants display bi-orientation defects (Fernius and Hardwick, 2007). In fission yeast Sgo2 is required for chromsome bi-orientation and efficient localisation of the chromosomal passenger proteins to kinetochores (Vanoosthuyse et al., 2007).

Bub1 protein levels vary throughout the cell cycle. Human Bub1 is degraded upon exit from mitosis in an APC-Cdh1 and KEN box dependent manner (Qi and Yu, 2007; Taylor et al., 2001). However, Bub1 degradation does not appear to be required for checkpoint silencing as stabilisation of Bub1 does not prevent mitotic exit (Qi and Yu, 2007).

1.6.2.1.3.6 Bub3

The spindle checkpoint protein Bub3 (budding uninhibited by benzimidazole 3) was first identified as a multi-copy suppressor of Bub1 in 1991 in budding yeast (Hoyt et al., 1991). It is required for the spindle checkpoint from budding yeast to vertebrates (Campbell and Hardwick, 2003; Kalitsis et al., 2000; Lopes et al., 2005). Fission yeast Bub3 was also initially thought to be essential for a functional spindle checkpoint due to loss of viability under spindle stress and benomyl sensitivity (Vanoosthuyse et al., 2004). Recent reports suggest otherwise and show that fission yeast Bub3 is not required for the spindle checkpoint but the regulation of spindle dynamics and the establishment of bi-orientation (Tange and Niwa, 2008; Windecker et al., 2009). Furthermore, Bub3 mutants display chromosome segregation defects under spindle stress (Tange and Niwa, 2008; Windecker et al., 2009).

Bub3 localises transiently to kinetochores from prometaphase until metaphase (Howell et al., 2004; Taylor et al., 1998) and is released from kinetochores after sister-chromatids are attached to the mitotic spindle (Gillett et al., 2004).

Bub3 interacts with Bub1 (Roberts et al., 1994; Taylor et al., 1998) and its kinetochore localisation is dependent on Bub1 that forms a scaffold at kinetochores (Kadura et al., 2005; Roberts et al., 1994; Vanoosthuyse et al., 2004). This interaction is required for the establishment of bipolar attachment (Windecker et al., 2009).

Bub3 is also required for Mad3 (Millband and Hardwick, 2002), Mad1 and Mad2 localisation to kinetochores (Windecker et al., 2009). Bub3 is present in a complex with Mad1 and Bub1, which is required for the spindle checkpoint (Brady and Hardwick, 2000). Bub3 is also part of the mitotic checkpoint complex (Mad2, BubR1, Cdc20) in budding yeast and vertebrates (Fraschini et al., 2001; Sudakin et al., 2001) that inhibits the APC during the spindle checkpoint. Importantly Bub3 does not appear to be part of the MCC in fission yeast (Sczaniecka et al., 2008).

1.6.2.1.4 Additional checkpoint proteins

1.6.2.1.4.1 Rod, Zw10 and Zwilch

Apart from the spindle checkpoint proteins described above additional checkpoint proteins have been identified in higher eukaryotes. Three of these checkpoint proteins are Rod (Rough Deal) (Karess and Glover, 1989), ZW10 (Zeste-white 10) (Starr et al., 1997; Williams et al., 1992) and Zwilch (Williams et al., 2003) that are essential for the spindle checkpoint (Basto et al., 2000; Chan et al., 2000) (for review (Karess, 2005)). Rod, Zw10 and Zwilch form a complex (Scaerou et al., 2001; Williams et al., 2003), which has been proposed to regulate the spindle checkpoint by recruiting Mad1 and Mad2 to kinetochores (Buffin et al., 2005; Kops et al., 2005a).

1.6.2.1.4.2 Aurora kinase

Another spindle checkpoint protein is Aurora kinase, which was identified in budding yeast in a screen for increase in ploidy 1 mutants (Ipl1) (Chan and Botstein, 1993) and was later shown to be a protein kinase required for accurate chromosome segregation (Francisco et al., 1994). Since then Ipl1 homologues in metazoans have been identified (Bischoff et al., 1998; Glover et al., 1995; Gopalan et al., 1997; Roghi et al., 1998) and characterised. Metazoans have three aurora kinases Aurora

A, B and C, whilst in yeasts only one aurora like kinase has been identified, Ipl1 in budding yeast and Ark1 in fission yeast, respectively. Aurora B is part of the chromosomal passenger complex (CPC), which has been reported to have roles in cytokinesis, chromosome alignment and the spindle checkpoint (for review see (Ruchaud et al., 2007)).

Aurora B activates the spindle checkpoint due to lack of tension at kinetochores (Biggins and Murray, 2001). One mechanism of how Ipl1 is thought to activate the spindle checkpoint is indirectly by creating unattached kinetochores once it senses tension defects. These unattached kinetochores then lead to the activation of the spindle checkpoint (Hauf et al., 2003; Pinsky et al., 2006; Tanaka et al., 2002). However, another study in budding yeast showed that Mad3 phosphorylation by Ipl1 is required for the spindle checkpoint due to lack of tension. This report suggests a more direct role for Ipl1 in the spindle checkpoint (King et al., 2007a). Furthermore, BubR1 phosphorylation and kinetochore localisation is dependent on Aurora B (Ditchfield et al., 2003; Hauf et al., 2003) and BubR1 relocalises to kinetochores upon loss of tension, which is also dependent on Aurora B (Ditchfield et al., 2003).

Ark1 (aurora related kinase 1) is the only Aurora kinase identified in fission yeast. It seems to comprise both functions of Aurora A and Aurora B. Ark1 has a role in chromosome condensation and chromosome segregation (Hauf et al., 2007; Leverson et al., 2002; Petersen and Hagan, 2003; Petersen et al., 2001). A study by Hauf et al. showed that Ark1 corrects kinetochore-microtubule mis-attachments and thus ensures correct bi-orientation of sister-chromatids during mitosis (Hauf et al., 2007). Whether Ark1 has a role in cytokinesis is controversial with two reports having opposing results (Leverson et al., 2002; Petersen and Hagan, 2003). Like human Aurora, Ark1 is a phosphoprotein and its kinase function requires the homologue of the CPC component Bir1/Cut17/Phb1 (Survivin) (Petersen and Hagan, 2003). Ark1 also interacted with the CPC component Pic1 (INCENP) in a two hybrid screen (Leverson et al., 2002). Furthermore, Ark1 is essential for the spindle checkpoint (Petersen and Hagan, 2003). However, unlike budding yeast Ipl1 and human Aurora B, Ark1 is required for the checkpoint activation and maintenance due to lack of

kinetochore attachment (Petersen and Hagan, 2003; Vanoosthuyse and Hardwick, 2009). This is consistent with a study in *Xenopus*, that also showed that Aurora is required for the establishment and maintenance of the spindle checkpoint (Kallio et al., 2002). Ark1 might have a role in APC inhibition as Mad2 localisation to kinetochores and Mad2-Mad3 complex formation requires Ark1 (Petersen and Hagan, 2003). However, Ark1 substrates and the physiological role of their phosphorylation in the spindle checkpoint still have to be determined.

1.6.2.1.5 APC inhibition upon spindle checkpoint activation

Upon activation of the spindle checkpoint the APC is inhibited in order to prevent degradation of securin and cyclin, which would lead to the onset of anaphase (Peters, 2002). Inhibition of the APC is regulated on different levels. To date five different mechanisms have been suggested to contribute to APC inhibition during the spindle checkpoint. These are phosphorylation of Cdc20, Cdc20 instability, the Mad3 pseudo-substrate model, Cdc20 sequestration by Mad2 and inhibition of the APC by the MCC (Figure 1.10).

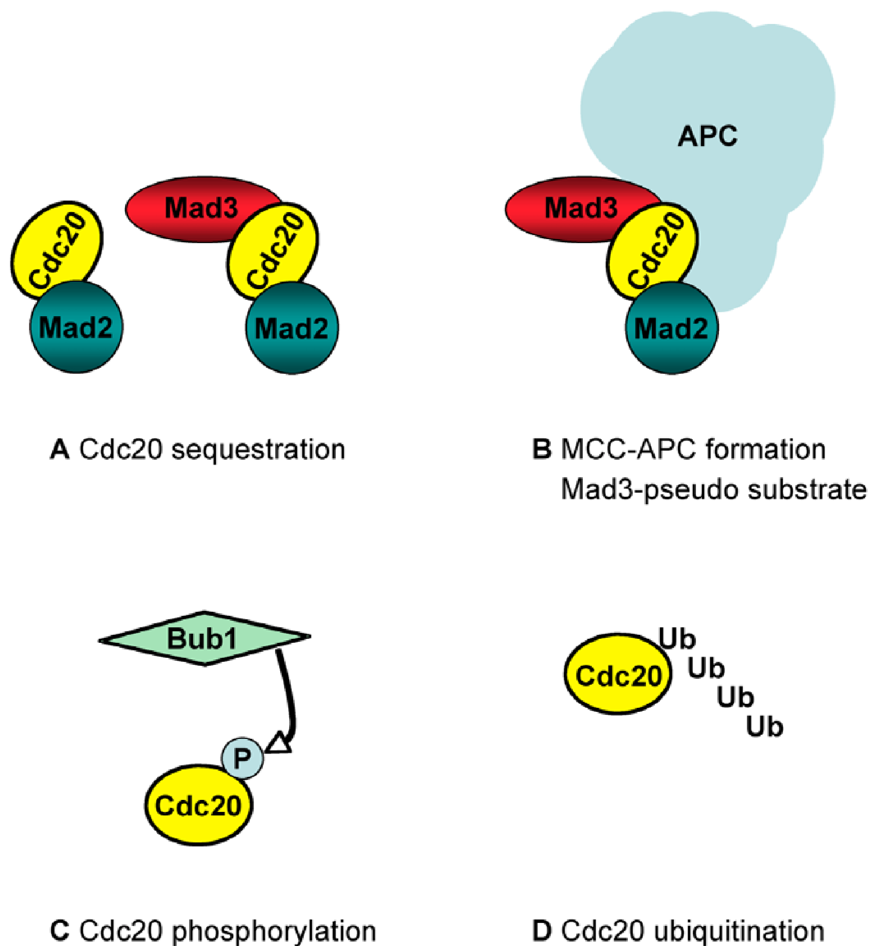


Figure 1.10: Model of modes of APC inhibition during the spindle checkpoint
A) Cdc20 sequestration; **B**) MCC binding to the APC and Mad3 acting as a pseudo-substrate; **C**) Cdc20 phosphorylation by Bub1; **D**) Cdc20 ubiquitination and degradation.

1.6.2.1.5.1 Phosphorylation of Cdc20

Cdc20 is a phosphoprotein and phosphorylation of Cdc20 by several kinases has been reported. Phosphorylation of Cdc20 by Cdk1 abolishes binding to the APC and increases its affinity to Mad2 *in vitro*, which suggest that Cdk1 phosphorylation of Cdc20 negatively regulates APC activity (D'Angiolella et al., 2003; Yudkovsky et al., 2000).

Cdc20 phosphorylation is required for the inhibition of the APC during a spindle checkpoint arrest in *Xenopus* and this is partially dependent on MAPK (Chung and Chen, 2003). Human Cdc20 is phosphorylated by Bub1. This phosphorylation is required for APC inhibition *in vitro* and a fully functional spindle checkpoint (Tang et al., 2004).

Furthermore, Cdc20 phosphorylation can influence its ability to bind Mad2 and Mad3/BubR1. Chung et al. reported that in *Xenopus* Cdc20 phosphorylation is required for efficient Mad2 and BubR1 binding (Chung and Chen, 2003). On the contrary Tang et al. showed in HeLa cells that phosphorylation of Cdc20 by Bub1 is not required for Mad2 and BubR1 binding (Tang et al., 2004). These discrepancies show that regulation of Cdc20 is complicated and that kinases appear to contribute to Cdc20 regulation in different ways. In addition, differences in Cdc20 regulation by phosphorylation between organisms is also possible.

1.6.2.1.5.2 Cdc20 ubiquitination

The role of Cdc20 degradation on the activity of the spindle checkpoint is controversial. Two reports suggest that human Cdc20 ubiquitination and deubiquitination controls the spindle checkpoint and that upon ubiquitination of Cdc20 Mad2 and BubR1 dissociate from the APC and the checkpoint is inactivated (Reddy et al., 2007; Stegmeier et al., 2007). On the contrary other reports in budding yeast and human argue that APC mediated degradation of Cdc20 ensures that Cdc20 levels are not too high and the spindle checkpoint can be maintained (Ge et al., 2009; Nilsson et al., 2008; Pan and Chen, 2004). Nillson et al. showed that an unubiquitinatable mutant of Cdc20 is not able to maintain a spindle checkpoint arrest (Nilsson et al., 2008). On balance it seems more likely that Cdc20 degradation is required for efficient maintenance of the spindle checkpoint.

1.6.2.1.5.3 Cdc20 sequestration and MCC binding to the APC

Herzog et al. detected two forms of inactive APC during an activated spindle checkpoint. One was APC bound to MCC and the second an apo-APC that was inactive because it lacked the activating co-factor Cdc20. Whereas apo-APC could be activated *in vitro* by Cdc20 or Cdh1, APC-MCC could not be activated by either Cdc20 or Cdh1 (Herzog et al., 2009). The proposed model for APC inhibition by the MCC proposes that the MCC binds to the APC in the same binding region where Cdc20 binds. Binding of the MCC induces conformational changes and puts the APC in a closed state and thus prevents binding of APC substrates (Herzog et al., 2009).

Initially it was thought that Mad2 was the major protein inhibiting the APC by sequestering Cdc20. However, later studies showed that Mad2 and Mad3 can both inhibit the APC *in vitro* independently of each other (Fang, 2002; Li et al., 1997; Tang et al., 2001a). Furthermore, Mad3 is a more potent inhibitor of the APC *in vitro* than Mad2 (Fang, 2002; Tang et al., 2001a). Mad3 can also inhibit the APC by acting as an APC pseudo-substrate and thus compete with other substrates for Cdc20 binding (Burton and Solomon, 2007; Malureanu et al., 2009). Both Mad2 and Mad3 interact with the APC during the spindle checkpoint (Li et al., 1997; Sczaniecka et al., 2008) and associate with Cdc20 (Fang, 2002; Fang et al., 1998a; Hardwick et al., 2000; Hwang et al., 1998; Kallio et al., 1998; Kim et al., 1998; Millband and Hardwick, 2002). In addition, Mad2 and Mad3 promote each others binding to Cdc20 and are interdependent (Burton and Solomon, 2007; Fang, 2002; Hwang et al., 1998; King et al., 2007b; Sczaniecka et al., 2008).

Furthermore, Mad2 and Mad3 are both part of the mitotic checkpoint complex (MCC), which was first identified by Sudakin et al. in HeLa cells. It is composed of Mad2, Mad3, Bub3 and Cdc20 and is capable of inhibiting the APC (Sudakin et al., 2001). Later analysis also identified MCC in budding yeast and fission yeast (Fraschini et al., 2001; Sczaniecka et al., 2008). Binding of the MCC to the APC is spindle checkpoint dependent (Morrow et al., 2005; Sczaniecka et al., 2008).

Importantly Bub3 does not appear to be part of the MCC in fission yeast (Sczaniecka et al., 2008).

Interestingly, MCC formation does not depend on kinetochores (Poddar et al., 2005; Sczaniecka et al., 2008; Sudakin et al., 2001). However, the MCC appears to be unable to inhibit the APC without checkpoint proteins being present at the kinetochore (Sczaniecka et al., 2008). These findings suggest that MCC components might be post-translationally modified in a way that is not required for MCC formation but is required for its ability to inhibit the APC. One possible post-translational modification being phosphorylation by one or more of the spindle checkpoint kinases.

Sudakin et al. showed that the MCC is a more potent inhibitor of the APC *in vitro* than Mad2 (Sudakin et al., 2001). However, a study in budding yeast showed that even though the components of the MCC (Mad2, Mad3, Bub3) are present in excess over Cdc20, only small quantities of MCC are present in a cell, which is not enough to inhibit all the APC present (Poddar et al., 2005).

All the mechanisms described above are important to ensure APC inhibition during a spindle checkpoint arrest. However, it is currently unclear whether these mechanisms act in parallel or under specific situations.

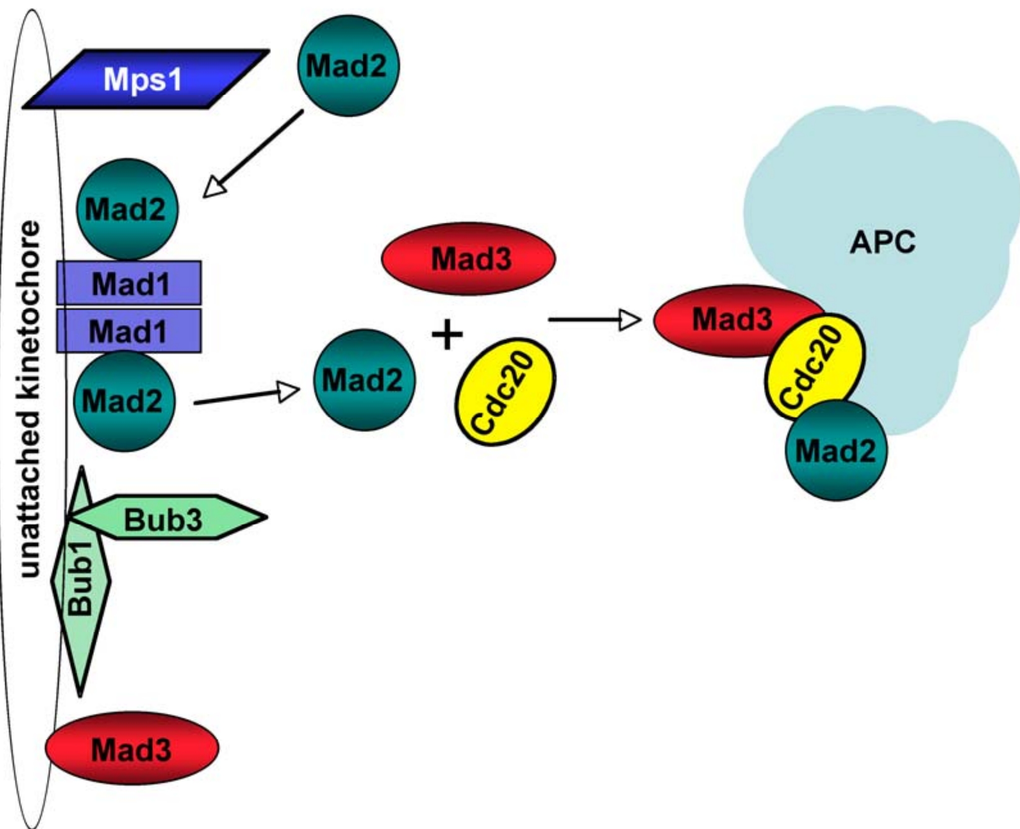


Figure 1.11: Model of the spindle checkpoint proteins at kinetochores
The kinetochore is thought to be the side where spindle checkpoint signalling is initiated, leading to the inhibition of the APC.

1.6.2.1.6 The role of the kinetochores in spindle checkpoint signalling

Kinetochores are large multi-protein structures that are assembled onto centromeres and serve as the site of kinetochore attachment during mitosis and link the sister-chromatids to the microtubules, which connect to the spindle-poles/centrosomes (for reviews (Cleveland et al., 2003; Santaguida and Musacchio, 2009). A budding yeast kinetochore contains at least 60 subunits and is built of 7 sub-complexes. In budding yeast one microtubule attaches to each kinetochore, whereas in fission yeast and higher eukaryotes several microtubules (kinetochore fibre) bind to each kinetochore. The kinetochores are thought to be the side where spindle checkpoint signalling is

initiated as the spindle checkpoint is activated even if a single unattached kinetochores is present or if there is a lack of tension at kinetochores (Li and Nicklas, 1995; Rieder et al., 1995). All spindle checkpoint proteins localise to unattached kinetochores during mitosis (Figure 1.11., 1.12) (Abrieu et al., 2001; Bernard et al., 1998; Chen et al., 1998; Chen et al., 1996; Taylor et al., 2001; Taylor and McKeon, 1997; Vanoosthuyse et al., 2004). The dynamics of spindle checkpoint proteins at kinetochores has been studied and showed that Bub1 and Mad1 and a fraction of Mad2 are stably associated with the kinetochores, whereas the other checkpoint proteins (Mad2, Mad3, Bub3, Mps1) are only transiently associated with the kinetochores (Howell et al., 2004; Rischitor et al., 2007; Shah et al., 2004).

Several kinetochore sub-complexes have been linked to the spindle checkpoint one of them being the Ndc80 complex, composed of Ndc80, Nuf2, Spc25 and Spc24. It is conserved between yeasts and vertebrates and associates with the centromere (Janke et al., 2001; McCleland et al., 2003; Wigge and Kilmartin, 2001). The Ndc80 complex is required for accurate chromosome segregation, chromosome alignment and microtubule-kinetochore interactions (Janke et al., 2001; McCleland et al., 2003; Wigge and Kilmartin, 2001). In addition components of the Ndc80 complex have been shown to be required for a functional spindle checkpoint (Janke et al., 2001; McCleland et al., 2003; Nabetani et al., 2001). In human Ndc80 is required for Mad1 and Mad2 localisation to kinetochores (Martin-Lluesma et al., 2002). Budding yeast Ndc80 interacts with and is phosphorylated by the spindle checkpoint kinase Mps1. This phosphorylation is required for a partially functional spindle checkpoint (Kemmler et al., 2009).

The hypothesis that the formation of spindle checkpoint complexes is initiated at kinetochores is contradicted by findings that the MCC can be formed independently of kinetochores (Fraschini et al., 2001; Sudakin et al., 2001). Nevertheless, this MCC does not appear to be able to inhibit the APC, which suggests that important post-translational modifications might be missing that could be delivered at the kinetochores (Sczaniecka et al., 2008). Furthermore, a BubR1 mutant that is not able to localise to kinetochores is still able to inhibit the APC (Malureanu et al., 2009).

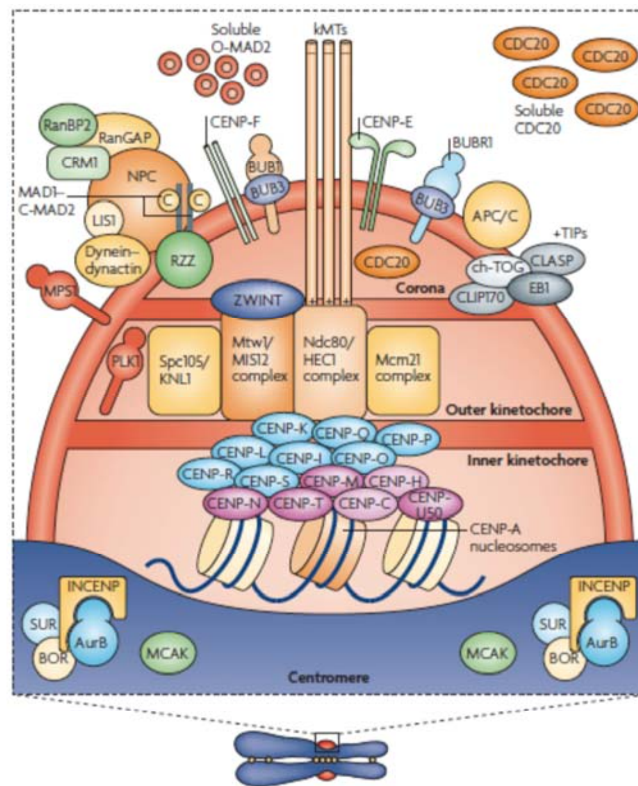


Figure 1.12: Model of the kinetochore. Taken from (Musacchio and Salmon, 2007)

1.6.2.1.7 Spindle checkpoint silencing

A mechanism as important as the activation of the spindle checkpoint is switching the spindle checkpoint off (silencing) after all the chromosomes have attached properly onto the mitotic spindle. Several silencing mechanisms have been reported.

One mechanism of checkpoint silencing proposed in budding yeast is degradation of the kinase Mps1 in anaphase and G1-phase. Budding yeast Mps1 is essential for establishment and maintenance of the spindle checkpoint (Jones et al., 2005) and degradation of Mps1 in anaphase and G1-phase by APC-Cdc20 and APC-Cdh1 has been proposed to inactivate the spindle checkpoint (Palframan et al., 2006).

A second mechanism is stripping Mad2 from attached kinetochores along spindle fibres via the motor-protein dynein (Howell et al., 2001). This process requires dephosphorylation of dynein by protein phosphatase 1 (PP1-γ) in vertebrates (Whyte

et al., 2008). PP1 has also been shown to be required for metaphase exit in fission yeast and budding yeast (Pinsky et al., 2009; Vanoosthuyse and Hardwick, 2009).

A third mechanism proposed by studies in *Xenopus* is checkpoint silencing via inhibition of BubR1 kinase activity by CENP-E. CENP-E activates BubR1 at kinetochores. Upon microtubule binding the interaction between CENP-E and BubR1 is altered and BubR1 is not active anymore and thus the checkpoint is silenced (Mao et al., 2003; Mao et al., 2005). However, this mechanism cannot be applied to yeast, as yeast Mad3/BubR1 does not contain a kinase domain.

A fourth mechanism is the inhibition of Mad2 promoted inhibition of the APC by p31/comet (Habu et al., 2002; Mao et al., 2003; Mapelli et al., 2006; Xia et al., 2004). Structural analysis of the Mad2-p31/comet complex showed that p31 binds to the Mad2 dimerisation interface and thus blocks APC inhibition (Yang et al., 2007). However, a homologue of p31 has not been identified in yeast.

Aims

The aim of this study was to analyse the role of Mph1 kinase activity in the spindle checkpoint and chromosome segregation.

1) Generate a kinase-dead allele of Mph1 and analyse the general role of Mph1 kinase activity in the spindle checkpoint and chromosome segregation.
2) Analyse the role of Mph1 kinase activity in the production of anaphase inhibitors, with a focus on binding of the mitotic checkpoint complex (MCC) to the APC.
3) Identify Mph1 substrates with spindle checkpoint functions, map and mutate Mph1 phosphorylation sites and analyse their physiological relevance in the spindle checkpoint.

Chapter 2

Materials and Methods

Chapter 2: Materials and Methods

2.1 Supplier Information

Chemicals used in this study were purchased from Sigma, Melford, Gibco BRL, Fisher, Boehringer Mannheim unless stated otherwise. Reagents for growth media were from Sigma, Oxoid, Difco, Biogene. Restriction enzymes used in this study were purchased from New England Biolabs.

2.2 General Information

2.2.1 Sterilisation

Solutions were filter sterilised using bottle top filters (Nalgene) and 0.45 μm syringe filters (Millipore) or autoclaved (120 °C, 15 pounds/inch^2, 15 min).

2.2.2 Buffers and Solutions

2.2.2.1 PVDF membrane Transfer Buffer (1X)

Tris-Cl	25 mM
Glycine	192 mM
Methanol	10 %

2.2.2.2 SDS Gel Running Buffer (1x)

Tris	50 mM
Glycine	384 mM
SDS	2 %

2.2.2.3 SDS Gel-loading Buffer (1x)

1 M Tris-Cl (pH 6.8)	20 ml
10 % SDS (w/v) (electrophoresis grade)	8 ml
70 % glycerol	4.28 ml
0.5 M EDTA (pH 8)	2 ml
Bromophenol blue (w/v)	to desired color

2.2.2.4 Nitrocellulose membrane Transfer Buffer (1x)

Tris-Cl	25 mM
Glycine	129 mM
Methanol	10 %

2.2.2.5 PBS-Tween 20

1 x PBS

0.02 % Tween 20

2.2.2.6 PonceauS stain

PonceauS	0.25 g
Acetic acid	12.5 ml
ddH_20	to 250 ml

2.2.2.7 Coomassie Blue stain

0.16 % Coomassie Blue in	4 volumes methanol
	4 volumes acetic acid
	5 volumes ddH_20

2.2.2.8 Coomassie Blue destain

4 volumes methanol

1 volume acetic acid

14 volumes ddH_20

2.2.2.9 10 x TBE

Tris	445 mM
Boric acid	445 mM
EDTA (pH 8)	100 mM

2.3 Molecular Biology

2.3.1 Bacterial strains

Cloning was carried out using XL1-Blue cells (electrocompetent or chemically competent) from Stratagene or DH5α cells prepared in the lab.

XL-1 Blue

XL1-Blue MRF′ Genotype: Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB* $lacI^{q}Z\Delta M15$ Tn*10* (Tet^{r})

DH5α

F^{-}*, φ80dlacZΔM15*, *Δ(lacZYA-argF)U169*, *deoR*, *recA1*, *endA1*, $hsdR17(rk^{-},mk^{+})$, *phoA*, *supE44*, λ^{-}, *thi-1*, *gyrA96*, *relA1*

2.3.2 Bacterial Media

LB

Bacto-tryptone	1 % (w/v)
Bacto-yeast extract	0.5 % (w/v)
NaCl	0.5 % (w/v)

pH adjusted to pH 7.2 with NaOH

2.3.3 Antibiotics

Ampicillin was added to bacterial growth medium or agar to a final concentration of 100 µg/ml from a 100mg/ml (1000x) stock solution.

2.3.4 Maintenance

Bacteria were either grown on plates or in liquid LB medium, containing the appropriate antibiotic for selection, at 37 °C.

2.3.5 Transformation

2.3.5.1 Transformation of XL1-Blue cells

Transformations of XL-1 Blue cells were carried out according to the protocol provided by Stratagene.

2.3.5.2 Transformation of DH5-α cells

To transform DH5-α competent cells the DNA was added to the cells and the mixture incubated for 30 min on ice. Cells were then heat shocked for 45 s at 42 °C, cooled down for 2 min on ice and resuspended in 400 µl LB. The cells were incubated shaking at 37 °C for 45 min for recovery, plated on LB agar plates containing the appropriate antibiotics and incubated overnight at 37 °C.

2.3.2 Fission yeast

Table 2.1: Fission yeast strains used in this study

Strain	Genotype	Source
AE247	*mad2Δ::ura4$^+$ leu1*	T. Matsumoto
AS232	*mad3-18A-GFP::leu1 mad3Δ::ura4$^+$ ade6-210 leu1-32 ura4-D18*	This lab
DMSP001	*mad3Δ::ura4$^+$ ade6-210 leu1-32 ura4-D18*	This lab
KP114	*ura4-D18 leu1-32 ade6-210*	R.Allshire
KP340	*nda3-KM311*	This lab
KP349	*lys1 ura4 cen2D107(::Kan-ura4-lacO) his7::lacI-GFP leu1 ad36-M210*	This lab
KP361	*cdc25-22 lid1-TAP::kan’ mad3-GFP::his3*	This lab
KP379	CH16 (*bub1Δ::ura4$^+$*)	This lab
KP434	*cdc25-22 lid1-TAP::kan’ mad2-GFP::his3 mad3-GFP::his3*	This lab
KP443	*cdc25-22 lid1-TAP::kan’ mad2-GFP::his3 mad3-GFP::his3 mph1Δ::NAT*	This lab
SP22	*mad3-szz*	This lab
VV414	*nmt::GFP-plo1 nda3-KM311*	H.Okhura
YJZ108	*mph1-kd::leu1 mph1-kd::NAT ura4-D18 ade6-210*	This study
YJZ124	*mad2-dimer::leu1 mad2Δ::ura4$^+$ cdc25-22 lid1-TAP::kan’ mad3-GFP::his3*	This study
YJZ128	*mad2Δ::ura4$^+$nmt::GFP-plo1 nda3-KM311*	This study
YJZ171	*mph1-kd::leu1 cen2D107(::Kan-ura4-lacO) his7::lacI-GFP*	This study
YJZ177	*mad2-5A::leu1 mad2Δ::ura4$^+$ cdc25-22 lid1-TAP::kan’ mad3-GFP::his3*	This study
YJZ181	*mad2-dimer::leu1 nda3-KM311 nmt::GFP-plo1*	This study
YJZ193	CH16 *mph1-kd::leu1* (*bub1Δ::ura4$^+$*)	This study
YJZ203	*nuf2-3::ura4$^+$ mad2Δ::ura4$^+$ mad2-5A::leu1*	This study
YJZ210	*mad3-18A-GFP::leu1 mad3Δ::ura4$^+$ cdc25-22 lid1-TAP::kan’ mad3-GFP::his3*	This study

YJZ213	*mad2-5A::leu1 nda3-KM311 KM311*	This study
YJZ217	*mad2-3D::leu1 nda3-KM311 KM311*	This study
YJZ218	*mad3-18A-GFP::leu1 nda3-KM311 mad3Δ::*$ura4^+$	This study
YJZ220	*mad2-3D::leu1 mad2Δ::*$ura4^+$ *cdc25-22 lid1-TAP::kan' mad3-GFP::his3*	This study
YJZ24	*mph1-kdSTAP::leu1 ura4-D18 leu1-32 ade6-210*	This study
YJZ4	*mph1STAP::leu1 ura4-D18 leu1-32 ade6-210*	This study
YJZ59	*mad3-szz mph1ΔK-GFP*	This study
YJZ67	*mph1-kd::leu1 ura4-D18 ade6-210*	This study
YJZ73	*mph1-kd::leu1 nda3-KM311*	This study
YJZ82	*mph1-kd::leu1 mph1-kd::NAT nda3-KM311*	This study
YJZ99	*mph1-kd::leu1 nda3-KM311 nmt::GFP-plo1*	This study
YLM202	*mph1Δ nmt::GFP-plo1 nda3-KM311*	This lab
YLM223	*mph1-STAP::kan' nda3-KM311*	This lab
YLM60	*mph1Δ*::NAT	This lab
YLM83	*mph1Δ nda3-KM311*	This lab
YVV20	*nuf2-3::*$ura4^+$ *his3Δ arg3Δ*	This lab

2.3.2.1 Fission yeast media

2.3.2.1.1 YES (yeast extract supplemented) (liquid and agar)

0.5 % yeast extract

3 % D-glucose, anhydrous

1 x supplements

add 2 % agar to YES

2.3.2.1.2 PMG (pombe minimal growth)

3 g/l Phtalic acid

2.2 g/l di-sodium hydrogen orthophosphate, anhydrous

3.75 g/l L-glutamic acid, monosodium salt

20 g/l D-glucose, anhydrous

1 x vitamins

1 x minerals

1 x salts

add 2 % agar to PMG

2.3.2.1.3 SPA (synthetic sporulation agar)

10 g/l glucose

1 g/l KH_2PO_4

1 x vitamins; added after autoclaving

1 x supplement (histidine, uracil, arginine, leucine, lysine, adenine); added after autoclaving

2 % (w/v) agar

Table 2.2: Fission yeast media supplements

50 x salts	53.5 g/l $MgCl_2$ 1 g/l $CaCl_2$ 50 g/l KCl 2 g/l Na_2SO_4
20 x supplement	1.5 g/l histidine, arginine, uracil, leucine, lysine, adenine
1000 x vitamin stock	1 g/l pantothenic acid (4.2 mM) 10 g/l nicotinic acid (81.2 mM) 10 g/l inositol (55.5 mM) 10 mg/l biotin (40.8 mM)
10000 x mineral stock	80.9 mM Boric acid 23.7 mM $MnSO_4$ 13.9 mM $ZnSO_4$ x 7 H_2O 7 mM $FeCl_3$ x 6 H_20) 2.47 mM Molybdic acid 6.02 mM KI 1.6 mM $CuSO_4$ x 5 H_2O 47.6 mM Citric acid
100 x thiamine stock	4 mg/ml thiamine in sterile, double distilled water
G418	G418 (Calbiochem) was dissolved in sterile, double distilled water and added to YES agar to a final concentration of 150 µg/ml.
CLONAT	Clonat/Nouroseothricin (Werner Bioagents) (200 mg/ml stock in ddH_2O); added to YES to a final concentration of 100 µg/ml.
Hygromycin	Hygromycin B (Formedium) (500 x stock in ddH_20); added to YES agar to a final concentration of 100 µg/ml.

