Sfi1p has multiple roles in the spindle pole body cycle

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Thesis presented for the degree of Doctor of Philosophy at the University of Edinburgh

2006
I declare that this thesis was composed by myself and that the research presented within is my own work, except where explicitly stated and acknowledgement is given.

Victoria Anderson

August 2006
Acknowledgements

Many thanks to Kevin Hardwick for support, direction and advice. I am also grateful to many past and present members of the Hardwick lab for making the lab a pleasant place to work. Special thanks to the 'Hardwick Harem' for gossip and 'Brian Molko and wine appreciation nights'.

Recognition for technical help and advice goes to Paul McLaughlin and Alistair Kerr for bioinformatics help, John Kilmartin (Cambridge) and Tom Giddings (Colorado) for EM advice and Vasilious Koutsokostas for help with the 2-hybrid screen.
Abstract

In order to identify new mitotic defects, a screen for mutants synthetic lethal with a mad1Δ deletion strain was performed in *S. cerevisiae*. Mad1p is a component of the spindle assembly checkpoint. Four of the mutants isolated in the screen were novel mutations in the essential *sf11* gene.

In this study, Sf1lp is shown to be a spindle pole body (SPB) protein that contains a conserved set of 21 amino acid repeats. Its phosphorylation is cell cycle regulated, with most phosphorylation being present in an alpha factor (G0/G1) arrest, the time of spindle pole body duplication.

The mutants from the screen all had mutations C-terminal to the conserved 21 amino acid repeats. This C terminal region is not conserved outwith the budding yeast, but within the budding yeasts family is the most conserved region. Deletion of the C terminal region is lethal, and results in mis-localisation of the protein. Complementary biochemical and 2-hybrid methods were used to search for binding partners of the C-terminal region but no significant results were obtained.

The *sf11-CT* mutants are lethal in combination with a range mutants in spindle checkpoint proteins, indicating that the defect is recognised by the spindle assembly checkpoint. The mutants are all temperature sensitive and benomyl resistant, and showed elevated levels of chromosome loss. The mutants showed weak genetic interactions with a selection of other spindle pole body genes (BBP1, MPS2, NDC1) and a microtubule motor (CIN8).

At all temperatures the *sf11-CT* mutants showed varying levels of large budded cells delayed in mitosis with spindle pole bodies very close together (0.2-0.4 μm) and few normal metaphase (1-1.5 μm) spindles. Examination of these abnormal SPB pairs by electron microscopy revealed many large budded cells containing sets of paired spindle pole bodies still attached by a half bridge. In these cases, both spindle pole
bodies appear morphologically normal and could nucleate nuclear and cytoplasmic microtubules. This is in contrast to other Sfi1p mutants in the conserved repeats, that result in a failure of spindle pole body duplication, and suggests that Sfi1p has multiple functions in both SPB duplication and separation.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>APC</td>
<td>Anaphase promoting complex</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>Bub</td>
<td>Budding uninhibited by benomyl (mutant)</td>
</tr>
<tr>
<td>Cdc</td>
<td>Cell division cycle (mutant)</td>
</tr>
<tr>
<td>Cdk</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CSM</td>
<td>Complete synthetic media</td>
</tr>
<tr>
<td>C-terminus</td>
<td>Carboxy-terminus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetra acetic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EGTA</td>
<td>1,2,-Di (2-aminoethoxy) ethane-N,N,N,N’-tetra acetic acid</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>5’FOA</td>
<td>5’Fluoroorotic acid</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GST</td>
<td>Gluthathione S-transferase</td>
</tr>
<tr>
<td>HU</td>
<td>Hydroxyurea</td>
</tr>
<tr>
<td>KT</td>
<td>Kinetochore</td>
</tr>
<tr>
<td>Mad</td>
<td>Mitotic arrest deficient (mutant)</td>
</tr>
<tr>
<td>N-terminus</td>
<td>Amino terminus</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>TAP</td>
<td>Tandem affinity purification</td>
</tr>
<tr>
<td>Tet</td>
<td>Tetracycline</td>
</tr>
<tr>
<td>SCF</td>
<td>Skp1-cullin-F box complex</td>
</tr>
<tr>
<td>SPB</td>
<td>Spindle pole body</td>
</tr>
<tr>
<td>YPD(A)</td>
<td>Yeast peptone with dextrose (and adenine)</td>
</tr>
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References
Chapter 1 - Introduction

1.1. Overview of the eukaryotic cell cycle
In order to grow organisms must replicate their cells. This involves creating enough material for two cells, and replicating and correctly segregating DNA into two daughter cells. Mistakes, especially in the correct segregation of genetic material can lead to diseases in higher organisms. It is of great use therefore, to understand the mechanisms of cell division.

Cell division is highly controlled and broadly called the cell cycle. The process of one cell producing two daughter cells is called mitosis. The cell has evolved a complex regulation and sequential order of events in order for this process to happen without errors. The mitotic cell cycle consists of 4 phases (figure 1.1A). The majority of the cycle is made up of two gap phases (G1 and G2) separated by S phase where genetic material is replicated, and the final phase, mitosis, is where the DNA and cell components are segregated equally into the two daughter cells. After completing mitosis, daughter cells are back in G1 phase.

One of the most important and potentially error-prone mitotic processes is the segregation of the genetic material. Each daughter cell must end up with one of each chromosome. In S phase the chromosomes are replicated to give two paired sister chromatids, these two sisters must become attached to opposite poles of the mitotic spindle to ensure correct segregation. Sister chromatids are held together by the cohesin complex (cohesin structure and function reviewed in (Nasmyth, 2005b)). Sister chromatid cohesion is important in order that the cell can identify sisters and ensure one goes to each daughter cell. From a mechanical point of view, sister chromatid cohesion is important to ensure tension between sisters attached to opposite spindle poles. This tension is an important way for the cell to monitor chromosome-spindle attachments (see section 1.2.3). Each chromatid is attached to spindle microtubules (section 1.3) via kinetochores (section 1.5). Kinetochores consist of special centromeric DNA sequences onto which a large multi-layered
multi-complex protein structure is assembled (reviewed in (McAinsh et al., 2003)). The spindle is bipolar, consisting of microtubules coming from two spindle poles (sections 1.6 and 1.7). Each of the two sister chromatids must be attached to opposite poles in order to be segregated correctly. There are mechanisms that ensure bipolar attachment (see section 1.2.3). The structure of the mitotic spindle is discussed in more detail in section 1.3.4.

Mitosis can in turn be divided into phases based on the behaviour of the DNA and the spindle (figure 1.1A). In prophase the DNA condenses and spindle formation is initiated. In most organisms prometaphase occurs when the nuclear membrane breaks down, attachment of kinetochores to the spindle starts and spindle formation continues. Metaphase is historically defined as the point when all sister chromatids are aligned at the centre of the spindle due to bipolar attachment. Upon correct attachment of all kinetochores to the spindle, the cohesin between chromosomes is dissolved and the sister chromatids are pulled to opposite poles. This is anaphase which can be further divided. In anaphase A the kinetochore-attached microtubules depolymerise, pulling chromosomes to the poles and in anaphase B the spindle poles separate, pulling the chromosomes with them into the daughter cells. In telophase DNA decondenses and the nuclear membrane reforms. The cells finally physically separate in cytokinesis. An overview of cytological cell cycle events is provided in (Alberts et al., 1993).

The budding yeast (Saccharomyces cerevisiae) cell cycle differs from that of metazoans. The cells do not divide equally - the mother cell forms a smaller bud that grows during the cycle. Like many fungi, they undergo closed mitosis, which means that the nuclear membrane never dissolves and the spindle is built in the nucleus. This means there is no visible distinction between S phase/G2/early mitosis, as a short spindle is present from S-phase until anaphase. It is also thought that kinetochores remain attached to microtubules during most of the cell cycle (Guacci et al., 1997; Jin et al., 2000; Tanaka et al., 2005). The DNA does not condense to the extent it does in higher organisms, and the chromosomes oscillate in the plane of the spindle until anaphase (He et al., 2000).
Figure 1.1A. The eukaryotic cell cycle. Phases of the vertebrate cell cycle including interphase (G1, S and G2), and stages of mitosis showing behavior of chromosomes and spindle.
Figure 1.1B. The budding yeast cell cycle highlighting biochemical events.
1.2. Control of the cell cycle

Progression through the cell cycle is controlled by cycles of protein synthesis/targeted proteolytic destruction and phosphorylation/dephosphorylation. The cyclin dependent kinases (Cdks) phosphorylate key regulators at different points in the cycle. Substrate specificity is determined by which cyclin subunit the Cdk is associated with (reviewed in (Morgan, 1997)). The anaphase promoting complex (APC) and Skp1-cullin-Fbox complex (SCF) are ubiquitin ligases that promote multi-ubiquitination of proteins, which targets them for proteolysis (reviewed in (Morgan, 1999)). The APC and SCF also associate with regulators of their specificity. They key biochemical events of the budding yeast cell cycle are illustrated in figure 1.1B.

1.2.1 Key players

Cell cycle regulators were first identified in budding yeast by Hartwell and colleagues as conditional mutants that arrested at the restrictive temperature with a uniform bud size, reflecting cell cycle stage. This large collection of mutants were named the cell division cycle (cdc) mutants. These mutants have since been allocated a wide range of functions. One particularly interesting mutant, cdc28, blocked budding, SPB duplication and DNA synthesis suggesting it was an important global regulator of the cell cycle (Hartwell et al., 1974; Hartwell et al., 1970). Similar work in fission yeast by Nurse and co-workers identified a similar global regulator, Cdc2p (Beach et al., 1982). These proteins have since been shown to homologues and the main cyclin-dependent kinases (Cdks) in respective yeasts, and to have homologues in all organisms that reflect the basic conservation of the Cdk cell cycle control mechanism.

As their name suggests, Cdks are regulated by activating subunits called cyclins. Cyclins were first identified in sea urchin eggs by Tim Hunt and colleagues. During a study on protein synthesis, cyclins were notable as proteins whose levels fluctuated through the cell cycle. Cyclin levels increase from fertilisation until mitosis, when levels drop abruptly, and this pattern continues through subsequent cell cycles (Evans et al., 1983). Cyclin levels are controlled by both periodic synthesis and
degradation. The relationship between cyclins and Cdk's was confirmed with the purification of MPF (maturation promoting factor), a protein kinase factor known to be the driving force behind embryonic cell cycles in *Xenopus* oocytes (Newport and Kirschner, 1984). It consists of Cdk and cyclin (Lohka et al., 1988). Cdk's associate with different cyclin subunits depending on cell cycle stage. Cyclins and Cdk's are reviewed in (Morgan, 1997).

The Cdk's co-ordinate one of the main processes driving the cell cycle - the cycle of phosphorylation and dephosphorylation of kinases and other cell cycle regulators. Other important kinases are polo like kinases (Cdc5p in budding yeast) (van Vugt and Medema, 2005; Xie et al., 2005) and the aurora kinases (Adams et al., 2001). In budding yeast the main Cdk is Cdc28p, and it associates with 9 different cyclins; Cln 1-3 in G1, Clb 5 and 6 in S-phase, and Clb1-4 in mitosis. The levels of cyclin subunits are cell cycle regulated through transcriptional control and targeted degradation. As well as regulation of cyclin subunit availability, Cdk's are controlled by inhibitors. Most notable of these in budding yeast is Sic1p, active from late anaphase until S phase, and particularly important at the G1-S transition, inhibiting Cdc28p/Clb1-4. Sic1p is inactivated by phosphorylation by Cdk/Clb (mainly Clb2p). This phosphorylation targets it for ubiquitination by the SCFCdc4 (see below). Sic1p transcription is activated by Swi5p, which is in turn deactivated by Cdk's. Vertebrates have two main cell cycle Cdk's, as well as additional non-cell cycle Cdk's (reviewed in (Morgan, 1997)). Cdk2/cyclin E controls G1/S and Cdk2/cyclin A is active in S-phase. Mitosis is controlled by Cdk1 (cdc2) with cyclins B and A.

Another important kinase cascade in budding yeast is that of the mitotic exit network (MEN), active at the end of mitosis. Most MEN mutants arrest at the end of mitosis with separated but uncondensed chromosomes and high Cdk activity. It consists of the kinases Cdc5p (Polo), Cdc15p, Dbf2p and Dbf20p as well as Tem1p, Let1p, Mob1p and the phosphatase Cdc14p. MEN activity is regulated by Tem1p, a GTPase, active when-GTP bound, and inactive when GDP-bound. It is activated by a 'GTP exchange factor' Lte1p (Shirayama et al., 1994), and inactivated by a 'GTPse
activating complex' of Bub2p and Bfa1p (Krishnan et al., 2000; Li, 1999). Activated Tem1p activates the Cdc15p kinase, which in turn activates a complex of the Dbf2p kinase and Mob1p (Asakawa et al., 2001; Mah et al., 2001). The mitotic exit network is reviewed in (Bardin and Amon, 2001; Pereira and Schiebel, 2001).

Opposing kinases are phosphatases, one of which is Cdc14p, which is activated in late mitosis. It is sequestered in the nucleolus until the appropriate time by Net1p/Ctf1p. Cdc14p release is inhibited by Pds1p (securin) and stimulated by the MEN, via the Dbf1p kinase, although it is not clear whether this is a direct interaction or not. Most MEN mutants can be rescued by overexpression of Cdc14p so it seems to be the essential regulatory target of the MEN (Jaspersen et al., 1998) (Shou et al., 2001). Key Cdc14p phosphatase targets (which it activates) are Cdh1p (APC activator, see below), Sic1p (Cdk inhibitor) and Swi5p (which in turn activates transcription of Sic1p (Visintin et al., 1998)).

Another key cell cycle control mechanism is control of protein levels by controlled transcription and targeted degradation. Most targeted degradation is mediated by two 'E3' ubiquitin ligase complexes, the APC and SCF. Ubiquitin is a small protein, added to the target protein in multi-ubiquitin chains that act as a signal for destruction by the 26S proteosome. Ubiquitin is added to targets by the action of 3 enzymes- an 'E1' ubiquitin activating enzyme, an 'E2' ubiquitin conjugating enzyme, and 'E3' ubiquitin ligases. The E3 ubiquitin ligases catalyse the transfer of a ubiquitin molecule from the E2 to a lysine residue of the target protein. The 'E3' adds the main layer of substrate specificity to the process. General aspects of ubiquitination are reviewed in (Hershko and Ciechanover, 1998).

One of these E3 ubiquitin ligases is the APC (anaphase promoting complex). Its activity is in turn regulated by substrate specific activators. These are WD repeat proteins named Cdc20p and Cdh1p in budding yeast (reviewed in (Morgan, 1999)). Cdc20p is active in mitosis and is controlled by transcription and the spindle checkpoint (see 1.2.3). Cdh1p is active from late anaphase through to S phase and is negatively regulated by phosphorylation. The APC is also pre-activated by
phosphorylation courtesy of Cdks, which is necessary for activity of APC$_{\text{Cdc20}}$ but not APC$_{\text{Cdh1}}$ (Shteinberg et al., 1999).

One of the key targets of the APC$_{\text{Cdc20}}$ at anaphase is Pds1p (securin). Securin holds separase (Esp1 in budding yeast) in its inactive state until anaphase. When released, separase cleaves Scc1p, a member of the cohesin complex that holds sister chromatids together, allowing sister separation (reviewed in (Nasmyth et al., 2000)). Pds1p also inhibits the release of Cdc14p from the nucleolus.

Another E3 ubiquitin ligase important in the cell cycle is the SCF (Skp1-Cullin-Fbox) complex (reviewed in (Nakayama and Nakayama, 2005)). It is another multi-subunit complex with ‘F-box’ proteins as substrate specific activators. SCF activity is controlled by phosphorylation of substrates rather than subunit availability. F-box proteins in budding yeast are Cdc4p (targets Sic1p, the Cdk inhibitor), Grr1p (targets Cln1-3) and Met30p (targets Swe1p).

1.2.2. Co-ordination of phosphorylation and ubiquitination drives the cell cycle

The first major point in the cell cycle that is controlled is commitment to S-phase. This G1 to S-phase transition is called ‘Start’ in unicellular eukaryotes and the ‘restriction point’ in multicellular organisms. Start is the main control and commitment point in the budding yeast cell cycle, it is where most $cdc28$ mutants arrest (Hartwell et al., 1974). When cells have reached critical size and nutrient availability, Cln3 is expressed, which activates Cdc28p to phosphorylate factors that activate transcription of Cln1-2 in a positive feedback loop (Nasmyth, 1996). The Cdc28/Cln1/2 complexes initiate spindle pole body duplication in yeast (Haase et al., 2001).

Later, the activity of S-phase Cdks rises (Cdc28p with Clb5/6 in budding yeast and Cdk2 with cyclins A and E in vertebrates). S-phase Cdks activate DNA replication, and in budding yeast promote SPB maturation and separation into a short spindle. Through phosphorylation of Cdh1p they inactivate the APC$_{\text{Cdh1}}$. This in turn allows activation of M-phase Cdks in late G2.
The next commitment point, more important in organisms other than budding yeasts, is the transition from G2 into mitosis. This is triggered by the activity of Cdc28p with Clb1-4 in budding yeast and Cdk1 with cyclin B in vertebrates. In vertebrates Cdk/CycB triggers nuclear envelope breakdown, chromosome condensation and the formation of a mitotic spindle. During prophase chromosomes become attached to the spindle via kinetochores, and in metaphase they are all attached and lined up in the centre of the spindle. The mitotic-Cdks promote the activity of the APC^{Cdc20} by phosphorylation. APC^{Cdc20} triggers the metaphase to anaphase transition through destruction of Pds1p (securin), mitotic cyclins and other factors. This destruction of regulators ensures that the cell cycle cannot go backwards.

After chromosome segregation, the cell must decondense chromosomes and cytokinesis must take place, physically separating the two daughter cells. Activity of mitotic Cdks inhibits this, so their destruction is important for the cell to exit mitosis. Cdk inactivation also allows re-licensing of DNA replication origins, so DNA is only able to re-duplicate once the cell has divided into two daughters. Release of Cdc14p at anaphase allows it to de-phosphorylate, and thereby activate Sic1p and Cdh1p. They both act to de-activate mitotic Cdks, Sic1p by direct inhibition, and Cdh1 by activation of the APC. APC^{Cdh1} remains active, along with Sic1p until the next G1-S transition. The cycle is complete.

1.2.3 Monitoring the spindle

At several key points in the cell cycle there are checkpoints which can halt the progress of the cell cycle if a defect is detected. The concept of cell cycle checkpoints was first proposed by Weinert and Hartwell, during discovery of the Rad9 mediated DNA damage checkpoint (Weinert and Hartwell, 1989; Weinert and Hartwell, 1988). This checkpoint arrests cells in response to DNA damage. This is important to prevent damaged or incorrect DNA proceeding into mitosis and being propagated into daughter cells. Another of these checkpoints is the spindle assembly checkpoint, which monitors microtubule-kinetochore attachments. The spindle checkpoint ensures that anaphase does not proceed until all chromosomes are
attached correctly to the bipolar spindle. If anaphase were allowed to proceed with incorrectly attached chromosomes, the chromosome may be unequally segregated, leading to aneuploidy. In yeasts this most likely results in death, but in higher organisms aneuploidy can lead to cancer, so study of the checkpoint is relevant to human disease. If a defect is detected the spindle assembly checkpoint arrests cells at the metaphase to anaphase transition by inhibition of the APC$^{\text{Cdc20}}$ (Hwang et al., 1998; Kim et al., 1998). This is illustrated in figure 1.2.

Components of the spindle assembly checkpoint (a.k.a. spindle checkpoint, mitotic checkpoint, kinetochore attachment checkpoint) were first discovered in yeast as mutants that progressed through mitosis in the presence of microtubule depolymerising drugs. This cell progression in the absence of a spindle led to gross chromosome mis-segregation and rapid death. These mutants are the mitotic arrest deficient (Mad1-3) and budding uninhibited by benomyl (Bub1and 3) mutants (Hoyt et al., 1991; Li and Murray, 1991). A protein kinase Mps1, originally identified by its role in spindle pole body duplication (Winey et al., 1991b), also displayed a role in the spindle checkpoint (Weiss and Winey, 1996). The spindle checkpoint proteins are not essential in budding yeast (with the exception Mps1), but they are in vertebrates, where they are thought to have a role mediating mitotic timing in every cell cycle (Meraldi et al., 2004a).

Mad1p is a phosphoprotein (Hardwick and Murray, 1995) and is constitutively bound to Mad2p. Mad2p is a member of many additional complexes, implicated at the effector end of the pathway. It was shown in both budding and fission yeast to bind Cdc20p, and cdc20 mutants that can no longer bind Mad2p do not have a functional checkpoint (Hwang et al., 1998; Kim et al., 1998). Complexes of Mad2p-Cdc20p form in vitro but require Mad1p to form in vivo (Hwang et al., 1998).
Signal from unattached kinetochore
Sensing unattachment or lack of tension

Activation of spindle checkpoint, including Mad1p, Mad2p, Mad3p, Bub1p, Bub3p, Mps1p

MCC (Cdc20p-Mad2p-Bub3p-Mad3p)  Mad2p-Cdc20p complex

APC Cdc20p

Metaphase  Anaphase

Pds1p and Clbs targeted by APC Cdc20 and degraded

Figure 1.2. The Spindle assembly checkpoint. Lack of tension or lack of microtubule attachment at a kinetochore activates the spindle checkpoint. The spindle checkpoint creates the MCC (mitotic checkpoint complex) and a Cdc20p-Mad2p complex which both inhibit the action of the APC Cdc20 in vitro. While the checkpoint is active APC cannot promote the metaphase to anaphase transition by destruction of Pds1p and cyclins.
Structures of Mad2p with peptides corresponding to Mad1p or Cdc20p have been solved by both NMR and crystallography as well as the structure of a Mad1p-Mad2-tetramer (Luo et al., 2000; Luo et al., 2002; Sironi et al., 2002). These structures are discussed later.

**Mad3p** and its mammalian homologue **BubRI** are also important for the inhibition of the APC\(^{\text{Cdc20}}\). Mad3p contains two regions of homology with Bub1, and BubRI additionally contains a kinase domain at the C-terminus. Region 1 of Mad3p binds Mad2p and Cdc20p and region II is for Bub3p binding throughout the cell cycle (Hardwick et al., 2000). **Bub3p** is a WD repeat protein that forms constitutive complexes with both Mad3p and Bub1p, binding them via the WD repeats. The crystal structure of *S. cerevisiae* Bub3p has been solved in two studies (Larsen and Harrison, 2004; Wilson et al., 2005). They reveal that the conserved seven WD repeats form a seven bladed propeller configuration. Mad3p and Bub1p binding sites overlap on the top side of the propeller and the two checkpoint proteins likely compete for Bub3p binding (Wilson et al., 2005).

**Bub1p** is required to recruit Bub3 and Mad3 to kinetochores in *S. pombe* (Millband and Hardwick, 2002). Kinetochore localisation is mediated via region I (homology with Mad3). The role of the kinase activity in the checkpoint remains controversial (Vanoosthuyse et al., 2004; Warren et al., 2002; Yamaguchi et al., 2003).

The **Mps1** kinase was discovered initially as a mutant in SPB duplication in budding yeast (Winey et al., 1991b). It was later shown to have a role in the spindle checkpoint (Weiss and Winey, 1996), and overexpression induces a spindle checkpoint arrest (Hardwick and Murray, 1995). The fission yeast homologue Mph1 is not essential and appears not to have a role in spindle pole body duplication (He et al., 1998). Mps1p homologues in other organisms are essential with spindle checkpoint roles, but there is conflicting data on whether they have a role in centrosome duplication (Fisk et al., 2003; Stucke et al., 2002).
In addition, extra checkpoint proteins not present in yeast are present in metazoans.
Rod, Zw10 and Zwillch form a complex and appear to be involved in interactions of
the Mad1-Mad2 complex with kinetochores. These proteins are reviewed in (Karess,
2005). Another is CENP-E (centromere-associated protein – E), a large kinesin that
is localised at kinetochore from prometaphase through anaphase A. It stabilises
kinetochore-microtubule attachments, helping with metaphase chromosome
alignment (Putkey et al., 2002). It is associated with BubR1 (Chan et al., 1998; Yao
et al., 2000) and controls BubR1 kinase activity dependent on microtubule binding to
its motor domain (kinase on when no microtubules binding, off when microtubules
bind) (Abrieu et al., 2000; Mao et al., 2003; Mao et al., 2005).

