Functional analysis of the budding yeast Bub1 kinase

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Declaration:

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

Josefin Fernius
2007
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Abbreviations:

AMP  Ampicillin
APC/C  Anaphase promoting complex/ Cyclosome
ATP  Adenosine tri-phosphate
bp  base pairs
Bub  Budding uninhibited by benzimidazole
ChIP  Chromatin immuno-precipitation
CDE  Centromere DNA element
Cdc  Cell division cycle
Cdk  Cyclin dependent kinase
CEN  Centromere
CSM  Complete synthetic media
C-terminus  Carboxy-terminus
DNA  Deoxyribonucleic acid
DTT  Dithiothreitol
EDTA  Ethylenediamine tetra acetic acid
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
FRET  Fluorescence resonance energy transfer
GFP  Green fluorescent protein
GST  Glutathione S-transferase
HRP  Horse radish peroxidase
IP  Immuno precipitation
Kan  Kanamycin
KT  Kinetochore
LB  Luria-Bertani medium
Mad  Mitotic arrest deficient
MBP  Myelin basic protein
MCAK  Mitotic centromere-associated kinesin
Min  Minutes
MT  Microtubule
N-terminus  Amino-terminus
OD  Optical density
ORF  Open reading frame
PAGE  Polyacrylamide gel electrophoresis
PBS  Phosphate buffered saline
PBS-T  Phosphate buffered saline with Tween
PCR  Polymerase chain reaction
PEG  Polyethylene glycol
PMSF  Phenyl methyl sulphonyl fluoride
RNA  Ribonucleic acid
S. cerevisiae  Saccharomyces cerevisiae
S. pombe  Schizosaccharomyces pombe
SCF  Skp1-cullin-F-box ubiquitin ligase protein complex
SDS  Sodium dodecyl sulphate
TAP  Tandem affinity purification
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<tr>
<td>SPB</td>
<td>Spindle pole body</td>
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<tr>
<td>UTR</td>
<td>Untranslated region</td>
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<tr>
<td>YPDA</td>
<td>Yeast peptone with dextrose (with adenine)</td>
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Publication

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Abstract

During cell division, all chromosomes must be segregated equally into each daughter cell. Errors in this process can have severe effects and give rise to aneuploidy, which is implicated in cancer progression and birth defects. The spindle checkpoint is a surveillance system that regulates the accuracy of chromosome segregation by inhibiting anaphase onset until all the sister-chromatids have established stable bi-polar attachments to microtubules emanating from opposite spindle poles. Bub1 is a conserved protein kinase that plays an essential role in the spindle checkpoint. The role of the Bub1 kinase activity remains elusive, and it is controversial whether it is required for spindle checkpoint arrest.

In this study, budding yeast was used as a model system to analyse the role of the Bub1 kinase domain, using a bub1ΔK allele lacking the C-terminal kinase domain. I showed that bub1ΔK cells are able to initiate and maintain a mitotic arrest induced by microtubule depolymerising drugs and kinetochore defects. Despite this, these cells display significant sensitivity to microtubule drugs and this is because they mis-segregate their chromosomes at high rates upon spindle re-formation. This phenotype is reminiscent of sgo1Δ cells in mitosis, and I showed that Bub1 kinase is required for accurate localisation of Sgo1 to kinetochores in metaphase. I demonstrate that both Bub1 kinase and Sgo1 are required for efficient chromosome bi-orientation, and I suggest that this is due to an inability to detect lack of tension at the kinetochores. Finally, I propose that Bub1 kinase targets Sgo1 protein to kinetochores to ensure efficient chromosome bi-orientation.
Chapter 1. Introduction

1.1 The eukaryotic cell cycle

1.1.1 Overview

Cell reproduction is a fundamental feature of life. The eukaryotic cell must divide in a highly accurate manner to ensure viability. The accuracy of cell division is therefore very tightly monitored by a number of highly complex control systems that have evolved over millions of years to ensure high fidelity of the cell cycle and to prevent disease.

The eukaryotic cell cycle is divided in four distinct phases: G1, S, G2 and M (Figure 1.1). These phases are defined according to the chromosomal events. In S phase, the chromosomes are replicated. The second major phase, M phase, consists of two main events: mitosis in which the replicated chromosomes are segregated and cytokinesis (cell division). In between S phase and M phase, there are two gap phases (G1 and G2). These phases are required to prepare for the main events in the cell, DNA replication and segregation. They also give time for the cell to grow in size and importantly to enable cells to regulate the entry into the next phase by monitoring pre-requisite steps. When cells are in G1, external and internal signals will decide whether the cell will commit to a cell cycle (START/ restriction point) or if it will execute exit from the cell cycle (also called G0).
Figure 1.1 The eukaryotic cell cycle. Schematic outline of the eukaryotic cell cycle. Although these events vary between organisms, typically the centrosomes (spindle pole bodies in yeast) are duplicated in S-phase, when chromosome replication occurs. G1 and G2 represent the gap phases that occur between S and M phase. The separate stages of mitosis are represented by images showing the main chromosomal events (modified from Cleveland et al, 2003).
The process of cell division is conserved in eukaryotic organisms, and it is therefore possible to study this important process in a variety of organisms. However, there are important differences between organisms and cell types that give advantages and disadvantages to the study of certain processes. Yeast has been an important model organism for the study of the cell cycle, and many cell cycle mutants (cdc) were originally identified in yeast (Hartwell et al., 1970; Hartwell et al., 1973; Hartwell and Smith, 1985; Weinert et al., 1994). Both budding yeast (Saccharomyces cerevisiae) and fission yeast (Schizosaccharomyces pombe) share the common advantage of the ease of genetic analysis. However, budding yeast differs from other eukaryotic organisms in some aspects. First of all, budding yeast (and fission yeast) undergoes a closed mitosis. The nuclear envelope never breaks down and the mitotic spindle is built inside the nucleus during S-phase. Importantly, this means that the chromosomes are attached to microtubules throughout the cell cycle (Tanaka et al., 2005), apart from during a short window of time when the centromeres are replicated. In contrast, animal cells undergo an open mitosis, in which the nuclear envelope breaks down to allow the spindle to form in the cytoplasm. Secondly, in budding yeast spindle pole body (SPB) duplication, DNA replication origin firing and bud emergence occur at START and spindle assembly starts immediately. Thus, the entry into mitosis in budding yeast is not as clearly defined as in other organisms (Figure 1.2).

1.1.2 Cell cycle controls

**CDKs and cyclins**

Cell cycle progression is carefully regulated by a large number of proteins and by parallel pathways. Many of these pathways are regulated by post-translational
Figure 1.2 Schematic diagram of the budding yeast cell cycle. (A) In G1 the single chromatids are clustered around the spindle pole bodies (SPB) and Cdc28/Cln3 help coordinate cell growth. (B) At START, SPB duplication and entry into S phase is initiated by Cdc28/Cln1,2. (C) S-phase cyclins Clb5-6 promotes SPB maturation and DNA replication (D) Late S phase/G2, Cdc28/Clb1-4 promotes bi-polar spindle formation to which the sister-chromatids attach. (E) Anaphase A, Pds1 destruction leads to poleward movement of chromosomes. (F) Anaphase B, the spindle elongates and pulls the sister-chromatids to opposite poles. (G) Cytokinesis and inactivation of Cdc28/Clb.
modifications of proteins such as phosphorylation, and by ubiquitin mediated proteolysis. Integral to this regulatory network are the Cyclin Dependent Kinases (Cdks) and their regulatory partner proteins, cyclins. The discovery of the fission yeast CDK (Cdc2) by Paul Nurse in the mid 1970s (Nurse, 1975), and that of cyclins by Tim Hunt in the 1980s (Evans et al., 1983) lead to a major breakthrough in the understanding of how cell cycle progression is controlled. The abundance of cyclin oscillates during the cell cycle due to regulation of their synthesis and ubiquitin dependent proteolysis. The Cdks drive cell cycle progression through interaction with stage specific cyclins that determine the Cdk localisation and substrate specificity. Cdks have no kinase activity in the absence of cyclin binding. Phosphorylation of Cdks themselves also stimulates their activity. For example, Cak1 is required for Cdc2 phosphorylation and activation in budding yeast (Espinoza et al., 1996). Cdc28 (Cdc2 in fission yeast) is the budding yeast homologue of Cdk1, and this single Cdk is able to regulate a diverse set of cell cycle transitions by association with specific cyclins. The cyclins that regulate Cdc28 in activity budding yeast are classified into G1 cyclins (Cln3), G1/S cyclins (Cln1-2), S cyclins (Clb5-6) and M cyclins (Clb1-4) and are summarised in figure 1.2. The regulation of the cell cycle in mammalian cells is far more complicated. There are at least nine Cdks, and four of them are directly involved in regulating cell cycle transitions. Mammalian cell cycle Cdks are Cdk1 (Cdc2 in fission yeast and Cdc28 in budding yeast) and Cdk2, which operate mainly in S- and M-phase.

**Ubiquitin mediated proteolysis**

Ubiquitin mediated proteolysis plays important roles in driving cell cycle transitions. Anaphase, for example, is triggered by activation of an E3 ubiquitin ligase called the
Anaphase Promoting Complex/ Cyclosome (APC/C) (reviewed in (Peters, 2006)). The ubiquitination of APC/C targets leads indirectly to the proteolytic cleavage of cohesin when cells enter anaphase (discussed in 1.2.3). While the APC/C acts mainly in mitosis and G1, the SCF (Skp1-Cullin-Fbox) is another E3 ubiquitin ligase, which is more versatile and does not only regulate proteolytic events in the cell cycle but also other cellular processes (reviewed in (Deshaies, 1999)).

**Kinases and phosphatases**

In addition to ubiquitin-dependent proteolysis, other post-translational modifications are also critical in the regulation of the cell cycle. Kinases like Polo-like kinase (Plks) and Aurora kinases (discussed in 1.6) are major cell-cycle regulators, which have been shown to regulate multiple processes throughout the cell cycle (reviewed in (Barr et al., 2004; Carmena and Earnshaw, 2003)). The sole budding yeast Plk (Cdc5) was first discovered by Kitada and colleagues (Kitada et al., 1993), and is a serine/threonine kinase required for many phases of the cell cycle, such as in entry of mitosis, bipolar spindle formation, chromosome segregation and cytokinesis. Plks represent a good example of how proteins can be subject to post-translational modifications themselves (phosphorylation and ubiquitination), which in turn increase or decrease their own activities towards other target proteins to control the cell cycle. Plks are involved in regulation of mitotic entry by stimulation of Cdk activity. In late mitosis, the budding yeast Cdc5 kinase activity is required for the activity of APC/C, and Cdc5 itself is a target of APC/C activity in late anaphase when it is degraded by the 26S proteasome (Shirayama et al., 1998). Phosphatases also play critical roles in cell cycle progression. For example the budding yeast Cdc14 phosphatase plays a role in the mitotic exit.
network (MEN), and Cdc5 is also involved in this process by localising Cdc14 by phosphorylation of its binding partner Net1 (Shirayama et al., 1998).

1.1.3 Sister-chromatid cohesion

When chromosomes are replicated in S-phase, their sister-chromatids need to remain cohesed to one another until the onset of anaphase. Mis-regulation of this pathway has severe consequences for the cell and the organism, as it gives rise to chromosome mis-segregation and aneuploidy. Hence, this pathways is subject to extremely tight cell cycle control in order to avoid aneuploidy and there are often overlapping, parallel pathways to avoid errors. During chromosome replication, a ring-like-structure called 'cohesin' is loaded onto the replicated chromatids, which holds them together. When cells enter mitosis, the sister-chromatids attach to the mitotic spindle in a bi-polar fashion. The metaphase-to-anaphase transition is a crucial point for the cell and is under tight regulation. The spindle checkpoint acts at this point (discussed in 1.4) and ensures that only once all sister-chromatids have established appropriate attachments to the spindle microtubules, is the cohesin is cleaved and cells enter anaphase.

Chromosome cohesion is established during DNA replication and is maintained until anaphase onset. The cohesin complex consists of four highly conserved proteins: Smc1, Smc3, Mcd1 (Scc1) and Scc3, and all four subunits are essential for sister-chromatid cohesion (Guacci et al., 1997; Losada et al., 1998; Michaelis et al., 1997) (reviewed in (Nasmyth and Haering, 2005) Figure 1.3 A-C). In budding yeast, cohesin is loaded during DNA replication initiation and its loading is dependent on the Scc2/Scc4 complex (Ciosk et al., 2000), amongst other factors (Figure 1.4). Nevertheless, it is not clearly understood exactly how the cohesin molecules hold sister-chromatids together,
Figure 1.3 Schematic diagram of cohesin molecules (Modified from Nasmyth, 2005). (A-C) Structure of the SMC proteins. Arrow indicates cleavage of Scc1 by separase (D). Suggested models of how cohesin holds sister chromatids together. The topological embracement model suggests that the two sister-chromatids fits within one cohesin ring, and the DNA helix with and a nucleosome bound to DNA is shown to scale, right. The other models suggest that either, cohesin binds directly to the sister-chromatids through the SMC-head subunits, or the dimer model suggests that each cohesin ring binds one sister-chromatid and the cohesin rings are connected through their hinge domains or through the Scc1 domains.
and this intriguing question has given rise to a variety of models of cohesin function during recent years (Figure 1.3) (reviewed in (Nasmyth and Haering, 2005)). In vertebrate cells, cohesin is removed in a step-wise manner (Losada et al., 1998; Waizenegger et al., 2000). Most of the cohesin on the chromosome arms is lost in the so-called ‘prophase pathway’ by the action of Polo (Plk) and Aurora B kinase activity by phosphorylation of the SA2-Scc3 subunit (Losada et al., 2002; Sumara et al., 2002). At the metaphase-to-anaphase transition the remaining centromeric cohesin is destroyed by proteolytic cleavage. This is governed by the APC/C-Separase pathway (described in section 1.2.3), which cleaves the α-kleisin subunit Mcd1 (Scc1) (Uhlmann et al., 1999). In yeast, both arm and centromeric cohesin is removed by cleavage of Mcd1 by Sephasase and complete cleavage requires phosphorylation of Mcd1 (Scc1) by Cdc5 (Alexandru et al., 2001).

1.2 Mitosis

Mitosis is a very elegant process by which the cell ensures the equal distribution of its genetic content to the two daughter cells. The regulation of mitosis is highly complex and its fidelity is crucial for cell survival.

1.2.1 The mitotic phases of budding yeast

Just like the cell cycle, mitosis is divided into distinct phases defined by chromosomal events. These stages are: prophase, metaphase, anaphase and telophase. In prophase, the sister-chromatids undergo structural changes known as condensation. This process is required for chromosome segregation. The ‘Condensin’ complex consists of five subunits: Smc2, Smc4, CAP-D2, CAP-G and CAP-H, however this complex is not
Figure 1.4 Cohesin loading and removal. (A) Cohesin loading and removal in yeast. The cohesin complex is loaded in G1, and holds the sister-chromatids together during DNA replication. Loading of cohesin is dependent on several factors. In addition to the Scc2/4 complex, loading is also dependent upon factors such as Eco1 acetyl transferase, the Ctf18/Dcc1/Ctf8 complex and Ctf4. The cohesin subunit Scc1 is then proteolytically cleaved by separase at anaphase onset, and this leads to sister-separation (Nasmyth, 2005). (B) Different cohesin removal pathways in yeast and vertebrates. (Modified from Cohen-Fix, 2000).
essential for chromosome condensation, but is required for the timing of chromosome condensation and for integrity of the chromosome structure (Hudson et al., 2003). However in budding yeast, which have rather small chromosomes, condensation of the DNA is less apparent but Condensin is still required for viability. In prometaphase the condensed chromosomes are involved in a search-and-capture process whereby kinetochores attach to spindle microtubules. In metaphase, the sister-kinetochores have established bi-polar attachments to microtubules emanating from opposite poles and as soon as all kinetochores are properly attached, the cell initiates anaphase. At anaphase, sister-chromatid cohesion is abruptly dissolved and sisters separate to each pole by means of two movements. Anaphase A is the poleward movement of the kinetochores, and Anaphase B is the increase in the pole-to-pole distance. In telophase, the elongated nucleus is pinched off during cytokinesis, the spindle disassembles and mitotic cyclins are degraded through the Mitotic Exit Network (MEN) (reviewed in (Bosl and Li, 2005)).

1.2.2 The mitotic spindle

Mitosis is highly dependent on a complex and dynamic structure, the mitotic spindle. The mitotic spindle is a bi-polar structure made up of microtubules, and is able to distribute the chromosomes to the daughter cells with amazing accuracy. The basic building blocks of microtubules consist of α- and β-tubulin heterodimers. These are joined up to create protofilaments, and 13 of these align to form a microtubule (Evans et al., 1985). The orientation in which these are ordered makes the microtubules polar. The side that exposes β-tubulin is called the ‘plus end’ and is the side that interacts with chromosomes, and conversely the ‘minus end’ exposes the α-tubulin and is present at the SPB (Desai and Mitchison, 1997) (Figure 1.5A). In addition, the polarity of
microtubules is important for movement of ‘cargo’ by microtubule motor protein. The microtubules originate from the ‘inner plaque’ of the spindle pole body (SPB) where the γ-tubulin (Tub4 in budding yeast) complex is present. This complex is required for microtubule nucleation (reviewed in (Jaspersen and Winey, 2004)).

One important aspect of microtubule behaviour in mitosis is its dynamic nature. Microtubules exhibit what is known as dynamic instability, reviewed in (Desai and Mitchison, 1997) (Figure 1.5B). It means that the ends can undergo dramatic changes from polymerising to de-polymerising states, with the minus end being slow growing and the plus end being fast growing in general. This dynamic nature is due to the fact that tubulin subunits are also GTPases, using the energy from conversion of GTP to GDP for growth and shrinkage. This dynamic nature of microtubules is important for several aspects of mitosis.

Budding yeast undergoes a closed mitosis and the SPBs are embedded in the nuclear envelope. In G1 astral microtubules emanate from the cytoplasmic face of the SPB. In early S-phase, the SPBs are duplicated and a bi-polar spindle forms by late S-phase (reviewed in (Segal and Bloom, 2001; Winey and O'Toole, 2001)). The orientation of the spindle depends on the site of bud-appearance. As discussed above, the basic structure of microtubules depends on self-assembly. However, the bi-polar spindle structure is also dependent on a number of other proteins, such as the MAPs (microtubule associated proteins) and motor proteins (Figure 1.5A). For example, bipolar spindle assembly in yeast is dependent on the plus-end directed kinesin–related motors Cin8, Kip1 (Hoyt et al., 1992; Roof et al., 1992). These generate out-ward forces that separate the SPBs (reviewed in (Kashina et al., 1997)).
Figure 1.5 Diagram of the mitotic spindle structure and associated proteins in budding yeast. (A) Structure of the budding yeast bi-polar spindle. The budding yeast spindle consists of 32 kinetochore microtubules (kMTs), 8 interpolar microtubules (iMTs) and astral microtubules (aMTs). Forces of the mitotic spindle are aided by the presence of kinesin related motor proteins and microtubule associated proteins (MAPS). These proteins play a role in SPB separation, at the kinetochore-microtubule attachment site and in spindle stability. (B) Microtubules exhibit dynamic instability (Modified from Desai, 1997).
1.2.3 The metaphase-to-anaphase transition

One of the critical events of the cell cycle is the metaphase-to-anaphase transition. Most cells wait until all kinetochores have established bi-polar attachments to the spindle, and only at this point is anaphase initiated. Errors in this process can lead to aneuploidy and death. Therefore, the cell has developed a highly complex mechanism that ensures that it does not prematurely enter anaphase until all prerequisite steps have been completed. This has become known as the ‘spindle checkpoint’ and is discussed in detail later.

However, once all kinetochores are accurately attached, the Anaphase Promoting Complex/Cyclosome (APC/C) is activated. The APC/C drives anaphase onset through targeted ubiquitination, and subsequent degradation of proteins by the 26S proteasome. The APC/C is a multi-subunit E3 ubiquitin ligase, and its activity is carefully regulated throughout the cell cycle (reviewed in (Peters, 2006)). Ubiquitination is the process by which small ubiquitin molecules are conjugated to proteins and target them for destruction by the 26S proteasome. This requires an E1 ubiquitin activating enzyme, E2 ubiquitin-conjugating enzyme(s) and an ubiquitin ligase. The APC/C E3 ubiquitin ligase also requires an activator. At the metaphase-to-anaphase transition, Cdc20 is the APC/C activator, but full APC/C activation also requires phosphorylation of APC/C by Cdk5. Cdc20 gets degraded in anaphase and is then replaced by the APC/C activator Cdh1 (Hct1) until the end of G1. At anaphase onset, APC/C<sup>Cdc20</sup> ubiquitinates a protein called Securin (Pds1 in budding yeast) (Cohen-Fix, 1996; Thornton and Toczyski, 2003; Yamamoto et al., 1996). The destruction of Pds1, in turn, releases the cysteine protease called Separase, which is responsible for Mccl cleavage and subsequent chromosome segregation at anaphase (Ciosk et al., 1998). At this point APC/C<sup>Cdc20</sup> also targets mitotic cyclins for destruction, however this process is not required for sister-
chromatid separation but for spindle disassembly and cytokinesis (Holloway et al., 1993) (reviewed in (Morgan, 1999)).

1.3 The kinetochore
Sister-chromatids attach to the mitotic spindle through a complex proteinacious machine called the kinetochore. The kinetochore varies in size and structure depending on the organism, but molecular components are conserved throughout eukaryotes. Kinetochores are made up of a hierarchy of protein complexes that assemble on centromeric DNA in a highly ordered fashion, to facilitate accurate attachments to microtubules and chromosome segregation (reviewed in (Cleveland et al., 2003; McAinsh et al., 2003; Westermann et al., 2007)). The kinetochore is a complex machine with several important roles: 1) the kinetochore is a dynamic structure that enables microtubule binding and resists pulling forces of the mitotic spindle 2) it is able to sense and correct inaccurate microtubule attachments 3) it is part of a surveillance system that ensures that anaphase is not initiated until all kinetochores have accurate bi-polar attachments. All these features of kinetochores are reflected in their dynamic and complex structure.

1.3.1 Kinetochore structure
The molecular make-up of the kinetochore is conserved amongst eukaryotes, however there are fundamental differences. Firstly the structure of the centromere upon which the kinetochores assemble differs greatly between, for example, humans, fission yeast and budding yeast (Figure 1.6 A). The budding yeast is the simplest eukaryotic centromere (~125 bp, point centromere) that encompasses the CDE I, II, and III DNA elements, which are necessary and sufficient for centromere function. In contrast, both
fission yeast and human have large centromeres with repetitive sequences, of up to 10 megabases in humans. Although these centromeres contain defined repetitive sequence elements, the centromere functions are determined by the epigenetic factors that they carry and not by simple DNA sequence (reviewed in (Karpen and Allshire, 1997)). The chromatin of centromeres consists of specialised nucleosomes with the histone H3 variant Cenp-A (Cse4 in budding yeast) (reviewed in (Black et al., 2007)).

Both in vertebrates and in yeast, the kinetochores are loaded in sub-complexes with many dependencies and in a hierarchical fashion (reviewed in (Chan et al., 2005; De Wulf et al., 2003; Maiato et al., 2004). Mammalian kinetochores are more complex and often have a higher copy number of kinetochore proteins than yeast kinetochores. Studies in budding yeast using comparative fluorescence intensity analysis, revealed a varied copy number of each kinetochore component. For example, Cse4 is present in two copies in metaphase, whereas the Dam1 and Ndc80 complexes are present in higher numbers, up to 20 copies (Joglekar et al., 2006). Lastly, a key feature that differs in budding yeast kinetochores is that it contains only one microtubule-binding site (Winey et al., 1995), compared to fission yeast and metazoans that contain many microtubule binding sites.

1.3.2 Kinetochore components in budding yeast

Fission yeast and vertebrate centromeres are dependent on repetitive heterochromatic structures (Pidoux and Allshire, 2004). However in budding yeast, the 125 bp centromere- CDE I, II and III elements are necessary and sufficient for kinetochore assembly. The budding yeast kinetochore consists of at least 65 proteins that are assembled in an orderly fashion, often in protein sub-complexes (Figure 1.6 B).
Figure 1.6 Centromeres and kinetochores. (A) Centromere organisation (Cleveland, 2003). (B) Structure of budding yeast kinetochore (adapted from Westermann, 2007), made up of inner, central and microtubule binding proteins. Protein complexes are marked by a c. following the protein name, e.g. Mtw1c. is the Mtw1 complex. EM shows Dam1 rings around microtubules.
Methods using tandem affinity purifications (TAP) have greatly aided the identification and characterisation of the budding yeast kinetochore (Cheeseman et al., 2002; Cheeseman et al., 2001; De Wulf et al., 2003; Westermann et al., 2003).

Similarly to vertebrate kinetochore organisation, the budding yeast components can be sub-divided into three classes: 1) inner kinetochore proteins, 2) central kinetochore proteins and 3) microtubule binding proteins (De Wulf et al., 2003; Westermann et al., 2007) (shown in Figure 1.6 A).

The inner-DNA binding kinetochore complexes

The CDE I DNA element in budding yeast is directly bound by the Cbf1 protein (Cai and Davis, 1990). The central region of the centromere, CDE II, is bound by the budding yeast homologue of CENP-A, Cse4, which is the centromeric histone H3-variant, essential for chromosome segregation (Basrai and Hieter, 1995). In addition, the CBF3 complex (Ndc10, Cep3, Ctf13, Skp1) binds directly to the CDE III centromeric region, and is an essential protein complex (Connelly and Hieter, 1996; Goh and Kilmartin, 1993) (reviewed in (McAinsh et al., 2003)). All other budding yeast kinetochore proteins depend on the CBF3 complex for their loading, suggesting that this complex is a determinant for kinetochore assembly (reviewed in (Westermann et al., 2007)). Cse4 protein also requires CBF3 complex for loading, but only a few kinetochore proteins have been shown to depend on Cse4.

The central and microtubule-kinetochore ‘linking’ complexes

The “linker” (central) kinetochore complexes in budding yeast consist of: the essential MIND complex (Mtw1, Dsn1, Nnf1, Nsl1), the Ndc80 complex (Ndc80, Spc43, Spc25,
Nuf2), the COMA complex (Ctf19, Okp1, Mcm21, Ame1), the Spc105 complex (Spc105, Ydr532c) and the Dam1 complex (Dam1, Duo1, Dad1-3, Spc19, Spc34, Ask1, Hsk1,3) (Cheeseman et al., 2002; Cheeseman et al., 2001) (reviewed in Westermann et al., 2007) and Figure 1.6 B). These linker-protein complexes are conserved from yeast to humans (Meraldi et al., 2006) apart from the Dam1 complex, which has not yet been identified in higher eukaryotes. Although they have been suggested to be microtubule-kinetochore ‘linker’ proteins, the Ndc80 and Spc105 homologues in C. elegans were found to bind directly to microtubules and that these interactions were crucial for kinetochore-microtubule binding (Cheeseman et al., 2006). Vertebrate studies also revealed that cells depleted of Ndc80 show severe alignment phenotypes (DeLuca et al., 2002). Furthermore RNAi studies in HeLa cells of the human Ndc80, homologue, Hec1 (highly enriched in cancer cells) (DeLuca et al., 2005), confirmed that this protein is essential for kinetochore-microtubule binding.

The Spc105-Ydr532c complex has in recent years proven to be an important structural component of the budding yeast kinetochore (Meraldi et al., 2006). This complex has been shown to interact both with the Ndc80 complex and the Mtw1 complex (Nekrasov et al., 2003). Also, the COMA complex has been shown to bind to the CBF3 complex and required Cse4 for localisation (Ortiz et al., 1999).

The protein complexes that are present at the kinetochore-microtubule interface, need to show two main features: 1) the ability to recruit and coordinate MAPS and microtubule motors that will generate force in an ATP-dependent manner, and 2) mechanically link the kinetochore with the dynamic microtubules and enable it to resist strong spindle pulling forces. These complexes consist of the Dam1 complex (Cheeseman et al., 2002;
Cheeseman et al., 2001) and the Chromosomal Passenger complex (Ipl1, Sli15, Bir1) (Sandall et al., 2006). The microtubule binding proteins Stu2, Bik1 and Bim1 are also present in this region and are required for microtubule-kinetochore attachments, as are the microtubule plus-end motors Cin8 and Kip1 (De Wulf et al., 2003). Interestingly, the Dam1 complex forms a ring-like structure that associates with microtubules (reviewed in (Westermann et al., 2007; Westermann et al., 2006) Figure 1.6 A). The Dam1 ring bundles microtubules and has been shown to bind preferentially to the growing end of microtubules (Westermann et al., 2005). The Dam1 rings appear to be mobile on the microtubule lattice, as opposed to stably interacting with specific regions of the microtubule. This feature has been suggested to be involved in force generation at the microtubule plus end. However, a vertebrate Dam1 homologue has yet not been identified.

1.3.3 Kinetochore-microtubule attachments

The fidelity of chromosome segregation relies on accurate kinetochore-microtubule attachments. Mutants that give rise to attachment defects (like ndc80 mutations) give rise to severe chromosome mis-segregation and death, underlining the importance of accurate chromosome attachments. Sister-chromatids ultimately need to attach to the mitotic spindle in a bi-polar fashion with the kinetochores attached to microtubules emanating from opposite spindle poles. However, due to the stochastic nature of microtubule capture, this process is error prone, and needs to be carefully regulated. Animal and fission yeast cells differ to budding yeast cells in this process because budding yeast kinetochores have been shown to only have one microtubule binding-site, whereas animal and fission yeast cells have several. When the DNA is replicated, it is thought that the kinetochores momentarily disassemble, but re-assemble again shortly
after. In budding yeast, the kinetochores remain attached to the spindle throughout most of the cell cycle (Guacci et al., 1997). Initially in G1, the single chromatids are attached to an array of microtubules emanating from the SPB. When SPBs are duplicated and a bi-polar spindle forms, the second, replicated chromatid gets attached to the opposite SPB and forms bi-polar attachments. However, this process is not always as simple as that. Several types of incorrect attachments can occur (Figure 1.7). Firstly, monopolar attachments can happen where only one sister-chromatid is attached, and the second kinetochore is still to be attached by a microtubule emanating from the opposite SPB. Secondly, syntelic attachments occur, where both sister-kinetochores are attached to microtubules arising from the same SPB (Tanaka, 2002). In animal and fission yeast cells, yet another attachment defect can occur due to the fact that they have multiple microtubule binding sites. It is then possible that one single kinetochore becomes attached to microtubules emanating from different poles. These are called merotelic attachments, and have been suggested to be the reason for lagging chromosomes that can occur in mammalian and fission yeast cells with compromised kinetochore structure or regulation (Cimini et al., 2001; Gregan et al., 2007). Correct bi-polar attachments, also called amphitelic attachments, occur when the kinetochores are attached to microtubules emanating from opposite poles. Intriguingly, bi-polar attachments in budding yeast give rise to transient separation of the centromere by spindle pulling forces and directional stability of microtubules (Goshima and Yanagida, 2000; He et al., 2000). This process can be visualised, by marking the CEN-DNA with GFP, and shows centromeric ‘breathing’ upon bi-orientation.

The cell requires both error detection mechanisms and the ability to correct these mal-oriented kinetochores. These mechanisms are starting to become elucidated and have
Figure 1.7 Modes of kinetochore-microtubule attachments. Monotelic, syntelic and merotelic attachments are incorrect and need to be corrected by the cell. Whereas monotelic and syntelic attachments give rise to lack of tension at kinetochores, merotelic attachments can still give rise to some tension. In budding yeast only monotelic and syntelic mis-attachments occur because there is only one microtubule binding site per kinetochore. However fission yeast and mammalian cells kinetochores can bind several microtubules, and merotelic attachments can occur.
been shown to rely on a number of kinetochore and microtubule associated proteins. Central to these are the Chromosomal Passenger proteins that can both detect and correct these incorrect attachments. This group of important proteins are discussed further in section 1.6.

1.4 The spindle checkpoint

1.4.1 Cell cycle checkpoints

The fidelity of the eukaryotic cell cycle is dependent on checkpoints. The concept of cell cycle ‘checkpoints’ was coined in the 1980s (Weinert and Hartwell, 1988) (reviewed in (Hartwell and Weinert, 1989)). Cell cycle checkpoints are mechanisms by which cell progression is actively halted in the absence of accurate completion of prerequisite steps. Initial studies identified several mutants (e.g. rad9) that failed to arrest the cell cycle in response to DNA damage. Since then, other cell cycle checkpoints have been described, for example the ‘DNA replication checkpoint’ that halts cell cycle progression in response to incomplete replication (Sanchez et al., 1997). The ‘spindle position checkpoint’ is required to ensure that the spindle has reached the bud-neck before exit from mitosis (Adames et al., 2001; Lee et al., 2001). Furthermore, the ‘NoCut checkpoint’ ensures that cytokinesis is prevented in the presence of spindle-midzone defects. This pathway involves the Aurora B homologue, Ipl1, and is essential for accurate chromosome segregation (Norden et al., 2006). In addition, the ‘spindle checkpoint’ plays a crucial role in regulating the metaphase-to-anaphase transition (discussed further below).

The spindle checkpoint
Essential for prevention of aneuploidy, the spindle checkpoint (also called the mitotic checkpoint, spindle assembly checkpoint or the metaphase checkpoint) monitors kinetochore-microtubule attachments and inhibits anaphase onset until all kinetochores have been attached in a bi-polar fashion (Figure 1.8). The core spindle checkpoint components, Mad1-3 (mitotic arrest deficient) and Bub1,3 (budding uninhibited by benzimidazole) were originally identified in separate budding yeast screens for mutants that are sensitive to spindle poisons (Hoyt, 1991; Li, 1991). In addition, Mps1 (monopolar spindles) was later identified as a spindle checkpoint component (Weiss and Winey, 1996; Winey et al., 1991). These protein components are conserved from yeast to man. The spindle checkpoint components are required for mitotic arrest in response to microtubule drugs in many organisms (Basu et al., 1999; Bernard et al., 1998; Gorbsky et al., 1998; Hardwick et al., 1999; Taylor and McKeon, 1997). One common feature of spindle checkpoint components is that they localise to kinetochores that are still to achieve stable bipolar attachments (Chen et al., 1998; Johnson et al., 2004; Millband and Hardwick, 2002; Taylor et al., 1998; Vanoosthuyse et al., 2004; Waters et al., 1998) (and reviewed in ). Similar to the kinetochore components, the spindle checkpoint proteins assemble on un-attached kinetochores in a highly hierarchical manner with many dependencies (discussed more in 1.5).

The main downstream target of the spindle checkpoint is the Cdc20, accessory subunit of the APC/C (Hwang et al., 1998; Kim et al., 1998). The spindle checkpoint proteins act in concert to inhibit this activator of the APC/C, until all kinetochores are appropriately attached (Figure 1.8). The role of kinetochores in the spindle checkpoint response has been under debate for many years. It is known that spindle checkpoint components localise to unattached kinetochores, which may act as a catalytic site for the
Figure 1.8 The spindle checkpoint. (A) The spindle checkpoint is activated by unattached kinetochores or by kinetochores that lack tension. The spindle checkpoint components inhibit the activity of the E3 ubiquitin ligase- APC/C. (B) The spindle checkpoint components localise to unattached kinetochores in an orderly fashion. Bub1 protein kinase acts as a scaffold and is required for localisation of many proteins. Spindle checkpoint proteins inhibit the activity of APC/C by many modes. They form inhibitory complexes, MCC, Mad2-Cdc20, but phosphorylation of Cdc20 by Bub1 may also be required in human cells.
‘wait anaphase’ signal (further discussed below, and reviewed in (Musacchio and Salmon, 2007) (Figure 1.8).

1.4.2 The spindle checkpoint: relevance to disease

The presence of a spindle checkpoint that ensures accurate chromosome segregation and prevents aneuploidy is also relevant to disease. Aneuploidy is a common characteristic of many human cancers (reviewed in (Kops et al., 2005b)). Also, meiotic aneuploidy occurring in the human germline has also been shown to lead to embryonic lethality, apart from trisomy 13, 18 and 21 (Down’s syndrome) that result in severe birth defects. A common feature of cancer cells is ‘chromosomal instability’ (CIN), where cells easily lose or gain extra chromosomes during cell division (Cahill et al., 1998). Although the molecular mechanism that gives rise to this phenotype of cancer cells is not clearly understood, defects most likely lie in mis-regulation of the mechanisms that ensure accurate chromosome segregation and control of the metaphase-to-anaphase transition. There is an ongoing debate about whether aneuploidy is directly contributing to tumour formation or is an indirect consequence (Weaver and Cleveland, 2006). Nevertheless, these features clearly demonstrate the importance to study the control of chromosome segregation in mitosis.

1.4.3 The spindle checkpoint components: an overview

Mad1 is a phospho-protein, which has been shown to bind Mad2 constitutively. Mad1 phosphorylation increases during spindle damage in budding yeast, and is dependent on Bub1, Bub3 and Mad2 (Hardwick and Murray, 1995). Mad2 is considered as one major downstream component in the spindle checkpoint pathway. Both Mad1-bound and free Mad2 have been shown to be required for spindle checkpoint arrest (Chung and Chen,
2002). Mad2 is found in complex with other spindle checkpoint proteins and with the APC/C activator, Cdc20 (Figure 1.8 B). It binds Cdc20 directly to inhibit APC/C activity, and mutations in cdc20 that affect Mad2-binding show a defective spindle checkpoint (Hwang et al., 1998; Kim et al., 1998). Mad2 is also part of a larger inhibitory complex called MCC (mitotic checkpoint complex), that in budding yeast consist of Mad2, Mad3, Bub3 and Cdc20 (Hardwick et al., 2000). These complexes have been proposed to bind to Cdc20 and inhibit anaphase in response to unattached kinetochores. Structural studies have revealed unusual conformational switches of Mad2 that are regulated depending on whether it is bound to Mad1 or Cdc20 compared to free Mad2 (De Antoni et al., 2005; Yu, 2006). When Mad2 is bound to Mad1 or Cdc20, it changes conformation from an ‘open’ form (oMad2), and instead adopts a ‘closed’ conformation (cMad2), which is thought to trap the binding protein. The ‘template model’ suggests that kinetochore-bound Mad1 binds cMad2, and that this complex recruits further oMad2 and converts it to cMad2, which in turn has higher affinity for Cdc20 (De Antoni et al., 2005; Sironi et al., 2001).