Benomyl	30 mg/ml stock in DMSO

2.3.2.2 Strain maintenance

Cells were kept on plates at 30 °C/ 32 °C/ 25 °C for short period of times depending on their genetic background. For longterm storage cells were resuspended in YES with the addition of 20 % glycerol and stored at -80 °C.

2.3.2.3 Crossing of fission yeast strains

Cells of opposite mating types were mixed on a SPA plate in 5 µl of sterile water. Plates were incubated at 30 °C (25 °C temperature sensitive mutants) until asci with spores (tetrads) were visible. A small amount of cells was then digested in a 2 % dilution of β-glucoronidase (MP Biomedicals) in water for 24 hrs at 37 °C. The digested cells were washed three times with sterile water, resuspended in 1 ml sterile water and 2 µl/20 µl of spore solution plated onto selective media.

2.3.2.4 Fission yeast transformation

2.3.2.4.1 Electroporation

An overnight culture of the strain to be transformed was grown to an OD_{595} of 0.5 - 1 in YES. Cells were centrifuged at 3000 rpm for 5 min at room temperature. Cells were washed once with ice-cold water and once with ice-cold 1 M sorbitol before being resuspended in 1 M sorbitol to a final concentration of 1 – 5 x 10^9 cells per ml. 40 µl of cell suspension was added to a pre-chilled 1.5 ml tube containing the DNA and incubated on ice for 5 min. Cells were electroporated using a BioRad electroporator and 2 mm cuvettes (EquiBio) with the following settings: 1.5 kV, 200 Ohm, 15 µF. After electroporation 500 µl ice-cold sorbitol was immediately added to

the cuvette and the cell suspension transferred to a 1.5 ml tube. Cells were centrifuged briefly, resuspended in sterile water and plated on selective media.

2.3.2.4.2 Lithium acetate

50 ml cultures were grown overnight to an OD_{595} of 0.2 - 0.5 (0.5 – 1 x 10^7 cells/ml). Cells were harvested at 2500 rpm for 3 min. Cells were washed in 50 ml of sterile water and centrifuged again (2500 rpm, 3 min). Cells were then resuspended in 0.1 M lithium acetate (pH 4.9) to a concentration of 1 x 10^9 cells/ml. 100 µl aliquots were incubated at 30 °C for 60 – 120 min. Cells were then mixed with the DNA and 290 µl of fresh 50 % (w/v) PEG (3350, Sigma) prewarmed to 30 °C. Cells were incubated at 30 °C for 30 min to 60 min followed by a 15 min heat-schock at 42 °C. Cells were then cooled down for 10 min at room temperature, briefly centrifuged, the supernatant removed and cells resuspended in 200 µl YES and plated on plates containing the appropriate antibiotics for selection or minimal plates.

2.3.2.5 Genomic DNA extraction

Overnight cultures were grown at 25 °C/30 °C. 1.5 ml culture was harvested at 8000 rpm. The cells were resuspended in 200 µl genomic DNA extraction buffer (2.5 M LiCl, 50 mM Tris-HCl (pH 8.0), 4 % Triton X-100, 62.5 mM EDTA (pH 8)) and moved to a screw cup tube containing 0.2 g 0.5 mm Zirconia/Silica beads (BioSpec Products). Phenol:Chloroform:IsoamylAlcohol 25:24:1 (Sigma) was added at an equal volume to the cell suspension. The cells were bead beat for 2 min and then centrifuged for 5 min at 14000 rpm. The upper phase was collected and the genomic DNA ethanol precipitated.

2.4 Fission yeast cell biology methods

2.4.1 Benomyl sensitivity assay

10 fold serial dilutions were plated on YES plates containing 0, 2, 4, 6, and 8 μg of the microtubule depolymerising drug benomyl (Sigma). Plates were incubated at 30 °C or 25 °C for 3 or 4 days.

2.4.2 General microscopy

For live cell microscopy 3 μl of culture were pipetted onto a slide and covered with a cover-slip.

To visualise GFP-Plo1 spots cells were briefly fixed in methanol, 3 μl pipetted onto a slide and a drop of vectashield (Vecatshield mounting medium with DAPI, Vector Laboratoreis Inc.) containing DAPI added.

To visualise DNA cells were briefly fixed in methanol, 3 μl pipetted onto a slide and a drop of vectashield containing DAPI added.

To visualise septa cells were briefly fixed in methanol, 3 μl pipetted onto a slide, 1.5 μl of calcufluor (fluorescent brightener, Sigma) and a drop of vectashield containing DAPI added.

2.4.3 Cell cycle arrests

2.4.3.1 Mitotic arrest using *nda3-KM311* strains

For *nda3-KM311* arrests cultures were grown at the permissive temperature of 30 °C overnight to mid-log phase and then shifted to the restrictive temperature of 18 °C for 6 hrs. Cells were fixed in methanol prior to microscopical analysis.

2.4.3.2 Mitotic arrest using pREP overexpression plasmids

To arrest cells in mitosis using pREP3x or pREP41x plasmids containing the gene of interest under an nmt (no message in thiamine) promoter were used. The gene of interest is only expressed in medium lacking thiamine. The pREP plasmids used in this study contain a LEU2 marker that enables for growth in medium lacking leucine. Plasmids were transformed into yeast using electroporation. Precultures were grown at 25 °C overnight in PMG-Leu with the addition of thiamine (40 μg/ml). The next day another set of overnight cultures was set up from the precultures in PMG-Leu. Prior to adding the cells from the preculture to the overnight culture the cells were washed three times with 1 ml PMG-Leu in order to remove all thiamine. The overnight cultures were grown for 18 hrs at 30 °C.

2.4.3.3 G2-phase arrest/release using *cdc25-22* strains

To arrest cells in G2-phase the *cdc25-22* mutant was used. Precultures were grown overnight at the permissive temperature of 25 °C. Overnight cultures were prepared from the precultures the following day and grown overnight at 25 °C to mid-log phase. Cultures were then arrested in G2-phase for 3.5 hrs at the restrictive temperature of 36 °C. Prior to release of the cells from the G2-phase arrest at 25 °C, cultures were quickly cooled down to 25 °C in ice water and then incubated at 25 °C.

2.4.4 TAT1 Spindle staining

Buffers used for TAT1 spindle staining

PEM	100 mM PIPES, pH7.6; 1 mM $MgSO_4$; 1 mM EGTA
PEMS	100 mM PIPES, pH7.6; 1 mM $MgSO_4$; 1 M sorbitol

Blocking solution/milk	5 % marvel dried milk; 1 x PBS; 0.02 % Tween-20; solution was centrifuged for 20 min, 4 °C, filter sterilised and stored at 4 °C.

Cells arrested in mitosis using pREP overexpression plamids were scored using methanol fixation followed by tubulin staining. Cells were methanol fixed by filtering 10 ml of yeast culture through a 0.22 µM filter membrane (Milipore) and immersion of the filter in 25 ml of methanol pre-chilled to -80 °C. Filters were removed prior to further processing of the sample. Alternatively 5 ml of yeast culture were directly added to 45 ml of methanol pre-chilled to -80 °C. Cells were spun down briefly for 1 min, 3000 rpm at 4 °C. The methanol was removed and the cell pellet resuspended in 1 ml PEM. (All future centrifuge steps were carried out for 15 s at 9000 rpm, the tubes turned 180 °C and centrifuged again for 15 s at 9000 rpm.) Cells were washed twice with PEM and then incubated in 1 ml PEMS with the addition of 0.4 mg/ml zymolyase (MP Biomedicals) for 30 min at 37 °C to digest the cells. The digested cells were washed once with 1 ml PEMS and then resuspended in 1 ml PEMS-TritonX-100 (1 %) for 30 seconds. Cells were once more washed with 1 ml PEM and then incubated in 200 µl blocking solution/milk rotating for at least 1 hr at room temperature or overnight at 4 °C. Following the blocking of unspecific antibody binding cells were incubated in 50 µl of milk with the addition of 1 µl TAT1 (anti-tubulin) antibody rotating overnight at 4 °C or for 5-6 hrs at room temperature. Following incubation with the primary antibody cells were washed once with 1 ml PEM and then incubated in 100 µl milk with the addition of 1 µl of anti-mouse (Alexa Fluor 594, Invitrogen) secondary antibody for 1 hr rotating at room temperature. The supernatant was then removed and the cells resuspended in 20-50 µl PEM prior to microscopic analysis.

2.4.5 Chromosome mis-segregation: chromosome 2 loss

To determine chromosome 2 (cen2-GFP) mis-segregation strains containing a GFP marked centromere on chromosome 2 were used. In this strain a tandem repeat of lac O is inserted close to the centromere of chromosome 2. GFP-tagged lacI is expressed and binds to lacO and thus enables visualising chromosome 2 as a green dot under the fluorescence microscope. If chromosomes are separated correctly one green spot at each spindle pole should be visible. If chromosome 2 is mis-segregated 2 spots are seen at one pole. Overnight cultures were grown to mid-log phase in YES and the rate of cen2-GFP mis-segregation scored in binucleate live cells.

2.4.6 Half sectoring assay: CH16 mini-chromosome loss

The half sectoring assay (Allshire et al., 1995) is based on the short linear mini-chromosome CH16 (530 bp) (Niwa et al., 1989). Ch16 contains an *ade6-216* mutant allele, which is complemented by the *ade6-210* allele in the parental strain. If CH16 is lost due to chromosome mis-segregation the adenine mutation *ade6-216* is not complemented anymore and cells will appear red due to a lack of adenine.
To score the loss of CH16 mini-chromosome loss overnight cultures were grown to mid-log phase in PMG-Ura,-Ade. The cell concentration was determined using a heamocytometer. 250 cells were plated on YES plates containing low adenine (1/15 of the usual adenine concentration). Plates were incubated for 4 days at 30 °C and the percentage of CH16 mini-chromosome loss scored as a percentage of half-sectored colonies to white colonies by eye or using a dissecting microscope (Olympus).

2.4.7 Colony forming assay

For colony forming assays cultures of *nda3-KM311* strains were grown overnight at 30 °C to an OD_{595} below 0.4. Cultures were then grown at the restrictive

temperature of 18 °C for 6 hrs. Samples were taken at 0 hrs (before shift to 18 °C) and after 6 hrs at 18 °C. A minimum of 81 single cells were plated on YES plates using a dissecting microscope and incubated at 32 °C for 3 or 6 days.

2.5 DNA methods

Table 2.3: Plasmids used in this study

Name	Description	Origin/Reference
pREP3x-*mad2*$^+$	Backbone: pUC; marker: LEU2; contains full-length *mad2*	Shelly Sazer (He et al., 1997)
pREP41x-*mph1kd*	Based on pREP41x-*mph1*$^+$, marker: LEU2, mutation: A1376C	This study
pREP41x-*mph1*$^+$	Backbone: pUC; marker: LEU2; contains full-length *mph1*	Shelly Sazer
pKW408	contains SZZ TAP tag	Karsten Weis
pJK *mph1-kd*	Backbone: shuttle vector pJK148; marker *leu1*; contains 408 to end of *mph1*, mutation: A1376C	Hardwick lab
pJK *mph1-kdSC*	Backbone: pJK *mph1-kd*; marker *leu1*; contains full-length *mph1*; 5'UTR: 338 bp, 3'UTR: 212 bp mutation: A1376C	This study
pJK Mph1KD-Stag	Backbone: shuttle vector pJK148; marker *leu1*; contains 408 to end of	This study

	mph1 and C-terminal SZZtag*;* mutation: A1376C	
pJK*mad2*	Backbone: shuttle vector pJK148; marker *leu1*; contains full-length *mad2,* 5'UTR: 259 bp; 3'UTR: 203 bp	This study
pJK *mad2-dimer*	Based on pJK*mad2*; marker *leu1*; mutations: R132E, Q133A	This study
pJK *mad2-5A*	Based on pJK*mad2*, marker *leu1*; mutations: S69A, S92A, S187A, T188A, S189A	This study
pJK *mad3-18A-GFP*	contains full-length *mad3*; 5'UTR: 500 bp, C-terminal GFP tag; marker *leu1*; mutations: (see Table 5.1)	Hardwick lab
pJK *mad2-3D*	Based on pJK*mad2*; marker *leu1*; mutations: S187D, T188D, S189D	This study
pQEMad2-His	Based on pQE30 (Qiagen); contains fission yeast Mad2 with a his tag	Hardwick lab

Table 2.4: Primers used in this study

Name	Sequence	Purpose
MPH BAHR	CTTTTAAAAGAGCGAAAAATT AAAGATTAAAAAAAAAAAGC CTTTAAGCTCCCTAAAAAAGT CGTTAACATTAAGAAACTCGT ATGATGTTTGGGAAACTGAAT T CGAGCTCGTATTAAAC	C-terminal SZZ-TAP tagging of Mph1 with pKW408
MPH1BAHF	GAAAGGTCGGGTGAGTTGAG TAAGCACAAGCGATTAAATA AGGAACTTATTGATAGCATGG CTTATGATTGCGTTAGCAATT TACGAAAAATGCCAGAACGG A TCCCCGGGTTAATTAA	C-terminal SZZ – TAP tagging of Mph1 with pKW408
mphkdf	GGAATTTGAAGCTGATTGCTT TTGGCATTGCCAAAGC	pREP41x-*mph1kd*
mphkdr	GCTTTGGCAATGCCAAAAGCA ATCAGCTTCAAATTC	pREP41x-*mph1kd*
Mph1_SacII_FWD	ATTATTCCGCGGTAAGGGCAA GGCGACCTAGGTG	pJK *mph1-kdSC*
Mph1_Pst1_REV	GGAGTGTTATGCAGCTGCAGA GG	pJK *mph1-kdSC*
M2_S189_FWD	CGAAGTTTTAGTACGGCAATG CACAAAATTGAT	pJK *mad2-5A*
M2_S189_REV	ATCAATTTTGTGCATTGCCGT ACTAAAACTTCGAAT	pJK *mad2-5A*
M2_S92AFWD	GTAATTACTAGTAAATGTGCG GGTGAAGATTTAGAGCGG	pJK *mad2-5A*
M2_S92AREV	CCGCTCTAAATCTTCACCCGC ACATTTACTAGTAATTAC	pJK *mad2-5A*
M2_BamH1_FWD	AATGGATCCCAAACTGACATT ATTACGATC	pJK*mad2*
M2_Pst1_REV	AATCTGCAGTAAAGAATGCA AAAGTATTAT	pJK*mad2*
S69FWD	CATTCGAAAAATTGTAGCGCA GTTACACAGTGAG	pJK *mad2-5A*
S69AREV	CTCACTGTGTAACTGCGCTAC AATTTTTCGAATG	pJK *mad2-5A*
M2_S187AT188A_FWD	GTTCAATTACGAAGTTTTGCA GCTGCAATGCACAAAATTGAT	pJK *mad2-5A*
M2_S187AT188A_REV	ATCAATTTTGTGCATTGCAGC TGCAAAACTTCGTAATTGAAC	pJK *mad2-5A*
M2RQFOR	GAAATTCAAGCTCTAATTGAA GCAATCACTGCTACAGTG	pJK *mad2-dimer*
M2RQREV	CACTGTAGCAGTGATTGCTTC AATTAGAGCTTGAATTTC	pJK *mad2-dimer*
M2_S187DT188DS289D_FWD	TGAACAAGTTCAATTACGAAG TTTTGATGACGATATGCACAA AATTGATTGTCAAGTTG	pJK *mad2-3D*
M2_S187DT188DS289D_REV	CAACTTGACAATCAATTTTGT GCATATCGTCATCAAAACTTC GTAATTGAACTTGTTCA	pJK *mad2-3D*

2.5.1 Polymerase Chain Reaction

For amplification of DNA polymerase chain reactions (PCR) were carried out. PCR reactions were carried out using a peltier thermal cycler from MJReasearch (PTC-200) or BioRad (DNA Engine, BS038051). The Expand High Fidelity PCR system (Roche) was used for cloning purposes. For diagnostic PCR reactions Taq polymerase was used.

Component	**Final concentration**
10 x PCR buffer	1 x PCR buffer
10 x dNTPs: 2.5 mM dATP, dCTP, dGTP, dTTP	1 x dNTPs
Sense primer	0.5 μM
Antisense primer	0.5 μM
template	yeast colonies, genomic DNA, plasmid DNA, *E.coli* colonies
Polymerase	1.75 U/25 μl reaction

Template DNA:

Yeast colony: For PCR from yeast a small amount of yeast was added to the PCR tube. The yeast was first heated for 2 min, 94 °C (PCR programme: step1) and then the reaction mix added to the tube.

E.coli colony: One conlony was dissolved in the PCR reaction mix prior to the PCR reaction.

Programme
Step

1		94 ºC	2 min
2	denaturing	94 ºC,	30 sec
3	annealing	50 – 57 ºC	30 sec
4	extension	72 ºC	1 min/kb

5 go to step 2, 29 x

6		72 ºC	7 min

2.5.2 Site directed mutagenesis

To perform site directed mutagenesis the QuikChange II Site-Directed Mutagenesis Kit from Stratagene was used.

PCR reaction mix: site directed mutagenesis

Component	Final concentration
10 x reaction buffer	1 μl
dNTP	0.2 μl
Sense primer	25 ng
Antisense primer	25 ng
ddH_2O	bring the final reaction volume to 10 μl
then add *PfuUltra* HF DNA polymerase	0.5 U

Programme
Step

1 95 ºC 1 min
2 95 ºC 30 sec
3 55 ºC 1 min
4 68 ºC, 1 min/kb of plasmid length
5 go to step 2 12 - 18 x

2.5.3 PCR purification

Purification of PCR reactions was carried out using the PCR purification kit from Qiagen or ethanol precipitation.

2.5.4 DNA Agarose Gel Electrophoresis

For separation of DNA fragments DNA agarose gel electrophoresis was carried out. Typically 1 % agarose gels (1 % agarose in TBE) containing 0.3 mg/ml ethidiumbromide (Sigma) were prepared. Samples were loaded with 4 x loading buffer and run in TBE electrophoresis buffer.

2.5.5 Gel purification

Purification of agarose gel was carried out using the gel extraction kit from Qiagen.

2.5.6 DNA Digestion with Restriction Endonucleases

To cut DNA at specific sites DNA digests were carried out. The DNA was mixed with the appropriate restriction enzyme(s) (NEB) and restriction buffer (NEB). The length of the digest and the temperature at which the digest was carried out were determined by the restriction enzyme and the purpose of the digests.

2.5.7 Ligation of DNA Fragments

To ligate specific DNA fragments DNA ligations were carried out. The fragments were mixed with the appropriate amount of T4 DNA ligase (NEB) and T4 DNA ligase buffer and incubated for at least 30 min at room temperature. The reaction mix was then used to transform *E.coli*.

2.5.8 Minipreps

3 ml *E.coli* overnight cultures were grown in selective LB medium containing antibiotics. Cells were harvested and plasmids isolated using the QIAGEN Miniprep Kit according to the provided protocol (QIAprep Spin Miniprep Kit Using a Microcentrifuge).

2.5.9 Sequencing

Sanger sequencing was carried out by "The GenePool", Ashworth Laboratories, University of Edinburgh with ABI 3730 capillary sequencer instruments. Sequencing reactions were set up using BigDye Terminator kit v3.1.

Sequencing reaction

2 µl BigDye Terminator kit v3.1
1.6 pmol primer
template DNA

add ddH_2O to 10 µl

Programme

Step

1	95 ºC	2 min
2	95 ºC	15 sec
3	58 ºC	1.5 min
4	go to step 2	30 x

2.5.10 Ethanol precipitation

The DNA sample was mixed with 2.5 times the volume of ethanol and 10 % of the DNA volume of NaOAc (pH 5.2) and incubated for a minimum of 1 hr at -20 °C. The DNA was pelleted (15 min, 4 °C, 14000 rpm), air dried, washed once with 70 % ethanol, pelleted again (15 min, 4 °C, 14000 rpm) air dried and resuspended in TE.

2.6 Protein Biochemistry

2.6.1 Protein extraction

2.6.1.1 Protein extracts

Protein extracts of proteins were prepared by growing 5 ml overnight cultures at 30 °C/25 °C (temperature sensitive strains). Cells were harvested for 3 min, 2500 rpm resuspended in 300 µl SDS Gel-loading buffer with the addition of 100 mM DTT and protease inhibitors (1 mM pefablock (Roche), 10 µg/ml leupeptin/pepstatin/chymostatin (Roche)). The cell suspension was added to a 1.5 ml tube containing 200 µl Zirconia/Silica beads (BioSpec Products) and bead beat twice for 30 seconds and immediately incubated at 95 °C for 5 min.

2.6.1.2 Denatured protein extracts

Denatured extracts of proteins were prepared by growing 5 ml overnight culture at 30 °C/25 °C (temperature sensitive strains). Cells were harvested for 3 min, 2500 rpm and immediately resuspendend in 5 % TCA (trichloroacetic acid) solution. The cell suspension was incubated for 10 min on ice, centrifuged for 1 min, 3000 rpm, 4 °C. The pellet was then washed once with 1 ml acetone and then centrifuged for 5 min, 14000 rpm. The pellet was air-dried for a minimum of 3 hrs prior to protein extract preparation. For the protein extract preparation 300 µl SDS Gel-loading buffer with the addition of 100 mM DTT and protease inhibitors (1 mM pefablock (Roche), 10 µg/ml leupeptin/pepstatin/chymostatin (Roche)) and 200 µl Zirconia/Silica beads (BioSpec Products) were added to the pellet and bead beat using a ribolyser twice for 30 seconds and immediately incubated at 95 °C for 5 min.

2.6.2 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Resolving Gel

Gel percentage	10 %	15 %
Resolving gel buffer (1.5 M Tris-HCl, pH 8.8)	3 ml	3 ml
Acrylamide (40 %) 29:1	3 ml	4.5 ml
10 % w/v SDS	120 µl	120 µl
2 % bisacrylamide	780 µl	516 µl
dd H_2O	5.1 ml	3.86 ml
add		
APS	120 µl	120 µl
TEMED	12 µl	12 µl

Stacking Gel

Stacking gel buffer (0.5 M Tris-HCl, pH 6.8)	1 ml
Acrylamide (40 %) 29:1	1 ml
2 % bisacrylamide	520 µl
dd H_2O	5.48 ml
add	
APS	80 µl
TEMED	8 µl

2.6.3 Western Blot Analysis

2.6.3.1 Protein Transfer

Proteins were transferred from SDS polyacrylamide gels to PVDF (Immobilon-P, Millipore) or nitrocellulose (GE Water & Process Technologies) membranes. Transfers were carried out according to the manufactureres protocol using a wet transfer tank (Hoefer, TE series)(65 V, 1.5 hrs) or a semi-dry transfer blotter (Hoefer, TE77) (180 mA, 1.5 hrs).

Following the transfer, membranes were stained with PonceauS. PonceauS stain was washed off the membrane with PBS-Tween-20 and the membranes then incubated in blocking solution (PBS-Tween-20, 5 % Marvel dry milk) for 30 min at room temperature. The membrane was then incubated in the appropriate primary antibody in PBS-Tween-20, 5 % marvel dry milk overnight at 4 °C or for 5 hrs at room temperature. The unbound primary antibody was washed off the membrane 3 times with PBS-Tween-20 (5 min, 5 min, 15 min). The membrane was then incubated with the appropriate secondary antibody (PBS-Tween-20, 5 % marvel dry milk) for 1 hr at room temperature. Unbound secondary antibody was washed off the membrane three times with PBS-Tween-20 (5 min, 5 min, 15 min).

2.6.3.2 Protein Detection

Proteins were detected using an ECL detection kit (Super Signal West Femto, Super Signal West Pico (Pierce), GE Healthcare) for western blotting. Membranes were incubated in a 1:1 mixture of detection reagent 1 and detection reagent 2 for 2-5 min. Detection reagents were removed and the membrane exposed to film.

Table 2.5: Antibodies used in this study

Antibody	Animal	Working concentration	Origin
anti-GFP	sheep	1:1000	Hardwick lab
anti-Cdc13	mouse	1:50	Ken Sawin
anti-Mad2	sheep	1:1000	Hardwick lab
anti-Mad1	rabbit	1:1000	Hardwick lab
anti-Mph1	rabbit	1:1000	Hardwick lab
Peroxidase-Anti peroxidase (PAP); recognises TAP tag		1:3000	SIGMA
Secondary α-sheep	F(ab) donkey anti-sheep HRP conjugate	1:20000	Jackson Immuno research
Secondary α-rabbit	ECL™ Anti-mouse IgG, Horseradish Peroxidase linked whole antibody (from sheep)	1:3000	GE Healthcare
Secondary α-mouse	ECL™ Anti-rabbit IgG, Horseradish Peroxidase linked F(ab')$_2$ fragment (from donkey)	1:2000	GE Healthcare
Alexa α-mouse secondary for immunofluorsecence	Alexa Fluor 594 goat anti-mouse IgG	1:100	Invitrogen
TAT1 (anti-tubulin)	mouse	1:50 for immunofluorescense 1:500 for western blot analysis	(Woods et al., 1989) Keith Gull

2.6.4 Immunoprecipitations

Lysis-buffer for immunoprecipitations

50 mM Hepes, pH7.5
75 mM KCl
1 mM $MgCl_2$
1 mM EGTA
0.1 % Triton-X100
1 mM $NaVO_4$
0.1 μM microcystin
1 mM pefablock (Roche)
10 μg/ml leupeptin/pepstatin/chymostatin (Roche)

Preparation of IgG Dynabeads for immunoprecipitations

0.2 mg IgG dynabeads were used per sample. Beads were washed twice with PBS-Triton-X (PBS, 0.1 % Triton-X100) and once with lysis buffer before being added to the cell lysate.

Immunoprecipitation

For all immunoprecipitations 300 μl lysis buffer and 200 μl Zirconia/Silica beads (BioSpec Products) were added to frozen pellets. The pellets were immediately bead beat twice for 20 seconds with a 30 second incubation on ice between the pulses. The lysate was centrifuged for 2 min, 14000 rpm at 4 °C, the supernatant moved to a new tube and centrifuged again using the same spinning parameters. 25 μl of the lysate was removed from the tube and mixed with SDS sample buffer for further analysis. Equal amounts of the remaining lysate were added to a tube containing 0.2 mg of IgG Dynabeads and then incubated for 30 min at 4 °C rotating. The beads were then washed 3 times with ice-cold lysis buffer (w/o phosphatase and protease inhibitors). To remove the immunoprecipitated protein of the beads, the beads were mixed with 20 μl SDS sample buffer (+ 100 mM DTT) and incubated for 15 min at room temperature.

2.6.4.1 Lambda phosphatase treatment

For lambda phosphatase treatment of samples IgG dynabeads coupled to the protein of interest were washed once with lambda phosphatase buffer. IgG dynabeads were then resuspended in 30 µl lambda phosphatase buffer, 400 units of lambda phosphatase (NEB) added and incubated for 30 min at 30 °C. To remove the immunoprecipitated protein of the beads, the beads were mixed with 20 µl SDS sample buffer (+ 100 mM DTT) and incubated for 15 min at room temperature.

2.6.5 Tandem affinity purifications (TAP-S-ZZ) Purifications

This protocol is based on (Cheeseman et al., 2001).

1 x Hyman Buffer	50 mM bis-Tris propane (pH 7), 100 mM KCl, 5 mM EGTA, 5 mM EDTA, 10 % glycerol
2 x Hyman Buffer	100 mM bis-Tris propane, 200 mM KCl, 10 mM EGTA, 10 mM EDTA, 20 % glycerol, protease inhibitors (1 mM pefablock, 10 µg/ml leupeptin/pepstatin/chymostatin (Roche))
TST	50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1 % Tween-20

Dynabead peparation

10 mg IgG Dynabeads/20 g cells were washed in batch twice with 15 ml TST buffer.

Lysate preparation

Cells were grown in 4 x YES to an OD_{595} below 10. Cells were pelleted at 4000 rpm for 10 min, washed once with ice-cold water, resuspendend in 1/5 pellet volume water and then drop frozen in liquid nitrogen and stored at -80 C. Prior to protein

purification cells were ground in liquid nitrogen using an automatic grinder or using a mortar and pestle. Ground cells can be stored at -80 C. For protein extraction the cell powder was resuspended in 2 x Hyman lysis buffer and then sonicated for 30 sec. Lysed cells were centrifuged 10 min, 4000 rpm to remove cell debris. The remaining supernatant was then first filtered through a 25 mm GD/X syringe filters (Whatman), pore size 2.6 μM and then a 25 mm GD/X syringe filters (Whatman), pore size 1.6 μM.

Bead binding and TEV cleavage

The clarified lysate was then incubated with IgG dynabeads (Invitrogen) for 30 min rolling at 4 °C to allow binding of the TAP tagged protein to the beads. IgG beads were washed 3 times with 15 ml 1 x Hyman buffer and then 3 times with 15 ml 1 x Hyman buffer with the addition of 1 mM DTT and 0.1 % Tween-20 in batch using a magnet (MagnaBot, Promega). The purified protein was cleaved off the beads in 1 ml 1 x Hyman buffer with the addition of 1 mM DTT and 0.1 % Tween-20 using 100 units AcTEV protease (Invitrogen) overnight rotating at 4 °C. The next day the supernatant was transferred to a new tube and the IgG Dynabeads washed once with 1 ml 1 x Hyman buffer (+ 1mM DTT, 0.1 % Tween-20).

S-protein agarose binding

The supernatant containing the cleaved protein was then incubated with S-protein agarose (Novagen) slurry (washed 3 times with 1 x Hyman buffer) for 3 hrs at 4 °C. S-protein agarose was then washed five times with 5 ml 1 x Hyman buffer, resuspended in 1 x Hyman buffer, aliquoted and stored at -80 °C.

2.6.6 2D-gel analysis

For instructions for the isoelectric focusing unit MulitphorII form Pharmacia Biotech see "Immobiline DryStripKit for 2-D Electrophoresis with Immobiline Dry Strip and ExcelGel SDS from Pharmacia Biotech".
For all buffers urea for electrophoresis from Sigma was used.

2.6.6.1 Preparing protein extracts

Approximately 300 mg of cell pellet were ground using a mortar and pestle for 15 min in liquid nitrogen. The cell powder was then added to 1.2 ml extraction buffer (7M urea, 2 M thioruea, 4 % CHAPS (USB Corporation), 10 mM DTT, 10 mM Hepes (pH 7.5)) and incubated at room temperature for 1 hr, centrifuged for 5 min at 20 °C and the supernatant then transferred to a fresh 1.5 ml tube. The extract was stored in aliquots at -80 prior to usage. The protein concentration was determined using the BioRad Protein Assay.

2.6.6.2 Determination of Protein Concentrations

Protein concentrations were determined using the BioRad Protein Assay using a standard curve created with the protein BSA.