The checkpoint signal
All checkpoint proteins have been seen at unattached kinetochores in various
systems. This ideally places them to monitor microtubule attachments, and/or
tension across sister chromatids upon bipolar attachment. In vertebrate cells Mad1
and Mad2 localise to only unattached kinetochores (Chen et al., 1998a; Hoffman et
al., 2001) and Bub1, BubR1 and Bub3 kinetochore binding decreases when the
checkpoint is switched off (Hoffman et al., 2001; Skoufias et al., 2001; Taylor et al.,
2001; Zhou et al., 2002) Cdc20p is also enriched at kinetochores (Fang et al., 1998;
Kallio et al., 1998). The only direct interaction of a checkpoint protein with a
kinetochore protein seen so far is that of BubR1 and CENP-E (Chan et al., 1998).

A complex of Mad2-Mad3-Bub3-Cdc20, known as the mitotic checkpoint complex
(MCC), has been seen in both budding and fission yeasts (Fraschini et al., 2001;
Hardwick et al., 2000; Millband and Hardwick, 2002). Nocodazole arrested HeLa
lysates contain an APC-Cdc20-Mad2 complex (Fang et al., 1998). Mad2 alone can
inhibit the APC in vitro, but in HeLa cells the MCC inhibits the APC 3000 fold
greater (Sudakin et al., 2001). Both Mad2 and BubR1 can bind Cdc20p. In
mammalian cells more than half the MCC or its subcomplexes are bound to the APC,
however upon anaphase the complexes disappear (Kallio et al., 1998; Kallio et al.,
2002). The MCC seems to be a key complex in checkpoint signalling.
A technique known as fluorescence recovery after photo bleaching (FRAP) can measure protein exchange at a particular site. A GFP tagged protein of interest can be photo-bleached by a laser. The rate by which fluorescence recovers is the rate at which new, unbleached protein is exchanged with bleached protein at the site. Studies of checkpoint proteins in PtK2 (kangaroo) and HeLa (human) cells at the kinetochore yielded evidence supporting a 'catalytic site' theory for generation of a checkpoint signal. Mad2, BubR1, Bub3, Mps1 and Cdc20 cycle rapidly at the kinetochore, whilst Mad1 and Bub1 are more stable (Howell et al., 2004; Shah et al., 2004). This supports the theory that the unattached kinetochore acts as a catalytic site for MCC formation. There is data that conflicts this simplistic idea, however. In budding yeast the Mad2p-Cdc20p and MCC complexes form in mitosis independently of checkpoint activation and kinetochore (Fraschini et al., 2001; Poddar et al., 2005). Poddar et al suggest that it is instead Cdc20p availability that limits complex formation. However, these complexes formed without checkpoint activation cannot inhibit the APC\(^{Cdc20}\), suggesting there is an extra layer of regulation yet to be characterised.

Howell et al find that 70% of Mad1 is stable at the kinetochore while 90% of Mad2 cycles in transiently transfected PtK2 cells (Howell et al., 2004). Shah et al suggest that a more modest 55% of Mad2 cycles in a stable Mad2-YFP HeLa cell line (Shah et al., 2004). Both agree that Mad2p exists in two populations, a stably kinetochore bound population, probably bound to Mad1p, and a rapidly cycling cytoplasmic pool. Mad1p is known to be important in regulating Mad2p, it is required in vivo for the formation of the Mad2-Cdc20 complex (Hwang et al., 1998). 50% of Cdc20p has same kinetics as Mad2 and other MCC components, so this portion probably reflects MCC formation (Howell et al., 2004).

Bub1 is stable at the kinetochore (Howell et al., 2004; Shah et al., 2004), and it is required for the kinetochore localisation of most other checkpoint proteins. This suggests it also acts as a scaffold for other checkpoint proteins at the kinetochore. Bub1 binds Bub3 and BubR1, of which 95% and 100% respectively cycle at the
kinetochores. 54% of BubR1 cycles with Bub3, suggesting this is the MCC population (Howell et al., 2004).

Structural studies have revealed a possible mechanism for the interactions of Mad1p, Mad2p and Cdc20p at the kinetochores. The NMR structure of Mad2p with a Cdc20p peptide (Luo et al., 2000), and crystal structure of Mad2p with a Mad1p peptide as well as a Mad2-Mad1 tetramer have been solved (Luo et al., 2002; Sironi et al., 2002). Mad1p and Cdc20p share a ten-residue Mad2p binding sequence that binds the same pocket of Mad2p, and Mad2p adopts the same conformation bound to either. Mad1p and Cdc20p appear to compete for Mad2p binding. An interesting structural feature is that the C-terminal tail of Mad2p acts as a ‘seat belt’ that closes over the Mad1p or Cdc20p bound in the pocket. This ‘seat belt’ must be unlocked and opened for substrate exchange in the pocket. Mad2p exists in two conformations - the closed ‘C’ state where either Mad1p or Cdc20p is bound, and an ‘open’ state, ready to receive either Mad1p or Cdc20p into its binding site (Luo et al., 2002). A previous ‘Mad2 exchange’ model suggested that Mad1p binding primed Mad2p for Cdc20p binding, requiring exchange of Mad1p and Cdc20p at the Mad2p pocket (Luo et al., 2004). It is hard to envision how this model fits with knowledge of the Mad2p C-terminal ‘seat belt’ locking substrates in the binding pocket.

Recently De Antoni et al published findings that updated the model ((De Antoni et al., 2005), reviewed in (Nasmyth, 2005a)). It had been known for some time that Mad2p formed dimers (Fang et al., 1998; Sironi et al., 2001). When De Antoni et al added Alexa labelled Mad2p to pre-formed Mad1p-Mad2p complexes, it was integrated into these complexes. A Cdc20p peptide dislodged the Alexa bound Mad2p, and Mad2p-Cdc20 peptide complexes were formed. However, the Cdc20p peptide did not remove Mad2p from the preformed Mad1p-Mad2p complexes. So only the additional Mad2p which was initially unbound to Mad1p could bind the Cdc20p peptide. The resulting model is that Mad1p-Mad2C complex is stable at the kinetochores, and this is reflected by the 50% stable population of Mad2p seen by FRAP by Shah et al. Mad2p from the cytoplasmic pool can bind to this Mad2C, but only if in the Mad2O conformation. This Mad2p in the open conformation at the
kinetochore is ready to receive Cdc20p. On Cdc20p binding, the conformational change of Mad2p from open to closed breaks the Mad2p-Mad2p interaction, resulting in dissociation of the Mad2p-Cdc20p complex. This results in free Mad2p-Cdc20p and leaves the stable Madlp-Mad2C at the kinetochore ready to recruit another Mad2O. These newly formed Cdc20-Mad2C complexes could also recruit additional Mad2O and act as catalyst much the same as Madlp-Mad2C, further propagating the checkpoint signal.

The current thinking is that the Mad2p-Cdc20p complex acts by sequestering Cdc20p away from the APC, whereas the MCC binds directly to the APC (Sudakin et al., 2001). Recently, evidence has been found for an additional checkpoint mechanism in budding yeast. The checkpoint appears to increase Cdc20p turnover, so that it reduces protein levels of this APC activator below a certain threshold (Pan and Chen, 2004). It is currently unclear whether this mechanism applies to other organisms.

The checkpoint gives cells time to correct attachment defects, but other factors are responsible for the actual correction. Of these Aurora B/Ipl1 is best understood. In response to a lack of tension it promotes turnover of microtubule-kinetochore attachments (see section 1.5.5).

**Attachment or tension?**

One of the most hotly debated issues is whether the checkpoint is monitoring attachment or tension across sister kinetochores. Two classic experiments support opposite cases. The argument for attachment alone is supported by an experiment in PtK1 (kangaroo) cells. The last unattached kinetochore was destroyed by laser ablation, and this was sufficient to relieve the checkpoint, even though presumably the sister kinetochore was not under tension (Rieder et al., 1995). The support for tension came from micromanipulation experiments in mantid spermatocytes. Unpaired chromosomes are attached but activate the checkpoint. Pulling on them with a needle to create tension across the un-paired kinetochore also relieved the checkpoint (Li and Nicklas, 1997). There is also some evidence to suggest different
checkpoint proteins respond to differing signals. Mad2p disappears from attached kinetochores in mammalian cells in the absence of tension whereas BubR1 and Bub1 remain on the kinetochore under conditions of attachment but not tension (Skoufias et al., 2001; Waters et al., 1998; Zhou et al., 2002). Other evidence suggests Mad2p is involved in a tension-sensing pathway (Shannon et al., 2002).

Since then, no definitive answer has been gained. There is evidence (reviewed in (Millband et al., 2002; Musacchio and Hardwick, 2002; Pinsky and Biggins, 2005)) to support both tension and attachment cases, and the solution is probably a combination of both, with the relative importance dependent on organism, cell type and other circumstances.

1.2.4 Mechanics of the cell cycle
This section has focused on the proteins that control the cell cycle. These proteins described in 1.2 control the mechanics of the cell cycle. The most important structure for accurate chromosome segregation is the mitotic spindle. It must mediate the segregation of one of each sister chromatid pair to each daughter cell. The rest of this chapter focuses on the proteins and complexes that make up the structure of the mitotic spindle. The mitotic spindle is made of microtubules (section 1.3) that are organised at their minus ends by spindle pole bodies (SPBs) in yeasts (section 1.6) and centrosomes in higher organisms (section 1.7). At their plus ends, the microtubules interact with the cell cortex, each other, or the sister chromatids via kinetochores (section 1.5). As well as proteins of the SPBs, centrosomes and kinetochore, other microtubule-associated proteins (MAPs) interact with microtubules. Some MAPs crosslink microtubules, contributing to mechanical strength in the spindle, while others control the movements and interactions of the microtubules (section 1.4). First I discuss the skeleton of the spindle, the microtubules.
Figure 1.3. A) Microtubule structure. (Adapted from Desai and Mitchison, 1997). B) Microtubule dynamics (Taken from Kline-Smith and Walczak, 2004).
1.3 Microtubules

1.3.1 Structure
Microtubules are cylindrical polymers of α and β tubulin. The smallest subunit is a heterodimer of α and β tubulin. These dimers assemble end on end to form linear 'protofibers' so that one end is alpha subunit and other is beta (figure 1.3A). These protofibers associate laterally into a hollow cylinder. *In vitro* the protofiber number varies from 10 to 15, but *in vivo* most microtubules have a protofiber number of 13 (Evans et al., 1985). The protofibers assemble with α contacting α, and β contacting β. In a 13 protofiber microtubule (and in the case of all odd numbers), this results in a 'seam' where subunits contact their opposite tubulin. The subunits interact laterally with an offset of 10° such that if contacting subunits are followed laterally along the microtubule they form a helix (Kikkawa et al., 1994; Mandelkow and Mandelkow, 1986; Song and Mandelkow, 1993). This helix is '3-start' which means that in one helical turn the length of 3 monomers has been travelled (figure 1.3A). Microtubule structure is reviewed in (Desai and Mitchison, 1997)

This structure results in the microtubule being polar – the end with exposed α subunits is the ‘minus’ end and the end with β exposed in the ‘plus’ end (Allen and Borisy, 1974; Amos and Klug, 1974). *In vitro*, the plus end polymerizes faster and is more dynamic than the minus end. In a cell factors bind to these ends and regulate the polymerisation and depolymerisation. This is discussed in section 1.4.

In yeast there are two α tubulin genes, *TUB1* and *TUB3* (Schatz et al., 1986a; Schatz et al., 1986b). *TUB3* is expressed at a much lower level. The protein products are 90% similar, and can substitute for each other if present in sufficient quantities. The only β tubulin gene is *TUB2* and it is essential (Neff et al., 1983).

1.3.2. Microtubule dynamics
After an initial nucleation step (see 1.3.3), polymerization begins from short protofibers, onto which dimers assemble until a sheet is gained. This then folds
to form a cylinder. *In vitro* observations of microtubules by electron microscopy show these sheets at the plus ends, suggesting this sheet is a standard precursor (Arnal et al., 2000; Chretien et al., 1995).

Tubulin is a GTPase whose binding activity is regulated by bound GDP/GTP (Erickson and O'Brien, 1992). GTP-tubulin binds the end of the microtubule with high affinity. The GTP binds a pocket of the β subunit at the plus end of the microtubule. GTP cannot be hydrolysed however, until an additional α subunit of the next dimer binds and contributes residues to the GTP binding pocket (Mitchison, 1993; Nogales et al., 1999). This means hydrolysis does not take place until the next dimer binds, promoting polymerisation. Tubulin in the bulk of the microtubules is GDP bound with a 'cap' of GTP bound dimers at the ends (Caplow and Shanks, 1996; Drechsel and Kirschner, 1994). GDP-tubulin is unstable and promotes depolymerisation of the microtubule. Once the process is reversed and the GTP bound cap removed, depolymerisation is rapid as the bulk of the microtubule is in the GDP bound form.

Current theories propose a structural change in the tubulin dependent on which nucleotide is bound. Shrinking ends have a frayed appearance with protofilaments curling away separately (often called 'rams-horns') (Arnal et al., 2000; Mandelkow et al., 1991). This is different to the sheet seen for polymerising microtubules. This suggests a structural change between GTP and GDP bound forms. The current model is that the GTP bound form creates straight protofilaments whereas the GDP bound form is naturally curved (Hyman et al., 1995; Melki et al., 1989). The straight GTP bound filaments bind easily into the lattice, however the GDP bound bulk is constrained, and the release of this conformational constraint is what allows depolymerisation to be rapid. Presumably these 'rams horns' are seen as the longitudinal bonds are slower to dissociate than the lateral bonds. These structural changes are illustrated in figure 13B. Microtubule dynamics are discussed in (Desai and Mitchison, 1997; Howard and Hyman, 2003; Kline-Smith and Walczak, 2004).
In vitro microtubules show dynamic instability, mostly at their plus ends (Mitchison and Kirschner, 1984). This is characterised by periods of growth and shrinkage switched between by catastrophe (growth to shrinkage) or rescue (shrinkage to growth). In vivo, the levels and direction of this dynamic instability are spatially and temporally controlled by many factors on the spindle and in the cytoplasm. Proteins that bind microtubules are called MAPs (Microtubule associated proteins), and these proteins are discussed in section 1.4.

1.3.3 Nucleation

In vivo, most microtubules are nucleated and stabilised at their minus ends by microtubule organising centres. The protein responsible for nucleation is another form of tubulin, γ-tubulin. Gamma tubulin was first discovered in *Aspergillus* (Oakley and Oakley, 1989) and has homologues in a wide range of organisms. The budding yeast protein, Tub4p was discovered by sequence homology (Sobel and Snyder, 1995).

γ-tubulin complexes

γ-tubulin exists in two complexes in metazoans. The γ-tubulin small complex (γ-TuSC) associates with other small complexes and extra proteins to form the larger γ-tubulin ring complex (γ-TuRC). In budding yeast, only the small complex is present and it is composed of two molecules of γ-tubulin along with one each of the Spc97p and Spc98p (Knop et al., 1997; Knop and Schiebel, 1997). Spc97p homologues are hGCP2 (*H. sapiens*) and Dgrip84 (*Drosophila*) and Spc98p homologues are hGCP3 (*H. sapiens*), Dgrip109 (*Drosophila*) and Xgrip109 (*Xenopus*). Although first discovered in budding yeast (Knop and Schiebel, 1997), this smaller complex was subsequently shown to be a component of the larger complex seen in higher organisms (Oegema et al., 1999).

The larger complex was first purified from *Xenopus* egg extracts (Zheng et al., 1998). There are 10-13 γ-tubulin molecules and at least eight additional proteins (Martin et al., 1998; Murphy et al., 2001; Oegema et al., 1999; Zheng et al., 1998). The complex is ring shaped, with a 25 nm diameter, which lead to the name γ-tubulin
ring complex (γ-TuRC) (Zheng et al., 1998). The γ-TuRC complex can enhance microtubule nucleation \textit{in vitro} more efficiently than the small complex (Oegema et al., 1999). All microtubules in budding yeast are nucleated by SPB associated γ-tubulin, so this small complex nucleating activity appears to be sufficient \textit{in vivo}. γ-tubulin complexes also ‘cap’ pre-formed microtubules, preventing dynamics \textit{in vitro} (Wiese and Zheng, 2000), and presumably allowing control of microtubule dynamics \textit{in vivo}.

Early studies on both \textit{Xenopus} and \textit{Drosophila} γ-tubulin ring complexes by EM showed a similar ‘ring’ conformation for both, suggesting the general structure is conserved. Higher resolution was provided by the examination of \textit{Drosophila} γ-TuRC by electron tomography (Moritz et al., 2000) (reviewed in (Moritz and Agard, 2001)). The ‘ring’ is not a flat ring but slanted with a helical arrangement of about 12 (from 9-14, but 12 most often) subunits forming the walls. The subunits appear to be in pairs, angled so they point together at top of ring and away at bottom of ring. Presumably these are made up of small complexes, 6 of which would give 12 gamma tubulin molecules. On the ‘top’ of the ring where the subunits point towards each other there is a globular cap sitting asymmetrically. This is most likely made up of Dgrips 163, 128 and 75, which are present at a lower stoichiometry (Oegema et al., 1999). It is likely that this cap faces away from the microtubule, and the other face provides the nucleation.

The crystal structure of monomeric human γ-tubulin was solved recently by Aldaz et al (2005). It is the first crystal structure of a monomeric tubulin. It also reveals that the structure of γ-tubulin to be broadly similar to that of α and β tubulins. The structure was solved with it bound to a non-hydrolysable GTP analogue. It reveals the γ-tubulin molecule has a similar curved conformation to GDP bound α and β tubulin. The crystal packing structure of γ-tubulins is similar to the lateral interactions seen between protofilaments in a microtubule. By providing a template as such, it might promote the weak lateral interactions of new protofilaments (Aldaz et al., 2005).
Microtubule nucleation

How do these structures fit into models of microtubule nucleation? All studies point towards a templating mechanism where the ring complex provides the first helix of tubulins so that the $\gamma$-tubulin molecules form longitudinal contact with $\alpha$ tubulins (reviewed by (Job et al., 2003; Moritz and Agard, 2001; Schiebel, 2000)). The exact number of small complexes in the $\gamma$-TuRC has not been determined, but as each small complex contains two $\gamma$-tubulin molecules there must be an even number of $\gamma$-tubulins in the ring complex. Presumably this is either 12 with a gap in the helix, or 14 with an overlap of two $\gamma$-tubulins. Immuno-EM studies on Xenopus $\gamma$-TuRCs bound to microtubules showed $\gamma$-tubulin restricted to one end of the microtubule, which supports this templating model (Keating and Borisy, 2000; Wiese and Zheng, 2000).

1.3.4. Microtubule dynamics in the mitotic spindle

Spindle microtubules

There are three categories of spindle microtubule; kinetochore microtubules, interpolar microtubules, and astral microtubules. Astral microtubules extend from the microtubule organising centre to the cell cortex and are involved in correctly positioning and orientating the spindle. In yeasts, which undergo closed mitosis, these are nucleated from the cytoplasmic side of the spindle pole body. From the nuclear face of the yeast SPB, or the centrosome, kinetochore microtubules extend to attach to kinetochores. In budding yeast there is only one microtubule per kinetochore, in fission yeast 2-5, and in higher organisms many, which sometimes bundle to form kinetochore fibres (K fibres). The structure of the budding yeast spindle is illustrated in figure 1.4.

The third category of spindle microtubules is interpolar microtubules. These extend from each organising centre and overlap in the centre of the spindle. Proteinaceous cross-bridges between these microtubules are seen by EM and are formed from several classes of microtubule associated proteins (section 1.4). These maintain the structure of the mitotic spindle.
Figure 1.4. Microtubule structure and associated MAPS in the budding yeast mitotic spindle.
3D modelling on the budding yeast spindle suggests in addition to just one microtubule per kinetochore (32, two for each of 16 chromatid pairs), there are on average 8 interpolar microtubules (O'Toole et al., 1999; Winey et al., 1995). The basic structure is the same for all organisms, although spindles vary greatly in size from the 1 μm metaphase spindle of the budding yeast to the ~1-20 μm spindles of mammals.

Tanaka et al propose the following in budding yeast: the kinetochores remain attached to the SPBs until S-phase when the DNA is replicated. The DNA is replicated and the spindle pole body duplicated. The kinetochores are re-assembled and microtubules from both poles search for kinetochores and capture them. Upon establishment of a bipolar spindle, if kinetochores are attached correctly tension promotes stability of the MT-KT attachment (Tanaka et al., 2005).

In animal cells bipolar attachment results in the condensed chromosomes lining up in the centre of the spindle – the ‘metaphase plate’ (Bajer and Mole-Bajer, 1986; Skibbens et al., 1993). In budding yeast there is no metaphase plate as such. Straight et al used GFP labelled centromeres to visualise kinetochore movements in budding yeast mitosis. In metaphase the kinetochores oscillated anywhere from the spindle centre to next to a SPB, and continued oscillating until anaphase (Straight et al., 1997). This suggests the microtubules are highly dynamic and undergo polymerisation and depolymerisation in metaphase. The two kinetochores from one chromatid pair can be resolved as two GFP spots and move independently, separating and coming back together in what is termed ‘breathing’, in a balance of forces between microtubules and centromeric cohesin (Goshima and Yanagida, 2000; He et al., 2000).

Anaphase constitutes two phases – A and B. In anaphase A the kinetochores move towards the poles due to shortening of the kinetochore microtubules. This is not a major factor in budding yeast as spindles are short (1-1.5 μm) and the kinetochores can be separated by up to 1 μm in metaphase.
In anaphase B the spindle lengthens by separation of the spindle pole bodies. In budding yeast there are two phases: an initial fast phase, and subsequent slower phase. Straight et al propose that the fast phase is the sliding of interpolar microtubules past each other, whereas the slow phase also requires polymerisation at the plus ends (Straight et al., 1997; Straight et al., 1998). In yeast, anaphase B is very important as the spindle can go from 1 μm to about 10 μm. O’Toole suggest that the actual force contribution from interpolar microtubules is likely to be small, as there are only 8 in total (O’Toole et al., 1999).

*In vivo* microtubule behaviour needs to be regulated appropriately for different cell cycle stage and functions. Dynamics are directed by a wide range of microtubule-associated proteins (MAPs), which in turn are controlled by the cell cycle regulators discussed in 1.2

**1.4 Microtubule associated proteins**

**1.4.1 Overview**
The first microtubule associated proteins (MAPs) were co-purified with microtubules from brain extract. Since then many classes have been characterised. They can be broadly characterised into those that use ATP to produce force on the microtubule (motors) or those that bind microtubules nucleotide-independently (non-motor MAPs).

Motor proteins have motor domains that bind microtubules. They either bind cargo and transport it along the microtubules, or bind and control microtubules. There are two major classes of motor proteins, the kinesins and dyneins. Dyneins are minus-end directed motor proteins; in yeast mitosis they are on the astral microtubules, controlling nuclear position. Kinesins are a family of motor proteins that all contain a conserved core motor domain that binds microtubules. The motor domain can be at C or N terminus, or internal. Recently a standardised nomenclature for the large collection of kinesins from different organisms was proposed (Lawrence et al., 2004). Families 5, 8, 13 and 14 are involved in the mitotic spindle. The basic
structure of kinesins is of a ‘head’ domain, which is the motor domain, followed by a long coiled coil neck domain, which is often used to multimerise.

There are many MAPs for all microtubule functions and cell cycle stages, here the ones key for mitosis are reviewed. An overview of the MAPs in the budding yeast spindle is shown in figure 1.4.

1.4.2 Sliding motors and midzone- spindle structure

**BimC family (kinesin 5)**

One of the main families of kinesins in the mitotic spindle is the BimC family, named after the BimC motor of *Aspergillus nidulans*. They were the first kinesins shown to have a role in the mitotic spindle. Family members include cut7 of *S. pombe*, Cin8p and Kip1 of *S. cerevisiae*, KLP61F of *Drosophila* and Eg5/HsEg5 of *Xenopus* and humans respectively. They act as homo-tetramers with two motor domains at each end. Instead of transporting cargo they cross link anti-parallel microtubules in the spindle. With both sets of motor domains moving towards the plus ends, the microtubules are slid past each other, causing spindle elongation. Recent work on Eg5 showed that it moves on both microtubules it crosslinks, creating a combined rate of 40 nm/s. This rate is comparable to that of spindle pole separation *in vivo* (Kapitein et al., 2005).