Mad3, and its human homologue BubR1, are also key components of the spindle checkpoint that show a direct role in inhibition of the APC/C^{Cdc20} by formation of a complex with Mad2 and Cdc20 (MCC) (Chen, 2002; Fang, 2002; Hardwick et al., 2000; Mao et al., 2003; Sudakin et al., 2001). Mad3 shows two homology domains with the N-terminus of Bub1 and BubR1 (Taylor et al., 1998). However Mad3 lacks the C-terminal kinase domain of the Bub1 and BubR1 homologues. Recent budding yeast studies have revealed that the two Mad3 KEN boxes (KEN30, KEN296) play a role in the spindle checkpoint and that deletion of KEN30 abolishes MCC formation and interaction with Cdc20 (Burton and Solomon, 2007; King et al., 2007b). Furthermore,
Mad3 has been shown to be phosphorylated by the budding yeast Cdc5 and the Aurora B homologue, Ipl1, and the latter is required for the spindle checkpoint response to lack of tension at kinetochores (King et al., 2007a; Rancati et al., 2005). The mammalian homologue of Mad3, **BubR1**, carries a kinase domain that is activated by the microtubule-associated protein CENP-E (Mao et al., 2003). Intriguingly, microtubule capture by the CENP-E motor domain appears to silence BubR1 kinase activity, and hence the checkpoint (Mao et al., 2005). In addition BubR1 also plays a critical role in the inhibition of APC/C by formation of MCC in human cells (Sudakin et al., 2001). **Bub3** is a WD repeat protein that binds constitutively with Bub1 and Mad3. The structure of Bub3 reveals a WD propeller structure. The Bub1 and Mad3 proteins bind on the top surface of this propeller structure. They are thought to compete for this binding site (Larsen et al., 2007; Larsen and Harrison, 2004; Wilson et al., 2005).

The spindle checkpoint also contains other kinases: the **Mps1** kinase and **Bub1** kinase (Bub1 is discussed in detail in section 1.5). Mps1 is an essential protein in budding yeast that acts upstream in the spindle checkpoint pathway. Over-expression of this kinase causes a spindle-damage independent metaphase delay that is dependent on all other Mad- and Bub1 proteins (Hardwick et al., 1996). Mps1 is itself regulated by phosphorylation, which peaks in mitosis and is essential for its spindle checkpoint function (Jones et al., 2005; Winey and Huneycutt, 2002). Mps1 was identified for its essential role in spindle pole body duplication (Winey et al., 1991). The fission yeast homologue, Mph1, does not have a role in SPB duplication and is not an essential protein, however it is required for spindle checkpoint function (He et al., 1998). The kinase domain of Mph1 has two functions: it is both required for efficient spindle
checkpoint arrest and for accurate chromosome segregation in an unperturbed mitosis (KH, LM, JZ, unpublished).

Interestingly, metazoans have additional spindle checkpoint components that are not present in yeast. For example the ZW10 (Zeste White), Rod (Rough deal) and Zwilch were discovered in *Drosophila*. Mutations in these give rise to lagging chromosomes and aneuploidy (Karess and Glover, 1989). These proteins form a complex and are required for kinetochore localisation of minus-end directed motors (dynein and dynactin). This event has been suggested to be required for pole-ward movement of chromosomes (Savoian et al., 2000; Sharp et al., 2000) (reviewed in (Karess, 2005)). Importantly the Zw10, Rod, Zwilch complex is also required to localise Mad1-Mad2 complex to the kinetochore (Buffin et al., 2005; Kops et al., 2005a).

Furthermore, in vertebrate cells the spindle checkpoint proteins also appear to be important for mitotic timing, and this could explain why these components are essential in mammals (Gorbsky et al., 1998; Meraldi et al., 2004; Taylor and McKeon, 1997). One study described a particular affect on mitotic timing when the MCC components Mad2 or BubR1 were depleted by RNAi (Meraldi et al., 2004), and it was suggested that Mad2 and BubR1 may act as cytosolic timers required to inhibit APC/C early in mitosis.

1.4.4 The spindle checkpoint signal

A classical laser ablation experiment showed that one single unattached kinetochore is sufficient to inhibit anaphase (Rieder et al., 1995). This suggests that the spindle checkpoint does not only need to monitor the attachments of all kinetochores, but it also
needs to transmit a global signal to inhibit the APC/C. The nature of this signal remains elusive and the role for the kinetochore in checkpoint signalling is still not fully understood (discussed further in 1.4.5). The kinetochore is, in one sense, a biochemical factory for inhibitory complexes but some studies have revealed that these complexes could be formed independently of the kinetochore (Fraschini et al., 2001a). There appear to be several modes in the cell by which APC/C can be inhibited (Figure 1.8 B). Firstly, Mad2 binds directly to Cdc20 and inhibits APC/C activity (Fang et al., 1998; Hwang et al., 1998; Kim et al., 1998). A similar mode of inhibition is carried out by the potent APC/C inhibitor, the mitotic checkpoint complex (MCC), which consists of Mad2, Mad3/BubR1, Bub3 and Cdc20 (Fraschini et al., 2001b; Hardwick et al., 2000; Milliband and Hardwick, 2002). The MCC appears to have a stronger inhibitory effect on the APC/C than Mad2-Cdc20 binding alone. It was shown that inhibition of the APC/C by MCC purified from HeLa cells was 3000-fold higher than recombinant Mad2 alone (Sudakin et al., 2001) (reviewed in (Musacchio and Salmon, 2007)). In addition, Bub1 has been shown to phosphorylate Cdc20 in human cells and this phosphorylation appears to be required for full APC/C inhibition (Tang et al., 2004a). Budding yeast experiments have also revealed another mode of regulation in Cdc20 turnover in the cell (Pan and Chen, 2004). This study showed that the stability of Cdc20 is increased in spindle checkpoint mutants, and that this leads to impaired spindle checkpoint function. Therefore, it seems likely that there are several, overlapping modes of APC/C inhibition carried out by the spindle checkpoint machinery.

1.4.5 The role for the kinetochore in the spindle checkpoint

The precise role for the kinetochore in spindle checkpoint functions remains controversial. A budding yeast study indicated that APC/C inhibitory complexes can
still form independently of the kinetochores, as shown in *ndc10* mutants (Fraschini et al., 2001a). In agreement with this, it was shown that the MCC still forms in budding yeast mitotic cells independently of spindle checkpoint activation (Poddar et al., 2005). Moreover, the spindle checkpoint in human cells is still functional when Aurora B is inhibited and the kinetochore targeting of BubR1, Mad2 and Cenp-E is abolished (Ditchfield et al., 2003). In contrast, some core kinetochore complexes were shown to be required for spindle checkpoint arrest, for example the core CBF3 and the Ndc80 complexes (Gardner et al., 2001; Janke et al., 2001).

Although it appears that MCC can still form independently of the kinetochores in some systems, it is not clear how potent this MCC is with regards to APC/C inhibition. The possibility remains that the kinetochore is required to activate the MCC, but not for its formation. It has been suggested that the kinetochores may not be required for formation of the MCC, but rather for ‘sensitizing’ the APC/C for MCC inhibition, or to convert the MCC to an active form (reviewed in (Musacchio and Salmon, 2007)).

1.4.6 Spindle checkpoint activation: Tension v attachment

Not only does the spindle checkpoint monitor the presence of unattached kinetochores, it also monitors tension that occurs at the kinetochores upon bi-orientation (Li and Nicklas, 1995; Nicklas et al., 1995). There appear to be two pathways for spindle checkpoint activation: one induced by un-attachment and one induced by lack of tension (reviewed in (Pinsky and Biggins, 2005). Although it has over recent years proven difficult to tease these two pathways apart, some studies have addressed these pathways separately. Original experiments in vertebrate cells using the drug taxol, showed that cells monitor tension. Taxol stabilises microtubules and although the kinetochores still
attach in these conditions, microtubules lose their dynamic behaviour and do not give rise to tension across the kinetochore (Waters et al., 1998). This study showed that Mad2 localises to kinetochores only when they are un-attached and not when they lack tension, whereas BubR1/Mad3 and Bub1 remained on kinetochores that are not under tension (Skoufias et al., 2001; Zhou et al., 2002). Furthermore, studies in yeast showed that Mad2 is required for spindle checkpoint arrest in response to un-attached kinetochores and to those that lack tension (Stern and Murray, 2001). Intriguingly, these two pathways appear to be linked through the passenger proteins, which detect kinetochores that are not under tension and thereby create unattached kinetochores that are detected by the spindle checkpoint (Pinsky et al., 2006) (reviewed in (Vagnarelli and Earnshaw, 2004)). Moreover, Mad3 phosphorylation by the budding yeast Aurora B homologue (Ipl1) kinase has been shown to be required for a mitotic delay in response to reduced cohesion (King et al., 2007a). The passenger proteins, and their role in tension sensing will be further described in section 1.6. In vertebrate cells, a phosphorylation dependent epitope, 3F3/2, appears on kinetochores that lack tension (Gorbsky and Ricketts, 1993), and this staining disappeared when tension was re-applied in grasshopper spermatocytes (Nicklas et al., 1995). The kinase responsible for this tension specific phosphorylation has since then been shown to be the Polo-like kinase Plk1 (Ahonen et al., 2005; Wong and Fang, 2005). However, the epitopes themselves remain unclear.

1.5 Bub1 kinase plays multiple roles in mitosis

The Bub1 kinase is essential for spindle checkpoint function, but also plays other major roles in mitosis. These functions are discussed below (roles for Bub1 are also summarised in figure 1.9 B)
Figure 1.9 Structure and function of Bub1. (A) Diagram of conserved domains in Bub1, Bub3 and BubR1. Light blue box shows the conserved Mad3-like N-terminal domain, which is required for kinetochore targeting. Blue box indicates the Bub3 binding region. Black box shows the C-terminal kinase domain. Mad3 lacks this kinase domain but carries two KEN-boxes (red box) that are also present in human BubR1. Recently, KEN boxes were also shown to be present in human Bubi (orange box) (Qi et al., 2007). (B) Overview of bub1 mutant phenotypes: 1) Bub1 is required for the spindle checkpoint (see benomyl sensitivity, Warren, 2002), 2) for chromosome segregation (see chromosome loss assay and lagging chromosomes in human cells, left, and in fission yeast, right (Warren, 2002; Jeganathan, 2007, V. Vanoosthuyse), 3) for regulation of Shugoshin localisation (see HeLa cell Bub1 RNAi. Kitajima, 2006) and 4) for sister-chromatid cohesion in vertebrate cells (see chromosome spreads. Tang, 2004).
1.5.1 Bub1 kinase, a spindle checkpoint component

*Bub1 structure*

Bub1 was originally identified as a protein required for mitotic arrest in response to spindle damage (Hoyt, 1991), and *bub1* mutants die rapidly when grown in the presence of microtubule drugs. It has since then been proven that Bub1 plays an essential role in the spindle checkpoint in all organisms tested (Roberts et al., 1994; Taylor and McKeon, 1997; Vanoosthuyse et al., 2004). The budding yeast Bub1 is a 118 kDa, conserved serine/threonine kinase that shares domains of homology with Mad3 and BubR1 (Roberts et al., 1994), described in Figure 1.9. The N-terminal region of Bub1 is required for localisation to kinetochores (Taylor et al., 1998; Taylor and McKeon, 1997; Vanoosthuyse et al., 2004) (Figure 1.9). The Bub3 binding region is essential for Bub3 binding and for also Bub1 and Bub3 localisation to kinetochores (Roberts et al., 1994; Taylor et al., 1998).

*Bub1 interactions*

Budding yeast Bub1 binds Bub3 throughout the cell cycle, and this binding is essential for function (Roberts et al., 1994). A mutation (*bub1-1*) in this region showed a very similar phenotype to that of a *BUB1* deletion, and was shown to be a single amino acid change in the Bub3 binding domain (amino acid 313-356) (Taylor et al., 1998; Warren et al., 2002). In fact, Bub3 was cloned as a multi-copy suppressor of this mutation (Hoyt, 1991). The C-terminal domain of Bub1 shares homology with other serine/threonine kinases. *In vitro* experiments reported auto-phosphorylation of Bub1 and also Bub1 dependent phosphorylation of Bub3 (Roberts et al., 1994). However this observation, or the relevance of this phosphorylation, has not been further investigated *in vivo*. In budding yeast, Bub1 is found in a complex with Mad1 and Bub3, during a
short window of time every cell cycle (Brady and Hardwick, 2000). This complex is also formed upon spindle checkpoint activation when kinetochore-microtubule attachments are still to be made (Brady and Hardwick, 2000). Mad1 was also found to bind and be phosphorylated by human Bub1 kinase \textit{in vitro} (Seeley et al., 1999), but further reports on Bub1-Mad1 interactions have yet to be made, and the physiological relevance of this interaction remains uncertain.

\textit{Bub1 regulation}

Many kinases are regulated by auto-phosphorylation or by phosphorylation by other kinases. This is also true for Bub1, which is phosphorylated in mitosis and shows hyper-phosphorylation upon spindle checkpoint activation (Brady and Hardwick, 2000; Chen, 2004; Taylor et al., 2001). The fission yeast Cdk (Cdc2) has been shown to phosphorylate Bub1 in mitosis and this phosphorylation is required for full spindle checkpoint arrest (Yamaguchi et al., 2003). Bub1 phosphorylation is also important in other organisms. MAPK dependent phosphorylation of Bub1 in \textit{Xenopus} chromosomal fractions was shown to be required for Bub1 to respond to weak attachment defects, such as those in late pro-metaphase or induced by low levels of microtubule drugs (Chen, 2004). Treatment with low drug concentrations in these extracts also results in less recruitment of Bub3, Mad1 and Mad2 in the Bub1 mutant that lacks MAPK phosphorylation.

\textbf{1.5.2 Bub1 is required for kinetochore recruitment of spindle checkpoint and kinetochore components}

Recruitment of spindle checkpoint components to the kinetochores is carefully regulated. Some spindle checkpoint and kinetochore proteins localise to kinetochores
only in mitosis. This could reflect their role in monitoring microtubule-kinetochore attachments, and levels of Mad2, Bub1 and BubR1 proteins on kinetochores decrease following microtubule capture (Chan et al., 1998; Jablonski et al., 1998; Taylor et al., 2001; Taylor and McKeon, 1997).

**Bub1 is required for localisation of outer kinetochore proteins**

Bub1 plays a crucial role in regulating the localisation of spindle checkpoint, and kinetochore components in many organisms (discussed below). Bub1 itself is recruited to unattached kinetochores or to those that lack tension in mitosis (Taylor et al., 2001), and it is sometimes referred to as a 'scaffold' protein. FRAP experiments indeed showed that Bub1 and Mad1 are stably associated with kinetochores, whereas Mad3/BubR1 and Mad2 show highly dynamic features at the kinetochores (Howell et al., 2004; Shah et al., 2004). Moreover, Bub1 was found to be required to localise outer kinetochore proteins such as BubR1, Cenp-E, Cenp-F and Mad2 in human cells (Johnson et al., 2004), and BubR1, Mad2, Mad1 and Cenp-E in *Xenopus* (Sharp-Baker and Chen, 2001).

**Bub1 is required for localisation of inner centromere proteins**

In addition, a recent study suggested a role for Bub1 in recruitment of inner centromere proteins in *Xenopus* egg extracts. This study suggests that Bub1 does not only recruit BubR1, Mad2, Bub3 and dynein-dynactin complexes to kinetochores, but is also required for accurate localisation of the chromosomal passenger proteins Aurora B and Borealin (Dasra A) to the inner centromere in both *Xenopus* egg extracts and in HeLa cells (Boyarchuk et al., 2007), in addition to Sgo1 and MCAK. In this study Aurora B and Survivin appeared to mis-localise to chromosome arms in Bub1 RNAi treated cells.
Biochemical studies implied that Bubi may phosphorylate INCENP and that this phosphorylation is required for stability of the passenger protein complex (the passenger proteins will be further discussed in section 1.6).

Interestingly, Bubi and Plk1 were shown to form a complex in in vitro binding assays from HeLa cells (Qi et al., 2006). This binding occurs through the polo-box domain, and phosphorylation of Bubi (T609) by Plk1 is required. This binding appears to be necessary for Plk1 to localise to kinetochores (Qi et al., 2006). Furthermore, Bubi, Mps1 and BubR1 have been found to be required for Plk1 (Plx1) loading in Xenopus extracts (Wong and Fang, 2006), and both Bubi and Mps1 kinase activities were shown to be required. This indicates yet another important scaffolding role for Bubi in Polo recruitment.

1.5.3 Bubi plays a role in chromosome segregation

Bubi also plays a separate role from that in the spindle checkpoint, in chromosome segregation and congression (Bernard et al., 1998; Johnson et al., 2004; Meraldi and Sorger, 2005; Perera et al., 2007; Vanoosthuyse et al., 2004; Warren et al., 2002). In budding yeast it was shown that, although the spindle checkpoint proteins are not essential for viability, mutations in these components give rise to increased chromosome loss in an un-perturbed mitosis (Warren et al., 2002). The level varies amongst spindle checkpoint mutants, but cells lacking Bubi shows the highest levels of chromosome loss. Moreover, fission yeast experiments have revealed that cells lacking Bubi kinase show defects in chromosome attachments. These cells display lagging chromosomes (Bernard et al., 1998; Vanoosthuyse et al., 2004), which are though to arise from un-corrected merotelic attachment (Gregan et al., 2007) (Figure 1.9 B).
Studies in HeLa cells also provide evidence that Bub1 is required for efficient chromosome congression by formation of stable end-on attachments (Meraldi and Sorger, 2005). The defects in chromosome congression are not simply a consequence of disruption of the spindle checkpoint per se, since Mad1, Mad2 or Bub3 depletion did not give rise to problems with chromosome congression. Cenp-E has previously been found to be required for chromosome congression, which agrees with the fact that Bub1 is required for Cenp-E localisation (Johnson et al., 2004; Sharp-Baker and Chen, 2001). However in the study by Meraldi and Sorger (Meraldi and Sorger, 2005), Cenp-E levels in Bub1 depleted human cells appeared unaffected, implying that the congression defect is not due to inability of these cells to localise Cenp-E and BubR1.

1.5.4 The role for Bubl kinase domain remains elusive

Although it has been clearly established that the N-terminal domains of Bub1 are essential for a spindle checkpoint arrest (Vanoosthuyse et al., 2004; Warren et al., 2002), the role for the C-terminal kinase domain in mitosis remains controversial. In my PhD, I have focused on this question and carried out a detailed analysis of the effects on budding yeast cells lacking the Bub1 kinase.

Initial budding and fission yeast studies implied that the kinase domain is essential for spindle checkpoint arrest (Roberts et al., 1994; Yamaguchi et al., 2003). Conversely, other studies suggested that the kinase domain was not required for the spindle checkpoint per se but required for accurate chromosome segregation (Vanoosthuyse et al., 2004; Warren et al., 2002). These conflicting results could partially be due to the fact that the \textit{bublK733R} mutant gives rise to instability of the Bub1 protein in budding
yeast (Warren et al., 2002). In addition to this, *Xenopus* experiments have shown that the recruitment of Mad1, Mad2, Bub3 and Cenp-E by Bub1 appears to be kinase independent (Sharp-Baker and Chen, 2001). Thus, it remains to be elucidated what role the Bub1 kinase domain plays in mitosis. One study however, provided evidence that Bub1 phosphorylates Cdc20, and it suggests that this phosphorylation is required for full inhibition of APC/C in human cells (Tang et al., 2004a). This has not been confirmed in other systems. To date, little is known of Bub1 substrates.

1.6 Chromosomal Passenger proteins

Another protein complex important for the spindle checkpoint, and for chromosome bi-orientation is the ‘chromosomal passenger complex’. These proteins were initially described in vertebrate cells (Cooke et al., 1987; Earnshaw and Bernat, 1991). During recent years, intensive research has revealed that these proteins regulate important mitotic events, and that they show dramatic and distinct localisation patterns throughout mitosis (reviewed in (Ruchaud et al., 2007) and described in figure 1.10 A).

1.6.1 Overview of the Chromosomal Passenger proteins

The central components of the Chromosomal Passenger Complex (CPC) are INCENP, Aurora B, Survivin and Borealin. INCENP (inner centromere protein) was the first passenger protein identified by a monoclonal antibody towards a protein that showed the distinct localisation pattern that these proteins exhibit (Cooke et al., 1987) (Figure 1.10 A).
Figure 1.10 The Chromosomal Passenger proteins. (A) Localisation pattern of Chromosomal Passenger proteins in HeLa cells (adapted from, Ruchaud et al., 2007). Aurora B (green) represents the typical localisation pattern of the Chromosomal Passenger proteins, which is conserved between organisms. Chromosomal Passenger proteins are present on the chromosome arms in prophase, at the inner centromere in metaphase, relocate to the spindle in anaphase and finally onto the spindle midzone in telophase. (B) Model for budding yeast Chromosomal Passenger proteins in tension sensing and error correction (Sandall, 2007).
The Chromosomal Passenger complex

The catalytic subunit of the passenger complex is Aurora B, which is a serine/threonine kinase that has many roles during mitosis (Carmena and Earnshaw, 2003). It binds to INCENP through the IN box of INCENP (Adams et al., 2000), and it has been shown to activate Aurora B kinase activity. Survivin also binds to Aurora B and is a target of Aurora B kinase activity. Borealin was identified as a chromosome binding protein (Gassmann et al., 2004; Sampath et al., 2004), and was found to bind to Survivin and INCENP by co-immunoprecipitation in mitotic HeLa cells (Gassmann et al., 2004). Most chromosomal passenger proteins are well conserved and have homologues in both budding and fission yeast: Aurora B/ Ipl1/Ark1, INCENP/Sli15/Pic1 and Survivin/ Bir1 respectively. However a yeast homologue of Borealin has not yet been identified. In yeast, it was found that Bir1 forms a complex with the core CBF3 components, and recent studies have revealed that the Bir1-Sli15 complex is required for microtubule binding to kinetochores (Gillis et al., 2005; Sandall et al., 2006).

The CPC has been shown to regulate many important aspects of mitosis, and depletion or mutations in these proteins have severe consequences for cells and organisms. During mitosis the CPC plays diverse roles such as in regulation of chromosome structure, spindle assembly, sister chromatid cohesion, establishment of accurate bipolar chromosome attachments, the spindle checkpoint and it also has late mitotic roles in cytokinesis (reviewed in (Ruchaud et al., 2007)). Here I will focus on the roles of the passenger proteins (in particular Aurora B/Ipl1) in the spindle checkpoint and chromosome bi-orientation.
1.6.2 The role for the Chromosomal Passenger proteins in tension sensing and chromosome bi-orientation

Studies in both yeast and vertebrates in recent years have revealed a role for the passenger proteins in the sensing and correction mechanism required for the establishment of chromosome bi-orientation (Adams et al., 2000; Gassmann et al., 2004; He et al., 2001; Pinsky et al., 2006; Tanaka, 2002) (reviewed in (Ruchaud et al., 2007; Tanaka, 2005)) (Figure 1.9 B). What is the mechanism in which the spindle checkpoint and Chromosomal Passenger proteins ensure proper chromosome bi-orientation? There has been a debate about whether these proteins respond to unattached kinetochores or to kinetochores that lack tension (discussed above). Interestingly, it appears that the Chromosomal Passenger proteins can sense the lack of tension at kinetochores that occur when they are mal-oriented (syntelic attachments), and ‘break’ these connections to create unattached kinetochores, which in turn activate the spindle checkpoint (Pinsky et al., 2006) (model in figure 1.9 B).

Budding yeast Ipl1-Sli15 complex is required for bi-orientation

The budding yeast Aurora B homologue, Ipl1, is required for establishment of chromosome bi-orientation (Biggins et al., 1999; Tanaka et al., 2002), and is required to respond to defective kinetochores (or kinetochores that lack tension), but is not required for metaphase arrest induced by anti-microtubule drugs (Biggins and Murray, 2001). Ipl1 is an essential protein in budding yeast. It is thought that kinetochores in budding yeast initially attach syntelically to microtubules from one spindle pole body. Ipl1 breaks these attachments and bi-orientation on the bi-polar spindle then occurs, hence this function is essential for viability in yeast (reviewed in (McAinsh et al., 2003)). Ipl1 also co-operates with the INCENP homologue, Sli15, in this process and ipl1 and sli15
mutants show severe chromosome mis-segregation. In these cells, most kinetochores appear to reside by one spindle pole, presumably due to unresolved syntelic attachments (Biggins et al., 1999; Chan and Botstein, 1993; He et al., 2001; Kim et al., 1999; Tanaka, 2002).

**Vertebrate Aurora B kinase**

Aurora B-INCENP complex also appears to play an important role in bi-orientation in higher eukaryotes, and disruption of Aurora B function gives rise to defects in chromosome congression in many systems (Adams et al., 2000; Ditchfield et al., 2003; Hauf et al., 2003; Kallio et al., 2002) (reviewed in (Carmena and Earnshaw, 2003)). Interestingly, mammalian Aurora B appears to play a role, similar to that in yeast, in delaying anaphase in response to tension defects. This is based on studies using the small molecule inhibitors of Aurora B (Hesperadin) (Hauf et al., 2003). HeLa cells in which Aurora B has been inactivated by Hesperadin showed an ability to arrest in mitosis in response to the microtubule poison nocodazole. In contrast, cells treated with Hesperadin and taxol (which stabilises microtubules) exited mitosis precociously indicating that Aurora B also plays a role in the spindle checkpoint in response to stabilised microtubules or tension defects (Hauf et al., 2003). Similar phenotypes were observed in another study using an independently identified Aurora B kinase inhibitor (ZM447439) (Ditchfield et al., 2003). This study also shows that cells treated with Aurora B inhibitor are able to respond to microtubule de-polymerising drugs but override the spindle checkpoint when microtubules are allowed to polymerise. In addition to the benefit of using these small molecule inhibitors of Aurora B in studies to try and elucidate the function of this kinase, these molecules have also been developed as potential anti-cancer drugs. This is due to encouraging observations from studies of
several Aurora B inhibitors, and of their anti-proliferative and apoptotic effects (Ditchfield et al., 2003; Girdler et al., 2006; Harrington et al., 2004).

**Mechanism of bi-orientation**

So what is the mechanism by which the passenger proteins monitor and correct defective microtubule-kinetochore attachments? Studies in both yeast and vertebrate cells have during recent years aided in the understanding of this complex mechanism. Interestingly, the budding yeast Ipl1-Sli15-Bir1 (Aurora B-INCENP-Survivin) complex has been shown to have not only functions in sensing inaccurate microtubule-kinetochore attachments, but also the physical correction mechanism in many organisms (Figure 1.10 B). Recently, the Bir1-Sli15 complex was identified in a biochemical purification as required for linking the yeast CBF3 complex to microtubules (Sandall et al., 2006). This study suggested that this linking mechanism would activate Ipl1 in the absence of tension. Ipl1 was shown to be dispensable for microtubules to bind to centromeres, but its activity is required for this. Interestingly, the Ipl1 activity induced by "lack-of-tension" has been shown to phosphorylate both the "tension-sensing" kinetochore-CBF3 attachments (phosphorylation of Sli15) and also components of the core kinetochore attachment proteins (e.g. Dam1 and Ndc80) (Cheeseman et al., 2002; Cheeseman et al., 2006; Kang et al., 2001; Sandall et al., 2006). The model proposed by Sandall and colleagues (Sandall et al., 2006) (Figure 1.10 B) suggests that when kinetochores establish bi-polar attachment and gain tension, Sli15 undergoes a conformational change so that the activating IN-box of Sli15 becomes unavailable for activation of Ipl1.
Phosphorylation of Dam1 by Ipl1 is crucial, and it was found that a dam1 phospho-mutant showed a phenotype reminiscent to that of ipl1 (Cheeseman et al., 2002). Hence, in addition to its role in tension sensing, the Ipl1-Sli15 complex has been directly implicated in the mechanism that releases mal-oriented kinetochores, to allow for correct attachments (Biggins et al., 1999; Pinsky et al., 2006; Tanaka et al., 2002).

Although a vertebrate equivalent of the Dam1 complex has not been identified, Aurora B has been shown to phosphorylate other targets at the vertebrate kinetochore that could contribute to regulation of bi-orientation. For example, Aurora B phosphorylates the kinesin, MCAK, in Xenopus egg extracts, and this phosphorylation inhibits the microtubule destabilising function of MCAK (Andrews et al., 2004). However, for Aurora B to carry out a correction function, we predict a phospho-target on the kinetochore-microtubule interface, which, when regulated by Aurora B phosphorylation will reduce microtubule-binding affinity to kinetochores. Aurora B was found to directly regulate microtubule-kinetochore attachments through phosphorylation of the conserved Ndc80 complex in C.elegans (Cheeseman et al., 2006). Aurora B phosphorylation of Ndc80 complex gives rise to reduced ability of this protein complex to bind microtubules. These data reflect an important, and conserved role for Aurora B kinase activity in the direct regulation of microtubule kinetochore attachments. In agreement with this, the human Ndc80 homologue (Hec1) was found to be a phospho-target of Aurora B (DeLuca et al., 2006). Interference with this phosphorylation event gives rise to an increase in lagging chromosomes (DeLuca et al., 2006), consistent with the fact that Aurora B phosphorylation is required to detach defective microtubule-kinetochore attachments.
Aurora kinase functions are unlikely to be confined to kinetochores. Indeed, Ipl1 was identified in a screen to identify spindle assembly proteins that are lethal when mutated in combination with cin8 mutants, suggesting a novel role for the Aurora B homologue (Ipl1) in spindle assembly (Kotwaliwale et al., 2007). In addition, the same study revealed that phosphorylation of the spindle midzone protein Ase1 is dependent on Ipl1, and that this phosphorylation is also required for spindle assembly in the absence of the Cin8 motor.

1.7 The Shugoshin family

The conserved Shugoshin (Japanese for 'guardian spirit') family of proteins have over recent years caused great research interest. These proteins were initially identified as protectors of centromeric cohesion in meiosis. They have since then been shown to play diverse roles in both meiosis and mitosis in many organisms, and a number of links with the chromosomal passenger proteins and Bub1 kinase have been proposed.

1.7.1 Overview of the Shugoshin proteins

The production of haploid gametes is a result of two rounds of chromosome segregation (meiosis I+II) following one round of DNA replication. In meiosis I, the homologues form chiasmata, which happens as a result of recombination (Buonomo et al., 2000). The sister-chromatid cohesion along the chromosome arms needs to be removed for the homologues to segregate in meiosis I, but the cohesion at the centromere is maintained until metaphase II (Figure 1.11 A). In meiosis, the cohesin subunit Scc1 is replaced by the subunit Rec8. At anaphase I, Rec8 is cleaved by separase, but centromeric Rec8 is protected until anaphase II. The discovery of the Shugoshin (Sgo) proteins was the
Figure 1.11 Schematic diagram of cohesin protection by Shugoshin in meiosis and mitosis (modified from Watanabe, 2005). (A) Pathways by which cohesin is removed and protected by Shugoshin in meiosis and mitosis. (B) Conservation of Shugoshin proteins between species. Shugoshin contains a conserved coiled-coil domain (orange) at the N-terminus and a basic region (blue) at the C-terminus.
beginning of the understanding as to how centromeric cohesin is protected from cleavage in meiosis I. The *Drosophila* MEI-S332 was the first protein found to play a direct role in the maintenance of centromeric cohesion (Kerrebrock et al., 1992). Since then, yeast genetic screens identified conserved coil-coiled proteins distantly related to MEI-S332, and these were shown to play a similar role in these organisms (Katis et al., 2004; Kitajima et al., 2004; Marston et al., 2004; Rabitsch et al., 2004) (Figure 1.11 B). Sgo-proteins have since then been found to have roles in mitotic cell division too (discussed further below).

*Sgo1 a protector of centromeric cohesion*

Sgo1 was identified as a protector of centromeric cohesion (Rec8) in meiosis I, in both budding and fission yeast (Kitajima et al., 2004; Marston et al., 2004; Rabitsch et al., 2004). Due to loss of centromeric cohesion, *sgo1* mutants prematurely separate their sister-chromatids at meiosis I. This leads to random segregation of sister-chromatids at meiosis II, which gives rise to chromosome loss and aneuploidy. Interestingly in vertebrate mitosis, a similar requirement for Sgo1-dependent protection of cohesin-cleavage is present (Figure 1.11 A). As discussed above, cohesin is removed in a step-wise manner, and the majority of arm cohesin is removed by phosphorylation of cohesin by Aurora B and Polo kinase (Losada et al., 2002). However some cohesin, mainly concentrated at the centromeres, remains on the chromosomes until anaphase when it is cleaved by separase. A series of studies showed that Sgo1 is also required for protection of centromeric cohesion in mitosis in human cells (Kitajima et al., 2005; McGuinness et al., 2005; Tang et al., 2004b) and in *Xenopus* (Salic et al., 2004), and that this protection involves a role for Sgo1 in preventing phosphorylation of cohesin by Polo (Kitajima et al., 2006).
It is therefore clear that Sgo1 is the protector of centromeric cohesion. So, what is the mechanism by which this is achieved? Sgo1 was shown to bind and recruit the protein phosphatase PP2A to centromeres (Kitajima et al., 2006; Riedel et al., 2006). Together these findings describe a role for Sgo1 and PP2A at centromeres, in preventing Polo-dependent phosphorylation of centromeric cohesin and thereby preventing its dissociation (Kitajima et al., 2006; Riedel et al., 2006; Tang et al., 2006) (reviewed in (Rivera and Losada, 2006)).

**Sgo1 and Sgo2**

Whereas MEI-S332 is the only copy of Sgo in *Drosophila*, mammalian and fission yeast cells carry two copies (Sgo1 and Sgo2) (Kitajima et al., 2004; Rabitsch et al., 2004) (Figure 1.11 A). The fission yeast Sgo1 is expressed only in meiosis, but Sgo2 appears to have functions both in meiosis and mitosis (Kitajima et al., 2004; Rabitsch et al., 2004). Both proteins are required for accurate chromosome segregation in meiosis, however Sgo2 is not required for protection of centromeric cohesion in mitosis or meiosis. Instead Sgo2 is required for proper disjunction of sister-homologues and for mono-orientation of sister-chromatids in meiosis (Rabitsch et al., 2004; Vaur et al., 2005).

In fission yeast mitosis, *sgo2Δ* cells show no apparent defect in chromosome segregation but still display significant sensitivity to microtubule drugs (Kitajima et al., 2006). Intriguingly, recent studies have revealed a function for Sgo2 protein in chromosome bi-orientation in mitosis (Kawashima et al., 2007; Vanoosthuyse et al., 2007). These studies also showed that Sgo2 is required for accurate localisation of passenger proteins, and for efficient formation of the INCENP-Survivin complex. Such
a role in Chromosomal Passenger regulation and targeting could nicely explain how Sgo2 could have a role in the error-correction machinery for mal-oriented kinetochores.

Although Sgo1 in budding yeast prevents Rec8 dissociation in meiosis (Katis et al., 2004; Kitajima et al., 2004; Marston et al., 2004; Rabitsch et al., 2004), it plays a different role in mitosis similar to that of Sgo2 in fission yeast mitosis (Indjeian et al., 2005). Cells lacking Sgo1 in budding yeast mitosis are sensitive to microtubule depolymerising drugs, but they can still arrest in response to these drugs. In contrast, sgo1 mutants are defective in the spindle checkpoint arrest in response to lack of tension. Thus, in yeast mitosis, Sgo1 and Sgo2 appear to be required for the spindle checkpoint in response to tension defects, and for chromosome bi-orientation.

1.7.2 Regulation of Sgo localisation to centromeres by Bub1 kinase

The spindle checkpoint protein Bub1 was first implicated in protection of sister-chromatid cohesion in a study of fission yeast meiosis by Bernard and colleagues (Bernard et al., 2001). This data was rather surprising because it suggested that Bub1, apart from the role in the spindle checkpoint and mitotic chromosome segregation, also plays a role in protection of cohesin (Rec8) in meiosis. Since then, it has become clear that Bub1 plays a role in regulating sister-chromatid cohesion in several organisms by recruiting Sgo proteins to centromeres (Kiburz et al., 2005; Kitajima et al., 2005; Kitajima et al., 2004; Riedel et al., 2006; Tang et al., 2004b). Initially, Bub1 was found to be required for Sgo1 localisation in fission yeast meiosis, and it was shown that Bub1 kinase activity was required (Kitajima et al., 2004). Moreover, Sgo2 localisation is also dependent on Bub1 and its kinase activity ((Kitajima et al., 2004) and Vanoosthuyse personal communication). It is interesting that, although Sgo1 and Sgo2 appear to play
different roles in meiosis and mitosis, Bub1 is nevertheless required to localise them both to centromeres. In budding yeast, Bub1 is required for localisation of both Sgo1 and the PP2A subunit, Rts1, to centromeres but the reverse is not true (Riedel et al., 2006). The same study provided evidence that Sgo1 and Rts1 are partly re-distributed onto chromosome arms in the *bub1* mutant, indicating that Bub1 is required to restrict Sgo1 and Rts1 to centromeres in budding yeast meiosis.