2.6.6.3 TCA precipitation

For TCA precipitations the appropriate amount of extract was mixed with TCA and ddH_2O so that the final concentration of TCA was 10 % and the final volume was 5 times the volume of the extraction buffer. The mix was incubated for 15 min on ice and then centrifuged for 15 min (14000 rpm, 4 C). The remaining pellet was washed once with 90 % acetone: 10 % 0.1N HCl, followed by one wash with acetone and a final wash with 90 % ethanol: 10 % 0.1 M Hepes (pH 7.5). The pellet was then air-dried for a maximum of 5 min and then resuspended in 100 µl rehydration solution

(8 M urea, 2 M thiourea, 4 % CHAPS, 2 % IPG buffer, 10 mM DTT, bromophenol blue).

2.6.6.4 Rehydration of Strips

18 cm Immobiline TM DryStrips pH 3-10 NL were rehydrated in 350 µl rehydration solution (8 M urea, 2 M thiourea, 4 % CHAPS, 2 % IPG buffer, 10 mM DTT, bromophenol blue) overnight at room temperature according to the manufacturers protocol.

2.6.6.5 Isoelectric Focusing

The appropriate amount of protein was loaded onto the strip using cups (see instruction protocol) and focused using the isolectric focusing unit MultiphorII (Pharmacia Biotech) with the following running protocol as recommended by the manufacturer for 18 cm strips pH 3 10 NL.

Step	**Voltage (V)**	**Time (h)**	**kVh**
1	500	0:01	
2*	500	6:00	3.0
3	3500	1:30	3.0
4	3500	10:10 - 13:00	35.5 - 45.5

* Step 2 can be omitted but step 4 should then be 2.4 kVh longer.

2.6.6.6 Equilibration of IPG strips

IPG strips were equilibrated prior to the SDS PAGE second dimension run. IPG strips were incubated in 10 ml equilibration buffer (50 mM Tris-Cl (pH 8.8), 6 M urea, 30 % glycerol, 4 % SDS, bromophenol blue) first with the addition of 100 mg DTT and then with the addition of 250 mg Iodoacetamide (Acros Organics) for 10 min at room temperature. The strip was then placed on top of a SDS PAGE, covered with overlay buffer (50 mM Tris-Cl (pH 8.8), 2 % SDS, 20 % glycerol) and the SDS PAGE running buffer carefully added to the gel tank.

2.6.6.7 Transfer

Transfers were carried out using PVDF membranes according to the manufacturers protocol using a wet transfer tank (Hoefer, TE Series). Gels were incubated in PVDF tansfer buffer for 30 min, with a buffer change after 15 min, prior to the transfer. Western blot analysis was carried out as described in section 2.6.3.

2.6.7 Purification of recombinant protein: Mad2-His

Mad2-His was expressed from the plasmid pQEMad2-His. Protein expression was induced overnight at 18 °C with 0.1 mM IPTG. Cells were harvested the following morning and ground in liquid nitrogen using a mortar and pestle. Mad2-His was purified following the "TALON Metal Affinity Resins User Manual" from Clontech Laboratories, Inc. Following the purification Mad2 was dialysed into the following buffer: 20 % glycerol, 50 mM Hepes, 200 mM KCl. Recombinant Mad2-His was stored at -80 C.

2.6.8 *In vitro* kinase assay

For kinase assays purified Mph1 kinase coupled to S-protein agarose beads was washed twice with 1 x kinase buffer (50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 0.5 mM DTT). 25 µl of kinase reaction buffer was then added to the beads (12.5 µl 2 x kinase buffer (100 mM Hepes, pH7.5, 20 mM $MgCl_2$, 1 mM DTT), 0.5 µl P^{32} gamma-ATP, 0.5 µl 1 mM ATP, x µl substrate, add ddH_2O to 25 µl). Reactions were typically carried out with 1.5 µg recombinant protein (substrate). The reaction was incubated at 30 °C for 30 min and the kinase reaction stopped by adding gel loading buffer to the reaction and incubation at room temperature for 5 min. Samples were run on 4 - 12 % precast NuPAGE gradient gels (Invitrogen) and then dried onto whatman paper and exposed to film.

2.6.9 Mass spectrometry

Phosphopeptide enrichment and mass spectrometry were carried out by H. Syred, (Okhura/Rappsilber lab, University of Edinburgh) unless stated otherwise. The following protocol was written by H.Syred.

2.6.9.1 Protein digestion and Phosphopeptide enrichment:

Proteins were electrophoresed into NovexNuPAGE 4-12% Bis-Tris gels (Invitrogen). Proteins were stained with a colloidal blue kit (Invitrogen). Proteins were excised and standard trypsin digestion procedure carried out as described previously (Maiolica et al., 2007). The supernatant of samples for MS analysis without phosphopeptide enrichment were loaded onto StageTips (Rappsilber et al., 2003). Supernatants of samples for phosphopeptide enrichment were removed and peptides further extracted from the gel using 3 % TFA/30% ACN solution followed by 100% ACN. Before enrichment approximately 3 mg TiO_2 beads were pre-incubated in 20µl of 85 mg/ml Lactic acid in 80 % ACN/0.1 %TFA. Pre-incubated bead mixture was added to the peptide mixture and incubated for 1 hour at room temperature. After washing the beads once with 10 % ACN/0.1 % TFA, and twice with 80 % ACN/0.1 % TFA, peptides were eluted with 2 % ammonium hydroxide in 40 % ACN (pH 10.5). Elute was concentrated to 100 µl and 100 µl of 2 % TFA added. All samples were loaded onto StageTips and stored at -20 ºC for MS analysis.

2.6.9.2 Nano-LC-MS/MS and Data analysis

An Orbitrap system (Finnigan LTQ-Orbitrap, Thermo electron), 1200 series nanoflow LC system for MS (Agilent technologies) and HTC PAL auto sampler (CTC analytics) were used for LC-MS/MS analysis. Mobile phases were (A) 5 % acetonitrile, 0.5 % acetic acid and (B) 99.5 % acetonitrile, 0.5 % acetic acid. A flow

rate of 300 nl/min was used with a spray voltage of 1.8 kV. Analysis was done using either a two-step linear gradient of 0 %-20 % B in 35 min, 20 %-80 % B in 4 min and 80 % B for 2 min or a two-step linear gradient of 0 %-20 % B in 75 min, 20 %-80 % B in 13 min and 80 % for 10 min. A column needle was prepared by packing 3µm Reprosil C18 materials (Germany) into Pico Tip Emitter silica tips (8±1µm) under the pressure of nitrogen. Each cycle consisted of one full MS scan acquired on the Orbitrap, followed by MS/MS of the six most abundant peptides on the ion trap.

2.6.9.3 Database searching

DTAsupercharge (V1.18) was used to create peak lists from raw data. Peak list were then used within Mascot daemon (V2.2.0) to conduct an automated database search against UniProt/SwissProt *Schizosaccharomyces pombe* database. Search parameters were set to: precursor mass tolerance of 10 ppm, fragment ion mass tolerance to 0.8 Da, enzyme as trypsin, allowing 3 missed cleavages. Peptide charge was set to 1+, 2+ and 3+. Carboamidomethylation of cysteine was set as a fixed modification, with oxidation of methionines, phosphorylation of serine, threonine, and tyrosine as variable modifications.

Chapter 3

The roles of Mph1 kinase activity in mitosis

Chapter 3: The roles of Mph1 kinase activity in mitosis

3.1 Introduction

Accurate chromosome segregation is crucial as mis-segregation leads to aneuploidy and this can result in severe diseases such as cancer. The spindle checkpoint monitors sister-chromatid attachment to the microtubules and inhibits the onset of anaphase until all chromosomes are correctly bi-oriented on the mitotic spindle. Among the core spindle checkpoint proteins are several kinases, one of them being Mps1. Mps1 (monopolar spindle 1) kinases, called Mph1 (monopolar spindle 1 like pombe homologue) in fission yeast, have been identified from yeast to vertebrates. They are required for a functional spindle checkpoint and accurate chromosome segregation (Abrieu et al., 2001; He et al., 1998; Liu et al., 2003; Stucke et al., 2002; Weiss and Winey, 1996). Furthermore it has been demonstrated that Mps1 kinase activity is essential for the spindle checkpoint (Abrieu et al., 2001; Jones et al., 2005; Tighe et al., 2008).

The first experiments investigating the role of Mps1 phosphorylation and kinase activity were performed in budding yeast. They demonstrated that budding yeast Mps1 is an active kinase *in vitro* and is hyperphosphorylated during a spindle checkpoint arrest (Hardwick et al., 1996; Lauze et al., 1995). Mps1 kinases identified to date all encode dual specificity kinases that phosphorylate serines, threonines and tyrosines. They are defined by a conserved C-terminal kinase domain, which can be structured into 11 subdomains as described by Hanks et al. (Fisk and Winey, 2001; Hanks et al., 1988). Subdomain VII of the kinase domain is highly conserved and contains the consensus sequence DFG (Figure 3.1, 3.2). A kinase-dead version of budding yeast Mps1 was cloned by substituting aspartic acid 580 in the consensus sequence with alanine. This amino acid substitution abolished Mps1 kinase activity *in vitro* and changed its gel mobility *in vivo* indicating *in vivo* autophosphorylation is probably lost in the mutant (Lauze et al., 1995).

Fission yeast Mph1 is a 678 amino acid dual-specificity kinase. He et al identified the *mph1* gene in fission yeast and studied *mph1* null mutants. They demonstrated that Mph1 is a structural and functional homologue of budding yeast Mps1. It has a C-terminal kinase domain that shares 52 % sequence homology to budding yeast Mps1 (Fisk and Winey, 2001; He et al., 1998). However, unlike *mps1* kinases in vertebrates and budding yeast, fission yeast *mph1* is not an essential gene and has no role in spindle pole/centrosome duplication. Yet, an *mph1* null mutant abolishes the spindle checkpoint and overexpression of Mph1 induces a metaphase arrest.

The only experiments analysing the role of Mph1 kinase activity in fission yeast were performed by L. Milne (Hardwick lab, unpublished data). She demonstrated that a truncation of the C-terminal kinase domain of *mph1* (*mph1KΔ*) abolishes the spindle checkpoint and leads to chromosome mis-segregation. However, the phenotypes observed could not only be due to the loss of kinase activity but rather to loss of the C-terminal kinase domain. The aim of this study was to analyse specifically the role of Mph1 kinase activity using a kinase-dead mutant of *mph1*.

In the following chapter, four questions were addressed. First, is Mph1 a phosphoprotein *in vivo* as well as *in vitro*? Second, what is the role of Mph1 kinase activity in the spindle checkpoint? Third, what is the role of Mph1 kinase activity in chromosome segregation? Fourth, what consequences does the loss of Mph1 kinase activity have on cell viability? The first question was answered by performing pull-downs of Mph1 in spindle checkpoint activated cells and *in vitro* kinase assays. To answer question two and three a kinase-dead version of Mph1 was cloned by substituting residue 459 aspartic acid with alanine. This mutation is based on the mutation made in budding yeast Mps1 (Lauze et al., 1995), which rendered budding yeast Mps1 kinase-dead. After analysing the *in vitro* kinase activity of this mutant the allele was analysed with regards to the spindle checkpoint and chromosome segregation. To answer this question four colony-forming assays with a kinase-dead *mph1* strain were carried out.

A

	% identity	e value
S.c. Mps1p	-	-
S.p. mph1	52%	$4x10^{-45}$
A.t. PPK1	41%	$9x10^{-39}$
D.m. CG7643	39%	$1x10^{-25}$
X.l. xMps1p	40%	$7x10^{-32}$
M.m. mMps1p	41%	$3x10^{-34}$
H.s. hMps1p	41%	$2x10^{-33}$

B

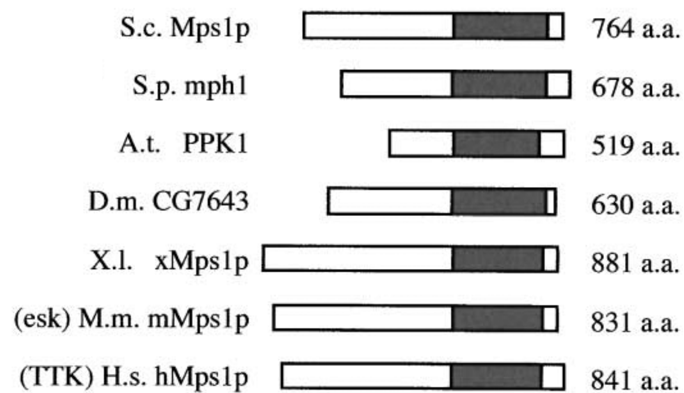


Figure 3.1: A Table of sequence identiy of Mps1 kinase domains of different species compared to budding yeast Mps1. Taken from Fisk et al., 2001. **B** Schematic of Mps1 kinases from different species. The kinase domains are coloured in grey. Taken from Fisk et al., 2001.

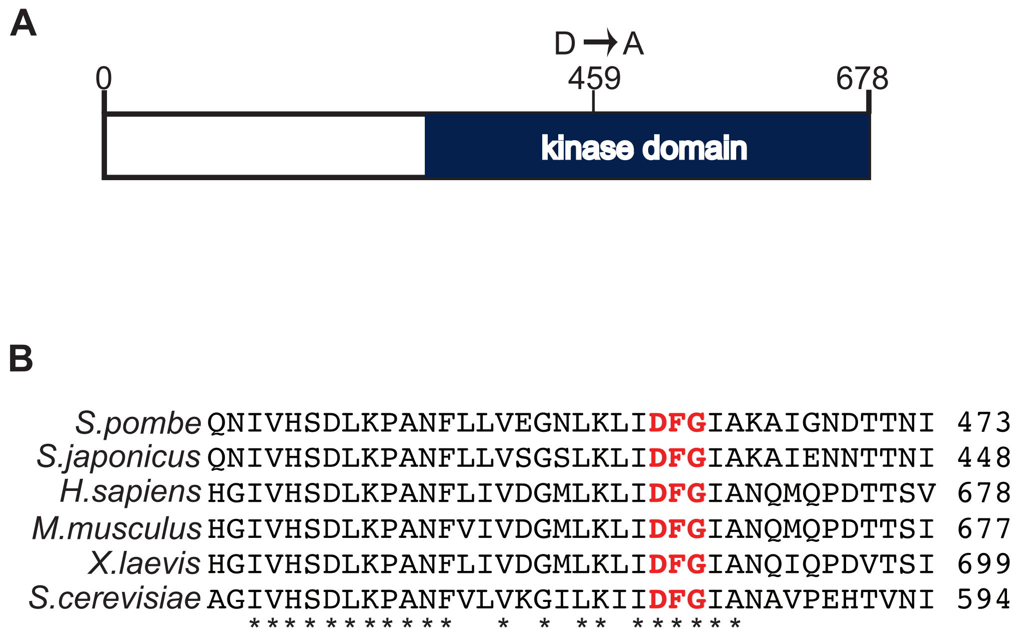


Figure 3.2: A Schematic of Mph1. The kinase-dead mutation is marked. **B** Sequence alignment of part of the kinase domain. The consensus sequence DFG is coloured in red.

3.2 Mph1 kinase is hyperphosphorylated during a spindle checkpoint arrest

In order to investigate, whether Mph1 is a phosphoprotein *in vivo* two approaches were used. The first approach was to carry out spindle checkpoint arrest/release time-courses followed by western blot analysis of extracts. In this way gel mobility shifts due to post-translational modification of Mph1 could be determined. Second, to investigate whether the post-translational modifications observed were due to phosphorylation, Mph1 pull-downs from cycling and mitotically arrested cells followed by lambda protein phosphatase treatment were carried out.

To arrest cells in mitosis *nda3-KM311* strains were used (Hiraoka et al., 1984). *nda3-KM311* encodes a cold- sensitive version of the β-tubulin gene. Cells grown at the restrictive temperature of 18 °C express a mutated form of β-tubulin and are not able to polymerise microtubules. This results in failure to assemble a mitotic spindle and due to the lack of attachment and tension at kinetochores the spindle checkpoint is activated and cells arrest in mitosis. This metaphase arrest is reversible and 6 - 10 min after shifting *nda3-KM311* cells to the permissive temperature they form a mitotic spindle and undergo anaphase (Kanbe et al., 1990).

For arrest/release time-courses *nda3-KM311* cells were arrested at the restrictive temperature of 18 °C for 6 hrs and samples taken every two hours. After six hours the cells were released from the mitotic arrest into anaphase by shifting the culture to the permissive temperature of 32 °C. Samples were taken at 5 min time-points for 20 min and then after 40 and 60 min. Denatured protein extracts of each sample were prepared and western blot analysis using α-Mph1 antibody carried out.

Figure 3.3A shows that during the 6 hrs of the arrest slower migrating forms of Mph1 appeared. After 6 hrs app. 70 % of cells were mitotically arrested and most of the Mph1 was modified and showed slower mobility on the gel. During the 60 min release of the cells from the mitotic arrest slower migrating forms of Mph1 disappeared and Mph1 shifted back down on the gel to its original position. This

gel-shift observed for Mph1 was likely to be due to phosphorylation. Phosphorylation of proteins can often be visualised on protein gels as the phosphorylated version of the protein migrates slower than its unphosphorylated counterpart.

To confirm that the gel shift observed was really a result of phosphorylation pull-downs of Mph1-STAP from cycling and mitotically arrested *nda3-KM311* cell extracts were performed followed by lambda protein phosphatase treatment. Figure 3.3B shows that even in cycling cells some of Mph1 was modified and migrated slower than Mph1. This shift was much more pronounced in mitotically arrested cells. The gel shifts present in cycling and arrested cells were eliminated upon lambda protein phosphatase treatment, which confirmed that they were due to phosphorylation. Having determined that Mph1 indeed is a phosphoprotein *in vivo* the next step was to look at Mph1 kinase activity by carrying out *in vitro* kinase assays.

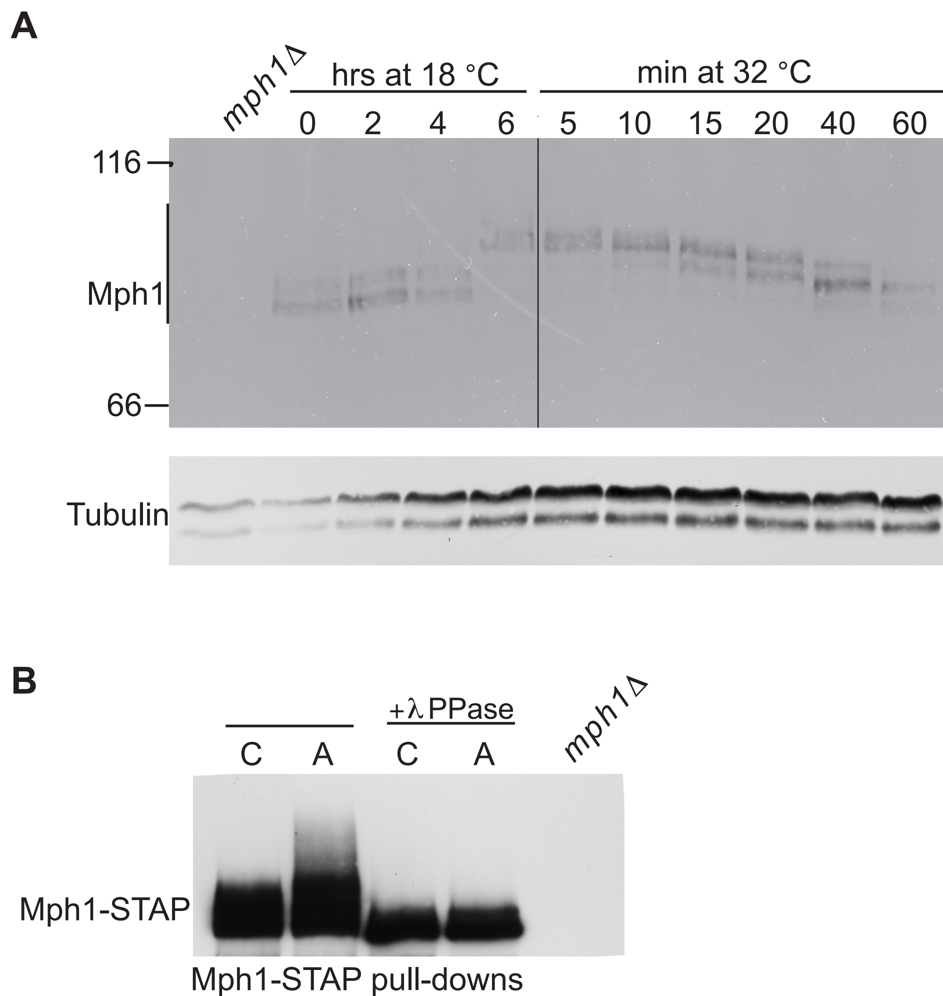


Figure 3.3: Mph1 kinase is hyperphosphorylated during a spindle checkpoint arrest A Western blot analysis of *nda-KM311* cells arrested in mitosis for 6 hrs at 18 °C and released from the arrest for 60 min at 32 °C. Immunoblot was probed with α-Mph1 antibody. **B** Pull-downs of Mph1-STAP from cycling (C) and mitotically arrested (A) *nda3-KM311* cells using IgG-Dynabeads with/without lambda protein phosphatase treatment. Immunoblot was probed with α-Mph1 antibody.

3.3 Tagging of Mph1 with the SZZ tandem affinity purification tag (STAP tag)

To be able to carry out *in vitro* Mph1 kinase assays Mph1 was purified from native yeast extracts. In order to do so efficiently a C-terminal SZZ-TAP (tandem affinity purification) tag was introduced using the plasmid pKW408 (K. Weis, (Cheeseman et al., 2001)) that is based on the Bahler cassette system (Bahler et al., 1998b), which allowed the purification of Mph1 from native fission yeast extracts. This tag is composed of an S tag, a TEV protease cleavage site and a ZZ tag (minimal protein A-binding domain) (Kim and Raines, 1993; Rigaut et al., 1999). Using the Bahler cassette system gene manipulations can be introduced including gene deletions and C- and N-terminal gene tagging. Bahler cassette plasmids all contain a tag and a drug resistance for selection. The tag and drug resistance are amplified with primers that contain gene specific sequences and plasmid specific sequences. The PCR product is transformed into yeast and integrates by homologous recombination with the gene specific sequence. For C-terminal tagging the forward primer contains a 60-80 bp gene specific sequence usually ending immediately before the stop codon followed by 20-25 bp sequence of the tagging plasmid. The reverse primer contains 60-80 bp gene specific sequence of the 3'UTR and 20-25 bp sequence of the tagging plasmid (Figure 3.4). In this study the gene of interest was Mph1, which was tagged at the C-terminus using primers MPH BAHR and MPH1BAHF (Table 2.4, Figure 3.4) and plasmid pKW408 that contains the SZZ-TAP tag sequence and *kanMX6* (G418 resistance) marker. Expression of Mph1-STAP was confirmed by western blot analysis and *in vitro* kinase assays (Figure 3.5).

Plasmid pKW408

Schematic of primer homology to Mph1

PCR

PCR product

transformation and integration by homologues recombination

Fission yeast genome

Figure 3.4: Schematic of introducing a SZZTAP (STAP) tag at the C-terminus of Mph1 using the plasmid pKW408 that is based on the Bahler cassette system.

3.4 Mph1 is an active kinase *in vitro* and autophosphorylates

Mps1 kinases are active *in vitro* and autophosphorylate (Jelluma et al., 2008a; Kang et al., 2007; Mattison et al., 2007; Weiss and Winey, 1996). To investigate whether this is the case in fission yeast *in vitro* kinase assays were carried out. Mph1-STAP was two step tandem affinity purified from native yeast extracts and kinase assays using γ–^{32}P-ATP carried out. Kinase assays were run on SDS protein gels and phosphorylation visualised on autoradiographs. Figure 3.5 shows that Mph1 phosphorylated the artificial substrate myelin basic protein (MBP). Furthermore a strong signal on the autoradiograph at the size of Mph1 could be detected. These results showed that Mph1 not only is an active *in vitro* kinase but also that it autophosphorylates. Knowing that Mph1 is an active kinase *in vitro* and hyperphosphoyrlated *in vivo* during a spindle checkpoint arrest, the next step was to create a mutant of Mph1 that lacks kinase activity to determine the role of kinase activity in the spindle checkpoint and chromosome segregation.

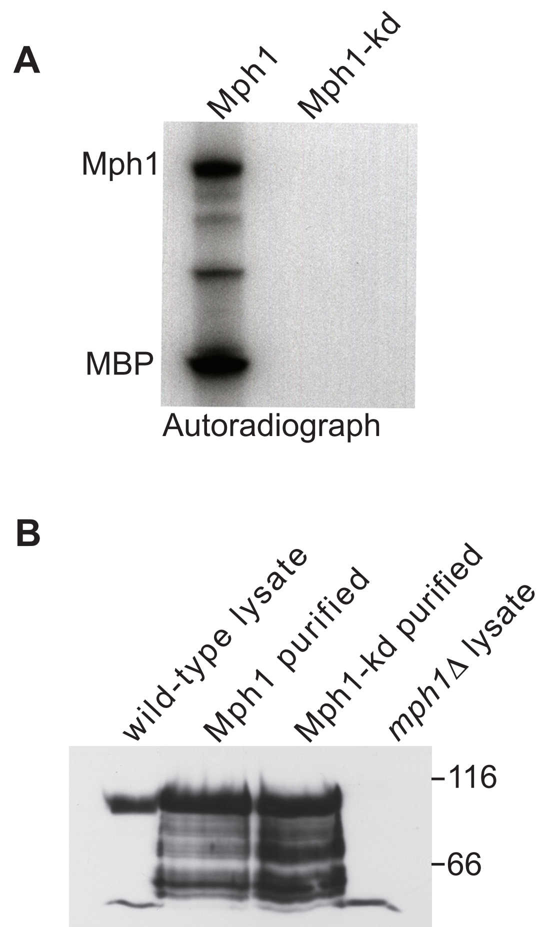


Figure 3.5: (**A**) *In vitro* kinase assays of Mph1-STAP and Mph1-kd-STAP affinity purified from yeast extracts and recombinant MBP (myelin basic protein). (**B**) Western blot analysis of tandem affinity purifications of Mph1-STAP and Mph1-kd-STAP from native yeast extracts probed with α-Mph1 antibody.

3.5 Creating Mph1 kinase-dead fission yeast strains

To create a strain carrying a kinase-dead version of Mph1 instead of wild-type Mph1 the fission yeast shuttle vector pJK*mph1-kd* was used. pJK*mph1-kd* is based on the vector pJK148 that contains an ampicillin resistance for growth in bacteria and a leu1 marker for growth in fission yeast. It contains the sequence of *mph1* (starting at 408 bp to the end of Mph1) with a mutation at 1376 bp from A to C. This point mutation changes the codon D (aspartic acid) to alanine in the consensus sequence DFG in subdomain VII of the catalytic domain. This amino acid substitution has been shown to abolish Mps1 kinase activity (Lauze et al., 1995). The mutated kinase-dead gene (*mph1-kd*) was integrated at the *mph1* locus and replaced wild-type *mph1*. Expression of *mph1-kd* was verified using western blot analysis with α-Mph1 antibody (Figure 3.6). Analysis of Mph1-kd protein levels showed that there were only approximately 50 % of wild-type Mph1 levels detectable. This could lead to mis-interpretation of results obtained from experiments with strains carrying the *mph1-kd* mutant, as the phenotypes observed could be due to reduced protein levels rather than lack of kinase activity. For that reason a second copy of *mph1-kd* was introduced at the leu1 locus in the strain already carrying *mph1-kd* at the mph1 locus. To do so the shuttle vector pJK*mph1-kdSC* was used. pJK*mph1-kdSC* contains full-length *mph1-kd* with 338 bp of 5'UTR containing the endogenous promoter of *mph1* and 212 bp of 3'UTR. This made expression of the second copy (*mph1-kdSC*) at the same levels as *mph1-kd* from the *mph1* locus possible. Expression of $mph1\text{-}kd^{2X}$ (Figure 3.6) was confirmed by western blot analysis of protein extracts of cycling cells of $mph1\text{-}kd^{2X}$ with α-Mph1 antibody. Figure 3.6 shows that $mph1\text{-}kd^{SC}$ was expressed, as the protein levels in the strain containing two copies of *mph1-kd* were similar to wild-type protein levels. Assays analysing the importance of kinase activity for the spindle checkpoint and viability performed with *mph1-kd* strains were also performed with $mph1\text{-}kd^{2X}$ strains to confirm that the phenotypes observed were due to a lack of kinase activity rather than reduced protein levels.

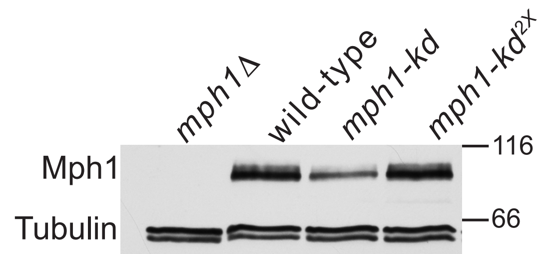


Figure 3.6: Protein stability analysis of Mph1-kd
Western blot analysis of native extracts from wild-type, *mph1-kd* and *mph1-kd*^{2X} interphase cells. Immunoblot was probed with α-Mph1 antibody and TAT1 (anti-tubulin, loading control) antibody.

3.6 Kinase-dead mutation abolishes kinase activity of Mph1

As described above wild-type Mph1 is active *in vitro* and autophosphorylates. In order to test the effect of the kinase-dead mutation on Mph1 kinase activity *in vitro*, kinase assays with Mph1-kd were carried out. Wild-type Mph1-STAP and Mph1-kd –STAP were two step tandem affinity purified from native yeast extracts and incubated in kinase assay buffer with or without the addition of the artificial substrate myelin basic protein (MBP). Figure 3.5 shows that Mph1-kd does not have any detectable *in vitro* kinase activity and unlike wild-type Mph1 neither phosphorylated myelin basic protein (MBP) nor autophosphorylated. These experiments also proved that the signals observed in Mph1 *in vitro* kinase assays (Figure 3.5) were due to intrinsic Mph1 kinase activity and not due to a contaminating co-purifying kinase. Knowing that the kinase-dead version of Mph1 has no kinase activity anymore, the effect of the loss of this kinase activity on mitosis was investigated looking at the spindle checkpoint and chromosome segregation.

3.7 Mph1 kinase activity is required for a functional spindle checkpoint

In order to determine the role of Mph1 kinase activity in the spindle checkpoint three different approaches were used. First, sensitivity of a strain carrying kinase-dead Mph1 (*mph1-kd*) to the microtubule destabilising drug benomyl was tested followed by an analysis of the ability of *mph1-kd* cells to spindle checkpoint arrest. And third, by overexpressing Mph1-kd it was determined whether Mph1 kinase activity is required to induce a mitotic arrest (He et al., 1998).

3.7.1 *mph1-kd* alleles are benomyl sensitive

Benomyl is commonly used to test mutants for spindle checkpoint defects. It is a microtubule depolymerising drug and leads to microtubule-kinetochore attachment defects and a perturbed mitotic spindle. Whereas wild-type cells with a functional spindle checkpoint grow on plates containing benomyl, mutants with a defective spindle checkpoint show growth impairment in the presence of benomyl, as the mutant cells initially divide rapidly and then die. It is noteworthy that benomyl assays are just an indicator for spindle checkpoint function of a protein. Benomyl sensitivity of a mutant can be due to defects other than an impaired spindle checkpoint such as chromosome mis-segregation. This is also the reason why benomyl sensitivity varies between different spindle checkpoint mutants (Vanoosthuyse et al., 2004). To assess whether cells lacking Mph1 kinase activity (*mph1-kd*) are spindle checkpoint defective *mph1-kd* benomyl sensitivity was compared to other known spindle checkpoint mutants. 10-fold serial dilutions of wild-type, *mph1-kd*, *mph1* null mutant, *mad2* null mutant and *mph1-kd*^{2X} cells, which contains two copies of the *mph1-kd* gene, strains were spotted on YES plates containing 0, 2, 4 or 6 μg/ml benomyl and grown at 30 °C for 3 days. *mph1* null mutant and *mad2* null mutant strains were used as positive controls as *mph1* null mutant cells are known to be hypersensitive to benomyl whereas *mad2* null mutant cells are less benomyl sensitive (Vanoosthuyse et al., 2004). As expected *mph1* null

mutant cells were much more benomyl sensitive than *mad2* null mutant cells (Figure 3.7). *mph1-kd* cells were slightly less benomyl sensitive than the *mph1* null mutant cells but more benomyl sensitive than *mad2* null mutant cells (Figure 3.7). Introduction of a second copy of *mph1-kd* did not rescue the hypersensitivity of *mph1-kd* cells to benomyl. *mph1-kd*^{2X} cells were as benomyl sensitive as the *mph1-kd* cells. These results showed that the loss of Mph1 kinase activity renders cells hypersensitive to benomyl. However, benomyl assays are just an indicator of spindle checkpoint function. For example, even though *mad2* is essential for the spindle checkpoint a *mad2* null mutant is less benomyl sensitive than an *mph1* null mutant. For this reason time-courses using *nda3-KM311* mutant strains were carried out. This mutant allows us to specifically determine the ability of mutants to arrest in metaphase due to the activation of the spindle checkpoint.