Cin8p (Hoyt et al., 1992) and Kip1p (Roof et al., 1991) are budding yeast members of the BimC family. Cin8p and Kip1p are individually non-essential at moderate temperatures, but the activity of at least one is required for viability. They appear to have overlapping redundant roles in the budding yeast mitotic spindle. *cin8Δ* cells are defective in the initial rapid separation of SPBs in anaphase B, whereas *kip1Δ* cells are defective in the second slow stage (Straight et al., 1998). Their activity is required to separate spindle poles in S-phase, and to maintain spindle integrity in mitosis (Hoyt et al., 1992; Roof et al., 1992; Saunders and Hoyt, 1992). Pre-anaphase spindles collapse when both proteins are inactivated, however, post anaphase spindles seem ok (Saunders and Hoyt, 1992). Overexpression of Cin8p
results in premature spindle elongation (Saunders et al., 1997b). In addition, both Cin8p and Kip1p are seen at kinetochores, and are thought to be involved in correct alignment and clustering of kinetochores through their microtubule crosslinking functions (Tytell and Sorger, 2006).

**Bundling**

There are also classes of non-motor MAPs that crosslink microtubules in the spindle midzone contributing mechanically to spindle structure. In budding yeast these are represented by Ase1p and Stu1p.

Ase1p (*Anaphase spindle elongation*) was first discovered as a midzone factor in budding yeast required for anaphase B (Pellman et al., 1995). It has a homologue in fission yeast and one named Prc1 in humans (Jiang et al., 1998).

Both the budding yeast and human forms are cell cycle regulated with low levels in G1 and a peak in mitosis (Jiang et al., 1998; Pellman et al., 1995). The budding yeast form is an important target of the anaphase promoting complex (Juang et al., 1997). A non-degradable form of the budding yeast protein delays spindle disassembly (Juang et al., 1997) and overexpression in an S-phase arrest results in abnormal spindle elongation (Schuyler et al., 2003). Correspondingly a loss of function causes premature spindle disassembly in anaphase. Analysis of fluorescently tagged Ase1p shows a slow rate of turnover at the midzone and low lateral diffusion. This suggests that Ase1p acts as a cross-bridge which gives the midzone matrix-like qualities (Schuyler et al., 2003).

The human form, Prc1 is a target of Cdks and is necessary for cytokinesis in addition to midzone functions (Jiang et al., 1998). The human and yeast proteins only share 40% similarity, so it is likely there are extra functions they do not share. As well as the spindle midzone, fission yeast Ase1p is seen at point of microtubule minus end microtubule overlap at interphase and the SPB (Loiodice et al., 2005). SpAse1p is also implicated in microtubule bundling in cytokinesis (Yamashita et al., 2005).
The other budding yeast midzone factor is Stulp, of the MAST/Orbit/CLASP family (Yin et al., 2002). Stulp temperature sensitive mutants fail in spindle pole body separation, and pre-built spindles collapse (Yin et al., 2002). This spindle collapse is similar to shorter spindles or unseparated SPBs seen in cin8 mutants (Roof et al., 1992; Saunders and Hoyt, 1992). In fact the stul mutants, but not the deletion, can be rescued by doubling expression of Cin8p (Yin et al., 2002).

These results are paralleled in work on Drosophila Mast/Orbit protein. MAST/Orbit localises to the spindle and is required for spindle organisation (Inoue et al., 2000; Lemos et al., 2000). Mast/Orbit deficient embryos build bipolar spindles that collapse after nuclear envelope breakdown (Maiato et al., 2002). This is similar to a phenotype seen the in Drosophila Cin8p family member, KLP62F (Sharp et al., 1999). This parallels the phenotype and co-operation of Cin8p and Stulp in crosslinking microtubules at the midzone in budding yeast.

1.4.3 MAPs that affect microtubule dynamics

Kin I kinesins (kinesin-13)
The Kin I (kinesin internal catalytic domain) are a family of motors that includes MCAK of humans. Instead of moving along or sliding microtubules they use the energy from ATP to cause depolymerisation by removing tubulin subunits. Overexpression of MCAK leads to almost complete loss of microtubules. Loss of Xenopus XKCM1 from egg extracts increases the size of arrays due to less microtubule catastrophe (Walczak et al., 1996).

MCAK is seen at kinetochores and probably contributes to the poleward movement of chromosomes in anaphase A. In vitro studies, however, suggest it can destabilise either end of microtubules with similar rates (Hunter et al., 2003), so activity must be regulated by where it is localised. It is thought that an overall increase in catastrophe, when balanced with promoting factors, maintains dynamic properties of the spindle and allows it to be remodelled correctly and quickly.
Work on the *Drosophila* MCAK homologue KLP59C, and on KLP10A another kinesin 13 found on centrosomes during anaphase, suggests that Kin I activity at both ends of the spindle microtubules is important in anaphase A (Rogers et al., 2004). This creates a poleward flux of microtubule carrying the chromosomes outward. However, work in vertebrates suggests the main role may be to depolymerise incorrectly attached microtubules (Maney et al., 1998).

Studies on the mechanism of Kin I kinesins have been done mainly on MCAK. It can cause processive depolymerisation at a rate of 20 tubulins/s (Hunter et al., 2003). Full depolymerisation activity can be gained from the motor domain and N-term alone in monomeric form (Maney et al., 2001) although it normally works as a dimer *in vivo*. Experiments suggest that it works by stabilising the curved, GDP bound form of tubulin protofilaments (Desai et al., 1999). MCAK has high affinity for microtubule ends, and finds ends faster than can be explained by diffusion or the rate of other kinesins. Hunter *et al* (2003) suggest that this is by ‘gliding’ along the microtubule.

**Kip3p (kinesin-8)**

Kip3p of *S. cerevisiae* is also a depolymerising kinesin (initially placed in the KinI family). The kinesin domain is actually N-terminal, and now it defines the kinesin-8 family along with Klp5 and Klp6 of *S. pombe* and KLP67A of *Drosophila* (Lawrence et al., 2004; Tytell and Sorger, 2006). It has similar depolymerising activity to the Kin I (kinesin 13) family, and therefore would be predicted to have a role in anaphase A movement. Anaphase A is less important in budding yeasts than higher organisms and anaphase defects are subtle in *kip3* mutants. Chromosomes are segregated correctly in *kip3Δ* cells, but kinetochore movement in anaphase is not as synchronised as in wild type (Tytell and Sorger, 2006). So Kip3p appears to be involved in co-ordinating anaphase kinetochore movement. The post-anaphase spindles in *kip3Δ* strains break down later than those of wild type and continue elongating so that they become longer than the cell and have to bend (Straight et al., 1998). This suggests that Kip3 is involved in destabilising the spindle microtubules at the end of mitosis in budding yeast.
XMAP215 and homologues

Another key family of MAPs that regulate microtubule dynamics are that of *Xenopus* XMAP215, and its homologues TOG (humans), Msps (*Drosophila*) and Stu2p in (*S. cerevisiae*) (Charrasse et al., 1998; Cullen et al., 1999; Garcia et al., 2001; Popov et al., 2001; Wang and Huffaker, 1997). Most homologues localise to spindle poles and the spindle, as well as the kinetochores in yeasts (Garcia et al., 2001; He et al., 2001; Nakaseko et al., 2001). Mutants in all organisms show various microtubule defects (reviewed in (Kinoshita et al., 2002; McNally, 2003; Popov and Karsenti, 2003)).

XMAP215 was first identified as a factor that promoted microtubule dynamicity *in vitro* (Gard and Kirschner, 1987). It has since been shown to have many abilities *in vitro*, both increasing polymerisation and depolymerisation rate, and reducing rescue events (Vasquez et al., 1994). These all lead to highly dynamic microtubules that can adapt to the situation. Other evidence suggests oppositely that it can also suppress catastrophe. In agreement with this, in an *in vitro* assay with purified proteins tubulin, XMAP and the Kin 1 kinesin alone can replicate microtubule dynamic behaviour *in vivo* (Kinoshita et al., 2001).

The budding yeast homologue, Stu2p shows slightly different behaviour. *In vitro* it slows the plus end polymerisation and depolymerisation, and increases catastrophe (van Breugel et al., 2003). van Brugel *et al* suggest it induces catastrophe by interfering with tubulin addition at plus ends. All family members appear to have slightly different activities, but this may be due to experiments *in vitro* vs *in vivo* and different cell cycle state and conditions. McNally suggests that the conserved family domain may be related more to specific plus end binding than a specific role in microtubule dynamics (McNally, 2003).

1.4.4. Dual functions – the Kar3 family (kinesin-14)

cin8/kip1 mutations in budding yeast can be partially rescued by a deletion or motor domain mutants of another motor, Kar3p (Hoyt et al., 1993). Kar3p has a C-terminal kinesin domain and has homologues including klpA in *Aspergillus* and Kl2p in *S.*
Overexpression of Kar3p results in shorter mitotic spindles (Saunders et al., 1997b). Work in *Drosophila* agrees with the yeast data suggesting Kar3p opposes the activity of Cin8p and Kip1p in mitotic spindle structure. The Kar3p homologue, Ncd opposes the action of the bimC homologue KLP61F (Sharp et al., 1999; Wilson et al., 2004).

Kar3p has other functions in addition to its role in the mitotic spindle. It was originally identified by virtue of its involvement in karyogamy, probably in shortening overlapped microtubules between the two mating nuclei (Rose, 1996), and may also have roles in regulating cytoplasmic microtubules in mitosis (Saunders et al., 1997a). It also has roles in meiosis (Bascom-Slack and Dawson, 1997). It was initially assumed that these functions were carried out by minus end directed sliding of microtubule, similar and opposite to those of the bimC family.

Kar3p was the first *S. cerevisiae* kinesin for which microtubule motor function was established *in vitro*. In a motility assay it moved microtubules at a rate of 1-2 μm/min. In these assays it also destabilised the minus ends of taxol-stabilised microtubules (Endow et al., 1994). This is an unusual phenotype for KinC family members, and the first report of a dual-function family of kinesins. After some confusion, this depolymerisation is now thought to be almost exclusively at plus ends *in vivo* (Sproul et al., 2005).

The mechanism of promoting depolymerisation is different to that of the Kin I kinesins. The Kar3p family do not promote catastrophe, instead each ATP hydrolysis is linked to the removal of one tubulin heterodimer, but the motor stays bound and continues toward the minus end (Sproul et al., 2005). The only homologue for which this depolymerisation activity has been confirmed *in vivo* is *S. pombe* Klp2 (Troxell et al., 2001), although *Drosophila* Ncd depolymerises plus ends with similar kinetics to Kar3p *in vitro* (Sproul et al., 2005).

Kar3p acts as a heterodimer with another non-motor protein, Cik1p, in the cytoplasm and Vik1p in the nucleus (Barrett et al., 2000; Manning et al., 1999). These binding
partners regulate Kar3p activity. Kar3p is seen mostly at SPBs in budding yeast, opposing the activity of Cin8p and Kip1p. It has recently been demonstrated that it is also present at unattached kinetochores, possibly functioning in microtubule kinetochore capture (Tanaka et al., 2005; Tytell and Sorger, 2006).

### 1.4.5 Other notable MAPs

**Spindle/nuclear position**

Similar factors operate on cytoplasmic microtubules. Astral microtubules are controlled at both their plus and minus ends, and serve to position the nucleus (yeast) or spindle correctly in mitosis. Astral microtubules also contribute to spindle elongation by pulling the spindle poles apart.

Budding yeast Dyn1p (dynein), Kip2p and Kip3p are involved in spindle positioning and anaphase spindle elongation through regulation of the astral microtubules (Cottingham and Hoyt, 1997; DeZwaan et al., 1997; Saunders et al., 1997a; Saunders et al., 1995; Yeh et al., 1995). Kip2p also positions the Biki1p plus end binding protein (Carvalho et al., 2004).

**Plus end tracking**

A subset of non-motor MAPS are ‘plus end tracking’ - found at the plus end and thought to stabilise interactions there. Two major families are represented in budding yeast as Bik1 (CLIP-170 in mammals) (Berlin et al., 1990; Pierre et al., 1992) and Bim1 (EB1 in humans) (Rogers et al., 2002; Schwartz et al., 1997).

Most functions of these proteins are in regulating cytoplasmic plus ends and their interactions with organelles and the cell cortex. A few mitotic roles have been described, however. CLIP-170 and its homologues in *Drosophila* and budding yeast have been seen at kinetochores (Dujardin et al., 1998; Dzhindzhev et al., 2005; Lin et al., 2001; Maiato et al., 2002).
Bik1p is not essential in budding yeast and there is partial functional overlap with Ase1p in its mitotic functions only (Pellman et al., 1995). Overexpression of Bik1p results in long cytoplasmic microtubules and short spindles. Its function appears to be microtubule plus end stabilisation during mitosis and karyogamy (Berlin et al., 1990; Pellman et al., 1995).

Kar9p is another MAP that links microtubules to actin at the cell cortex (Miller et al., 1999). It is involved in spindle positioning in S. cerevisiae. It interacts with Bim1p, Bik1p, Stu2p (XMAP215) and Kip2p.

1.5 Kinetochores

1.5.1. Overview
Clearly if the function of the spindle is to facilitate correct segregation of the chromosomes, then correct and regulated attachment of the chromosomes to the spindle is very important. There must be one and one only microtubule attachment site per sister chromatid and mechanisms for ensuring and monitoring correct attachment. Sites on sister chromatids must be able to monitor events at the other sister in order to ensure correct bi-orientation (attachment of one to each pole). The site at which microtubules bind DNA is a specific region of heterochromatin called the centromere, upon which a large protein structure, the kinetochore, is built.

Kinetochores are large and complicated structures. Even that of budding yeast, one of the simplest and best characterised, contains over 65 proteins organised into at least 17 sub-complexes (De Wulf et al., 2003). The yeast kinetochore is approximately 5 megadaltons, but still the best understood. The complexes of the budding yeast kinetochore are illustrated in figure 1.5A.

Broadly speaking there are four categories of kinetochore proteins; those that bind centromere DNA, those that link these inner proteins to the microtubule binding complexes, the microtubule interactors, and control elements (coloured differently in figure 1.5A). If the kinetochore is assumed to be layered structure of these sets of
Figure 1.5. The budding yeast kinetochoore. A) Proteins and complexes of the budding yeast kinetochoore. Taken from McAinsh et al. (2003). Complexes are colour coded according to layer. B) Model for interaction of the Dam1 ring complex with microtubules. Taken from Salmon (2005). The Dam1 ring complex surrounds microtubules held in place by electrostatic interactions and is tethered to the kinetochoore via the Ndc80 complex.
proteins, then we would expect each subsequent layer to be dependent on the last, but independent of other members of its layer. Therefore the inter-dependencies between proteins and complexes can help solve the structural puzzle. Kinetochore structure is also reviewed in (Cheeseman et al., 2002b; McAinsh et al., 2003; Measday and Hieter, 2004).

1.5.2 Centromeres
In budding yeast the centromere is only 125 bp, but those of higher organisms are several megabases and thought to be determined by epigenetic mechanisms rather than a specific sequence (Choo, 2001). The 125 bp sequence on each budding yeast chromosome is made up of regions CDE I-III (Fitzgerald-Hayes et al., 1982). CDEII is an 80 bp AT rich region in the centre and chromatin packaged with a centromere specific nucleosome - Cse4p (CENP-A in vertebrates) instead of histone H3 (Keith et al., 1999; Meluh et al., 1998). A dimer of Cbf1p binds the CDEI region and is thought to induce DNA bending (Mellor et al., 1991).

The ‘Cbf3’ complex (NdclOp, Cep3p, Cfl13p, Skp1p) binds the CDE III region (Lechner and Carbon, 1991). Cse4p nucleosomes are dependent on this complex, although CBF3 can bind independently of Cse4p (Measday et al., 2002; Ortiz et al., 1999), suggesting counter intuitively that the CBF3 complex may encourage the centromere specific binding of Cse4p rather than the other way round. The Cbf3p complex is required for binding of virtually every kinetochore component, so is a key factor. The Ndc10p subunit is the only one to show DNA binding in vitro (Espelin et al., 2003).

1.5.3 Protein complexes of the central kinetochore
The area of the kinetochores between the CBF3 complex and microtubule binding complexes is the most complex and least understood. It is made up, in part, of several independent subcomplexes.

The Ndc80 complex is named after its most well characterised component; it also contains Nuf2p, Spc24p and Spc25p. All components are essential and localise
exclusively to kinetochores (He et al., 2001; Janke et al., 2001; Wigge and Kilmartin, 2001). Ndc80p and Nuf2p are conserved and also required for chromosome segregation in humans and *Xenopus* (DeLuca et al., 2002; Hori et al., 2003; Howe et al., 2001; Martin-Lluesma et al., 2002; McCleland et al., 2003; Wigge and Kilmartin, 2001; Zheng et al., 1999). In mutants of the Ndc80 complex, kinetochores are completely detached from the microtubule suggesting it is important in mediating interactions between the DNA bound and microtubule bound complexes (De Wulf et al., 2003).

Initially, twelve proteins co-purified with Ctf19, but different centromere binding properties of individual subunits suggested more complicated organisation. De Wulf *et al* using a number of techniques clarified the organisation further into subcomplexes (De Wulf et al., 2003).

Ctf19p itself was present in a smaller ‘COMA’ complex, a heterotetramer with Okp1p, Mcm21p and Ame1p. It seemed to be made from assembly of two independent dimers of Okp1p-Ame1p and Mcm21p-Ctf19p. Only Okp1p and Ame1p are essential, and Ame1p localises in the absence of the Mcm21p-Ctf19p dimer. The authors suggest that the latter may regulate timing, as the full complex is not present in G1. Mtw1p was in the ‘MIND’complex, also including Nsf1p, Nsl1p, Dsn1p and possibly another protein or extra copy of the above. Assembly intermediates lacking the extra protein (named M2) and also lacking Dsn1p (named M1) were found. Unlike Ndc80 complex mutants, kinetochores of MIND and COMA mutants are still attached to microtubules, but are deficient in the forces that pull chromatids apart transiently in metaphase (De Wulf et al., 2003).

In addition there are several other proteins (Iml1p, Chl4p, Nkp1p1, Nkp2p, Mcm16p, Mcm22p and Ctf3p) that occupy the “grey area” between centromeric binding and microtubule binding complexes. Three non-essential components Ctf3p, Mcm16p, and Mcm22p immuno-precipitate together (Measday et al., 2002) and are currently thought to be involved in centromeric chromatin assembly. The *S. pombe*
homologue of Ctf3p, Mis6 is one of the factors required for centromeric recruitment of Cse4p homologue Cnp1 (Takahashi et al., 2000a).

Concurrent with the ‘layer’ model De Wulf et al suggest COMA, MIND and Ndc80 complexes can all associate with centromeric DNA independently. Experimental data so far has proved that neither MIND nor COMA require the Ndc80 complex, and COMA does not require MIND (De Wulf et al., 2003). All four complexes discussed depend on the CBF3 complex for kinetochore localisation and Cse4p (De Wulf et al., 2003; He et al., 2001; Janke et al., 2001; Ortiz et al., 1999) except Ndc80 which does not require the Cse4p nucleosome (Gardner et al., 2001; McCleland et al., 2003; Measday et al., 2002).

1.5.4 Microtubule attachment

A major microtubule binding complex at the kinetochores is the Dam1 complex (Dam1p, Duo1p, Dad1p, Spc19p, Spc34p, Ask1p, Dad2p, Dad3p, Dad4p, Hsk3p). This complex requires the Ndc80 complex in order to bind kinetochores (He et al., 2001) defining a further outer layer of the kinetochore.

Dam1 complex mutants show monopolar attachment, but not all chromosomes go to the same pole (Janke et al., 2002). This suggests that kinetochores can capture microtubules, but not withstand the tension generated by bipolar attachment. The complex is present along the mitotic spindle but concentrates at kinetochores during metaphase (Jones et al., 2001).

There has been recent progress on the structure and microtubule binding of the Dam1 complex. Two independent groups discovered that 12-15 dimers of Dam1 complexes assemble to form rings around the microtubules (Miranda et al., 2005; Westermann et al., 2005). The ten subunits co-expressed in bacteria, when mixed with microtubules in vitro, assemble the ring structure even at low concentrations. The complex can bundle microtubules, probably through ring-ring interactions (Westermann et al., 2005).
The rings bind the C-termini of both α and β tubulin, and have preference for the GTP bound tubulin (Westermann et al., 2005). This preference for the GTP cap explains how they may recognise the ends of growing microtubules. Electron microscopy on ring complexes sectioned end-on suggested 16 fold symmetry, which is out of phase with the 13 fold symmetry of the microtubule. It is thought the interaction is electrostatic, the Dam1 ring exposing positive charges that interact with the negative charges on the surface of the microtubule (Westermann et al., 2006).

As the microtubules depolymerise in vitro, fluorescently labelled Dam1 complex can be seen to slide along (Westermann et al., 2006). As described earlier, the GDP bound protofilaments on depolymerising microtubules curve out, releasing the conformational constraint enforced in the microtubule lattice. It is currently thought that this outward force powered by the release of constraint pushes the Dam1 complex along the microtubule, and it slides along due to its electrostatic interactions (Salmon, 2005; Westermann et al., 2005; Westermann et al., 2006). The model for interaction of Dam complex with microtubules is illustrated in figure 1.5B.

How the Dam1 ring complex of budding yeast applies to higher organisms is not yet clear. Homologues have been found in fission yeast only (Liu et al., 2005) but it is unclear if the complex and structure are the same. Kinetochores are complicated in most other organisms by the need to bind many, instead of just one microtubule and these must all come from the same pole.

Many of the MAPs already discussed in section 1.4 are seen at the kinetochores or thought to function there. Cin8p, Kip1p and Kip3 motors associate with centromeric DNA (McAinsh et al., 2003). Cin8p and Kip1p, of the bimC plus end crosslinking family might help to cluster microtubules in metaphase, and their kinetochore association depends on the Ndc80 complex (Tytell and Sorger, 2006). Kip3p, may play a role in poleward kinetochore movement in anaphase A. Kip3p recruitment to the kinetochore is not dependent on Ndc80, implying it is a core kinetochore protein (Tytell and Sorger, 2006).
Stu2p, the XMAP215 homologue is localised to kinetochores (He et al., 2001). Stu2p destabilises plus ends (van Breugel et al., 2003) and microtubules are less dynamic in *stu2* cells (Kosco et al., 2001). *stu2* cells can make bipolar attachments but no sister chromatid separation is seen in metaphase (He et al., 2001), suggesting microtubules can attach but not generate force from depolymerisation.

Plus end tracking proteins Bim1 (EB1) and Bik1 (CLIP-170) are also seen at kinetochores in either yeast or higher organisms (McAinsh et al., 2003), so are likely regulating microtubule dynamics there.

1.5.5. Signalling at the kinetochore

The kinetochores are the point where microtubule attachment and tension across sister chromatids can be monitored. Correspondingly, many checkpoint proteins localise to unattached kinetochores (see 1.2.3). Spindle checkpoint function is dependent on the CBF3 complex (Gardner et al., 2001), Spc24p and Spc25p (Janke et al., 2001; McCleland et al., 2003).

In addition to checkpoint proteins, the budding yeast aurora kinase, Ipl1p is also required in the pathway that sense tension across the kinetochores (Biggins and Murray, 2001). Aurora kinases were originally identified as components of a 'chromosomal passenger complex', a group of proteins who are seen on the chromosomes in prophase, centromere in metaphase and the spindle midzone in anaphase. In addition to aurora kinases these include INCENP/Sli5p and Survivin / Bir1p. There are three aurora kinases in humans: A, B and C, the single budding yeast member, Ipl1p is analogous to aurora B (Adams et al., 2001).

Ipl1p regulates microtubule-kinetochore attachment. Kinetochores are unable to detach in *ipl1* mutants (Tanaka et al., 2002) suggesting it promotes turnover. The point where Ipl1p function is required is before bipolarity is established. If a bipolar attachment is established and then Ipl1p is inactivated, the bipolar attachment is unaffected. The current explanation is that Ipl1p senses a lack of tension and generates unattached kinetochores in order to sever incorrect attachments and also
activate the spindle checkpoint (Pinsky et al., 2006). It may also activate the spindle checkpoint through additional mechanisms such as phosphorylation of Mad3p (E. King, personal communication).