In agreement with this, Bub1 is also required for Sgo1 localisation to centromeres in human mitotic cells (Tang et al., 2004b, Kitajima, 2005 #101). One study also revealed that Sgo1 redistributes to the chromosome arms in Bub1 RNAi cells and that this gives rise to ectopic protection of cohesin on the arms (Kitajima et al., 2005). The phenotype of these cells suggests that the centromeric cohesion in vertebrate mitotic cells is also lost in the absence of Bub1. Similar results were obtained from Tang and colleagues, who showed the loss of centromeric cohesion in Bub1 and Sgo1 RNAi cells (Tang et al., 2004b). However, this hypothesis remains controversial because the loss of Bub1 has also been described to give rise to spindle checkpoint defects, and hence premature sister-chromatid separation (Perera et al., 2007). Therefore it is not clear whether the loss of centromeric cohesion in Bub1 RNAi cells was due to lack of protective mechanism through Sgo1, or whether it was simply a defect in spindle checkpoint function.
1.8 Objectives

The Bub1 kinase plays many crucial roles in mitosis and is also implicated in disease. I have specifically discussed the importance of the Bub1 protein kinase in spindle checkpoint function, in chromosome segregation, and in the protection of centromeric cohesion by regulation of Sgo1 localisation. However, to date, the precise role of the Bub1 kinase domain remains elusive. In my PhD, I set out to investigate the role of the budding yeast Bub1 kinase domain. Specifically, my aims were to 1) establish the role for the kinase domain in the spindle checkpoint, 2) analyse functions of the kinase domain in chromosome segregation, and 3) investigate any role for Bub1 kinase domain in Sgo1 regulation in budding yeast mitosis.
Chapter 2. Materials and methods

2.1 Supplier information

Chemicals were bought from the following companies unless stated otherwise: Fisher, Gibco, BDH, Boehringer Mannheim, Sigma, GE Healthcare. DNA polymerases and enzymes were purchased from Roche, Invitrogen, Stratagene, Lonza and Promega. Reagents for cell growth were purchased from Biogene, Difco, Oxoid, Sigma and Formedium.

2.2 General Information

2.2.1 Sterilisation methods

Media, glassware and other equipment was autoclaved at 120°C and 15 pounds/ inch$^2$ for 15 minutes. Alternatively, media and solutions were sterilised using Nalgene bottle top filters (0.2μm), or for small amounts Sartorus, Minisart (0.45 μm) syringe filters were used.

2.2.2 Common buffers and solution

<table>
<thead>
<tr>
<th>DNA Buffers</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x TBE</td>
<td>445 mM Tris, 200 mM glacial acetic acid, 100 mM EDTA pH 8.0</td>
</tr>
<tr>
<td>TE pH 8.0</td>
<td>10 mM Tris-HCl, 1 mM EDTA</td>
</tr>
<tr>
<td>DNA loading buffer</td>
<td>75 mM EDTA, 25% (w/v) sucrose, 0.1% (w/v) bromophenol blue</td>
</tr>
</tbody>
</table>
2.3 Microbiological methods

2.3.1 Bacterial strains, media, growth conditions and transformations

2.3.1.1 Bacterial strains

XL1-Blue cells were used for cloning, and BL21 cells were used for expression of GST fusion proteins.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>XL1-Blue</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’ proAB lacI ZΔM15 Tn10 [Tet’]]</td>
<td>Stratagene</td>
</tr>
<tr>
<td>BL21</td>
<td>E.coli B F’ dcm ompT hsdS(rB–mB–) gal</td>
<td>Stratagene</td>
</tr>
</tbody>
</table>

Table 2.2. Bacterial strains used in this study

2.3.1.2 Bacterial growth conditions and storage

Bacterial cells were grown at 37°C in LB media (1% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto yeast extract, 0.5% (w/v) NaCl, pH adjusted to 7.2 with 5 M NaOH. For solid
media 2% (w/v) agar was added before autoclaving the LB media. To maintain plasmids, the media contained the appropriate antibiotic (for plasmids used in this study, 100 μg/ml ampicillin). Bacterial strains were stored, long term, in LB media with 20% glycerol at -80°C.

2.3.1.3 Bacterial transformation by heat-shock

Transformation of XL1-Blue cells was performed according to manufacturers instructions.

2.3.2 S. cerevisiae strains, media, growth conditions and transformation

2.3.2.1 S. cerevisiae strains and media

All strains, unless otherwise stated, are of the W303 yeast background (R.Rothstein). (*) indicates that the strains are of the S228C yeast strain background.

<table>
<thead>
<tr>
<th>Table 2.3</th>
<th>Yeast strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>JF001 a</td>
<td>trp1-1::lacO::URA, his3-11::pCUP-12-lacI12::HIS PdsI-Myc18::LEU ade2-1 leu2-3,112</td>
</tr>
<tr>
<td>JF002 α</td>
<td>trp1-1::lacO::URA his3-11::pCUP-12-lacI12::HIS PdsI-Myc18::LEU ade2-1 leu2-3,112</td>
</tr>
<tr>
<td>JF004 a</td>
<td>trp1-1::lacO::URA his3-11::pCUP-12-lacI12::HIS PdsI-Myc18::LEU ade2-1 leu2-3,112</td>
</tr>
<tr>
<td>JF006 α</td>
<td>trp1-1::lacO::URA his3-11::pCUP-12-lacI12::HIS PdsI-Myc18::LEU bub1L779G::TRP Bub3-myc13::kanMX ade2-1 leu2-3,112</td>
</tr>
<tr>
<td>JF007 a</td>
<td>trp1-1::lacO::URA his3-11::pCUP-12-lacI12::HIS PdsI-Myc18::LEU bub1L779G::TRP Bub3-myc13::kanMX ade2-1 leu2-3,112</td>
</tr>
<tr>
<td>JF009 a</td>
<td>trp1-1::lacO::URA his3-11::pCUP-12-lacI12::HIS PdsI-Myc18::LEU Bub3-myc13::kanMX ade2-1 leu2-3,112</td>
</tr>
<tr>
<td>JF010</td>
<td>trp1-1::lacO::URA his3-11::pCUP-12-lacI12::HIS PdsI-Myc18::LEU Bub1L779G::TRP ade2-1 leu2-3,112</td>
</tr>
<tr>
<td>JF011</td>
<td>trp1-1::lacO::URA his3-11::pCUP-12-lacI12::HIS PdsI-Myc18::LEU Bub1L779G::TRP ade2-1 leu2-3,112</td>
</tr>
<tr>
<td>JF016 a</td>
<td>Pds1-myc::LEU lacO::TRP GFPlac1::HIS3 pGAL-MCD1::kanMX bar1 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1</td>
</tr>
<tr>
<td>JF017 a</td>
<td>Pds1-myc::LEU lacO::TRP GFPlac1::HIS3 pGAL-MCD1::kanMX madΔ::URA bar1 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1</td>
</tr>
<tr>
<td>JF019 a</td>
<td>Pds1-myc::LEU lacO::TRP GFPlac1::HIS3 pGAL-MCD1::kanMX mad3Δ:: bar1 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1</td>
</tr>
<tr>
<td>JF023 a</td>
<td>Pds1-myc::LEU lacO::TRP GFPlac1::HIS3 pGAL-MCD1::kanMX bub1ΔK::HPH bar1 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1</td>
</tr>
</tbody>
</table>
ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1

SGOJ-13myc::kanMX6 bub1ΔK::HPH ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1

SPC105-13myc::kanMX6 bub1ΔK::HPH ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1

SGOJ-13myc::kanMX6 bub1ΔK::HPH ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1

SPC105-13myc::kanMX6 bub1ΔK::HPH ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1

SGOJ-13myc::kanMX6 bub1ΔK::HPH ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1

SPC105-13myc::kanMX6 bub1ΔK::HPH ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1

SGOJ-13myc::kanMX6 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1

CtfJ9-CFP::HIS/Ndc8O-CFP::I ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1

SGOI-6HA::TRP bub1AK::HPH ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1

MCDJ-6HA bub1AK::HPH ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1

MCDJ-6HA bub1AK::HPH ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1

pURA3-tetR::GFP::LEU2 cenlV::tetO(x448):: URA3 METprom-CDC20:: URA

Spc42-Tomato::NAT sgo1Δ::kanMX6 bub1AK::HPH ade2-1 leu2-3 ura3-1 trp1-1 his3-11,15

Pdsl-myc::LEU lacO::TRP GFPlacl::HIS3 pGAL-MCDJ::kanMX bub1ΔK::HPH

bar1 ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1
<table>
<thead>
<tr>
<th>Media</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>YPDA</td>
<td>1% (w/v) yeast extract, 2% (w/v) bacto-peptone, 2% glucose, 0.003% (w/v) adenine sulphate</td>
</tr>
<tr>
<td>YEP (GAL/RAFF)</td>
<td>1% (w/v) yeast extract, 2% (w/v) bacto- peptone, 2% (w/v) galactose, 2% (w/v) raffinose, 0.003% (w/v) adenine sulphate</td>
</tr>
<tr>
<td>Minimal media</td>
<td>0.7% (w/v) yeast nitrogen base w/o amino acids, 2% (w/v) glucose</td>
</tr>
<tr>
<td>CSM— drop out (BIO101)</td>
<td>0.7% (w/v) yeast nitrogen base w/o amino acids, 2% (w/v) glucose. This media was supplemented with appropriate</td>
</tr>
</tbody>
</table>
amino acids from a 100 x stock solutions as below (per 100 ml):
- Adenine 0.6 g stored at 4°C
- Methionine 0.2 g stored at 4°C
- Uracil 0.3 g store at room temperature
- Histidine 0.6 g stored at 4°C
- Tryptophan 0.8 stored in dark at room temperature
- Leucine 0.8 g stored at 4°C
- Lysine 0.3 g stored at 4°C

Sporulation media 0.3% (w/v) potassium acetate, 0.02% (w/v) raffinose

Table 2.4 Common growth media for S. cerevisiae

<table>
<thead>
<tr>
<th>Nutrients/supplements</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-factor</td>
<td>10 µg/ml for BAR+ strains, 1 µg/ml for bar- strains. Stock solution at 10 mg/ml in DMSO</td>
</tr>
<tr>
<td>(Peptide Protein Research Ltd)</td>
<td></td>
</tr>
<tr>
<td>Benomyl</td>
<td>8-10 µg/ml in agar, as stated in text. 30 µg/ml in liquid. Added to boiling media. Stock at 30 mg/ml in DMSO.</td>
</tr>
<tr>
<td>Nocodazole</td>
<td>15 µg/ml in liquid media. Stock at 10 mg/ml in DMSO.</td>
</tr>
<tr>
<td>Clonat (WERNER BioAgents)</td>
<td>100 µg/ml in agar. Stock at 200 mg/ml in dH2O.</td>
</tr>
<tr>
<td>Hygromycin (Calbiochem)</td>
<td>300 µg/ml in agar. Purchased from Calbiochem.</td>
</tr>
<tr>
<td>G418</td>
<td>300 µg/ml in agar. Stock at 300 mg/ml in DMSO.</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>10 µg/ml in agar. Stock at 5 mg/ml in 50% (v/v) ethanol.</td>
</tr>
<tr>
<td>Zymoypase (ICN Pharmaceuticals Inc.)</td>
<td>1 mg/ml. Stock at 5 mg/ml in H2O.</td>
</tr>
</tbody>
</table>

Table 2.5 Nutrients and supplements for S. cerevisiae media.
2.3.2.2 *S. cerevisiae* growth conditions and storage

Wild-type *S. cerevisiae* yeast strains were typically grown at 30°C, and liquid cultures were grown on shaking incubator platforms at 180-200 rpm. Temperature sensitive strains were grown at permissive temperature of 25°C, and at restrictive temperature of 36°C. Cold sensitive strains were grown at permissive temperature of 30°C, and at restrictive temperature of 18°C. To maintain plasmids containing auxotrophic markers, yeast cells were grown in CSM- media lacking the appropriate amino acid. *S. cerevisiae* strains were stored at -80°C long term, in YPDA with 30 % glycerol.

2.3.2.3 Lithium acetate transformation of *S. cerevisiae* cells (Ito et al., 1983)

A 5 ml over-night culture of cells was used to inoculate a 50 ml culture to OD<sub>600</sub> 0.5. This culture was then grown for 3 hours and the cells were collected by centrifugation at 1000 g for 3 minutes and washed twice 1 ml of lithium acetate mix (100 mM LiOAc, 10 mM Tris pH 7.4, 1 mM EDTA). The cells were then resuspended in 500 µl of lithium acetate mix. 15 µl of boiled salmon sperm DNA (10 mg/ml) was mixed with 1-2.5 µg of transformation DNA. The DNA was then mixed with 100 µl of cells, and 700 µl of PEG mix (40% (w/v) PEG 2000, 100 mM LiOAc, 10 mM Tris pH7.4, 1 mM EDTA) and incubated at room temperature for 30 minutes. The transformation mix was then heatshocked at 45°C for exactly 15 minutes and cells were centrifuged at 1000 g for 3 minutes. Cells were then resuspended in 200 µl YPDA and plated out on appropriate media. For integrative transformations using drug-resistance markers, cells were allowed to recover on rich media over-night, then replica-plated onto media containing the appropriate drug.

2.3.3 *S. cerevisiae* cell cycle arrests and synchronisation
2.3.3.1 G1 arrest using the mating pheromone α-factor

When arresting and synchronising cells in G1 using α-factor, *S. cerevisiae* cells of mating type a (Mat a) were grown to mid log-phase (OD$_{600}$ 0.3-0.6) in liquid YPDA media. To arrest cells in larger volumes, cells were resuspended in 1/10 volume for the arrest. For *BARI* cells 10 μg/ml α-factor was used, and for *barI* cells 1 μg/ml α-factor was used. The cells were allowed to arrest for 3 hours at 30°C, and for *BARI* cells, α-factor was re-added after 1.5 hours into the arrest. For synchronous release from G1 the α-factor was carefully washed out 3 times in YPDA.

2.3.3.2 Mitotic arrest using nocodazole and/or benomyl

To arrest cells in metaphase using the microtubule destabilising drugs nocodazole and benomyl, cells were grown at 30°C to mid log-phase (OD$_{600}$ 0.3-0.6). The cells were then centrifuged at 1000 g for 3 minutes and resuspended in the appropriate drug: 15 μg/ml nocodazole for smaller cultures, and 30 μg/ml benomyl for larger cultures. The arrests were carried out at 23°C for 3 hours unless stated otherwise.

2.3.4 Mating of haploid yeast strains

Strains of opposite mating types were grown over-night on YPDA agar plates. If the two strains carried two different auxotrophic markers, then the cells were mixed on a YPDA plate in 10 μl of water, and left at 30°C over-night. The mated cells were then streaked out on double selective CSM media lacking both amino acids corresponding to the two markers in both the strains. Only diploids should then grow. However, if one strain carries an auxotrophic marker and the other strain a drug resistance marker, then only a very small amount of yeast carrying the drug resistance marker was mixed with a larger amount of the strain carrying the auxotrophic marker, and incubated at 30°C.
over-night as above. The cells were then streaked out for single colonies on plates containing the drug corresponding to the drug resistance cassette carried by one of the strains. The diploid cells were then incubated at 23°C in 5 ml of sporulation media for 4 days, or until tetrads appeared.

2.3.5 Tetrad dissection

1 ml of sporulated cells were collected by centrifugation at 1000 g for 2 minutes. The cells were resuspended in 50 μl of 1 mg/ml zymolyase in dH2O, and left at room temperature to digest for 15 minutes. The cells were then placed on a YPDA agar plate and the tetrads were dissected using a Singer Instruments MSM System Series 300 micromanipulator.

2.4 Nucleic acid methods

2.4.1 Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Information</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pRS304</td>
<td>TRP</td>
<td>(Sikorski and Hieter, 1989)</td>
</tr>
<tr>
<td>pJF01</td>
<td>Contains <em>BUBI</em> a.a. 1285- 1824 linked through 8 glycines to <em>SGOl</em> ORF. Fusion construct cloned in at <em>EcoRI</em>. 500 bp of <em>BUBI</em> 3'UTR was also cloned in to allow homologous recombination.</td>
<td>This study</td>
</tr>
<tr>
<td>YCPlac22</td>
<td>CEN, single copy vector</td>
<td>(Gietz and Sugino, 1988)</td>
</tr>
<tr>
<td>pJF02</td>
<td><em>SGOl</em> ORF cloned into YCPlac22 using <em>BamHI</em> and <em>HindIII</em> sites</td>
<td>This study</td>
</tr>
</tbody>
</table>
2.4.2 Phenol / chloroform extraction of yeast genomic DNA

5 ml of over-night yeast culture was collected by centrifugation and resuspended in 200 µl of yeast 'DNA extraction buffer (2.5 M LiCl, 50 mM Tris pH 8.0, 62.5 mM EDTA, 4% (v/v) Triton X-100). 200 µl of phenol / chloroform / isoamyl alcohol (25:24:1) (Sigma) and glass beads were then added to the cells, which were then homogenised using a mini bead beater (BioSpec Products) for 2 minutes, followed by centrifugation at 18'000 g for 10 minutes at 4°C. The aqueous layer containing the genomic DNA was then removed and precipitated (see 2.4.3), then resuspended in 20 µl of TE.

2.4.3 Precipitation of DNA

2.5 times the volumes of ethanol were added to the DNA and incubated at 4°C for 10 minutes, followed by centrifugation at 18'000 g for 10 minutes. The DNA pellet was
then washed in 70% cold ethanol then centrifuged at 18'000 g for 5 minutes. The ethanol was then removed and the pellet allowed to dry for 5 minutes at room temperature. The DNA was then resuspended in 20 µl of TE.

2.4.4 Plasmid purification using Qiagen Miniprep by spin column
Purification of plasmid DNA from *E. coli* was performed using Qiagen™ mini-prep kit, according to manufacturers instructions. The plasmid was typically eluted in 50 µl EB buffer or dH2O.

2.4.5 Agarose gel electrophoresis
DNA fragments were separated using agarose gel electrophoresis. Typically 1% agarose was dissolved in TBE buffer by heating. 0.3 mg/ml ethidium bromide (Sigma) was added to the agarose solution, in order to visualise DNA. DNA loading buffer was added at 1/10 dilution to the DNA samples before loading it on the gel. 1 kb DNA ladder (Gibco) was used as a DNA fragment size marker. Electrophoresis was performed using TBE running buffer at 90-120 Volts depending on gel size.

2.4.6 Extraction of DNA from agarose gels
DNA fragments from agarose gels were purified using Qiagen™ gel extraction kit according to manufacturers instructions.

2.4.7 Restriction digests
Restriction digests of plasmids and DNA fragments were performed according to New England Biolab’s instructions for the specific enzymes. Typically 5 units of enzyme was used for 1 µg of DNA, and digested at 37°C for 1-2 hours. Before further use of the
DNA, the enzymatic reaction was stopped by incubation at -20°C, then put through a Quiagen™ PCR clean-up kit (according to manufacturers instructions), or purified from an agarose gel (see 2.4.6).

2.4.8 Ligation of DNA

Ligation of digested DNA fragments was performed using T4 DNA ligase kit (Roche), according to manufacturers instructions. Typically, the ratio of insert:vector was 3:1, and the reaction was incubated at 18°C from 4 hours to over-night, then 3 µl of each reaction was transformed into competent XL-1 Blue cells (Stratagene, see 2.3.2).

2.4.9 PCR, oligos and sequencing methods

2.4.9.1 PCR methods

General PCR reaction:

Template DNA  10-30 ng plasmid DNA/ 10-500 ng yeast genomic DNA
3' primer  0.5 µM
5' primer  0.5 µM
1X dNTPs
1X PCR buffer
Taq polymerase  1 µl/ 50 µl reaction
dH₂O to final volume

General PCR programme:

Step 1  95°C  5 minutes
Step 2  Denaturing  95°C  30 seconds
Step 3  Annealing  55°C**  30 seconds

(**typically 55°C but varies depending on the primer)

Step 4  Extension  72°C  1 minute/ kb
(Typically Step 2- 4: 30 cycles)
Step 5  72°C  10 minutes
For yeast and *E. coli* colony PCR, a small amount of cells were added to the reaction as template DNA. 10x stocks of dNTPs (2.5 mM dATP, dCTP, dGTP, dTTP) and PCR buffer were stored at -20°C. Taq polymerase was used for diagnostic PCR, whereas proof-reading enzyme Expand Long Template (Roche) or Expand High Fidelity (Roche) were used for cloning purposes or for amplification of long PCR products for homologous recombination. All PCR reactions were carried out using a PTC-200 DNA Engine (MJ Research).

### 2.4.9.2 Oligonucleotides used in this study

<table>
<thead>
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<td>ATTTCTAAGCATTGAAAGAGGATTTATCAGATATTTAAAGTATA</td>
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</tr>
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<td>SGO1SEQ3</td>
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</tbody>
</table>

Table 2.7 Oligos used in this study

2.4.9.3 PCR based sequencing
PCR mediated sequencing was performed using BigDye® Terminator v3.1 Cycle (Applied Biosystems), and reactions were made up to 10 μl total volume.

Reaction content for 10 μl.

~50 ng template DNA
1.6 pmol sequencing primer
2 μl BigDye® Terminator v3.1 Cycle (Applied Biosystems)
dH$_2$O up to 10 μl

Sequencing Programme:
Step 1 94°C 2 minutes
Step 2 94°C 15 seconds
Step 3 58°C 1.5 minutes
30 cycles.

2.5 Protein methods

2.5.1 Crude total cell lysate preparation from yeast
5 ml of cells, grown over-night at 30°C, were collected by centrifugation at 1000 g for 5 minutes. To allow for equal protein loading, the cell pellets were equalized by weight and 300 μl of sample buffer (2%(w/v) SDS, 80 mM Tris pH 6.8, 10% Glycerol, 10 mM EDTA, trace bromophenol blue) was added to the pellet. Protease inhibitors (1 mM pefablo/), 10 μg/ml leupeptin/pepstatin/chymostatin) and 100 mM DTT were added fresh to the sample buffer each time. 0.5 mm Silica beads (BioSpec Products) were added to equal size of the pellet and the samples were lysed using a Hybaid RiboLyser for 20 seconds at setting 4. Protein samples were then centrifuged at 6000 g for 5 minutes at 4°C, then boiled for 5 minutes and centrifuged yet again at 6000 g for 5 minutes at 4°C.
2.5.2 Immunoprecipitation (IP)

<table>
<thead>
<tr>
<th>Reagent (stock concentration)</th>
<th>Working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Hepes, pH 7.6</td>
<td>50 mM</td>
</tr>
<tr>
<td>3 M KCl</td>
<td>75 mM</td>
</tr>
<tr>
<td>1 M MgCl₂</td>
<td>1 mM</td>
</tr>
<tr>
<td>1 M EGTA</td>
<td>1 mM</td>
</tr>
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<td>20% Triton-X</td>
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</tr>
<tr>
<td>0.1 M Pefablock</td>
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</tr>
<tr>
<td>1 mM Na Vanadate</td>
<td>1 mM</td>
</tr>
<tr>
<td>0.1 mM Microcystin (Alexis)</td>
<td>0.1 μM</td>
</tr>
<tr>
<td>0.1 M PMSF in EtOH</td>
<td>1 mM</td>
</tr>
<tr>
<td>LPC</td>
<td>1/1000</td>
</tr>
<tr>
<td>1 M Na F (make fresh each time)</td>
<td>50 mM</td>
</tr>
<tr>
<td>1 complete mini EDTA-free protease inhibitor tablet / 10 ml lysis buffer</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.8 Lysis buffer for Immuno-precipitation

Typically, for immunoprecipitation, 300 mg of mid-log phase yeast cells were collected by centrifugation at 1000 g for 5 minutes. The cell pellet was washed once in cold dH₂O. Lysis buffer (Table 2.8), silica beads were added (as in 2.5.1) and the cells were lysed by bead-beating for 1 minute. The lysates were then centrifuged at 18'000 g for 5 minutes at 4°C. The supernatant was transferred to a clean Eppendorf tube and centrifuged again as previously. 1 mM DTT was added to the cleared lysate. Beads (9E10 pre-coupled beads, Santa Cruz Biotechnology, for myc IPs) were washed 3 times in lysis buffer, then added as a 1:1 slurry of beads:lysis buffer to the lysates (~30 μl beads to 500 μl lysate), and incubated at 4°C for 2-4 hours. The beads were then
washed 3 times in lysis buffer. 30 μl protein sample buffer was added to the beads and boiled 5 minutes before loading on a SDS-PAGE gel.

2.5.3 Lambda-phosphatase treatment

After the beads from 2.5.2 were washed 3 times in lysis buffer, they were washed once in lambda phosphatase (NEB) buffer, and the beads were split in two. The beads were collected by centrifugation at 500 g and resuspended in 50 μl of lambda-phosphatase buffer (NEB). 0.5 μl of lambda-phosphatase was added to one set and they were incubated at 30°C for 30 minutes. The beads were then collected by centrifugation as above, and resuspended in 30 μl SB + 5% β- mercaptoethanol, and boiled for 5 minutes before loading on a SDS-PAGE gel.

2.5.4 Chromatin immunoprecipitation (ChIP)

<table>
<thead>
<tr>
<th>Solution</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formaldehyde Diluent</td>
<td>143 mM NaCl, 1.43 mM EDTA, 71.43 mM HEPES-KOH</td>
</tr>
<tr>
<td>2X FA lysis buffer</td>
<td>100 mM HEPES-KOH, pH 7.5, 300 mM NaCl, 2 mM EDTA, 2% Triton X-100, 0.2% Na Deoxycholate</td>
</tr>
<tr>
<td>TBS</td>
<td>20 mM Tris-HCl pH 7.5, 150 mM NaCl</td>
</tr>
<tr>
<td>ChIP washing 1.</td>
<td>1X FA buffer + 0.1% SDS, 275 mM NaCl</td>
</tr>
<tr>
<td>ChIP washing 2.</td>
<td>1X FA buffer + 0.1% SDS, 500 mM NaCl</td>
</tr>
<tr>
<td>ChIP washing 3.</td>
<td>10 mM Tris pH 8, 250 mM LiCl, 1 mM EDTA, 0.5% (v/v) NP-40, 0.5% Na Deoxycholate (v/v)</td>
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<tr>
<td>ChIP washing 4.</td>
<td>10 mM Tris pH 8.0, 1 mM EDTA</td>
</tr>
<tr>
<td>TES buffer</td>
<td>50 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% SDS</td>
</tr>
</tbody>
</table>

Table 2.9 Solutions for chromatin immuno-precipitation
2.5.4.1 Chromatin-protein crosslinking

50 ml of cells at OD$_{600}$ 0.6 were treated with 1% formaldehyde by adding 5 ml of 11% formaldehyde (made freshly by adding 1.5 ml 37% formaldehyde to 3.5 ml of formaldehyde diluent), straight to the culture and incubated at room temperature for 2 hours, swirling. The cells were then collected by centrifugation at 1500 g at 4°C for 3 minutes, then washed two times in 10 ml ice-cold TBS and once in 10 ml ice-cold FA lysis buffer with 0.1% SDS. The cells were then transferred to pre-chilled screw-cap tubes, snap frozen in liquid nitrogen and stored at -80°C until processed.

2.5.4.2 Lysis, sonication and immuno-precipitation

The cell pellet was resuspended in 200 μl of ice-cold FA buffer with 0.5% SDS, 1 mM PMSF, 1x protease inhibitors-P.I. (diluted from 50X stock made by resuspending one pellet of EDTA-free protease inhibitors (Roche) in 1 ml of dH$_2$O). An equal volume of silica beads was added, and the cells were lysed using Hybaid RiboLyser at setting 6.5 for 30 seconds then placed on ice for 10 minutes and ribolysed once more as previously. The cell lysate was separated from the beads by piercing the bottom of the screw-cap tube and placing it on top of a new screw cap tube, which was put in a 15 ml Falcon tube and centrifuged at 1000 g for 3 minutes at 4°C. The lysate (both pellet and supernatant) was then centrifuged at 18'000 g for 15 minutes at 4°C on a table-top centrifuge. The supernatant was removed and a 'glass-like' transparent top layer of the pellet indicates that cross-linking has occurred. The pellet was resuspended, by stirring with a pipett-tip, in 1 ml FA lysis buffer with 0.1% SDS, 0.1 mM PMSF, 1X P.I., and centrifuged again at 18'000 g for 15 minutes at 4°C. The supernatant was discarded and the pellet resuspended as above in 500 μl FA lysis buffer with 0.1% SDS, 0.1 mM PMSF, 1X P.I. The lysates were then sonicated (Branson Digital Sonifier) four times for
10 seconds ON and 45 seconds OFF at power setting 50%, ~10% amplitude. The sheared DNA was collected by centrifugation at 18’000 g for 15 minutes at 4°C and 500 μl FA lysis buffer with 0.1% SDS, 0.1 mM PMSF, 1X P.I. was added to the 500 μl supernatant collected after centrifugation. 100 μl of FA lysis buffer was added to the lysate. After mixing, 1/10 was taken off and stored at -80°C as INPUT DNA. The lysate was then pre-cleared for 2 hours at 4°C with 20 μl of Immobilized Protein G beads (Pierce), which had been washed 3 times in FA buffer. The solution was then centrifuged at 500 g for 2 minutes and the supernatant was added to a new screw-cap tube. The appropriate ChIP antibody (7.5 μl/ sample 12CA5 HA antibody in this study) was then added and incubated with the lysate at 4°C over night. The next day 20 μl of washed (as above) Immobilized Protein G beads were added to the chromatin-antibody mixture and incubated rocking at 4°C for 3 hours. The beads were then washed by rocking at room temperature, for 5 minutes (four times) in 1 ml of ChIP washing buffer: 1, 2, 3 and 4 respectively. The beads were centrifuged at 500 g for 2 minutes between each wash. After the last wash, the beads were eluted with 200 μl TES buffer at 65°C for 10 minutes. The beads were briefly vortexed and incubated yet another 10 minutes 65°C. The supernatant was collected, after centrifugation at 18’000 g for 3 minutes, and added to a new tube. The beads were then washed in 200 μl TE at room temperature for 15 minutes and pelleted again at 18’000 g for 3 minutes. Supernatant was added to the rest of the eluate. 300 μl of TE was then added to the 100 μl of ‘INPUT’ DNA to achieve 400 μl samples.

2.5.5.3 De-crosslinking, DNA purification and ChIP PCR

To de-crosslink the protein and the DNA, 40 μl of Proteinase K (10mg/ ml) (Sigma) was added to the 400 μl of ChIP/ INPUT samples from 2.5.5.2. These samples were
then incubated at 42°C for 1 hour, then over night at 65°C. To purify the DNA a 'Wizard® SV Gel and PCR Clean-Up System' (Promega) was used according to manufacturers instructions. The DNA was eluted in 100 µl of nuclease free dH2O and 100 µl of TE was added to these and stored at -20°C.

ChIP PCR reaction:
Each reaction was made up to 20 µl with the following components:

Template DNA  4 µl of ChIP sample, 4 µl of 1:500 INPUT DNA
3' primer  0.5 µM
5' primer  0.5 µM
Ex Taq™ Hot start DNA polymerase (Lonza)
1X dNTP and 1X TAKARA buffer
up to 20 µl dH2O

ChIP PCR programme:

Step 1  95°C  5 minutes
Step 2  Denaturing  95°C  30 seconds
Step 3  Annealing  55°C  30 seconds
Step 4  Extension  72°C  1 minute/kb
(Step 2-4: 26 cycles)
Step 5  72°C  10 minutes

3 µl of 6X ChIP DNA loading dye (0.1% Orange G (w/v), 10% glycerol, 1 mM EDTA pH8.0) were added to each PCR sample and run out on a 2% in TBE agarose gel. The bands were quantified using the program Image J.

2.5.5 SDS-PAGE
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<td>1 ml</td>
</tr>
<tr>
<td>2% bis-acrylamide</td>
<td>624 μl</td>
<td>780 μl</td>
<td>520 μl</td>
</tr>
<tr>
<td>1.5 M Tris pH 8.8</td>
<td>2.4 ml</td>
<td>3 ml</td>
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</tr>
<tr>
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<tr>
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<td>-</td>
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<td>9.6 μl</td>
<td>12 μl</td>
<td>8 μl</td>
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Table 2.10 Gel solutions for SDS-PAGE

2.5.6 Western blotting

The proteins from the SDS-PAGE gel was transferred onto nitrocellulose membrane using a Hoefer TE77 semi-dry transfer apparatus (Amersham Biosciences) according to the manufacturers instructions. The membrane was then blocked for 30 minutes at room temperature with Blotto (5% Marvel dry milk, 0.1% Tween-20 in PBS). The membrane was typically incubated with primary antibody at 4°C over-night then washed three times in PBS-T (0.1% Tween-20 in PBS). If the antibody gave rise to a lot of background staining, the membranes was washed in PBS-T with 0.5 M NaCl. The membrane was then incubated with secondary antibodies at room temperature for 1 hour and washed three times with PBS-T, before exposing the membrane to ECL (Amersham Biosciences) according to manufacturers instructions.

2.5.7 TAP-S-ZZ Purifications

This protocol was modified from (Cheeseman et al., 2001).
Table 2.11 Solutions for TAP-S-purification

EGTA, 10 mM EDTA, 20% glycerol
TST 50 mM Tris HCl pH 7.4, 15 mM NaCl, 0.1% Tween

2.5.7.1 Preparation of lysates

The yeast was grown to \( \text{OD}_{600} \approx 0.8 \) and harvested by centrifugation, and the cells were resuspended with 0.2 volumes of cold water. They were then drop-frozen in liquid nitrogen. The yeast was lysed by grinding for 15 minutes, using a RM-100 grinder (Retsch). For each TAP purification, 30 g of ground yeast powder was used. To 30 g of powder, 30 ml of 2X Hyman buffer containing 1X protease inhibitors (LPC), 1 mM PMSF, 1 tablet EDTA free protease inhibitor (Roche) per 10 ml lysis buffer, 5 mM Na azide, 10 mM NaF, 0.4 mM Na orthovanadate, 20 mM β-glycerophosphate and 1/10000 microcystin (Alexis Biochemicals), was added. The cell lysate was thawed quickly in a water-bath, and 12.5 ml of 10% Triton X-100 (1% final) was added, then sonicated for 30 seconds. The lysate was then centrifuged using the Ti45 ultra-centrifuge rotor at 12'000 g for 30 minutes, then the supernatant was transferred to Ti70 ultra-centrifuge bottles and centrifuged at 20’000 g for 30 minutes.

2.5.7.2 IgG sepharose bead preparation, binding and TEV cleavage

0.4 ml IgG sepharose beads (GE Healthcare) per purification were washed in the following solutions: 10 ml TST, 1 ml 0.5 M NH₄OAc (pH 3.4), 5 ml TST, 1 ml 0.5 M NH₄OAc (pH 3.4), 5 ml TST, 5 ml 1X Hyman buffer. The beads were added to the lysate and incubated, rocking, at 4°C for 4 hours. The beads were then placed in a column (Bio-Rad) and washed with 15 ml 1X Hyman buffer (adjusted to 300 mM KCl), and with 25 ml 1X Hyman buffer (adjusted to 300 mM KCl) + 1 mM DTT, 0.1% Tween-20. The beads were then resuspended in 1.5 ml of 1X Hyman buffer with 1 mM
DTT, 0.1% Tween-20. To cleave the protein complexes off the sepharose beads, 10 μl of TEV protease (NEB) was added and incubated, rocking, at 4°C over-night. The supernatant was collected by centrifugation of the beads at 500 g for 3 minutes. An additional volume of 1 ml of 1X Hyman buffer / 300 mM KCl + 1 mM DTT, 0.1% Tween-20 was added to elute any remaining protein off the beads.

2.5.7.3 CBD- binding and elution (CBD-TAP tag)

This protocol was modified from Yeast Resource Centre TAP Protocol and (Rigaut et al., 1999). For TAP-tags containing a Calmodulin Binding Domain (CBD), the cleaved-off protein in the supernatant from section 2.5.7.2 was mixed with three volumes of Calmodulin Binding Buffer- CBB (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM Mg acetate, 1 mM imidazole, 2 mM CaCl₂, 10 mM β-mercaptoethanol) and 3 μl of 1 M CaCl₂ per ml of IgG eluate. Then 300 μl of Calmodulin Sepharose 4B (GE Healthcare) in CBB was added to the supernatant and incubated at 4°C for 4 hours. The sepharose was then transferred to a Bio-Rad column and washed two times with 1 ml of CBB (0.1% NP-40) then with 1 ml of CBB (0.02%NP-40). The beads were then split 50:50. Half the beads were eluted with 100 μl of 8 M urea buffer and used for mass-spectrometry. The other half was used for silver staining and kinase assays.

2.5.7.4 S-protein agarose binding and elution (S-TAP tag)

For TAP-tags containing the S-protein binding domain, the cleaved protein in the supernatant from section 2.5.7.2 was added to 60 μl of S-protein agarose (Novagen) slurry per purification, which had been washed three times with 1X Hyman buffer. This binding was allowed to occur at 4°C for 3 hours. The resin was then washed with 10 ml
1X Hyman buffer. For kinase assays the beads were frozen in 1X Hyman buffer with 30% glycerol at -80°C.

2.5.8 Kinase Assays

The S protein beads containing Bub1-Bub3-TAP protein complex (see section 2.5.8) were washed two times in Kinase Buffer (50 mM HEPES pH 7.6, 5 mM MgCl2, 2.5 mM MnCl2, 5 mM β-glycerophosphate, 1 mM DTT). The buffer was removed and 30 μl of Kinase buffer + 20 μM cold ATP and 5 μCi γATP (~0.5 μl of 0.5 MBq 32P) was added to the beads and incubated at 30°C for 30 minutes. To inactivate the reaction, 30 μl protein sample buffer was added and the samples were then boiled for 5 minutes. These samples were run out on a pre-cast NuPAGE gel (Invitrogen) transferred onto nitrocellulose membrane and exposed overnight.