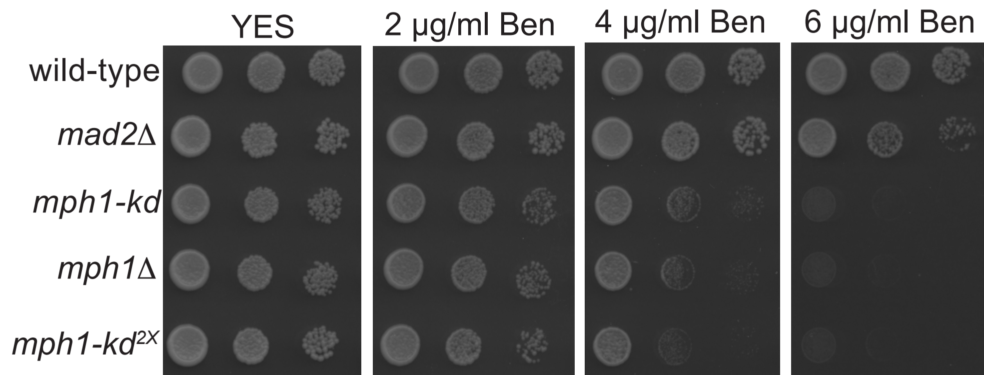


Figure 3.7: Mph1 mutants are benomyl sensitive. Benomyl sensitivity assays of wild-type, *mad2Δ*, *mph1-kd, mph1Δ* and *mph1-kd*^{2X} strains. 10-fold serial dilutions were spotted on YES plates containing 0, 2, 4, 6 μg/ml benomyl. Plates were incubated for 3 days at 30 °C.

3.7.2 *mph1-kd* alleles fail to checkpoint arrest

To investigate whether a kinase-dead mutant of Mph1 (*mph1-kd*) is able to spindle checkpoint arrest *mph1-kd* was crossed into an *nda3-KM311* background strain. As described above the cold sensitive β-tubulin mutant *nda3-KM311* leads to a spindle checkpoint arrest in wild-type cells upon growth of cells at the restrictive temperature of 18 °C due to a perturbed spindle. However, spindle checkpoint deficient mutants do not spindle checkpoint arrest in the presence of lack of kinetochore-microtubule attachment and tension. They proceed through mitosis into anaphase without correctly bi-oriented chromosomes. This leads to chromosome mis-segregation and ultimately cell death. To score mitotic cells, the strains analysed contained GFP fused to polo kinase (Plo1) (GFP-Plo1). Plo1 localises to spindle pole bodies (SPB) during mitosis but not interphase (Bahler et al., 1998a; Mulvihill et al., 1999). Mitotic cells were scored as cells containing two green spots at the SPBs (Figure 3.8A).

Cells were incubated at the permissive temperature of 30 °C overnight and then shifted to the restrictive temperature of 18 °C for 6 hrs. Samples were taken at 1 hr time-points, cells fixed and the number of mitotic cells determined scoring localisation of GFP-Plo1 to SPBs using microscopy. Time-courses were carried out with wild-type, *mph1-kd*, *mph1Δ* and *mad2Δ* strains. *mph1Δ* and *mad2Δ* are known to be spindle checkpoint defective and were used as negative controls. As expected over the 6 hrs the mitotic index in wild-type rose steadily until after 6 hrs 91 % were arrested in mitosis. *mph1Δ* and *mad2Δ* cells did not arrest and the mitotic index was never higher than 18 % and 22 % respectively (Figure 3.8B). A similar inability to arrest was observed in *mph1-kd* (14 %) cells with a mitotic index similar to *mph1Δ* and *mad2Δ* (Figure 3.8B).

The same assay as described above was performed with *nda3-KM311* strains carrying wild-type, *mph1-kd* and *mph1-kd*^{2X}. Instead of using strains carrying GFP-Plo1, septation was scored. Time-points were taken before the temperature-shift (0

hrs) and after 6 hrs. The cells were methanol fixed, stained with calcofluor to visualise septa and analysed using microscopy. Cells that are arrested in metaphase do not contain a septum. Therefore, septa were scored as an indicator of cells that were not arrested in metaphase but had progressed through anaphase and initiated cyotkinesis. Figure 3.8C shows preliminary results for wild-type, *mph1-kd* and *mph1-kd*^{2X}. After 6 hrs 1 % of wild-type cells had a septum, compared to 17.5 % septation in cycling cells (0 hrs). The septation index of *mph1-kd* cells, that do not spindle checkpoint arrest was 23 % after 6 hrs. The septation index of *mph1-kd*^{2X} was similar to *mph1-kd* with 30 %, indicating that *mph1-kd*^{2X} cells did not spindle checkpoint arrest. These results showed that Mph1 kinase activity is required for a spindle checkpoint arrest due to lack of microtubule-kinetochore attachment and tension.

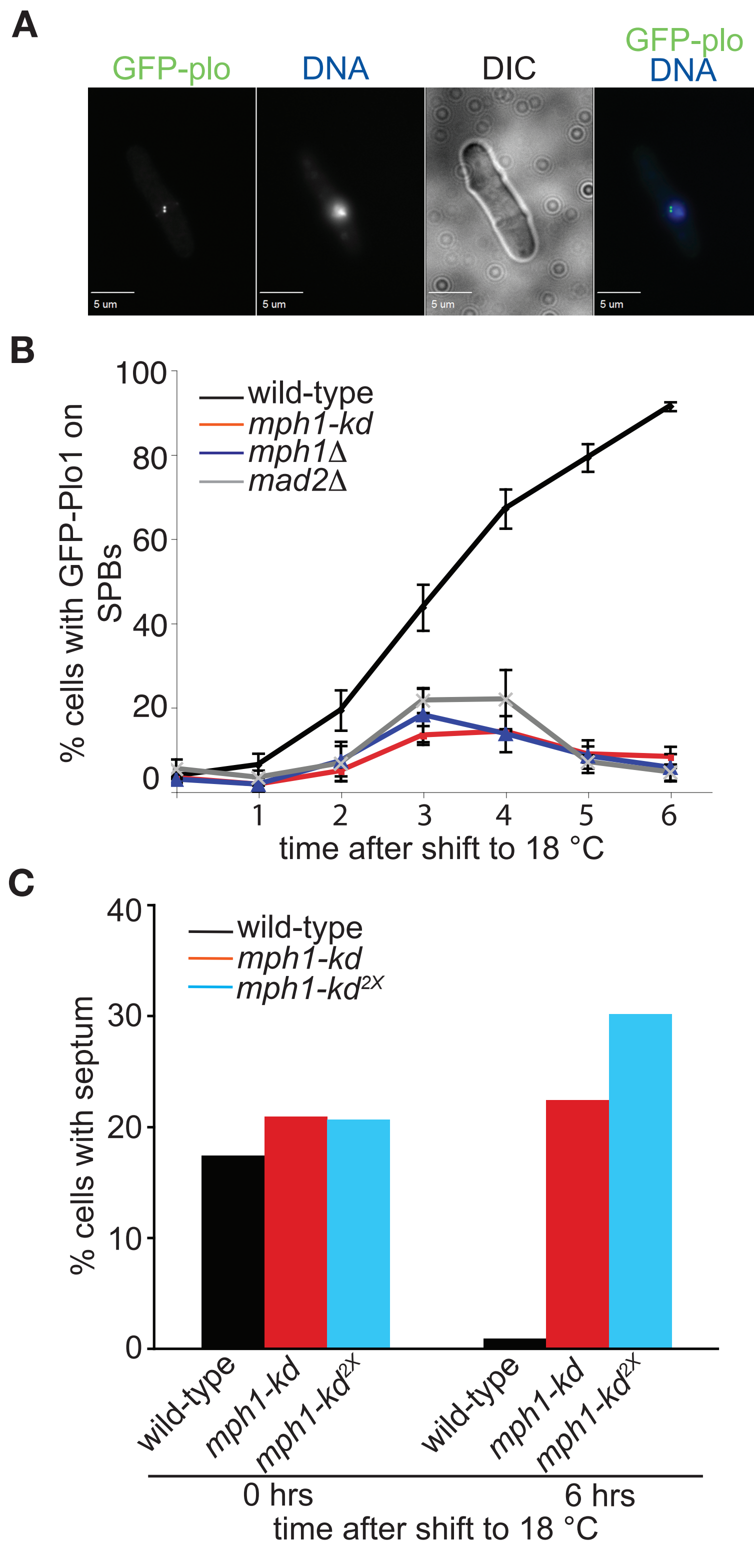
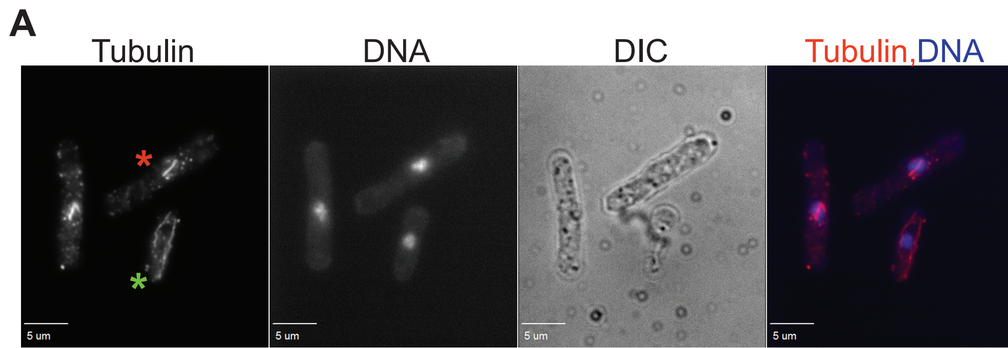


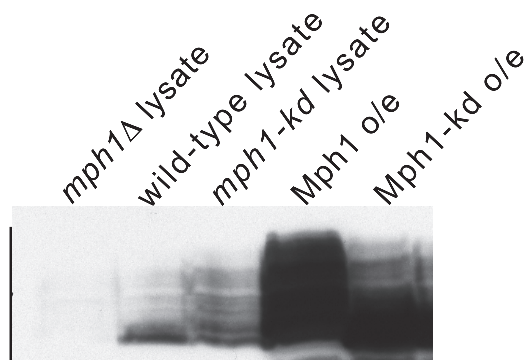
Figure 3.8: Mph1 kinase activity is required for a functional spindle checkpoint. A Representative mitotically arrested cell with GFP-Plo1 spots at the spindle pole body **B** Mitotic arrest time-courses with *nda3-KM311* strains of wild-type, *mph1-kd, mph1*Δ and *mad2*Δ. Mitotic cells were scored as a percentage of cells with GFP-Plo1 localisation to spindle pole bodies. (n=3 $\pm$ stdev) **C** Mitotic arrest time-courses with *nda3-KM311* strains of wild-type, *mph1-kd* and *mph1-kd*^{2X}. Cells that were not in mitosis were scored as a percentage of cells with a septum. wild-type: n=1; *mph1-kd* (n=2); *mph1-kd*^{2X} (n=2).

3.7.3 Kinase activity of Mph1 is required for an overexpression induced metaphase arrest

Overexpression of Mph1 in a wild-type background, both in fission yeast and budding yeast, leads to a metaphase arrest (Hardwick et al., 1996; He et al., 1998). To test whether Mph1 kinase activity is required for this arrest an overexpression plasmid containing *mph1-kd* was constructed. Using site directed mutagenesis (QuikChange kit, Stratagene) the kinase-dead mutation was introduced into the Mph1 expression plasmid pREP41x-*mph1* (He et al., 1998). In a pREP41x plasmid the gene of interest is under the control of a medium strength nmt (no message in thiamine) promotor, which is repressed in the presence of thiamine (Craven et al., 1998). pREP41x-*mph1* and pREP41x-*mph1-kd* were transformed into wild-type strains. Precultures were grown in medium containing thiamine so that only endogenous levels of Mph1 were present in the cells. To overexpress Mph1 and Mph1-kd the thiamine was washed out of the cells and cells cultured in medium lacking thiamine for 18 hrs at 30 °C. Cells were then methanol fixed and stained for tubulin using anti-tubulin (TAT1) antibody. Metaphase arrested cells were scored counting short metaphase spindles (Figure 3.9A). Cells overexpressing wild-type Mph1 had a mitotic index of 56 %, which was consistent with the numbers determined in previous studies (He et al., 1998). Overexpression of Mph1-kd failed to arrest cells in mitosis with a mitotic index of 3 % (Figure 3.9B).

The results described above are consistent with observations made for kinase-dead versions of Mph1 homologues in other organisms that showed that Mps1 kinase activity is required for a functional spindle checkpoint. Next it was tested whether Mph1 kinase activity is also required for accurate chromosome segregation in fission yeast.





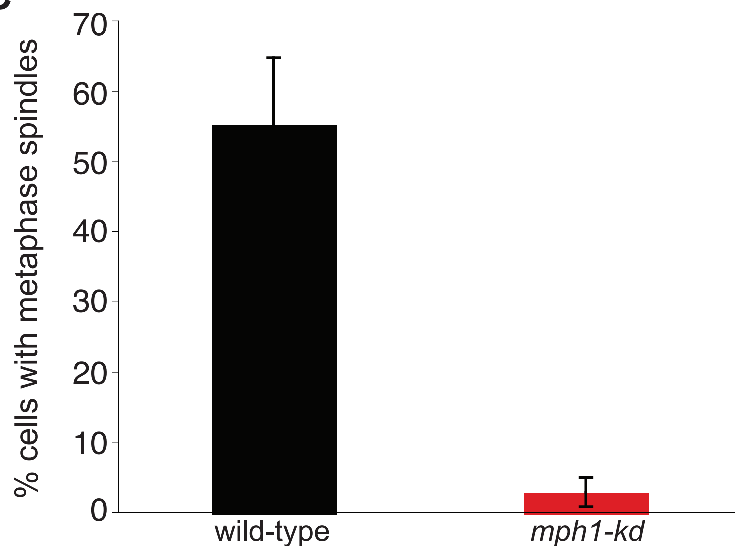


Figure 3.9: Mph1 kinase activity is required for an overexpression induced metaphase arrest. (**A**) Microscopy pictures of metaphase and interphase cells stained with α-TAT1 antibody to visualise tubulin (red). DNA (blue) was stained with DAPI. (**B**) Western blot analysis of strains overexpressing (o/e) Mph1 and Mph1-kd from the expression vectors pREP41x-*mph1* and pRPE41x-*mph1-kd* probed with α-Mph1 antibody. (**C**) Mph1 overexpression arrest using the expression vectors pREP41x-*mph1* and pRPE41x-*mph1-kd*. Mitotic arrest was scored as a percentage of cells with short metaphase spindles (n=3 $\pm$stdev).

3.8 Mph1 kinase activity is required for accurate chromosome segregation

Two different approaches were used to determine whether Mph1 kinase activity is required for accurate chromosome segregation. First the rate of mis-segregation of chromosome 2 was determined. Secondly, a half-sectoring assay that determines the loss rate of the artificial minichromosome CH16 was carried out (Niwa et al., 1989).

3.8.1 Chromosome 2 loss

The first approach used to determine whether Mph1 kinase activity is required for proper chromosome segregation was to count mis-segregation of chromosome 2. In this assay an *mph1-kd* strain was used containing a GFP marked centromere on chromosome 2. In this strain a tandem repeat of lac O is inserted close (~ 5kb) to the centromere of chromosome 2 (Yamamoto and Hiraoka, 2003). GFP-tagged lacI is expressed and binds to lacO and thus enables visualising chromosome 2 as a green dot under the fluorescence microscope. If chromosomes are separated correctly one green spot at each spindle pole should be visible. If chromosome 2 is mis-segregated 2 spots are seen at one pole. In wild-type cells chromosome mis-segregation occurs in 0 % of cells (Vanoosthuyse et al., 2004). Analysis of *mph1-kd* strains showed that 2 % (n = 983) of cells lacking Mph1 kinase activity mis-segregated chromosome 2 (Figure 3.10). Interestingly this is lower than an *mph1* null mutant that has about 8 % (n = 992) chromosome 2 mis-segregation (data for *mph1* null mutant: L.Milne, Hardwick lab, unpublished data). It is noteworthy that not all spindle checkpoint proteins have chromosome segregation defects. The only other spindle checkpoint mutant identified to date with chromosome segregation defects is Bub1 (5.3 %; n = 1002) (Vanoosthuyse et al., 2004).

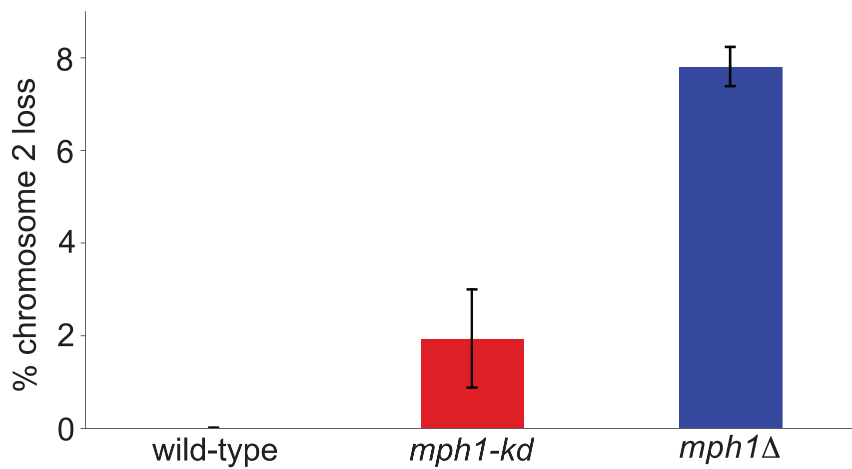


Figure 3.10: Mph1 kinase activity is required for accurate chromosome segregation. Percentage chromosome 2 loss (cen2-GFP) of wild-type, *mph1-kd* and *mph1Δ* strains**.** Data for wild-type are from Vanoosthuyse et al., 2004. Data for *mph1Δ* are from L.Milne (Hardwick lab, unpublished data).

3.8.2 Half sectoring assay (CH16 mini-chromosome loss)

The second approach used to determine whether Mph1 kinase activity is required for proper chromosome segregation was a half sectoring assay (Allshire et al., 1995). This assay is based on the short linear mini-chromosme CH16 (530 bp) (Niwa et al., 1989). Ch16 contains an *ade6-216* mutant allele, which is complemented by the *ade6-210* allele in the parental strain. If CH16 is lost due to chromosome mis-segregation the adenine mutation *ade6-216* is not complemented anymore and cells will appear red due to a lack of adenine. To score CH16 chromosome loss cells are plated on low adenine plates. Three different types of colonies can be observed. White colonies represent cells that did not loose CH16. Red colonies mean that CH16 was lost from the cell in the preculture before the cells were plated out. Half-sectored colonies indicate loss of CH16 in the first division after plating of the cells (Figure 3.11A). Wild-type CH16 cells and *mph1-kd* CH16 cells were plated on plates containing low adenine. After incubation for three days at 30 °C CH16 loss was scored as a percentage of half-sectored colonies to white colonies. Red colonies were not counted as they lost CH16 before the first division on the plate. Figure 3.11B shows that 0 % of wild-type cells lost minichromosome CH16 whereas 5 % of *mph1-kd* cells lost minichromosome CH16.

The results above showed that Mph1 kinase activity is required for a functional spindle checkpoint and accurate chromosome segregation. It is known that loss of the spindle checkpoint and chromosome mis-segregation leads to aneuoploidy and ultimately cell death. This led to the question of what consequences loss of Mph1 kinase activity has on cell viability in fission yeast.

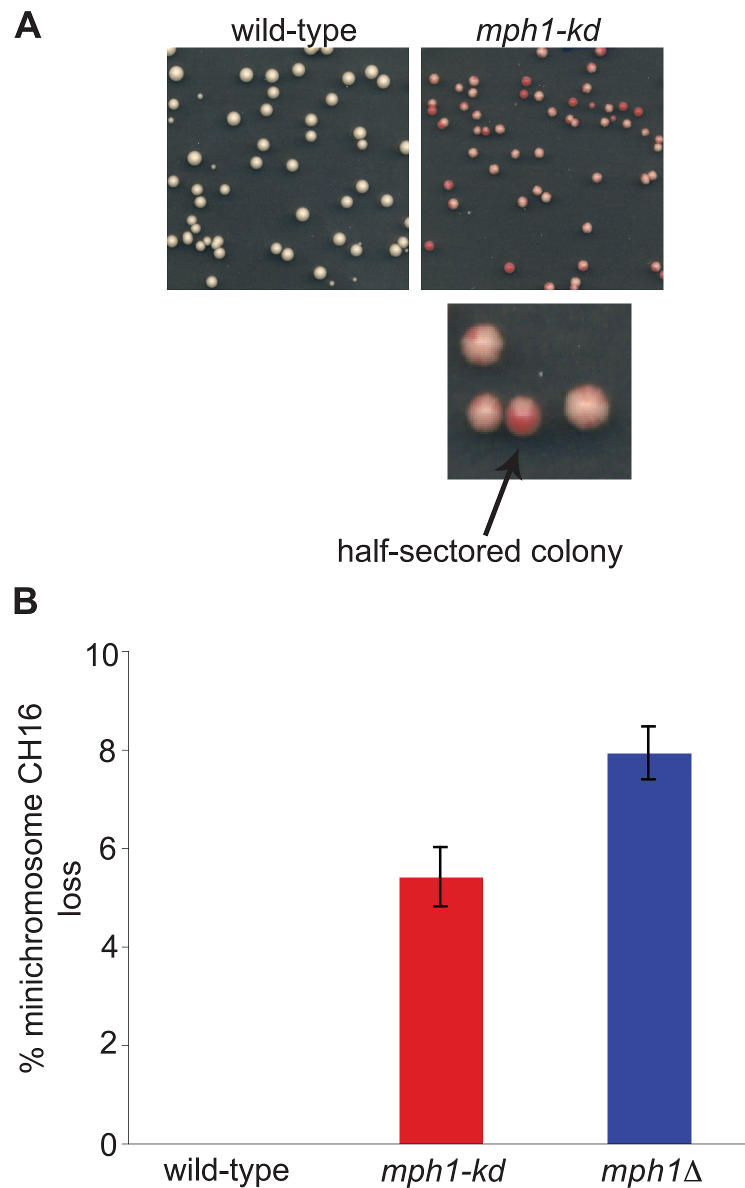


Figure 3.11: Mph1 kinase activity is required for accurate chromosome segregation. Half sectoring assay of wild-type and *mph1-kd* strains. Representative plates shown (**A**). **B** Percentage minichromosome CH16 loss of wild-type, *mph1-kd* and *mph1*Δ strains**.** Data for *mph1*Δ are from L.Milne (Hardwick lab, unpublished data).

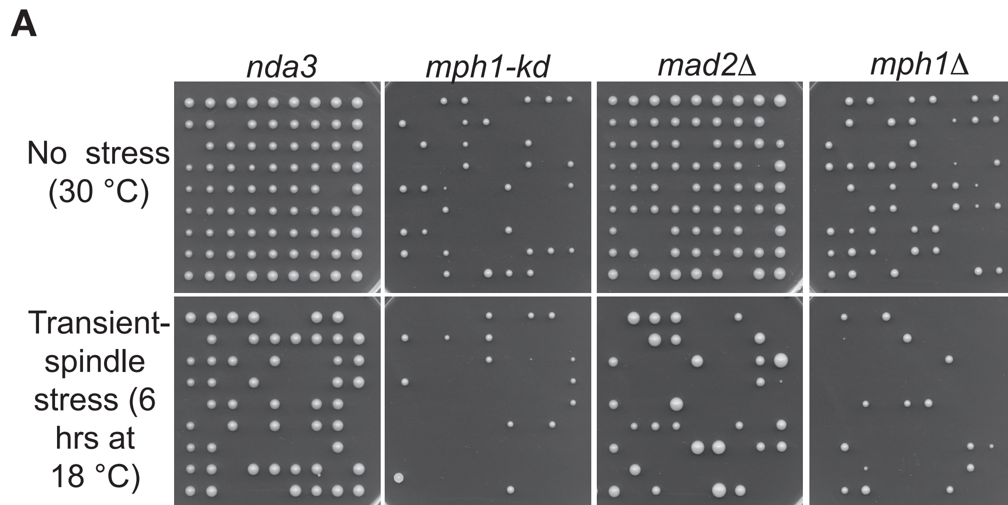
3.9 *mph1-kd* cells die under spindle stress

To determine the viability of *mph1-kd* cells upon spindle stress colony forming assays were carried out. *nda3-KM311* strains as described above were used. Cultures were grown at the permissive temperature of 30 °C overnight and then shifted to 18 °C for 6 hrs to induce spindle stress. A minimum of 81 single cells were isolated and plated on solid media prior to the temperature shift without any spindle stress and after 6 hrs of spindle stress at 18 °C. Plates were incubated for 3 or 6 days at 32 °C and the percentage of cells able to form a colony scored as a total of the cells plated out. A *nda3-KM311* metaphase arrest is reversible and shortly after shifting *nda3-KM311* cells to the permissive temperature cells form a mitotic spindle and undergo anaphase (Kanbe et al., 1990). Unlike wild-type cells spindle checkpoint defective mutants do not arrest and progress into anaphase without spindle or correctly bi-oriented chromosomes. This leads to the cut phenotype, chromosome mis-segregation, aneuploidy and ultimately cell death.

Colony-forming assays were carried out with *nda3-KM311* strains carrying *mph1-kd*, *mph1-kd*^{2X}, *mph1*Δ, *mad2*Δ and *mad3*Δ. Unlike *mph1* mutants, *mad2*Δ and *mad3*Δ strains do not have chromosome segregation defects in addition to spindle checkpoint defects (Vanoosthuyse et al., 2004). They were used to compare viability of spindle checkpoint mutants that only have a non-functional spindle checkpoint to mutants that also comprise chromosome segregation (*mph1* mutants) defects under spindle stress. Figure 3.12A shows representative plates of wild-type, *mph1-kd*, *mph1*Δ and *mad2*Δ strains. Wild-type and *mad2*Δ colonies were bigger and healthier looking than *mph1-kd* and *mph1*Δ colonies, both with and without spindle stress, perhaps due to cell inviability during colony formation. Scoring colony formation (Figure 3.12B) confirmed this observation. Without spindle stress 97 %, 88 % and 91 % of wild-type, *mad2*Δ and *mad3*Δ cells form colonies respectively, whereas all three *mph1* mutants (*mph1-kd*, *mph-1kd*^{2X}, *mph1*Δ) only had about 42 %, 39 % and 52 % colony formation/cell survival, respectively. After six hours of spindle stress the wild-type had 71 % colony formation. The spindle checkpoint mutants *mad2*Δ

and *mad3*Δ showed a lower viability with 36 % and 32 %, respectively. All three Mph1 mutants had very low cell viability and only formed 17 %, 14 % and 15 % colonies. These results showed that mutants with a non-functional spindle checkpoint die under spindle stress. This loss of viability appeared to be higher in mutants that also have chromosome segregation defects.

A



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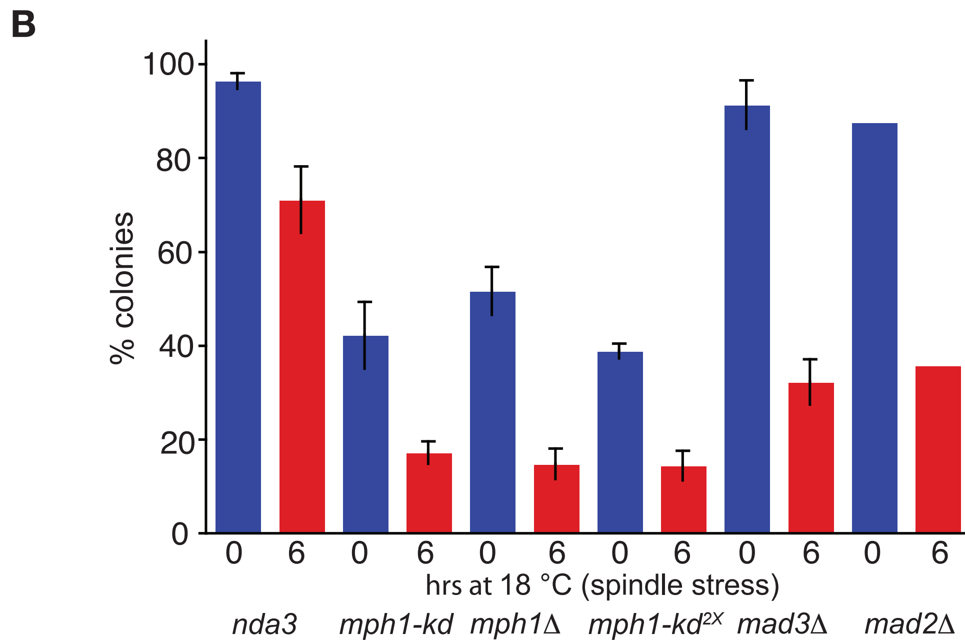

Figure 3.12: *mph1-kd* cells die under spindle stress Viability assay of *nda3-KM311* strains of wild-type, *mph1-kd*, *mph1Δ*, *mph1-kd*^{2X}, *mad3Δ* and *mad2Δ*. A minimum of 81 cells were spotted onto YES plates from cultures grown at 30 °C and under 6 hrs of spindle stress (18 °C) and incubated at 32 °C for 3 or 6 days. (wild-type, *mph1-kd*, *mph1Δ*: n=3 ± stdev; *mph1-kd*^{2X}, *mad3Δ*: n=2 ± stdev; *mad2Δ*: n=1)

3.10 Summary and Discussion

Members of the Mps1 kinase family are phosphoproteins with a peak in phosphorylation during metaphase. Their kinase activity is required for a functional spindle checkpoint and chromosome segregation (Abrieu et al., 2001; Jelluma et al., 2008a; Jones et al., 2005; Kang et al., 2007). Fission yeast Mph1 is essential for the spindle checkpoint but it was not clear whether this was due to its kinase activity. Furthermore, it has not been investigated yet whether Mph1 kinase activity is required for accurate chromosome segregation.

The aim of this chapter was to answer four questions. First, is Mph1 a phosphoprotein *in vivo* as well as *in vitro*? Secondly, what is the role of Mph1 kinase activity in the spindle checkpoint? Third, what is the role of Mph1 kinase activity in chromosome segregation? Fourth, what consequences does the loss of Mph1 kinase activity have on cell viability?

The results obtained answered these questions as follows. First, Mph1 is a phosphoprotein that is hyperphosphorylated during a spindle checkpoint arrest and has *in vitro* kinase activity. Second, lack of Mph1 kinase activity abolishes the spindle checkpoint. Third, Mph1 kinase activity is also required for accurate chromosome segregation. And fourth, cell viability of cells lacking Mph1 kinase activity is severely impaired. Figure 3.13 at the end of this chapter shows a model of the functions of Mph1 kinase activity in mitosis.

The mitotic arrest/release time-courses performed in this study showed that fission yeast Mph1 is hyperphosphoryated during a spindle checkpoint arrest. These data are consistent with data from budding yeast and human in which, hyperphosphorylation of Mps1 has been reported previously (Hardwick et al., 1996; Lauze et al., 1995; Liu et al., 2003; Stucke et al., 2002). It is not clear from the results obtained in this study whether the hyperphosphorylation observed was due to autophosphorylation or phosphorylation by other kinases. It is likely that Mph1 is phosphorylated by other spindle checkpoint kinases such as Bub1 or Ark1/Aurora as

phosphorylation of the Mph1 homologue Mps1 in *Xenopus* by mitogen activated kinase (MAPK) is required for its checkpoint activity (Zhao and Chen, 2006). However, Mps1 hyperphosphorylation in budding yeast has also been shown to be partly due to autophosphorylation (Lauze et al., 1995). The *in vitro* kinase assays carried out in this study showed that Mph1 autophosphorylates *in vitro*, which suggests that Mph1 phosphorylation *in vivo* is at least partly due to autophosphorylation. This ability of Mps1 to autophosphorylate is important for its own activity and function in the spindle checkpoint (Jelluma et al., 2008a; Kang et al., 2007; Mattison et al., 2007). This study showed that Mph1 autophosphorylates *in vitro* and mass spectrometric analysis of Mph1 kinase assays identified several autophosphorylation sites (Figure S.1, S.2, Table S.1)). One of these sites (T676 in human Mps1) has been shown to be important for Mps1 kinase activity and the spindle checkpoint in human and budding yeast (Jelluma et al., 2008a; Kang et al., 2007; Mattison et al., 2007). This study did not investigate the role of autophosphorylation of Mph1 in the spindle checkpoint but focused on analysing the role of Mph1 kinase activity in the spindle checkpoint using a kinase-dead allele of Mph1.

This kinase-dead allele of *mph1* has a substitution of aspartic acid 459 to alanine in the consensus sequence DFG in subdomain VII of the kinase domain. It renders Mps1 kinases inactive (Abrieu et al., 2001; Lauze et al., 1995). This was also the case in fission yeast (Figure 3.5). Mph1 kinase-dead purified from native yeast extracts had no detectable *in vitro* kinase activity towards the artificial substrate myelin basic protein (MBP) or itself (autophosphorylation). Using the kinase-dead mutant the role of Mph1 kinase activity in the spindle checkpoint was analysed.

Hypersensitivity of *mph1-kd* to the microtubule destabilising drug benomyl strongly supported the hypothesis that Mph1 kinase activity is required for a functional spindle checkpoint as null mutants of spindle checkpoint proteins all display benomyl sensitivity (Vanoosthuyse et al., 2004). However, benomyl sensitivity assays are just an indicator for spindle checkpoint function of a protein. For example, even though Mad2 is essential for the spindle checkpoint, a *mad2* null

mutant is less benomyl sensitive than an *mph1* null mutant strain. These differences in benomyl sensitivity can be due to other defects in addition to spindle checkpoint defects such as chromosome mis-segregation. Chromsome mis-segregation leads to aneuploidy and cell death, which can explain the severe growth impairment of *mph1* mutants on benomyl plates.