Ipl1p phosphorylates Ndc10p, Cse4p, Ndc80p, Dam1p, Spc34p and Ask1p (Biggins et al., 1999; Buvelot et al., 2003; Cheeseman et al., 2002a; Kang et al., 2001). Dam1p mutants that cannot be phosphorylated mimic ipll mutants, indicating that this phosphorylation of Dam1p is one of Ipl1p’s most important functions (Cheeseman et al., 2002a). The phosphorylation of Dam1p by Ipl1p appears to weaken the interaction between the Dam1 complex and the rest of the kinetochore (Shang et al., 2003). This would promote turnover of kinetochore-microtubule interactions until bipolar attachment is achieved, using tension as an indicator of bipolar attachment. However, unattached chromosomes and spindle checkpoint activation in the dam1 phospho deficient mutant still require Ipl1p, suggesting additional Ipl1 sites on Dam1 or other targets (Pinsky et al., 2006).

1.6 The budding yeast SPB

1.6.1. Microtubule organising centres

At the other end of spindle microtubules from the kinetochores are microtubule organising centres (MTOCs). These nucleate and anchor microtubule minus ends of both spindle and astral microtubules. It is important the spindle is organised with exactly two poles, otherwise the DNA cannot be segregated equally and correctly. Interphase cells have one MTOC which must be duplicated once and only once before formation of the mitotic spindle. The two spindle poles end up one in each daughter cell, pulling the correctly segregated DNA with them. Budding yeast has only one MTOC, the SPB. Other organisms have many in different parts of the cell. S. pombe has three, interphase MTOCs, the equatorial MTOC that organises the post-anaphase array, and the spindle pole body. Higher organisms have many microtubule organising centres, but the mitotic spindle poles are organised by centrosomes (section 1.7). Only a few components are conserved among all mitotic MTOCs; the γ-TuSC, calmodulin, centrin, spc110/kendrin and Stu2/XMAP215. In
this section the *S. cerevisiae* SPB is examined in detail, and section 1.7 deals with MTOCs in other organisms.

### 1.6.2. SPB structure and composition

**Overview**

The structure of the budding yeast spindle pole body was first observed by electron microscopy. These so-called 'spindle plaques' were observed as large layered electron dense structures that spanned the nuclear membrane and nucleated microtubules on both their nuclear and cytoplasmic faces. Initially three electron dense layers, or plaques, were described: a central layer that spans the nuclear membrane, an outer plaque that nucleates cytoplasmic microtubules, and an inner plaque that nucleates nuclear spindle microtubules (Byers and Goetsch, 1974; Byers and Goetsch, 1975). In the last few years electron tomography (O'Toole et al., 1999) and cryo-electron microscopy (Bullitt et al., 1997) have identified further layers. Between the outer plaque and central plaque are two intermediate layers, IL1 and IL2. Associated with one side of the SPB in the plane of the membrane is an electron dense structure of about 80 nm called the half bridge. The new SPB begins assembly as a spherical satellite at furthest tip of the half bridge. The half bridge appear to be composed of two continuous folded layers of membrane, and an additional layer of electron dense material on the cytoplasmic face (O'Toole et al., 1999) and a less dense thin layer sometimes seen on nuclear side (Adams and Kilmartin, 1999). The half bridge can also nucleate cytoplasmic microtubules during G1 and nuclear fusion. O'Toole et al observed two hook like structures associated with the central plaque that are proposed to anchor the SPB in the nuclear membrane. An electron micrograph of a spindle and schematic diagram of a haploid interphase SPB are shown in figure 1.6A and B.

The spindle pole body has a constant height of 150 nm (Byers and Goetsch, 1974) but expands and contracts laterally through the life of the cell. It is 80 nm wide in G1 and expands to 110 nm in mitosis in haploid cells, but is 160 nm in an interphase diploid cell. The diameter therefore seems to be related to the number of nuclear
microtubules required. A recent FRAP study suggested that it is a dynamic structure with constant exchange of some components through most of the cell cycle (Yoder et al., 2003).

It was some years after description of the structure before the first molecular characterisation of components of the SPB. Rout and Kilmartin used purified preparations of spindle pole bodies and spindle matter to raise monoclonal antibodies against components (Rout and Kilmartin, 1990; Rout and Kilmartin, 1991). These two studies identified 5 proteins between them, which later were found to be Spcl10p (110 kDa component, (Kilmartin et al., 1993)), Spc42p (42 kDa component, (Donaldson and Kilmartin, 1996)), Spc98p (90 kDa component, (Geissler et al., 1996)), Spc72p (85 kDa component (Wigge et al., 1998)), and Ndc80p (80 kDa component, which has since been shown to actually be a kinetochore protein (Wigge and Kilmartin, 2001)).

Other components were identified by varied methods. M. Winey did a genetic screen for mutants with monopolar spindles (Jaspersen et al., 2002; Winey et al., 1991a; Winey and Byers, 1993). Karlp, a half bridge component was discovered in genetic screen for karyogamy (nuclear fusion) mutants (Rose and Fink, 1987). Many were discovered by synthetic lethal or suppressor screens with existing mutants – Spc98 (Geissler et al., 1996), Spc97 (Knop and Schiebel, 1997) and Bbp1 (Schramm et al., 2000). A mass spectroscopy analysis by Wigge et al identified many more. Highly enriched SPB preparations were run on SDS-gels and bands identified by MALDI peptide mapping (Wigge et al., 1998). Lastly, components have been identified by their physical interactions through 2-hybrid (Spc72p (Chen et al., 1998b; Knop and Schiebel, 1998)).

At the time of starting this study there were seven known structural components of the main SPB (Spc42p, Spc29p, Cmdlp, Cnm67p, Nudlp, Spc72p, Spcl10p), and three half-bridge proteins (Cdc3lp, Karlp, Mps3p) in addition to the γ-tubulin complex and several membrane and microtubule associated factors.
The central plaque/IL2

One of the major components of the central plaque of the SPB is Spc42p. It was first discovered as a 42 kDa protein that reacted with antibodies raised against SPB preps (Rout and Kilmartin, 1991). Overexpression of Spc42p results in a large electron dense polymer layer resembling the central plaque extending laterally along the outer face of the nuclear membrane (Donaldson and Kilmartin, 1996). Although it extends laterally it has the same thickness as the central plaque. Further studies on this super-polymer of Spc42p revealed it is a crystal with a hexagonal mesh conformation (Bullitt et al., 1997).

A recent FRET (fluorescence resonance energy transfer) study (Muller et al., 2005) has yielded detailed information about the orientation of central and IL2 proteins. With the N terminus of Spc42p at the inner face of the central plaque, the protein extends through both central plaque and IL2 before turning round back through IL2 ending with its C-terminus on the inner face of IL2. The central coiled coil domain spans the gap between the two layers. IL2 is therefore made mostly of a hexagonal lattice of Spc42p, plus a C-terminal portion of Cnm67p (see outer plaque, and figure 1.6C).

The central plaque also contains Spc29p (Elliott et al., 1999), Cmdl the yeast calmodulin (Davis et al., 1986) and the C-terminus of Spc110p (see below). Spc29p binds the N-terminus of Spc42 (Elliott et al., 1999) and its N and C-termini are both at the inner face of the central plaque (Muller et al., 2005). Muller et al propose that Spc42p and Spc29p form a ring through which the C terminus of Spc110p enters the central plaque. The C-terminus of Spc110p binds Spc29p and Cmd1p (see nuclear face). The organisation of IL2 and the central plaque according to Muller et al is illustrated in figure 1.6C.

Cmd1p is the sole yeast calmodulin. Calmodulins are one of the most highly conserved families, present in animals, plants and fungi. The sequences from all multicellular organisms are more than 90% identical (Moncrief et al., 1990), although S. cerevisiae calmodulin is only 60% identical to that of vertebrates. The
family are composed of four calcium binding helix-loop-helix motifs called ‘EF hands’. Only three of them in the Cmd1p can bind Ca$^{2+}$. Calmodulins have many, many roles, mostly in Ca$^{2+}$ mediated signalling. None of the Ca$^{2+}$ dependent roles are essential in budding yeast, strains with Cmd1p that cannot bind calcium show minimal disruption to growth (Geiser et al., 1991). The role of Cmd1p in the spindle pole body is not essential providing the Cmd1p binding site on Spc110p is also removed. It is one of the few proteins conserved in the centrosome of higher organisms (Geiser et al., 1993). It is proposed to help in stabilising the structure formed by the other proteins (Muller et al., 2005).

**The nuclear face**

Spc110p joins the central plaque to the inner plaque and gamma tubulin complex. Spc110p acts as a spacer protein first seen as a filamentous connection by EM, with its C-terminus in the central plaque and its N-terminus at the inner plaque (Rout and Kilmartin, 1990). Its central coiled-coil domain determines the space between plaques. A classic experiment illustrated this point. Kilmartin *et al* made strains that had deletions in the coiled-coil region of Spc110p. In these strains there was a proportionate decrease in the spacing of central and inner plaques as seen by EM (Kilmartin *et al*., 1993).

FRET studies suggest that the C-terminal portion of Spc110p traverses the central plaque (Muller et al., 2005). Two regions of the C-terminus have been characterised, region II and III. Region III binds Cmd1p (Stirling *et al*., 1994; Sundberg and Davis, 1997). This is required to localise Cmd1p to the SPB (Spang *et al*., 1996; Sundberg and Davis, 1997; Sundberg *et al*., 1996). This binding may be regulated by Ca$^{2+}$ (Stirling and Stark, 2000). Spc29p binds a region that overlaps region III, and has been proposed to regulate Cmd1p binding to Spc110p (Elliott *et al*., 1999). Mutations in region II result in detachment of the inner plaque upon bi-orientation (Sundberg *et al*., 1996; Yoder *et al*., 2005). Spc42p and Spc29p remain in the SPB but Cmd1p is pulled out with the defective Spc110p, and they together form microtubule-nucleating aggregates in the nucleus.
The N-terminus (region I) of Spc110p binds Spc98p and Spc97p, anchoring the gamma tubulin complex to the nuclear side of the spindle pole body (Knop and Schiebel, 1997; Nguyen et al., 1998; Sundberg and Davis, 1997) and thereby providing the nuclear microtubule nucleation site.

The outer plaque

The C-terminus of Spc42p binds the C-terminus of Cnm67p at the inner face of IL2. Cnm67p has a coiled-coil domain that acts as a spacer between IL2 and the outer plaque in a similar fashion to Spc110p (Schaerer et al., 2001).

The N-terminus of Cnm67p binds Nud1p (Elliott et al., 1999), which in turn binds Spc72p via interactions of their C-termini (Gruneberg et al., 2000). A Cnm67p-Spc72p fusion protein can fulfil the astral microtubule nucleation function of Nud1p. This suggests the sole microtubule nucleation role of Nud1p is to link Spc72p to Cnm67p (Gruneberg et al., 2000). Nud1p has another important function in recruiting signalling proteins to the SPB during mitotic exit (see section 1.6.5).

The N-terminus of Spc72p binds the gamma tubulin complex (Knop and Schiebel, 1998), resulting in nucleation of cytoplasmic microtubules from this face. Spc72p also binds Kar1p at half bridge – during G1 and mating astral microtubules are nucleated here too (Adams and Kilmartin, 1999; Brachat et al., 1998; Byers and Goetsch, 1974; Byers and Goetsch, 1975; Pereira et al., 1999). Spc72p binds Kar1p via a different region of the C-terminus to Nud1p (Gruneberg et al., 2000).

Spc72p binds Stu2p (Chen et al., 1998b; Usui et al., 2003) through an adjacent domain to the one it binds the γ-tubulin complex. Stu2p appears to form a complex with Spc72p and the γ-tubulin complex (Usui et al., 2003). Stu2p is a member of the MAP215/Dis1/TOG family of MAPS. The most well characterised roles of these proteins are at the plus ends of microtubules (see section 1.4), but Stu2p also seems to have a role at outer plaque.
The half bridge

This structure was first described as an electron dense ‘bridge’ joining paired spindle pole bodies (Byers and Goetsch, 1974; Byers and Goetsch, 1975). When SPBs separate it leaves a horseshoe shaped structure adjacent to the SPB, termed the half bridge.

The first component to be localised to the half bridge was Cdc31p (Spang et al., 1993). Cdc31p belongs to a conserved family of proteins called centrins (Baum et al., 1986) that were first identified in green algae (Salisbury et al., 1984). Centrins have been identified in a wide range of organisms including humans and higher plants. They belong to a larger group of ‘EF hand’ Ca\(^{2+}\) binding proteins including calmodulin. Most importantly centrin homologues all have a function in microtubule organising centres such as the SPB, centrosome and basal bodies. *Chlamydomonas* centrin is a component of fibres that contract in the presence of Ca\(^{2+}\) (Wright et al., 1985). Budding yeast Cdc31p shares 54% identity with human centrin, and human centrin is 68% identical to *Chlamydomonas* (Schiebel and Bornens, 1995). This suggests a conserved role for this contractile role in MTOCs. This contractile property may well be used in the half bridge as it expands and contracts during SPB duplication, although there is no definitive proof of this yet.

The next half bridge component to be identified was Kar1p, a protein originally identified by its role in karyogamy (Rose and Fink, 1987). It showed physical and genetic interactions with Cdc31p and localised to the half bridge (Biggins and Rose, 1994; Spang et al., 1995; Vallen et al., 1994). The physical interaction is required to recruit Cdc31p to the half bridge (Spang et al., 1995) and this binding may be affected by Ca\(^{2+}\) concentration (Geier et al., 1996). Kar1p is composed of three functional domains (Vallen et al., 1992a). A central domain targets it to the SPB and is not required for nuclear fusion. The N-terminus is required for nuclear fusion and localisation of the Kar3p ‘Kin C’ motor to the half bridge during karyogamy. The third domain, including the C-terminus, is sufficient to target Kar1p to the nuclear membrane and is required for both mitosis and karyogamy (Vallen et al., 1992a; Vallen et al., 1992b). Together these suggest Kar1p is tethered to the membrane via
its C-terminus and several protein-binding domains extend into the cytoplasm. One of these domains is important for recruiting Cdc31p.

Mps3 was discovered as a monopolar spindle mutant that arrested without a half bridge (Jaspersen et al., 2002). The Mps3p protein also binds Cdc31p, and Cdc31p localisation is partially dependent on Mps3p.

How these proteins make the half bridge structure or associate with the main SPB is not clear. Both Mps3p and Kar1p have a trans-membrane domain (Jaspersen et al., 2002; Spang et al., 1995; Vallen et al., 1992a), but as yet no interaction between half bridge proteins and core SPB proteins has been documented.

Since the experimental work in this thesis was started, a new binding partner of Cdc31p was identified (Kilmartin, 2003). Kilmartin pulled down Sfi1p with Cdc31p, and multiple Cdc31p molecules bind Sfi1p. Two temperature sensitive mutants sfi1-3 and sfi1-7 were created by PCR mutagenesis. This paper will be discussed in detail at relevant points during this thesis.

Non-structural SPB associated proteins.
Ndc1p was first discovered as the mutant ndc1-1 in which all chromosomes were segregated to one pole of the spindle. It has 6-7 putative trans-membrane domains and localises by immuno-fluorescence to the nuclear envelope (Winey et al., 1993). Immuno-EM localises Ndc1p to the SPB and nuclear pores (Chial et al., 1998). The amount of Ndc1p protein is controlled, as overexpression results in similar defects to the ndc1-1 mutant (Chial and Winey, 1999). This protein is common to both nuclear pores and the SPB suggesting that they may have similar mechanisms of inserting or tethering in the membrane (also see ‘insertion in the membrane’ later).

Another protein shown to be an integral membrane protein and associated with the SPB is Mps2p (Munoz-Centeno et al., 1999). Mps2p binds another protein, Bbp1p (Le Masson et al., 2002; Schramm et al., 2000). Bbp1p is not integral membrane, but binds the core protein Spc29p and half bridge protein Kar1p (Schramm et al., 2000).
2000). This is interesting, as no direct interaction between core and half bridge has been identified yet, so this complex may mediate this. Recently a fourth protein, Nb1p, was shown to bind both Ndc1p and Mps2p (Araki et al., 2006). It appears to be part of the Mps2p-Bbp1p complex and links the previously distinct Ndc1p and Mps2p-Bbp1p complex.

This evidence suggests that the Mps2p-Bbp1 complex, and perhaps Ndc1p are involved in tethering the spindle pole body in the membrane, as none of the core components contain a trans-membrane domain. Perhaps they form the hook structures seen by electron tomography (O'Toole et al., 1999) that are proposed to anchor the SPB in the nuclear membrane?

1.6.3 Spindle pole body duplication

Spindle pole body duplication takes place in budding yeast in early S or even late G1 phase. Careful EM studies by Byers and Goetsch, and later Adams and Kilmartin categorised the four main stages of SPB duplication, illustrated in figure 1.7 (Adams and Kilmartin, 1999; Byers and Goetsch, 1974; Byers and Goetsch, 1975).

Satellite assembly

In G1 the half bridge elongates to about 150 nm and a spherical mass of material accumulates on the distal cytoplasmic tip (Byers and Goetsch, 1974). This structure is known as the satellite and so far is known to include Spc42p, Nud1p, Cnm67p and Spc29p (Adams and Kilmartin, 1999). Most mutants in half bridge proteins (Cdc31p, Kar1p, Mps3p) cannot complete this step (Byers, 1981b; Jaspersen et al., 2002; Rose and Fink, 1987). As yet no interaction between proteins of the half bridge and the satellite have been identified, which leaves the question open of how the satellite is tethered to the half bridge.
Figure 1.7. Spindle pole body duplication. Schematic diagram of *S. cerevisiae* SPB duplication. 1) Satellite assembly, 2) Expansion into duplication plaque, 3) Insertion into membrane, 4) Separation and building of spindle. The genes whose products are required for each step are shown underneath. (Taken from Jaspersen and Winey, 2004)
If cells are arrested with the mating pheromone alpha factor, they arrest with the satellite assembled (Byers and Goetsch, 1974; Byers and Goetsch, 1975), indicating this stage takes place before DNA replication is initiated. Initiation of SPB duplication requires the activity of Cdc28p with the G1 cyclins (Cln1-3) (Haase et al., 2001).

**Satellite expands into duplication plaque**

The next stage seen by EM is appearance of the ‘duplication plaque’ that appears morphologically similar to the cytoplasmic face of the SPB (central and outer plaques). It contains the same components as the satellite (Adams and Kilmartin, 1999) so the current model is that the satellite expands laterally into this ‘duplication plaque’, although intermediates have not been seen. The assembly of Spc42p into this plaque is thought to be by a similar method to which the super plaque is assembled during Spc42p overexpression (Bullitt et al., 1997). Cnm67p, Nud1p and Spc72p are not required for Spc42p assembly into the central plaque, but are required for the duplication plaque to assemble correctly. Presumably in these mutants the duplication plaque lacks the outer plaque equivalent.

Spc29p is found in the satellite and duplication plaque, but spc29 mutants can build a second defective SPB (Adams and Kilmartin, 2000; Elliott et al., 1999) so it is not essential for the satellite or duplication plaque to form. When overexpressed, Spc29p is a nuclear protein (Elliott et al., 1999). This suggests the vast majority of Spc29p is assembled onto the central plaque from the nuclear side after the duplication plaque is inserted into the membrane.

**Insertion into membrane**

Following assembly of the duplication plaque, it is inserted into the membrane by a poorly characterised mechanism. In a few EM pictures the lipid bilayers at the distal end of the half bridge appear to have fused to create a pore in the membrane (Adams and Kilmartin, 1999). One theory is that the lipid bilayers including this pore retract under the half bridge exposing the duplication plaque to the nucleoplasm.
Mutants in Mps2p, Ndc1p, Nbp1p and Bbp1p fail in insertion (Araki et al., 2006; Schramm et al., 2000; Winey et al., 1991b; Winey et al., 1993). Bbp1p exists in a complex with Mps2p and also binds the central plaque and duplication plaque protein Spc29p, and the half bridge protein Kar1p (Schramm et al., 2000).

Nuclear pore complexes are often seen near duplicating SPBs (Adams and Kilmartin, 1999) and Ndc1p is localised to NPCs as well as SPBs and is required for their assembly (Chial et al., 1998; Lau et al., 2004). This suggests that Ndc1p may be involved in a common membrane insertion technique, although it is dispensable for nuclear pore function (Chial et al., 1998).

There is a homologue of Ndc1p, Cut11, in fission yeast. The *S. pombe* SPB shuttles in and out of the membrane during the cell cycle (see section 1.7.1), and Cut11 is only associated with the SPB when it is inserted in the membrane (West et al., 1998). Ndc1p can partially rescue a temperature sensitive *cut11* mutant. This suggests a common mechanism for membrane insertion/association between structurally different yeast SPBs.

Some Mps1p mutants also show insertion defects (Schutz and Winey, 1998) suggesting the Mps1p kinase is involved in controlling this step.

**Assembly of inner plaque**

Following insertion, the nuclear components Cmd1p, Spc110p, Spc29p and the γ-tubulin complex assemble in the nuclear plaque. Studies on Spc110p suggest that in addition to this initial assembly step, the spindle pole body is not a stable structure but is dynamic with exchange of Spc110p occurring especially in G1/S (Yoder et al., 2003). In *spc110* mutants defective in the C-terminal Region III Cmd1p binding and region II domains, assembly of the nuclear plaques is defective, and there are often aberrant microtubule organising bodies in the nucleus (Kilmartin and Goh, 1996; Sundberg et al., 1996; Yoder et al., 2005).
One C-terminal mutant, *spc110-220*, is synthetic lethal with a deletion in Mlp2p, one of a family of filamentous proteins associated with the nucleoplasmic side of the nuclear pore. They have been proposed to form a peripheral nuclear network, with a potential role in nuclear-cytoplasmic transport (Strambio-de-Castillia et al., 1999). Mlp2p binds Spc110p, Spc29p and Spc42p directly, and in *mlp2Δ* cells there are SPB assembly defects (Niepel et al., 2005). *mlp2* cells often contain multiple microtubule nucleating bodies in the nucleoplasm, containing both Spc42p and Spc110p that has failed to insert correctly into the SPBs and the functional SPBs contain less Spc42p. Niepel *et al.* suggest that Mlp2p recruits Spc110p and Spc42p to the nuclear face of the SPB for insertion or exchange.

**Separation**

Once two functional spindle pole bodies are assembled, they are separated by bridge cleavage and pulled apart by spindle microtubules. The physical process of bridge cleavage is not understood.

The activity of Cdc28p, with S-phase cyclins Clb5-6, as well as Clb1-4 promotes maturation of side-by-side SPBs into a spindle. Mutants in the SCF^{Cdc4} arrest with side-by-side un-separated SPBs (e.g. *cdc4* (Byers and Goetsch, 1974)). The main role of SCF^{Cdc4} is the destruction of Sic1p, the Cdk/Clb inhibitor. SCF^{Cdc4} mutants probably arrest due to there being too little Cdk activity to promote SPB separation.

Mutants in the microtubule plus end bimC motor proteins Cin8p and Kip1p, and the midzone microtubule interdigitating protein Stu1p fail to build a spindle (Hoyt et al., 1992; Hoyt et al., 1993; Pasqualone and Huffaker, 1994; Saunders and Hoyt, 1992). Less severe mutants of the two BimC motors can build a spindle, but it is shorter (Hoyt et al., 1992; Hoyt et al., 1993; Saunders and Hoyt, 1992; Straight et al., 1998). This suggests that the role these proteins have is purely in force generation across interpolar microtubules and not a specific role at the bridge.
**SPBs in the mitotic spindle**

After SPB separation in S-phase a short spindle remains until metaphase. Intermediates between side-by-side SPBs and a short (~600 nm) spindle with SPBs facing have been hard to observe (Winey and O'Toole, 2001). The spindle lengthens to a metaphase length of 1-2 μm. The function of the SPBs is to nucleate the ~20 microtubules each required for the mitotic spindle, along with the cytoplasmic microtubules that orientate the nucleus and pull the SPBs apart in anaphase B.

**1.6.4 Regulation of SPB duplication and co-ordination with the cell cycle**

**Transcription**

The transcription of many spindle pole body proteins is restricted to G1/S phase by a promoter containing a Mlu1 cell cycle box (MCB) (Jaspersen and Winey, 2004; McIntosh et al., 1991). Protein levels appear constant though, and overexpression SPB proteins does not induce extra rounds of duplication, so it is unlikely this is a major control mechanism. The promoter of \(SPC110\) contains a consensus for the fork head transcription factor Hcm1p. Deletion of \(HCM1\) does not affect the basal level of \(SPC110\) transcription, but reduces the induction seen in late G1 (Zhu and Davis, 1998).

Mutations in Hsf1, a heat shock transcription factor result in an arrest with an unduplicated spindle pole body and enlarged half bridge (Zarzov et al., 1997). Few SPB components have the Hsf1 element in their promoters. It promotes transcription of chaperones such as Hsp90, so the effect of Hsf1 is likely an indirect one through chaperones that promote assembly of the spindle pole body satellite.