2.5.9 GST protein purification

<table>
<thead>
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<th>Buffer</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis Buffer</td>
<td>PBS with 1 M NaCl, 0.5 M Pefablock, 2 Protease Inhibitor tablets (Roche) per 50 ml, 0.5% Tween-20 (v/v)</td>
</tr>
<tr>
<td>Washing Buffer</td>
<td>PBS with 0.05% Tween-20 (v/v), 0.5 mM DTT, 0.25 M KCl</td>
</tr>
<tr>
<td>Elution Buffer</td>
<td>50 mM Tris pH 8.0, 0.25 M KCl, 5 mM reduced glutathione</td>
</tr>
<tr>
<td>Dialysis Buffer</td>
<td>50 mM HEPES pH 7.6, 0.1 M KCl, 30% glycerol (v/v)</td>
</tr>
</tbody>
</table>

Table 2.12 Buffers for GST purification

3 litres of LB media containing 75 μg/ml ampicillin was inoculated with BL-21 bacterial cells from a pre-culture to the OD600 0.1. The cells were grown at 37°C until OD600 reached 0.8. IPTG was then added to 0.1 mM. The temperature was shifted to 18°C, and induced protein expression was allowed to occur overnight. The cells were collected by centrifugation at 10'000 g using a Beckman centrifuge. The cells were resuspended in 1/10 volume of lysis buffer and drop frozen in liquid nitrogen.
frozen cell pellets were then ground in liquid nitrogen using Retsch R100 Grinder, for 20 minutes. The powder was transferred to a glass beaker, allowed to warm up until the edges of the powder were thawed, then five volumes of Lysis buffer was added to the powder. This was left stirring with a magnet at 4°C for 5 minutes, then sonicated for 1 minute. DTT was then added to 10 mM (final concentration) and the lysate was centrifuged at 20'000 g for 1 hour at 4°C using an ultra-centrifuge. The supernatant was collected and 5 ml of glutathione agarose beads (Sigma) was added, and incubated rocking at 4°C for 2 hours. The lysate and beads were transferred to a Bio-Rad column and washed with 50-100 ml of Washing buffer until no protein was washed off, assayed using Bradford reagent (Bio-Rad). Then the column was washed with 2 column volumes of Washing buffer lacking Tween-20. To elute the bound protein, 1 ml Elution buffer was added to the column, and several of these 1ml fractions were collected and assayed for protein content using Bradford reagent. The fractions highest in protein were pooled and dialysed over-night.

2.5.10 Small scale affinity purification of antibodies

~20 µg of purified protein was run out on a SDS-PAGE gel and transferred onto a nitrocellulose membrane. After staining with Ponceau S, the protein band was cut out and washed in PBS with 0.1% Tween-20, then blocked with Blotto (5% marvel dry milk, 0.1% Tween-20 in PBS). The protein membrane-strip was then incubated with 1 ml of serum in Blotto overnight at 4°C. The antibodies were eluted 3 times for 30 seconds each with 400 µl elution buffer (50 mM glycine-HCl pH 2.3, 0.5 M NaCl, 0.5% Tween-20 (v/v), 100 µg/ml BSA, 0.1% azide). The eluted protein solution was neutralized directly into 40 µl of neutralization buffer (50 mM Na₂HPO₄). The antibody was stored at +4°C.
### 2.5.11 Antibodies used in this study

<table>
<thead>
<tr>
<th>Primary Antibody</th>
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<td>Roche</td>
</tr>
<tr>
<td>A14 (rabbit myc)</td>
<td>Western 1:1000</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>anti-PGK1 (mouse)</td>
<td>Western 1:1000</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>9E10 (mouse myc)</td>
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<td>Covance</td>
</tr>
<tr>
<td>Mad1 (Rabbit)</td>
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<td>K. Hardwick</td>
</tr>
<tr>
<td>Cdc20</td>
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<td>Santa Cruz Biotechnology</td>
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</tr>
<tr>
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<td>Invitrogen</td>
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<td>Roche</td>
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<td>HA11</td>
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<td>GE Healthcare</td>
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<td>GE Healthcare</td>
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<tr>
<td>Donkey anti-sheep/goat</td>
<td>Western 1:5000</td>
<td>Oxford Biotechnology</td>
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<td>Invitrogen</td>
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<tr>
<td>Alexa Fluor® 488 anti-mouse *cross absorbed</td>
<td>Fluorescence 1:1000</td>
<td>Invitrogen</td>
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Table 2.13 Antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody Type</th>
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<td>Alexa Fluor® 488 Goat anti-rabbit cross absorbed</td>
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<td>Invitrogen</td>
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<tr>
<td>Alexa Fluor® 594 Goat anti-mouse cross absorbed</td>
<td>Fluorescence 1:1000</td>
<td>Invitrogen</td>
</tr>
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</table>

2.6 Spindle Checkpoint assays

2.6.1 Benomyl sensitivity

Yeast strains were plated out on rich media over-night and then plated out in 10-fold serial dilutions on YPDA media and YPDA media containing 6 µg/ml, 8 µg/ml and 10 µg/ml benomyl. Plates were typically incubated at 23°C for 3 days.

2.6.2 Nocodazole viability assay

Overnight cultures were diluted back to OD$_{600}$ 0.2 and grown in YPDA media at 30°C for 3 hours. The yeast was then incubated in YPDA media containing 15 µg/ml nocodazole during a time course of 5 hours. Each hour, ~ 500 cells per culture were plated out on YPDA media lacking nocodazole and the viability, as judged by the number of colonies formed, were calculated for each time point and plotted on a graph.

2.6.3 Sister-chromatid separation assay

The yeast cells were grown to log-phase and synchronized in G1 using α-factor then released into 20 µg/ml nocodazole and incubated at 23°C for 3 hours. Cells were fixed
in 50 μl of methanol at time-point zero (G1) and after 3 hours. Cells were then analysed by fluorescence microscopy (see section 2.8.1).

2.6.4 Cell morphology analysis

Yeast cells were streaked out on a YPDA agar plate and grown over night at 30°C. These cells were then patched onto YPDA plates containing 20 μg/ml or 80 μg/ml benomyl. 50 single cells per strain were then picked using a dissecting microscope (MMS Singer Instruments) and cells that formed a large-budded (arrested) shape were scored in contrast to the cells that went through the arrest and re-budded. The percentage of cells remaining large-budded during the time course was scored and plotted. The cells were incubated at 23°C during the time course and scored every hour.

2.6.5 Tension checkpoint assays

2.6.5.1 GAL-MCD1 experiment

The yeast cells were grown over night in YEP (2%GAL, 2%RAFF) media. The next day the culture was diluted so that the cells were in mid-log phase the following morning. These cells were then arrested in G1 in YEP (GAL/RAFF) media containing α-factor at 30°C for 3 hours. Then the cells were washed three times in YPDA media and resuspended in YPDA media containing α-factor and incubated at 30°C for 2 hours to deplete Mcd1. Cells were then washed 3 times in YPDA, and to release cells from G1, they were incubated in YPDA at 30°C during the 3 hour time course. After the 60 minute time point, α-factor was added back to the cultures to prevent cells entering into the next cell cycle. At each time point, samples were taken for immunobloting to assess the levels of Pds1-myc.
2.6.5.2 Linear Mini Chromosome (LMC) assay

This assay was performed as in (Indjeian et al., 2005). Cells were grown over-night on CSM-LEU plates. The cells were then spotted in 10-fold dilutions on CSM-LEU plates of CSM-LEU plates containing 10 μg/ml doxycycline (to repress the expression of the dominant \textit{CDC20-127}) and assayed for ability to grow on these plates.

2.6.6 \textit{mtwl-1} kinetochore defect assay

This experiment was performed based on methods from (Pinsky et al., 2003). Yeast cells were grown in YPDA over-night at the permissive temperature of 23°C. The cells were diluted to OD$_{600}$ 0.2 in the morning, and grown at 23°C for 2 hours. The cultures were then treated with α-factor to synchronise the cells in G1 for 2 hours and 30 minutes. At this point the cultures were switched to the restrictive temperature of 36°C and incubated for another 30 minutes. The cells were then washed 3 times in YPDA and released from G1 into a three-hour time course at 36°C. Pds-lmyc levels were monitored by immunoblotting.

2.7 Cell biological assays

2.7.1 Whole cell immunofluorescence

Cells were fixed for 1 hour in 3.7% formaldehyde and washed with 0.1 M potassium phosphate, pH 7.5. Then they were treated with 50 μg/ml Zymolyase 100,000 in 0.1 M potassium phosphate / 0.7 M Sorbitol. The general immunofluorescence protocol was performed as previously described in (Hardwick and Murray, 1995). Cells were analysed using an Intelligent Imaging Innovations (3i) Marianas System (Denver, CO). This system uses a Zeiss Axiovert fluorescence microscope (Thornwood, NY), a
coolSNAP HQ charge-coupled device camera (Photometrics, Woburn, MA) and Slidebook software (3i, Photometrics). Cells were analysed for the presence of one GFP focus (sister chromatid cohesion) or two GFP foci (sister chromatid separation).

2.7.2 Chromosome spreads and immuno-fluorescence

<table>
<thead>
<tr>
<th>Solution</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution 1</td>
<td>2% (w/v) KAc, 0.8% (w/v) sorbitol</td>
</tr>
<tr>
<td>Solution 2</td>
<td>0.1 M MES, 1mM EDTA, 0.5 mM MgCl₂ pH 6.4; 1 M sorbitol</td>
</tr>
<tr>
<td>Fixative</td>
<td>4% (w/v) paraformaldehyde, 3.4% (w/v) sucrose</td>
</tr>
<tr>
<td>Blocking Buffer</td>
<td>0.2% (w/v) gelatin, 0.5% (w/v) BSA in PBS</td>
</tr>
</tbody>
</table>

Table 2.14 Buffers for chromosome spreading

2.7.2.1 Chromosome spreading

Chromosome spreads were performed based on methods from (Loidl et al., 1998; Nairz and Klein, 1997). 5 ml of cycling or arrested cells were collected by centrifugation for 3 minutes at 2500 rpm. The pellet was resuspended in 200 μl of solution 1 with 2 μl DTT and 2.6 μl zymolyase (10 mg/ml). The cells were then digested at 37°C for 5-10 minutes until the cells showed “chewed” edges. Then the tube was filled with ice-cold Solution 2. Cells were collected by centrifugation at 800 g for 4 minutes, and resuspended in 200 μl of Solution 2 and placed on ice. Cells were spread out with a glass rod on ethanol washed glass slides as follows:

- 15 μl cell suspension : 30 μl Fixative : 60 μl 1% Lipsol (gift from A.Marston) ;
- 60 μl Fixative

The slides were placed in a hood overnight to dry then placed at -80°C until processed.
2.7.2.2 Chromosome spreads: immunofluorescence

Slides were placed 10 minutes in PBS, 10 minutes in Blocking Buffer (under coverslip), then rinsed briefly in PBS. Primary antibody was diluted in Blocking buffer and slides were incubated with diluted antibody over-night under a coverslip in a humid chamber at 4°C. The next day the slides were washed 5 minutes in PBS then incubated with secondary antibodies under coverslip for 2-4 hours at room temperature. The slides were then rinsed with PBS and 1 drop of Vectashield containing DAPI (Vector) was placed under the coverslip.

2.7.3 Chromosome loss assays

2.7.3.1 Colony sectoring assay

Quantitative chromosome mis-segregation assays (sectoring assay) were performed as previously described (Hieter et al., 1985; Warren et al., 2002).

2.7.3.2 GFP-Chromosome loss assay

Strains containing GFP-marked chromosome IV were pre-synchronised in G1 and a sample was fixed in 3.7% formaldehyde for 5 minutes. The remaining cells were then released into 30 µg/ml nocodazole and grown at 23°C for 3 hours. The nocodazole was washed out, three times with YPDA media and arrested in the following G1, and another sample was fixed as above. Using fluorescence microscopy, the cells containing two copies of the GFP-marked chromatid (defined by the presence of two GFP foci) in G1 were scored as chromosome mis-segregation.
The same strain was used to analyse chromosome segregation at the first anaphase after nocodazole arrest. Cells were pre-synchronised in G1 and arrested with nocodazole as above. Cells were released and fixed for 1 hour in 3.7% formaldehyde and washed with 0.1 M potassium phosphate pH 7.5. Then they were treated with 50 μg/ml Zymolyase 100,000 in 0.1 M potassium phosphate / 0.7 M Sorbitol. General immunofluorescence protocol was performed as previously described in (Hardwick and Murray, 1995). Cells were analysed using fluorescence microscopy, as above, and cells with anaphase spindles containing 2 GFP-dots in one daughter cell, were scored for chromosome mis-segregation.

2.7.4 Chromosome attachment (Mtw1-GFP, Tub-CFP) assay

Strains containing Tub1-CFP and Mtw1-GFP were grown over-night in YPDA. The cells were then diluted back to OD₆₀₀ 0.2 and grown in YPDA for 3 hours at 30°C. Then the cells were arrested in mitosis using 15 μg/ml nocodazole for 3 hours. The nocodazole was then washed out and the cells were allowed to re-build their microtubules for 30 minutes then fixed in 3.7% formaldehyde for 5 minutes. The cells were then analysed using fluorescence microscope. The cells with Mtw1-GFP foci off the spindle axis were scored as cells with microtubule-kinetochore attachment defects.

2.7.5 Chromosome bi-orientation assay

This experiment was based on a method from (Indjeian and Murray, 2007). Strains were arrested in G1 with 10 μg/ml α-factor in medium lacking methionine for 3 hours, then transferred to YPDA media + 8 mM methionine + 10 μg/ml α-factor for 2 hours at 30°C to deplete Cdc20. The α-factor was subsequently washed out and cells were then incubated at 23°C for 3 hours in media containing 30 μg/ml benomyl and 15 μg/ml
nocodazole to depolymerise microtubules and 8 mM methionine to deplete Cdc20. The microtubule drugs were then washed out, and the spindle was allowed to reform at 30°C in YPDA media + 8 mM methionine to maintain the metaphase arrest. Cells were fixed at the indicated times in 3.7% formaldehyde for 5 minutes. The GFP-dots were analysed only in cells that had a short bi-polar spindle (i.e. 2 SPBs) to score for bi-orientation. Such experiments were repeated 3 times and at each time point 100 cells were counted.
Chapter 3. Analysis of the role for Bub1 kinase domain in the spindle checkpoint

3.1 Background

Although Bub1 kinase activity has been shown to be required for accurate chromosome segregation in both budding and fission yeasts (Vanoosthuyse et al., 2004; Warren et al., 2002) the roles of the Bub1 kinase domain have not been clearly established. To date it remains controversial whether the kinase activity is required for a spindle checkpoint arrest, in either organism. In some budding and fission yeast studies Bub1 kinase activity was thought to be necessary for checkpoint arrest (Roberts et al., 1994; Yamaguchi et al., 2003), but in others it was not (Vanoosthuyse et al., 2004; Warren et al., 2002). This controversy might partly be due to the use of a ‘kinase-dead’ point mutation (bub1K733R) which has since been shown to destabilise budding yeast Bub1p (Warren et al., 2002), and could therefore display a phenotype similar to that of a bub1Δ. In addition, a novel K733M kinase-dead allele was previously created in our lab, but it was found that this protein was also unstable (KGH, data not shown). Therefore, I decided to carry out a detailed analysis of the role of the Bub1 kinase domain, using a truncated Bub1 kinase allele (containing amino acids 1-608) in S. cerevisiae. This allele lacks the whole kinase domain, and has already been shown to express a stable protein (Warren et al., 2002), and to efficiently bind Bub3p and Mad1p (Brady and Hardwick, 2000). A similar Bub1 truncation is stable, and is able to localise to kinetochores in S. pombe (Yamaguchi et al., 2003) (and Vincent Vanoosthuyse, personal communication).

3.2 Construction of the bub1ΔK allele
Due to the controversy in the field regarding the role for the Bub1 kinase domain in mitosis, and specifically in the spindle checkpoint, I wanted to carry out experiments that would address this directly. Specifically, I wanted to investigate whether cells lacking this domain 1) are sensitive to microtubule drugs 2) are able to initiate and maintain a mitotic arrest in response to unattached and/or defective kinetochores and 3) show increased chromosome loss and aneuploidy. Due to the fact that kinase-dead point mutations have proven to de-stabilise Bub1 protein, an allele that would remove the whole kinase domain was used. However, by doing so we are not directly testing the role for the kinase activity, but instead analysing the role for the conserved C-terminal domain. This allele \((bub1\Delta K)\) was created by replacing the kinase domain (amino acid 608-1021) with a hygromycin resistance cassette (Goldstein and McCusker, 1999) (Figure 3.1 A-C). Primers were designed so that the PCR product contained homologous sequence to the final 90 bases of the \(BUB1\) kinase domain before amino acid 608, followed by a stop codon and the forward sequence of the pFA6a-HPH cassette. The reverse primer was designed to have the reverse complement in the 3'UTR of \(BUB1\) followed by the reverse sequence of the cassette. Hence, the PCR product will recombine at the \(BUB1\) locus and replace the kinase domain with the hygromycin marker, allowing selection for the targeted replacement by hygromycin resistance. Accurate integration was confirmed by colony PCR (Figure 3.1 D-F) and sequencing.

3.3 \(bub1\Delta K\) cells are sensitive to microtubule depolymerising drugs

Spindle checkpoint mutants are hyper-sensitive to microtubule destabilising drugs due to their inability to arrest in metaphase in response to weakly attached or unattached kinetochores. The precocious separation of sister chromatids in these mutants gives rise
A. 

\[ \text{5'UTR} \quad \text{BUB1} \quad \text{Kinase Domain} \quad 3'UTR \]

\[ \text{STOP} \quad \text{Forward Primer} \quad \cdots \]

\[ \text{P-TEF} \quad \text{hph} \quad \text{T-TEF} \]

B. 

\[ \text{P-TEF} \quad \text{hph} \quad \text{T-TEF} \]

\[ \text{5'UTR} \quad \text{BUB1} \quad \text{Kinase Domain} \quad 3'UTR \]

C. 

\[ \text{5'UTR} \quad \text{BUB1} \quad \text{P-TEF} \quad \text{hph} \quad \text{T-TEF} \quad 3'UTR \]
Figure 3.1 Construction of bub1ΔK allele using Pringle- pFA6a Hygromycin resistance cassette (Goldstein et al 1999). (A) Primer design. The forward primer was designed to cover ~90 bases before the start of the kinase domain at amino acid 608. A stop codon was designed into the primer, followed by the forward sequence of the cassette. The reverse primer was designed with ~90 bases in the 3'UTR followed by the reverse sequence of the cassette. (B-C) A PCR reaction created a product containing homologous regions within BUB1 and the resistance marker followed by homologous sequence of the 3'UTR of BUB1. Transformation of the PCR product gave rise to double crossover (B) homologous recombination and replacement of the kinase domain with hygromycin resistance marker. (D-F) To confirm correct integration, primers as shown in D and E were used.
to unequal segregation of chromosomes and aneuploidy. Yeast cells that have lost one or more chromosomes die as a consequence, and do not form colonies. Spindle checkpoint deletion mutants show varied sensitivity to the microtubule destabilising drug, benomyl, where *bub1Δ* mutant cells show the highest sensitivity and *mad3Δ* mutants show a lower sensitivity. Therefore microtubule drug sensitivity can be a useful method to test whether the spindle checkpoint is functional in a particular mutant. Wild-type yeast can grow on low levels of benomyl due to their ability to delay each mitosis to ensure correct microtubule-kinetochore interactions before onset of anaphase. Despite being a useful method to assay for functional spindle checkpoint, benomyl sensitivity is not a specific assay for the spindle checkpoint. The sensitivity can also be due to other chromosome missegregation defects, unrelated to the spindle checkpoint per se.

3.3.1 Cells lacking Bub1 kinase domain show intermediate benomyl sensitivity compared to other spindle checkpoint mutants

We wanted to test the *bub1ΔK* mutant on benomyl and compare it to other spindle checkpoint deletion mutants. Cells were plated out in 10-fold dilutions on YPDA media and on YPDA media containing benomyl and incubated at 23°C for 3 days. On YPDA media containing low concentrations of benomyl, *bub1ΔK* cells showed an intermediate sensitivity to the drug compared to other spindle checkpoint mutants, and it was clear that cells with the *bub1ΔK* mutation were not as sick as the complete *bub1Δ* (Figure 3.2).
Figure 3.2. *bub1ΔK* cells show an intermediate sensitivity to benomyl compared to wild-type and other spindle checkpoint mutants. The indicated strains were plated out in 10-fold serial dilutions on YPDA media and on YPDA media containing 8 μg/ml benomyl, and grown at 23°C for 3 days.
3.3.2 Cells lacking Bub1 kinase domain die rapidly in liquid cultures containing nocodazole

Spindle checkpoint mutants die rapidly compared to wild-type cells in liquid cultures containing microtubule drugs (Straight et al., 1996), so I asked how long \textit{bub1ΔK} cells remain viable under such conditions. Cells were grown in media containing 30 μg/ml nocodazole at 23°C and then plated on rich media lacking microtubule drugs. Viability was scored as the percentage of cells able to form colonies. In contrast to wild-type cells, \textit{bub1ΔK} behaved like \textit{bub1Δ} and \textit{mad2Δ} cells and showed rapid death in this viability assay (Figure 3.3). After 1 hour, 55% of \textit{bub1ΔK} cells were already inviable when plated out on YPDA media. These data show that cells lacking the Bub1 kinase domain are sensitive to microtubule drugs and that they die rapidly, but also that \textit{bub1ΔK} is not a complete loss of function (null) allele.

3.4 The Bub1 kinase domain is dispensable for robust spindle checkpoint arrest

\textit{bub1ΔK} cells could be sensitive to microtubule depolymerising drugs for various reasons. They may be unable to arrest in mitosis, as the \textit{bub1Δ} mutant, or they could be unable to recover properly from the arrest. To distinguish between these possibilities, I examined cell morphology, the level of sister-chromatid cohesion and levels of Securin (Pds1) in \textit{bub1ΔK} cells in response to unattached or defective kinetochores.

3.4.1 \textit{bub1ΔK} cells arrest with a ‘large-budded’ phenotype on plates containing benomyl

Wild-type \textit{S. cerevisiae} cells that arrest in mitosis adopt a specific ‘large-budded’ shape. As an indication of a functional spindle checkpoint, a morphological assay was
Figure 3.3. *bub1ΔK* cells rapidly lose viability in cultures containing nocodazole. The indicated strains were grown in YPDA media containing 30 μg/ml nocodazole at 23°C for 5 hours. At each time point, ~500 cells were plated out and the percentage of viable cells (judged by the total number able to form colonies) was plotted against time.
performed, scoring the percentage of cells able to maintain this 'large-budded' state on plates containing 20 μg/ml and 80 μg/ml benomyl (Figure 3.4). Both wild-type and bub1ΔK cells remained large-budded (which is an indication of mitotic arrest) for up to 6 hours on benomyl compared to the spindle checkpoint mutants mad2Δ and bub1Δ, which did not respond to microtubule depolymerisation and re-budded prematurely (Figure 3.4 A-B).

3.4.2 Bub1 kinase domain is not required to stabilize Securin (Pds1) - or to maintain sister-chromatid cohesion in response to unattached kinetochores

As discussed in the introduction, when the APC/C is activated it ubiquitinates Securin (Pds1), which gets degraded by the 26S proteasome. This event releases Separase (Esp1), which in turn cleaves Mcd1 (also called Scc1), and the sister-chromatids segregate to daughter cells. Therefore, the levels of Pds1 in the budding yeast cell act as an indicator of the cell cycle stage. If high levels of Pds1 are maintained it indicates mitotic arrest, whereas a peak of Pds1 in mitosis followed by rapid degradation indicates activation of the APC/C. This can be used to biochemically investigate whether the spindle checkpoint can be activated and maintained in bub1ΔK cells in the presence of unattached kinetochores as a result of nocodazole treatment. To test this, the cells were synchronised in G1 with α-factor and released into YPDA with or without nocodazole, and the levels of Pds1-18myc were monitored every 20 minutes by immunoblotting (Figure 3.5 A). As expected, there was no Pds1 present in G1 (indicates efficient synchronisation) and the levels peaked at ~60 minutes when the cells went into mitosis. In the cells that were released into media containing nocodazole, the levels of Pds1-18myc were maintained in both wild-type and bub1ΔK cells for the duration of the time-course, indicating an efficient mitotic arrest. To confirm that cells lacking the
Figure 3.4. *bub1ΔK* cells arrest as large-budded cells on plates containing benomyl. Single cells (50 per strain) were picked and placed on YPDA agar containing (A) 20 μg/ml benomyl or (B) 80 μg/ml benomyl and incubated at 23°C. Cell morphology was followed, and at each time-point the percentage of 'large-budded' cells (cells arrested in mitosis) was plotted against time.
Figure 3.5. Bub1 kinase domain is dispensable for nocodazole arrest initiation-and maintenance. (A) Strains containing Pdsl-myc were synchronised in G1, released into YPDA or YPDA + nocodazole and assayed for levels of Pds1-myc by immunoblotting (using A14 anti-myc antibody and anti-PGK1 antibody as loading control) at the indicated timepoints. (B-C) The indicated strains containing a GFP-marked chromatid, were synchronised in G1 and released into YPDA + nocodazole and incubated at 23°C for 3 hours. Grey bars represent the time of release from G1, and black bars represent 3 hours in nocodazole. The percentage of cells which had separated their GFP-marked sister chromatid (i.e. 2 GFP dots), as opposed to maintained cohesion (1 GFP dot), is plotted in the graph (n=100 cells per experiment, error bars indicate standard deviation from 3 separate experiments).
Bubi kinase domain do not precociously enter anaphase in the presence of microtubule drugs, a yeast strain expressing lacI-GFP and that contains a lac-operator array on chromosome IV thereby marking this chromosome with GFP (Figure 3.5 B) was used. This enabled me to visualize whether the sister-chromatids were still cohesed (one GFP dot) or had separated pre-maturely (two GFP dots) in the presence of anti-microtubule drugs. Cells were synchronized in G1 with α-factor, and released into media containing nocodazole. bub1ΔK cells behaved like wild-type and efficiently maintained sister-chromatid cohesion for three hours in cultures containing nocodazole (Figure 3.5 C). However, as expected, the bub1Δ and the mad3Δ mutants separated their sister-chromatids precociously.

3.4.3 Cells lacking the Bub1 kinase domain arrest in mitosis in response to defective kinetochores

In the assays described above, anti-microtubule drugs (benomyl and nocodazole) were used to activate the spindle checkpoint pathway that recognizes un-attached kinetochores. Complete microtubule de-polymerisation is not a very common physiological situation, thus bub1ΔK cells were therefore analysed in other situations. Ipl1 kinase activates the spindle checkpoint by creating un-attached kinetochores in response to mutations in several kinetochore components that are thought to create reduced tension at centromeres (Pinsky et al., 2006). To investigate whether the kinase domain of Bub1 is required to arrest cells containing such defective kinetochores, strains were used carrying the temperature sensitive mutation of the kinetochore protein Mtw1 (mtw1-1), and Pds1-18myc to enable me to analyse the levels of Securin (Pds1) in a time course at the restrictive temperature. The cells were synchronised in G1 at the permissive temperature of 23°C then shifted to the restrictive temperature of 36°C for
30 minutes before the release from G1. Levels of Pds1-18myc were monitored at indicated time points, and PGK1 was used as a loading control (Figure 3.6). Pds1 levels peaked in wild-type cells at 60 minutes after G1 release, and Pds1 was degraded as the cell cycle continued, then the levels of Pds1 remained low throughout the time-course due to re-addition of α- factor after 60 minutes to prevent another cell cycle. However, cells containing the mtwl-1 mutation arrested in mitosis and stabilised Pds1 in response to the kinetochore defect. This is in contrast to the ipl1-321, mtwl-1 double mutant cells, which are unable to arrest as previously shown (Pinsky et al., 2003). Cells containing the mtwl-1 mutation in combination with bub1ΔK were still able to respond to the kinetochore defect and stabilise Pds1-18myc (Figure 3.6) as in the mtwl-1 single mutant. Similar results were obtained when using the more severe ndc80-1 kinetochore mutant in combination with bub1ΔK (data not shown). These results suggest that Bub1 kinase is not required to "sense" these defective kinetochores, nor to activate Ipl1p in these mutants, or to respond to the unattached kinetochores that Ipl1p kinase activity creates (Pinsky et al., 2003).

3.4.4 bub1ΔK cells are not sensitive to microtubule drugs due to inability to release from mitotic arrest

One explanation as to why cells lacking Bub1 kinase domain are sensitive to microtubule poisons, yet able to arrest, could be if they were unable to release from the arrest. To test this, the cells were arrested in mitosis using microtubule drugs, which were then carefully washed out and the levels of Pds1-18myc were monitored by immunoblotting (data not shown). The results showed that bub1ΔK cells are indeed capable of releasing from nocodazole arrest and degrade Pds1.
Figure 3.6 The Bub1 kinase domain is not required to arrest cells in response to defective kinetochores. Strains carrying the temperature sensitive allele *mtw1-1* and Pds1-18myc were synchronised in G1 for 2.5 hours at the permissive temperature of 23°C, then incubated for 30 minutes at the restrictive temperature (36°C). Cells were washed and released from G1, and the levels of Pds1-18myc were monitored at the indicated timepoints by immunoblotting using anti-myc (A14 antibody) and anti-PGK1 antibody as loading control.
3.5 Bub1 kinase is required for accurate chromosome segregation

The above experiments strongly suggest that the C-terminal kinase domain of Bub1 is not required to initiate or maintain spindle checkpoint arrests induced by unattached or defective kinetochores. In addition, nocodazole release experiments monitoring Pds1-18myc levels show that they are able to degrade Pds1 after the microtubule drugs have been washed out. The fact that bub1ΔK cells are hypersensitive to anti-microtubule drugs, yet are able to arrest efficiently, suggests that the Bub1 kinase domain could be required for accurate recovery from spindle damage. To understand the sensitivity of bub1ΔK cells to anti-microtubule drugs, I investigated whether they have increased chromosome loss following nocodazole arrest.

3.5.1 bub1ΔK cells mis-segregate chromosomes at high frequency during the recovery from a spindle checkpoint arrest induced by spindle drugs

Chromosome segregation was followed during the first anaphase after nocodazole release, using the GFP-marked chromosome strain described above (Figure 3.5 B). Accurate chromosome segregation should give rise to one GFP dot in each daughter cell. The cells were arrested in metaphase for three hours with nocodazole, then released into anaphase. After 30 minutes, the cells were fixed and stained with α-tubulin antibody to monitor spindle elongation. The results showed 33% mis-segregation of the GFP-marked chromatid in bub1ΔK cells (Figure 3.7 A, lower panel) compared to 2% in wild-type (Figure 3.7 A, upper panel). The same strain was then used to score a larger number of cells for chromosome mis-segregation, and compared unchallenged G1 cells with G1 cells that had been released from a nocodazole arrest. In G1, the cells have unreplicated chromosomes and should therefore only contain one GFP marked chromatid.
Figure 3.7 *bub1ΔK* cells display high levels of chromosome mis-segregation upon nocodazole release. (A) Wild-type and *bub1ΔK* cells were released from G1 into media containing 30 μg/ml nocodazole and incubated at 23°C for 3 hours. Cells were subsequently released into anaphase by washing out the nocodazole. Samples were fixed in 3.7% formaldehyde for 1 hour, 30 minutes after release, and stained with anti-GFP antibody (GFP-marked chromosome) and anti-tubulin (red spindle) antibody. The DNA was stained with DAPI (blue). Percentage of non-disjunction of the GFP marked chromatid at the first anaphase following nocodazole arrest was 2% in wild-type cells and 33% in *bub1ΔK* cells (n ≥ 50 anaphase cells). Scale bar represents 3 μm. (B) Indicated strains were synchronised in G1 as previously described and cells with 1 GFP dot (D, empty triangle) versus two dots (D, filled triangle) were counted (n=400) (C). The cells were then released and incubated in media containing 30 μg/ml nocodazole at 23°C for 3 hours, and released into media containing α-factor to score cells in the following G1. The number of cells with one GFP dot versus two dots were scored (n=400) Scale bar represents 2 μm.
(i.e. 1 GFP dot). In a control experiment, cells were arrested in G1 using α-factor and the number of cells with one GFP focus (Figure 3.7 D, empty triangle) versus two GFP foci (Figure 3.7 D, filled triangle) was counted. As expected, most wild-type cells had one GFP dot (Figure 3.7 B), representing one copy of chromosome IV, in both wild-type (0% had 2 GFP dots, n=400) and bub1ΔK cells (2% had 2 GFP dots, n=400). The small number of bub1ΔK cells with two copies of this chromosome probably reflects a background level of aneuploidy, frequently observed in bub1 mutants. However, when cells were released from G1 into media containing nocodazole for three hours, then released and trapped in the following G1, there was a marked increase in the number of cells containing two GFP foci in bub1ΔK (30% had two GFP dots, n=400) compared to wild-type (where only 4% had two GFP dots, n=400) (Figure 3.7 C). This confirms that there was a significant defect in segregating chromosome IV faithfully during the anaphase following nocodazole release. Due to the high incidence of chromosome mis-segregation following treatment with nocodazole, and considering that only one of the 16 budding yeast chromosomes was scored in this analysis, I propose that the reason why bub1ΔK cells are sensitive to anti-microtubule drugs, despite showing the capacity to arrest in metaphase, is due to chromosome loss following the release.

3.5.2 The chromosome mis-segregation phenotype is not due to inability of bub1ΔK to cleave the cohesin subunit—Mcd1

Since co-segregation of the GFP marked sister-chromatids was detected in a significant number of cells during nocodazole release (Figure 3.7 C), I wondered whether this could be due to a defect in the cleavage of the Cohesin sub-unit Mcd1 upon anaphase, which could explain sisters-chromatids co-segregating. Therefore, a strain was employed carrying MCD1-6HA that enabled detection of cleavage products of Mcd1
upon nocodazole washout. The cells were arrested in nocodazole and benomyl at 23°C for 3 hours. The drugs were then washed out and samples were taken at the indicated time-points for immunoblotting with α-HA antibody and α-PGK1 as a loading control (Figure 3.8). The results indicate that bub1ΔK cells cleave Cohesin at similar rates and levels as wild-type cells. I therefore suggest that it is not due to an inability to cleave Cohesin (Mcd1) that sister-chromatids mis-segregate in bub1ΔK mutant cells.

3.5.3 The Bub1 kinase domain is required for accurate chromosome segregation during an unperturbed mitosis

Bub1 has been implied to have a function in chromosome segregation, which is separate to its function in the spindle checkpoint (Vanoosthuyse et al., 2004; Warren et al., 2002). These studies suggest that the Bub1 kinase domain is involved in this function as opposed to the N-terminal domains, which are required for the spindle checkpoint. To investigate whether the budding yeast Bub1 kinase domain also plays a role in chromosome segregation in unperturbed mitosis, a chromosome loss sectoring assay was employed, which has previously been used to analyse many mitotic and checkpoint mutants (Hieter et al., 1985; Spencer et al., 1990; Warren et al., 2002).

The strain used carries an ade2-101 mutation, which blocks the adenine biosynthesis pathway and gives rise to red colonies on YPD plates. The ade2-101 mutation gives rise to a premature (ochre- UAA) stop codon, that can be suppressed to wild-type by a single copy of the gene, SUP11, encoding a mutant tyrosine tRNA. This tRNA recognises the mutant stop codon, coded by ade2-101 and inserts a tyrosine, which then gives rise to translation of the Ade2. Thus the colonies appear white. This ade2-101 strain also carries an artificial, non-essential test chromosome carrying the SUP11 gene.
Figure 3.8. Mcd1 cleavage is not defective in \textit{bub1ΔK} cells. The indicated strains were arrested in mitosis using 15 μg/ ml nocodazole and 30 μg/ml benomyl at 23°C for 3 hours. The drug was washed out and samples for immunoblotting with 12CA5 (anti-HA antibody) were taken at indicated timepoints. There was no obvious difference in timing or amount of Mcd1 cleavage detected. Blot shows a representative experiment. The experiment was repeated 3 times.
and a URA marker. When cells are grown in media containing uracil, there is no selective constraint for the cells to keep the chromosome, and loss of this chromosome can be scored by appearance of red-white sectoring of colonies (Figure 3.9 A). The loss of this chromosome was scored at the first division by counting colonies that were half red and half white (half-sectored colonies, Figure 3.9 B, filled triangle). Consistent with published data (Warren et al., 2002), in an unchallenged cell cycle bub1Δ cells lost the test chromosome at a rate of 41 per 1000 division compared to 0.5 per 1000 in wild-type. bub1KΔ cells also showed chromosome loss in a normal mitosis but at a lower rate than bub1Δ, at 28 per 1000 divisions (Figure 3.9, B). These results show that the Bub1 kinase domain plays a role in chromosome segregation, which becomes very important upon challenge with microtubule drugs. I suggest that bub1Δ cells have an even higher chromosome loss rate, because in addition to their segregation defects, these cells have also lost their ability to checkpoint arrest.

3.6 Discussion

Bub1 is an essential protein for spindle checkpoint function, but it is not required for viability in S. cerevisiae. It has previously been shown that the N-terminal domains of Bub1 are required for spindle checkpoint arrest, by recruiting other spindle checkpoint components to the kinetochores (Vanoosthuyse et al., 2004). However, it was still unclear whether Bub1 kinase activity plays a role in spindle checkpoint arrest, and there is conflicting evidence in the field.