To investigate the role of Mph1 kinase activity specifically in the spindle checkpoint the cold-sensitive β-tubulin mutant *nda3-KM311* was used. When grown at the restrictive temperature this mutant does not polymerise microtubules and thus does not build a functional spindle. This leads to the activation of the spindle checkpoint as a result of lack of microtubule-kinetochore attachment and tension. Using the *nda3-KM311* mutant this study showed that kinase activity of Mph1 is required for a spindle checkpoint arrest induced by lack of attachment. *mph1-kd* showed a similar phenotype to an *mph1* null mutant and a *mad2* null mutant. These results are consistent with studies of Mps1 homologues in other organisms that reported that Mps1 kinase activity is required for a functional spindle checkpoint (Abrieu et al., 2001; Jones et al., 2005; Tighe et al., 2008). Using an *nda3-KM311* mutant it is not possible to distinguish between defects in the spindle checkpoint due to lack of attachment or tension. In budding yeast it is possible to carry out assays that allow to specifically determine the ability of mutant to spindle checkpoint arrest due to a loss of tension (King et al., 2007a). Such assays have been carried out in fission yeast (Meadows and Millar, 2008). In the future we will try and analyse *mph1* mutants using these assays. An *mph1* kinase-dead mutant showed the same spindle checkpoint phenotype as an *mph1* null mutant. In chapter 5 candidate spindle checkpoint substrates for Mph1 are investigated in detail.

Western blot analysis of strains carrying *mph1-kd* or wild-type *mph1* showed that the Mph1-kd protein is less stable than wild-type Mph1. One reason could be that the amino-acid substitution alters the structure of Mph1 and renders it less stable. A second reason could be that autophosphorylation of Mph1 is required for its own stability. Mph1-kd has no detectable kinase activity and does not autophosphorylate, which could explain its instability. Nevertheless a second copy of *mph1-kd* did not

rescue the phenotypes observed with strains carrying a single copy of *mph1-kd*. This leads to the conclusion that the mitotic defects observed are due to the loss of Mph1 kinase activity and not loss of protein. This hypothesis was further supported by overexpression experiments. Overexpression of Mph1 in fission yeast and budding yeast leads to a metaphase arrest (Hardwick et al., 1996; He et al., 1998). In this study overexpression of a kinase-dead version of Mph1 was not able to induce a metaphase arrest, which suggests that the Mph1 induced overexpression arrest in fission yeast is dependent on Mph1 kinase activity.

L.Milne observed that *mph1* null mutants also have chromosome segregation defects in addition to the defects in the spindle checkpoint (L.Milne, unpublished data). In this study it was tested whether chromosome segregation defects are also present in a kinase-dead *mph1* mutant.

Two approaches were used to investigate chromosome segregation in this mutant, loss of the minichromosome 16 (CH16) and chromosome 2 loss. Both assays showed that kinase activity of Mph1 is required for accurate chromosome segregation. These results led to the conclusion that Mph1 might have a role at kinetochores distinct from its role in the spindle checkpoint. With Mph1 being a kinase regulation could occur via phosphorylation of kinetochore subunits or proteins localised to kinetochores. This hypothesis is supported by several studies. Budding yeast Mps1 directly interacts and phosphorylates the kinetochore subunit Ndc80 (Kemmler et al., 2009). Human Mps1 phosphorylates the chromosomal passenger complex (CPC) subunit Borealin (Jelluma et al., 2008b) that monitors correct bi-orientation of chromosomes on the mitotic spindle.

Importantly, L Milne (Hardwick lab) showed that a null mutant of *mph1* has a significantly higher rate of chromsome mis-segregation (unpublished data) than the *mph1-kd* mutant analysed in this study. This suggests that even though Mph1 kinase activity is required for accurate chromosome segregation Mph1 must have additional segregation roles independent from its kinase function. L.Milne analysed a kinase mutant of Mph1 that lacks the whole C-terminal kinase domain. This mutant has a

similar phenoptype to the kinase-dead *mph1* mutant with regards to chromosome segregation. This suggests that the additional role of Mph1 does not lie in the C-terminal kinase domain of Mph1 but in the N-terminus.

There are two likely roles for the N-terminus of Mph1. First, the N-terminus could be required for kinetochore targeting of Mph1. Unfortunately, it was not possible yet to visualise Mph1 at kinetochores microscopically or to prove kinetochore binding using biochemical methods such as CHIP analysis. However, kinetochore localisation of Mph1 homologues has been demonstrated and has been shown be dependent on the N-terminus (Abrieu et al., 2001; Liu et al., 2003; Stucke et al., 2004; Stucke et al., 2002). Secondly, Mph1 could have scaffolding functions at kinetochores and recruit other checkpoint proteins. This hypothesis is supported by studies in *Xenopus* and human, in which Mps1 regulates Mad1 and Mad2 binding to kinetochores (Abrieu et al., 2001; Kang et al., 2007; Liu et al., 2003).

The inability to delay anaphase until chromosomes are correctly bi-oriented on the mitotic spindle and the additional chromosome segregation defects can result in aneuploidy that ultimately leads to cell death. The kinase-dead *mph1* mutant displays both these phenotypes. For that reason viability of cells carrying kinase-dead *mph1* under spindle stress was tested. In the colony-forming assays carried out in this study cells were exposed to spindle stress caused by a lack of microtubule-kinetochore attachment and tension. In wild-type cells this leads to the activation of the spindle checkpoint. Spindle checkpoint defective mutants such as *mad2* null mutants, *mad3* null mutants and *mph1* mutants proceed through mitosis into anaphase without properly bi-oriented chromosomes. This leads to chromosome mis-segregation, aneuploidy and ultimately cell death. This explains the data obtained from this study in which null mutants of the spindle checkpoint proteins *mad2* and *mad3* have a higher rate of cell death upon spindle stress. Nevertheless, the rate of cell death in the *mph1* null mutant and the kinase-dead *mph1* mutant are even higher than in *mad2* and *mad3* null mutants. This phenotype can be explained by the additional chromosome segregation defects *mph1* mutants have. The results obtained from this study were consistent with results obtained in previous cell

viability studies of *mad2*, *mad3* and an *mph1* null mutants (He et al., 1997; Millband and Hardwick, 2002) (L.Milne, unpublished data).

In conclusion the work described in this chapter showed that Mph1 kinase activity is required for a functional spindle checkpoint and chromosome segregation. However, many questions on Mph1 phosphorylation and its role in mitosis remain unanswered. Experiments to further analyse Mph1 are discussed in chapter 5 and 6.

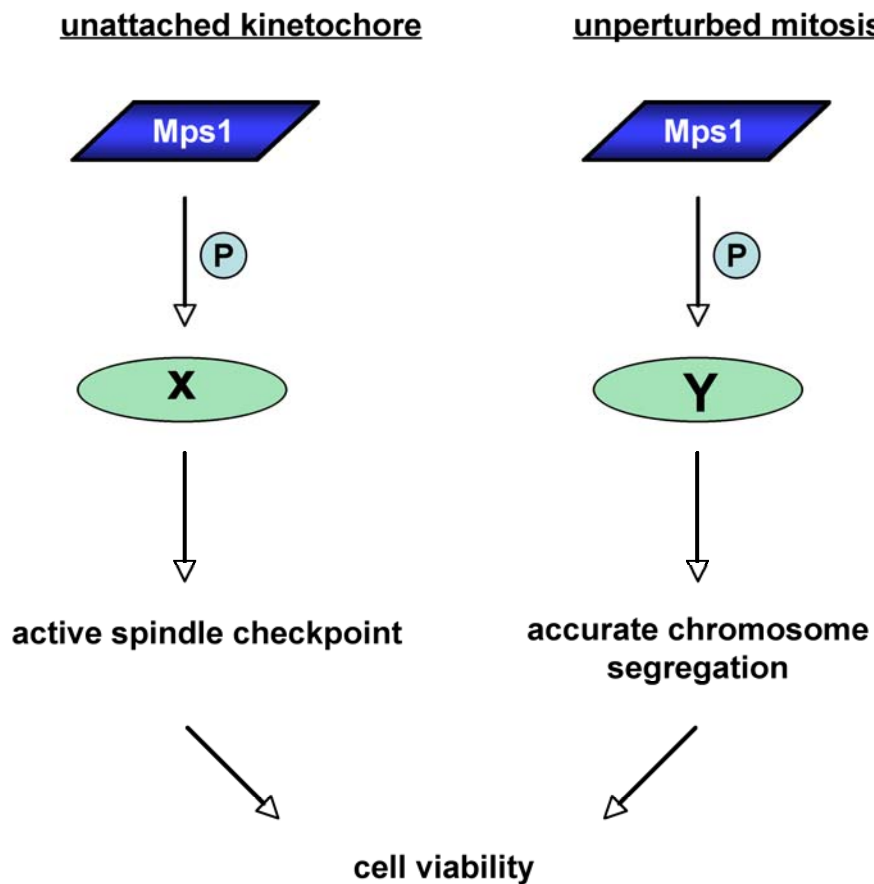

Figure 3.13: Model of the functions of Mph1 kinase activity in mitosis.

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Chapter 4

The role of Mph1 kinase in Mad2 and Mad3 binding to the APC

Chapter 4: The role of Mph1 kinase in Mad2 and Mad3 binding to the APC

4.1 Introduction

The anaphase promoting complex (APC) is an E3 ubiquitin ligase that ubiqutinylates proteins and thus targets them for degradation by the 26 S proteasome. During mitosis the two major substrates of the APC are the separase inhibitor securin/Cut2 and the Cdk1 activating subunit cyclin B/Cdc13. Degradation of securin and cyclin B is required for cells to exit mitosis (Cohen-Fix et al., 1996; Funabiki et al., 1996; Murray et al., 1989). Upon activation of the spindle checkpoint the APC is inhibited in different modes, one of them being inhibition by the mitotic checkpoint complex (MCC). Once the APC is inhibited securin and cyclin B are not degraded and the onset of anaphase delayed until all sister-chromatids are bi-oriented on the mitotic spindle (Morrow et al., 2005; Sczaniecka et al., 2008). The MCC is composed of the spindle checkpoint proteins Mad2, Mad3, Bub3 (not in fission yeast) and the APC activator Cdc20 (Slp1 in fission yeast, which will be referred to as Cdc20). The current model for APC inhibition by the MCC in fission yeast is that Mad2 in a complex with Cdc20 recruits Mad3 and that the Mad2-Cdc20-Mad3 complex then binds to the APC and inhibits its activity (Sczaniecka et al., 2008).

It is not clear to date how MCC binding to the APC is regulated. One likely regulatory mechanism is phosphorylation of components of the MCC, Cdc20 or the APC. Phosphorylation of Mad2, Mad3 or Cdc20 have not been demonstrated in fission yeast. However, phosphorylation of Cdc20 by MAPK and Bub1 has been shown to be required for a functional spindle checkpoint in *Xenopus* and human respectively (Chung and Chen, 2003; Tang et al., 2004).

The spindle checkpoint kinase Mph1is required for a functional spindle checkpoint in fission yeast (He et al., 1998) (see chapter 3). Thus it is possible that components of the MCC, Cdc20 or the APC are targets of Mph1.

Two main questions were addressed in the following chapter. First, do Mad2 and Mad3 bind to the APC only upon spindle checkpoint activation or also in an unperturbed mitosis? If yes, does this binding depend on Mph1 kinase activity? Secondly, what role does the spindle checkpoint kinase Mph1 have in Mad2 and Mad3 binding to the APC during a spindle checkpoint arrest? To answer these questions APC pull-downs were carried out in synchronised and spindle checkpoint arrested cells and Mad2/Mad3 binding to the APC monitored by western blot analysis.

4.2 Mad2 and Mad3 bind to the APC during mitosis. This interaction is dependent on Mph1 kinase activity

In order to determine the role of Mph1 kinase activity in the binding of Mad2 and Mad3 to the APC "APC binding assays" were carried out. Using the temperature sensitive *cdc25-22* mutant cells were blocked in G2-phase at the restrictive temperature of 36 °C. Cells were then synchronous released into mitosis by shifting the temperature to the permissive temperature of 25 °C. Samples were taken at 15 min time-points after release into mitosis for up to 4 hrs and pull-downs of the APC (Lid1-TAP/APC4-TAP) carried out. Co-immunoprecipitation of Mad2 and Mad3 was monitored by western blot analysis with α-GFP antibody. To monitor exit and entry into mitosis Cdc13 levels were determined by western blot analysis with α-Cdc13 antibody. Cyclin B/Cdc13 is the activating subunit of Cdk1/Cdc2. Its protein levels peak during metaphase before it is degraded at the end of mitosis (Murray et al., 1989; Yamano et al., 1998).

Figure 4.1A shows an APC binding assay of a wild-type strain released from a G2-phase arrest into mitosis. Mad2/Mad3 binding to the APC was monitored for two cell cycles (4 hrs). Mad2 and Mad3 associated with the APC 30 min after the release into mitosis. Mad2/Mad3 levels steadily rose and peaked around 45-90 min after release into mitosis. Mad2/Mad3 binding levels then dropped significantly after 105 min. Mad2/Mad3 then associated with the APC again upon entry into the subsequent cell cycle (Figure 4.1A). Cdc13 levels present in the cell, changed in a similar pattern to Mad2/Mad3 binding to the APC, showing that Mad2 and Mad3 associated with the APC during mitosis and dissociated from the APC once Cdc13 was degraded.

Performing an APC binding experiment under the same conditions as described above but in the absence of Mph1 kinase activity (*mph1-kd*) showed that Mad2/Mad3 binding to the APC was greatly reduced in this mutant (Figure 4.1B). Cdc13 immunoblots confirmed that the *mph1-kd* cells went through mitosis at the same time as wild-type cells did.

These results showed that Mad2 and Mad3 bind to the APC during an unperturbed mitosis and that this binding is dependent on Mph1 kinase activity. We conclude that the spindle checkpoint is activated every cell cycle in fission yeast. This led to the question whether Mad2 and Mad3 binding to the APC increases upon spindle checkpoint activation and if yes this binding is also dependent on Mph1 kinase activity.

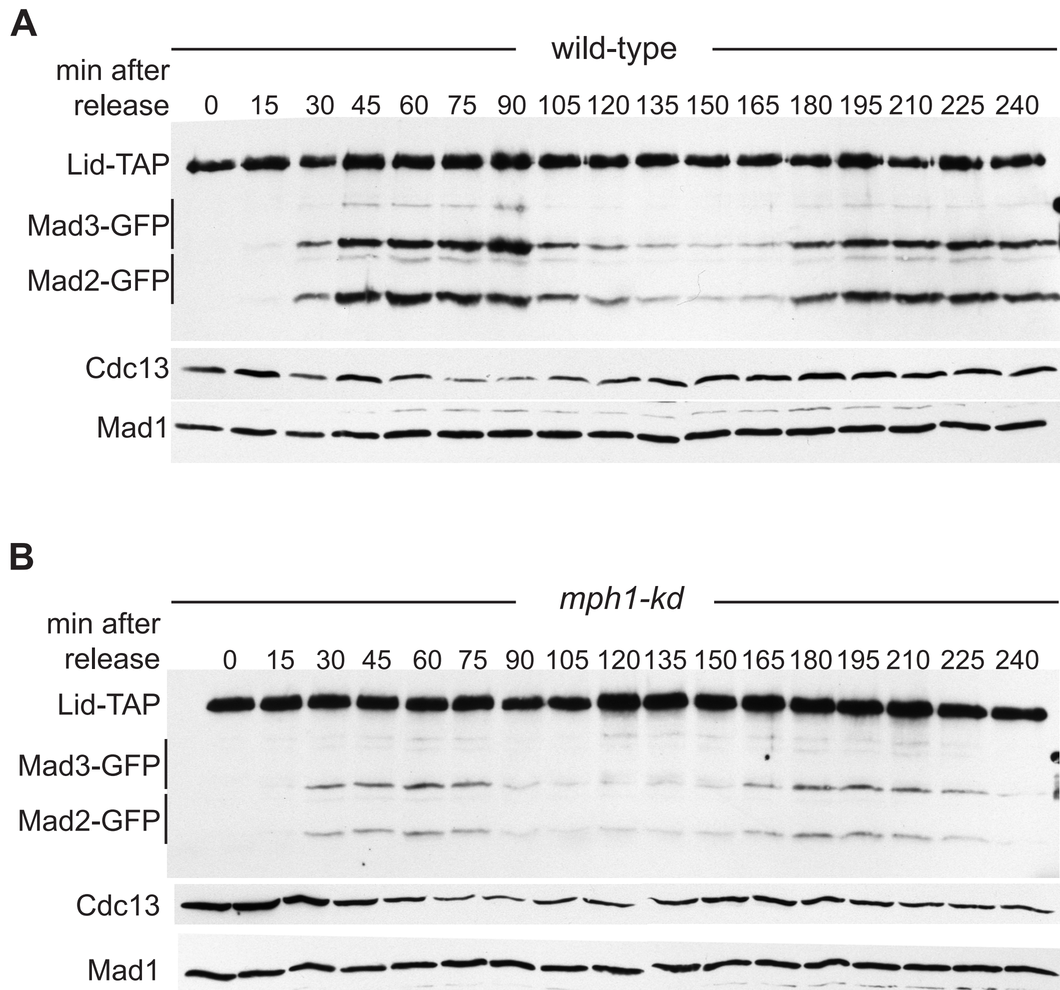

Figure 4.1: APC binding assays of wild-type (**A**) and *mph1-kd* (**B**) strains released into mitosis from a G2 arrest. Western blot analysis of Lid1-TAP (APC) pulldowns from native yeast extracts probed with α-GFP antibody to monitor co-immunoprecipitation of Mad2-GFP and Mad3-GFP. Immunoblots of lysates were probed with α-Cdc13 antibody to monitor entry and release from mitosis and α-Mad1 antibody as a loading control.

4.3 Mph1 kinase activity is required for Mad2 and Mad3 binding to the APC during an activated spindle checkpoint

In order to determine Mad2/Mad3 binding to the APC in spindle checkpoint activated cells, "APC binding assays" were carried out as described above. To maintain activation of the spindle checkpoint the drug carbendazim (CBZ) was added to the culture 20 min after release from G2-phase, when the cells had already entered mitosis. CBZ is a microtubule destabilising drug that leads to unattached kinetochores and lack of tension and thus activates the spindle checkpoint (Walker, 1982). In order to determine whether cells arrested in metaphase upon addition of CBZ cells were analysed microscopically using DAPI and calcofluor to stain DNA and septa, respectively.

Figure 4.2A shows Mad2/Mad3 binding to the APC in wild-type cells after release from a G2-phase arrest into mitosis and activation of the spindle checkpoint. Compared to cycling cells the levels of Mad2 and Mad3 bound to the APC were significantly higher in spindle checkpoint arrested cells. Unlike in cycling cells the levels of Mad2/Mad3 binding did not drop but stayed high throughout the 2 hrs time-course, which represents inhibition of the APC during the spindle checkpoint arrest. On the contrary, levels of Mad2/Mad3 binding in *mph1-kd* and *mph1Δ* cells remained as low as in cycling cells (Figure 4.2B). Monitoring the spindle checkpoint arrest using microscopy showed that the wild-type cells arrested in mitosis, whereas *mph1-kd* and *mph1Δ* cells failed to arrest (data not shown). These results showed that Mph1 kinase activity is required for efficient Mad2/Mad3 binding to the APC in an unperturbed mitosis as well as in spindle checkpoint activated cells.

Mph1 is only one of three known spindle checkpoint kinases. The next section describes the role of the spindle checkpoint kinase Ark1 in Mad2 and Mad3 binding to the APC.

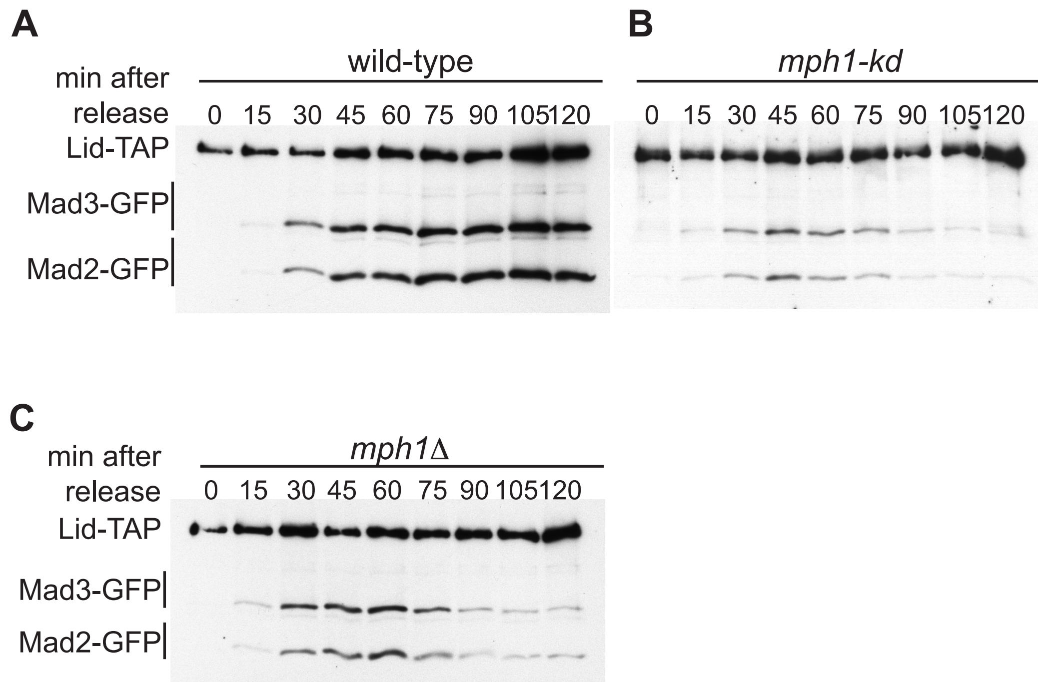

Figure 4.2: APC binding assays of wild-type (**A**), *mph1-kd* (**B**) and *mph1*Δ (**C**) released from a G2 arrest into mitosis with the addition of CBZ after 20 min to activate the spindle checkpoint. Western blot analysis of Lid1-TAP (APC) pulldowns from native yeast extracts probed with α-GFP antibody to monitor co-immunoprecipitation of Mad2-GFP and Mad3-GFP.

4.4 Ark1 kinase activity is required for the binding of Mad2 and Mad3 to the APC

In order to determine the role of the kinase activity of the spindle checkpoint kinase Ark1/Aurora in Mad2/Mad3 binding to the APC "APC binding assays" were carried out as described above. All experiments were performed with spindle checkpoint activated cells.

Ark1 experiments were performed by Kevin Hardwick using an analogue-sensitive version of Ark1 (*ark1-as*) (Hauf et al., 2007). Kinase activity of ark1-as can be inhibited with the ATP-analogue 4-amino-1-tert-butyl-3-(1-napthylmethyl)pyrazolo[3,4-d]pyrimidine (1NM-PP1). Ark1 is required for maintenance of the spindle checkpoint and inhibiting Ark1 kinase activity releases cells from a spindle checkpoint arrest (Vanoosthuyse and Hardwick, 2009).

Figure 4.3 shows an APC binding assay of an *ark-as* strain released from a G2-phase arrest into mitosis. To inhibit Ark1 kinase activity 1NMPP1 was added 15 min after release from G2-phase into mitosis. CBZ was then added 5 min later in order to activate the spindle checkpoint. Figure 4.3 shows that Mad2/Mad3 binding levels to the APC in *ark-as* cells were similar to wild-type without the inhibition of Ark1 kinase activity. However, upon inhibition of Ark1 kinase activity binding levels of Mad2 and Mad3 dropped significantly to a level similar observed for *mph1-kd* and *mph1Δ* (Figure 4.2). Monitoring the spindle checkpoint arrest, by scoring septation, it was apparent that *ark1-as* cells were immediately released from the arrest after the inhibition of kinase activity (data not shown).

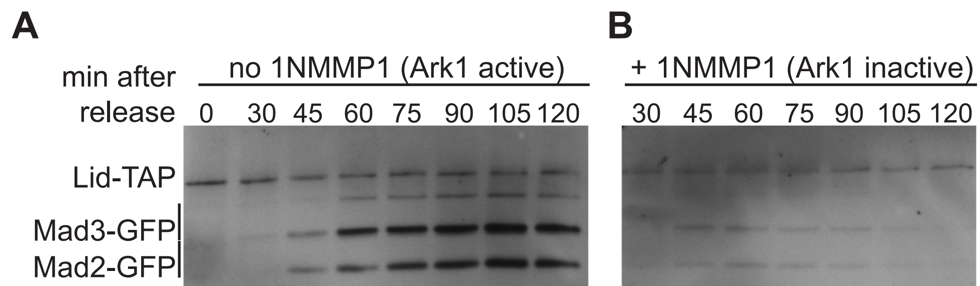

Figure 4.3: APC binding assays of *ark1-as* cells released from a G2 arrest into mitosis with (**A**) the addition of CBZ after 20 min to activate the spindle checkpoint and (**B**) the addition of 1NMMP1 (15 min) to inactivate Ark1 and CBZ after 20 min to activate the spindle checkpoint. Western blot analysis of Lid1-TAP (APC) pulldowns from native yeast extracts probed with α-GFP antibody to monitor co-immunoprecipitation of Mad2-GFP and Mad3-GFP.

4.5 Summary and Discussion

The anaphase promoting complex (APC) is an E3 ubiquitin ligase that drives cells into anaphase by targeting the separase inhibitor securin/Cut2 and cyclin B/Cdc13 for degradation by the 26 S proteasome (Cohen-Fix et al., 1996; Funabiki et al., 1996). Upon activation of the spindle checkpoint the APC is inhibited by the mitotic checkpoint complex (MCC) (Morrow et al., 2005; Sczaniecka et al., 2008). In fission yeast this inhibition is dependent on Cdc20, Mad2 and Mad3 (Sczaniecka et al., 2008).

The aim of this chapter was to answer two main questions. First, does Mad2 and Mad3 binding to the APC take place in an unperturbed mitosis? If yes, is Mph1 kinase activity required for this binding event? Second, are the kinase activities of the spindle checkpoint kinases Mph1 and Ark1 required for Mad2/Mad3 binding to the APC during an activated spindle checkpoint?

The questions were answered as follows. First, Mad2 and Mad3 bind to the APC during an unperturbed mitosis and this binding is dependent on Mph1 kinase activity. Second, the kinase activities of Mph1 and Ark1 are required for efficient Mad2-Mad3 binding in spindle checkpoint activated cells. Figure 4.4 at the end of this chapter shows a model of the role of Mph1 kinase activity in formation and binding of the MCC to the APC.

The observations described above showed that Mad2 and Mad3 bind to the APC not only during an activated spindle checkpoint but also during an unperturbed mitosis. These findings are consistent with previous studies in fission yeast and human that showed that Mad2 and Mad3 bind to the APC every mitosis (Herzog et al., 2009; Sczaniecka et al., 2008). These results led to the hypothesis that in fission yeast the spindle checkpoint is active during an unperturbed mitosis like in vertebrate cells. The APC is inhibited to some extent during every mitosis, maybe to prevent premature anaphase onset before all the sister-chromatids are bi-oriented on the mitotic spindle.

In this chapter the role of Mph1 and Ark1 kinase activity during an activated spindle checkpoint was investigated. The results showed that the kinase activitiy of Mph1 and Ark1 is required for efficient Mad2/Mad3 binding to the APC. However, whilst the results from this study showed that Mad2/Mad3 binding to the APC is dependent on the kinase activity of Mph1 and Ark1 they do not prove that it is due to direct phosphorylation of spindle checkpoint proteins. There are several possibilities as to how the kinase activity of Mph1 and Ark1 could regulate Mad2/Mad3 binding to the APC. First, Mph1 and Ark1 could directly phosphorylate spindle checkpoint proteins, Cdc20 or the APC. Second, they could phosphorylate other kinases, such as each other or Bub1, and activate them so they can phosphorylate spindle checkpoint proteins, Cdc20 or the APC. Third, they could phosphorylate spindle checkpoint scaffolding proteins such as Mad1 or Bub1.

The MCC can be formed independently of kinetochores (Poddar et al., 2005; Sczaniecka et al., 2008). However, the MCC appears to be unable to inhibit the APC without checkpoint proteins being present at the kinetochore (Sczaniecka et al., 2008). This suggests that the MCC requires post-translational modifications in order to be functional. One possible site where components of the MCC could be modified is the kinetochore.

The current model postulates that the Mad1-Mad2 complex is localised to kinetochores and catalyses Mad2-Cdc20 binding and thus the inhibition of the APC (De Antoni et al., 2005, Sironi et al., 2002). It is known that Mad1 localisation to kinetochores depends on Mps1(Abrieu et al., 2001; Fisk and Winey, 2001; Liu et al., 2003) and Bub1 (Sharp-Baker and Chen, 2001). Phosphorylation of Mad1 or Mad2 by Mph1 could regulate Mad1-Mad2 complex formation and thus APC inhibition. Mad1 phosphorylation has not been demonstrated in fission yeast, but Mad1is phosphorylated by Mps1 in budding yeast (Hardwick et al., 1996) and by human Bub1 kinase *in vitro* (Seeley et al., 1999). Furthermore Mph1 could phosphorylate Mad2 or Mad3 at kinetochores or after the MCC has formed. This study showed that Mad2 and Mad3 are *in vitro* substrates of Mph1 and that phosphorylation of Mad2 by Mph1 is required for efficient Mad2/Mad3 binding to the APC (see chapter 5).

These results are supported by findings in mammalian studies, that showed that BubR1/Mad3 phosphorylation is dependent on Mps1 and that this phosphorylation is required for APC inhibition *in vitro* (Huang et al., 2008). Mad3 in budding yeast has been identified as an Ipl1/Aurora B substrate and phosphorylation of Mad3 by Ipl1/Aurora B is required for the spindle checkpoint. However, the role of Mad3 phosphorylation with regards to APC inhibition still has to be determined (King et al., 2007a).

Another component of the MCC is Cdc20. In fission yeast Mad2 and Mad3 binding to the APC is dependent on Cdc20. The current model is that Mad2 and Mad3 bind to the APC indirectly through Cdc20 (Sczaniecka et al., 2008). Cdc20 phosphorylation could be required to mediate this binding. Cdc20 is phosphorylated in fission yeast but the physiological role of Cdc20 phosphorylation has not been determined yet. However, Bub1 has already been shown to regulate APC activity by phosphorylating human Cdc20 (Chung and Chen, 2003; Tang et al., 2004) and Cdc20 phosphorylation is required for the spindle checkpoint in *Xenopus* (Chung and Chen, 2003).

Furthermore, phosphorylation of the APC itself could regulate Mad2 and Mad3 binding to the APC. Phosphorylation of APC subunits in fission yeast has been described before (Yamada et al., 1997; Yoon et al., 2006). Even though phosphorylation of the APC by Mps1 has not been reported, human Mps1 interacts with the APC subunits Cdc16/Cut9 and Cdc27/Nuc2 (Liu et al., 2003).

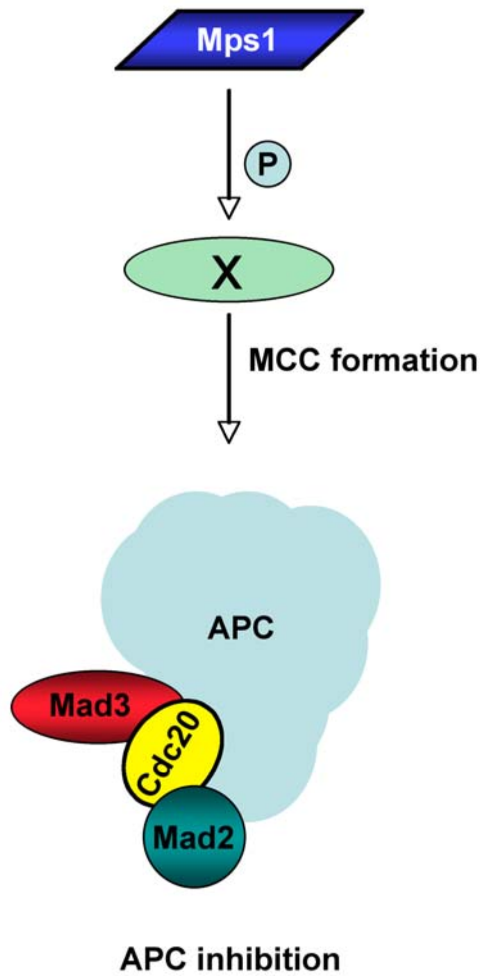

Figure 4.4: Model of the role of Mph1 kinase activity in formation and binding of the mitotic checkpoint complex (MCC) to the APC.

Chapter 5

Analysis of Mph1 spindle checkpoint substrates

Chapter 5: Analysis of Mph1 spindle checkpoint substrates

5.1 Introduction

Fission yeast Mph1 kinase activity is essential for the spindle checkpoint, correct chromosome segregation and cell viability (see chapter 3). Furthermore, Mph1 kinase activity is required for efficient Mad2 and Mad3 binding to the APC (see chapter 4). These findings strongly support the hypothesis that Mph1could regulate the spindle checkpoint by phosphorylating spindle checkpoint proteins, APC subunits and/or kinetochore subunits.

Only a few substrates of Mps1 in the context of the spindle checkpoint have been identified. These include the spindle checkpoint protein Mad1 and the kinetochore subunit Ndc80 in budding yeast (Hardwick et al., 1996; Kemmler et al., 2009). Furthermore, human Mps1 phosphorylates the chromosomal passenger complex component borealin (Jelluma et al., 2008b). However, to date no substrates of Mph1 in fission yeast have been identified. The aim of this study was to identify Mph1 substrates and analyse the physiological role of their phosphorylation with regards to the spindle checkpoint.