**Phosphorylation**

Many SPB proteins are phosphorylated *in vivo* (Spc42p –(Donaldson and Kilmartin, 1996), (Ubersax et al, 2003)(Ficarro et al., 2002), Spc110p (Friedman et al., 1996), Nud1p (Gruneberg et al., 2000), Spc98p (Pereira et al., 1998), Cnm67p (Schaerer et al., 2001), Tub4p (Vogel et al., 2001) (Wigge et al., 1998)). For many of them this is constitutive, although a few such as Spc42p and Spc110p seem to be cell cycle
regulated with an increase in G1/S phase (Donaldson and Kilmartin, 1996; Friedman et al., 1996; Stirling and Stark, 1996).

**Cdc28p**

Cdc28p activity is required for all stages of SPB duplication. Cdc28/Cln complexes initiate duplication, and Cdc28/Clb1-6 complexes promote separation of SPBs into a spindle (Haase et al., 2001). This requires de-phosphorylation of tyrosine-19 by Swe1p (Lim et al., 1996). Mutants in SCFCdc4 do not separate their SPBs (Byers and Goetsch, 1974; Mathias et al., 1996). This is most likely because they cannot degrade the Cdk/Clb inhibitor Sic1p and Cdk/Clb activity stays low.

Mitotic cyclins Clb1-4 are involved in ‘re-licensing’ the SPBs for duplication in the next cycle. If there is no Clb1-4 activity (quadruple deletion strains), but Clb5-6 present the SPBs will re-duplicate many times. If a single Clb1-4 cyclin is added back, correct duplication is restored (Haase et al., 2001). Therefore Cdk/Clb1-4 activity prevents re-licensing until Clbs are destroyed in anaphase.

In a proteomic screen Spc42p, Spc110p, Spc29p, Mps2p and Bbp1p were identified as good Cdc28p substrates (Ubersax et al., 2003). Many other SPB proteins have potential Cdc28p phosphorylation sites, for example Cnm67p and Kar1p (Jaspersen and Winey, 2004).

The only SPB protein where the function of phosphorylation has been carefully investigated is Spc42p. Jaspersen et al used the formation of the Spc42p super plaque upon overexpression as an assay. They showed that Cdc28p, probably with Clns 1 and 2, phosphorylates serine 4 and threonine 6 of Spc42p and that this phosphorylation promotes assembly of Spc42p into the plaque (Jaspersen et al., 2004). Cdc28 also phosphorylates Mps1p on threonine 29, which stabilizes it. Mps1p in turn plays a major part in Spc42p phosphorylation and promotes super-plaque assembly, although this may be direct rather than through phosphorylation (Jaspersen et al., 2004).
**Mps1p**

The other major G1/S SPB duplication kinase is Mps1p (Lauze et al., 1995). Different Mps1p mutants have different defects suggesting Mps1p is involved in controlling multiple steps such as satellite assembly (Castillo et al., 2002) and insertion (Schutz et al., 1997; Schutz and Winey, 1998). Unlike Cdc28p, Mps1p has no consensus target sequence, because only 2 substrates other than Spc42p have been identified: Spc98p (Pereira et al., 1998) and Spc110p (Friedman et al., 2001). The phosphorylation of Spc110p in the N-terminus was the first Mps1 site mapped. The N-terminus of Spc110p is the section that interacts with the gamma tubulin complex, and a non-phosphorylatable alanine substitution mutant is synthetic lethal with spec97 (Friedman et al., 2001). Spc98p is also phosphorylated after SPB duplication, most likely by Mps1p (Jaspersen and Winey, 2004; Pereira et al., 1998). This suggests a role of Mps1p mediated phosphorylation in nuclear microtubule nucleation, but as yet the exact nature is not clear.

**Other kinases**

Tub4p is phosphorylated on tyrosine 445 by an as yet un-identified kinase in GI (Vogel et al., 2001).

Another important mitotic kinase is polo, or Cdc5p in budding yeast. Overexpression of Cdc5p results in multiple Spc42p containing microtubule organising centres in the nucleus, suggesting that Cdc5p may be involved in the licensing to duplicate (Song et al., 2000). Cdc5p is targeted to the spindle pole body via interaction of its ‘polo-box’ with the N-terminus of Bbp1p (Park et al., 2004).

Mitogen-activated proteins kinases have a role in basal body regulation (Berman et al., 2003), but as yet have not been implicated in SPB function in yeast.
1.6.5 Other functions of the SPB

**Karyogamy**

Karyogamy is the term for the nuclear fusion that occurs when two haploid yeast cells mate. After membrane fusion events, the SPBs form one larger diploid SPB. Many budding yeast spindle associated proteins were first identified due to their role in karyogamy.

The outer plaque and half-bridge nucleate microtubules that pull the two nuclei together. During this process Spc72p localises to the half bridge by binding Kar1p, allowing microtubule nucleation by its recruitment of the \(\gamma\)-tubulin complex (Pereira et al., 1999).

The site of SPB fusion is the satellite, which is already formed in mating factor arrest (Byers and Goetsch, 1975). SPB fusion must also require fusion of the membrane areas forming the pore in which the SPB sits. This probably involves pinching down between inner and outer membranes such that two pores become one (reviewed in Rose, 1996).

**Mitotic exit and the spindle position checkpoint**

The spindle position checkpoint arrests cells in late anaphase/telophase in response to a mis-oriented spindle, via inhibition of the mitotic exit network (MEN). In budding yeast the signal is not mis-orientated *per se*, but the entire nucleus (i.e both poles) being in the mother cell. The Tem1p MEN activator is inactivated by the Bfa1-Bub2 GAP and activated by the Lte1 GEF. Tem1p and Bfa1p/Bub2p are specifically localised to the bud bound SPB (Pereira et al., 2000) via interaction with Nud1p (Gruneberg et al., 2000). Localisation of Tem1p to the SPB is dependent on Bub2p at this point in the cell cycle. Lte1p is present in the bud cortex (Bardin et al., 2000; Pereira et al., 2000). Microtubules are important in the interaction of the SPB with the bud cortex (Adames et al., 2001; Pereira et al., 2001). Only when the SPB enters the bud can Lte1p activate Tem1p, allowing progression of the MEN. The
localisation of another MEN kinase, Cdc5p, to the SPB via Bbp1, is also important for mitotic exit (Park et al., 2004). The role of the SPB in mitotic exit is reviewed in (Pereira and Schiebel, 2001).

1.7 MTOCs in other organisms
Organisms other than budding yeast have many MTOCs. In this section I discuss the mitotic spindle organisers of other organisms.

1.7.1 Schizosaccharomyces pombe SPB
The spindle pole body of fission yeast is quite different to that of budding yeast. Characterisation is 20 years behind that of the budding yeast spindle pole body. A detailed EM study of spindle pole body cycle in fission yeast was first undertaken in 1997 (Ding et al., 1997). The most striking difference between yeasts, is that the SPB of fission yeast spends most of the cell cycle in the cytoplasm, and is only inserted into the nuclear membrane for mitosis.

The bulk of the SPB consists of an ordered central layer, surrounded by fibrous material in an ellipsoid shape (Ding et al., 1997). One surface contacts the nuclear membrane. This area of the membrane appears to be specialised and is associated with an accumulation of material on the inner membrane face. There are no nuclear microtubules during interphase, but most of the nuclear γ-tubulin and the kinetochores are associated with this membrane region.

If cells are arrested in G1 by starvation there is only one SPB (Uzawa et al., 2004). A bridge like structure can be seen, but as yet no satellite SPB has been seen by EM (Ding et al., 1997). The next stage seen by EM is two identical SPBs separated by the bridge. They are both smaller in size than the G1 SPB, leading to the suggestion that material from the old is used to make the new, or that they both exchange with a common pool of material (McIntosh and O'Toole, 1999). Duplication requires activity of the S. pombe Cdk, cdc2, similar to budding yeast (Krapp et al., 2001; Uzawa et al., 2004). An S-phase arrest reveals duplicated but not yet matured SPBs
The two SPBs enlarge in G2. Later, a ‘fenestra’ in the membrane opens and the two SPBs and associated material are inserted side by side. The dark material on the membrane surface appears more structured at this point (McIntosh and O'Toole, 1999). Both SPBs nucleate microtubules that interdigitate to form a spindle as in budding yeast (Ding et al., 1997). In anaphase the fenestra begin to close around the remaining 2-6 microtubules excluding the SPBs from the nucleus. In late telophase the pores finally shut and the SPBs are back in the cytoplasm. The spindle pole body cycle in fission yeast is illustrated in figure 1.8.

The SPBs show some parallels between yeasts. In both organisms they are assembled on the tip of a bridge, mostly or entirely in the cytoplasm. These large structures (entire SPBs or central plaques) are inserted into the membrane by a specialised pore or fenestra. SPB duplication is initiated by S-phase Cdks in both cases.

Molecular characterisation of the *S. pombe* SPB is limited. Components conserved from budding yeast so far discovered are Cdc3lp, Cut11p (Ndc1) and Cdc11p (Nud1p). Others components are Cut12, on the nuclear surface (Bridge et al., 1998) and Sad1 and Kms1 are membrane bound SPB proteins (Hagan and Yanagida, 1995; Miki et al., 2004; Shimanuki et al., 1997).

Fission yeast Cdc31p is also on the bridge like structure between SPBs, and mutants fail in SPB duplication (Paoletti et al., 2003). This suggests a very similar role to budding yeast Cdc31p.

Cdc11p shows homology to Nud1p and similar to Nud1p is involved in anchoring the MEN, Cdc11 interacts with components of the equivalent network in fission yeast, the septation initiation network (SIN) (Krapp et al., 2003; Krapp et al., 2001).

One of the most interesting components shared between organisms is Ndc1p/Cut11. The fission yeast protein, Cut11p, localises to spindle pole bodies and nuclear pores (West et al., 1998), as does the budding yeast homologue Ndc1 (Chial et al., 1998).
Figure 1.8. The spindle pole body cycle in *Schizosaccharomyces pombe*. (Taken from Ding *et al.*, 1997)
They share a modest 49% similarity and 22% identity, but Ndc1 can partially rescue a cut11 mutant (West et al., 1998). Cut11p has seven potential trans membrane domains, as does Ndc1p. The spindle pole bodies in cut11 mutants fail to anchor in the membrane and are often free in the nucleoplasm (West et al., 1998). As discussed, ndc1 mutants fail in the insertion of the new SPB into the budding yeast nuclear membrane (Winey et al., 1993). Thus the two yeasts appear to share a common SPB-membrane interaction mechanism.

1.7.2. Centrosomes and basal bodies

Structure
The centrosomes of higher organisms have a quite different structure to the spindle pole bodies of yeasts. The centrosome is 1-2 μm in diameter (for comparison, that is the size of a budding yeast metaphase spindle), and composed of hundreds of proteins (Andersen et al., 2003; Doxsey, 2005) as opposed to the ~20 core structural components of the budding yeast SPB.

The centrosome is composed of a pair of centrioles at right angles, and surrounding pericentriolar material (PCM). Each centriole is composed of nine triplets of microtubules assembled into a short (400 nm) tube. In interphase the proximal end of each centriole seems joined by fibrous material. The older centriole – the ‘mother’ has two sets of nine appendages. The ‘subdistal’ appendages appear to be involved in microtubule anchoring, whereas the function of the ‘distal’ appendages is unclear. It is the fibrous PCM that recruits the γ-tubulin complexes for microtubule nucleation, the centrioles appear to simply focus the PCM. The ‘procentriole’ – the younger daughter centriole, lacks appendages and has a cartwheel shaped scaffold inside and this is continuous with fibrous material linking the centrioles. Centrioles are similar in structure to basal bodies, which organise cilia and flagella in motile cells. In some cells they are one and the same dependent on cell cycle stage.

Centrosome structure is illustrated on figure 1.9A.
Figure 1.9. A) **Centrosome structure** showing orientation and structure of centrioles. PCM not shown. (Take from Lange and Gull, 1996)

B) **Centrosome duplication**
Behavior of centrioles during centrosome duplication. (Taken from Kramer and Ho, 2001)
In G1 phase centrioles separate slightly and loose perpendicular orientation. Duplication starts in co-ordination with DNA replication, a pre-cursor procentriole appears on the proximal end of the centriole where the fibrous link between centrioles was. The procentriole lengthens until M phase, and the centrosomes in the spindle go through mitosis with a mature centriole and an immature daughter centriole. The original daughter centriole finally matures by gaining appendages and γ-tubulin, ninein, cenexin amongst others (Palazzo et al., 2000). Centrosome duplication is illustrated in figure 1.9B.

**Molecular characterisation**

Less is known about the components of the centrosome than the budding yeast spindle pole body. Molecular characterisation initially relied heavily on antibodies generated to centrosome components, either by design, or by use of human autoimmune disorders.

A proteomic analysis of proteins that co-fractionate with known human centrosome components estimated more than 500 proteins (Andersen et al., 2003). Very few of these have been identified, and even fewer have a known function. The two areas best characterised are the MAPs, and the proteins that nucleate and anchor microtubules.

‘Coiled coil’ domains are intertwined parallel right hand α-helices with a repeating pattern of hydrophilic and hydrophobic interactions. It is an evolutionarily conserved protein-protein binding motif. Many centrosome proteins contain coiled coils and act as huge scaffolds for microtubule nucleation and signalling.

Other than the γ-tubulin small complex components, the only budding yeast SPB proteins with homologues in vertebrates are calmodulin and centrin. Still, there are several conserved features of centrosomes, discussed below.
**Functions**

Centrosomes are not absolutely required to organise the mitotic spindle in the way yeast spindle pole bodies are. During vertebrate female meiosis many cells have acentrosomal spindles and higher plants lack centrosomes altogether. These spindles are organised by chromatin and MAPs (reviewed in (Compton, 1998; Gergely, 2002; Quimby and Dasso, 2003)). It seems these centrosome independent spindle organising mechanisms also operate in cells with centrosomes. When one or both centrosome are destroyed in a mammalian cell line by laser ablation, a bipolar spindle with one or two acentrosomal poles still forms (Khodjakov et al., 2000). These spindles can undergo anaphase, but are mis-orientated which leads to cytokinesis and chromosome segregation defects (Khodjakov and Rieder, 2001). Alternatively the centrosome can be removed by microsurgery. If this is in S or G2, again, a functioning but mis-orientated bipolar spindle can form (Hinchcliffe et al., 2001). When *Drosophila* spermatocytes are treated so that the centrosomes remain at the cell periphery too far away for astral microtubules to extend to chromosomes, a centrosome independent bi-polar spindle forms around the chromosomes (Rebollo et al., 2004). This along with other evidence (reviewed in (Wadsworth and Khodjakov, 2004)) suggests that an acentrosomal, chromatin and MAP mediated pathway does act in centrosomal cells, the centrosome acts as a dominant force that focuses and defines the poles when present. Supernumerary centrosomes do result in multipolar spindles, although in ‘wild type’ acentrosomal spindles multipolarity is rare, suggesting there is a mechanism for establishing bipolarity to which centrosomes are dominant if present.

Although the role of centrioles/centrosomes in the spindle is still unclear there is a growing body of evidence giving them a much larger role in cell cycle control than the SPBs of yeast.

In the microsurgery experiment explained above, if the centrosomes are removed in G1 phase, the cells do not progress into S phase (Hinchcliffe et al., 2001). This suggests a spindle-independent role for centrosome activity in the G1-S transition. Centrosomes are a large scaffold of coiled-coil proteins, ideal for providing a large platform for signalling pathways. Centrosomes provide a platform for a pathway
analogous to the MEN, as well as their role in the G1-S transition. In addition roles in cytokinesis, entry into mitosis, and the metaphase to anaphase transition have been implicated (reviewed in (Doxsey et al., 2005a; Doxsey et al., 2005b)).

1.7.3. Parallels in spindle organisers of all organisms
Some parallels can be drawn between spindle pole body duplication and centriole duplication. Both new SPBs and centrioles start life as a smaller satellite version at a set distance from the mother organelle. The maternal mother and new organelle may always contain centrin (see later). This daughter organelle then matures through the cell cycle to become competent for microtubule nucleation. There are also parallels in the basic control of division by cyclin dependent kinases (Winey, 1999).

Both the centrosome and spindle pole bodies act as scaffolds for signalling mechanisms and proteins that control microtubule dynamics. Although centrosomes have many extra cell control roles, SPBs and centrioles all have a role in mitotic exit, and homologues scNud1/spCdc11 and centriolin are all involved in recruiting the signalling pathway to the organelle. There are other conserved components of all mitotic microtubule organisers that have been characterised in detail. These are the \( \gamma \)-tubulin complexes along with orthologues of the \( \gamma \)-tubulin complex binding protein Spc110p, and the centrin/Sfl1 fibres. These parallels are discussed below.

\( \gamma \)-tubulin and microtubule nucleation
The best characterised conserved component of all MTOCs is the \( \gamma \)-TuSC. GCP2 is the homologue of Spc97p, GCP3 of Spc98p (Murphy et al., 1998; Tassin et al., 1998). Budding yeast have two \( \gamma \)-TuSC binding proteins, Spc72p and Spc110p that bind Spc97/98 but not \( \gamma \)-tubulin directly (Knop and Schiebel, 1997; Nguyen et al., 1998; Sundberg and Davis, 1997).

An orthologue for Spc110p was first proposed with the discovery that a centrosomal epitope recognised by an Spc110 antibody could be found in human and *Xenopus* (Tassin et al., 1997). Spc110p binds the yeast calmodulin, Cmd1p, and it was by homology to this calmodulin binding domain, that the first mammalian orthologue,
Kendrin (pericentrin - B) was discovered in humans (Flory et al., 2000; Li et al., 2001). This calmodulin-binding domain is sufficient for centrosome targeting and was named the PACT (pericentrin-AKAP450-centrosomal targeting) domain (Gillingham and Munro, 2000).

Apart from the PACT binding domain, kendrin/pericentrin bear little similarity to Spc110p on a sequence level. On a functional scale it recruits γ-tubulin by binding GCP2 and/orGCP3 with its N terminus (Takahashi et al., 2002), as does Spc110 by binding Spc98p (Knop and Schiebel, 1997). The N-terminus of kendrin shares homology with mouse 'pericentrin', and it was later shown that it was a larger alternative splice variant of the pericentrin gene in both humans and mice (Flory and Davis, 2003). There may be as many as 10 splice variants of the pericentrin gene dependent on tissue type. Pericentrin was the first mammalian γ-TuRC binding protein to be characterised (Dictenbèrg et al., 1998). Loss of pericentrin results in reduction of astral microtubules and monopolar spindles (Zimmerman et al., 2004).

Another mammalian protein that shares the Spc110p like 'PACT' calmodulin binding domain in its C-terminus is CG-NAP (or AKAP450, AKAP350, Hyperion) (Gillingham and Munro, 2000; Takahashi et al., 1999). CG-NAP (centrosome and Golgi-localised PKN-associated protein) is also a large coiled coil protein that binds GCP2/3 of the γ-TuRC through its N terminal domain (Takahashi et al., 2002). It localises to the centrosome throughout the cell cycle, and also the Golgi in interphase. As well as recruiting the γ-TuRC it associates with many protein kinases and phosphatases, acting as a scaffold (Takahashi et al., 2000b; Takahashi et al., 1999).

Both CG-NAP and Kendrin localise to the centre of microtubule asters, but their centrosome localisation is independent of microtubules. Microtubule aster formation from isolated centrosomes is suppressed by antibodies to either protein (Takahashi et al., 2002).
Spc110p like calmodulin and γ-TuSC binding proteins have also been found in fission yeast, *Aspergillus* and *Drosophila*, based on sequence homology with the ‘PACT’ domain (Flory et al., 2002; Kawaguchi and Zheng, 2004; Martinez-Campos et al., 2004). All orthologues are seen at centrosomes/SPBs and show roles in microtubule nucleation.

**Centrins and the spacer element**

Centrins are a family of small (~20 kDa) EF hand Ca\(^{2+}\) binding proteins that are highly conserved from yeast to human, including higher plants. Budding yeast have just one centrin, Cdc3lp, that has several roles, but some other organisms have several centrins that perform different roles. Human centrin shares 45% sequence identity with budding yeast Cdc3lp (Schiebel and Bornens, 1995). Human centrin 3 cannot complement a *cdc3l* mutant but overexpression of human centrin 3 in budding yeast disrupts SPB duplication, possibly by titrating away centrin binding proteins (Middendorp et al., 2000).

All centrins localise to the centrosome or equivalent, although in animal cells much of it is in additional locations. The centrin of the alga *Spermatozopsis similis* localises to fibres connecting the pro-basal body to the mother basal body (Lechtreck and Grunow, 1999). Budding yeast Cdc3lp is on the half bridge and *cdc3l* mutants are disrupted in SPB duplication. *Chlamydomonas* centrin mutants display disrupted basal body duplication (Taillon et al., 1992), and reduction of centrin amounts blocks basal body duplication (Koblenz et al., 2003). *Tetrahymena* CEN1 is required for basal body duplication (Stemm-Wolf et al., 2005). Human centrins 2 and 3 are involved in centriole duplication (Middendorp et al., 2000; Salisbury et al., 2002).

So, centrins have a conserved role in duplication of microtubule organising centres.

Similar to the assembly of the satellite on distal end of the half bridge, a new centriole starts as a smaller satellite at a set distance from the proximal end of the mother centriole. During basal body assembly, when there is no cell cycle control restricting duplication to once per cell cycle, up to eight new basal bodies (~centrioles) are seeded at once (as opposed to one daughter centriole during
centrosome duplication). They are all a set distance from the existing mother basal body, suggesting there is mechanism for determining this set distance (Anderson and Brenner, 1971).

The first centrin-like protein to be discovered was in *Vorticella conrallaria*. The centrin like protein, spasmin, is present in an organelle called the spasmoneme (Amos et al., 1975). The spasmoneme is a Ca\(^{2+}\) dependent contractile organelle, but it has elastic properties independent of Ca\(^{2+}\) (Weis-Fogh and Amos, 1972). In *S. similis*, one location of centrin is in the basal body distil connecting fibres. These fibres contract and re-orient basal bodies in response to Ca\(^{2+}\) (McFadden et al., 1987). These two pieces of evidence suggests that one role of centrins is in creating contractile fibres responsive to Ca\(^{2+}\) concentration. The half bridge of the yeast SPB must also expands and contract during SPB duplication (Adams and Kilmartin, 1999), although this seems to be Ca\(^{2+}\) independent. The fibres that separate the mother and daughter centriole expand and contract during the course of the cell cycle (Vorobjev and Chentsov Yu, 1982).

All together this suggests a conserved role for centrins in Ca\(^{2+}\) dependent and independent contractile fibres. One conserved role of these fibres seems to be in separating mother and daughter organelles involved in microtubule organisation, and possibly also determining the distance between them.

This model has been updated during the course of this study by the discovery of a new Cdc31p binding protein, Sfi1p, in budding yeast (Kilmartin, 2003). The updated model is discussed in chapter 7.

### 1.7.4. Spindle pole abnormalities and cancer

**Cancer**

Cancer is an abnormal growth of cells. Cancerous cells show loss of control of cell number (hyperplasia), abnormal morphology (dysplasia and metaplasia) and invasion of surrounding tissue (metastasis). Some cancers also show loss of differentiation
and defects in cytoplasmic organisation, tissue architecture and cell orientation. There are three broad ways by which cells become ‘transformed’, the term for this loss of regulation. These are through gain/abnormal activation of a growth promoting gene (oncogene e.g. Ras, Myc), loss of both alleles of a growth inhibiting gene (tumor suppressor, e.g. p53, Rb) or deregulation of the cell cycle. Although there are some genes commonly mutated in cancers, multiple genetic alterations are required for full development of cancer. This suggests that an important step in cancer progression is mutation that causes genetic instability, and promotes the development of other mutations.

Most human cancers display genetic instability, most commonly aneuploidy – an incorrect number of chromosomes (reviewed in (Cimini and Degrassi, 2005)). Although other genetic instabilities exist in cancers aneuploidy is the most common (reviewed in (Lengauer et al., 1998)). In addition to defects in other areas of the mitotic spindle, abnormal centrosomes have been implicated in some cases of aneuploidy and cancer.