I decided to create a truncated allele of Bub1, lacking the whole kinase domain. This allele truncates the last 609-1021 residues of Bub1. The kinase domain is thought to
Figure 3.9. The Bub1 kinase domain is required for accurate chromosome segregation during an unperturbed mitosis. (A) Schematic diagram of the strains containing the SUP11 mutation suppressing the ade2-101 mutation. When grown in YPD media, cells can lose the artificial SUP11 carrying chromosome and colonies appear red. (B) Strains were grown over-night in CSM-URA media, then diluted back to early log-phase and grown in YPDA media at 30°C for 3 hours. Cells were then plated out on YPD at a density of ~500 cells per plate. Only colonies that were at least half red (B, filled triangle) were scored as losing the test chromosome at the first division ('half-sectors') on the plate.
begin at amino acid 710, however the 609-1021 truncation was previously shown to be stable (Warren et al., 2002), hence this allele was chosen. *bub1ΔK* cells were analysed on YPDA plates containing different concentrations of benomyl. Consistent with previously published data (Warren et al., 2002), the *bub1Δ* mutant was the most sensitive. It was clear from the data that the *bub1ΔK* mutant strain did not show as strong sensitivity to benomyl as the complete Bub1 deletion (*bub1Δ*) but it showed significantly more sensitivity to benomyl than wild-type yeast. This is in agreement with the analysis of the fission yeast *bub1K762M* ‘kinase-dead’ point mutant (Vanoosthuyse et al., 2004), which also showed an intermediate benomyl sensitivity compared to wild-type and *bub1Δ*. These data indicate that there are defects in the *bub1ΔK* mutant, but also shows that this is not a complete loss of function allele, as a complete loss of function would give similar phenotype on benomyl as the *bub1Δ* strain. I then asked how long *bub1Δ* cells can remain viable in liquid media containing microtubule drugs then plated on rich media. The results showed that these cells die at the same rate as *bubΔ* and *mad2Δ* cells. Taken together, these results clearly show that *bub1ΔK* cells are sensitive to microtubule drugs.

Despite being sensitive to microtubule drugs, here I showed that this is not because these cells are spindle checkpoint deficient in their response to these drugs. Firstly, *bub1ΔK* cells showed a similar response to wild-type in our morphological benomyl assay and in their ability to stabilise Pds1 in response to microtubule drugs. In agreement with this, *bub1ΔK* cells were also able to maintain sister-chromatid cohesion for 3 hours in nocodazole. There was a slight increase in the number of separated sisters. This could either be due to a very slight defect in the maintenance of the nocodazole arrest, or background aneuploidy levels.
In the experiments discussed so far, microtubule drugs were used to induce a mitotic arrest. The levels of spindle drugs that were used would destabilise microtubules, and give rise to unstable and unattached kinetochores. However, this is a very strong activator of the spindle checkpoint, and I saw that Bub1 kinase domain is not required for this arrest. However, it has been suggested in other systems that the kinase activity of Bub1 might be required for full APC/C inhibition in human cells (Yu and Tang, 2005) and required for spindle checkpoint arrest in Xenopus extracts in response to low (but not high) doses of nocodazole (Chen, 2004). Therefore, I tested whether Bub1 kinase might be required in situations other than complete spindle damage. The Aurora B homologue, Ipl1 is not required for nocodazole arrest, but has previously been shown to be required to arrest cells in response to kinetochore defects such as those in the mtw1-1 temperature sensitive allele (Pinsky et al., 2003). It is not exactly clear what the state of the microtubule-kinetochore attachments are in this mutant, but it has been suggested that there are more subtle defects in which Ipl1 is required for the arrest of the cell cycle. The ipl1-321 mutant was used as a control to investigate whether bub1ΔK cells are able to arrest in response to this kinetochore defect. The results of this experiment showed that bub1ΔK cells are clearly able to detect the mtw1-1 induced defect and stabilise Pds1 for 200 minutes. Even wild-type cells appeared to come out of the arrest quicker than bub1ΔK cells, indicating that the Bub1 kinase domain is not required for the arrest induced by spindle damage, nor by kinetochore defects. It was concluded from these experiments that the reason why bub1ΔK cells are benomyl sensitive is not due to an inability to checkpoint arrest, in contrast to the bub1Δ mutant.
We considered the two possibilities that \textit{bub1ΔK} cells can either not recover from the checkpoint arrest at all, or that they fail to recover in an accurate manner. Therefore, the ability of \textit{bub1ΔK} cells to degrade Pds1 after washing out the nocodazole was analysed, and no difference was detected between wild-type or the mutants, suggesting that APC/C can still be activated after a prolonged arrest. Chromosome segregation in \textit{bub1ΔK} cells was then analysed and there was a dramatic increase in mis-segregation of the GFP marked chromosome during recovery from the arrest. However, this was not due to an inability to cleave the Mcd1-Cohesin subunit, which could lead to co-segregation of sisters upon anaphase. My data also show that the Bub1 kinase domain is required for chromosome segregation in a normal mitosis. These results are in agreement with other studies that suggest that Bub1 plays a role in chromosome segregation distinct from that in the spindle checkpoint (Warren et al., 2002). We know that the N-terminal domains of Bub1 are required for the spindle checkpoint, and previously it was not clear whether the C-terminal kinase domain was important for checkpoint function. In this study I clearly show that the kinase domain has no apparent role in the initiation or maintenance of spindle checkpoint arrest, but that it is required for accurate recovery from spindle damage. I propose that the kinase domain also plays a role every mitosis by ensuring accurate chromosome segregation. This mechanism may or may not be the same that ensures accurate chromosome segregation following spindle damage (discussed later).

Despite being unable to definitely show whether it is actually the lack of kinase activity or some other function of the C-terminal domain that gives rise to this phenotype of the \textit{bub1ΔK} cells, this approach has enabled us to make targeted approaches to find direct
or indirect targets of Bub1 kinase. The next two chapters will provide evidence for- and discuss the possible mechanisms that give rise to these phenotypes.
Chapter 4. Bub1 kinase and Sgo1 act together to establish accurate chromosome bi-orientation

4.1 Background

The survival of cells depends on the accurate transmission of DNA to each daughter cell. This event is highly dependent on cells being able to: 1) sense unattached kinetochores or kinetochores that lack tension and arrest the cells in response to these defects, and 2) repair any defects for accurate cell cycle progression. The replicated sister-chromatids need to establish accurate bi-polar attachments to the mitotic spindle before the onset of anaphase. These bipolar attachments of the kinetochores give rise to ‘tension’ across the centromere, which in turn gives rise to centromeric ‘breathing’ (Goshima and Yanagida, 2000; He et al., 2000) where the centromere is transiently pulled apart by the forces of microtubules (for review see (Tanaka, 2005)). The budding yeast Aurora B homologue (Ipl1) is involved in establishing bi-orientation and has been shown to break improper attachments (Pinsky et al., 2006), for example when both kinetochores are attached to the same spindle pole (syntelic attachments). This process is absolutely required for biorientation, and ipl1 (and slj15) mutants show an increased number of sister-chromatids that remain nonbioriented in the vicinity of one SBP, which leads to severe chromosome mis-segregation at anaphase (Tanaka et al., 2002).

Ipl1 (and other spindle checkpoint proteins (Biggins and Murray, 2001; Indjeian et al., 2005; King et al., 2007a; Stern and Murray, 2001)) is also required to sense the ‘lack of tension’ between kinetochores and delays anaphase in response to this. It is thought that Ipl1 de-stabilises weak attachments or attachments that do not give rise to tension. This event gives rise to unattached kinetochores that then activates the spindle checkpoint
(Pinsky et al., 2006). Hence, the ‘tension’ and the ‘attachment’ pathways appear to be intimately linked through the Chromosomal Passenger protein 1p11.

In Chapter 3, the phenotype of budding yeast cells lacking the kinase domain of Bub1 was described. Importantly, it was established that this kinase domain is not required for spindle checkpoint arrest per se, but is required for accurate recovery from spindle damage. *bub1ΔK* mutant cells display high levels of chromosome loss after spindle damage, and also significant levels of chromosome loss in a normal mitosis. This phenotype closely resembles that of *sgo1Δ* mutant cells in budding yeast mitosis (Indjeian et al., 2005). The Sgo1 protein is a homologue of the Shugoshin ('guardian spirit') *Drosophila MEI-S322* protein (for review see (Watanabe and Kitajima, 2005)).

Several studies have recently described Sgo1 and its role in protection of centromeric cohesin in meiosis I (Kitajima et al., 2004; Marston et al., 2004; Rabitsch et al., 2004). Subsequent studies also described a role for Sgo proteins in mitosis (Kitajima et al., 2005; Kitajima et al., 2004; McGuinness et al., 2005; Salic et al., 2004; Tang et al., 2004b). Whereas some organisms have two orthologues of Shugoshin (Sgo1 and Sgo2), budding yeast only has one (Sgo1), which has been shown to have a role in mitosis that is distinct from its meiotic role in the protection of centromeric cohesion. In a study by Indjeian and colleagues (Indjeian et al., 2005), they set out to identify components of the tension-sensing machinery by screening for mutants that “ignored” the presence of Linear Mini Chromosomes (LMCs). These chromosomes segregate poorly, probably due to their inability to resist tension, and activate the spindle checkpoint in wild-type cells. Interestingly, it was also shown in this study that *sgo1Δ* mutant cells were able to arrest in response to microtubule drugs and also that they mis-segregate chromosomes at high rates upon the release. The striking similarity of the phenotypes of *sgo1Δ* and
bub1ΔK cells in budding yeast mitosis prompted us to investigate whether these two proteins are in fact part of the same pathway to ensure accurate chromosome segregation in mitosis.

4.2 The chromosome loss phenotype of bub1ΔK and sgo1Δ is not due to general microtubule-kinetochore attachment defects

I wanted to investigate the reason behind the chromosome loss phenotype of bub1ΔK and sgo1Δ cells in mitosis. It is known that (unlike in vertebrate cells that lack Sgo1) budding yeast sgo1Δ cells do not show a major defect in mitotic sister-chromatid cohesion (Indjeian et al., 2005). Other core kinetochore proteins such as Ndc80, when deleted, show severe chromosome attachment defects (Cheeseman et al., 2006; Pinsky et al., 2006; Wigge et al., 1998) (reviewed in (Maiato et al., 2004)), and this leads to serious chromosome mis-segregation and death. Therefore I wanted to investigate whether bub1ΔK and sgo1Δ cells show increased chromosome-microtubule attachment defects following the release from microtubule drugs. For this, a strain carrying Tub-CFP to mark the spindle and Mtw1-3xGFP to label all the kinetochores was employed. Cells were arrested in mitosis with the microtubule drugs benomyl and nocodazole. These drugs were then carefully washed out. Time was allowed for spindle re-assembly (20-30 minutes), then the position of the kinetochores were analysed. Any Mtw1-3xGFP staining off the spindle axis was scored as an unattached kinetochore. As previously described, the ndc80-1 mutation gives rise to many unattached kinetochores at the restrictive temperature (Figure 4.1). However, in the bub1ΔK and sgo1Δ cells, an increase in unattached kinetochores was not detected (Figure 4.1).
Figure 4.1 Cells lacking Bub1 kinase do not display major chromosome attachment defects. (A) The indicated strains were arrested in 15 μg/ml nocodazole and 30 μg/ml benomyl at 23°C for 3 hours. The cells were then washed 3 times and released into YPDA media for 30 minutes. The control ndc80-1 cells were grown at 36°C for one hour before fixation. The cells were then fixed in 3.7% formaldehyde for 5 minutes. We scored cells which had kinetochores (Mtw1-3GFP) off the spindle axis at this point as “unattached kinetochores”. (B) Table showing the numbers of unattached kinetochores in the different strains.
4.3 The Bub1 kinase domain is required to respond to lack of tension of kinetochores

One major finding in the Indjeian et al study (Indjeian et al., 2005) was that the Sgo1 protein plays a role in tension-sensing in budding yeast mitosis. Lack of tension over sister-kinetochore pairs can occur for various reasons: either when only one kinetochore is attached to a microtubule (mono-orientation), or when both kinetochores are attached to microtubules emanating from the same spindle pole body (syntelic attachment), or it may also occur in association with some types of kinetochore defects. Since Sgo1 protein has been shown to be required to arrest cells in response to tension defects, I decided to compare it to the bublΔK mutant in these assays.

4.3.1 Bub1 kinase, like Sgo1, is required to delay anaphase in response to reduced cohesion

The first assay makes use of a strain which carries a glucose repressible promoter for MCD1 (GAL-MCD1), which means that when cells are grown in glucose, Mcd1 expression is turned off and cohesin is lost between sister-chromatids (Figure 4.2 A). However in this situation the kinetochores are still attached to microtubules so the effect will be a 'loss of tension' as opposed to 'loss of attachment'.

The cells were arrested in G1 in media containing 2% galactose and 2% raffinose for 3 hours to keep the expression of Mcd1 turned on. The cells were then kept in G1, but transferred to media containing 2% glucose to switch off the expression of Mcd1 for 2 hours. Then, the α-factor was washed out and the cells released into the cell cycle. The cells were allowed to go through S-phase and replicate their chromosomes, but in the
Figure 4.2 Bub1 kinase is required to respond to lack of tension at kinetochores
(A) Schematic diagram of the basis of the experiment. (B) The indicated strains were
arrested in G1 with α-factor with media containing 2% rafﬁnose and 2% galactose for 3
hours. The cells were then kept in G1 but the expression of Mcd1 was switched off by
incubating them in 2% glucose, for 3 hours. The cells were washed 3 times and released
in media containing 2% glucose. Samples for immuno-blotting were taken at indicated
timepoints and levels of Pds1 were monitored using anti-myc (A14) antibody. Anti-PGK1
was used as a loading control. (C) Bub1 kinase is required for proper arrest in response to
linear mini chromosomes. The indicated strains were plated out in 10-fold serial dilutions
on -LEU plates (dominant Cdc20, ON) and on -LEU plates containing 10 μg/ml doxycycline
(dominant Cdc20, OFF).
absence of Mcd1. In mitosis the sister-chromatids attach as normal to the spindle but they will not be cohesed (Figure 4.2 A). Samples were taken for immunoblotting with anti-myc antibody (A14) to detect Pds1-myc, and anti-PGK1 antibody as a loading control. Mad2 has previously been shown to be required for the anaphase delay that occurs in this assay in wild-type cells (Indjeian et al., 2005), so a mad2Δ strain was used as a control. As expected, wild-type cells maintained Pds1 levels for 120 minutes, but mad2Δ cells had already degraded Pds1 at 80 minutes, consistent with previously published data. The sgo1Δ cells also showed clear evidence of premature degradation of Pds1 (as described in (Indjeian et al., 2005)), however possibly at a slightly later time-point than mad2Δ cells (Figure 4.2 B). which could be due to their sick, aneuploid phenotype. Finally, bub1ΔK cells also displayed premature degradation of Pds1, indicating that the kinase domain of Bub1 is, indeed, also required to delay anaphase in response to lack of tension.

4.3.2 bub1ΔK cells are defective in their response to linear mini chromosomes

To confirm the role for Bub1 kinase in tension-sensing, the bub1ΔK mutant was tested in the Linear Mini Chromosome (LMC) assay. This assay was initially described in (Wells and Murray, 1996), and uses a budding yeast strain harbouring LMCs. These chromosomes activate the spindle checkpoint in wild-type cells, due to their proposed inability to resist the forces of microtubules (i.e. they can not resist tension). This anaphase delay is lethal in wild-type cells carrying the CDC28-VF mutation that reduces APC/C activity (Rudner et al., 2000). However, this delay can be overcome by a mutation in the APC/C activator, Cdc20. This mutation (CDC20-127) is dominant, does not respond to Mad2 inhibition and therefore over-rides the spindle checkpoint.
The cells were plated in 10-fold serial dilutions on -LEU plates (expression of dominant CDC20-127), or on -LEU plates containing doxycycline (repression of dominant CDC20-127). On the -LEU plates, all yeast strains could grow due to the presence of dominant Cdc20. However, on the –LEU plates containing doxycycline, the dominant Cdc20 allele is repressed and only cells that ignore the LMCs can grow. The results show that mad2Δ (as previously shown (Indjeian et al., 2005)) and bub1ΔK cells can grow in the absence of dominant Cdc20, indicating that Mad2 and Bub1 kinase are required to delay anaphase in response to LMCs (Figure 4.2 C). This result further strengthens the hypothesis that Bub1 kinase is involved in tension-sensing.

4.4 Bub1 kinase and Sgo1 act in concert to establish efficient chromosome bi-orientation

One downstream affect of the inability to detect tension defects could be failure to bi-orient chromosomes, which would in turn lead to chromosome mis-segregation. It was therefore tested whether Bub1 kinase and Sgo1 are involved in establishing chromosome bi-orientation. In budding yeast, this can test this directly. A strain was employed, which carries an array of tet-operators integrated close to the centromere (2kb away) on chromosome IV and expresses a Tet-repressor-GFP fusion protein. This strain also has the SPBs marked with Spc42-tomato (constructed using pFA6a-ttdTomato::NAT cassette, gift from K. Sawin). In addition, a repressible promoter for CDC20 (pMET-CDC20) enabled us to deplete Cdc20 in the presence of methionine, which causes a metaphase arrest independent of microtubule drugs or spindle checkpoint activity. This is because the APC/C cannot get activated in mitosis if Cdc20
is depleted. The cells were synchronised in G1 and depleted of Cdc20. They were then released into media containing microtubule drugs and methionine to maintain the repression of Cdc20 for three hours. The microtubule drugs were then washed out and the spindle was allowed to re-assemble (see experimental outline in Figure 4.3 A), but the cells were still arrested in metaphase due to the lack of Cdc20. The rate of bi-orientation (amphitelic attachments) was scored as the percentage of cells with 2 GFP dots in between 2 SPBs (Figure 4.3 B and C, filled triangle). The ‘2 GFP dots’ on the spindle axis appear due to centromeric stretching (‘breathing’) that occurs upon bi-orientation due to spindle forces (i.e. tension). The level of bi-orientation in this assay will however be underestimated since the breathing-characteristic of the kinetochores means that it can (despite being bi-oriented) also be seen as a single GFP dot in between two SPBs (Figure 4.3 C, white empty triangle). A significant defect was observed in all the mutant strains in their ability to bi-orient following microtubule drug treatment. When the position of the ‘single GFP dots’ was scored it was apparent that in the mutants they more often resided close to the SPB than in between the SPBs. The bub1ΔK, sgo1Δ double mutant showed no synthetic effect when compared to the single mutants.

4.5 bub1ΔK, sgo1Δ double mutant displays no synthetic interaction

‘Synthetic sickness’ (or lethality) can be a useful genetic tool to analyse whether two proteins are part of the same pathway or not. The theory is that if two proteins act in the same pathway, then deleting both of them will not give rise to an increased severity of the phenotype. However, if the mutants show similar phenotypes but the double mutant
Figure 4.3 Bub1 kinase and Sgo1 are both required for efficient chromosome bi-orientation. (A) Schematic diagram of the experimental outline. Cells were arrested in G1 in media lacking methionine to maintain the expression of Cdc20. Then the cells were kept in G1 but Cdc20 expression was switched off with the addition of 8 mM methionine for 3 hours to ensure Cdc20 depletion. The spindle was then disassembled with the addition of benomyl and nocodazole + 8 mM methionine for 3 hours. The microtubule drugs were subsequently washed out and the spindle was allowed to re-form in the presence of 8 mM methionine to keep cells in metaphase due to the absence of Cdc20. (C-D) Time-points were taken as indicated, and cells with 2 GFP dots between two SPBs (filled triangle) were scored as 'bi-oriented' whereas 1 GFP dots (either by one SPB- red triangle, or in the middle- white empty triangle) were scored as non-bioriented. Error bars indicate SD of the mean from 5 experiments with all strains apart from the double mutant, which was performed twice along with the other strains. Scale bar represents 2 μm. (E) The position of every 'single GFP dot' was monitored and plotted in a graph. The percentage of cells with a single GFP dot residing by one SPB was calculated from the total number of single GFP dots. This experiment was carried out twice and the mean is plotted.
show synthetic sickness, then one presumes that these proteins have roles in distinct pathways. Due to the very similar phenotypes of \( \textit{bub1}\Delta \text{K} \) and \( \textit{sgo1}\Delta \) cells, I propose that they act together in the same pathway to ensure accurate bi-orientation. In agreement with this, the \( \textit{bub1}\Delta \text{K} \), \( \textit{sgo1}\Delta \) double mutant showed no additive effect in the bi-orientation assay. To further strengthen this hypothesis, the \( \textit{bub1}\Delta \text{K} \), \( \textit{sgo1}\Delta \) double mutant was tested in other assays. First, the double mutant was tested for benomyl sensitivity (Figure 4.4 A). Second, the double mutant was assayed for chromosome loss using the sectoring assay, described in figure 3.5.3. In agreement with the bi-orientation data, no synthetic phenotype was observed in the \( \textit{bub1}\Delta \text{K} \), \( \textit{sgo1}\Delta \) double mutant either on benomyl or in chromosome loss rate (Figure 4.4).

4.6 Is \( \textit{SGO1} \) a multi-copy suppressor of the \( \textit{bub1}\Delta \text{K} \) mutant?

As discussed in chapter 1, Bub1 has been shown to be required for Sgo1 localisation in other systems. Presuming this is true in budding yeast mitosis, Bubi kinase could regulate localisation and/or phosphorylation of Sgol (investigated in chapter 5), and this could be critical for its function in chromosome bi-orientation. Here, it was tested whether \( \textit{SGO1} \) is a multi-copy suppressor of the \( \textit{bub1}\Delta \text{K} \) mutation.

4.6.1 Cloning of \( \textit{SGO1} \) into YCPlac22 –CEN plasmid

First, I tested whether one extra copy of \( \textit{SGO1} \) could suppress the benomyl sensitivity of the \( \textit{bub1}\Delta \text{K} \) mutant. The promoter and open reading frame (ORF) of \( \textit{SGO1} \) were cloned into a centromere vector (YCPlac22). The \( \textit{SGO1} \) fragment was created by PCR and digested with \( \textit{HindIII} \) and \( \textit{BamHI} \) restriction enzymes. The empty vector was also digested with the same restriction enzymes, using restriction sites present in the
Figure 4.4 *bub1ΔK, sgo1Δ* double mutant shows no synthetic phenotype. (A) Benomyl sensitivity. The indicated strains were plated out as 10-fold serial dilutions on YPDA plates and on YPDA plates containing 6 μg/ml benomyl, and incubated at 23°C for 3 days. (B) The chromosome loss (sectoring) assay was employed to quantify chromosome loss of the double mutant. No additive chromosome loss or benomyl sensitivity was detected.
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Figure 4.5 One extra copy of SGO1 does not rescue the bub1ΔK phenotype. The promoter and the open reading frame of SGO1 were cloned into a 'single copy' YCPlac22 vector and transformed into the indicated strains. These strains were then tested for benomyl sensitivity by plating out as 10-fold serial dilutions on rich media (YPDA) and on YPDA containing 6 or 8 µg/ml benomyl. To show that the SGO1 construct was functional we tested rescue of a sgo1Δ strain (lower panel). One extra copy of SGO1 did not appear to rescue benomyl sensitivity of bub1ΔK mutant.
multiple-cloning site. The ligated construct was sequenced and transformed into the indicated strains (Figure 4.5). Trp-positive clones were re-streaked on -TRP media and assayed for benomyl sensitivity (Figure 4.5). The results showed that this construct (i.e. one extra copy of Sgo1) was not sufficient to rescue the benomyl sensitivity of bub1ΔK cells (Figure 4.5, upper panel). To show that the pSGOI clone was functional and can rescue sgo1Δ cells, it was transformed it into a sgo1Δ strain. The results confirmed full rescue of sgo1Δ cells with the pSGOI on benomyl plates.

4.6.2 Cloning of SGOI into pRS425 – 2 micron plasmid

I learned from the experiment in 4.5.1 that one extra copy of SGOI in the cell does not make up for the loss of Bub1 kinase domain, and did not rescue the benomyl sensitivity of bub1ΔK cells. Therefore, SGOI was sub-cloned from the YCPlac22 vector into a pRS425 multi-copy (2 micron) vector. It is known that major over-expression of Sgo1 (GAL-SGOI) makes cells very sick (A. Marston, personal com.). However, the use of a 2 micron vector does not usually give as high over-expression as when using GAL-SGOI. The plasmid was transformed into the indicated strains (Figure 4.6) using -LEU selection. The transformants were re-streaked on -LEU plates and assayed for benomyl sensitivity. Intriguingly, this plasmid appeared to partially suppress the benomyl sensitivity of bub1ΔK cells (Figure 4.6, upper panel). Again, I wanted to test that the construct could rescue sgo1Δ cells, so the plasmid was transformed into two separate sgo1Δ strains. This was done because sgo1Δ cells show high levels of aneuploidy and sgo1Δ strains arising from different crosses or transformations can appear slightly different on benomyl. The lower panel in figure 4.6 shows two separate sgo1Δ strains with or without the pSGOI (2 micron) plasmid. The results show that this pSGOI (2
Figure 4.6 Multiple copies of SGO1 partially suppress the bub1ΔK benomyl phenotype. The promoter and open reading frame of SGO1 was cloned into a 'multi-copy' pRS425-2 micron vector and transformed into the indicated strains. These strains were then tested for benomyl sensitivity by plating out in 10-fold serial dilutions on rich media (YPDA) and on YPDA containing 6 or 8 mg/ml benomyl. To show that the SGO1 construct was functional we tested rescue of two separate sgo1Δ strains (see lower panel). The results indicate that pSGO1 (2 micron) may partially suppress the bub1ΔK benomyl sensitivity (see upper panel).
micron) efficiently suppresses the benomyl sensitivity of sgo1Δ strains (Figure 4.6, lower panel).

4.7 Discussion

In this chapter, the link between Bub1 kinase and Sgo1 in their function(s) in budding yeast mitosis was addressed. Initially, I showed that the reason why these mutants display high levels of chromosome loss is not simply due to kinetochore-microtubule attachment defects, in contrast to the ndc80-1 kinetochore mutant. However, I demonstrate that Bub1 kinase domain is required to respond to 'lack of tension' defects, as shown for sgo1Δ (Indjeian et al., 2005) in budding yeast mitosis. These data support the idea that the spindle checkpoint can respond to two separate defects: those in which the kinetochores lack attachments to microtubules (e.g. by microtubule drugs) and those in which they lack tension. Thus, it appears that the N-terminal domains of Bub1 are sufficient to arrest cells through the first pathway (that of lack-of-attachment) and the kinase domain is required for the second pathway (that of lack-of-tension).

It has been proposed that the ‘tension-sensing’ machinery is dependent on outer kinetochore proteins, as opposed to core kinetochore proteins (Kitagawa et al., 2003). A previous study showed that the interaction of Bub1 with the kinetochore protein Skp1 is essential for a mitotic delay in response to reduced cohesion in the ctf8Δ mutant (Kitagawa et al., 2003). These data are consistent with ours in the respect that Bub1 plays a role in the tension-sensing machinery. Kitagawa et al propose in their study that it is through the interaction with Skp1 that Bub1 senses tension at kinetochores. This binding occurs through a small binding region close to the Bub3 kinetochore-binding
domain in the N-terminus of Bub1. Thus, it appears that both Skp1 binding to Bub1, and the Bub1 kinase domain are required for tension sensing, but it is not clear what the relationship between these functions is (this study, and (Kitagawa et al., 2003)).

Another interesting observation also implicated the Bub1 kinase domain in tension sensing. In that study they treated Xenopus egg extracts with low levels of microtubule drugs and showed that the bub1ΔK mutant was defective in the spindle checkpoint response to low (but not high) levels of microtubule poison (Chen, 2004). They proposed that the inability of bub1ΔK to arrest in response to low levels of these drugs reflects a defect in responding to the lack of tension, since they suggest that these levels of microtubule drugs may not cause unattached kinetochores but instead induce tension defects. This would fit nicely with the data presented in this chapter, and in chapter 3, and it would suggest a conserved function of the Bub1 kinase domain in sensing the lack of tension at kinetochores.

To try and understand in more detail the defects in these mutants, an assay was employed to test for chromosome bi-orientation (Figure 4.3). A significant defect was observed in bub1ΔK and sgo1Δ cells in their ability to establish chromosome bi-orientation following spindle damage. An increase in non-bioriented sister-chromatids (1 GFP dot) that resided close to one SPB was also detected in the mutants, compared to wild-type cells. This favours the idea that the single-GFP dots observed in most mutant cells, appeared as 'single dots' due to their inability to bi-orient as opposed to sister-chromatids that have bi-oriented on the spindle but fail to 'breathe'. In wild-type cells, out of all the cells with only one GFP foci, most of the single GFP dots were present in-between the SPBs. This hypothesis is further supported by results from an assay in which the GFP-chromatid was followed by live microscopy. In this assay, wild-type
cells showed ‘breathing’ of the GFP-chromatid in 88% of the cells, whereas only 56% of the cells showed this characteristic in the mutants during the time observed (KH, data not shown). Our interpretation is that sister-chromatids that initially manage to bi-orient in the mutants, are capable of breathing and show no major defects in microtubule-kinetochore attachments. However, the sister-chromatids that fail to initially bi-orient are unable to correct this and re-orient. These chromosomes will remain mal-oriented and eventually will mis-segregate their sister-chromatids at anaphase.

Interestingly, the fact that bublΔK and sgo1Δ, mutants fail to respond to ‘lack of tension’ defects fits in this model very nicely. Based on these findings and those of others, I propose a model in which bublΔK and sgo1Δ mutants fail to detect/or break chromosome attachments that are not under tension. Therefore, after spindle ablation and re-assembly, syntelically attached sister-chromatids never bi-orient and this leads to chromosome mis-segregation (Figure 4.7). However, the few chromosomes that do initially manage to bi-orient on the first try, are capable of centromeric breathing and will segregate accurately. These conclusions are strongly supported by a recent study from the Murray lab (Indjeian and Murray, 2007) (discussed in Chapter 6).

In the model (Figure 4.7), I propose alternative pathways for chromosome bi-orientation. We know from other studies that the Chromosomal Passenger proteins play a crucial role in bi-orientation. The Aurora B kinase homologue, Ipl1, is involved in tension-sensing, and has also been shown to be physically involved in destabilising microtubule attachments that do not give rise to tension. It does this through phosphorylation of the kinetochore proteins Dam1 and Ndc80 (Cheeseman et al., 2002; Cheeseman et al., 2006). It may seem logical to propose that Bubi and Sgo1 act through...
Figure 4.7 Model: Bub1 kinase and Sgo1 act together to establish chromosome bi-orientation. Bub1 kinase and Sgo1 act in concert in a novel fashion to establish chromosome bi-orientation following spindle damage. The middle section shows Bub1 kinase and Sgo1 in a linear pathway that ensures bi-orientation. The upper section shows an alternative pathway in which Bub1 kinase and Sgo1 may act through the Ipl1-Sli15 passenger complex to ensure bi-orientation. Finally, the lower section proposes that Bub1 and Sgo1 may act directly upon kinesins or microtubule proteins to break / destabilise inappropriate microtubule-kinetochore attachments. This model does not show any direct interactions, but proposes several parallel (not mutually exclusive) pathways which will be more closely investigated in chapter 5.
Ipl1 to establish bi-orientation. In fact, a previous study investigated the bi-orientation efficiency of an \textit{ipl1} mutant in metaphase and showed that 50-60\% of sister-chromatids reside next to one SPB in \textit{ipl1} mutant cells compared to 10-20\% in wild-type (Tanaka et al., 2002). These results are strikingly similar to what was seen in the experiment where the position of the non-bioriented GFP-chromatid was analysed. However, Ipl1 is an essential protein and the \textit{ipl1} mutants show much stronger phenotypes than that of \textit{bub1ΔK} or \textit{sgo1Δ} mutants. This suggests that Bub1 kinase and Sgo1 may have a partial affect through the Chromosomal Passenger protein pathway. Alternatively, Bub1 kinase and Sgo1 may act in a parallel pathway to the Chromosomal Passenger proteins. They could directly be involved in sensing and/ or breaking inappropriate attachments. In fact, one study shows the involvement of human Sgo2 in kinesin (MCAK, which is an aurora B substrate) recruitment to kinetochores (Huang et al., 2007), and other studies show direct binding of Sgo1 to microtubules and its ability to alter microtubule dynamics in vertebrate cells (Salic et al., 2004). This underlines the complexity of the mechanism that ensures bi-orientation. This is unlikely to involve one simple, linear pathway but more complex parallel pathways.

The suppression studies reported here suggest that Sgo1 is not simply a dosage suppressor of the \textit{bub1ΔK} mutation, although the \textit{pSGO1-2} micron plasmid appeared to alleviate some of the benomyl sensitivity of \textit{bub1ΔK} cells. I propose that Bub1 kinase and Sgo1 act in the same pathway (supported by lack of synthetic phenotype of the double mutant) to establish chromosome bi-orientation (Figure 4.7, middle part). Due to the similarity with the \textit{ipl1} mutant in the tension- and bi-orientation experiments, it is reasonable to predict that Bub1 and Sgo1 may partially act through the same pathway as Ipl1 to establish bi-orientation (Figure 4.7, upper part). Finally, results from other
studies support the fact that Sgo proteins themselves can be involved in breaking mal-
oriented attachments directly (Figure 4.7, lower part). In chapter 5, I aim to investigate
the interactions between Bub1 kinase, Sgo1, the Chromosomal Passengers and other
kinetochore proteins to enable me to re-fine the model.
Chapter 5. Analysis of candidate Bub1 kinase targets

5.1 Background

Having established that the role for Bub1 kinase domain in budding yeast lies not in spindle checkpoint activation or maintenance but rather in the recovery from spindle checkpoint arrest, I wanted to identify downstream targets of Bub1 kinase. The phenotypic study showed a role for Bub1 in chromosome segregation and in bi-orientation after spindle ablation. This knowledge enabled us to use a targeted approach with the aim to find protein binding partners and/or substrates. Candidate targets such as Sgo1, the Chromosomal Passenger proteins, Spc105-Ydr532 and certain other kinetochore proteins were analysed for Bub1 dependencies.

Despite great research interest of Bub1 in recent years, there is little evidence of Bub1 kinase substrates. One study reported that budding yeast Bub1 phosphorylates itself (autophosphorylation) and also its binding partner Bub3 (Roberts et al., 1994) \textit{in vitro}. However the relevance of this has never been reported \textit{in vivo}. In HeLa cells, Bub1 has been shown to phosphorylate Cdc20 and it was proposed that this phosphorylation was important for full inhibition of APC/C activity (Tang et al., 2004a). In addition, human Bub1 phosphorylates Mad1 \textit{in vitro} (Seeley et al., 1999), again this was not further studied \textit{in vivo}.

The phenotypic analysis of \textit{bub1ΔK} cells performed in chapter 3 and 4 did not directly test the loss of kinase activity of Bub1, since this allele removed the whole C-terminal kinase domain of Bub1. Potentially, this domain could have other functions apart from
kinase activity, for example in protein-protein interactions. However, as previously discussed, 'kinase-dead' point mutations in the budding yeast BUB1 kinase domain have been shown to destabilise the protein, and were therefore not used in this study.

The similar phenotypes of bub1ΔK and sgo1Δ mutant cells prompted us to investigate whether Sgo1 is a downstream target (direct or indirect) of Bub1 kinase. In the model in figure 4.7, Sgo1 was placed in a linear pathway downstream of Bub1. This is due to the fact that there is so far no evidence in the field that Sgo1 would regulate Bub1. However, there is emerging evidence in meiosis, and also in mitosis, that Bub1 plays a role in Sgo1 localisation (Kiburz et al., 2005; Kitajima et al., 2005; Kitajima et al., 2004; Riedel et al., 2006; Tang et al., 2004b).

This chapter describes attempts to find downstream targets of Bub1 kinase. Candidate targets such as Sgo1, the Chromosomal Passengers (Ipl1, Sli15) and certain kinetochore proteins were investigated. Here, I report evidence that Bub1 shows little kinase activity in vitro compared to another spindle checkpoint kinase, Mph1. Preliminary experiments showed no major phospho-dependent gel-mobility shift of Sgo1 in bub1ΔK mutant cells. Chapter 5 however, reports a clear role for the Bub1 C-terminal kinase domain in the accurate localisation of Sgo1. I propose that the localisation of Sgo1 to centromeres (although probably not through direct phosphorylation) is the main role for the Bub1 kinase domain in mitosis.

5.2 The Bub1 kinase domain is required for accurate localisation of Sgo1 to kinetochores
Recent years of intensive research in the field of Shugoshin proteins have revealed that Bub1 plays an important role in Sgo1 localisation in several organisms. Firstly, studies have shown the dependency of Bub1 for Sgo1 localisation to centromeres in meiosis, in both budding yeast and fission yeast (Kiburz et al., 2005; Kitajima et al., 2004; Riedel et al., 2006). In addition, Bub1 is also required for localisation of Sgo1 in mitosis in human cells (Kitajima et al., 2005; Tang et al., 2004b), and for Sgo2 localisation in humans (Huang et al., 2007) and fission yeast (Kitajima et al., 2004) (and V.Vanoosthuyse, personal com.) The role for Sgo1 and Bub1 in chromosome segregation in vertebrate cells, however, remains controversial but it has been suggested that Bub1 and Sgo1 are required to maintain sister-chromatid cohesion of mitotic chromosomes (Kitajima et al., 2005). Conversely, in budding yeast mitosis there is no evidence for this role of Sgo1 (Indjeian et al., 2005). Furthermore, a recent study investigated the phenotypes of a bub1 mouse knockout strain, and they suggest that the cohesion defects seen in bub1 mutants in vertebrate mitosis are rather a spindle checkpoint defect than a defect in sister-chromatid cohesion (Perera et al., 2007). The role for Bub1 kinase activity in Sgo-localisation remains elusive. Importantly, nothing is known about the role of the Bub1 kinase domain in Sgo1 regulation in budding yeast mitosis.