Mad2 and Mad3 are essential components of the spindle checkpoint (He et al., 1997; Millband and Hardwick, 2002) that localise to unattached kinetochores during metaphase (Ikui et al., 2002; Millband and Hardwick, 2002). Mad2 localisation to kinetochores is dependent on Mps1 and the current model is that Mad2 is activated at kinetochores for its role in APC inhibition (Mad2 template model) (De Antoni et al., 2005) (see section 1.6.2.1.3.3). Furthermore, Mad2 and Mad3 binding to the APC is dependent on Mph1. Therefore, this study concentrated on Mad2 and Mad3 as Mph1 candidate substrates.

Three questions were addressed. First, are Mad2 and Mad3 Mph1 substrates? Second, if yes, which residues are phosophorylated by Mph1? Third, are phosphorylation mutants of Mad2 and Mad3 defective in spindle checkpoint activity

and APC binding? The three questions were addressed as follows. First, *in vitro* kinase assays were carried out to test whether Mad2 and/or Mad3 are Mph1 substrates. Second, Mph1 phosphorylation sites in Mad2 and Mad3 were determined *in vitro* and/or *in vivo* using mass spectrometry. Third, phosphorylation mutants were created and analysed for their functionality in the spindle checkpoint performing benomyl sensitivity assays, mitotic arrest time-courses and APC binding assays.

5.2 Mad2 and Mad3 are *in vitro* substrates of Mph1

As Mad2 and Mad3 recruitment to the APC is dependent on Mph1 kinase activity *in vitro* kinase assays were carried out in order to determine whether Mad2 and Mad3 are substrates of Mph1. *In vitro* kinase assays using γ–^{32}P-ATP were carried out as described previously using recombinant Mad2-His and Mad3-MBP as substrates. An *in vitro* kinase assay with the tag MBP (maltose binding protein) was done as a negative control to show that Mad3 phosphorylation was specific to Mad3. Mad2-His, Mad3-MBP and MBP were expressed and purified from *E.coli* (Mad2: this study, Mad3 and MBP: Sjaak van der Sar). Mph1-STAP and Mph1-kd-STAP were two step tandem affinity purified from native yeast extracts. Mph1-kd-STAP was used as a negative control to verify that phosphorylation of substrates was specific to Mph1 and not a co-purified contaminating kinase. Kinase assays were run on SDS protein gels and phosphorylation visualised on autoradiographs.

Figure 5.1 shows that Mph1 phosphorylated Mad2 and Mad3 *in vitro*. No signal could be detected for MBP on the autoradiograph, which confirmed that the signal detected for Mad3 was specific.

These results showed that Mad2 and Mad3 are substrates of Mph1 *in vitro*. To determine whether phosphorylation of Mad2 and Mad3 by Mph1 is relevant *in vivo*, phosphorylation sites were mapped so that mutants could be created and analysed with regards to the spindle checkpoint.

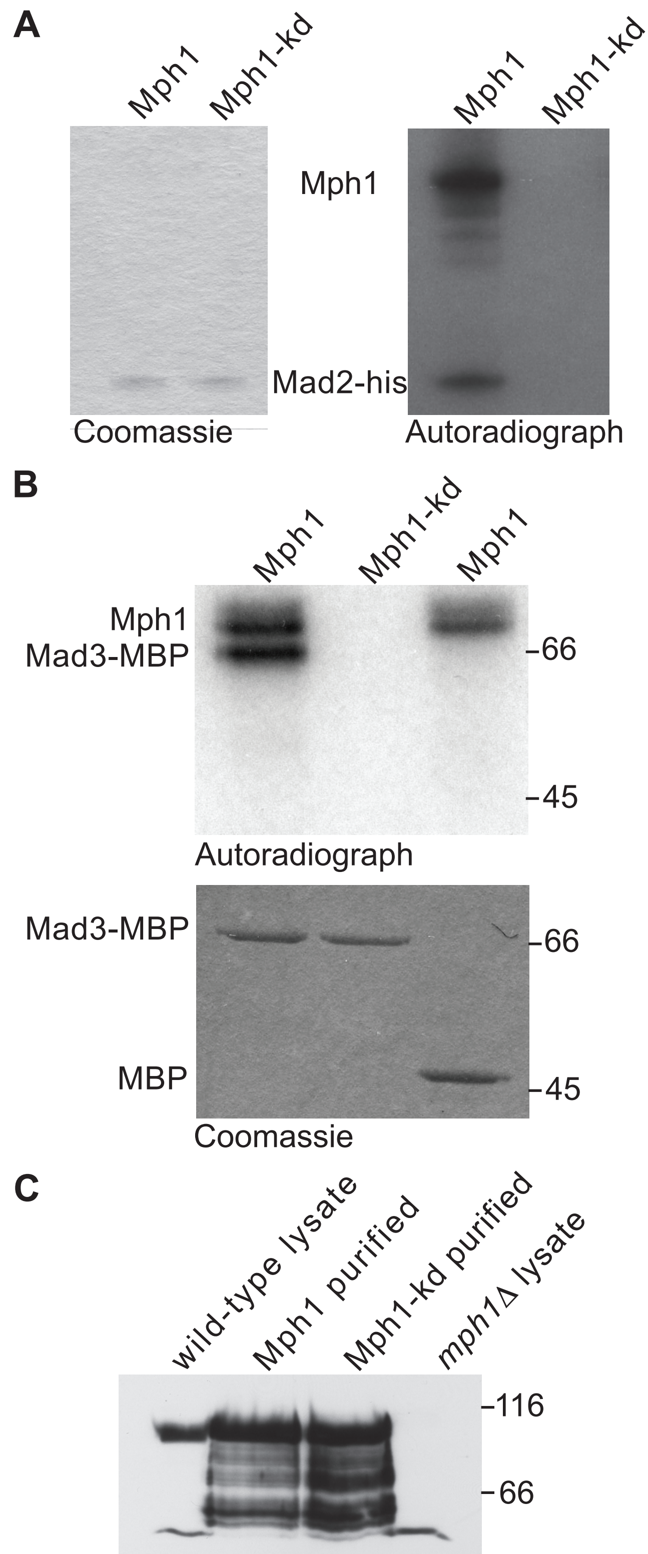

Figure 5.1: Mad2 and Mad3 are in vitro substrates of Mph1. *In vitro* kinase assays of Mph1-STAP or Mph1-kd-STAP with recombinant Mad2-his (**A**) and Mad3-MBP (**B**) (MBP-maltose binding protein). (**C**) Western blot analysis of tandem affinity purifications of Mph1-STAP and Mph1-kd-STAP from native yeast extracts probed with α-Mph1 antibody.

5.3 Analysis of the Mph1 substrate Mad3

5.3.1 *in vivo* Mad3 phosphorylation is dependent on Mph1

Figure 5.1 showed that Mad3 is an *in vitro* substrate of Mph1. To determine whether *in vivo* Mad3 phosphorylation is also dependent on Mph1 two dimensional gel (2D-gel) analysis was carried out. A wild-type strain carrying Mad3-TAP and an Mph1 kinase activity deficient strain (*mph1KΔ-GFP*) carrying Mad3-TAP were mitotically arrested using the pREP-*mad2*$^+$ overexpression plasmid. Proteins were first separated according to their isoelectric point using isoelectric focusing. Each phosphate group added to a protein changes its isoelectric point, which allows the separation of different phosphorylated forms of a protein. Following isoelectric focusing the proteins were run on a SDS page (second dimension) and western blot analysis carried out using PAP (peroxidase-coupled anti-peroxidase) antibody.

Figure 5.2 shows that several spots were visible for Mad3 in a strain containing active Mph1 kinase. However, Mad3-TAP in the Mph1 kinase activity deficient background showed a different pattern with less spots visible. This suggested that one or more phosphorylated forms of Mad3 were not present in the absence of Mph1 kinase activity and that phosphorylation of Mad3 *in vivo* is partially dependent on Mph1 kinase activity. These results were confirmed by carrying out 2D-gel analysis as described above with strains carrying Mad3-GFP instead of Mad3-TAP and western blot analysis using α-GFP antibody (data not shown).

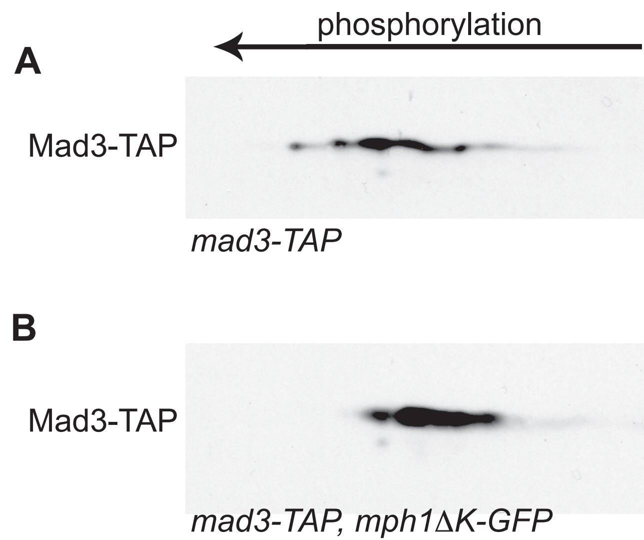

Figure 5.2: Mad3 phosphorylation is dependent on Mph1. 2D-gel analysis of Mad3-TAP in a (**A**) wild-type background and a (**B**) Mph1 kinase activity deficient background (*mad3-TAP*, *mph1ΔK-GFP).* Immunoblots of Mad3-TAP were probed with PAP antibody (recognises TAP tag).

5.3.2 Identification of phosphorylation sites

In order to analyse the physiological role of Mad3 phosphorylation by Mph1, phosphorylation sites were identified and phosphorylation mutants created. To identify phosphorylation sites, *in vitro* kinase assays were carried out as described previously using Mph1 kinase and recombinant Mad3-MBP. Phosphopeptide enrichment, followed by mass spectrometry was carried out with the proteins present in the *in vitro* kinase reaction. In order to carry out mass spectrometry the whole kinase assay reaction was run on a precast gel about 5 mm into the gel. The gel was coomassie stained, to visualise the proteins, and the section of the gel containing the proteins cut out and an in gel tryptic digest performed. Phosphopeptide enrichment was carried out on some samples prior to mass spectrometric analysis. (Tryptic digest, phosphopetide enrichment and mass spectrometry were carried out by H. Syred, Okhura/Rappsilber lab, University of Edinburgh.)

Sixteen *in vitro* Mph1 phosphorylation sites in Mad3 were identified (Table 5.1, Figure 5.3). Furthermore, mass spectrometric analysis of spindle checkpoint complexes (Sjaak van der Sar, Hardwick lab, unpublished data; mass spectrometry: YRC, San Diego) from native yeast extracts, confirmed six of the phosphorylation sites identified *in vitro* and identified two additional sites that were not identified *in vitro* (Table 5.1). To be able to analyse the physiological role of these phosphorylation sites a phosphorylation site mutant was generated, that had the sixteen *in vitro* and the additional two *in vivo* sites replaced with alanines.

Amino acid	Peptide identified	Identified		
		in vitro	*in vivo*	
			mitotic arrest	interphase
S19	NWVHMDVIEQ**S**KENIEPR	+	+	
S31	KAGH**S**ASALAK	+	+	
S33	KAGHSA**S**ALAK	+	+	
S38	AGHSASALAKSS**S**RNHTEKEVAGLQK	+		
S40	AGHSASALAK**S**SSRNHTEKEVAGLQK	+		
T44	NH**T**EKEVAGLQK	+		
T64	KIE**T**SESLDDPLQVWIDYIK	+		
T93	**T**SGLVTLLER	+		
S94	T**S**GLVTLLER	+		
T195	YQQF**T**HR	+		
S246	FKFSVY**S**DADGSGK			+
S251	FKFSVYSDADG**S**GK		+	+
T259	DGQPG**T**WQTLGTVDQR	+	+	
T262	DGQPGTWQ**T**LGTVDQRR	+		
T265	DGQPGTWQTLG**T**VDQR	+		
S276	ENNI**S**ATSWVGEKLPLK	+	+	
T278	ENNISA**T**SWVGEKLPLK	+	+	
S279	ENNISAT**S**WVGEKLPLK	+	+	

Table 5.1: Mph1 phosphorylation sites in Mad3. Phosphorylation sites were identified by mass spectrometry after phosphopetide enrichment. *In vitro* sites were identified from *in vitro* kinase assays with Mph1 kinase and recombinant Mad3-MBP. *In vivo* sites were identifed from spindle checkpoint complexes purified from native yeast extracts. For alignment of Mad3 protein sequences from different species see Supplementary Figure S.3.

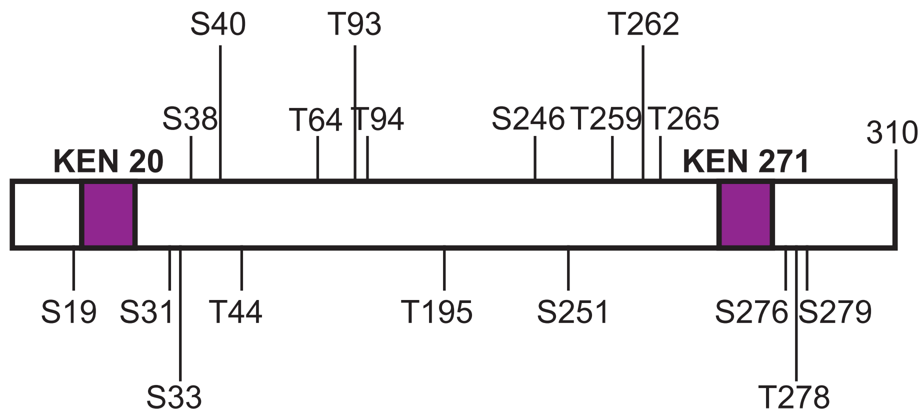

Figure 5.3: Schematic of Mad3 showing the phosphorylation sites identified by mass spectrometry. The two conserved KEN boxes in the N- and C-terminus of Mad3 are coloured in purple.

5.3.3 Cloning of a Mad3 phosphorylation mutant

To create a strain carrying a phosphorylation mutant of Mad3 the whole *mad3* gene with the18 identified phosphorylation sites (serines and threonines) substituted to alanines, was synthesised by Geneart (Table 5.1). The gene was then cloned into the pJK148 vector that contains a leu1 marker for growth in fission yeast (A.Sochaj, Hardwick lab). 500 bp of 5'UTR of *mad3* were inserted in front of the *mad3-18A* sequence, containing the endogenous promoter to enable expression of *mad3-18A* from the leu1 locus. Furthermore, a C-terminal GFP tag was introduced to enable detection of Mad3 by western blot analysis, as currently no good Mad3 antibody is available in the lab. *mad3-18A-GFP* was integrated at the leu1 locus into a strain lacking wild-type Mad3 (*mad3Δ*) and expression and stability of Mad3-18A-GFP was confirmed by western blot analysis (Figure 5.4).

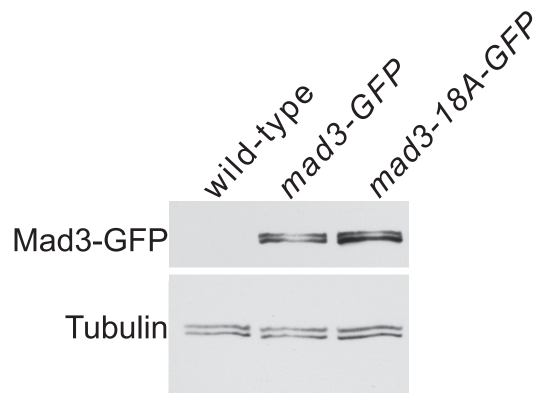

Figure 5.4: Protein stability analysis of Mad3-18A-GFP Western blot analysis of protein extracts from wild-type, *mad3-GFP* and *mad3-18A-GFP* cells. Immunoblot was probed with α-GFP antibody and anti-tubulin (TAT1, loading control) antibody.

5.3.4 Analysis of *mad3-18A-GFP*

In order to analyse the role of Mad3 phosphorylation by Mph1 in the spindle checkpoint, three assays were carried out with the phosphorylation mutant *mad3-18A-GFP*. First, sensitivity of *mad3-18A-GFP* to the microtubule depolymerising drug benomyl was tested. Second, time-courses were carried out with the temperature sensitive tubulin mutant *nda-KM311* and the ability of *mad3-18A-GFP* to spindle checkpoint arrest was determined. Third, APC binding assays were carried out to test whether binding of Mad3-18A-GFP to the APC was perturbed.

5.3.4.1 *mad3-18A-GFP* is not benomyl sensitive

In order to determine whether *mad3-18A* is sensitive to the microtubule depolymerising drug a benomyl sensitivity assay was carried out as described previously. 10-fold serial dilutions of wild-type, *mph1* null mutant, *mad3* null mutant, *mad3-18A-GFP* and *mad3-GFP* cells were spotted on YES plates containing 0 or 6 μg/ml benomyl and incubated for three days at 25 °C. The *mph1* null mutant and *mad3* null mutant strains were used as positive controls as *mph1* null mutant cells are known to be hypersensitive to benomyl whereas *mad3* null mutant cells are less benomyl sensitive (Vanoosthuyse et al., 2004).

Figure 5.5 shows that as expected wild-type cells and *mad3-GFP* cells were not benomyl sensitive, whereas *mph1* null mutant cells were hypersensitive to benomyl. The *mad3* null mutant cells showed an intermediate phenotype. *mad3-18A-GFP* did not show any obvious benomyl sensitivity. However, benomyl sensitivity is not a very sensitive assay and is just a rough indicator for spindle checkpoint mutants. Therefore, further analysis of *mad3-18A-GFP* with regards to spindle checkpoint function was carried out.

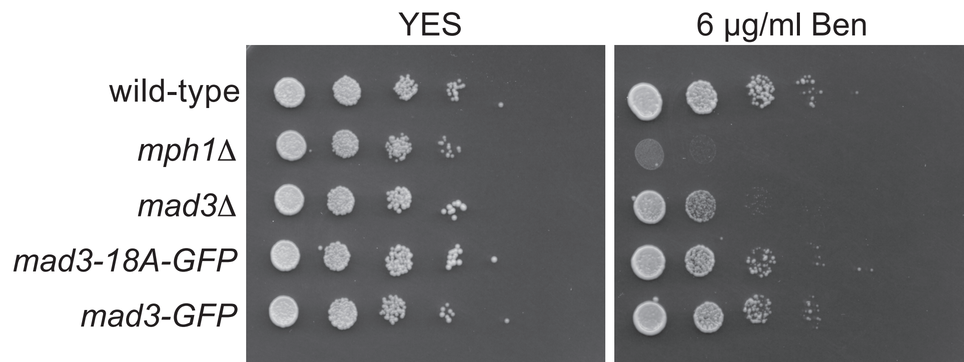

Figure 5.5: Benomyl sensitivity of mad3 mutant strains.
Benomyl sensitivity assays of wild-type, *mph1*Δ, *mad3*Δ and *mad3-18A-GFP* strains. 10-fold serial dilutions were spotted on YES plates containing 0 or 6 μg/ml benomyl. Plates were incubated for 3 days at 25 °C.

5.3.4.2 *mad3-18A-GFP* has a spindle checkpoint maintenance defect

To determine the ability of *mad3-18A-GFP* to spindle checkpoint arrest in response to unattached kinetochores *nda3-KM311* time-courses were performed as described previously. Cultures of *mad3-18A-GFP* were grown overnight at the permissive temperature of 30 °C and then shifted to the restrictive temperature of 18 °C for 8 hrs. Time-points were taken before the temperature-shift (0 hrs) and after 6 and 8 hrs. The cells were methanol fixed, stained with calcofluor to visualise septa and analysed using microscopy. Cells that are arrested in metaphase do not contain a septum. Therefore, septa were scored as an indicator of cells that were not arrested in metaphase but had progressed through anaphase and initiated cyotkinesis.

Figure 5.6 shows that after 6 hrs 1 % of wild-type cells had a septum, compared to 17.5 % present in cycling cells. Preliminary results for the *mad3-18A-GFP* mutant showed that *mad3-18A-GFP* cells had a higher percentage of septation (8 %). To determine whether this higher percentage of sepated cells in *mad3-18A-GFP* cells was due to inability to arrest or due to inability to maintain the arrest cultures were kept at 18 °C and another sample taken after 8 hrs. Figure 5.6 shows that the wild-type cells stayed arrested in metaphase with a low percentage of septation (4.5 %) after 8 hrs. However, the percentage of septation increased significantly in *mad3-18A-GFP* cells to 44.5 % after 8 hrs.

These results suggested that *mad3-18A-GFP* has a spindle checkpoint arrest maintenance defect. To determine whether this defect was due to defective binding of Mad3-18A-GFP to the APC, “APC binding assays” were carried out.

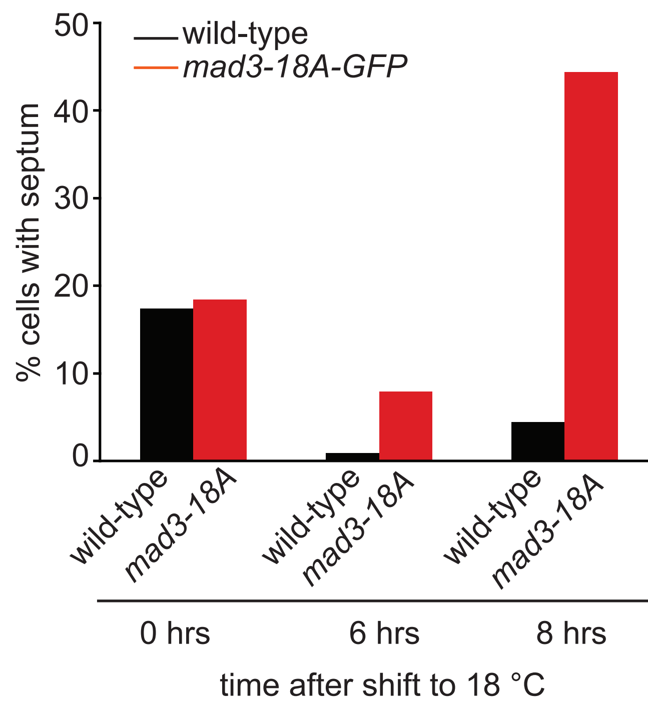

Figure 5.6: Mad3-18A-GFP causes a spindle checkpoint maintenance defect. Mitotic arrest time-courses of *nda3-KM311* strains carrying wild-type or *mad3-18A-GFP.* Cells that were not in mitosis were scored as a percentage of cells with a septum. The data in this graph are preliminary but reproducible. The numbers shown are from one of two experiments performed.

5.3.4.3 Analysis of Mad3-18A-GFP binding to the APC

In order to determine the ability of Mad3-18A-GFP to bind to the APC during an activated spindle checkpoint "APC binding assays" were carried out as described previously. The temperature sensitive *cdc25-22* mutant carrying *mad3-18A-GFP* was used. Cells were grown at 25 °C to mid-log phase and then blocked in G2-phase at the restrictive temperature of 36 °C for 3.5 hrs. Cells were then synchronously released into mitosis by shifting the temperature back to the permissive temperature of 25 °C. To activate the spindle checkpoint the drug carbendazim (CBZ) was added to the culture 20 min after release from G2-phase, when cells had already entered mitosis. CBZ is a microtubule destabilising drug that leads to unattached kinetochores and lack of tension and thus activates the spindle checkpoint (Walker, 1982). Samples were taken at 15 min time-points for 2 hrs after release from G2-phase into mitosis.

Figure 5.7 shows that the binding pattern of Mad3-18A and Mad2 binding to the APC in the *mad3-18A-GFP* strain was similar to Mad2 and Mad3 binding in a wild-type strain. However, there was a subtle difference between the two strains. The assay was performed twice and both times at the last time-point after 120 min the levels of Mad2-GFP and Mad3-18A-GFP binding to the APC decreased more in *mad3-18A-GFP* cells than in wild-type cells. Importantly, the difference observed was only subtle and to determine whether this difference is significant further analysis will have to be carried out in the future with later time-points. However, these results suggested that *mad3-18A-GFP* might have a spindle checkpoint maintenance defect and strengthen the result from the *nda3-KM311* spindle checkpoint assay. Scoring of the arrest by septation showed that *mad3-18A-GFP* cells arrested in metaphase at a similar level to wild-type cells upon treatment with CBZ (Figure 5.7).

Taken together the results described above showed that the phosphorylation mutant *mad3-18A-GFP* appears to have a spindle checkpoint maintenance defect. However,

Mad2 and Mad3 binding to the APC does not seem to be perturbed at early time-points during a CBZ induced spindle checkpoint arrest.

The second protein identified as an Mph1 substrate *in vitro* was Mad2. In the second half of the chapter the physiological role of Mad2 phosphorylation by Mph1 will be discussed using the same assays that were used to analyse *mad3-18A-GFP*.

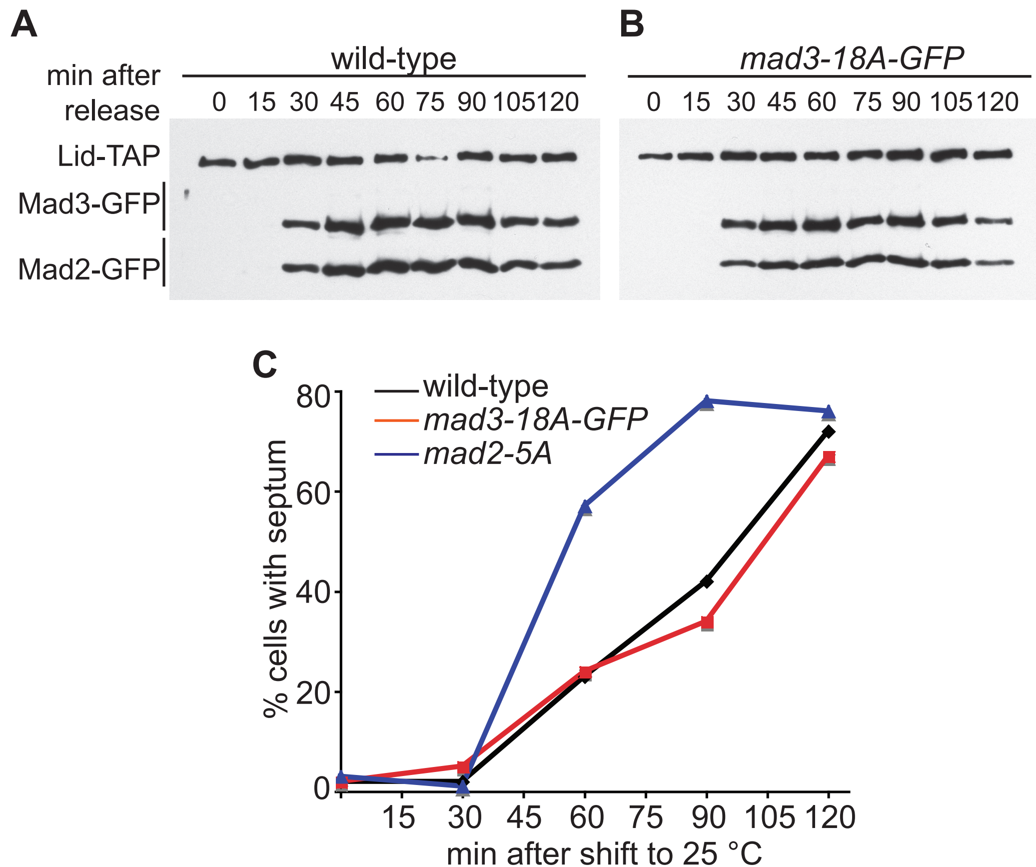

Figure 5.7: APC binding assays of wild-type (**A**) and *mad3-18A-GFP* (**B**) released from a G2 arrest into mitosis with the addition of CBZ after 20 min to activate the spindle checkpoint. Western blot analysis of Lid1-TAP (APC) pulldowns from native yeast extracts probed with α-GFP antibody to monitor co-immunoprecipitation of Mad2-GFP and Mad3-GFP/Mad3-18A-GFP. (**C**) Percentage of cells with septum after release from G2 into mitosis scored for wild-type, *mad3-18A-GFP* and *mad2-5A* (data for latter mutant taken from Figure 5.13).

5.4 Analysis of the Mph1 substrate Mad2

5.4.1 Identification of phosphorylation sites

In order to be able to analyse the physiological role for Mad2 phosphorylation by Mph1 phosphorylation sites were identified and then phosphorylation mutants created. Two approaches were used to identify Mad2 phosphorylation sites. First, *in vitro* kinase assays were carried out as described previously with Mph1 kinase purified from native yeast extracts and recombinant Mad2-His. Phosphopeptide enrichment followed by mass spectrometry was performed as described above (mass spectrometry: H. Syred, Okhura/Rappsilber lab, University of Edinburgh). Second, in order to confirm that these *in vitro* phosphorylation sites were also modified *in vivo*, Mad2 was purified from denatured yeast extracts and mass spectrometry was carried out (protein purification: A. Sochaj, Hardwick lab, mass spectrometry: H.Syred).

Table 5.2 shows the peptides identified *in vitro* and *in vivo*. Three phosphorylated peptides were identified *in vitro*. Peptide one showed phosphorylation on residue S69, peptide two on residue S92 and the third peptide identified was phosphorylated on three different sites (S187, T188, S189). This last peptide was also detected in the *in vivo* experiment but only showed phosphorylation on S187 and S189.

To be able to analyse the physiological role of these phosphorylation sites two phosphorylation mutants were cloned. The *mad2-5A* mutant, that had the five phosphorylation sites identified *in vitro* replaced with alanine. And in order to investigate the consequences of constant phosphorylation of Mad2 a phosphomimic mutant was cloned that contained substitution of S187, T188 and S189 to aspartic acid (*mad2-3D*).

Amino acid	Peptides identified	Identified *in vitro*	Identified *in vivo*: mitotic arrest	Identified *in vivo*: interphase
S69	IV**S**QLHK	+	-	-
S92	LILVITSKC**S**GEDLER	+	-	-
S187	SF**S**TSMHK	+	+	+
T188	SFS**T**SMHK	+	-	-
S189	SFST**S**MHK	+	+	-

Table 5.2: Mph1 phosphorylation sites in Mad2. Phosphorylation sites were identified by mass spectrometry after phosphopetide enrichment. *In vitro* sites were identified from *in vitro* kinase assays with Mph1 kinase and recombinant Mad2-his. *In vivo* sites were identifed from Mad2 purified from denatured yeast extracts.