**Centrosome abnormalities**

The role of centrosomes in this transformation was first proposed by Boveri in the 1800s. His theories were re-visited in the last 10 years by the discovery of centrosomal aberrations in many human cancers. In 1998 three studies on tumours and tumour cell lines documented centrosome abnormalities. Lingle et al studied human breast cancer tissue after surgery, Weber et al cerebral primitive neuroectodermal tumors and Pihan et al a variety of tumours and cell lines (Lingle et al., 1998; Pihan et al., 1998; Weber et al., 1998). The centrosome abnormalities seen were an increase in centrosome number and volume, accumulation of excess pericentriolar material, supernumerary centrioles and aberrant protein phosphorylation (Lingle et al., 1998).

Supernumerary centrosomes have been seen in virtually every type of cancer (Nigg, 2002). The two most likely causes of supernumerary centrosomes are loss of control of duplication, resulting in overduplication, or aborted mitosis where duplicated
centrosomes and DNA remain in the one cell. They may also arise from cell fusion (promoted by viruses) or de novo formation (Khodjakov et al., 2002).

Centrosome duplication takes several hours, so in order for centrosome to re-duplicate before cell division a pre-anaphase cell cycle delay is needed. Some tumour cell lines (e.g. U2OS) can be made to over-replicate centrosomes with a DNA damage checkpoint mediated S-phase arrest (Balczon et al., 1995; Meraldi et al., 1999) whereas others arrest both DNA replication and centrosome replication (Meraldi et al., 2002). Centrosome duplication is initiated by Cdk2 along with cyclins E and A and overexpression of cyclin A or loss of Cdk inhibitors does promote centrosome overduplication (Meraldi et al., 1999). Vertebrate aurora A kinase (BTAK/STK15) is involved in centrosome maturation and promotion of microtubule nucleation (Brittle and Ohkura, 2005; Meraldi et al., 2004b) and is amplified in many cancers including 12 % of primary breast cancers (Salisbury et al., 1999). Transient transfection of aurora A in NIH 3T3 and MCF7 cells does indeed result in centrosome amplification (Zhou et al., 1998).

The other major potential mechanism that would result in supernumerary centrosomes is aborted cell division. Cells either abandon mitosis resulting in one cell with double ploidy and centrosomes, or divide before the spindle is ready, resulting in one aploid cell and one with two centrosomes and double ploidy (tetraploid in the case of a diploid organisms such as humans). In support of this as a clinically relevant mechanism, tetraploidy is not uncommon in solid human tumours (Nigg, 2002).

After these long arrests or aborted divisions, the tumour suppressor protein p53 is key in the decision to continue arrest, die (apoptosis) or re-enter S-phase. p53 is a transcription factor that mediates cell cycle arrest or apoptosis in response to DNA damage. Its inactivation in a wide variety of cancers is well documented (Duensing and Duensing, 2005). There is some evidence that loss of p53 function can affect centrosome number. p53 null mouse embryonic fibroblast cells show multiple centrosomes, and reduced mitotic fidelity (Fukasawa et al., 1996). In papillomas and
metastatic skin carcinomas of p53 null mice, 75% of cells have three or more centrosomes (Wang et al., 1998). However, centrosome amplification is not an inevitable consequence of p53 loss in vivo and targeted inactivation of p53 in diploid human cells doesn’t cause aneuploidy, but some tetraploidy (Bunz et al., 2002; Goepfert et al., 2000).

More subtle alterations in centrosome structure and function are also seen in cancers. Overexpression of centrosomal proteins pericentrin, TACC, CEP135 and C-NAP1 in cultured cells results in centrosomal abnormalities similar in appearance to those seen in tumours (reviewed in (Nigg, 2002)). Overexpression of pericentrin in prostate epithelial cells produces the same changes, particularly the loss of cellular architecture (Pihan et al., 2001). Abnormalities in microtubule nucleation are also seen, but seem to be due to defects in control mechanisms rather than nucleation capacity (Salisbury et al., 1999).

**Affects of centrosome aberrations on chromosome segregation**

There is a strong correlation between centrosomal abnormalities and chromosome aberrations in tumours. Centrosomes have an important role in determining organisation of the mitotic spindle, so any abnormalities will directly impact the mitotic spindle. Multiple centrosomes or acentriolar bodies from PCM may organise multipolar spindles, which would clearly mis-segregate DNA. Both multipolar and monopolar spindles are common in tumours (Brinkley, 2001; Griffin et al., 2000; Lingle and Salisbury, 2000; Pihan and Doxsey, 1999).

In most cases of multipolar spindles, resulting aneuploid cells will not be viable. It would not necessarily be a selective advantage for a tumour cell to always go through multipolar mitosis. On occasion an aneuploid progeny might end up with the right combination of chromosomes to transform in a tumour cell, but most of a population will gain mechanisms to restore bipolar mitosis. Bipolar spindles are seen with anything from zero to many centrioles clustered at each pole (Brinkley et al., 1981; Ring et al., 1982; Sharp et al., 1982), suggesting some mechanism selects for organisation of extra centrosomal bodies into just two spindle poles, or only two
are active. A second method may be that a non-centrosomal extra pathway enforces bipolarity.

As well as the mitotic spindle, the centrosome has an important role in determining the cell axis. The orientation of the cleavage plane and symmetry of cell division, as well as cytoplasmic architecture and directional vesicular trafficking may all be disrupted if there are multiple centrosomes in cell. This could lead to cell division defects as well as the disorganisation and loss of differentiation seen in some tumours.

There is debate about whether centrosome abnormalities are a primary cause of cancer, or a consequence of aneuploidy and cell disregulation (reviewed in (Nigg, 2002)). Centrosome abnormalities are found not only in invasive but also in low-grade and in situ cancers (early in cancer progression) suggesting they are an early factor in chromosome instability (Nigg, 2002). No matter how centrosome abnormalities originally arise, they will undoubtedly encourage chromosome missegregation.
Chapter 2 - Materials and Methods

2.1 Supplier information
Chemicals were purchased from the following sources, except where stated otherwise: BDH, Boehringer Mannheim, Fisher, Fisons, Gibco BRL, Melford and Sigma.

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

Reagents for all growth media were obtained from the following sources, except where stated otherwise: Biogene, Difco, Oxoid, Sigma.

2.2 Commonly used buffers

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBE</td>
<td>445 mM Tris</td>
</tr>
<tr>
<td></td>
<td>445 mM Boric Acid</td>
</tr>
<tr>
<td></td>
<td>100 mM EDTA pH 8.0</td>
</tr>
<tr>
<td>TE (pH 7.5 or 8.0)</td>
<td>10 mM Tris</td>
</tr>
<tr>
<td></td>
<td>1 mM EDTA</td>
</tr>
<tr>
<td></td>
<td>pH adjusted as appropriate</td>
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<tr>
<td>DNA loading buffer</td>
<td>75 mM EDTA</td>
</tr>
<tr>
<td></td>
<td>25% (w/v) Sucrose</td>
</tr>
<tr>
<td></td>
<td>0.1% (w/v) Bromophenol Blue</td>
</tr>
<tr>
<td>SDS PAGE Running Buffer</td>
<td>50 mM Tris</td>
</tr>
<tr>
<td></td>
<td>384 mM Glycine</td>
</tr>
<tr>
<td></td>
<td>0.5 % (w/v) SDS</td>
</tr>
<tr>
<td>Transfer Buffer</td>
<td>25 mM Tris Base</td>
</tr>
<tr>
<td><strong>192 mM Glycine</strong>&lt;br&gt;20 % (v/v) Methanol</td>
<td><strong>25 mM Tris</strong>&lt;br&gt;192 mM Glycine&lt;br&gt;0.1% (w/v) SDS&lt;br&gt;20 % (v/v) Methanol</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td><strong>Towbin transfer buffer</strong></td>
<td><strong>137 mM NaCl</strong>&lt;br&gt;2.7 mM KCl&lt;br&gt;10.1 mM Na₂PO₄&lt;br&gt;1.76 mM KH₂PO₄&lt;br&gt;Adjust to pH 7.2</td>
</tr>
<tr>
<td><strong>PBS</strong></td>
<td><strong>extra 0.5M NaCl</strong></td>
</tr>
</tbody>
</table>
| **Protein Sample buffer**                 | **80 mM Tris, pH 6.8**<br>10 mM EDTA<br>10% (w/v) SDS<br>Bromophenol Blue<br>10% (v/v) Glycerol | Added immediately before use: 1 mM DTT, 100 mM Pefabloc, 20 µM Pepstatin, 20 µM Leupeptin, 20 µM Chymostatin
Optional phosphatase inhibitors: 50 mM sodium fluoride, 2 mM sodium pyrophosphate, 1 mM sodium vanadate, 100 nM microcystein (Alexis Biochemicals) |
2.3 Microbiology

2.3.1 E.coli

2.3.1.1 Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>F- φ80lacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rk, mk) phoA supE44 thi-1 gyrA96 relA1 λ-</td>
<td>M. Brady</td>
</tr>
<tr>
<td>XL1- Blue</td>
<td>F':::TnI0 proA⁺B⁺ lacI⁴ Δ(lacZ)M15/recA1 end A1 gyrA96 (NaIr) thi hsdR17 (rₘ₉⁺m₉⁻) glnV44 relA1 lac</td>
<td>Stratagene</td>
</tr>
<tr>
<td>BL21</td>
<td>B F- ompT hsdS(rB⁻ mB⁻) dcm⁺ Tetr gal (DE3) endA Hte</td>
<td>Stratagene</td>
</tr>
</tbody>
</table>

2.3.1.2 Media

<table>
<thead>
<tr>
<th>Media</th>
<th>Bacto-tryptone 1% (w/v)</th>
<th>Bacto-yeast extract 0.5% (w/v)</th>
<th>NaCl 0.5% (w/v)</th>
<th>pH adjusted to 7.2 with 5 M NaOH.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOC</td>
<td>2% Tryptone</td>
<td>0.5% Yeast Extract</td>
<td>0.5 g/L NaCl</td>
<td>10 mM MgCl₂</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>10 mM MgSO₄</td>
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<td></td>
<td></td>
<td></td>
<td>0.4% (w/v) Glucose</td>
</tr>
<tr>
<td>Ampicilin (Amp)</td>
<td></td>
<td></td>
<td></td>
<td>to 100 μg/ml</td>
</tr>
</tbody>
</table>
Strains were stored long term by freezing fresh cultures in LB with 30 % glycerol and storing at -80 °C.

2.3.1.3. Transformation

Preparation of chemically competent DH5α

Reagents

- TfbI - 30 mM KAc, 100 mM RbCl2, 10 mM CaCl2, 50 mM MnCl2, 15% (v/v) Glycerol
- TfbII - 10 mM MOPS, 75 mM CaCl2, 10 mM RbCl2, 15% (v/v) Glycerol

A 5ml overnight culture of DH5α were diluted 1:200 into 50ml LB and grown with shaking at 37°C until an O.D₆₀₀ of 0.5 was reached. This was then further 1:50 into 200ml LB and grown again to an O.D₆₀₀ of 0.48. The culture was chilled on ice before being collected by centrifugation (5 min at 5000 rpm, 4 °C). The supernatant was decanted and the pellet was resuspended in 80 ml cold TfbI and incubated on ice for 5 min. The cells were again collected by centrifugation (10 min at 10000 rpm, 4 °C), resuspended in 6 ml TfbII and left on ice for 15 min. Cells were divided in aliquots of 100-200 µl and immediately transferred to - 80 °C for storage.

Transformation of chemically competent DH5α by heat shock

An aliquot of competent DH5α cells was thawed in ice, and then 1-5 µl of DNA added and left on ice for 30 min. After 30 min the cells were heat shocked at 42 °C for 90 sec. 1 ml of LB was then added and the cells allowed to recover at 37 °C for 30-60 min. 200 µl cells were then plated out on appropriate selective LB plate and grown at 37 °C overnight.

Electroporation

Competent XL1-Blue or BL21 were transformed according to manufacturers instructions. Typically 0.5 µl of plasmid DNA or 1/2 volume of a ligation was transformed into 40 µl of cells.
### 2.3.2.1 Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mating type</th>
<th>Genotype</th>
<th>Source/Notes</th>
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<tr>
<td>KH 34</td>
<td>a</td>
<td>ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100</td>
<td>K. Hardwick. W303</td>
</tr>
<tr>
<td>KH 35</td>
<td>α</td>
<td>ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100</td>
<td>K. Hardwick W303</td>
</tr>
<tr>
<td>KH 321</td>
<td>a</td>
<td>ura3-1, leu2, 3-112, his3-11, trp1-1, ade2-1, can1-100, CFII URA3 SU11</td>
<td>Chromosome loss, in W303 background K. Hardwick</td>
</tr>
<tr>
<td>SLM-65 Ua</td>
<td>a</td>
<td>sfl-65, mad1Δ:HIS ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,</td>
<td>K. Hardwick</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p(MAD1, URA)</td>
<td></td>
</tr>
<tr>
<td>SLM-65 Wα</td>
<td>α</td>
<td>sfl-65, mad1Δ:HIS ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,</td>
<td>K. Hardwick</td>
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<tr>
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<td>p(MAD1, TRP)</td>
<td></td>
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<tr>
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<td>p(MAD1, URA)</td>
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<tr>
<td></td>
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<td>sfl-273, mad1Δ:HIS ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,</td>
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<tr>
<td>SLM-273 Wα</td>
<td>α</td>
<td>sfl-273, mad1Δ:HIS ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,</td>
<td>K. Hardwick</td>
</tr>
<tr>
<td></td>
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<td>p(MAD1, TRP)</td>
<td></td>
</tr>
<tr>
<td>VEA 1</td>
<td>a</td>
<td>SFII-YFP:HIS, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,</td>
<td>This study</td>
</tr>
<tr>
<td>VEA 2</td>
<td>a</td>
<td>SFII-YFP:HIS, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p(MTW1-CFP, LEU)</td>
<td></td>
</tr>
<tr>
<td>VEA 3</td>
<td>a</td>
<td>SFII-YFP:HIS, SPC42-CFP::TRP, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,</td>
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</tr>
<tr>
<td>VEA 4</td>
<td>a</td>
<td>SFII-YFP:HIS, TUB-CFP::URA ura3-1, leu2,3-112, his3-11,</td>
<td>This study</td>
</tr>
</tbody>
</table>
This study

VEA 5  a  sfl-65, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,

VEA 6  a  sfl-65, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,

VEA 7  a  sfl-120, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,

VEA 8  a  sfl-120, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,

VEA 9  a  sfl-229, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,

VEA 10  a  sfl-229, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,

VEA 11  a  sfl-273, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,

VEA 12  a  sfl-273, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,

VEA 13  a  TUB1-GFP::URA, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,

VEA 14  a  TUB1-GFP::URA, SPC42-CFP::TRP, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,

VEA 15  a  sfl-60, TUB1-GFP::URA, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,

VEA 16  a  sfl-65, TUB1-GFP::URA, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,

VEA 17  a  sfl-120, TUB1-GFP::URA, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,

VEA 18  a  sfl-229, TUB1-GFP::URA, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,

VEA 19  a  sfl-273, TUB1-GFP::URA, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,

VEA 20  a  sfl-65, SPC42-GFP::TRP, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,

VEA 21  a  sfl-120, SPC42-GFP::TRP, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,

VEA 22  a  sfl-229, SPC42-GFP::TRP, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,

VEA 23  a  sfl-273, SPC42-GFP::TRP, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,

VEA 24  a  SPC42-GFP::TRP, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,

VEA 25  a  sfl-65, Chromosome loss

VEA 26  a  sfl-120, Chromosome loss

VEA 27  a  sfl-229, Chromosome loss

VEA 28  a  sfl-273, Chromosome loss

VEA 29  a  cdc4, SPC42-GFP::TRP

VEA 30  a  cdc4, SPC42-GFP::TRP

VEA 31  a  cdc4, SPC42-GFP::TRP

VEA 32  a  cdc4, SPC42-GFP::TRP

VEA 33  a  cdc4, SPC42-GFP::TRP

VEA 34  a  sfl-65, mad1Δ:HIS, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(MAD1, URA)

VEA 35  a  sfl-65, mad3Δ, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(MAD3, URA)

VEA 36  a  sfl-65, bub1Δ:HIS, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(BUB1, URA)

VEA 37  a  sfl-120, mad1Δ:HIS, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(MAD1, URA)

VEA 38  a  sfl-120, mad3Δ, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(MAD3, URA)

VEA 39  a  sfl-120, mad3Δ, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(MAD3, URA)

VEA 40  a  sfl-120, mad1Δ:HIS, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(MAD1, URA)

VEA 41  a  sfl-120, mad3Δ, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(MAD3, URA)

VEA 42  a  sfl-120, mad1Δ:HIS, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(MAD1, URA)

VEA 43  a  sfl-120, mad3Δ, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(MAD3, URA)
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<td>sjfl-120, bub1Δ::HIS, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(MAD3, URA)</td>
<td>This study</td>
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<tr>
<td>VEA40</td>
<td>sjfl-229, mad1Δ::HIS, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(MAD1, URA)</td>
<td>This study</td>
</tr>
<tr>
<td>VEA41</td>
<td>sjfl-229, mad3Δ, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(MAD3, URA)</td>
<td>This study</td>
</tr>
<tr>
<td>VEA42</td>
<td>sjfl-229, bub1Δ::HIS, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(BUB1, URA)</td>
<td>This study</td>
</tr>
<tr>
<td>VEA43</td>
<td>sjfl-273, mad1Δ::HIS, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(MAD1, URA)</td>
<td>This study</td>
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<tr>
<td>VEA44</td>
<td>sjfl-273, mad3Δ, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(MAD3, URA)</td>
<td>This study</td>
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<tr>
<td>VEA45</td>
<td>sjfl-273, bub1Δ::HIS, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(BUB1, URA)</td>
<td>This study</td>
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<tr>
<td>VEA55</td>
<td>sjfl-3, SPC42-GFP::TRP</td>
<td>Integration of SPC42 plasmid at TRP locus into strain JKY1724</td>
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<tr>
<td>VEA56</td>
<td>sjfl-3, mad1Δ, p(MAD1, URA)</td>
<td>This study</td>
</tr>
<tr>
<td>VEA57</td>
<td>sjfl-3, mad3Δ, p(MAD3, URA)</td>
<td>This study</td>
</tr>
<tr>
<td>VEA58</td>
<td>sjfl-3, bub1Δ, p(BUB1, URA)</td>
<td>This study</td>
</tr>
<tr>
<td>VEA59</td>
<td>EK11, SPC42-mCherry::NAT</td>
<td>This study, PCR tagging SPC42 with mCherry</td>
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<td>VEA60</td>
<td>SBY215, SPC42-mCherry::NAT</td>
<td>This study</td>
</tr>
<tr>
<td>VEA63</td>
<td>sjfl-65, GFP chromosomes (TRP, HIS), SPC42-mCherry::NAT, bar-</td>
<td>This study</td>
</tr>
<tr>
<td>VEA64</td>
<td>sjfl-120, GFP chromosomes (TRP, HIS), SPC42-mCherry::NAT, bar-</td>
<td>This study</td>
</tr>
<tr>
<td>VEA65</td>
<td>sjfl-229, GFP chromosomes (TRP, HIS), SPC42-mCherry::NAT, BAR+</td>
<td>This study</td>
</tr>
<tr>
<td>VEA66</td>
<td>sjfl-273, GFP chromosomes (TRP, HIS), SPC42-mCherry::NAT, bar-</td>
<td>This study</td>
</tr>
<tr>
<td>VEA78</td>
<td>sjfl-120-mCherry::NAT, SPC42-GFP::TRP, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100,</td>
<td>This study</td>
</tr>
<tr>
<td>VEA79</td>
<td>sjfl-Δ770-mCherry::NAT, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(SFII, URA)</td>
<td>sjfl-Δ770 tetrad</td>
</tr>
<tr>
<td>VEA80</td>
<td>ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(SFII, URA)</td>
<td>sjfl-Δ770 tetrad</td>
</tr>
<tr>
<td>VEA81</td>
<td>ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(SFII, URA)</td>
<td>sjfl-Δ770 tetrad</td>
</tr>
<tr>
<td>VEA82</td>
<td>sjfl-Δ770-mCherry::NAT, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(SFII, URA)</td>
<td>sjfl-Δ770 tetrad</td>
</tr>
<tr>
<td>VEA83</td>
<td>ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(SFII, URA)</td>
<td>sjfl-Δ801 tetrad</td>
</tr>
<tr>
<td>VEA84</td>
<td>sjfl-Δ801-mCherry::NAT, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(SFII, URA)</td>
<td>sjfl-Δ801 tetrad</td>
</tr>
<tr>
<td>VEA85</td>
<td>sjfl-Δ801-mCherry::NAT, ura3-1, leu2,3-112, his3-11, trp1-1, ade2-1 can1-100, p(SFII, URA)</td>
<td>sjfl-Δ801 tetrad</td>
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### 2.3.2.2 Media and growth conditions

<table>
<thead>
<tr>
<th>Media</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>YPDA</strong></td>
<td>1% (w/v) Yeast extract</td>
</tr>
<tr>
<td></td>
<td>2% (w/v) Bacto-Peptone</td>
</tr>
<tr>
<td></td>
<td>2% (w/v) Glucose</td>
</tr>
<tr>
<td></td>
<td>0.003% Adenine sulphate</td>
</tr>
<tr>
<td><strong>CSM (dropouts)</strong></td>
<td>0.7% (w/v) Yeast Nitrogen Base</td>
</tr>
<tr>
<td></td>
<td>2% (w/v) Glucose</td>
</tr>
<tr>
<td></td>
<td>Appropriate 'Complete synthetic media' (CSM) powder from Q-Biogene (used at 2x recommended concentration)</td>
</tr>
<tr>
<td></td>
<td>Sometimes supplemented with amino acids -</td>
</tr>
<tr>
<td></td>
<td>Adenine - 120 mg/l</td>
</tr>
<tr>
<td></td>
<td>Histidine - 120 mg/l</td>
</tr>
<tr>
<td></td>
<td>Leucine - 160 mg/l</td>
</tr>
<tr>
<td></td>
<td>Lysine - 60 mg/l</td>
</tr>
</tbody>
</table>
Methionine - 40 mg/l
Tryptophan - 160 mg/l
Uracil - 60 mg/l

SPO 0.3% (w/v) Potassium Acetate
0.02% Raffinose

FOA - URA CSM plus:
20 mg/l Uracil
5 g/l Ammonium sulphate
1 g/l 'Fluoro-orotic acid (FOA)

α factor
10 µg/ml for bar+ strains, 1 µg/ml for bar- strains
stock – 16.6 mg/ml in 0.1M sodium acetate, pH 5.2 (zymo research)

Benomyl
10 – 15 µg from 30 mg/ml stock in DMSO, added to agar when boiling. 30 µg/ml used in liquid media

Nocodazole
15 µg/ml used in liquid media, stock 10 mg/ml in DMSO

'ClonNAT'
100 µg/ml Nourseothricin (Werner Bioagents), stock 200 mg/ml in water

**Growth conditions**

*S. cerevisiae* strains were typically grown on solid media overnight at 30 °C or in the case of *sf1l* mutants at 23 °C for 1-2 days.

**Storage**

Strains were stored long term by freezing fresh cultures in YPDA with 30 % glycerol and storing at -80 °C.

**2.3.2.3 Transformation**

- LiOAc mix - 100 mM LiOAc, 10 mM Tris pH 7.4, 1 mM EDTA
- PEG mix - 40% PEG 2800, 100 mM LiOAc, 10 mM Tris (pH 7.4), 1 mM EDTA
The lithium acetate transformation was based on the method of (Ito et al., 1983). A 3ml culture of the strain to be transformed was set up in the appropriate media and grown overnight. This was diluted the following morning to OD\textsubscript{600} 0.1 (5ml per transformation) and grown at 30 °C (or 23 °C for very ts mutant) until OD\textsubscript{600} of roughly 0.5 was obtained. The cells were spun down and washed 3 times with LiOAc mix and then resuspended in 100 µl LiOAc mix per 5 ml of original culture. 100 µl cells were added to 1-5 µg of DNA for transformation along with 15 µl of boiled salmon sperm DNA (@ 10 mg/ml). To this 700 µl of PEG mix was added and gently mixed by pipetting. The mix was left at 30 °C for 30 min followed by a heat shock at 42 °C (37 °C or no heat shock for some temperature sensitive strains) for 15 min. The cells were pelleted by centrifugation and resuspended in 200 µl YPDA then plated out on the appropriate selective media.

For the 2-hybrid screen 0.5 µl library DNA and 1 µl pVA5 were used per 100 µl cells.