5.2.1 Sgo1 is mis-localised on mitotic chromosome spreads in the bub1ΔK mutant

It is known that Sgo1 localises to the kinetochores from pro-metaphase until anaphase when it disappears (Kiburz et al., 2005). To analyse Sgo1 localisation in bub1ΔK cells chromosome spreads were performed. Chromosome spreading techniques were used because individual budding yeast kinetochores are difficult to visualise in whole-cells, or by using Sgo1-GFP. However, attempts to analyse Sgo1-GFP live were carried out,
but the signal was considered too weak to characterise (data not shown). The chromosome spreads were performed on cells arrested in mitosis with nocodazole and benomyl for three hours at 23°C. The cells were then harvested and processed for chromosome spreading. Immunofluorescence of the spreads from arrested cells revealed full co-localisation of Sgo1-9myc with the kinetochores (Ndc10-6HA) in 70% of wild-type spreads (Figure 5.1 A). In wild-type cells, not all chromosome spreads showed obvious Ndc10 or Sgo1 staining, thus only cells with a clear Ndc10 staining pattern were counted. Chromosome spreads of *bub1ΔK* cells revealed a striking reduction to 13% of cells that showed full co-localisation of Sgo1-9myc to the kinetochores (Ndc10-6HA). There also appeared to be more chromosome spreads with a diffuse Sgo1 staining, or staining that did not co-localise with Ndc10 in the mutant (Figure 5.1 A, lower panel). This could be due to nonlocalised Sgo1 residing in the nucleus, or mis-localised to a non-kinetochore position in the mutant. Despite having treated the cells with microtubule depolymerising drugs, the kinetochores tended to cluster together in the spreads from wild-type and mutant cells. This is probably due to short stable microtubules emanating from the SPBs to which kinetochores cluster. This made it more difficult to categorize the staining pattern. In addition, the same chromosome spreading and immuno-staining were performed on cycling cell populations of wild-type, *bub1ΔK* and *bub1Δ* cells. Consistent with the nocodazole treated cells, there was a clear reduction of full co-localisation of Sgo1 to Ndc10 in the mutant cells (Figure 5.1 B). The data plotted here excluded any cells that did not show any Sgo1 staining, which was ~40% of wild-type, ~50% of *bub1ΔK* and ~60% *bub1Δ* cells.

**5.2.2 ChIP analysis confirms the reduced localisation of Sgo1 to the centromere and pericentromere of mitotic *bub1ΔK* cells**
Figure 5.1 The Bub1 kinase domain is required for accurate localisation of Sgo1 to kinetochores. (A) Chromosome spreads were performed on indicated strains carrying Sgo1-9myc and Ndc10-6HA (and no tag), that had been arrested in mitosis with 15 μg/ml nocodazole and 30 μg/ml benomyl at 23°C for 3 hours. Anti-myc antibodies (CM100, Gramsch) were used to detect Sgo1-9myc, and anti-HA antibodies (HA11, BabCO) were used to detect the kinetochore protein Ndc10-6HA. The cells were analysed and the cells with most of the Ndc10-6HA staining co-localising with Sgo1-9myc were categorized as ‘full co-localisation’, whereas the cells with only partial overlap of Ndc10-6HA and Sgo1-9myc were categorized as ‘partial co-localisation’. Wild-type showed 70% full co-localisation and there was 13% full localisation in cells expressing bub1ΔK. Scale bar represents 2 μm. (B) Bub1 is required for accurate Sgo1 localisation in cycling cells. Chromosome spreads were performed, as in A, on a cycling cell population with the indicated strains. The localisation pattern was categorized as in A and plotted in the graph. Error bar represents standard deviation of the mean from 3 separate experiments.
To confirm the reduction of Sgo1 localisation to kinetochores in \textit{bub1\Delta K} mutant cells, chromatin-immunoprecipitation was performed on cells arrested in mitosis. Previously it was shown that Sgo1-6HA is associated with the centromere and the pericentromere of budding yeast chromosomes (Kiburz et al., 2005). The cells were arrested in mitosis with nocodazole and benomyl for three hours at 23°C. 50 ml yeast cultures were used for each ChIP, and duplicate samples for each strain were processed in parallel. PCR reactions were performed on ChIP samples using primer-sets that amplified the centromere, pericentromere and one negative arm region of chromosome III (Figure 5.2 A-B). Due to slight variability between PCR reactions, several PCRs were performed on the same ChIP sample, and on separate ChIP samples and the standard deviation of the mean was calculated and plotted in a graph (Figure 5.2 C). The results showed consistency with the chromosome spread data and showed a clear reduction of Sgo1-6HA associating with the centromere and pericentromere in the \textit{bub1\Delta K} mutant. The ChIP PCR using centromere primers, however, gave a slightly higher signal than ‘no-tag’ in \textit{bub1\Delta K} cells but this signal was much reduced from wild-type cells. There was no difference between the \textit{bub1\Delta K} and the ‘no tag’ control of the pericentromeric ChIP PCRs. Furthermore, the \textit{bub1\Delta K} mutant showed a slight increase in association with the arm region compared to wild-type and ‘no tag’ cells. However this difference was not statistically significant. These data confirm that there is a marked reduction of Sgo1 at centromeres in the \textit{bub1\Delta K} mutant, although it cannot be ruled out that there is still some Sgo1 on the centromeres.

### 5.2.3 Sgo1 mislocalises to spindle pole bodies in \textit{bub1\Delta K}

The above data confirmed that Bub1 kinase domain plays a role in Sgo1 localisation in mitosis. In figure 5.1 the staining pattern of Sgo1 in wild-type and \textit{bub1\Delta K} cells was
Figure 5.2 Bub1 kinase regulates Sgo1 association with the centromere in budding yeast mitosis. (A) Schematic diagram of primer design for Sgo1-6HA ChIP. CEN3 primers amplify a centromeric region of chromosome III. R3 primers amplify a region in the pericentromere ~800 bp to the right of the core centromere, and c281 is an arm region. (B) PCR on ChIP with the indicated strains. The indicated cells carrying Sgo1-6HA (and no tag) were arrested in metaphase with 15 μg/ml nocodazole and 30 μg/ml benomyl at 23°C for 3 hours. 50 ml of yeast culture was processed for each ChIP. For each PCR reaction 4 μl of ChIP DNA and 4 μl of a 1:500 dilution of the input DNA was used. (C) The graph shows quantification (using Image J software) of the ChIP PCR data as the 'binding ratio'. The binding ratio was calculated as the relative intensity of ChIP PCR signal to 1:500 diluted input PCR signal. This did not give us the absolute percentage of binding but the relative binding for each strain. Each quantified signal was normalised for the background. The error bars indicate SD of the mean from 5 separate PCR reactions from 2 separate experiments.
described. The data revealed that there was still distinct staining of Sgo1-9myc in most mutant cells. This was no longer co-localising with the kinetochores as observed in wild-type. I wondered if this distinct staining could be Sgo1, mis-localising to the SPBs. Immunofluorescence on chromosome spreads was performed to detect Sgo1-9myc (as in figure 5.1) and anti-Spc110 monoclonal antibodies were used to detect the SPB component Spc110. These results provide evidence that in cells lacking the Bub1 kinase domain, Sgo1 protein mis-localises to the SPBs in most cells. Although Sgo1 staining sometimes coincided with the SPBs in wild-type, the Sgo1 staining most often appeared to be present around the SPBs as opposed to co-localising with them, indicating that the kinetochores were present around the SPBs rather than Sgo1 co-localising to them (Figure 5.3).

5.3 *bub1ΔK*- Sgo1 fusion protein

The data presented hitherto suggests that the Bub1 kinase domain plays a role in Sgo1 localisation, and I propose that this activity is essential for chromosome bi-orientation after spindle ablation. Lack of a synthetic phenotype in the *bub1ΔK, sgo1Δ* double mutant implies that this is the main function of Bub1 kinase in mitosis, although it can not be excluded that it has other minor roles. To test this hypothesis, a fusion protein was constructed that fuses the N-terminal part of Bub1 (same as *bub1ΔK*, a.a. 1-608) to Sgo1. This construct simply replaced the Bub1 kinase domain with the Sgo1 protein, and had a flexible poly-glycine (8 Gly) linker between the two (Figure 5.4). Such a fusion protein would bring Sgo1 to the sites of Bub1 localisation at the kinetochores. Hence, if the only role for Bub1 kinase domain is to accurately localise Sgo1, then
Figure 5.3 Sgo1 mislocalises to the SPBs in the *bub1ΔK* mutant. Chromosome spreads were performed on cells that had been arrested in mitosis with 15 μg/ml nocodazole and 30 μg/ml benomyl at 23°C for 3 hours. Anti-myc antibodies (CM100, Gramsch) were used to detect Sgo1-9myc, and anti-Spc110 antibodies (gift from J.Kilmartin) were used to detect the SPBs. In *bub1ΔK* most cells (90%) showed Sgo1 staining that only co-localised to SPBs, whereas in wild-type cells, most cells (96%) showed a Sgo1 staining pattern as shown in the upper panel, with multiple Sgo2 foci around the SPBs. Scale bar represents 2 μm.
fusing Sgo1 to the Bub1-N terminal may be a substitute for the absence of the kinase domain.

5.3.1 Construction of bub1ΔK-Sgo1 fusion protein

Cloning of the bub1ΔK-SGO1 fusion construct was performed by cloning of a fusion-PCR product of bub1ΔK-SGO1, described in Figure 5.4 (A-C). Firstly, 500 base pairs of the BUB1 3’UTR was cloned into vector pRS304 by standard cloning techniques using SalI and EcoRI sites. The cloning of 500 base pairs of BUB1 3’UTR into the vector enabled integration of the construct by homologous recombination. Then, two PCR reactions were performed to amplify the N-terminal regions of the BUB1 and SGO1 ORFs separately (Figure 5.4 A-B). The primers were designed to create an overlapping region between the 3’ part of the BUB1 fragment and the 5’ region of the SGO1 fragment (Figure 5.4 A, blue line). This region also includes a sequence encoding eight glycines to serve as a flexible linker between the two proteins. This was done to increase the chances of Sgo1 being functional despite being fused to Bub1. The two PCR fragments were then used as a template to amplify the complete fusion construct (Figure 5.4 A-B). 10 cycles amplified the full-length fusion product (2331 base pairs), and most of the template DNA appeared to have been converted to the full-length fusion at 2331 base pairs (Figure 5.4 B). This fusion PCR product was then digested with EcoRI and BamHI and ligated into the vector, and the insert was sequenced. The construct is shown in (Figure 5.4 C). The vector was then linearized by EcoRI digestion and transformed into yeast. Homologous recombination of the BUB1 coding region and the BUB1 3’UTR region resulted in replacement of BUB1 kinase domain with SGO1 and the TRP marker (Figure 5.4 D). Expression of this construct should therefore result in endogenous expression levels (for Bub1) of bub1ΔK linked through 8 glycines to
Figure 5.4 Construction of *bub1ΔK-Sgo1* fusion protein. (A) Schematic diagram of the basis for fusion PCR reaction. Primer A and D contained restriction sites to enable ligation of the fusion construct into the vector. Two separate reactions were carried out to amplify the 3’ regions of *BUB1* (primer A+B) and *SGO1* (primers C+D) (PCR1A,B). The primers were designed to create an overlap between the two fragments, indicated by the blue ‘fuzzy’ line. In addition, an 8-glycine linker sequence was also engineered to link the two fragments. A third round of PCR (PCR2), using the two fragments as template gave rise to a larger, fusion fragment with restriction fragments at each end. (B) PCR of the two *Bubi* (584 bp) and *Sgo1* (1817 bp) fragments, 2 left lanes. The right lanes show the fusion PCR (2331 bp) with 5 or 10 cycles. 10 cycles appeared to be the minimum number of cycles that amplified the fusion from the two smaller fragments. (C) Diagram of the final plasmid pJF01. (D) Schematic diagram of integration of the fusion construct. The plasmid was digested with *EcoRI* and the construct integrated at the *BUB1* locus by double cross-over. This event replaced the kinase domain with a poly-glycine linker and *Sgo1*. (E) Schematic diagram of final protein. (F) PCR reactions that confirmed the correct integration of the construct. Left panel shows a PCR band of correct integration using a forward primer in the *BUB1* promoter and a reverse primer in the *SGO1* ORF, that was not present in wild-type. Conversely, the right panel shows loss of a PCR band amplified using a primer in *BUB1* kinase domain and a reverse primer in the 3’UTR.
Sgo1 (Figure 5.4 E). The accurate integration of the construct was analysed by PCR using two sets of primers (Figure 5.4 F).

5.3.2 Analysis of cells carrying the bub1ΔK- Sgo1 construct

Both wild-type and sgo1Δ cells were transformed with the bub1ΔK-Sgo1 fusion construct. This meant that the wild-type cells contained the N-terminal region of Bub1 fused to Sgo1 (lacking the BUB1 kinase domain), plus having one endogenous copy of Sgo1, whereas transformed sgo1Δ cells only had one copy of Sgo1 that was fused to the Bub1 N-terminal domain. These cells were analysed for benomyl sensitivity (Figure 5.5.A). The benomyl phenotype of bub1ΔK-Sgo1 cells (Figure 5.5. A, upper panel) showed very similar sensitivity to bub1ΔK cells. This was also true for sgo1Δ cells carrying the construct. Despite showing no major rescue of benomyl sensitivity, this construct gave rise to slightly better growth on YPDA plates and a very slight rescue on benomyl. Therefore these cells were also analysed for chromosome loss, using the sectoring assay (Figure 5.5.B), again, no major rescue of chromosome loss was observed, but there was consistently slightly less chromosome loss in the cells carrying the bub1ΔK-Sgo1-fusion (1-6% rescue with bub1ΔK-Sgo1 in wild-type; 12-28% rescue in sgo1Δ cells). These results imply that the function that Bub1 kinase domain plays in a role in normal cell cycle and in chromosome segregation may partially be substituted by bub1ΔK-Sgo1 fusion. This function, however, may be different to that one that is required for proper growth on benomyl, which could not be supported by the bub1ΔK-Sgo1 fusion construct. Alternatively, the bub1ΔK-Sgo1 construct was not fully functional, perhaps due to mis-folding or instability of the fusion protein, and therefore only showed a slight rescue.
Figure 5.5 Analysis of cell carrying the *bub1ΔK-Sgo1* fusion construct. (A) Benomyl sensitivity of cells carrying the *bub1ΔK-Sgo1* fusion construct. The indicated yeast strains were plated out as 10-fold serial dilutions on YPDA media or on YPDA media containing 6 μg/ml or 8 μg/ml benomyl and allowed to grow for 3 days at 23°C. (B) Chromosome loss analysis of cells carrying the *bub1ΔK-Sgo1* fusion construct.
5.4 Biochemical analysis of Sgo1

5.4.1 Sgo1 phosphorylation: Introduction

I have reported that Sgo1 localisation to kinetochores is dependent on Bub1 kinase domain. As previously discussed, a truncation of Bub1 has in this study been used that does not directly test the effect of the lack of kinase activity. In this section I look for evidence that Bub1 may regulate Sgo1 through phosphorylation, either directly or indirectly.

5.4.2 Is Sgo1 a phospho-protein?

Phosphorylation of proteins can sometimes be detected by SDS-gel mobility shifts. Most often, phosphorylated proteins migrate slower on SDS-PAGE. Not much is known about the phosphorylation state of Sgo1 in budding yeast mitosis, therefore gel-mobility assays were used to investigate whether there was a change in phospho-dependent gel mobility of Sgo1-myc in cells lacking the Bub1 kinase domain. Initially, Sgo1-13myc was analysed by immunoblotting of cycling cells. The Sgo1-13myc signal appeared similar in wild-type and bub1ΔK cells (Figure 5.6 A). There was an indication of slower migrating bands (‘fuzzy’ band) in the Sgo1-13myc staining, which could indicate phospho-dependent gel shifts. Therefore, immunoprecipitations (IPs) were carried out to test this. Cells were arrested in mitosis with nocodazole and benomyl for three hours and samples were taken for IP. Half the IPs were treated with lambda protein-phosphatase to remove any phosphorylation of the protein. The eluted IP was then run on a 10% gel and probed with anti-myc antibody to detect Sgo1-13myc. The results showed faint indications of a phospho-dependent band-shift that disappeared in the phosphatase-treated samples (Figure 5.6 B). The ‘no tag’ control showed no Sgo1-
Figure 5.6 Biochemical analysis of Sgo1. (A) Immunoblotting of Sgo1-13myc. The indicated strains were harvested in log phase, and blotted with anti-myc (A14) antibodies to detect Sgo1-13myc and with anti-Mad1 antibody as a loading control. (B) The same strains were used for IP to detect phospho-dependent gel-shifts. The cells were grown to log phase then arrested in mitosis with 15 μg/ml nocodazole and 30 μg/ml benomyl at 23°C for 3 hours. IPs were then carried out using c-Myc 9E10 sepharose beads (Santa Cruz Biotechnology). The IPs were split in two, and half was treated with lambda-phosphatase to remove any phosphorylation. The IPs were then run out on a 10% SDS-PAGE and blotted with anti-myc (A14) antibody. (C) Same as in B but using anti-Mad1 as a loading control of inputs. IPs were run on a 8% SDS gel.
13myc signal. In order to resolve the band-shift further, the IP was repeated and run on an 8% gel. These results showed a more obvious shift due to further separation of the bands, and again a clear shift of the band in the phosphatase-treated samples. Although the \textit{bub1ΔK} mutant cells still showed a phospho-dependent shift, as in wild-type, this shift appeared slightly weaker than in wild-type. Analysis of the extracts for Mad1 levels indicated equal IP input. These results imply that although Sgo1 phosphorylation appear to be intact in the \textit{bub1ΔK} mutant, it cannot be ruled out that Bub1 gives rise to some Sgo1 phosphorylation. Preliminary 2D gel analysis confirms no obvious difference of phospho-Sgo1, between wild-type and \textit{bub1ΔK} cells (data not shown).

5.5 Analysis of the kinetochore proteins Spc105 and Ydr532

5.5.1 Spc105 and Ydr532: Introduction

The fact that no major difference was observed in phospho-dependent band-shifts of Sgo1 in mitosis in the mutant cells (or on 2D gel analysis, data not shown), suggests that Bub1 is not the main kinase that phosphorylates Sgo1 in mitosis. Studies of \textit{Drosophila MEI-S332} show that phosphorylation of this protein is dependent on Polo kinase (Clarke et al., 2005), and it is quite possible that this kinase is responsible for Sgo1 phosphorylation in budding yeast mitosis also. I therefore suggest that there may be two other possibilities: either, Bub1 phosphorylates another protein (protein X) that lies between Bub1 and Sgo1 in the kinetochore targeting pathway, or the Bub1 C-terminus plays another non-catalytic role in Sgo1 targeting. Spc105 was identified in spindle pole body enriched preparations in budding yeast (Wigge et al., 1998), but was also co-purified with a protein of unknown function (Ydr532) in a study by Nekrasov et al (Nekrasov et al., 2003). In this study, they confirm that its localisation resides close
to the SPB on the nuclear face, but also that Spc105 is indeed a centromere protein, as shown by ChIP. The Spc105-Ydr532 complex has also makes physical interactions with Mtw1, Ndc80 and microtubule plus end tracking complexes in budding yeast (Goshima and Yanagida, 2000; Kerres et al., 2004; Nekrasov et al., 2003). Importantly, *spc105*, *ydr532* and *bubi* alleles were all identified in the same screen that originally identified Sgo1 as a tension-sensor, and all these proteins appear to have similar phenotypes (Indjeian et al., 2005)(and Indjeian, personal com; and this study). Therefore I wondered if Spc105 (or Ydr532) could be factor X and a Bubi substrate.

### 5.5.2 Analysis of phospho-dependent gel-mobility shift of Spc105

First, immuno-blotting was performed on whole-cell lysates from cycling cells. The results showed that both in wild-type and *bub1ΔK*, the Spc105-13myc band appeared as a doublet, with the majority being a slow-migrating form. This could indicate post-translational modifications of Spc105, for example phosphorylation. Although the band-shifts were similar in wild-type and the mutant, the levels of Spc105-13myc protein level consistently appeared to be increased in the mutant. To investigate whether the band-shift was due to phosphorylation, IPs using c-Myc 9E10 sepharose (Santa Cruz Biotechnology) were performed. The IPs were carried out on cells that had been treated with benomyl and nocodazole for three hours at 23°C, and were performed as in 5.4.1. Treatment with lambda-protein phosphatase gave rise to a faster migrating band, which indicates that Spc105 is a phospho-protein (Figure 5.7 B-C). Dilutions of the phosphatase-treated *bub1ΔK* extracts were run on a gel to roughly estimate protein content (Figure 5.7 C-D). Also, cells carrying both Spc105-13myc, and Sgo1-13myc were analysed by IP (Figure 5.7 D). The result indicates a small, but consistent increase
Figure 5.7 Biochemical analysis of Spc105 and Ydr532. (A) Immunoblotting of Spc105-13myc. The indicated strains were harvested in log phase, and blotted with anti-myc (A14) antibodies to detect Spc105-13myc and anti-Mad1 antibody as a loading control. (B) The same strains as in A were used for IP to detect phospho-dependent gel-shifts. The cells were grown to log phase and arrested in mitosis with 15 μg/ml nocodazole and 30 μg/ml benomyl at 23°C for 3 hours. IPs were then carried out using c-Myc 9E10 sepharose beads (Santa Cruz Biotechnology). The IPs were then split in two, and half was treated with lambda-phosphatase (λ) to remove any phosphorylation. The IPs were then run out on a 10% SDS-PAGE and blotted with anti-myc (A14) antibody. (C) Repeat IP as in B but samples were run of a 8% gel to separate bands further, also using anti-Mad1 as a loading control of inputs. (D) IPs were carried out as in C, with strains containing both Sgo1-13myc and Spc105-13myc. The lambda phosphatase treated mutant sample were run out using 1/3 or 2/3 of the IP. (E) IPs were carried out on cells that had been treated with microtubule drugs, as in B, with strains carrying YDR532-13myc or no tag and run on a 10% SDS gel.
in Spc105 protein levels in the *bub1ΔK* mutant. In addition, a vague fast-migrating band was detected in the mutant that was not as abundant in wild-type IPs (Figure 5.7 C-D, green star). This could be due to loss of one or more phosphorylation events in the *bub1ΔK* mutant, or it could arise due to the fact that there may be more protein present.

To analyse this further, 2D gel analysis was performed, but unfortunately I failed to detect Spc105-13myc using this assay (data not shown). Although I only detected subtle biochemical differences of Spc105, this needs to be investigated further to rule out any phosphorylation by Bub1 kinase. Alternatively, recombinant Spc105 could be used in *in vitro* Bub1 kinase assays.

### 5.6 Analysis of Chromosomal Passenger protein localisation in *bub1ΔK* cells

In chapter 1 and 4, the role of the Chromosomal Passenger proteins in tension-sensing and in chromosome bi-orientation was discussed. I wondered whether I could find evidence that Bub1 kinase acts through the Ipl1 (or Ipl1-Sli15) pathway to ensure bi-orientation. There are no indications in the field that Ipl1 is a Bub1 kinase target. However, there is some data arguing that Sgo1 is required for accurate Ipl1 localisation in meiosis (Yu and Koshland, 2007). Furthermore, fission yeast Sgo2 has been shown to be required for Chromosomal Passenger protein localisation in mitosis (Kawashima et al., 2007; Vanoosthuyse et al., 2007).

Here, it was tested whether the lack of Bub1 C-terminus gives rise to loss of Ipl1 on kinetochores in mitosis. Cells expressing Ipl1-GFP and also Ndc80-CFP and Ctf19-CFP (to mark the kinetochores) were treated with nocodazole and benomyl at 23°C for three hours. The cells were fixed and analysed by fluorescence microscopy (Figure 5.8 A).
Figure 5.8 Analysis of the Chromosomal Passenger proteins- (Ipl1 and Sli15) localisation in \textit{bub1}\textDelta K mutant. (A) The indicated strains were arrested in mitosis in CSM media containing 15 \(\mu\)g/ml nocodazole and 30 \(\mu\)g/ml benomyl. Cells were fixed in 3.7\% formaldehyde for 5 minutes and washed with PBS. Cells were then analysed by microscopy. Kinetochores were marked using Ctf19-CFP and Ndc80-CFP. (B) Strains carrying Sli15-GFP were arrested in mitosis as in A. The microtubule drugs were then washed out and cells allowed to enter anaphase. Sli15-GFP signals were detected on what appeared to be metaphase kinetochores and also on the anaphase spindle.
Most cells, both wild-type and mutant, showed clear co-localisation of Ipl1-GFP with the kinetochores. However, in some mutant cells there may have been a slightly weaker Ipl1-GFP signal but this proved difficult to quantify, and the difference was (if any) very subtle. Similarly, in preliminary experiments analysing Sli15-GFP during a spindle checkpoint release, no noticeable difference was observed (Figure 5.8 B). As expected, Sli15-GFP showed a staining that co-localised with kinetochores on a metaphase spindle (Figure 5.8, upper panel), whereas it transferred onto the spindle in anaphase in both wild-type and mutant cells (Figure 5.8 B, lower panel). These data together indicate that there is no dramatic difference in localisation of the Chromosomal Passenger proteins Ipl1 and Sli15 in cells expressing bublΔK during microtubule drug treatment.

5.7 Purification of Bub1 and Bub3 by Tandem Affinity Purification (TAP)

Affinity purification is a useful method for identification of novel protein-protein interactions, and also for purification of active kinases for in vitro kinase assays. In this section a Tandem Affinity Purification (TAP) method (Rigaut et al., 1999) was used in order to purify active Bub1 protein kinase. This method has proven very efficient in the purification of many complexes in recent years, for example budding yeast kinetochore complexes (Cheeseman et al., 2001). The TAP purification method was used because it allows a stepwise, tandem purification. The first step involves binding of the Protein A (ZZ) tag to IgG-coupled beads, followed by cleavage and release of any bound proteins using a TEV cleavage site in the TAP tag. The second binding step depends on what TAP tag is used. S-TAGs allow binding of the S-tag to S-sepharose, and another version allows a calmodulin binding domain (CBD) to bind to calmodulin beads. The tandem
purification should give rise to cleaner purifications compared to one-step purifications, and especially for kinase assays where it is important not to have any background contaminating kinases present.

5.7.1 Bubi antibody production

To enable us to detect Bubi by immuno-blotting, an N-terminal region of Bubi was used as an antigen for antibody production in sheep and rabbit. A plasmid, containing the first 460 residues of \( \text{BUB1} \) fused to GST (pEK1, pGEX6p-\( \text{BUB1.1-460-GST} \)), was transformed into BL-21 \( \text{E.coli} \) cells. The construct was over-expressed by IPTG induction then purified from the bacteria (for method, see 2.5.9). The purified protein was then injected into a sheep and a rabbit. The crude serum was tested on wild-type, Bubi over-expression and on \( \text{bub1A} \) cell extracts. Unfortunately, both the sheep and rabbit anti-Bubi serum showed a signal of correct size (118kDa) only in cells over-expressing Bubi. However, purification of the antibody reduced the background but this affinity-purified antibody was still only sensitive enough to detect Bubi in IPs.

5.7.2 TAP tagging of Bubi and Bub3

TAP purifications followed by mass-spectrometry have proven a very efficient way to detect protein complexes in recent years. Here, TAP tagging of Bubi and Bub3 was used to try and identify novel interactors. Firstly a small scale, one-step TAP purification was performed using Bubi-TAP (Figure 5.9). The cells were grown to log-phase and arrested in mitosis with microtubule drugs. Following cell lysis, protein extracts were prepared and were then incubated with IgG sepharose, and the bound protein eluted with sample buffer. To confirm that the purification had worked, the extracts were probed with anti-Bubi and anti-Mad1, because Mad1 has previously been
Figure 5.9 Bub1,3-TAP constructs and Bub1-TAP purification. (A-B)
Schematic diagram of TAP tag constructs. (A) These TAP tags contain a ZZ Protein A binding domain (Protein A BD), which binds to IgG-sepharose beads.
TEV cleavage site, which enables cleaving of the construct with TEV protease is indicated. Finally, a calmodulin binding domain (CBD) binds calmodulin beads in the second step of the purification. (B) Diagram shows an alternative TAP tag (S-TAP), which has the calmodulin binding domain replaced by an S-tag. Constructs were made using tagging cassettes (pKW804 S-TAG, gift from K. Weiss, University of California) and integrated at the C-terminus of Bubi and Bub3. (C) One step Bub1-TAP purification. Purification of Bubi was carried out by incubating protein extracts from the indicated strains with IgG-sepharose beads. The protein was eluted from beads with sample buffer and run in SDS-PAGE. Immuno-blotting with purified anti-Bubi antibody and anti-Mad1 antibody showed co-purification of Mad1 with Bubi-TAP.
shown to bind to Bub1 upon checkpoint arrest (Brady and Hardwick, 2000). The results also show slower migrating bands of Mad1, which indicates that Bub1 pull-downs are enriched for phospho-Mad1. This is entirely consistent with previously published data (Brady and Hardwick, 2000).

5.7.3 Bub3-TAP purifications

Purification of Bubi was also performed using a Bub3-TAP strain. We know that Bubi and Bub3 are closely associated in a complex throughout the cell cycle and this strain was therefore used to 1) analyse the protein complex and hopefully identify novel components 2) pull down purified Bubi with the hope that Bubi kinase is more active when pulled down with Bub3-TAP than when tagged itself. Initially, a one-step Bub3-TAP purification was carried out (Figure 5.10 A). This experiment made use of two separate Bub3-TAP strains: one using W303 background (background yeast commonly used in cell cycle studies) and one in another strain background (JB811), which lacks the proteases Proteinase C, B and Pep4, i.e. is partially protease deficient. The cells were grown to log-phase then arrested with nocodazole for 3 hours. Following the purification and SDS-PAGE the purified proteins were probed with antibodies against proteins that have already been shown to bind in a Bub3 complex upon mitotic arrest to confirm the efficiency of the purification. In budding yeast, the MCC consists of Bub3, Mad3, Mad2 and Cdc20 (Hardwick et al., 2000). Indeed Bub3-TAP clearly pulled down Bubi, phospho-Mad1, Cdc20, and there was also evidence for Mad3 and Mad2 binding (Figure 5.10 A). This is consistent with published data that Bubi and Bub3 form a complex with Mad1 upon checkpoint activation (Brady and Hardwick, 2000), and that Bub3 is also, separately, in a complex (MCC) with Mad2, Mad3 and Cdc20. These results showed that using the protease deficient strain proved a lot more efficient for
A) Bub3-TAP

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α-Bub1
**Figure 5.10 Bub3-TAP purification.** (A) The indicated strains were grown to log-phase and arrested with 30 μg/ml benomyl for 3 hours. The cells were collected and frozen in liquid nitrogen. The frozen cells were ground for 15 minutes and added to lysis buffer. The lysate was then centrifuged at high speed. The IP using IgG sepharose was incubated at 4°C for 3 hours. After washes, the beads were eluted with sample buffer and the samples were run on a 10% gel and blotted with anti-Bub1 (sheep), anti-Mad1 (rabbit), anti-Mad2 (sheep), anti-Mad3 (rabbit) and anti-Cdc20 (sheep) (B) The indicated strains were grown to log-phase then arrested in mitosis with 30 μg/ml benomyl. Protein extracts were then incubated with IgG sepharose for 3 hours. IPs were eluted in sample buffer and run on a 10% SDS-gel. Anti-Bub1 (Sheep), anti-Mad1 (rabbit) and PAP antibodies were used. (C) IPs were performed as in B and run on a 10% SDS-gel and blotted with anti-Bub1 (sheep) antibody. (D) 9 L of Bub3-S-TAP yeast strain was arrested using the tub2-123 mutation at 16°C for 3 hours (90% large budded cells). This gave rise to 60 g dried frozen yeast which was used for purification by two-step S-TAP purification according to the method described in 2.5.7.1-2.5.7.2 and 2.5.7.4. The eluted proteins were sent to the J.Yates lab at the Yeast Resource Centre for MS/MS. The results are shown in the table. The sequence count indicates the number of peptides identified and the % coverage indicates the percentage of total sequence coverage of the protein.
purifications (Figure 5.10 A). This is probably due to less protein degradation of proteins during the purification process. These data confirm the interaction of Bub3 with Bub1 and also with the MCC. Therefore, I was confident that these TAP-purifications were efficient and could hopefully enable me to identify novel components by mass-spectrometry, and also use this method to purify active Bub1 for kinase assays.

As a control for those kinase assays, the creation of a Bub3-TAP, bub1ΔK stain was necessary. Therefore, Bub1 kinase domain was truncated in a Bub3-TAP, protease deficient strain. To confirm the expression of bub1ΔK and its ability to bind Mad1, a small scale, one-step purification was performed as above. The cells were arrested in mitosis and the purification was performed (Figure 5.10 B). The purified extracts were then probed with anti-Bub1, anti-Mad1 and with PAP-antibody (which detects the TAP tag). The results showed the presence of Bub1 at the correct size (118kDa) in Bub3-TAP pull-down (Figure 5.10 B, left panel). bub1ΔK was also clearly pulled down with Bub3-TAP and the position of the band corresponds to the correct size predicted for truncated bub1 (67kDa). The results also suggest that the truncated bub1 is slightly stabilised, and may possibly have an altered phosphorylation state judged by the lack of a couple of slower-migrating bands (Figure 5.10 B, left panel). Immunoblotting with anti-Mad1 confirms that bub1ΔK still forms a complex with Mad1. Mad1 also showed a distinct ladder on the gel, which again indicates phosphorylation of Mad1 upon checkpoint activation, however these gel-shifts did not change with the lack of Bub1 kinase. Bub3-TAP was detected in both Bub3-TAP and Bub3-TAP, bub1ΔK pull-down using PAP antibody (Figure 5.10 B, right panel).
Despite the ability to purify Bub1, Mad1, Cdc20, Mad3 and Mad2 using Bub3-TAP in the protease deficient strain, there were indications that purification using this TAP tag gave rise to rather large amounts of unspecific binding (discussed later). A modified TAP tag (S-TAP, see Figure 5.9 B) has previously been developed and it was considered to give rise to cleaner, more specific purifications (Cheeseman et al., 2001; Kim and Raines, 1993). Therefore, Bub1-S-TAP, Bub3-S-TAP and Bub3-S-TAP, \( bubi\Delta K \) strains (Figure 5.19 B) strains were constructed. These strains were then used as previously, in a one-step purification on mitotically arrested cells and immunoblotted with Bub1. This was done to confirm the efficiency of the purification of Bub1. The results showed clear signals for Bub1 in the different strains (Figure 5.10 C). Again, the purification of the truncated \( bubi \) appeared to lack a couple of slower migrating bands.

Using the Bub1-S-TAP for one-step purifications gave rise to a very strong Bub1 signal by immunoblotting (5.10 C). A large-scale, two-step purification was therefore performed (for method see 2.5.7.2 and 2.5.7.4). The eluate was then sent for mass-spectrometry (J.Yates lab, Yeast Resource Centre). Unfortunately, the mass-spectrometry revealed that the purification had failed, possibly due to loss of proteins during the second purification step. Bub3-S-TAP was therefore used for large-scale, two-step purification (as above), and also sent off for mass-spectrometry (YRC, J.Yates). This purification proved more successful and the results are shown in Figure 5.10 D. These data are in agreement with the immunoblot analysis of Bub3-TAP purification shown in 5.10 and confirmed the presence of the checkpoint proteins tested apart from Mad2 (i.e. Bub1, Mad1, Mad3 and Cdc20). The other ‘hits’ appeared to show no obvious relevance and could be contaminants. In conclusion, Bub3-S-TAP
seems a reliable method to purify Bub1 protein and the MCC, however mass-spectrometry of the purified complexes detected no novel interactions.

5.8 Analysis of Bub1 kinase activity using Bub1-TAP and Bub3-TAP

We showed in chapter 5.7 that TAP purifications efficiently pull down Bub1, both using Bub1-TAP and Bub3-TAP. To try to establish a reliable kinase assay for Bub1, the complexes from these purifications were assayed for Bub1 kinase activity. Firstly, the proteins purified in a one-step IgG pull-down using both Bub1-TAP and Bub3-TAP side-by-side were assayed along with an un-tagged W303 control strain. The cells were grown to log-phase, then half of each culture was arrested in mitosis with microtubule drugs. The cells were frozen and ground in liquid nitrogen. Extracts were prepared and incubated with IgG-sepharose beads at 4°C for three hours. The beads were washed and added to kinase buffer including γ-ATP. The kinase reaction was then carried out at 30°C for 30 minutes. Sample buffer was added to the kinase reaction, boiled, then run on 10% SDS-PAGE (Figure 5.11 A). The results show a strong signal of around 175 kDa, only in Bub3-TAP samples. There was a slightly stronger signal in the nocodazole arrested cells (Figure 5.11 A,*noc) compared to cycling cells.