A

```
      S.pombe  VKTYIRKIVSQLHKWMFAKKIQKLILVITSKCSGEDLERWQ  99
  S.japonicus  VKTYIRKIISQLHRWTCGGKIQKVALVITNKDSGEDLERWQ  100
 S.octosporus  VKAYIRRIIAQLHRWMYRGKIQKLVVVITDKDTGDDLERWQ  101
S.cerevisiae   LKDYIRKILLQVHRWLLGGKCNQLVLCIVDKDEGEVVERWS  95
   H.sapiens   LIKYLNNVVEQLKDWLYKCSVQKLVVVISNIESGEVLERWQ  101
                  *      *   *            *     *   ****

      S.pombe  ···· RDAEQVQLRSFSTSMHKIDCQVAYRVNP--  202
  S.japonicus  ···· KNAEQVQLRSFSTNMHKIDCQVAYRFDP--  203
 S.octosporus  ···· QNAEQVQLRSFSTNMHKIDCQVAYRMN---  205
S.cerevisiae   ···· PDGEVVQFKTFSTNDHKVGAQVSYKY----  196
   H.sapiens   ···· TNSEEVRLRSFTTTIHKVNSMVAYKIPVND  205
                       * *    ***  **    * *
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B

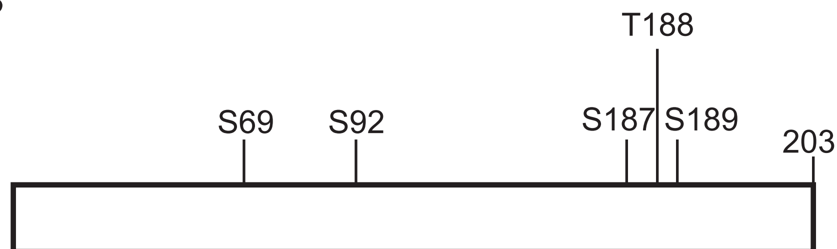


Figure 5.8: (**A**) Alignment of Mad2 protein sequences from different species. Phosphorylation sites identified by mass spectrometry in *S.pombe* are marked in red. For an alignment of the full Mad2 protein sequence see Supplementary Figure S.4. (**B**) Schematic of Mad2 showing the phosphorylation sites identified by mass spectrometry.

5.4.2 Cloning of Mad2 phosphorylation mutants

Fission yeast strains containing phosphorylation mutants of Mad2 were created using the pJK148 vector. pJK148 is a yeast shuttle vector that contains ampicillin resistance for selection in *E.coli* and a leu1 marker for growth in fission yeast. All *mad2* mutants were based on the plasmid pJK*mad2* that contained the *mad2* gene with the addition of 259 bp of 5'UTR containing the endogenous promoter of *mad2*, that enabled expression of *mad2* at the leu1 locus and 203 bp of 3'UTR. *mad2* (+5'UTR and 3'UTR) was amplified from fission yeast genomic DNA by PCR and cloned into the pJK148 vector using common molecular biology methods.

Using pJK*mad2,* three different *mad2* mutants were constructed by introducing amino acid substitutions using QuikChange site directed mutagenesis. pJK*mad2-5A* contained substitutions of the five phosphorylation sites identified by mass spectrometry (Table 5.2). pJK*mad2-3D* contained substitutions of S187, T188 and S189 to aspartic acid. In addition to these two phosphorylation mutants, a *mad2* mutant predicted to be unable to form a "conformational" dimer (*mad2-dimer*) was cloned as a negative control in addition to the *mad2* null mutant already present in the lab. The formation of a "conformational" Mad2 dimer is required for a functional spindle checkpoint in both human and budding yeast (De Antoni et al., 2005; Nezi et al., 2006). pJK*mad2-dimer* was based on mutants previously analysed in human and budding yeast that are unable to form a conformational dimer (De Antoni et al., 2005; Nezi et al., 2006). It contained substitutions of R132 to E and Q133 to A.

The three plasmids were integrated at the leu1 locus into a *mad2* null mutant strain that does not express wild-type *mad2*. Western blot analysis of protein extracts was carried out to confirm stable protein expression (Figure 5.9).

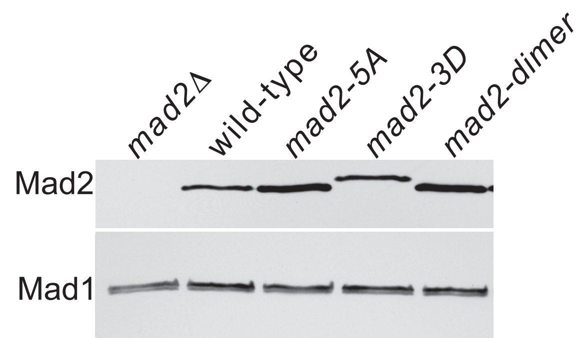


Figure 5.9: Protein stability analysis of Mad2 mutants Western blot analysis of protein extracts from *mad2Δ*, wild-type, *mad2-5A, mad2-3D* and *mad2-dimer* cells. Immunoblot was probed with α-Mad2 antibody and α-Mad1 antibody (loading control).

5.4.3 Analysis of Mad2 phosphorylation mutants

In order to analyse the importance of Mad2 phosphorylation by Mph1 in the spindle checkpoint three assays were carried out with the phosphorylation mutants *mad2-5A, mad2-3D* and the dimerisation mutant *mad2-dimer*. First, sensitivity of *mad2-5A, mad2-3D* and *mad2-dimer* to the microtubule destabilising drug benomyl was tested. Second, time-courses with the tubulin mutant *nda-KM311* carrying *mad2-5A*, *mad2-3D* or *mad2-dimer* were carried out to test the ability of the *mad2* mutants to spindle checkpoint arrest. In addition to time-courses with *nda3-KM311*, the ability of *mad2-5A* to spindle checkpoint arrest was assayed using the temperature sensitive *nuf2-3* mutant, that contains defective kinetochores when grown at the restrictive temperature (Nabetani et al., 2001). Third, APC binding assays were carried out to determine whether binding of Mad2-5A and Mad2-3D to the APC is perturbed.

5.4.4.1 Benomyl sensitivity of *mad2* mutants

In order to determine whether *mad2-5A*, *mad2-3D* and *mad2-dimer* are sensitive to microtubule destabilising drugs, benomyl sensitivity assays were carried out as described previously. 10-fold serial dilutions of wild-type, *mph1* null mutant, *mad2* null mutant, *mad2-5A*, *mad2-3D* and *mad2-dimer* cells were spotted on YES plates containing 0 or 6 μg/ml benomyl and incubated for three days at 25 °C. The *mph1* null mutant and *mad2* null mutant strains were used as positive controls as *mph1* null mutant cells are known to be hypersensitive to benomyl whereas *mad2* null mutant cells are less benomyl sensitive (Vanoosthuyse et al., 2004).

As expected wild-type cells did not show any benomyl sensitivity, *mph1* null mutant cells were very benomyl sensitive and *mad2* null mutant cells displayed an intermediate phenotype (Figure 5.10). *mad2-5A* cells were not sensitive to benomyl, whereas *mad2-3D* and *mad2-dimer* cells were as benomyl sensitive as *mad2* null mutant cells (Figure 5.10).

These results suggested that *mad2-5A* is not particularly spindle checkpoint defective, whereas *mad2-3D* and *mad2-dimer* displayed a *mad2* null mutant phenotype. However, benomyl sensitivity is not a very sensitive assay and is just a rough indicator for spindle checkpoint mutants. Therefore, further analysis of *mad2-5A* and *mad2-3D* with regards to spindle checkpoint function were carried out.

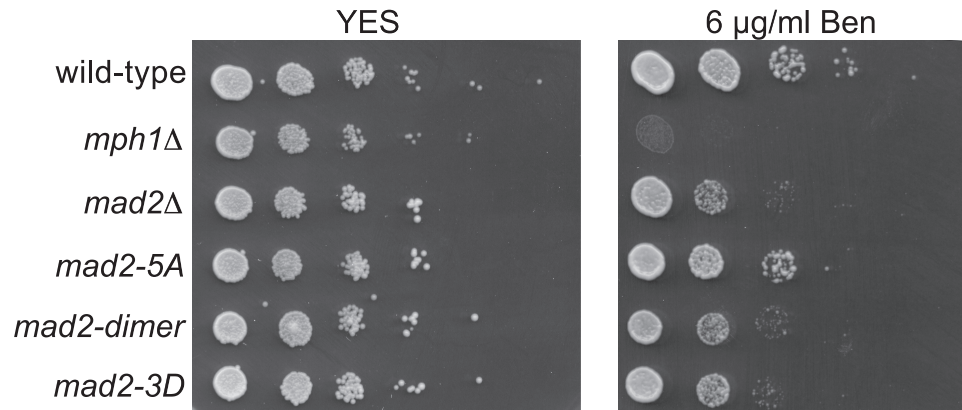


Figure 5.10: Benomyl sensitivity of mad2 mutant strains
Benomyl sensitivity assays of wild-type, *mph1*Δ, *mad2*Δ, *mad2-dimer*, *mad2-5A* and *mad2-3D* strains. 10-fold serial dilutions were spotted on YES plates containing 0 or 6 μg/ml benomyl. Plates were incubated for 3 days at 25 °C.

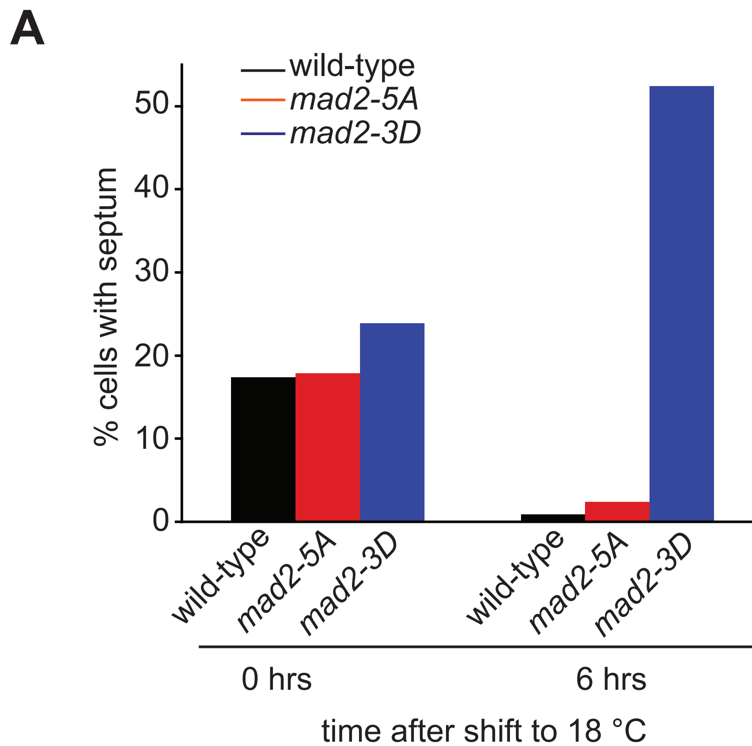
5.4.4.2 Analysis of *mad2* mutants for spindle checkpoint defects

5.4.4.2.1 *nda3-KM311* arrests

To determine the ability of the *mad2* mutants to spindle checkpoint arrest in response to completely unattached kinetochores *nda3-KM311* time-courses were performed. Overnight cultures of *mad2-5A*, *mad2-3D* and *mad2-dimer* were grown at the permissive temperature of 30 °C and then shifted to the restrictive temperature of 18 °C for 6 hrs. Samples were taken before the temperature shift (0 hrs) and after 6 hrs. The cells were methanol fixed, stained with calcofluor to visualise septa and analysed using microscopy. Cells that are arrested in metaphase do not contain a septum. Therefore, septa were scored as an indicator of cells that were not arrested in metaphase but had progressed through anaphase and initiated cyotkinesis.

Figure 5.11A shows that after 6 hrs 1 % of wild-type cells had a septum, compared to 17.5 % septation in cycling cells (0 hrs). The septation index of the *mad2-5A* mutant was similar to wild-type with 18 % septation at 0 hrs and 3% septation after 6 hrs. These results were confirmed carrying out *nda3-KM311* time-courses as described previously scoring spindle checkpoint arrested cells using localisation of polo kinase (Plo1) fused to GFP (GFP-Plo1) to spindle poles. Plo1 localises to spindle pole bodies (SPB) during mitosis but not interphase (Bahler et al., 1998a; Mulvihill et al., 1999). Mitotic cells were scored as cells containing two green spots at the SPBs. Figure 5.11B shows that after 6 hrs 88 % of wild-type cells were arrested in mitosis. *mad2-5A* also arrested in mitosis with a mitotic index of 87 %. *mad2-dimer* cells and *mad2Δ* cells did not arrest and the mitotic index was never higher than 22 % (Figure 5.11B). Preliminary results for the *mad2-3D* mutant suggested that they were unable to arrest in an *nda3-KM311* mutant, similar to a *mad2* null mutant or *mad2-dimer* mutant, with a septation index of 53 % after 6 hrs (Figure 5.11A).

These results showed that *mad2-5A* did not appear to have a spindle checkpoint defect using the *nda3-KM311* mutant, whereas the *mad2-dimer* and the *mad2-3D* mutants were clearly spindle checkpoint defective.



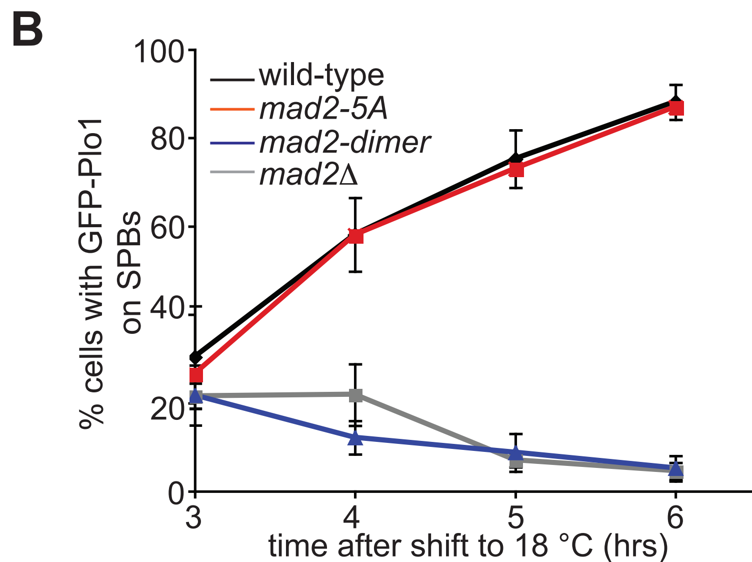


Figure 5.11: NDA3-KM311 spindle checkpoint time-courses of Mad2 mutants. Mitotic arrest time-courses with *nda3-KM311* strains of wild-type, *mad2-5A*, *mad2-dimer*, *mad2-3D* and *mad2Δ*. **A** Cells that were not in mitosis were scored as a percentage of cells with a septum. The data in this graph are preliminary and show numbers from one of two experiments performed. **B** Mitotic cells were scored as a percentage of cells with GFP-Plo1 localisation to spindle pole bodies.

5.4.4.2.2 *nuf2-3* arrests

The results from the *nda3-KM311* time-courses suggested that *mad2-5A* is able to arrest when all kinetochores are unattached. However, a second assay was carried out scoring its ability to arrest in metaphase in the presence of defective kinetochores. For this assay the temperature sensitive kinetochore mutant *nuf2-3* was used. *nuf2-3* cells grown at the restrictive temperature of 36 °C spindle checkpoint arrest in metaphase with short metaphase spindles (Nabetani et al., 2001).

To investigate whether the phosphorylation mutant *mad2-5A* is able to spindle checkpoint arrest *mad2-5A* was crossed into a *nuf2-3* background strain. In this study *nuf2-3* strains carrying wild-type *mad2* and *mad2-5A* were grown overnight at the permissive temperature of 25 °C. Cells were then shifted to the restrictive temperature of 32 °C for 4.5 hrs. After 4.5 hrs cells were methanol fixed and stained for tubulin using an anti-tubulin (TAT1) antibody. Metaphase arrested cells were scored counting short metaphase spindles.

Preliminary results both for wild-type and *mad2-5A* cells showed that after 4.5 hrs 35 % of wild-type cells had short-metaphase spindles, whereas only 10 % of *mad2-5A* cells had short metaphase spindles (Figure 5.12).

These preliminary results suggested that *mad2-5A* may be defective in responding to certain kinetochore defects. Further analysis will be carried out with the *mad2-5A* mutant to confirm the results described above. The next step taken was to determine the ability of the Mad2 mutants to bind to the APC during an activated spindle checkpoint.

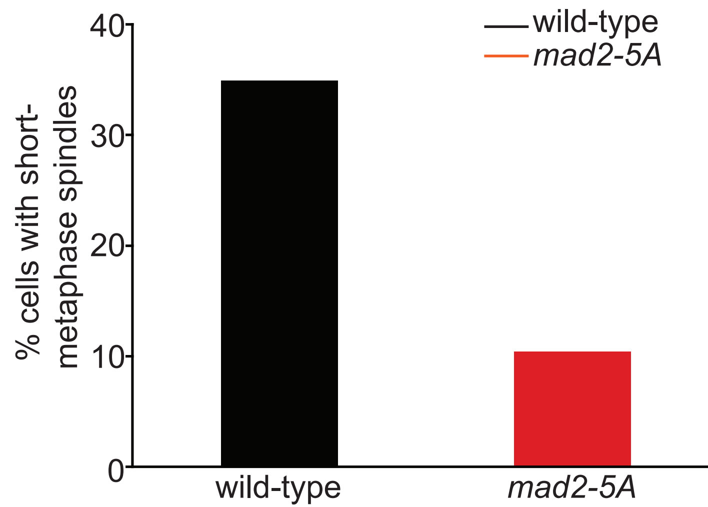


Figure 5.12: Mad2-5A causes a spindle checkpoint defect. Preliminary data of mitotic arrest time-courses with *nuf2-3* strains of wild-type and *mad2-5A.* Mitotic arrest was scored as the percentage of cells with short metaphase spindles.

5.4.5 *mad2-5A* and *mad2-3D* binding to the APC is perturbed

In order to determine the ability of Mad2-5A and Mad2-3D to bind to the APC during an activated spindle checkpoint "APC binding assays" were carried out. The temperature sensitive *cdc25-22* mutant carrying *mad2-5A*, *mad2-3D* or *mad2-dimer* was used. Cells were blocked in G2-phase at the restrictive temperature of 36 °C. Cells were then synchronously released into mitosis by shifting them to the permissive temperature of 25 °C. To activate the spindle checkpoint the drug carbendazim (CBZ) was added to the culture 20 min after release from G2-phase, when cells had already entered mitosis. CBZ is a microtubule destabilising drug that leads to unattached kinetochores and lack of tension and thus activates the spindle checkpoint (Walker, 1982). Samples were taken at 15 min time-points for 2 hrs after release from G2-phase into mitosis.

Figure 5.13 shows Mad2 and Mad3 binding to the APC in wild-type cells after release from a G2-phase arrest into mitosis and activation of the spindle checkpoint. The levels of Mad2 and Mad3 binding did not drop but stayed high throughout the 2 hrs time-course, which represents inhibition of the APC during the spindle checkpoint arrest. On the contrary, levels of Mad2-5A and Mad3 binding in *mad2-5A* were lower and did not remain high. In *mad2-3D* cells no Mad2-3D and very little Mad3 was detectable. This was also the case for the *mad2-dimer* mutant. Due to antibody issues only Mad3 binding to the APC has been determined for this mutant.

Scoring of the arrest by septation showed that neither *mad2-5A* cells nor *mad2-3D* cells spindle checkpoint arrested effectively upon treatment with CBZ (Figure 5.13).

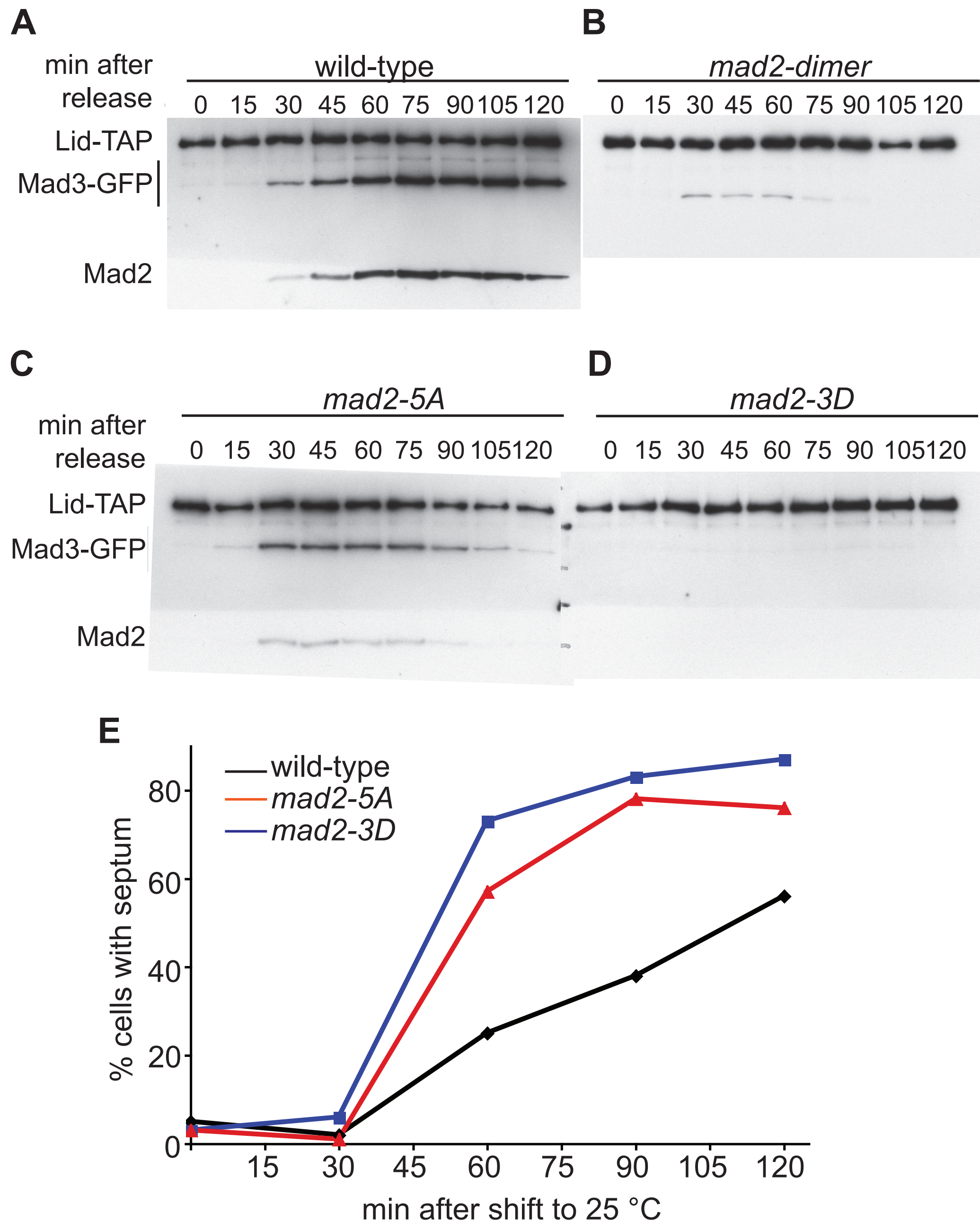


Figure 5.13: APC binding assays of wild-type (**A**), *mad2-dimer* (**B**), *mad2-5A* (**C**) and *mad2-3D* (**D**) released from a G2 arrest into mitosis with the addition of CBZ after 20 min to activate the spindle checkpoint. Western blot analysis of Lid1-TAP (APC) pulldowns from native yeast extracts probed with α-GFP antibody to monitor co-immunoprecipitation of Mad3-GFP and α-Mad2 antibody to monitor co-immunoprecipitation of Mad2. (**E**) Percentage of cells with septum after release from G2 in to mitosis scored for wild-type, *mad2-5A* and *mad2-3D.*

5.5 Summary and Discussion

This study showed that Mph1 kinase activity is required for a functional spindle checkpoint (see chapter 3) and furthermore that it is required for efficient Mad2 and Mad3 binding to the APC during an activated spindle checkpoint (see chapter 4). Therefore, there was a strong possibility that Mad2 and Mad3 could be Mph1 substrates.

Three questions were posed at the beginnning of this chapter. First, are Mad2 and Mad3 substrates of Mph1? Second, if yes which sites are phosphorylated by Mph1? Third, are phosphorylation mutants of Mad2 and Mad3 defective in spindle checkpoint signalling and APC binding? The questions were answered as follows. First, kinase assays showed that Mad2 and Mad3 are *in vitro* substrates of Mph1. Furthermore, 2D-gel analysis showed that Mad3 phosphorylation is also partially dependent on Mph1 *in vivo*. Second, phosphorylation sites for Mph1 were identified in Mad2 and Mad3 using mass spectrometry and phosphorylation mutants were created (*mad2-5A*, *mad2-3D*, *mad3-18A-GFP*). Third, analysis of the phosphorylation mutants showed that all three mutants are spindle checkpoint defective to different extents. Figure 5.14 at the end of this chapter shows a model of the regulation of the spindle checkpoint through Mph1 phosphorylation of Mad2 and Mad3.

The results described above showed that Mad2 and Mad3 are *in vitro* substrates of Mph1, while 2D-gel analysis showed that Mad3 phosphorylation is partially dependent on Mph1 *in vivo*. However, the 2D-gel result does not prove direct phosphorylation of Mad3 by Mph1 *in vivo* but it is consistent with a recent study by Huang et al., that demonstrated that human BubR1 phosphorylation *in vivo* is dependent on Mps1 (Huang et al., 2008). The 2D-gel results from this study suggest that Mad3 is not only phosphorylated by Mph1. This finding is supported by other studies that demonstrated Mad3/BubR1 phosphorylation by Aurora and Polo kinase (Ditchfield et al., 2003; King et al., 2007a; Rancati et al., 2005). Currently A. Sochaj (Hardwick lab) is studying phosphorylation of Mad3 by Ark1 in fission yeast.

In order to analyse the physiological role of Mad2 and Mad3 phosphorylation by Mph1 in the spindle checkpoint, phosphorylation mutants were created and analysed. Phosphorylation sites were identified performing mass spectrometry after phosphopetide enrichment of *in vitro* and *in vivo* samples.

Six phosphorylated peptides were identified in Mad2, which showed phosphorylation on S69, S92, S187, T188, S189. The four latter sites are conserved between fission yeast and human (Figure S.4). Wassman et al. analysed a phosphorylation mutant of human Mad2 that had a substitution of the corresponding residue to S92 in fission yeast to alanine. However, this mutant did not show a phenotype with the assays performed (Wassmann et al., 2003).

18 phosphorylated peptides were identified in Mad3. Three of the sites identified are conserved between fission yeast and human and two sites are conserved between budding yeast and fission yeast. Only one site, S94 is conserved in budding yeast and human. It is noteworthy that there are two patches of phosphorylation sites close to the N-terminal and C-terminal KEN boxes (Figure S.3).

Importantly, the conditions in an *in vitro* experiment do not represent the conditions in a cell. Therefore, unspecific phosphorylation sites could be identified by mass spectrometry that are not present *in vivo*. In addition, sites that are phosphorylated *in vivo* might not get phosphorylated *in vitro* as the protein has to be presented in a certain conformation or needs to be present in a complex. Therefore, mass spectrometric analysis of Mad2 affinity purified from denatured yeast extracts was carried out, which confirmed two of the sites identified *in vitro* (S187, S189). Currently we are in the process of purifying Mad3 from denatured fission yeast extracts to identify further *in vivo* phosphorylation sites.

Preliminary results for *mad3-18A-GFP* suggests that this mutant has a spindle checkpoint maintenance defect. However, *mad3-18A-GFP* does not appear to be particularly defective in APC binding during an active spindle checkpoint or disrupt Mad2 binding to the APC. Apart from the last time-point in the APC assay that

suggested that Mad3-18A-GFP binding was further reduced than wild-type Mad3, Mad3-18A-GPF and Mad2 binding to the APC looked similar to wild-type. This suggests that Mad3-18A-GFP can still form the MCC and inhibit the APC. However, Mad3-18A-GFP association with other proteins required for the spindle checkpoint or Mad3-18A-GFP localisation to kinetochores might be slightly perturbed. A more detailed analysis of *mad3-18A-GFP* and confirmation of the data described above will be carried out in the future. This will include longer APC binding time-courses to determine whether Mad3-18A-GFP and Mad2 binding to the APC is reduced at later time-points and viability assays. In addition *nuf2-3* arrests will be carried out to determine the ability of *mad3-18A-GFP* cells to arrest in the presence of defective kinetochores.

Mad2 was the second *in vitro* substrate of Mph1 identified in this study. Two different phosphorylation mutants, a phosphorylation deficient (*mad2-5A*) and a phosphomimic (*mad2-3D*) mutant were created and analysed with regards to the spindle checkpoint.

In order to determine the ability of *mad2-5A* to spindle checkpoint arrest three assays were carried out and somewhat surprisingly gave different results depending on the assay being employed. In an *nda3-KM311* background, in which microtubule attachment to kinetochores is abolished, *mad2-5A* cells were able to spindle checkpoint arrest. On the contrary, *mad2-5A* did not arrest in the presence of defective kinetochores as it failed to arrest in a *nuf2-3* background. However, the results from the *nuf2-3* experiments are preliminary and need to be confirmed. Nevertheless, *mad2-5A* also failed to arrest due to depolymerised spindles upon treatment with CBZ, which supports the data received from the *nuf2-3* experiment. We do not know why there are these discrepancies between the different assays. One explanation could be severity of the attachment defect. *nda3-KM311* cells are grown under conditions that activate the spindle checkpoint for six hours and all microtubules are fully detached. In an *nda3-KM311* arrest up to 90 % of wild-type cells spindle checkpoint arrested. The attachment defect in a *nuf2-3* mutant or in cells treated with CBZ might not be as severe as in *nda3-KM311* cells. Only 35 % of

wild-type cells were arrested in metaphase in the *nuf2-3* arrest. Therefore, a spindle checkpoint mutant that is only partially spindle checkpoint defective might still be able to arrest under the conditions in the *nda3-KM311* mutant but not in the *nuf2-3* mutant or upon CBZ treatment. A similar phenomenon was observed in *Xenopus.* A Bub1 phosphorylation mutant spindle checkpoint arrested upon treatment with high doses of nocodazole but not lower doses (Chen, 2004).

In addition to spindle checkpoint assays, "APC binding assays" were carried out that showed that in *mad2-5A* cells, Mad2-5A and Mad3 binding to the APC is perturbed upon spindle checkpoint activation. However, this could be due to failure of *mad2-5A* cells to spindle checkpoint arrest.

In addition to *mad2-5A* a phosphomimic mutant *mad2-3D* was analysed. One expectation one could have from a phosphomimic mutant would be that if the non-phosphorylatable mutant fails to arrest the phosphomimic mutant might lead to a constant activation of the spindle checkpoint. However, the results retrieved in this study do not support this hypothesis. Preliminary results suggest that *mad2-3D* cells do not arrest in an *nda3-KM311* background or upon treatment with CBZ. Furthermore, in *mad2-3D* cells, Mad2-3D and Mad3 binding to the APC was nearly completely abolished. The results from the APC time-course are consistent with a report from Wassmann et al., that showed that a human Mad2 phosphomimic mutant can not bind to Mad1 or the APC anymore (Wassmann et al., 2003). This could also explain the inability of *mad2-3D* to spindle checkpoint arrest as the formation of the Mad1-Mad2 complex is required for a functional spindle checkpoint (Chen et al., 1999).

There could be several reasons for the spindle checkpoint defects observed in the *mad2-5A* and *mad2-3D* mutants. First, complex formation with Mad1 could be perturbed in the two mutants, which is essential for the spindle checkpoint (Chen et al., 1999). We are currently analysing whether Mad2-5A and Mad2-3D still form a complex with Mad1. Second, kinetochore targeting of Mad2-5A and Mad2-3D could be perturbed. However, preliminary results showed that Mad2-5A can at some

level still localise to kinetochores. Further analysis regarding kinetochore targeting of Mad2-5A and Mad2-3D will be carried out. A third possibility is that Mad2-5A and Mad2-3D can build a complex with Mad1 (Mad1-C-Mad2) and localise to kinetochores but that recruitment of the open conformer of Mad2-5A and Mad2-3D is perturbed. Furthermore, the conformational change upon dimerisation or the release from kinetochores could be perturbed. If Mad2-5A and Mad2-3D localise to kinetochores FRAP analysis will be carried out to determine the kinetics of Mad2-5A and Mad2-3D at kinetochores. Fourth, localisation of human Mps1 to kinetochores requires Nuf2 (Stucke et al., 2004). If Mad2-5A is phosphorylated by Mph1 at sites in addition to the ones identified in this study this could "rescue" mad2-5A spindle checkpoint defects. The inability of *mad2-5A* to arrest in the *nuf2-3* mutant could then be explained by a lack of Mph1 at kinetochores. However, Mph1 localisation to kinetochores has not been demonstrated in fission yeast yet.

In addition to the *mad2* phosphorylation mutants, a *mad2* mutant predicted to not be able to form a conformational dimer was analysed. The Mad2 dimerisation mutant created in this study was based on mutants previously analysed in human and budding yeast (De Antoni et al., 2005; Nezi et al., 2006). Mutations of residues R133 and Q134 at the Mad2 binding interface in human and the corresponding residues in budding yeast abolished formation of a conformational Mad2 dimer. The *mad2* dimerisation mutant in this study contained mutations of the corresponding residues in fission yeast. The analysis performed to date showed that *mad2-dimer* cells were not able to spindle checkpoint arrest and had phenotypes similar to *mad2* null mutant cells, which was in agreement with the result in human and budding yeast (De Antoni et al., 2005; Nezi et al., 2006).

Furthermore, this study showed that Mad3 binding to the APC was nearly completely abolished in the *mad2-dimer* mutant. Due to antibody issues Mad2-dimer binding to the APC has not been tested in depth but preliminary results suggest that Mad2-dimer does not bind to the APC. These results suggest that the formation of a conformational dimer in fission yeast is required for Mad2 and Mad3 binding to the APC. One reason for the inability of Mad3 to bind to the APC in *mad2-dimer*

cells could be due to the inability of Mad2-dimer to bind to the Mad1-Mad2 complex as the Mad2 dimerisation mutants in humans and budding yeast are unable to bind to the Mad1-Mad2 complex that is localised at kinetochores (De Antoni et al., 2005; Nezi et al., 2006). We are currently analysing Mad2-dimer for its ability to bind to Mad1. Another reason could be that the Mad2-dimer cannot bind to Slp1/Cdc20 efficiently, as was reported in budding yeast (Nezi et al., 2006). In the future we will test whether in *mad2-dimer* cells Mad2-dimer and Mad3 bind to Slp1/Cdc20.

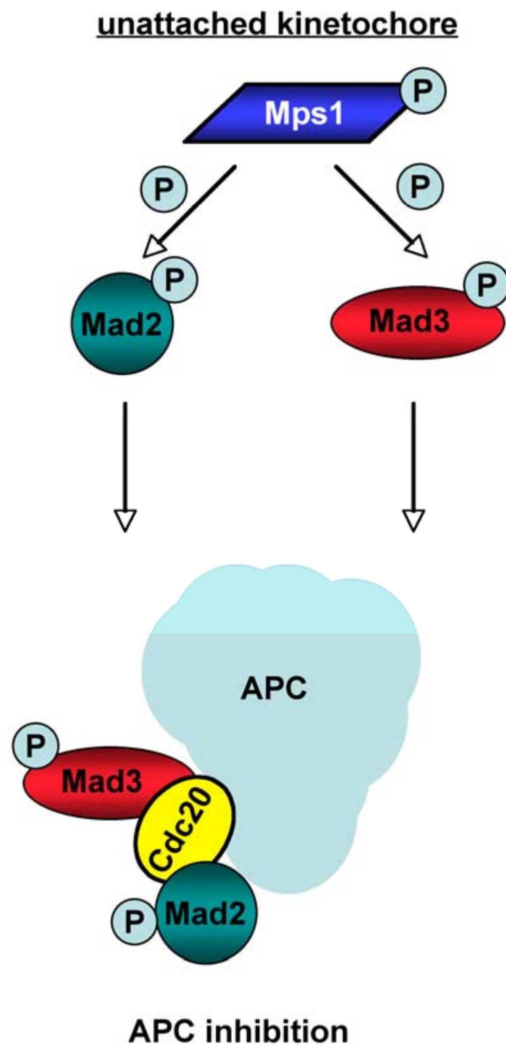


Figure 5.14: Model of the regulation of the spindle checkpoint through Mph1 phosphorylation of Mad2 and Mad3.

Chapter 6

Final Discussion

Chapter 6: Final Discussion

Accurate chromosome segregation is crucial as mis-segregation results in aneuploidy and this can lead to severe diseases such as cancer. The spindle checkpoint controls chromosome segregation during mitosis by monitoring sister-chromatid attachment to the mitotic spindle. It inhibits the onset of anaphase until all chromosomes are correctly bi-oriented on the mitotic spindle and thus prevents chromosome mis-segregation. Upon activation of the spindle checkpoint the anaphase promoting complex (APC) is inhibited among other mechanisms by the mitotic checkpoint complex (MCC), composed of the spindle checkpoint proteins, Mad2, Mad3 and Cdc20. Inhibition of the APC prevents degradation of securin and cyclin and thus delays the onset of anaphase. Events of the spindle checkpoint, including complex or scaffold formation and the inhibition of the APC are regulated by post-translational modifications such as phosphorylation and ubiquitination. One of the main kinases essential for the spindle checkpoint is Mps1, whose kinase activity has been shown to be essential for the spindle checkpoint (Abrieu et al., 2001; Jones et al., 2005; Kang et al., 2007; Tighe et al., 2008).

The aim of this study was to analyse the role of Mph1 kinase activity in the spindle checkpoint and chromosome segregation in fission yeast. Three questions were asked. First, what is the general role of Mph1 kinase activity in the spindle checkpoint and chromosome segregation? Second, what is the role of Mph1 kinase activity in Mad2 and Mad3 binding to the APC? Third, what is the physiological role of phosphorylation of Mph1 substrates with regards to the spindle checkpoint?

Chapter 3, 4 and 5 answered these questions as follows. First, Mph1 kinase activity is required for a functional spindle checkpoint and accurate chromosome segregation. Furthermore, loss of the spindle checkpoint and chromosome mis-segregation due to lack of Mph1 kinase activity leads to reduced cell viability. Second, Mph1 kinase activity is required for Mad2 and Mad3 binding to the APC. Third, Mad2 and Mad3 were identified to be *in vitro* substrates of Mph1. Analysis

of Mad2 and Mad3 phosphorylation mutants showed that phosphorylation of both proteins by Mph1 is required for a fully functional spindle checkpoint.

Prior analysis of fission yeast Mph1 mutants determined that Mph1 is required for the activation of the spindle checkpoint ((He et al., 1998), L.Milne, Hardwick lab, this study). However, no studies in fission yeast have been carried out to determine whether Mph1 kinase activity is required to maintain the spindle checkpoint. Preliminary data for the Mad3 phosphorylation mutant analysed in this study suggest that Mph1 might be required for spindle checkpoint maintenance (see chapter 5). To investigate the role of Mph1 in spindle checkpoint maintenance an analogue sensitive mutant of Mph1 could be used. Analogue sensitive kinase mutants contain a mutation in the ATP binding pocket that allows binding of an ATP analogue called 1NMPP1 (4-amino-1-tert-butyl-3-(1-napthylmethyl)pyrazo- lo[3,4-d]pyrimidine). Binding of 1NMPP1 prevents binding of ATP and thus inhibits kinase activity. This allows switching off the kinase at a specific point in the cell cycle. Analogue sensitive mutants of Mps1 have been cloned and analysed and showed that Mps1 kinase activity is required for the maintenance of a spindle checkpoint arrest in budding yeast and human (Jones et al., 2005; Tighe et al., 2008). The cloning of an analogue sensitive mutant was attempted before based on the mutants made in budding yeast (John Doig, Hardwick lab). However, the Mph1 "analogue sensitive" mutant was benomyl sensitive even in the absence of 1NMPP1, which suggests that this mutation perturbed the function of Mph1.

This study showed that Mph1 is a phosphoprotein like its homologues in other organisms (Hardwick and Murray, 1995; Lauze et al., 1995; Liu et al., 2003; Stucke et al., 2002). However, we do not know whether Mph1 phosphorylation is due to autophosphorylation or phosphorylation by other kinases, such as Bub1, Ark1, Polo kinase, Cdk or MAPK. To analyse autophosphorylation of Mph1 pull-downs of wild-type Mph1 and kinase-dead Mph1 cells could be performed and the gel migration pattern compared. To analyse Mph1 phosphorylation by other kinases double kinase mutants of Mph1 with for example Bub1 and/or Ark1 could be created

and analysed. Furthermore, *in vitro* kinase assays with Mph1-kd as a substrate and the kinases of interest, such as Bub1 and Ark1 could be carried out.
Several *in vitro* Mph1 autophosphorylation sites have been determined during this study. To investigate the role of Mph1 autophosphorylation in the spindle checkpoint and chromosome segregation autophosphorylation mutants could be created and analysed. The autophosphorylation sites identified in this study are mainly situated in the N-terminus, which has been shown to be required for Mps1 kinetochore targeting (Abrieu et al., 2001; Liu et al., 2003) (Table S.1, Figure S.1, S.2). Furthermore, autophosphorylation on two sites in the N-terminus have been demonstrated to be required for kinetochore targeting in human cells (Xu et al., 2009). Unfortunately, the sites analysed by Xu et al. were not identified in this study. However, the corresponding residue of T676 in human has been identified and substitution of this residue with alanine has been demonstrated to impair Mps1 kinase activity and the spindle checkpoint (Jelluma et al., 2008a; Kang et al., 2007; Mattison et al., 2007). In the future, we could generate and analyse autophosphorylation mutants of Mph1, for example a mutant of residue T676 to test whether phosphorylation ot this residue is required for Mph1 kinase activity and the spindle checkpoint in fission yeast.

The results from this study suggest that the MCC binds to the APC during every mitosis, which is consistent with human data and a recent study in fission yeast (Herzog et al., 2009; Sczaniecka et al., 2008). These findings led to the hypothesis that in fission yeast the spindle checkpoint is active in an unperturbed mitosis like in vertebrates. Importantly, binding of Mad2 and Mad3 to the APC was dependent on Mph1 and Ark1 kinase activity. The role of Ark1 kinase activity was not further analysed in this study so it is not clear whether Ark1 directly phosphorylates spindle checkpoint proteins to regulate Mad2 and Mad3 binding to the APC. Mad3 is a good candidate substrate of Ark1, as budding yeast Aurora/Ipl1 kinase phosphorylation of Mad3 is required for the spindle checkpoint (King et al., 2007a). A. Sochaj is currently studying phosphorylation of Mad3 by Ark1 kinase and its role in the spindle checkpoint.

This study analysed phosphorylation of Mad2 and Mad3 by Mph1 and showed that both proteins are substrates of Mph1. Phosphorylation mutants of both proteins were partially spindle checkpoint defective. Whereas, a phosphorylation mutant of Mad3 did not show major defects in APC binding a Mad2 phosphorylation mutant and phosphomimic mutant showed defects in APC binding. Importantly, phosphorylation mutants of *mad2* and *mad3* did not display a null mutant phenotype. However, a phosphomimic mutant of *mad2* appeared to have spindle checkpoint defects similar to a *mad2* null mutant. It would be interesting to know at what stages during the cell cycle Mad2 and Mad3 are phosophorylated at the identified sites. To test this western blot analysis with phosphospecific antibodies could be carried out. Furthermore, phosphospecific antibodies could be used to try and determine the localisation of the phosphorylated proteins in the cell using microscopy.

The 2D-gel analysis results for Mad3 and the fact that both the Mad2 and Mad3 phosphorylation mutants were not completely spindle checkpoint defective suggests that Mad2 and Mad3 are also phosphorylated by other kinases, such as Ark1 or Bub1. In addition it is likely that not all Mph1 phosphorylation sites were identified by mass spectrometry.

Mad2 and Mad3 are also most likely not the only substrates of Mph1. In budding yeast Mad1 and Ndc80 have been identified as Mps1 substrates (Hardwick et al., 1996; Kemmler et al., 2009). A phosphorylation mutant of budding yeast Ndc80 is partially spindle checkpoint defective, whereas a phosphomimic mutant “rescued” this phenotype and led to a constitutively active spindle checkpoint (Kemmler et al., 2009). Furthermore, budding yeast Ndc80 has been shown to interact with Mps1 and it would be interesting to test whether this is also the case in fission yeast (Kemmler et al., 2009). However, Mph1-TAP was purified several times from native fission yeast extracts and co-immunoprecipitated proteins identified by mass spectrometry (Hardwick lab). Unfortunately, neither Ndc80 nor any other relevant proteins were identified during these analyses. Another Mps1 substrate is the chromosomal passenger complex component Borealin (Jelluma et al., 2008b). Phosphorylation of human Borealin by Mps1 is required for chromosome alignment and attachment. A

phosphomutant of Borealin showed chromosome alignment defects and reduced Aurora B activity, whereas a phosphomimic mutant restored chromosome alignment (Jelluma et al., 2008b). I performed an *in vitro* kinase assay with the fission yeast homologue of Borealin (purified by Lisa Reynolds, Hardwick lab), which showed that it is an *in vitro* substrate of Mph1. It would be very interesting to test whether Mad1 and Ndc80 or other kinetochore components are also substrates of fission yeast Mph1. Other possible substrates of Mph1 are other kinases, such as Ark1 or Bub1, the APC co-factor Slp1/Cdc20 or APC subunits. We are able to purify fission yeast APC from fission yeast extracts and therefore *in vitro* kinase assays could be carried out to test phosphorylation of APC subunits by Mph1.

It is very important to identify the kinases and their substrates that regulate the spindle checkpoint. Nevertheless, it is also important to identify the phosphatase(s) that dephosphorylate these proteins. Recently Vanoosthuyse et al reported that protein phosphatase 1 ($PP1^{Dis2}$) is required for spindle checkpoint silencing in fission yeast (Vanoosthuyse and Hardwick, 2009). It would be very informative to test whether Mad2 and Mad3 or other spindle checkpoint proteins are direct substrates of $PP1^{Dis2}$. To test this, an *in vitro* kinase assay with the substrate of interest and Mph1 could be performed. The kinase assay reaction could then be treated with phosphatases (e.g. $PP1^{Dis2}$) and the reduction of phosphorylation determined.

Wild-type Mad2 and Mad3 localise to unattached kinetochores (Ikui et al., 2002; Millband and Hardwick, 2002). It would be informative to know whether the phosphorylation mutants still localise to kinetochores and, if yes, to determine their exchange rate at kinetochores carrying out fluorescence recovery after photobleaching (FRAP) analysis. This has been previously done for wild-type fission yeast proteins (Rischitor et al., 2007). In vertebrates a stable pool of app. 50 % of Mad2 is stably localised to kinetochores, whereas the other 50 % only localise transiently (Shah et al., 2004). If for example a phosphorylation mutant of Mad2 would perturb Mad2-Mad2 binding or Mad2-Mad1 binding one would predict that the dynamics of Mad2 at the kinetochore would be different. The same applies for Mad3. The dynamics of a phosphorylation mutant of Mad3 at kinetochores might be

different to wild-type Mad3 that only localises to kinetochores transiently (Rischitor et al., 2007).

To date the analysis of Mph1 has mainly been focusing on the C-terminal kinase domain. This study and L. Milne (Hardwick lab) showed that Mph1 kinase activity and the C-terminal kinase domain of Mph1 are required for accurate chromosome segregation. However, an *mph1* null mutant has a significantly higher rate of chromosome mis-segregation than a kinase-dead mutant or a C-terminal kinase domain truncation mutant (L Milne, Hardwick lab, unpublished data). This suggests that Mph1 has additional roles apart from its kinase function and that these lie in the N-terminus. It would be interesting to analyse the role of the N-terminus of Mph1. There are several possible functions for the N-terminus of Mph1. It could be required for kinetochore localisation of Mph1 to kinetochores. Mps1 homologues in other organisms localise to kinetochores (Abrieu et al., 2001; Fisk and Winey, 2001; Liu et al., 2003) and the N-terminus has been demonstrated to be required for this localisation (Abrieu et al., 2001; Liu et al., 2003). To be able to analyse the role of the N-terminus of fission yeast Mph1 it would be helpful to know whether it localises to kinetochores. L. Milne (Hardwick lab) already investigated Mph1 kinetochore localisation. Unfortunately, she was not able to detect Mph1 at kinetochores at endogenous expression levels and overexpression of Mph1 only resulted in a diffuse nuclear staining and no specific localisation. In the future we will try to further analyse Mph1 localisation by microscopy using different tags and more sensitive microscopes. Furthermore, CHIP analysis could be carried out.

During the course of this study I started to set up an *in vitro* APC assay with fission yeast APC. In addition, Ors et al. recently published a fission yeast *in vitro* APC assay (Ors et al., 2009). Using these assays recombinant Mad2 and Mad3 phosphorylation mutants could be individually or in combination be tested for their ability to inhibit the APC *in vitro*.

Taken together the analysis in this thesis presented new information about the role of Mph1 kinase activity in the spindle checkpoint and chromosome segregation. Nevertheless, many more questions about Mph1 remain to be answered.

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Supplementary Figures