2.3.2.4 Mating

**Mating**

Fresh (overnight growth) cultures of the two strains to be mated grown on solid media were mixed in a drop of YPDA on a YPDA plate and left at room temperature. If diploids could be selective then he mix was streaked on selective media after 24 hour. If not, after 3-4 hours zygotes were picked using a dissecting microscope.

**Sporulation**

Diploid strains were streaked out a grown overnight on YPDA or selective media if absolutely necessary (i.e. to keep plasmid) and then scraped of plate and added to 2 mls SPO media and left shaking at room temperature. After 4-7 days tetrads were picked and dissected onto a YPDA agar plate using a Singer MSM dissecting microscope. To prepare for dissection 250 µl SPO mix was spun down and
resuspended in 500 μl 100 μg/ml zymolyase in water and incubated at room temperature for 20-30 min.

2.4 Nucleic acids

2.4.1 Plasmids

<table>
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<tr>
<th>Name</th>
<th>Info</th>
<th>Source</th>
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<tr>
<td>pVA3</td>
<td>pGEX-6P - SFII-CT</td>
<td>This study, GST tagged C-term fragment</td>
</tr>
<tr>
<td>pVA4</td>
<td>pGEX-6P - SFII-120-CT</td>
<td>This study, as above</td>
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<tr>
<td>pVA5</td>
<td>pAS1-CYH2 - SFII-CT</td>
<td>This study, 2hybrid Bait</td>
</tr>
<tr>
<td>pVA6</td>
<td>pAS1-CYH2 - SFII-120-CT</td>
<td>as above</td>
</tr>
<tr>
<td>pGEX - 6p</td>
<td>GST tagging</td>
<td>Pharmacia Biotech</td>
</tr>
<tr>
<td>pAS1-CYH2</td>
<td>2-hybrid Bait vector</td>
<td>Clontech</td>
</tr>
<tr>
<td>pGAD-C1,2,3</td>
<td>2-hybrid library</td>
<td>(James et al., 1996)</td>
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<tr>
<td>pIA29</td>
<td>SPC42-GFP::TRP integrating,</td>
<td>from Ian Adams</td>
</tr>
<tr>
<td>pOK-MTW1-CFP</td>
<td>Mtw1-CFP</td>
<td>from Oliver Kerscher</td>
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<tr>
<td>pRS-SPC42-CFP</td>
<td>SPC42-CFP integration</td>
<td>from Elmar Schiebel</td>
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<tr>
<td>pDH5</td>
<td>YFP-HIS3 tagging cassette</td>
<td>Yeast resource centre</td>
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<tr>
<td>pFA6a-mCherry-natMX6 (pKS391)</td>
<td>mCherry-NAT tagging cassette</td>
<td>K. Sawin</td>
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### 2.4.2 Oligonucleotides

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<th>Function/Location</th>
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<tr>
<td>VEA1</td>
<td>S.c. tagging via PCR method</td>
<td>CGCCAATTCTCTCAAATCGATCAAAGATATGATATTATAAGAGAGCATGATAAATCCCGTTAAGTCGTAACGTCGTAAGATGGGATGTTTAAAGTAACAGGAGATCACGGGATTTAACAA</td>
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<td>VEA2</td>
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<td>ACGAAAAATATAGTAGATGGATGATTTTCAAGCTGGAAATATTATAGTAATATTGATGCACTCTGAAGACGACAGAATGAGTAAACGTGTAACCGGGATTCGTTTAACCA</td>
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<td>VEA4</td>
<td>GFP rev at 3’ end</td>
<td>CGATGACGATGGTTGTAATCCCG</td>
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<td>5'UTR scSfi</td>
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<td>VEA7</td>
<td>3'UTR scSfi</td>
<td>gcctcaggaagcaagag</td>
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<tr>
<td>VEA8</td>
<td>as VEA7 but better primer</td>
<td>CCATTAGTCTCACAACGGAACACG</td>
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<td>ctccggtagctgctagctattcaag</td>
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<td>as 13</td>
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<td>opposite of VEA5 for seq</td>
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<td>C term cloning BamH1 starts @ 770</td>
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<td>Rev EcoRI plus</td>
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<td>VEA18</td>
<td>VEA16 but NdeI</td>
<td>cgcctagctgaatgagacgctggg</td>
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<td>VEA19</td>
<td>VEA17 but BamH1</td>
<td>gcggattctcatgtgcgtatcagag</td>
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<td>VEA23</td>
<td>5' forward SPC42 tagging primer for mCherry cassette</td>
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<td>forward primer for sf1-Δ770 truncation (use with VEA2)</td>
<td>acacggatgcattataaataaacatcataaatcataaaatctgtaaaatgagatgggatagtctccgcttacatcataaaatagagatgg</td>
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<td>SiM</td>
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<td>aattgcagacgaagtcgg</td>
</tr>
</tbody>
</table>
2.4.3 DNA extraction

2.4.3.1 Extraction of Plasmid DNA from *E. coli*

**Miniprep**
Plasmid DNA was prepared from 1.5 ml overnight cultures using a spin-column kit (Qiagen), according to manufacturer’s instructions. Typically 30-50 µl of DNA in H₂O was obtained from 1.5 ml culture.

**Maxi -prep by rapid alkaline lysis method**

**Reagents**
- GTE (solution I) - 50 mM Glucose, 25 mM Tris-Cl, pH 8.0, 10 mM EDTA
- Solution II - 1% (w/v) SDS, 0.2 M NaOH
- Solution III - 3 M KOAc, 2 M HOAc
- PEG/NaCl – 20 % (w/v) PEG, 2.5 M NaCl.

100-400 ml of LB AMP media was inoculated with the plasmid bearing *E. coli* strain (DH5α or XL1-Blue) and grown overnight at 37 °C. Cells were harvested by centrifugation (4000 rpm, 5-10 minutes), the supernatant decanted and the pellet resuspended in 20 ml of GTE. To this 20 ml of solution II was added and the sample mixed gently until clear and viscous. To stop lysis, 15 ml of solution III was added. In order to separate out the cell debris the samples were centrifuged (5000, 5-10 min)
and then the supernatant filtered through cheesecloth. 45 ml of propan-2-ol was added to precipitate the nucleic acids, which were pelleted by centrifugation (8000, 10 min). This pellet was resuspended in 4 ml TE. 4 ml of 5 M LiCl was added to this and the samples chilled on ice for 10 min. The precipitated RNA was pelleted out by centrifugation (10000, 10 min). The supernatant from this centrifugation was added to 8 ml Propan-2-ol, mixed, and centrifuged (10000, 10 min). The pellet from this was washed with 70% (v/v) ethanol, air dried, and resuspended in (typically 200 μl) TE. the protocol was finished here for crude plasmid preps

(optional PEG precipitation)
RNAase A was added to 40 μg/ml and incubated at 37 °C for 15 min. 0.5 vol PEG/NaCl was added and the mixture left on ice for 15 min. The mixture was the spun and the DNA pellet dissolved in 400 μl TE. Phenol/chloroform extraction (see section) was performed and the ethanol precipitation of DNA (see section). The final DNA was resuspended in 500 μl TE.

2.4.3.2 Yeast DNA- Extraction

- Yeast DNA extraction buffer - 2.5 M LiCl, 50 mM Tris, pH 8.0, 62.5 mM EDTA, 4% (v/v) Triton X-100.
- Ph/Chl - Phenol/Chlorform/isoamyl alcohol (25:24:1) (Sigma)

Yeast DNA extraction followed the method in (Ward, 1990). Cells were harvested by centrifugation from 1.5 ml overnight culture in appropriate media. The supernatant was aspirated off and to the pellet of cells 200 μl yeast DNA extraction buffer, 200 μl of Ph/Chl and approx 200 μl of glass beads was added. The mixture was homogenised in a mini bead beater (BioSpec products) for 2 min at 4 °C, followed by centrifugation (14000 rpm, 10 min, 4°C). The upper aqueous phase was removed with a pipette and ethanol precipitated. The pellet was air dried and resuspended in typically 20 μl TE.
2.4.4 General DNA methods and cloning

**Ethanol precipitation**

10% volume of 3M NaAcetate, pH 5.2 and 2 volumes of 96% ethanol were added to solution and incubated on ice to precipitate DNA. Mixture was then centrifuged at 4 °C for 10 min at maximum speed. The DNA pellet was washed with 70% ethanol, air dried, and typically resuspended in TE.

**Cloning**

All restriction digests used enzymes and corresponding buffer form New England Biolabs, according to instructions. Ligations used the T4 DNA ligase kit from NEB according to manufacturers instructions.

**Polymerase chain reaction**

All reactions used a PTC-gradient cycler (MJ Research).

**General PCR**

PCR-mix:

- Template – 10-30 ng plasmid DNA/ 10-500 ng yeast genomic DNA
- 0.5 μM each primer
- 2.5 mM dNTPs
- PCR buffer – 10 mM Tris, pH 8.3, 50 mM KCl, 2 mM MgCl2, 0.01% Gelatin
- Taq (made in lab), 1 in 100

Typical program:

Initial template denaturation – 95 °C, 1 minute

30 cycles:

Denaturation - 95°C 30 sec

Annealment – 55 °C 30 sec

Extension – 72 °C, 1 min per kb of product required

Final extension – 72 °C 5 min
Yeast colony PCR
Typically these were 50 μl. A small blob of cells from fresh (grown overnight) strain on solid media were put into a 500 μl tube with a toothpick. These were baked (dry) in the tube at 96 °C for 10 min. 50 μl PCR mix (see above) was then added and the typical PCR program run.

Long template PCR for *S. cerevisiae* genomic integration
Using the ‘Expand long template’ kit from Roche according to instructions, with buffer number 3.

Program:
94 °C 2 min
10 cycles of:
92 °C 30 sec
50 °C 30 sec (determined experimentally to be optimum for primers VEA1 and VEA2)
68 °C 4 min

then 19 cycles of:
92 °C 10 sec
50 °C 30 sec
68 °C for 4 min plus 20 sec per cycle
Final elongation 68 °C 7 min

Sequencing
Sequencing was done using ‘Big Dye’ 3.1 (ABI). A typical reaction was 4 μl Big Dye, 1 μl primer (@ 1.6 pmol/μl) and 3-5 μl of DNA to a total volume of 10 μl. For plasmids 15-45 ng was used, PCR products – a 50 μl reaction was cleaned up/extracted into 30 μl H₂O and 3-5 μl used for sequencing.

Agarose gel
Typically agarose gels were 1% (w/v) in TBE, containing ~ 0.2 mg/ml ethidium bromide. DNA loading dye was added ~ 1/10 to samples before loading. Gels were run in TBE at 90 – 120V. A 1 kb DNA ladder (Gibco) was run in parallel.

**Gel extraction**

Fragments were visualised with a UV transluminator and cut out with a sterile scalpel. DNA was extract from agarose fragments using Qiagen gel extraction kit.

### 2.5 Protein Methods

#### 2.5.1 SDS page and western blotting

**Whole cell extracts**

Typically 1.5 ml of cell culture was spun down. The pellet was resuspended in 300 µl protein samples buffer with an equal volume of glass beads (425-600 µm). Samples were homogenised either in a ‘bead beater’ (BioSpec products) for 1 min or Hybaid Rybolyster for 20 sec at setting 4. Samples were centrifuged at full speed for 1-5 min and then boiled for 5 min.

**SDS polyacrylamide gels**

Resolving gel:
- 10 % acrylamide
- 0.13 % Bis-acrylamide
- 0.375 M Tris pH 8.8
- 0.1 % Sodium dodecyl sulphate (SDS)

Stacking gel:
- 5.36 % acrylamide
- 0.13 % Bis-acrylamide
- 0.125 M Tris pH 6.8
10 % APS was added 1/10 and TEMED 1/100 in order to polymerise the gel.

Gels were run at 150-180V in running buffer, in Anachem gel tanks. In the case of Sfi1p blots, gels were run at 180V for 15 min after the blue dye had run off the bottom of the gel.

Gels were either stained with GelCode blue (Pierce), or western blotted as below.

**Western blotting**

Protein was transferred to ‘Protran’ 0.2 μm nitrocellulose membrane (Whatman) by wet transfer (Hoefer) in Towbin buffer, for 90 min at 65 V. Membranes were washed in water and stained with Ponceau staining to reveal size markers. Membranes were blocked with 5% milk in PBS for 1 hour. Primary antibodies were incubated at 4 °C overnight. Blots were washed 3 x 10 min with PBST. HRP conjugated secondary antibodies were incubated at room temperature for 1-2 hours. Blots were again washed 3 x 10 min. HRP was detected with ECL (Amersham), and Biomax Mr-light film.

2.5.2 TAP pull down

- Lysis buffer – 50 mM Hepes, pH 7.6, 75 mM KCl, 1 mM MgCl2, 1 mM EGTA, 0.1% Triton-X100, LPC, 1mM Pefabloc, 50 mM NaF, 2mM Na pyrophosphate, 1 mM Na Vanadate, 100 nM microcystin.

Cells were grown in the appropriate conditions. 100-300 mg of cells were pelleted and resuspended in 300 μl lysis buffer per 100 mg cells. Glass beads were added, and cells ribolysed as for whole cell extract preparation. Samples were spun at ~700 rpm in a mini-centrifuge at 4 °C, and the supernatant collected. DTT was added to 0.5 mM, and samples spun and decanted again several times until no visible pellet was gained.
10 μl IgG sepharose (Amersham) was washed three times with PBS/0.1 % Triton, and once with lysis buffer. The cell extract from above was added to the beads and incubated for 2 hours at 4 °C with rotation. The beads were then washed three times with lysis buffer and two times with PBS. The beads were split into two, one samples was frozen. The other sample was washed once in lambda phosphatase buffer (NEB), and then resuspended in 50 μl phosphatase buffer, along with 0.25 μl lambda phosphatase and incubated at 37 °C for 30 min. After this, the beads were washed once with phosphatase buffer and twice with PBS, and then frozen. Samples were thawed in 20 μl protein sample buffer (with 5% β-mercaptoethanol instead of DTT) on the bench for 15 min. Samples were then boiled fro 5 min, and ready to be loaded on an SDS PAGE gel.

2.5.3 Antibodies used in this study

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<td>J. Blyth</td>
</tr>
<tr>
<td>Sfi1-C (Rabbit)</td>
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<td>this study</td>
</tr>
<tr>
<td>HA (12CA5) (Mouse)</td>
<td>1 in 500</td>
<td>Sigma</td>
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<td>Clb2p (Rabbit)</td>
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<td>D. Kellogg (UCSC)</td>
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<tr>
<td>Mad1p (Rabbit)</td>
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<td>K. Hardwick</td>
</tr>
<tr>
<td>Anti Rabbit HRP</td>
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<td>Amersham</td>
</tr>
<tr>
<td>Anti Mouse HRP</td>
<td>1 in 5000</td>
<td>Amersham</td>
</tr>
</tbody>
</table>

2.5.4 Protein purification - GST from bacteria

- Lysis Buffer – PBS with 0.5% (v/v) Tween–20, 1 mM Pefablock, 1 M NaCl, LPC (1 tablet (company) per 50 ml).
- Wash Buffer – PBS with 0.05% (v/v) Tween-20, 0.5 mM DTT, 0.25 M KCl.
- Elution Buffer – 50 mM Tris pH 8.1, 0.25 M KCl, 5 mM reduced glutathione
- Dialysis Buffer - 50 mM Hepes pH 7.6, 100 mM KCl, 3- % (v/v) Glycerol.
4 litres of GST expressing BL-21 were grown up in LB Amp at 37 °C to an OD₆₀₀ of 0.8. IPTG was then added to a concentration of 0.1 mM to induce expression and left with shaking at 18 °C overnight. The cells were then spun down by centrifugation and resuspended in a small amount as possible of lysis buffer and the mixture pipetted into liquid nitrogen to freeze as ‘pellets’. These pellets were then ground in a Retsch R100 grinder with liquid nitrogen, to a fine powder. The powder was allowed to thaw slightly at room temperature, and then dissolved in 200 ml lysis buffer by leaving to mix at 4 °C. The mixture was lysed further by sonication until viscosity was reduced. DTT was added to 10 mM and the lysate spun at 35000 rpm for 60 min in a Beckman ultracentrifuge. The supernatant was mixed with 5-10 ml of glutathione agarose beads (Sigma G-4510) and left gently mixing to bind at 4 °C for 1-2 hours. The agarose beads were loaded onto a column and washed with 50-100 ml wash buffer or until the flow through from the column contains no proteins (assayed by Bradford reagent). Then washed with 2 column volumes of wash buffer without Tween - 20. Protein was eluted off the column in fractions by pipetting 1 ml elution buffer at a time and collecting the elutate in 1.5 ml tubes. Protein concentrations of the fractions were assayed by Bradford reagent and the peak fractions pooled and dialysed in Dialysis buffer. Protein in dialysis buffer was stored at -80 °C.

2.5.5 Affigel coupling

- Coupling Buffer – 50 mM Hepes pH 7.6, 100 mM KCl.
- Affigel 10 (BioRad)

The required amount of Affigel 10 was allowed to warm to room temperature then washed 3-4 times in ice cold coupling buffer. The protein solution (from GST purification, above) was added to the Affigel to give a 1:1 slurry with a protein concentration of 1-5 μg. This was spun down and 5 μl sample taken for a Bradford assay. The slurry was allowed to mix at 4°C. 25 μl samples were taken every 5 minutes until the O.D. for the Bradford assay was the same as the 0 time. This meant that 80 % coupling had been achieved. The coupling reaction was stopped by adding
1 M Tris pH 7.5 to a final concentration of 50 mM. The affigel was washed with whatever elution buffer would be used before use.

2.4.6 Antibody purification

- Wash buffer – PBD containing 0.5 M NaCl, 0.1% Tween-20.
- Elution buffers— 100 mM triethylamine pH 11.5 OR 100 mM Glycine pH 2.5.
- Dialysis buffer – PBS containing 50 % glycerol.

An affigel column of 2-5 ml with 1-15 mg protein was prepared and washed with both elution buffers and then equilibrated with PBS. The rabbit serum was centrifuged at 15000 rpm for 10 min and then Sodium Azide added to 0.04%. The supernatant was first run through a GST pre-column by gravity, then pumped through the Sfi1 column at a rate of 5 column volumes per hour. The column was then washed with 50 volumes of wash buffer, followed by 2 volumes of 0.2X PBS. Antibodies were first eluted with 0.5 ml aliquots of Triethylamine into tubes containing 200 μl 1M Tris pH 6.8. The column was washed again with several volumes of 0.2X PBS and elution repeated with Glycine into Tris at pH 8.3. The eluted fraction were pooled and dialyzed into dialysis buffer.

2.5.7 Yeast extract and affinity chromatography

- Extraction Buffer – 50 mM Hepes (KOH) pH 7.6, 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 1 mM PMSF, LPC protease inhibitors.
- Wash Buffer – 50 mM Hepes (KOH) pH 7.6, 50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 10 % Glycerol, LPC.
- Elution Buffer – 50 mM Hepes (KOH) pH 7.6, 1 mM EGTA, 1 mM MgCl₂, 1 mM DTT, LPC, 10% Glycerol. KCl (variable concentration)

4 litres of JB811 cells were grown in YPDA to saturation then pelleted by centrifugation and resuspended in minimum volume of extraction buffer and frozen
in pellets by pipetting into liquid nitrogen. The pellets were ground with liquid nitrogen with a Retsch grinder until a fine powder was obtained. 30 – 50 ml extraction buffer was added and the powder allowed to dissolve with stirring at 4 °C. This was then centrifuged at 40000 for an hour at 4 °C. DTT was added to the supernatant to 1mM. The supernatant was then pre-cleared by passing through a 30 ml BSA on affigel column. An affigel column of the protein of interested was equilibrated with extraction buffer, and then the flow through from the BSA column passed over at 8 column volumes per hour with a pump. The column was then washed with 8 column volumes of wash buffer or until no protein was coming off in the flow through (assessed by Bradford assay). The protein was eluted off with elution buffer pipetted 1 ml at a time with increasing concentrations of KCl from 100 mM to 1 M and collected in 1.5 ml tubes. The protein in each eluted fraction was precipitated with 10 % trichloroacetic acid and left on ice for 10 min. The precipitate was pelleted by centrifugation at 4 °C for 10 min. The supernatant was removed and the pellets resuspended in protein samples buffer made with non-pH’d Tris. These samples were then run on an SDS-PAGE gel.

2.6 Cell Biology

2.6.1 Microscopy

Cell Culture
Cells were typically grown for microscopy in CSM with extra adenine, or the appropriate CSM dropout media. Live cells were spun down and mixed (~1:1) with 0.5 % low melting temperature agarose in CSM. Fixed cells were fixed by adding 1/10 37% formaldehyde (BDH) for 10 min. Samples were then washed twice and stored at 4 °C in PBS/1M sorbitol.
**Imaging**

Microscopy was performed with the ‘Marianas’ digital microscope system from Intelligent Imaging Innovations (www.intelligent-imaging.com/marianas/main.php), with a Coolsnap HQ CCD camera. Images were taken and manipulated using the ‘Slidebook’ software also from Intelligent Imaging Innovations.

**2.6.2 Electron microscopy**

Cells were grown to log phase at 23 °C in YPDA and then arrested in alpha factor. Cells were then released into fresh YPDA at 37 °C for 3 hours. They were high pressure frozen in a BAL-TEC HPM 010 as described in (Giddings et al., 2001). Frozen samples were freeze-substituted in 2 % osmium tetroxide, 0.1 % uranyl acetate in acetone and -80 °C for 3-4 days, then embedded in Spurr’s resin. Serial thin sections (80 nm) were collected on formavar coated slot grads and stained with uranyl acetate and lead citrate. The sections were viewed in a Philips CM10 transmission electron microscope operating at 80 KV. The electron microscopy showed in this study was performed at the University of Colorado, Boulder by T. Giddings.
3.1 Introduction

One of the most important events in the life of a cell is dividing mitotically to form two new daughter cells with the correct DNA content. There have been many approaches to identifying genes involved in mitosis. The yeasts *Saccharomyces cerevisiae* (Budding yeast) and *Schizosaccharomyces pombe* (Fission yeast) are particularly useful due to their short generation time and genetic techniques available.

The first screens for mitotic genes in budding yeasts focussed on the cell cycle itself. In a famous early screen, Hartwell and co-workers screened for temperature sensitive mutants that ceased to cycle at the restrictive temperature. These were named the *cdc* (cell division cycle) mutants (Hartwell, 1973; Hartwell et al., 1970; Hereford and Hartwell, 1974). These have since been shown to be involved in many aspects of cell division, both structural components of the spindle (e.g. Cdc31p) and control elements (e.g. Cdc28p, Cdc20p). Later screens focussed on the most important event of mitosis, the correct segregation of genetic material. The most basic function of mitosis is to correctly segregate DNA to two daughter cells. This chromosome segregation can be assayed directly. Various assays were used and yielded the *mcm* (Mini-chromosome maintenance, (Maine et al., 1984)), *mif* (Mitotic fidelity, (Meeks-Wagner et al., 1986)), *chl* (chromosome loss, (Kouprina et al., 1988)) and *ctf* (chromosome transmission fidelity (Spencer et al., 1990)) mutants.

Mitosis, and in particular chromosome segregation requires co-ordination of so many processes there are likely many genes still to be discovered. Mitotic defects that affect the integrity and function of the spindle are recognised by the spindle assembly checkpoint (section 1.2.3). By screening for new mutants that require a functional checkpoint to be viable, we screen for new mutants that impinge on the mitotic spindle either directly or indirectly. To this end, a screen for new mutants
that were synthetic lethal with a dead checkpoint \((mad1\Delta)\) was performed. Investigating the mutated genes can reveal new factors required in mitosis.

3.2 The \(mad1\Delta\) synthetic lethal screen

3.2.1 Synthetic lethal screens
In order to identify new mitotic defects a screen for genes synthetic lethal with the \(mad1\Delta\) mutation was performed. Synthetic lethal screens are a way to search for genetic interactions between genes. Mutations in the two genes may not be lethal in isolation but become so in combination. This is most often because the gene products act in complimentary pathways that can partially compensate for the loss of the other. In this case we are looking for mitotic mutations that would normally be recognised by the spindle checkpoint allowing the cell to arrest and resolve the defect. In absence of a functional spindle checkpoint the cell continues through mitosis and divides aberrantly. This will be lethal especially if it results in the mis-segregation of chromosomes or other factors such as the spindle pole bodies. Spindle checkpoint mutants are not usually synthetically lethal with each other, as the checkpoint is eliminated with mutation of just one, so further defects have no effect.