Because of the result in figure 5.11 A, I chose to carry out Bub1 kinase assays using Bub3-TAP purifications. It may be that C-terminal tagging of Bub1 interferes with the kinase activity. Since no other kinases are known to co-purify with Bub3, I thought this would be the most efficient way to screen for potential substrates. As previously discussed, Bub1 has chromosome segregation roles that are independent of the spindle checkpoint. Also, I have shown that Bub1 plays a role in chromosome bi-orientation
Figure 5.11 Bub1 kinase assays. (A) A kinase assay on Bub3-TAP purification gives rise to a signal of ~175 kDa by autoradiography. The indicated strains were used for one-step purification by IgG sepharose binding. The cells were grown to log-phase and the strains marked (noc) were arrested with anti-microtubule drugs at 23°C for 3 hours. 50 ml of cells were then frozen and ground in liquid nitrogen. 1 ml of yeast powder was used for each IP. Extracts were prepared then incubated with IgG sepharose. The beads were washed and kinase buffer was added. The kinase reaction (with 32P) was carried out at 30°C for 30 minutes. Half the reaction was loaded on a 10% SDS gel. (B) The same frozen yeast as above was used in a kinase assay testing 3 different kinetochore complexes (gift from Barnes/Drubin lab). Added per reaction: ~4 μg of Dam1 complex (Ask1, Dam1, Spc19, Dad2, Dad-4, Hsk3), ~1 μg of Mtw1 complex (Dsn1, Mtw1, Nnf1, Nsl1) or ~1 μg of Ndc80 complex (Ndc80, Nuf2, Spc25, Spc24). The bands with a 'red star' are the ones that we think correspond to Ask1, Dam1, Dsn1 and Ndc80 judging from their sizes. (C) Samples from one step purifications (as above) and samples that were prepared by two-step S-TAP purifications were compared side-by-side in a kinase assay including the Mtw1 protein complex. (D) Two-step purified fission yeast Mph1-S-TAP, Bub3-S-TAP and Bub3-S-TAP, bub1ΔK were tested for kinase activity against Myelin Basic Protein (MBP). The Bub3-S-TAP samples were prepared from a large-scale purification and kinase assay carried out on the S-beads. (Mph1-S-TAP was used as a positive, high kinase activity control. gift from J. Zich).
after spindle damage. Considering this, a targeted approach was carried out to test whether kinetochore proteins are potential Bub1 targets. Four kinetochore complexes purified from bacteria or insect cells were used. These are the Dam1, Mtw1 and Ndc80 complexes including 22 proteins in total (kind gift from Drubin/ Barnes lab, Berkley). The protein purifications were performed as in 5.11 A, and the different kinetochore complexes were added to separate kinase reactions. At first it appeared very interesting that each kinase reaction gave rise to one or more specific signal. These were what I thought corresponded to Ask1, Dam1, Dsn1 and Ndc80, according to their molecular weights (Figure 5.11 B). Unfortunately, repeat experiments showed these to be unspecific signals. Any repeats of this experiment showed the same signals in all the strains, including the controls ('no tag' yeast or a strain containing the truncated Bub1 allele (data not shown)). These results suggested that there might have been contaminating kinases in the Bub3-TAP purification or in the purified kinetochore complexes, that were responsible for the signals. Moreover, preliminary experiments using fission yeast Mph1 as a kinase, gave very similar banding patterns, again indicating that these bands are unspecific (KH, data not shown).

Due to the problems with Bub1-independent phosphorylation signals in the in vitro kinase assays, repeat experiments were performed using the Bub3-S-TAP. I hoped that this purification would be more specific due to the very tight binding of the S-tag to S-protein sepharose and would give rise to less background. A one-step purification using IgG-sepharose was performed, but also a two-step S-TAP purification and compared the two purifications on kinase assays containing the Mtw1 complex (Figure 5.11 C). The results showed a lot more background signals in the one-step purification (same signal in untagged and Bub3-S-TAP) compared to the two-step purification. Unfortunately,
even the phosphorylation signal detected after two-step purifications was present in both control and Bub3-S-TAP samples, indicating that these are still background bands and not due to Bub1 activity. To investigate whether Bub1 shows any specific activity towards the artificial substrate, Myelin Basic Protein (MBP), a large-scale Bub3-S-TAP purification was carried out, along-side controls (Bub3-S-TAP, bub1ΔK and JB811-no tag) (Figure 5.11 D). The kinase assay was carried out on S-beads and the results showed a rather clean kinase assay, compared to those seen previously. In addition, an aliquot of S-TAP-purified fission yeast Mph1-S-TAP was used as a ‘high activity kinase’ control. These results showed that, as expected, the Mph1 kinase activity towards MBP was much higher than Bub1 kinase (Figure 5.11 D). It appeared that Bub1 kinase did show activity towards MBP, but again the bub1ΔK control still gave a weak signal. However this was much weaker than wild-type Bub1. These results indicate that Bub1 shows some activity towards MBP \textit{in vitro} but much weaker that the other spindle checkpoint kinase, Mph1. The fact that a weak signal was still detected in the bub1ΔK control suggests that there was another kinase co-purifying with the complex. Until I find out what this kinase is, this approach may prove difficult in the analysis of potential Bub1 substrates.

5.9 Discussion

5.9.1 The Bub1 kinase domain is required for accurate Sgo1 localisation to centromeres in budding yeast mitosis

In chapter 3 and 4, I established the phenotype of \textit{bub1ΔK} cells and concluded that these cells behave in a very similar manner to \textit{sgo1Δ} cells in budding yeast mitosis (Indjeian et al., 2005). This obvious correlation, made us wonder whether Sgo1 is a
Bub1 kinase target. Sgo1-research has been fast moving in recent years. There have been several studies implicating Bub1 in regulating Sgo1 localisation in budding- and fission yeast meiosis (Bernard et al., 2001; Kiburz et al., 2005; Kitajima et al., 2004; Riedel et al., 2006). In addition, Bub1 has been shown to be required for accurate Sgo1 localisation in human cells (Kitajima et al., 2005; Tang et al., 2004b). However, thus far it has not been established whether Bub1 kinase domain is required for Sgo1 localisation to centromeres in budding yeast mitosis. I hypothesised that Bub1 could be required for Sgo1 localisation in budding yeast mitosis and that this activity could be required for chromosome bi-orientation. Hence, an important role for Bub1 would be to localise Sgo1 to centromeres. In this chapter, this was directly tested using chromosome spreads and ChiP analysis. In addition, biochemical analysis was carried out to search for any evidence that Sgo1 (or other kinetochore proteins) was a Bub1 kinase target.

Sgo1 is mis-localised to spindle pole bodies in cells lacking Bub1 kinase domain

Firstly, chromosome spreads were performed on cycling cells and on cells that had been arrested in mitosis with microtubule drugs. Using the kinetochore marker Ndc10-6HA, I tested the degree to which Sgo-9myc appeared to co-localise with kinetochores in the presence or absence of Bub1 kinase. The results showed that most wild-type cells displayed a clear overlap of the kinetochore marker with the Sgo1 staining (Figure 5.1). Despite having incubated the cells with high levels of microtubule drugs, the kinetochores tended to cluster, probably around one or two SPBs. The SPBs may in this situation have short stable microtubules still emanating from them, to which kinetochores could cluster. In the bub1ΔK mutant, the kinetochores were usually clustered, as seen in wild-type, but in most cells the Sgo1-9myc staining did not show accurate co-localisation with Ndc10-6HA. The staining pattern was categorized and the
cells were counted that showed major overlap of Ndc10-6HA and Sgo1-9myc, or cells that showed partial overlap. In the mutant, most spreads appeared to show only partial overlap. In addition, there appeared to be more Sgo1 staining that did not co-localise to any kinetochores, compared to wild-type cells (Figure 5.1). In fission yeast meiosis (Riedel et al., 2006), and in also in human mitosis (Kitajima et al., 2005), it was shown that Sgo1 re-localises to the chromosome arms in bub1 mutants. The non-kinetochore staining that was observed could therefore be due to Sgo1 re-localising to the arms.

ChIP analysis allowed us to investigate the localisation of Sgo1 more accurately. In fact, Sgo1 has been shown to localise to a broader area than the budding yeast 125 base pair centromeric region. Sgo1 was recently shown to spread out over a pericentromeric region of approximately 50 kb of meiotic chromosomes (Kiburz et al., 2005; Riedel et al., 2006). Therefore, ChIP analysis was performed using primer sets to amplify the centromeric, pericentromeric and an arm region of chromosome III. The results confirmed the chromosome spread data and revealed a significant reduction of Sgo1 on centromeres and the pericentromeric region in mitotic cells. The ChIP levels in the mutant were similar to the 'no tag' control indicating that there was no, or very little, Sgo1 on these regions in the mutant. Interestingly, there appeared to be a slight increase of Sgo1 on the chromosome arm region compared to the 'no tag' control and wild-type cells. This difference was not statistically significant, although it would fit with data from other organisms, and with the fact that evidence of non-kinetochore staining Sgo1 was observed in the bub1ΔK mutant. This observation could be confirmed by using ChIP on chip analysis of Sgo1, as described in (Kiburz et al., 2005).
The fact that some Sgo1 staining was detected on chromosome spreads indicated that Sgo1 may still be present in the nucleus, although mis-localised from the kinetochores. 'Fuzzy' nuclear staining, as discussed above, could indicate relocation to arm regions or punctate nuclear staining. However, the Sgo1 staining patterned appeared to localise to specific foci close to the kinetochore clusters in most of the bub1ΔK cells, and I therefore wondered whether Sgo1 mis-localised to SPBs. Indeed, this was the case judging from chromosome spreads co-stained with Spc110 and myc-antibodies to detect Sgo1-9myc. Therefore, I concluded that the 'partial co-localisation' of Sgo1 and Ndc10, was in fact mostly co-localisation of Sgo1 with SPBs in the mutant. This interpretation is in agreement with the fact that kinetochores do reside very close to SPBs in nocodazole treated cells. However, it cannot be ruled out that there is still some centromeric staining of Sgo1-9myc in the mutant.

5.9.2 Analysis of cells carrying the bub1ΔK-Sgo1 fusion construct

The hypothesis so far was that Bub1 kinase domain targets Sgo1 to centromeres to ensure accurate chromosome bi-orientation. A fusion construct was created to try and test whether the only role for Bub1 kinase domain is to localise Sgo1. If that were true, then replacing the kinase domain with Sgo1 may rescue the phenotype of bub1ΔK if it would bring Sgo1 to centromeres, independently of Bub1 kinase, and make Sgo1 carry out its role at the kinetochore. This construct was created and a poly-glycine linker was placed between the two proteins to increase the chances of Sgo1 being functional. This construct was transformed into wild-type cells and into sgo1Δ cells. In wild-type cells, the integration of the construct replaced the kinase domain with Sgo1, however these cells will still carry the endogenous Sgo1. This is in contrast to the construct integrated in sgo1Δ cells, which would only have a copy of Sgo1-fused to the N-terminus of Bub1.
The resulting strains showed very similar benomyl sensitivity to \textit{bublΔK} cells, indicating that the construct cannot replace the function of Bub1 kinase domain. This can be interpreted in two ways: 1) the \textit{bublΔK-Sgo1} construct is non-functional. The function of Sgo1 could be inhibited by fusion to Bub1, or the stability of the construct could be affected. However one would anticipate a benomyl phenotype more similar to \textit{bublΔ} if that were the case. 2) Bub1 kinase has other roles in mitosis in addition to localising Sgo1. This option seems less likely since the \textit{bublΔK, sgo1Δ} double mutant showed little if any synthetic effects. However, this fusion construct did appear to partially suppress poor growth of the \textit{bublΔK} and \textit{sgo1Δ} mutants on rich media. This could reflect that there may be Bub1 kinase functions, during an un-perturbed cell cycle that are rescued by this fusion. This would also agree with the fact that there was slightly lower chromosome loss in the cells carrying the \textit{bublΔK-Sgo1} fusion, compared to \textit{bublΔK} and \textit{sgo1Δ} cells.

\textbf{5.9.3 Sgo1 and Spc105 are phospho-proteins in mitosis}

During this study, I have carefully established the phenotypes of \textit{bublΔK} and \textit{sgo1Δ} cells in budding yeast mitosis. If Bub1 kinase ensures Sgo1 localisation to centromeres, then is Sgo1 a direct kinase target? My approach to investigate this began with the biochemical analysis of Sgo1 in mitosis. The results showed that Sgo1 is a phospho-protein, but that this phospho-dependent gel mobility shift is not entirely dependent on Bub1 kinase. In some experiments, a slightly weaker signal of the ‘fuzzy’ Sgo1 signal was observed in the \textit{bublΔK} mutant. One dimensional SDS-PAGE does, however, not always give rise to gel-shifts of phosphorylated proteins and despite the lack of an obvious difference in gel-shifts of Sgo1 between wild-type and mutant, this does not rule out that Sgo1 is a Bub1 target. Therefore, 2D gel electrophoresis was performed.
(preliminary data not shown). These results are in agreement with Sgo1 being a phospho-protein, and there were weak indications that Bub1 may give rise to phospho-shifts of Sgo1, however these data were not strongly reproducible and need to be repeated. The novel kinetochore proteins Spc105 and Ydr532 were to us, obvious candidates to analyse next. This is due to the fact that spc105 and ydr532 mutants show very similar phenotypes with bublΔK and sgo1Δ in mitosis (Indjeian, personal com.), and may take part in the same pathway. In addition, the S. pombe homologue of Spc105, Spc7, was recently shown to have a role in re-orientation of mis-aligned kinetochores (Kerres et al., 2007; Kerres et al., 2004). A similar biochemical study was performed on Spc105-13 myc and its binding partner Ydr532-13myc, as with Sgo1. The results showed that the majority of Spc105 is phosphorylated in mitosis. Importantly, the bublΔK mutant showed no obvious lack of phospho-shift of Spc105, indicating that Spc105 is still phosphorylated in the absence of Bub1 kinase. However, subtle differences were observed which might prove important if confirmed. In several experiments Spc105 protein levels seemed to be slightly increased in the bublΔK mutant (Figure 5.7A-D). No co-precipitation of Bub1 was detected in these IPs (data not shown). However, I know that the bublΔK allele slightly stabilises Bub1 (see 5.9 B, left panel), and if Bub1 would bind Spc105, the possibility remains that the bublΔK mutant also stabilises Spc105. However these are currently only speculations. One other minor observation was that in the non-lambda phosphatase treated Spc105-13myc IPs, there was a very faint band that did not appear to be phosphorylated in the bublΔK mutant. This band consistently appeared to be slightly stronger in the bublΔK mutant compared to wild-type (Figure 5.7 B-D, green star). Again, this difference could not be quantified, and analysis of any phospho-depentent shifts would have to be confirmed by 2D gel electrophoresis or mass-spectrometry. Although these were attempted with
Spc105-13myc, the data were inconclusive (data not shown). In addition, in preliminary experiments the Spc105 binding partner, Ydr532, showed no phospho-dependent gel mobility shifts and there was no difference between wild-type and mutant (Figure 5.7 E). Whilst still a possibility, I have little evidence at present that modifications of Sgo1, Spc105 or Ydr532 are Bubi kinase dependent.

5.9.4 Chromosomal Passenger protein localisation

Other candidate targets for Bubi would be the Chromosomal Passenger proteins that are known to play roles in bi-orientation and in tension sensing as previously discussed. The model in figure 4.7 suggests interplay between the Bubi kinase → Sgo1-pathway with the Chromosomal Passenger proteins in the function for chromosome bi-orientation. Here, the localisation of Ipl1 (and preliminary Sli15 data) was tested and no apparent loss of Ipl1 or Sli15 localisation with kinetochores was detected. However, this study certainly does not rule out that Bubi kinase subtly affects the Chromosomal Passenger proteins (kinase dependent/ or independent), and it would be interesting to investigate this possibility further. Only localisation studies of Ipl1 and Sli15 were carried out and it would, for example, be interesting to see whether any Chromosomal Passenger proteins show any phospho-dependent gel shifts in bub1ΔK mutant. In addition, there may in fact have been a slight reduction of Ipl1-GFP staining on some kinetochores in the mutant, but careful quantification or ChIP analysis would be needed to confirm this. The lack of a clear affect on Ipl1 localisation in cells lacking Bubi kinase was, however, somewhat expected. If there was been a major reduction of Ipl1 on centromeres, one would expect a more severe phenotype, since Ipl1 function is essential for the cell. One would also expect another outcome for bub1ΔK in the mtwl-1
experiment, since Ipl1 function is required to delay cells in metaphase in response to the
mtwl-1 mutation, whereas Bub1 kinase was not.

5.9.5 TAP purifications
TAP purifications of Bub1 and Bub3 were carried out to try identify novel Bub1 protein
interactions and/or substrates. In addition, this method was used to purify active Bub1
kinase for in vitro kinase assays. Initially, a TAP tag containing the calmodulin-binding
domain (CBD) was used, both to identify interacting proteins by mass-spectrometry and
for kinase assays. Whereas this TAP tag proved efficient to pull-down already known
interactors (Bub1 and Mad1 with Bub1-TAP, and Bub1, Mad1-3 and Cdc20 using
Bub3-TAP) shown by immunoblotting, the S-TAP tag was chosen for large-scale
purification and mass-spectrometry. The reason why the large scale Bub1-TAP
purification did not work is unknown. Maybe there was too much protein lost during the
second step of the purification. Alternatively, Bub1 has proven to be rather unstable and
sensitive to proteases and may have been degraded during the two-step protocol. The
Bub1 signal shown in figure 5.9 is from a ‘one-step’ purification. The Bub3-S-TAP,
large-scale, purification and mass-spectrometry confirmed results observed in figure 5.9
A. The strongest ‘hits’ by mass-spectrometry consisted of Bub1 and the MCC
components Mad3, Bub3 and Cdc20. Mad2 was not detected by this method but may
have been missed by mass-spectrometry due to its small size (22kDa). The other
components detected, appeared to be common contaminants as for example protein
chaperones and ribosome components.

5.9.6 Does budding yeast Bub1 have kinase activity in vitro?
The initial studies using Bub3-TAP purified Bub1 were promising, because they showed a strong signal in Bub3-TAP at a molecular weight size that could potentially correspond to auto-phosphorylated Bub1 (Figure 5.11 A-B). In addition, the kinetochore proteins tested appeared to show specific signals to proteins estimated to be Dam1, Ask1, Dsn1 and the Ndc80. However, at this point, I realised that the ‘no tag’ control, was in fact from W303 yeast background and not the protease-deficient, JB811, as the Bub3-TAP samples were. Unfortunately, when a ‘no tag’ JB811 strain or Bub3-TAP, bub1ΔK strains were used, the signal appeared the same in the ‘no tag’ and the Bub3-TAP stains. This strongly indicates that whatever gave rise to phosphorylation of proteins in these kinase assays was, in fact, not Bub1 but some other contaminating (or co-purifying) kinase. One-step and two-step S-TAP purified Bub1 was also tested side-by-side, and these results showed that a lot of background signal certainly disappeared during the second step of purification but there was still background signal. This led us to wonder whether Bub1 is active at all *in vitro*? Therefore, the artificial substrate (MBP) was tested from a large-scale, two-step, Bub3-S-TAP purified Bub1 alongside purified Mph1 which has been shown to be a very active kinase *in vitro* (J. Zich, personal communication). Bub1 showed very weak activity towards MBP, however the protein levels of Mph1 and Bub1 were not compared side-by-side. Moreover, the Bub3-S-TAP, bub1ΔK still gave a signal for MBP phosphorylation. These data, taken together suggest that Bub1 has a very weak activity *in vitro* and the signals detected in figure 5.11 are probably due to a contaminating kinase. To identify Bub1 substrates *in vitro* other means of purification would be needed to improve these assays. For example, major over-expression and purification from bacteria may boost the kinase activity. However, previous attempts to co-express Bub1 and Bub3 in bacteria showed that Bub1 is very prone to degradation (KH, personal communication). Alternatively, the kinase
buffer may need to be further optimised for increased Bub1 activity. Despite poor
evidence that Sgo1 or Spc105 (amongst others) are Bub1 kinase targets, it would be
interesting to test these proteins directly *in vitro*. These proteins could be purified from
yeast, or bacteria, and tested in kinase assays.

Despite little evidence for any targets of Bub1 kinase activity this study demonstrates
that the C-terminus, including the kinase domain, is very important in Sgo1 localisation.
I therefore propose, that this domain itself (regardless of its activity) plays a very
important role in mitosis, and loss of it results in lethal events.
Studies of the Bub1 kinase in many organisms have over recent years shed light on the crucial roles this protein plays in mitosis. Bub1 was originally identified as a protein essential for the spindle checkpoint arrest, and bub1Δ cells showed a failure to halt the cell cycle in response to spindle damage. Since then, an impressive amount of data has revealed other equally important roles for Bub1. Studies in yeast and metazoans have revealed that Bub1 is essential for chromosome segregation independently of its checkpoint function, and also that it plays a role in localisation of Shugoshin proteins that protect centromeric cohesion. Bub1 is an essential protein in mammals and is also implicated in tumour progression (Cahill et al., 1998; Jeganathan et al., 2007), underlining the importance of studying Bub1 function in mitosis.

A precise role for the Bub1 kinase domain, however, remained unclear. There are conflicting reports on its role in the spindle checkpoint, and there is surprisingly poor evidence of any Bub1 kinase targets. Therefore, this study set out to carefully investigate the roles for the Bub1 kinase domain in budding yeast mitosis. In this thesis I provide evidence that the Bub1 kinase domain is not required for spindle checkpoint arrest but is required chromosome segregation, especially after spindle disruption and re-formation (Chapter 3). I further show that, like Sgo1, Bub1 kinase is required to detect tension defects at kinetochores, and that both Bub1 and Sgo1 are important for chromosome bi-orientation following spindle damage (Chapter 4). Finally, I present data describing the role for the Bub1 kinase domain in Sgo1 association with kinetochores, but no clear evidence of Bub1 kinase substrates was found (Chapter 5).
It has been established that Sgo proteins are required for protection of centromeric cohesin in meiosis I, but it is not still not clear what the role for these proteins are in mitosis. Some studies suggest that they also play a role in protection of centromeric cohesion in mitosis. For example, human RNAi studies revealed that sister-chromatids lose cohesion in the absence of Sgo1 in mitotic cells, and this was also true for Bub1 RNAi cells (Kitajima et al., 2005; McGuinness et al., 2005; Tang et al., 2004b). It was therefore suggested that Sgo1 and Bub1 play a role in centromeric cohesion protection in mitosis. However, budding yeast Sgo1 (and fission yeast Sgo2) is not required for protection of centromeric cohesion in mitosis, but is required to sense the lack of tension between sister-chromatids, and for chromosome bi-orientation (Indjeian and Murray, 2007; Indjeian et al., 2005; Kawashima et al., 2007; Vanoosthuyse et al., 2007). Interestingly, cells lacking Sgo1 in budding yeast mitosis show benomyl sensitivity. This is not due to inability to arrest, but rather the failure to release in an accurate manner, presumably due to failure to detect and/or correct aberrant kinetochore-microtubule attachments. The data I present in this thesis shows that this phenotype closely mirrors that of \textit{bub1ΔK}.

\textit{Mislocalisation of Sgo1 in cells lacking Bub1 kinase}

Due to the similar phenotypes of \textit{bub1ΔK} and \textit{sgo1Δ} cells, and indications from other studies that Bub1 is important for Sgo localisation, the localisation of Sgo1 in cells lacking the Bub1 kinase domain was analysed. These results clearly showed that Sgo1 is displaced from the kinetochores in the \textit{bub1ΔK} mutant in mitosis (section 5.2), which is in agreement with other studies. It was further showed that Sgo1 was still present in these mutant cells, but that it mis-localised to the spindle pole bodies. The \textit{bub1ΔK}, \textit{sgo1Δ} double mutant displayed no synthetic phenotype in benomyl sensitivity- or
Figure 6.1 Model: Bub1 kinase and Sgo1 act together to establish chromosome bi-orientation. Bub1 kinase and Sgo1 act in concert in a novel fashion to establish chromosome bi-orientation following spindle damage. The middle section shows Bub1 kinase and Sgo1 in a linear pathway that ensures bi-orientation, in which Bub1 localises Sgo1 to the centromeres. The upper section shows a parallel mechanism ensuring chromosome bi-orientation, containing Ipl1-Sli15 passenger protein complex. We propose that Bub1 and Sgo1 may act directly upon kinesins or microtubule proteins to break / destabilise inappropriate microtubule- kinetochore attachments. This model does not show any direct interactions, but proposes several parallel (not mutually exclusive) pathways. Possible interactions are indicated by dotted lines. Although no biochemical evidence was detected for a role of Bub1 kinase activity, there is still a possibility that Bub1 phosphorylation plays a role in this process (labelled with a P).
chromosome bi-orientation assays. Furthermore, the presence of multiple copies of SGO1 appeared to slightly suppress the benomyl sensitivity of bub1ΔK cells. These observations lead me to hypothesise that Bub1 regulates Sgo1 localisation in a linear pathway, and that this is important for chromosome bi-orientation. Despite this strong correlation, I failed to detect any biochemical relationship between Bub1 kinase and Sgo1, although it cannot be excluded from these experiments that Sgo1 is a Bub1 kinase substrate. However, despite much evidence in the field that Bub1 regulates Sgo1 localisation, no study has demonstrated that Sgo1 is directly phosphorylated by Bub1, or indeed that they biochemically interact. In my model (Figure 6.1) I therefore propose that the Bub1 kinase domain regulates Sgo1 by a phosphorylation independent mechanism or, by so far undetected phosphorylation of Sgo1. Alternatively, Bub1 may regulate another protein that lies between Bub1 and Sgo1 in this pathway. Interestingly, the kinetochore protein, Spc105, was detected in the same screen that identified Sgo1 as a tension-sensor (Indjeian et al., 2005) (Indjeian, personal com). Because the phenotype of this spc105 mutant was very similar to that of sgo1 (Indjeian et al., 2005) and bub1ΔK (this study), we wondered whether Spc105 could be a Bub1 kinase target. Unfortunately, we found no evidence for such an interaction and we failed to detect Bub1 in Spc105 immunoprecipitates. Despite this, we do not rule out that there are subtle or transient biochemical interactions between these three proteins.

A previous study used the ‘kinase-dead’ point mutant of budding yeast Bub1 and it was argued that the kinase activity was required for Sgo1 localisation (Riedel et al., 2006), however we know that this point mutation gives rise to a destabilised Bub1 protein and may reflect a phenotype similar to that of bub1Δ cells. In addition, a bub1 ‘kinase-dead’ point mutant in fission yeast showed defects in Sgo2 localisation, indicating a role for
the fission yeast Bub1 kinase activity in Sgo2 localisation to kinetochores. However, there is no *in vitro* or *in vivo* evidence for direct phosphorylation of Sgo2 by Bub1 in fission yeast. Hence, it is still not clear whether phosphorylation of Sgo proteins is important for their localisation by Bub1.

*Does Sgo1 re-distribute onto chromosome arms in the bub1ΔK mutant?*

The Sgo1 localisation studies indicated that there might be some re-distribution of Sgo1 away from the kinetochores and onto the chromosome arms in the *bub1ΔK* mutant. This statement is based on a small increase in ChIP signal using a primer set for a chromosomal arm region, and also on the chromosome spreads that appeared to show an increase in non-kinetochore, non-spindle pole body staining of Sgo1 in the mutant. This would agree with vertebrate studies, which showed that Sgo1 mis-localises to chromosome arms in the absence of Bub1 (Kitajima et al., 2005). This gave rise to ectopic protection of cohesin cleavage. Our data suggest no major defect in cohesin (Mcd1) cleavage in the *bub1ΔK* mutant (Figure 3.5.2), but there did appear to be consistently slightly higher levels of full-length Mcd1 present. This suggests that although the majority of Mcd1 in the cell is cleaved upon anaphase entry in *bub1ΔK* mutant cells, there may be a slight reduction in such cleavage. To confirm whether Sgo1 is re-distributed onto chromosome arms in *bub1ΔK* cells, it would be very useful to perform ChIP on chip analysis to globally investigate the localisation pattern of Sgo1. It would also be interesting to analyse cohesin levels in the mutant, using the same technique.

*What are the mechanisms by which Bub1 kinase and Sgo1 regulate chromosome bi-orientation?*
In order to ensure accurate chromosome segregation the cells needs to be able to detect aberrant kinetochore-microtubule attachments, e.g. syntelic attachments, and arrest the cell cycle progression in response to these. The cell also needs to be able to correct these incorrect attachments. The study by Indjeian and colleagues (Indjeian et al., 2005) described Sgo1 as a tension-sensor based on the requirement of Sgo1 to arrest cells in response to reduced tension at kinetochores. In this thesis, I provide evidence showing a similar requirement for Bubi kinase, supporting the hypothesis that they may act together to establish bi-orientation. Other studies, both in yeast and in vertebrates, have also suggested a role for Bubi in tension-sensing. Bubi was shown bind to the inner centromere protein Skp1, and this interaction proved important for cells to arrest in response to reduced cohesion (Kitagawa et al., 2003). In human cells, the kinetochore localisation of Polo kinase, which is responsible for phosphorylation of the tension-sensing kinetochore epitope 3F3/2, is dependent on Bubi (Qi et al., 2006). These observations suggest a conserved function of Bubi in sensing tension at kinetochore.

But what is the mechanism of that ensures bi-orientation? We know that both the Bubi C-terminus and Sgo1 are required to sense tension defects at the kinetochore and delay anaphase in response to this. So far there is no clear evidence that Bubi and Sgo1 play a direct role in the correction mechanism in yeast. However, observations from other organisms have indicated that this may in fact be the case. Salic and colleagues showed that Sgo1 in vertebrate cells, can directly bind- and modulate microtubule stability (Salic et al., 2004). In addition, the human Sgo2 protein has been shown to regulate MCAK localisation, which is important for microtubule dynamics (Huang et al., 2007). An important parallel here is that of Aurora B and its regulation of MCAK, which has
been suggested to be an important mechanistic feature of error-correction through regulation of microtubule dynamics (reviewed in (Ruchaud et al., 2007)).

The Chromosomal Passenger proteins are known to be essential for both tension-sensing and the kinetochore-microtubule attachment correction processes (Cheeseman et al., 2006; Pinsky et al., 2006; Sandall et al., 2006). *sli15* and *ipl1* mutants show severe chromosome mis-segregation due to defects in correction of syntelic attachments (Biggins et al., 1999; Sandall et al., 2006; Tanaka et al., 2002). Therefore, the possibility remains that Bub1 and Sgo1 may act through the Chromosomal Passenger proteins to establish bi-orientation. Is there any evidence for this? This study tested whether Ipl1 (and Sli15) is accurately localised in the *bub1ΔK* mutant, and no obvious difference between wild-type and *bub1ΔK* cells was detected. This is in agreement with vertebrate experiments in which Bub1 RNAi showed chromosome congression defects, yet levels of Aurora B on kinetochores were unaffected (Johnson et al., 2004; Meraldi and Sorger, 2005). My result was not very surprising, and we did not expect a complete abolishment of Ipl1 from kinetochores in the *bub1ΔK* mutant. This is because, even though there are similarities between *bub1ΔK* and *ipl1* mutants, there are also important differences. Firstly Ipl1 is an essential protein, in contrast to Bub1 and Sgo1. Also, although it is dispensable for an arrest in response to microtubule drugs, Ipl1 is absolutely required for an arrest in response to kinetochore defects (such as those in *mtw1-1* and *ndc80-1* (Pinsky et al., 2003)), whereas Bub1 kinase and Sgo1 are not (this study, and (Pinsky et al., 2006)).

So what are the differences and similarities between the requirement for Ipl1 during a normal cell cycle and the mechanism that requires Bub1 and Sgo1 for bi-orientation
upon spindle damage? In the recent study by Indjeian and colleagues, they make an important observation in this regard (Indjeian and Murray, 2007). They show that when microtubule drugs are added to budding yeast cells directly released from G1, the sgo1 mutant cells die due to un-resolved syntelic attachments followed by chromosome mis-segregation upon drug washout. However, if the spindle was allowed to form and the SPBs separated before the addition of the microtubule drug, this could rescue the lethality of the sgo1 cells. The conclusion from this observation was that sister kinetochores show a bias to bi-orient on a bipolar mitotic spindle, and that the position of the SPBs is crucial for survival of sgo1 mutants.

So, if Ipl1 is required to break syntelic attachments made before a bipolar spindle is formed, then why is Ipl1 function not sufficient in the absence of Bub1 or Sgo1? What are the differences that make Ipl1 function in error-correction essential, and the role for Bub1 and Sgo1 only essential after spindle damage? It remains possible that Ipl1 activity is differently regulated in G1 and in mitosis, or that Bub1 and Sgo1 partially regulate Ipl1 activity and this requirement becomes essential upon spindle damage. Alternatively, it is possible that Bub1 and Sgo1 play another or an additional role in the integrity of the mitotic spindle or in spindle repair.

Implications of these findings in the understanding of bi-orientation in other systems

How do these findings compare to bi-orientation in higher organisms? It appears that Bub1 plays a conserved role in Sgo1 localisation to kinetochores (Kiburz et al., 2005; Kitajima et al., 2005; Kitajima et al., 2004; Riedel et al., 2006; Tang et al., 2004b). To date, the evidence suggests that this localisation of Sgo1 is required for accurate chromosome bi-orientation. There is no strong evidence indicating that Bub1 and Sgo1
act through the Aurora B-INCENP pathways to establish this. Instead, data suggest that Sgo1 plays a direct role in regulating chromosome-microtubule attachments by directly binding to microtubules and affecting their stability in mitosis (Salic et al., 2004). In addition, as mentioned above the human Sgo2 homologue has been shown to play a role in MCAK localisation and therefore presumably plays a role in microtubule dynamics. It would be interesting to investigate whether yeast Sgo1 has microtubule binding abilities, and whether this binding is affecting microtubule dynamics. In contrast, the fission yeast Sgo2 protein, which has been described to have a similar role in bi-orientation, does affect the passenger proteins both by enhancing complex formation and their kinetochore localisation (Kawashima et al., 2007; Vanoosthuyse et al., 2007).

In our study, the loss of the Bub1 kinase domain in yeast did affect Sgo1 localisation, but it did not appear to cause major defects in sister-chromatid cohesion, consistent with the study of budding yeast Sgo1 in mitosis (Indjeian et al., 2005). There is an ongoing debate about the roles for Sgo1 and Bub1 in sister-chromatid cohesion in mitosis in vertebrate cells. Some RNAi based studies argue that the loss of cohesion at centromeres reflects a role for Sgo1 and Bub1 in retaining centromeric cohesion, similar to that in meiosis. However a recent study using cells derived from a mouse knockout of Bub1, has addressed the possibility that cells lacking Bub1 lose cohesion due to a defective spindle checkpoint function rather that defects in centromeric cohesion (Perera et al., 2007). This conclusion is based on the critical observation that cells lacking Bub1 did not lose cohesion in the presence of the proteasome inhibitor (MG132), which prevents cells from entering anaphase. This study, using a mouse knockout model, also provided important evidence for a direct role of Bub1 in spindle checkpoint activation in vertebrate cells, especially considering the presence of previous
conflicting Bubi RNAi studies. It would be very interesting to see the phenotypes of specific *bub1* (e.g. kinase dead mutants) in such mouse cell lines.

**Future directions**

To gain further understanding of the processes that Bub1 kinase and Sgo1 regulate to establish chromosome bi-orientation (described in this thesis), I see two main future directions: 1) To further elucidate the role for Bubi and Sgo1 in tension-sensing and bi-orientation, it seems most logical to investigate any direct roles Sgo1 might play in these processes, particularly if our hypothesis holds that the main role for the Bub1 kinase is to localise Sgo1 to kinetochores. It would be interesting to see whether Sgo1 in budding yeast binds directly to microtubules, and if it affects microtubule stability. Further it would be useful to establish whether Sgo1 in budding yeast is required for formation of the Chromosomal Passenger protein complexes or for their localisation. 2) It would also be interesting to establish whether the Bubi kinase activity plays a catalytic role in the bi-orientation pathway, and in that case what the substrates are. It also seems reasonable to suggest that Bubi may phosphorylate targets at the kinetochore, since the Bubi C-terminus appears to play a role in accurate chromosome segregation in a normal mitosis. In my hands, the *in vitro* kinase assays did not prove very useful since there appear to be a contaminating kinase in the purifications used for these assays. Therefore, there are other approaches that we could take to further analyse Bub1 kinase targets. Firstly, it would be useful to employ a ‘Shokat allele’ (ATP analogue sensitive allele) of Bub1, which would enable us to specifically down-regulate the Bub1 kinase activity with the addition of an ATP analogue, and ask whether it has the same phenotype as the *bub1ΔK* cells. This would directly test the affect of Bubi kinase activity. In addition, peptide arrays or phosphorylation site-mapping using mass-
spectrometry techniques would be useful tools to investigate any Bub1 dependent phosphorylation of candidate targets such as kinetochore proteins, Sgo1 and Spc105.


Introduction

The fidelity of chromosome segregation is dependent upon correct bipolar attachment of sister chromatids to the spindle microtubules (for review see [1]). These attachments are mediated through complex, molecular machines called kinetochores, which assemble at the centromere of each chromosome (for reviews see [2,3]). Accurate chromosome segregation is crucial: any errors lead to aneuploidy, which is characteristic of many diseases and a hallmark of tumour progression [4,5]. Cells have evolved a number of control mechanisms to prevent segregation errors. One of the most important is the spindle checkpoint, which is a surveillance system that tightly regulates the metaphase-to-anaphase transition. It ensures that all kinetochores have established proper bipolar (also known as amphiletic) attachments, where sister kinetochores are attached to microtubules emanating from opposite spindle pole bodies (SPBs), before anaphase is initiated [3,6,7]. The spindle checkpoint consists of a set of conserved proteins (Mad1-3p, Bub1p, Bub3p, and Mps1p [8-10]) that form distinct complexes and localise to unattached kinetochores in a highly ordered manner [11,12]. The downstream target of these proteins is Cdc20p [13,14], an activator of the E3 ubiquitin ligase known as the Anaphase Promoting Complex or Cyclosome (APC/C) (for review see [15]). Securin (Pds1p in budding yeast) and cyclin B are the key APC/C substrates and polyubiquitination of these, and their ensuing proteolytic destruction, is required for anaphase onset and mitotic exit [16-18]. Thus when the spindle checkpoint is active, the APC/C is inhibited, Securin levels remain high, and anaphase onset is delayed.

The spindle checkpoint responds to unattached kineto-
required for localisation of several checkpoint proteins to unattached kinetochores [12,28,29]. It is well established that the N-terminal domains of Bub1p, which include the kinetochore targeting and Bub3p-binding domains, are required for spindle checkpoint arrest [29,30]. However the role of the C-terminal kinase domain remains elusive, although it has been suggested that phosphorylation of Cdc20 by human Bub1 is required to enhance the inhibition of APC/C [31]. Several lines of evidence suggest role(s) for Bub1 in addition to its spindle checkpoint function. Bub1 is required for accurate chromosome segregation in both budding and fission yeasts [29,30] and to maintain ploidy in fission yeast [32]. In addition, there is evidence that Bub1 plays a role in chromosome congression in mammalian cells [28,33].