```
S.pombe          ------------------------------------------------------------
S.japonicus      ------------------------------------------------------------
S.cerevisiae     -----------------MSTNSFHDYVDLKSRTNTRQFSDDEEFTTPPKLSNFGSALLSH 43
H.sapiens        MESEDLSGRELTIDSIMNKVRDIKNKFKNEDLTDELSLNKISADTTDNSGTVNQIMMMAN 60

S.pombe          ---------------------------MSKRNPPVTNIADLVS--DSSLDEDSLS--FLE 29
S.japonicus      ---------------------------MSKPDLPRTGFEDLSL--DSSFDEETLS--FLR 29
S.cerevisiae     TEKTSASEILSSHNNDKIANRLEEMDRSSSRSHPPPSMGNLTSGHTSTSSHSTLFGRYLR 103
H.sapiens        NPEDWLSLLLKLEKNSVPLSDALLNKLIGRYSQAIEALPPDKYGQNESFARIQVRFAELK 120
                                             .  . .   :        .:  .  :    *.

S.pombe          ELQDPE--------------------LYFKNDTFSSKSSHSDGTVTGDTLRRQSS----- 64
S.japonicus      NIDDET--------------------STPIDYKTVTKTAFSS---TSEGLKKSDD----- 61
S.cerevisiae     NNHQTS--------------------MTTMNTSDIEINVGNSLDKSFERIRNLRQNMKED 143
H.sapiens        AIQEPDDARDYFQMARANCKKFAFVHISFAQFELSQGNVKKSKQLLQKAVERGAVPLEML 180
                   .:                          :      .  ..     . :..

S.pombe          -----GATALERLVSHPRTKNFDLQGNGGQ-NSALKE-VNTPAYQSMHH----------- 106
S.japonicus      -----GSS--EKTIQSRRSVTLSAPTTVKRPATLLNE-KKTPTFKASSG----------- 102
S.cerevisiae     ITAKYAERRSKRFLISNRTTKLGPAKRAMTLTNIFDEDVPNSPNQPINA----------- 192
H.sapiens        EIALRNLNLQKKQLLSEEEKKNLSASTVLTAQESFSGSLGHLQNRNNSCDSRGQTTKARF 240
                           :: :   .  .             :.        :

S.pombe          -FEHLITPLPSTNAS--------HSEVSLSAGVNDLNSNSEHDLLPKSVNKTP-----GS 152
S.japonicus      -SESLITPLGLDSHS--------KSFGLNSVGPTEHDGDAVTHGTVKSHNG-------GS 146
S.cerevisiae     -RETVELPLEDSHQTNFKERRENTDYDSIDFGDLNPIQYIKKHNLPTSDLPLI-----SQ 246
H.sapiens        LYGENMPPQDAEIGYRNSLRQTNKTKQSCPFGRVPVNLLNSPDCDVKTDDSVVPCFMKRQ 300
                        *                       *          .   .:           .

S.pombe          LSISRRRRIGRIGLGPPKRAEYTLTDPSKTSD---------TKNST---EADEDIEMKSR 200
S.japonicus      TVSSRWRRIGRIGLGPPKRAEYSLAD-VKASD---------SQESG---AEDDMHEGKQN 193
S.cerevisiae     IYFDKQREENRQAALRKHSSRELLYKSRSSSSSLSSNNLLANKDNS---ITSNNGSQPRR 303
H.sapiens        TSRSECRDLVVPGSKPSGNDSCELRNLKSVQNSHFKEPLVSDEKSSELIITDSITLKNKT 360
                    .. *     .          * .  . ..          :..      ..

S.pombe          EVSPASNSVAATTLKPLQLHNTPLQTSQEHPKPSFHPSQFESSFSPRVQFDHDVERRASE 260
S.japonicus      HHVPAAGSDRAN--AEAEIEDEHLRSRKLR---ALSVSKAGLDFSKFAEVAPQTNASKKP 248
S.cerevisiae     KVSTGSSSSKSSIEIRRALKENIDTSNNSNFNSPIHKIYKGISRNKDSDSEKREVLRNIS 363
H.sapiens        ESSLLAKLEETKEYQEPEVPESNQKQWQSKRKSECINQNPAASSNHWQIPELARKVNTEQ 420
                 .    :    :.      : :      : .            . .

S.pombe          LHSRPVTVFQEPQRS----------------------ASQPYESHALSPKVAPLFDNSQA 298
S.japonicus      LNHN--SLLNAQQGQ----------------------MANPFLD---TPVVVP------Q 275
S.cerevisiae     INANHADNLLQQENKRLKRSLDDAI-------TNENINSKNLEVFYHRPAPKPPVTKKVE 416
H.sapiens        KHTTFEQPVFSVSKQSPPISTSKWFDPKSICKTPSSNTLDDYMSCFRTPVVKNDFPPACQ 480
                  :      .   . .                        .        *

S.pombe          TPIPKR---------------------------QQDVVTVANLQFIKLGVVGKGGSSMVY 331
S.japonicus      TLFPG----------------------------AEAVTISGHTFIKLGVIGKGGSSKVF 306
S.cerevisiae     IVEPAKSASLS--------------------NNRNIITVNDSQYEKIELLGRGGSSRVY 455
H.sapiens        LSTPYGQPACFQQQQHQILATPLQNLQVLASSSANECISVKGRIYSILKQIGSGGSSKVF 540
                    *                               : ::: .  :  :  :* **** *:

S.pombe          RIFSPDNSRLYALKEVNFINADQTTIQGYKNEIALLRKLSGN-DRIIKLYAAEVNDTLGQ 390
S.japonicus      RIIAPENKVIYALKEVDFENADYAAVQGYKNEIALLKKLSGH-ERIIRLYAAEVNDIKGQ 365
S.cerevisiae     KVKGSGN-RVYALKRVSFDAFDDSSIDGFKGEIELLEKLKDQ-KRVIQLLDYEMGD--GL 511
H.sapiens        QVLNEKK-QIYAIKYVNLEEADNQTLDSYRNEIAYLNKLQQHSDKIIRLYDYEITD--QY 597
                 ::    :  :**:* *.:   *  :::.::.**  *.**. : .::*:*   *: *

S.pombe          LNMVMECGETDLANLLMKNMKKPINLNFIRMYWEQMLEAVQVVHDQNIVHSDLKPANFLL 450
S.japonicus      LSMVMEYGECDMAHLLAKNSHRPINLHFIRLYWQQMLQAVQVVHEQNIVHSDLKPANFLL 425
S.cerevisiae     LYLIMECGDHDLSQILNQRSGMPLDFNFVRFYTKEMLLCIKVVHDAGIVHSDLKPANFVL 571
H.sapiens        IYMVMECGNIDLNSWLKKK--KSIDPWERKSYWKNMLEAVHTIHQHGIVHSDLKPANFLI 655
                 : ::** *: *:   * :.   .::    : * ::** .::.:*: .***********::
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S.pombe        VEGNLKLIDFGIAKAIGNDTTNIHRDSHIGTINYMAPEALTDMNAHTNSG----VKLVKL 506
S.japonicus    VSGSLKLIDFGIAKAIENNTTNIHRDTHVGTVNYMAPEALIDTNADATTN----IKLVKL 481
S.cerevisiae   VKGILKIIDFGIANAVPEHTVNIYRETQIGTPNYMAPEALVAMNYTQNSENQHEGNKWKV 631
H.sapiens      VDGMLKLIDFGIANQMQPDTTSVVKDSQVGTVNYMPPEAIKDMSSSRENG----KSKSKI 711
               *.* **:******: :  .*..: :::::** ***.***:   .    .      .  *:

S.pombe        GRPSDVWSLGCILYQMVYGRAPFAHLK-MIQAIAAIPNEQYHIHFPEVALPANAVQEKEG 565
S.japonicus    GRPSDVWSLGCILYQMVYGHAPFAHLN-MIKAIAAIPDVRARINFPETAVPARPAPGSNQ 540
S.cerevisiae   GRPSDMWSCGCIIYQMIYGKPPYGSFQ-GQNRLLAIMNPDVKIPFPEHTSNNEKIPKS-- 688
H.sapiens      SPKSDVWSLGCILYYMTYGKTPFQQIINQISKLHAIIDPNHEIEFPDIPEKD-------- 763
               .  **:** ***:* * **:.*:  :    . : ** :   .* **: .

S.pombe        SLP--GVTVGPDLMDVMKRCLERDQRKRLTIPELLVHPFLNP-LPSYLTPLAKKPLPVSG 622
S.japonicus    VNPKLAVPVPPDLIRVMKSCLERDQRKRLTIPELLRDPFLHPGEAPTTTAVAERPTRPD- 599
S.cerevisiae   ------------AIELMKACLYRNPDKRWTVDKVLSSTFLQP------------------ 718
H.sapiens      ------------LQDVLKCCLKRDPKQRISIPELLAHPYVQIQTHPVNQMAKGTTEEMK- 810
                              ::* ** *:  :* :: ::*  .:::

S.pombe        HTNNAHPLRLSTEISASQLSMIIERSVELSKHK-RLNKELIDSMAYDCVSNLRKMPE-- 678
S.japonicus    ----------ATEISASQLSMIIERSVELSRQR-LLSKDVIFALAKDCISNLQKHPRQ- 646
S.cerevisiae   -----------FMISGSIMEDLIRNAVRYGSEKPHISQDDLNDVVDTVLRKFADYKI-- 764
H.sapiens      ------------YVLGQLVGLNSPNSILKAAKTLYEHYSGGESHNSSSSKTFEKKRGKK 857
                            : .. :     .::  . .      .           .: .
```

Figure S.1: Alignment of Mps1 protein sequences from different species. Mph1 autophosphorylation sites identified by mass spectrometry are marked in red.

Amino acid	Peptides identified
T53, T58	SSHSDG**T**VTGD**T**LRR
S64	RQS**S**GATALER
T67	QSSGA**T**ALER
S74	LV**S**HPRTK
S152	TPG**S**LSISR
T175	AEY**T**LTDPSK
T177	RAEYTL**T**DPSK
S199	NSTEADEDIEMK**S**R
S263, T267	ASELH**S**RPV**T**VFQEPQR
Y280	SASQP**Y**ESHALSPK
S286	SASQPYESHAL**S**PK
T470	AIGND**T**TNIHR
T471	AIGNDT**T**NIHR

Table S.1: Mph1 autophosphorylation sites. Phosphorylation sites were identified by mass spectrometry after phosphopetide enrichment from *in vitro* kinase assays.

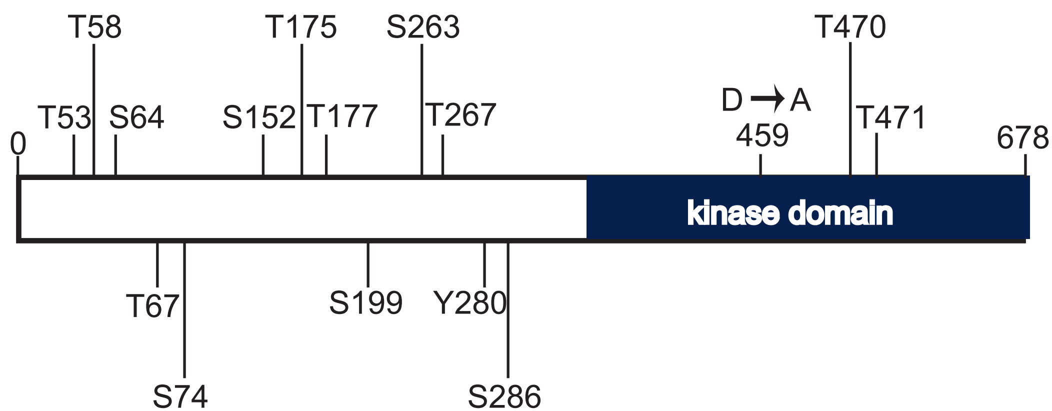

Figure S.2: Schematic of Mph1 showing the autophosphorylation sites identified by mass spectrometry.

```
S.pombe         ----------MEPLDAGKNWVHMDVIEQSKENIEPRKAGHSASALAKSSS 40
S.octosporus    ----------MN-TTAKQTQTSIDIIEQAKENIEPRRQGHSAAAISKTFS 39
S.japonicus     --------------MSGSKIVNIETIEFQKENIEPRREGHRARALEKAFT 36
S.cerevisiae    MKAYAKKRISYMPSSPSQNVINFEEIETQKENILPLKEGRSAAALSKAIH 50
Human           -----AAVKKEGGALSEAMSLEGDEWELSKENVQPLRQGRIMSTLQGALA 45
                               .       :  *  ***: * : *:   ::  :

S.pombe         RNHTEKEVAGLQKERMGHERKIETSE-SLDDPLQVWIDYIKWTLDNFPQG 89
S.octosporus    KDHEQKVSNQLHEERISFEEKLQIAD-REEDPLQVWIDYIQWTLNSYPQG 88
S.japonicus     RDPSESAIKDIEATKQSYEEAIQNTG-TTDDPLEPWLKYIQWTLETFPQG 85
S.cerevisiae    QP-----LVEINQVKSSFEQRLIDELPALSDPITLYLEYIKWLNNAYPQG 95
Human           QESACNNTLQQQKRAFEYEIRFYTGN----DPLDVWDRYISWTEQNYPQG 91
                :          .     .*  :        **:  :  **.*  : :***

S.pombe         -ETKTSGLVTLLERCTREFVRNPLYKDDVRYLRIWMQYVNYID-----EP 133
S.octosporus    -NTSESGLLSLLERCSQQFVKSPIYKNDIRYLRIWMQYAKYVE-----DP 132
S.japonicus     -DSNVSEFVRLLERCTQHFLKDPLYQNDIRYLKVWLRYAPYTN-----DP 129
S.cerevisiae    GNSKQSGMLTLLERCLSHLKDLERYRNDVRFLKIWFWYIELFTRNSFMES 145
Human           --GKESNMSTLLERAVEALQGEKRYYSDPRFLNLWLKLGRLCN-----EP 134
                   . * :  ****.   :     * .* *:*.:*:            :.

S.pombe         VELFSFLAHHHIGQESSIFYEEYANYFESRGLFQKADEVYQKGKRMKAKP 183
S.octosporus    AELFSFLSLHEIGTNFSLYYEEFAGYFESNGLYKKAEDVYQKGFLRKAKP 182
S.japonicus     AELFSFLEVHKIGLQFSIYYEEYANYFESKGLYAKALSIYNRGQERHARP 179
S.cerevisiae    RDIFMYMLRNGIGSELASFYEEFTNLLIQKEKFQYAVKILQLGIKNKARP 195
Human           LDMYSYLHNQGIGVSLAQFYISWAEEYEARENFRKADAIFQEGIQQKAEP 184
                 ::: ::  : ** . : :* .::     .  :  *  : : *   :*.*

S.pombe         FLRFQQKYQQFTHRWLEFAPQS-----FSS-NTNSVNPLQTTFESTN-IQ 226
S.octosporus    FARFQQRYDQFLHRKVIYAPDT-----ITMRQTNEYPPLQTTFQLSNPHQ 227
S.japonicus     ALRFEERRREFLYRCMEKAPDC-----LKE-QTLPETALQIKFENTLSLG 223
S.cerevisiae    NKVLEDRLNHLLRELGENNIQLGNEISMDSLESTVLGKTRSEFVNRLELA 245
Human           LERLQSQHRQFQARVSRQTLLALEKEEEEEVFESSVPQRSTLAELKSKGK 234
                   ::.:  .:  .

S.pombe         EISQS------------RTKIS---------KPKFKFSVYSDADGS---- 251
S.octosporus    QNGRS------------EVSVS---------DAR-RISVFSDTEGTSSTN 255
S.japonicus     SDSSS------------SSTLSSHAAAHFRKPVQKRITVFSDASGD---- 257
S.cerevisiae    NQNGT------------SSDVNLTKNNVFVDGEESDVELFETPNRG---- 279
Human           KTARAPIIRVGGALKAPSQNRGLQNPFPQQMQNNSRITVFDENADEAS-T 283
                .   :                .           .  . ::.

S.pombe         GKDGQPGTWQTLGTVDQRRKENNISATSWVG------------------- 282
S.octosporus    GRSTNPTSWENFGTVEQKRKENTVPSRAWVG------------------- 286
S.japonicus     PSSTLDTAWEQFGSRAVRRKENTISATPWVG------------------- 288
S.cerevisiae    ---VYRDGWENFDLKAERNKENNLRISLLEANTNLGE------------- 313
Human           AELSKPTVQPWIAPPMPRAKENELQAGPWNTGRSLEHRPRGNTASLIAVP 333
                           :     : *** :

S.pombe         -------------EKLPLKSPRKLDPLGKFQVHC-----DEEVSKE---- 310
S.octosporus    -------------ETLQTHSSRKVDPLNTFSVYQ-----DESSSH----- 313
S.japonicus     -------------VTLPIKS-RKSTTSHKLHVYR-----DEQIPLQQTLP 319
S.cerevisiae    ------------LKQHEMLSQKKRPYDEKLPIFR-----DSIGRSDPVYQ 346
Human           AVLPSFTPYVEETARQPVMTPCKIEPSINHILSTRKPGKEEGDPLQRVQS 383
                                   :  *     .  :       :.
```

Figure S.3: Alignment of Mad3 protein sequences from different species. Phosphorylation sites identified by mass spectrometry in *S.pombe* Mad3 are marked in red. KEN boxes are marked in blue.

```
S.pombe         --SSVPIRTNFSLKGSSKLVSEFFEYAVNSILFQRGIYPAEDFKVVRKYGLNMLVSVDEE 58
S.octosporus    -MAAVPIRSNLSLRGSSRLISEFFEYAVNSILFQRGIYPPEDFKVVRKYGISMLVTVDEE 59
S.japonicus     MATTVPTRSSLSLKGSAKLVSEFFEYAVNSILFQRGIYPPEDFKVVRKYGINMLITIDDE 60
S.cerevisiae    ------MSQSISLKGSTRTVTEFFEYSINSILYQRGVYPAEDFVTVKKYDLTLLKTHDDE 54
Human           MALQLSREQGITLRGSAEIVAEFFSFGINSILYQRGIYPSETFTRVQKYGLTLLVTTDLE 60
                         .::*:**:. ::***.:.:****:***:**.* *  *:**.:.:* : * *

S.pombe         VKTYIRKIVSQLHKWMFAKKIQKLILVITSKCSGEDLERWQFNVEMVDTADQFQNIGNKE 118
S.octosporus    VKTYIRKIISQLHRWTCGGKIQKVALVITNKDSGEDLERWQFNVEILQKDTPISPENTAQ 119
S.japonicus     VKAYIRRIIAQLHRWMYRGKIQKLVVVITDKDTGDDLERWQFNVEILCKNEDSIGEESKE 120
S.cerevisiae    LKDYIRKILLQVHRWLLGGKCNQLVLCIVDKDEGEVVERWSFNVQHISGNSNGQDDVVDL 114
Human           LIKYLNNVVEQLKDWLYKCSVQKLVVVISNIESGEVLERWQFDIECDKTAKDDSAPREKS 120
                :  *:..:: *:: *    . ::: : * .   *: :***.*:::

S.pombe         D--ELRVQKEIQALIRQITATVTFLPQLEEQ--CTFNVLVYADKDSEVPTDWVDSDPRIL 174
S.octosporus    D--DTRIQKEVQALIRQITATVTFLPQLEGR--CTFNVLVYADRDSEVPTDWVDSDPRLI 175
S.japonicus     AKPEKEIQNEIQALIRQVTATITFLPQLDTR--CTFNVLVYADKDSEVPTDWVDSDPRQL 178
S.cerevisiae    N----TTQSQIRALIRQITSSVTFLPELTKEGGYTFTVLAYTDADAKVPLEWADSNSKEI 170
Human           Q---KAIQDEIRSVIRQITATVTFLPLLEVS--CSFDLLIYTDKDLVVPEKWEESGPQFI 175
                       *.:::::***:*:::**** *      :* :* *:* *  ** .* :*..: :

S.pombe         RDAEQVQLRSFSTSMHKIDCQVAYRVNP-- 202
S.octosporus    KNAEQVQLRSFSTNMHKIDCQVAYRFDP-- 203
S.japonicus     QNAEQVQLRSFSTNMHKIDCQVAYRMN--- 205
S.cerevisiae    PDGEVVQFKTFSTNDHKVGAQVSYKY---- 196
Human           TNSEEVRLRSFTTTIHKVNSMVAYKIPVND 205
                 :.* *::::*:*. **:.. *:*:
```

Figure S.4: Alignment of Mad2 protein sequences from different species.
Phosphorylation sites identified by mass spectrometry in *S.pombe* are marked in red.

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