In this paper we characterise the role of the Bub1p kinase domain in budding yeast mitosis. We show that bub1ΔK cells die rapidly in the presence of microtubule drugs despite being able to initiate and maintain a spindle checkpoint arrest. This rapid cell death is due to chromosome mis-segregation following release from antimicrotubule drugs. In addition, we demonstrate a role for Bub1 kinase in accurate Sgo1p localisation to mitotic centromeres. Sgo1p is the sole budding yeast member of the Shugoshin/MEL-S332 family (for review see [34]). Members of this family are important protectors of centromeric cohesion, particularly in meiosis [35–38]. However, budding yeast Sgo1p does not regulate cohesion in budding yeast mitosis, and it has been proposed that this protein is a tension sensor at kinetochores [39]. Here we demonstrate that the Bub1 kinase domain and Sgo1p act together to ensure efficient chromosome biorientation.

Results

Although Bub1 kinase activity has been shown to be required for accurate chromosome segregation in both budding and fission yeasts [29,30], the roles of the Bub1 kinase domain have not been clearly established. Indeed, it remains controversial whether the kinase activity is required for a spindle checkpoint arrest, in either organism. In some budding and fission yeast reports, Bub1 kinase activity was thought to be necessary for checkpoint arrest [26,40], but in others it was not [29,30]. This controversy might partly be due to the use of a “kinase-dead” point mutation (K733R), which has since been shown to destabilise budding yeast Bub1p [31] and could therefore display a phenotype similar to that of bub1Δ. We generated a novel K733M kinase-dead allele, but found that this protein was also unstable (K. G. Hardwick, unpublished data). Therefore, we chose to carry out a detailed analysis of the role of the Bub1 kinase domain by using a truncated Bub1 kinase allele (containing amino acids 1–608) in Saccharomyces cerevisiae. This allele lacks the whole kinase domain and has already been shown to express a stable protein [30], and to efficiently bind Bub3p and Mad1p [27]. A similar Bub1 truncation is stable and is able to localise to kinetochores in S. pombe ([40] and V. Vanooystuyse, personal communication).

Cells Lacking the Bub1 Kinase Domain Die Rapidly in the Presence of Microtubule Depolymerising Drugs

Spindle checkpoint mutants are hypersensitive to microtubule destabilising drugs because of their inability to arrest in metaphase in response to unattached kinetochores. The precocious separation of sister chromatids gives rise to unequal segregation of chromosomes and aneuploidy, which is lethal in yeast. On rich media containing low concentrations of the microtubule drug benomyl, bub1ΔK cells showed an intermediate sensitivity to the drug compared to other spindle checkpoint mutants. For example, it was clear that the bub1ΔK mutation was not as benomyl sensitive as the complete bub1Δ but that it was more sensitive than mad3Δ (Figure 1A). Spindle checkpoint mutants die rapidly in liquid cultures containing microtubule drugs [41], so we asked how long bub1ΔK cells remain viable under such conditions. Cells were grown in liquid media containing 30 μg/ml nocodazole and then plated on rich media lacking microtubule drugs. Viability was scored as the percentage of cells able to form colonies. In contrast to wild-type cells, bub1ΔK behaved like bub1Δ and mad2Δ cells and showed rapid death in this viability assay (Figure 1B). After 1 h, 55% of bub1ΔK cells were already inviable. These data show that cells lacking the Bub1 kinase domain are sensitive to microtubule drugs and that they die rapidly, but that bub1ΔK is not a complete loss of function (null) allele.

The Bub1 Kinase Domain Is dispensable for a Robust Spindle Checkpoint Arrest in Response to Unattached Kinetochores

bub1ΔK cells could be sensitive to microtubule depolymerising drugs for various reasons. They may be unable to arrest in mitosis, or they may fail to recover properly after spindle checkpoint arrest. To distinguish between these possibilities, we examined cell morphology and the level of sister chromatid cohesion in bub1ΔK cells in response to microtubule drugs. First we performed a morphological assay scoring budding of cells on plates containing 20 μg/ml and 80 μg/ml benomyl (see Figure S1). Both wild-type and bub1ΔK cells remained large-budded (which is an indication of mitotic arrest) for up to 6 h on benomyl, compared to the spindle checkpoint mutants, mad2Δ and bub1Δ, which did not respond to microtubule depolymerisation and rebudded prematurely. This confirms the previous report of a large-
The Bub1 Kinase Domain Is Necessary for the Checkpoint Response to Reduced Cohesion

In the assays described above, we used antimicrotubule drugs to activate the spindle checkpoint pathway that recognizes unattached kinetochores. Complete microtubule depolymerisation is not a very common physiological situation, and we were therefore interested to analyse bub1AK cells in other situations. Ipl1 kinase activates the spindle-checkpoint by creating unattached kinetochores in response to mutations in several kinetochore components that were thought to create reduced tension at centromeres [24]. We wondered whether the kinase domain of Bub1 is required to arrest cells containing such defective kinetochores. In contrast to an ipl1-321, mit1-1 double mutant, cells containing the mit1-1 mutation in combination with bub1AK were able to respond to the kinetochore defect and stabilise Pds1-myc (Figure 2A). Similar results were obtained when using the more severe ndc80-1 kinetochore mutant in combination with bub1AK (unpublished data). These results show that Bub1 kinase is not required to “sense” these defective kinetochores, nor to activate Ipl1p in these mutants, nor to respond to the unattached kinetochores that Ipl1p kinase activity creates.

Ipl1p (Aurora kinase), Mad3p phosphorylation, and Sgo1p have all been demonstrated to be necessary for the checkpoint response to reduced cohesion, even though they are not necessary for the response to unattached kinetochores [23,25,39]. To test whether the Bub1 kinase domain is necessary for the response to reduced cohesion, bub1AK,GAL-MCD1 cells were synchronised in G1, depleted for cohesin by turning off MCD1 expression with glucose addition, and then

Figure 1. Cells Lacking Bub1 Kinase Domain Show Sensitivity to Microtubule Depolymerising Drugs despite Being Spindle Checkpoint Proficient

(A) Wild-type (KH186), mad1Δ (MB076), mad2Δ (KH141), mad3Δ (KH173), bub1Δ (KH127), bub3Δ (MB003), and bub1ΔK (JF098) strains were plated out in 10-fold serial dilutions on YPDA media or on YPDA media containing 8 μg/ml benomyl.

(B) The indicated strains were grown in YPDA media containing 30 μg/ml nocodazole, and the viability of the cells was measured as percentage of cells able to form colonies on YPDA media lacking microtubule drugs.

(C) Wild-type (JF004), bub1ΔK (JF125), bub1Δ (JF140), and mad3Δ (EK013) strains containing a GFP-marked chromosome were synchronised in G1 using α-factor, then released into YPDA with 30 μg/ml nocodazole at 23 °C. We tested ability of the cells to maintain a spindle checkpoint arrest by scoring cells that could keep their GFP-marked sister chromatids cohesed for 3 h in nocodazole (i.e., one GFP dot). The percentage of cells with two GFP dots was counted at the release from G1 (grey bars) and after 3 h in nocodazole (black bars) (n = 100 cells for each repeat experiment). Error bars indicate standard deviation.

(D) Wild-type (JF004) and bub1ΔK (JF125) strains containing Pds1-18myc were arrested in G1 using α-factor and synchronously released into YPDA media or YPDA media containing 30 μg/ml nocodazole at 23 °C. Samples were taken at indicated times. Levels of Pds1 were monitored by immunoblotting using A14 α-myc antibody and α-PGK1 as a loading control.

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The Bub1 Kinase Domain Is Necessary for the Checkpoint Response to Reduced Cohesion

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<td>bub1(AK)</td>
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<td>sgo1Δ</td>
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C

linear mini-chromosomes

- LEU - LEU + doxycycline

CDC20-117 on CDC20-117 off

released into the cell cycle. Immunoblotting for Securin levels (Pds1) showed that neither bub1\(AK\) nor sgo1 mutants were able to maintain a metaphase arrest as well as wild-type cells (Figure 2B). They did not degrade Pds1p quite as quickly as the mad2Δ control, but we believe that was because they grow more slowly, most likely because of their aneuploid phenotype. We also analysed the ability of bub1\(AK\) cells to grow in the presence of poorly segregating linear chromosomes. Such chromosomes have been shown to delay mitosis in a checkpoint-dependent manner [42] and were employed in a screen that identified sgo1 alleles [39]. For the screen, the linear chromosome-induced delay becomes lethal as the strains also contain Cdc28-VF, a mutation in Cdc28/Cdk1 that reduces APC activity. This lethality is rescued by checkpoint mutations, as no mitotic delay is imposed. Figure 2C shows that bub1\(AK\) cells grow far better than wild type in the presence of the short linear chromosomes, indicating that this checkpoint response is also defective. We conclude that whilst the Bub1 kinase domain is not necessary for the response to unattached or defective kinetochores, it is necessary for the budding yeast checkpoint response to a lack of tension at mitotic kinetochores.

The above experiments strongly suggest that the C-terminal kinase domain of Bub1 is not required to initiate or maintain spindle checkpoint arrests induced by unattached or defective kinetochores, but that it is necessary to respond to a lack of tension. The bub1\(AK\) phenotype closely mirrors that of sgo1Δ, which is also necessary for the tension response [39] but not the response to mtw1-1- or nde80-1-induced kinetochore defects [24]. The bub1\(AK\) and sgo1Δ phenotypes differ from that displayed by ip1 mutants. Ip1p is required for the tension response but also for the response to mtw1-1- or nde80-1-induced kinetochore defects [24], and Ip1p is an essential protein [43].

The Bub1 Kinase Domain Plays a Role in Sgo1 Localisation

Bub1p is important for Sgo1p localisation at centromeres in meiosis in budding and fission yeast [36,37,44] and regulates Sgo1 localisation in mitosis in human cells [45,46]. Because of this and the similarity in the bub1\(AK\) and sgo1Δ phenotypes, we tested whether the Bub1 kinase domain has a role in localising Sgo1p to kinetochores in budding yeast mitosis. Unfortunately Sgo1-GFP gives a rather weak signal, so we did this by performing immunofluorescence on fixed chromosome spreads. We used a strain containing Ndc10-6HA to label kinetochores and asked whether Sgo1-9Myc co-localised with Ndc10p in wild type, bub1\(Δ\), and bub1\(AK\) cells. We analysed unbiased populations of cycling cells (see Figure S2) and cultures that had been arrested in mitosis for 3 h with nocodazole. Whilst wild-type cells showed multiple foci with

Figure 2. Bub1 Kinase Domain Is Required to Arrest in Response to Reduced Cohesion
(A) Wild-type (JF004), mtw1-1 (SBY1646), mtw1-1, bub1\(AK\) (JF100), and mtw1-1, ip1-321 (SBY1724) strains carrying the temperature-sensitive allele mtw1-1 and Pds1-18Myc were synchronised in G1 for 2.5 h then incubated at the restrictive temperature 36 °C for 30 min before release. The levels of Pds1 were monitored by immunoblotting with A14 anti-myc antibody and anti-PGK1 as a loading control.
(B) Wild-type (V8545), bub1\(AK\) (JF023), mad2Δ (V8560), and sgo1Δ (JF224) strains, containing Pds1-myc, were synchronised in G1 with α-factor in media containing 2% galactose and 2% raffinose to maintain Mcd1 expression. Mcd1 was then turned off by incubating them in media containing glucose and α-factor for 3 h. The cells were then released into rich media containing glucose, and samples for immunoblotting were taken at indicated times. Pds1 and PGK1 levels were monitored as previously described.

\(\text{DOI:10.1371/journal.pgen.0030213.g002}\)
Overlapping localization for Sgo1p and Ndc10p in many spreads, this was rarely the case for bub1AK cells (Figure 3A). Categorization revealed such colocalization of Sgo1p and Ndc10p in only 13% of mutant cells (compared with 2% in wild type). In bub1AK cells there were often one or at most two bright punctate signals for Sgo1p. To determine whether these could be SPBs, we carried out double label staining for Sgo1p-9Myc and the 110-kD component of the SPB. Colocalization was observed in many cells (Figure 3B), suggesting that significant levels of Sgo1p localise to the SPB in the bub1AK mutant. We cannot rule out the possibility that some of this signal is due to centromeres that remain associated with the SPB in these nocodazole-treated cells. To confirm the decreased association of Sgo1p with kinetochores in bub1AK cells, we employed chromatin immunoprecipitation (ChIP). We reproducibly observed decreased association of Sgo1p and centromeres, using both centromeric and pericentromeric primer sets, in bub1AK cells (Figure 3C-3E).

These results confirm that the kinase domain of Bub1p plays an important role in localising Sgo1p to budding yeast centromeres in mitosis. This could explain why the sgo1A and bub1AK strains display such similar phenotypes.

**bub1AK Cells Mis-Segregate Chromosomes during Recovery from a Checkpoint Arrest**

The fact that bub1AK cells are hypersensitive to antimicrotubule drugs, yet able to arrest efficiently, suggested to us that the Bub1 kinase domain could be required for proper recovery from spindle damage. To test this, we first asked whether they have increased chromosome loss following nocodazole arrest. We followed chromosome segregation during the first anaphase after nocodazole release, using the GFP-marked chromosome strain described above. Accurate chromosome segregation should give rise to one GFP spot in each daughter cell. We arrested cells in metaphase for 3 h with nocodazole then released them into anaphase. After 30 min, cells were fixed and stained with α-tubulin antibody to monitor spindle elongation. The results showed 33% mis-segregation of the GFP-marked chromatids in bub1AK cells (Figure 4A, lower panel) compared to 0.2% in wild type (Figure 4A, upper panel).

We then used the same strain to score a large number of cells for chromosome mis-segregation and compared unchallenged G1 cells with G1 cells that had been released from a nocodazole arrest. Cells were arrested in G1 using α-factor, and the number of cells with one GFP foci (Figure 4C, empty triangle) versus two GFP foci (filled triangle) were counted. As expected, most cells had one GFP dot, representing one copy of Chromosome IV, in both wild-type (0% had two GFP dots, n = 400) and bub1AK cells (2% had two GFP dots, n = 400). The small number of bub1AK cells with two copies of this chromosome (Figure 4B) reflects a background level of aneuploidy, frequently observed in bub1 mutants. However, when cells were released from G1 into media containing nocodazole for 3 h, then released and trapped in the following G1, there was a marked increase in the number of cells containing two GFP foci in bub1AK (30% had two GFP dots, n = 400) compared to wild type (where only 4% had two GFP dots, n = 400) (Figure 4C). This confirms that there was a significant defect in segregating this chromosome faithfully during the anaphase following nocodazole release. Because of the high incidence of chromosome mis-segregation following treatment with nocodazole, and considering that we only scored one of 16 budding yeast chromosomes in this analysis, we propose that the reason why bub1AK cells are sensitive to antimicrotubule drugs, despite showing capacity to arrest in metaphase, is because of chromosome loss.

To more accurately quantify their chromosome-loss rate, and to compare it with that of sgo1A mutants, we employed sectoring assays, which have previously been used to analyse many mitotic and checkpoint mutants [30,47]. We used a strain containing a nonessential test chromosome that carries the SUP11 (ochre-suppressing tRNA) gene that makes colonies that are normally red, because of the ade2-1 mutation, white. We scored loss of this chromosome at the first division by counting colonies that are half red and half white. Consistent with published data [30], in an unchallenged cell cycle, bub1AK cells lost the test chromosome at a rate of 41 per 1,000 divisions compared to 0.5 per 1,000 in wild type. bub1AK cells also showed chromosome loss in a normal mitosis, but at a lower rate than bub1A, at 28 per 1,000 divisions (Figure 4E). The loss rate in sgo1A was very similar: 32 per 1,000 divisions.

If the Bub1 kinase domain and Sgo1p carry out the same function, one might predict little, if any, synthetic phenotype when combining the two mutations in bub1AK, sgo1A cells. Alternatively, if the double mutant was significantly sicker than that would suggest that the Bub1 kinase domain has other functions, in addition to Sgo1p targeting. Our analysis of the double mutant, both in terms of sensitivity to microtubule drugs and chromosome-loss rates, strongly supports the former option. We found that the double mutant is no more benomyl sensitive than the bub1AK strain (Figure 4D) and that the rate of chromosome loss was no higher than that of the bub1AK and sgo1A single mutants (Figure 4E).

These results show that the Bub1 kinase domain plays a role in chromosome segregation that becomes very important upon challenge with microtubule drugs. The lack of a synthetic genetic interaction suggests that the Bub1 kinase domain function is closely related to that of Sgo1p, consistent with the idea that the major role of the kinase is to efficiently target Sgo1p to centromeres (Figure 3). Note, bub1A cells have an even higher chromosome-loss rate because, in addition to their segregation defects, these cells have also lost their ability to checkpoint arrest.

**The Bub1 Kinase Domain Ensures Proper Chromosome Biorientation**

As bub1AK cells have chromosome segregation defects, we tested whether they displayed kinetochore attachment defects. To do this we employed a bub1AK strain containing Tub1-cyan fluorescent protein (CFP) to label spindle microtubules and Mtw1-3xGFP to mark all kinetochores. Cells were arrested in mitosis with benomyl and nocodazole, which were then washed out, and the ensuing anaphase analysed. We identified no more unattached kinetothes (indicated by Mtw1-GFP foci off the spindle axis) in bub1AK cells than in wild-type cells (8%, see Figure 5A). Similar results, with low levels of unattached kinetothes, were obtained for sgo1A cells. ndc80-1 mutants were used as positive controls for this experiment, and as expected these contain many unattached kinetothes at their restrictive temperature (Figure 5A [24,48]). From this we conclude that bub1AK and sgo1A cells...
Figure 3. Bub1 Kinase Domain Is Necessary for Accurate Sgo1 Localisation to Kinetochores

(A) Wild-type (AMY1110) and bub1ΔK (JF038) strains containing Sgo1-9Myc and Ndc10-6HA (to mark the kinetochores) were arrested in metaphase with 15 μg/ml nocodazole and 30 μg/ml benomyl at 23 °C for 3 h. Chromosome spreads were performed and stained with α-myc antibody (CM-100), α-HA antibody (HA11), and DAPI to recognize the DNA. Spreads with clear Ndc10-6HA staining were categorized as showing colocalization with Sgo1-9-myc only if they contained multiple (>2) overlapping foci. Fifty spreads per strain were analysed.

(B) Sgo1-9myc colocalises with the SPB in the bub1ΔK mutant. Spreads were prepared as in (A) and stained with α-myc antibody, anti-Spc110 antibody to detect SPBs, and DAPI. Scale bar represents 2 μm.

(C) Schematic of primers sets for Sgo1-6HA ChIP analysis showing the CEN3, centromeric region; R3, pericentromeric region; and c281, the negative arm region.

(D) PCR on ChIPs of “no tag” (KH186), Sgo1-6HA (AMY209), and bub1ΔK, Sgo1-6HA (JF211) strains arrested with 15 μg/ml nocodazole and 30 μg/ml benomyl at 23 °C for 3 h, showing reduced Sgo1p levels associated with CEN3 and R3 regions in the bub1ΔK mutant compared to wild type.

(E) The graph shows quantification of the ChIP data from the “no tag” (KH186), wild-type (AMY209), and bub1ΔK (JF211) strains. The “binding ratio” was calculated as a ratio of the ChIP PCR signal to the PCR signal from a 1:500 diluted input fraction. The error bars indicate standard deviation (SD) of the mean from five different PCR reactions from two separate experiments.

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Bub1 Kinase and Bio-orientation

Figure 4. bub1ΔK Cells Display High Levels of Chromosome Mis-segregation upon Nocodazole Release

(A) Wild-type (JF004) and bub1ΔK (JF125) cells were released from G1 into media containing 30 μg/ml nocodazole and incubated at 23 °C for 3 h. Cells were subsequently released into anaphase by washing out the nocodazole. Samples were fixed in 3.7% formaldehyde for 1 h, 30 min after release, and stained with z-GFP (GFP-marked chromosome) and z-tubulin (red spindle) antibodies. DNA was stained with DAPI (blue). Percentage of nondisjunction of the GFP-marked chromatid at the first anaphase following nocodazole arrest was 2% in wild-type cells and 33% in bub1ΔK cells (n ≥ 50 anaphase cells). Scale bar represents 3 μm.

(B) Wild-type (JF004) and bub1ΔK (JF125) strains were synchronised in G1 as previously described and cells with one GFP dot (empty triangle) versus two dots (filled triangle) were counted (n = 400). Scale bar represents 2 μm.

(C) Cells from (B) were then released and incubated in media containing 30 μg/ml nocodazole at 23 °C for 3 h and released into media containing α-factor to score cells in the following G1. The number of cells with one GFP dot versus two dots were scored (n = 400).

(D) Wild-type (KH186), bub1Δ (KH127), bub1ΔK (JF098), sgo1Δ (JF188), and bub1Δ, sgo1Δ (JF185) strains were plated out in 10-fold serial dilutions on rich media and on rich media containing 8 μg/ml benomyl.

(E) Strains carrying the SUP11 artificial chromosome were grown overnight in CSM-URA media, then diluted back to OD₆₀₀ 0.2 and grown in YPDA media at 30 °C for 3 h. Cells were then plated out on YPD at a density of ~500 cells per plate. Only colonies that were at least half red were scored for losing the test chromosome at the first division.

<table>
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<tr>
<td>bub1Δ</td>
<td>41</td>
</tr>
<tr>
<td>bub1ΔK</td>
<td>28</td>
</tr>
<tr>
<td>sgo1Δ</td>
<td>32</td>
</tr>
<tr>
<td>bub1ΔK, sgo1Δ</td>
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doi:10.1371/journal.pgen.0030213.g004

do not display significant numbers of unattached kinetochores, even after spindle ablation and re-formation.

Next we wanted to test whether the bub1ΔK cells contain more subtle kinetochore attachment problems, such as defects in biorientation. If, during spindle reassembly, both sister kinetochores attach to microtubules from the same spindle pole (syntelically) perhaps the bub1ΔK cells would be unable to detect or correct this defect. Note syntelic attachments are unlikely to result in tension across sister kinetochores. To test this possibility, we used strains that carry an array of tet operators integrated only 2 kb from the centromere on Chromosome IV and express a Tet repressible GFP fusion protein. In addition, their spindle poles are marked with Spc42-tomato, and the strain has a methionine repressible promoter for CDC20 (pMET-CDC20). By depleting Cdc20p, we can induce a metaphase arrest that is independent of spindle damage and the checkpoint. Using such strains we could directly test the ability of cells to establish proper bipolar attachment after nocodazole treatment. Amphitelic attachments were visualised as pairs of GFP-centromere (CEN) spots pulled apart by the microtubule forces of the bipolar spindle (see Figure 5B) [49-51], whereas monotelic or syntelic attachments (both monopolar) remained as single GFP-CEN spots, on the spindle axis. Cells were arrested in G1, depleted of Cdc20p, and then released into media containing benomyl and nocodazole for 3 h. The microtubule drugs were
A

<table>
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B

![Diagram](image9.png)

D

![Graph](image10.png)

E

![Graph](image11.png)
then washed out, cells kept in metaphase by continued repression of Cdc20p, and the level of biorientation was scored as two GFP dots in between two red SPBs (filled white triangle, Figure 5C). This procedure gave us the relative levels of biorientation, although these numbers will be underestimated, since, at the time of fixation, some cells that have established biorientation will only show one GFP dot because of the “breathing” characteristics of mitotic centromeres [49,50]. Quantitation of these images revealed a significant defect in establishing proper biorientation in both bub1ΔK and sgo1Δ mutants, after nocodazole release, indicating that Bub1 kinase and Sgo1p both work to ensure efficient chromosome biorientation (Figure 5D). In addition, the bub1ΔK,sgo1Δ double mutant showed a very similar quantitative defect in biorientation to the two single mutants, suggesting that the Bub1 kinase domain and Sgo1p are necessary for the same biorientation function.

The position of the chromosomes in these images, relative to the SPBs (Figure 5E), is consistent with them being syntetically attached: both sisters are attached to the same spindle pole and therefore localise very close to it. We have carried out live-cell analysis of these cells (unpublished data) in which we scored the numbers of cells in which the GFP-marked chromosomes were “breathing” on a short mitotic spindle. Whilst 88% of chromosomes were observed to breathe in wild-type cells, only 56% did in the bub1ΔK and sgo1Δ mutants during the time observed. We conclude that these chromosomes are most likely syntetically attached, rather than being amphitelic attachments that fail to be stretched, and that the bub1ΔK and sgo1Δ mutants are unable to correct these attachments. Such a defect could account for much of the chromosome loss detailed in Figure 4. These findings agree well with recent live-cell imaging from the Murray lab showing that sgo1-100 mutants frequently fail to correct syntelic attachments [52].

Discussion

Bub1 Kinase Is Not Required for Most Spindle Checkpoint Arrests, but Is Required for the Response to Reduced Cohesion

In this study we have carried out a detailed analysis of whether the kinase domain of Bub1 is required for a robust spindle checkpoint, using a truncated Bub1 kinase allele. Note, our truncation removes the last 413 residues of Bub1p (residues 609-1,021), which include 83 residues before the start of the conserved kinase domain. bub1ΔK cells showed sensitivity to benomyl, although not as severe as bub1Δ cells, and died rapidly in a nocodazole viability assay. However, our data clearly show that the Bub1 kinase domain is not required to initiate or maintain spindle checkpoint arrests induced by unattached or defective kinetochores. Robust arrests were observed in the presence of antimicrotubule drugs (either benomyl or nocodazole), and in the mtw1Δ and ndc80Δ knockout mutants (Figure 2A). In addition, we have found that the Bub1 kinase domain is unnecessary for the arrest induced by overexpression of the Mps1 protein kinase (GAL-MPS1, unpublished data and [53]). Thus, the Bub1 kinase domain is not necessary for a wide range of spindle checkpoint arrests.

However, like Sgo1p [39] and Ipl1p [23], the Bub1 kinase domain is necessary to delay anaphase onset in cells with reduced cohesion (Figure 2B). The simplest interpretation of this result is that these three functions are all needed for the checkpoint response to a lack of tension at kinetochores.

The Sensitivity of bub1ΔK Cells to Microtubule Drugs Is Due to Chromosome Mis-Segregation after the Release from Nocodazole

In agreement with previous work [30] we found that bub1ΔK cells display chromosome mis-segregation in an unpaused mitosis, at a level between those of wild-type and bub1Δ cells. The bub1ΔK chromosome-loss rate becomes far higher upon spindle damage (Figure 4). We have also demonstrated that bub1ΔK cells die rapidly when released from nocodazole. Importantly, this was not because of an inability to arrest in nocodazole, but rather to an inability to segregate chromosomes accurately upon spindle reassembly (see Figures 4 and 5). We propose that the Bub1 kinase domain has a role in regulating chromosome segregation every cell cycle, and that this role becomes particularly important after spindle damage. Such segregation defects could reflect the inability of bub1ΔK cells to respond to a lack of tension at kinetochores (Figure 2).

Our genetic studies support the idea that Bub1 kinase and Sgo1p act in the same pathway: whilst the complete gene
Overall, our data agree with work from other groups showing a role for Bub1 in localisation of Sgo1 to centromeres in mitosis in human cells [45,46], and in fission yeast [36]. Data are also in agreement with the finding that Bub1 kinase activity is required for centromeric localisation of Sgo1p (and the protein phosphatase PP2A sub-unit Rts1p) in budding yeast meiosis [44]. That study also showed that Bub1p localisation is independent of Sgo1p and PP2A [44].

Is Sgo1 a Bub1 Kinase Substrate?

Because sgo1Δ and bub1ΔΔ cells have such similar phenotypes and Sgo1p is mislocalised in bub1ΔΔ, Sgo1p is a strong candidate to be a mitotic Bub1 kinase substrate. We have carried out preliminary experiments showing that Sgo1p is a phosphoprotein, but there is little if any effect on the phosphorylation state of Sgo1p in bub1ΔΔ mutants (J. Fernius, unpublished data). We also failed to observe a gel mobility change for fission yeast Sgo2p in bub1ΔΔ mutants (V. Vanoothuiysen, personal communication). Therefore we think it is likely that there is an unknown Bub1 kinase substrate (Factor X in our models, see Figure 6) that is required for Sgo1p targeting to centromeres.

Amongst the candidates for Factor X are the Chromosomal Passenger proteins (Aurora B, INCENP, and Survivin, see [54] for review). In Drosophila meiosis, incenp mutants perturb Mei-S332 (Sgo) localisation, and Mei-S332 was shown to be a good Aurora B substrate [55]. In budding yeast meiosis, Ipl1 kinase and the monopolar complex are key regulators of kinetochore orientation [56]. However, only partial perturbation of Sgo1p was observed upon Ipl1p depletion, and it was shown that Sgo1p actually recruits Ipl1p to meiotic centromeres [57]. Fission yeast studies also demonstrated an interdependence between Passenger proteins and Sgo2p targeting to centromeres, and in those experiments Survivin appears to be the Passenger protein most closely linked to Sgo2p [58,59].

Because of these links with the Passenger proteins, we have carried out preliminary experiments, looking at the Ipl1p and Sli15p in budding yeast mitotic cells that lack the Bub1 kinase domain. Whilst there may be subtle effects on the efficiency of recruitment of the Passenger proteins to mitotic centromeres, we find these to be far less significant than the effects on Sgo1p (Figure 5, and unpublished data). Thus we doubt that Factor X is one of the Passenger proteins (see models in Figure 6). Experiments to identify this factor are ongoing.

Bub1 Kinase and Sgo1 Are Required for Efficient Chromosome Bi-orientation following Nocodazole Treatment

We have demonstrated that both bub1ΔΔ and sgo1ΔΔ mutants have a defect in chromosome bi-orientation upon nocodazole release (Figure 5). In many of the mitotic cells we observed chromosomes that fail to biorient, lying close to one of the SPBs. One explanation could be that these mutants fail to detect or respond to inappropriate attachments, for example syntelic attachments that lack tension. This hypothesis is supported by the fact that bub1ΔΔ and sgo1ΔΔ cells lack the ability to delay anaphase onset in response to tension defects induced by reduced cohesion (Figure 2 and [59]). Alternatively, bub1ΔΔ and sgo1ΔΔ cells may sense these defects but be unable to break the inappropriate kinetochore-microtubule attachments. The Yen lab have recently demon-

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**Figure 6. Models for Bub1 Kinase and Sgo1p Functions**

(A) Simple linear pathway: Bub1 kinase phosphorylates Factor X in such a way that Sgo1 is efficiently targeted to centromeres. This in turn ensures efficient recruitment of the Passenger proteins, which act to monitor and correct any inappropriate kinetochore-microtubule attachments.

(B) Parallel pathways: Bub1 kinase, Sgo1p, and the Passenger proteins act in a concerted fashion to ensure efficient biorientation of sister kinetochores. This model better explains why localization of the different proteins are not entirely dependent upon one another. It also suggests that Bub1 kinase, and/or Sgo1p, might act to break inappropriate kinetochore attachments independently of Aurora kinase. doi:10.1371/journal.pgen.0030213.g006
strated that human Sgo2 is not required to recruit Passenger proteins to centromeres but is needed to recruit the kinesin MCAK [60]. The activity of that kinesin, which is an Aurora B substrate, is known to be important for breaking inappropriate kinetochore attachments in some systems [61,62]. In addition, Sgo proteins have been shown to bind directly to microtubules in yeast cells and to modulate kinetochore-microtubule dynamics [63], so it is also possible that Sgo1p has a direct role in breaking microtubule attachments.

Bub1 kinase and Sgo1p could be part of the pathway that employs the Passenger proteins to destabilise kinetochore-microtubule attachments that lack tension. If so, one would expect similar phenotypes between bub1Δ, sgo1Δ, and Passenger protein mutants. There are striking similarities: (1) ipi1 and sli15 mutants can arrest the cell cycle in response to unattached kinetochores because of nocodazole treatment, and (2) ipi1 mutants fail to delay anaphase onset when cohesion is reduced and there is a lack of tension at kinetochores [23,64]. However, the Passenger proteins are essential, and are required for response to “core” kinetochore attachment defects, such as those present in ndc80-1 and mut2-1 [24]. Figure 6 contains models that attempt to explain these observations.

In certain circumstances, such as those found during spindle assembly upon nocodazole washout, the activity of Bub1 kinase and Sgo1p become necessary to ensure complete biorientation. Our data show that Bub1 kinase is required for efficient localisation of Sgo1p to centromeres, which in turn may aid efficient targeting of the Passenger proteins or their targets. Such a model of action places Bub1p, Sgo1p and the Passengers in a simple linear pathway (Figure 6A). However, due to the lack of a clear effect on Passenger protein localisation, we currently favour an alternative model in which Bub1p, Sgo1p and the Passengers act cooperatively, but in distinct pathways, to ensure efficient biorientation (Figure 6B). The identification of direct Bub1 kinase substrates will be key to a deeper understanding of the role(s) of Bub1 kinase in the complex regulation of kinetochore attachment and error correction in mitosis.

Materials and Methods

Yeast strains, media, and standard techniques. The yeast strains used in this study are derivatives of W303 (ade2-1 his3-11 leu2-112 trp1-1 can1-100 sst2-d2) and are listed in Table 1. Yeast strains were grown in YPD or selective media, and other basic yeast methods have been previously described [70,71]. Sgo1-9myc was detected using rabbit anti-myc antibody (CM-100, Gramsch) at a 1:800 dilution, and anti-rabbit Alexa Fluor 488 (Invitrogen) at a 1:1,000 dilution. Ndc10-6HA was detected using a mouse anti-HA antibody (HA11, BabCO) at a 1:200 dilution and anti-mouse Alexa Fluor 594 (Invitrogen) at a 1:1,000 dilution. The spindle pole bodies were marked using an anti-Spc110 antibody at a 1:500 dilution (kind gift from John Kilman) and anti-mouse Alexa Fluor 594 (Invitrogen) at a 1:1,000 dilution.

ChIP. Cells were arrested for 3 h in 15 μg/ml nocodazole and 30 μg/ml benomyl, and 50 μl cells were collected for ChIP. Sgo1-6HA ChIPs were performed using 12CA5 anti-HA antibody (Roche), and the general protocol including primers was performed as described in [72].

Chromosome attachment assay. Similar experiments to those described in [24] were performed, where the mitotic spindle is labelled with tubulin-CFP and the kinetochores with Mtw1-3XFPR. Strains were treated with 15 μg/ml nocodazole and 30 μg/ml benomyl at 23 °C for 3 h. Cells were then washed three times and grown in YPDA at 30 °C for 20 min, then fixed in 3.7% formaldehyde for 5 min. ndc80-1 cells were grown for the last 90 min at the restrictive temperature of 36 °C.

Chromosome biorientation assay. Strains were arrested in G1 in medium lacking methionine for 3 h, then transferred to YPDA media plus 8 mM methionine for 2 h at 30 °C to deplete Cdc20. The β-factor was subsequently washed out, and cells were then incubated at 29 °C for 3 h in media containing 30 μg/ml benomyl, 30 μg/ml nocodazole, and 8 mM methionine. The microtubule drugs were then washed out, and the spindle was allowed to reform at 30 °C in YPDA media plus 8 mM methionine. Cells were fixed at indicated times in 3.7% formaldehyde for 5 min. The GFP dots were analysed only in cells that had a short bipolar spindle (i.e., two SBPs) to score for biorientation. These experiments were repeated at least three times, and at each time point 100 cells were counted.

Supporting Information

Figure S1. bub1Δ Cells Initiate and Maintain a Robust Checkpoint Arrest in Response to Antimicrotubule Drugs

(A) Wild-type (KH186), bub1Δ (JF098), bub1Δ (KH127), and mada2A (KH141) yeast strains were analysed for growth on YPDA media containing (A) 20 μg/ml or (B) 80 μg/ml benomyl grown at 25 °C. We scored the percentage of cells that remain arrested (large-budded) throughout the time course (n = 50 cells). The bub1Δ cells initiate and maintain a robust checkpoint arrest.

Found at doi:10.1371/journal.pgen.0030213.sg001 (49 KB PDF).

Figure S2. Sgo1 Localisation to Kinetochores (Ndc10) Is Defective in bub1Δ

(A) Wild-type (AMY1110), bub1Δ (F038), and bub1Δ (AMY1379) cells were harvested in log-phase, and staining was performed on chromosome spreads. Anti-α-tubulin (CM-100) was used to detect Sgo1-9myc, and anti-HA (HA11) was used to detect Ndc10-6HA. Cells with clear Ndc10-6HA staining were categorised as either having colocalisation with Sgo1-9myc (left panel), or only partial colocalisa-
tion that could be due to spindle pole bodies (right panel). The spreads with no Sgo1 staining are not shown but were similar in number in wild type and bub1ΔK mutant.

(B) Quantification of spreads scoring percentage of spreads that showed colocalisation of Sgo1 to the Ndc10 kinetochore marker. Error bars indicate standard deviation of the mean.

Found at doi:10.1371/journal.pgen.0030213.s002 (66 KB PDF).

Figure S3. Mdc1 Cleavage Is Not Defective in bub1ΔK Cells

Wild-type (AMY1145) and bub1ΔK (JF216) strains were arrested in mitosis using 15 μg/ml nocodazole and 30 μg/ml benomyl. Kinetochore positions were marked using α19-CIA and Ndc80-3CFP. There was no significant difference in timing or amount of Mdc1 cleavage detected. Blot shows a representative experiment. The experiment was repeated three times.

Found at doi:10.1371/journal.pgen.0030213.s003 (124 KB PDF).

Figure S4. Kinetochore Localisation of Ipl1 Kinase Is Not Significantly Perturbed in bub1ΔK Cells

Wild-type (T5241) and bub1ΔK (JF169) strains were arrested in mitosis using 15 μg/ml nocodazole and 30 μg/ml benomyl. Kinetochore positions were marked using α19-CIA and Ndc80-3CFP. There was no significant difference in timing or amount of Mdc1 cleavage detected. Blot shows a representative experiment. The experiment was repeated three times.

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