\(mad1\Delta\) cells are viable under normal growth conditions. Their growth only becomes compromised under conditions that would normally induce a checkpoint arrest. In previous synthetic lethality experiments with the checkpoint \(mad1\Delta\) has shown a wider range of interactions and synthetic stronger phenotype than e.g. \(mad3\) (Hardwick et al., 1999). Mutations synthetic lethal with \(mad1\Delta\) most likely have mitotic defects normally detected by the spindle checkpoint.

3.2.2 The screen
Many screens in budding yeast take advantage of a feature of the adenine biosynthetic pathway. The product of the \(ADE3\) gene creates a red pigmented intermediate. This is then processed by Ade2p back into an unpigmented intermediate. Cells that are \(ade2\) but \(ADE3\) therefore, are blocked in the pathway
and accumulate the red intermediate. This gives the yeast colony a red colour easily detected by eye. This is used to provide a way to assay the presence of a plasmid or artificial chromosome (Hieter et al., 1985; Spencer et al., 1990) containing the ADE3 gene in an ade2 strain background.

Strains were constructed containing a mad1 deletion (mad1Δ) as well as mutations in the ade2 and ade3 genes. The assay strain also contained a plasmid with both wild type MAD1 gene and ADE3, as well as a TRP1 or URA3 marker. Due to the ADE3 gene the plasmid gives the colonies a red colour. As mad1Δ is not lethal, these cells can lose this plasmid under non-selective conditions and the colonies are made up of cells with and without the plasmid. Due to the way colonies grow they appear sectored red and white.

Upon EMS mutagenesis however, any mutants that arise that are synthetic lethal with the mad1Δ cannot lose the plasmid and remain red, even on non-selective media. Mutant strains can therefore be identified by eye. A schematic of the screen is shown in figure 3.1.

The identity of the mutant gene can be found by a screen with a library of plasmids. Those plasmids that restore sectoring are rescuing the synthetic lethal phenotype. In this case a W303 genomic DNA library (Hardwick and Murray, 1995) was used and any potential plasmids sequenced. An additional MAD1 plasmid would allow sectoring also. Any MAD1 encoding plasmids were identified by PCR and restriction digest analysis and discounted. The plasmids could contain several genes, and the rescuing gene could be a suppressor so it was important to confirm gene identity by linkage analysis. The synthetic lethal mutants were crossed with strains containing a URA3 marked gene of interest, and haploid progeny scored for the mutation and URA marker. If the two never or rarely were found in the same strain then there was a low rate of recombination between them, and it was concluded they were very close in the genome, mostly probably the same gene. In some cases the mutant genes were also sequenced.

The screen was carried out by K.Hardwick, and he and various honours and summer students carried out complementation studies, candidate plasmid rescue and genomic library rescue.
Figure 3.1. The madl synthetic lethal screen A) A schematic of the screen. B) Results of the screen (K. Hardwick). ts = temperature sensitive, cs = cold sensitive, benS = benomyl sensitive, benSS = benomyl super sensitive, benR = benomyl resistant. Mutant genes confirmed by plasmid rescue (plasmid) then some by additional genetic mapping (mapping) then a subset additionally by sequencing of gene (seq). The most specific level of gene identification is indicated.
<table>
<thead>
<tr>
<th>Mutant</th>
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<td>1</td>
<td>$TUB1$ - mapping</td>
<td>cs, benS</td>
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<tr>
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3.2.3 Results of the madlΔ synthetic lethal screen

20 synthetic lethal mutants were identified in the screen, so far the gene identity has been discovered for 13 of these. The results are shown in figure 3.1B. The genes identified are TUB1 (α tubulin), CIN8 (a microtubule plus end motor), STU1 (a microtubule associated protein) and 5 alleles mapped to SFII, a protein that little was known about. SFII was discovered as a suppressor of a mutation in fermentation induced loss of stress resistance (Ma et al., 1999). SFII is an essential gene, when put under GAL promoter (gene expression on in galactose, off in glucose) and expression shut off with glucose cells arrest in G2/M. The other three genes identified in the madlΔ screen all have a role in the spindle. It was decided to further investigate the role of Sfi1p in mitosis, and this is the subject of my PhD.

3.3 Sequence of Sfi1p

3.3.1 Sfi1p has a conserved set of repeats

A BLAST search reveals that Sfi1p is not well conserved, however it contains a series of 20 short (~15) amino acid repeats that contain a conserved tryptophan residue. These repeats can also be identified in potential homologues of Sfi1p a range of organisms including Homo sapiens. An alignment using the T-Coffee program of all repeats from S. cerevisiae and S. pombe, along with sample repeats from Neurospora crassa, Aspergillus fumigatus, and H. sapiens is shown in figure 3.2A. These repeats make up 60% of S.cerevisiae Sfi1p. It also has extensions of roughly 200 amino acids both N and C terminal to these repeats which are not conserved past budding yeasts and are predicted to be unstructured.

3.3.2 Sequencing of sf1l mutants

Primers were designed to PCR amplify overlapping fragments of SFII from genomic yeast DNA (Wild type W303 and the sf1l mutants) and also sequence these fragments in both directions. The sf1l alleles from each strain were sequenced in both directions several times from different fragments/sequencing primers. The following differences in wild type W303 were found from the wild type S288C strain in the SGD database (www.yeastgenome.org):
Figure 3.2. Structure of Sfl1p. A) Schematic of S. cerevisiae Sfl1p showing position of CT mutants (65, 120, 229, 273). B) Alignment of all Sfl1 repeats from Saccharomyces cerevisiae (Sc) and Schizosaccharomyces pombe (Sp) plus 1 each from Neurospora crassa (Nc), Aspergillus fumigatus (Af) and Homo sapiens (Hs) (Paul McLaughlin). Hydrophobic residues are highlighted in blue (orange - glycine, green - serine/threonine, turquoise - uncharged polar residues, yellow - proline).
84 (silent GAT-GAC), P97L, 153 (silent cgc-cgg), 184 (silent CAC-CAT), L278P, 393 (silent GAA-GAG), G541D, V623I, 860 (TCC-TCT), E945G. Some of the sequencing was performed by J. Luquet. The mutations from WT303 were found to be sfi1-65 - P856L, sfi1-120 - S855N, sfi1-229 - G886STOP, and sfi1-273 - R789K. A summary of these mutations including their position is shown in figure 3.2B. The synthetic lethal mutations are all in the C-terminus of Sf1p, not the repeats, indicating this poorly conserved portion may still have an important role in budding yeast.

No mutation from wild type W303 SF11 could be found in sfi1-60 in either the coding region or 200 bp either side. The mutation did however map to the SF11 region by linkage analysis (see above) and plasmid rescue, and there are no other obvious candidate genes in the region (10 genes either side of SF11). For these reasons sfi1-60 was not used further in this study.

For the remainder of this study sfi1-65, sfi1-120, sfi1-229 and sfi1-273 will collectively be referred to as 'sfi1-CT' mutants to distinguish from other published mutations (Kilmartin, 2003).

3.4 The sfi1-CT mutants are synthetic lethal with a range of checkpoint mutants

3.4.1 Checkpoint proteins and synthetic lethality
The sfi1-CT mutants are synthetic lethal with madΔ, most probably because they cause a mitotic defect that activates the spindle checkpoint. To exclude the possibility of a madΔ specific interaction it was important to verify the sfi1-CT are synthetic lethal with other spindle checkpoint genes. Two representative other checkpoint proteins, Mad3p and Bub1p were used. Often mutations that are synthetic lethal with other checkpoint proteins are not with mad3Δ (Daniel et al., 2006; Hardwick et al., 1999; Lee and Spencer, 2004). This is discussed further in section 3.7.

3.4.2 Testing synthetic lethality
The sfi1-CT mutants strains were crossed with strains that were madΔ, mad3Δ or bub1Δ and contained a URA3 marked plasmid containing the corresponding
checkpoint protein gene. The *URA3* gene can be selected against by supplementing the media with 5-fluoroorotic acid (5’FOA). The *URA3* gene encodes orotidine -5’-phosphate decarboxylase, part of the uracil biosynthetic pathway, but converts 5’FOA to the toxic 5-fluorouracil (Guthrie, 2004). If strains contain a *URA3* plasmid they must first lose it in order to grow on 5’FOA. Strains that cannot lose the *URA3* plasmids (in this case due to synthetic lethality) cannot grow on the 5’FOA plates. Serial dilutions of strains were spotted out on YPDA, -URA and 5’FOA plates and grown at room temperature for 2 or 3 days. As can be seen from the results in figure 3.3 all four *sf1l-CT* mutants are synthetic lethal with all the spindle checkpoint deletions at 23°C, indicating that the synthetic lethality with *mad1Δ* is most likely due to its spindle checkpoint role.

3.5 *sf1l-CT* mutants are temperature sensitive but benomyl resistant

3.5.1 Temperature sensitivity

Although the *sf1l-CT* mutants are synthetic lethal with checkpoint deletions even at lower temperatures, their growth on both rich media and -URA media seems to be relatively normal as long as the checkpoint is functional (see other panels of Figure 3.3). A simple way of assaying this in *S. cerevisiae* is to spot out serial dilutions and grow replica plates at different temperatures. The *sf1l-CT* mutants show similar growth to wild type at both 30°C and 18°C (data not shown), but grow much slower at 37°C (Figure 3.4A). The temperature sensitivity varies amongst the mutants with *sf1l-229* showing virtually no phenotype and *sf1l-120* having the most severe one. Even *sf1l-120* has a mild phenotype compared to a more tight temperature sensitive allele that does not grow at all at 37°C (*sf1l-3* shown as example).

When grown in liquid culture at 37°C the *sf1l-CT* mutants seem to have normal morphology, but are enriched for large budded cells (data not shown). This suggests a mitotic defect. This may well be a spindle checkpoint induced arrest/delay as the mutants were discovered by virtue of their synthetic lethality with a spindle checkpoint component. This arrest is investigated further in chapter 6.
Figure 3.3. The sfi1-CT mutants are all synthetically lethal with mad1Δ, mad3Δ and bub1Δ. Serial dilutions of strains were replica spotted onto YPDA, CSM-URA and 5′FOA plates and grown for 2-3 days at 23 °C. Failure to grow on 5′FOA indicates synthetic lethality between the sfi1 mutant and the checkpoint deletion indicated.
YPDA

sfi1-120
sfi1-120, p(MAD1, URA3)
sfi1-120, p(MAD3, URA3)
sfi1-120, p(BUB1, URA3)
sfi1-120, mad1Δ, p(MAD1, URA3)
sfi1-120, mad3Δ, p(MAD3, URA3)
sfi1-120, bub1Δ, p(BUB1, URA3)
wt, p(MAD1, URA3)

sfi1-229
sfi1-229, p(MAD1, URA3)
sfi1-229, p(MAD3, URA3)
sfi1-229, p(BUB1, URA3)
sfi1-229, mad1Δ, p(MAD1, URA3)
sfi1-229, mad3Δ, p(MAD3, URA3)
sfi1-229, bub1Δ, p(BUB1, URA3)
wt, p(BUB1, URA3)

sfi1-273
sfi1-273, p(MAD1, URA3)
sfi1-273, p(MAD3, URA3)
sfi1-273, p(BUB1, URA3)
sfi1-273, mad1Δ, p(MAD1, URA3)
sfi1-273, mad3Δ, p(MAD3, URA3)
sfi1-273, bub1Δ, p(BUB1, URA3)
wt, p(MAD3 URA3)
Figure 3.4. Phenotypes of *sfil-CT* mutants. A) *sfil-CT* mutants are mildly temperature sensitive. Serial dilutions of strains were replica spotted on YPDA and grown for 2 days at indicated temperature. B) *sfil-CT* mutants are benomyl resistant, Serial dilutions were spotted out as above onto YPDA containing indicated concentration of benomyl, and grown at 23 °C for 3 days.
3.5.2 Benomyl sensitivity
Another assay commonly used in the study of mitotic checkpoint defects is benomyl sensitivity. Benomyl is a drug that depolymerises microtubules. At low levels wild type cells recognise microtubule disturbance, arrest via the spindle checkpoint until the defect is resolved, and then continue dividing. Thus wild type cells can grow on low levels of benomyl, but do so slowly. Many checkpoint deficient strains initially grow fast as they do not recognise the defect and fail to arrest, divide aberrantly, losing chromosomes. This is lethal within 2-3 divisions, and these strains are therefore sensitive to benomyl. The checkpoint proteins vary in their sensitivity but one of the most (bud1Δ) and least (mad3Δ) sensitive strains are shown as examples. The strains were replica spotted on YPDA containing a range of concentrations (0-15 μg/ml) of benomyl and grown at 23°C for 3 days. A representative selection is shown in figure 3.4B. As can be seen from the plate with 15 μg/ml benomyl, sfi1-CT mutants are more resistant to benomyl than wild type. Another sfi1 mutant, sfi1-3 grows as wild type at this temperature and at the range of benomyl concentrations tested. Benomyl resistance is a relatively rare phenotype, and aside from mutations involving transport of the drug across the cell membrane, most often seen in microtubule structural genes such as α (Richards et al., 2000; Schatz et al., 1988) and β (Reijo et al., 1994) tubulins. The benomyl drug destabilises microtubules, so often the resistant mutants are those that cause stabilization of the microtubule polymer. Benomyl resistance is therefore also seen in mutants of microtubule destabilising factors such as Kip3p and Kar3p (Cottingham et al., 1999; Cottingham and Hoyt, 1997; Saunders et al., 1997a). This result suggests that Sfi1p is directly or indirectly involved in microtubule structure or dynamics.

3.6 sfi1-CT mutants show elevated chromosome loss

3.6.1 Chromosome loss
The most important thing for the cell to achieve during mitosis is correct segregation of its genetic material. In the end most mitotic defects that are deleterious to the cell are so because they affect the fidelity of this process. Although sfi1-CT mutants are temperature sensitive with regards to growth and grow as wild type at 23°C, they
were isolated by virtue of being synthetic lethal with a \textit{mad1}Δ at 23°C. This means they must still have some mitotic defects at this ‘permissive’ temperature. These defects may result in chromosome loss.

Chromosome loss arises when sister chromatids are incorrectly segregated, with both going to one daughter cell (2:0 segregation), or one chromosome completely lost (1:0 segregation). Loss of a chromosome is most often lethal in yeast, but aneuploidy (incorrect number of chromosomes) in higher organisms has other consequences, for example nearly all human cancers are aneuploid (Lengauer and Wang, 2004).

Mis-segregation is often due to incorrect bi-orientation of the chromosomes at metaphase. In wild type cells, the spindle checkpoint (section 1.2.3) will detect any defects in chromosome bi-orientation, and arrest the cells until it is resolved. This ensures that the rate of chromosome loss is low (roughly 1 per 100 000 divisions for a natural chromosome (Hartwell and Smith, 1985). In spindle checkpoint mutants defects are not resolved and the rate of loss is higher, 50 fold higher \textit{bub1}Δ, and 3-5 fold higher in \textit{mad} mutants (Warren et al., 2002). Many other mitotic factors can influence chromosome loss, anything that affects the structure and function of the spindle, plus cytoplasmic factors involved in positioning the nucleus correctly between mother and bud.

Several different assays have been developed to monitor chromosome loss (Biggins et al., 2001; Hegemann et al., 1999; Hieter et al., 1985; Shero et al., 1991; Spencer et al., 1990), and the one used here is described below.

\textbf{3.6.2 The chromosome loss assay}

I used the chromosome loss assay devised by Hieter \textit{et al} (Hieter et al., 1985), also (Spencer et al., 1990). Similar to the sectoring screen, this assay utilises the \textit{ade2} mutation which blocks the adenine biosynthetic pathway at a point where a red intermediate accumulates in the cells allowing red/white screening by eye. The assay strain contains an \textit{ade2-101} mutation that is a premature ochre ‘stop’ codon. This stop codon can be suppressed by the product of the \textit{SUP11} gene, which encodes
a mutant tyrosine tRNA that recognises the premature STOP codon (and instead of stopping inserts a tyrosine and translation continues). This results in translation of full length, functional Ade2p, no build up of red intermediate, and a white colony. The assay strain contains the SUP11 gene encoded on an artificial chromosome (145 kb) along with a URA3 marker in a genomic ade2-ochre background. When grown on rich media the artificial chromosome is not required for growth and its loss can be followed by the appearance of sectors with red colouring. In order to quantitate the chromosome loss, the first division after plating is assayed – i.e. those colonies that are at least half red will have lost the artificial chromosome at the first division. The percent chromosome loss is therefore the number of half (or more) red colonies divided by the total colonies counted (x 100 to get %). Any completely red colonies are discounted as they have lost the chromosome previous to plating out. It should be noted that this is loss of a small artificial chromosome so the values obtained only apply to this particular artificial chromosome. It has been shown however, that mutants that affect the artificial chromosome also affect the stability of Chromosome III (Spencer et al., 1990), indicating that this assay is a reliable indicator of natural chromosome loss. The screen is explained diagrammatically in Figure 3.5A

3.6.3 Chromosome loss in sfil-CT mutants

Strains that were MAD1 with the sfil-CT mutants (obtained by backcrossing mutants from screen with wild type 303) were crossed with the chromosome loss strain. After tetrad dissection, spores that were URA+ and predominantly white (some red sectors on rich media) were picked and presence of the sfil-CT mutation was confirmed by sequencing.

For the assay, white sections were picked and grown in CSM-URA overnight, followed by dilution and growth to log phase. The cell density was calculated using a haemocytometer and the cells diluted and plated on YPD (low adenine enhances development of the red pigment) with a density of 200-300 cells per plate. At least 2000 colonies were counted for each strain/condition. The plates were left at either 23°C or 30°C (the sfil-CT mutants do not form colonies from single cells at 37°C) until colonies had formed and then counted once the red colour had developed.
Figure 3.5. sfi1-CT mutants show increased levels of chromosome loss. A) Schematic of the chromosome loss assay. B) Comparison of sfi1-CT chromosome loss strains with wild type. Colonies on YPD 30°C displaying relative levels of sectoring, and the rarity of half-sectored colonies. C) Results. Cells were grown to log phase in CSM-URA, then diluted back and plated out at roughly 200 cells per plate. Total colonies (excluding entirely red) and those at least half red were counted. % chromosome loss = 100 x (half sectored/total).
The results are shown in figure 3.5C. Example colonies for each strain, showing different levels of sectoring are shown in figure 3.5B. This also show the relative rarity of half-sectored colonies, as even in the most extreme case make up less than 2% of all colonies. The sfl-CT strains all showed elevated levels of chromosome loss compared to wild type, and this increased in severity at 30°C compared to 23 °C. The strains did not grow at all at 37°C but following the trend it would be expected to be more severe, maybe so severe it contributes to cell death. Interestingly the chromosome loss rates do not correlate in severity with temperature sensitivity. For example sfl-120, which is temperature sensitive, has a lower rate than sfl-229, the least temperature sensitive. All strains have chromosome loss rates higher than the checkpoint mutants mad1Δ, mad2Δ and mad3Δ, but lower than the more severe bub1Δ (Warren et al., 2002), which indicates significant chromosome segregation defects, even at the so-called ‘permissive’ temperature of 23°C.

3.7 Discussion

Many previous synthetic lethal screens with the checkpoint proteins have been limited to non-essential genes as they involve using banks of strains deleted for all the non-essential genes (Daniel et al., 2006; Lee and Spencer, 2004). The advantage of the sectoring screen is that it can screen both essential and non-essential genes and more subtle point mutations.

Other than alleles of sfl, alleles of three other genes were identified. Tub1p is the main alpha tubulin in S. cerevisiae, a structural component of microtubules. Cin8p is a plus-end directed microtubule sliding motor required for spindle integrity and creating outward spindle forces, for example those separating SPBs (Hoyt et al., 1992). cin8Δ has shown synthetic lethality with mad1Δ in several previous screens (Daniel et al., 2006; Hardwick et al., 1999; Lee and Spencer, 2004). Stu1p is a microtubule associated non-motor protein that associates with β tubulin. It is also required for the outward forces in the spindle such that mutants in it arrest with side-by-side SPBs (Pasqualone and Huffaker, 1994; Yin et al., 2002).
The screen also identified 4 novel alleles of *sf1*, which had a previous indication of a mitotic role (Ma et al., 1999). Sf1p contains an internal set of amino acid repeats conserved across all organisms. *S. cerevisiae* Sf1p also has a c-terminal extension which is not conserved outside of budding yeasts. It is to this region however, that all mutations identified in the *mad1Δ* synthetic lethal screen have been located by sequencing.

The *sf1-CT* mutants were also synthetic lethal with *mad3Δ* and *bub1Δ*. There have three other investigations into synthetic lethal interactions with the checkpoint. Hardwick *et al* tested synthetic interactions between deletions of *mads 1,2* and *3* and specific mitotic factors (Hardwick et al., 1999). *tub2* (beta tubulin), *mps2* (SPB membrane insertion), and *cin8* (plus end sliding motor), were synthetic lethal with all three, but *kar3* (minus end motor) and *ctfl* (kinetochore protein) were synthetic lethal with only *mad1Δ* and *mad2Δ*. Lee and Spencer did a screen with *mad1Δ* and tested all positives with *mad2Δ* and *mad3Δ* (Lee and Spencer, 2004). *mad2Δ* showed the same amount of sickness or lethality as *mad1Δ*, whereas *mad3Δ* had a lesser phenotype with the majority of the mutants. In a screen of all non-essential genes with *mad1Δ*, *mad2Δ*, *mad3Δ* and *bub3Δ* (Daniel et al., 2006), *mad3Δ* had far fewer synthetic interactions (12 vs 60 or more for the other three), and shared very few of those of the other three checkpoint genes. The other three mutants shared a significant number of interactions, yet each checkpoint mutation also has several specific synthetic interactions.

There are two main views on why these differences in interactions between checkpoint mutants exist. Lee and Spencer make a case for Mad3p having a distinct limited role in the checkpoint. They use an assay utilising a *cdc6* mutant that results in unreplicated chromosomes. In mitosis these chromosomes can attach to the spindle, but there is no bipolar attachment or tension. This would normally cause a checkpoint-induced arrest. They claim that while *mad1Δ* and *mad2Δ* mutants fail to recognise this defect, that *mad3Δ* mutants still can, and arrest as checkpoint proficient cells do. They suggest this as evidence that Mad3p has a role in an attachment sensing pathway only, a sub-pathway of the spindle checkpoint.
Daniel et al propose a different explanation. They suggest that the interactions shown by mad3Δ reflect the core checkpoint interactions. Additional interactions shown by the other mutants may reflect additional non-checkpoint roles for these genes. An example of this is that human Mad1p and Mad2p are seen at nuclear pores in interphase and may have an additional function here (Campbell et al., 2001). In budding yeast, mad1Δ does show synthetic lethality with some pore proteins (Daniel et al., 2006). However, 50-60% of the mutants identified by Daniel et al were either benomyl sensitive or showed elevated chromosomes loss, showing most had a role in microtubule functions or chromosome segregation.

This debate reflects a limitation of synthetic lethality as an assay. Death (or the inability to form colonies) is not a very specific assay, and knowing that the loss of two gene products leads to death is not always that illuminating on their function. The checkpoint mutants were all initially isolated due to one phenotype - their sensitivity to the microtubule drug benomyl. Maybe the shared interactions simply reflect mutations that most closely resemble the microtubule destabilisation seen with these drugs, and all other interactions represent the complex network of additional defects that the checkpoint monitors.

Whatever the explanation, the sf1-CT mutants are notable in their synthetic lethality with mad3Δ. Other mutants shown to be synthetically lethal with both mad1Δ and mad3Δ are tub2 (beta tubulin), mps2 (spindle pole body) (Hardwick et al., 1999), ctf18 (sister chromatid cohesion), ctf19 (kinetochore protein), cik1 (Kar3p activator) (Daniel et al., 2006). cin8, kar3 and bim1 were identified as synthetic lethal with mad3Δ by Lee and Spencer, and at least one of the other two studies. The fact the sf1-CT mutants are synthetic lethal with three checkpoint deletions including mad3Δ adds weight to the conclusion that the mutants have a defect which activates the checkpoint.

Benomyl resistance is a relatively rare phenotype, which makes it interesting. Benomyl is a drug that destabilizes microtubules and therefore resistance is most...
common in mutants that cause incorrect stabilisation of the microtubules. The most well documented are mutants of alpha or beta tubulin, in fact one beta tubulin mutant can only grow in presence of benomyl (Machin et al., 1996; Thomas et al., 1985). At the levels used in this study, microtubules are destabilised but not abolished completely, as wild type cells can grow, albeit slowly. This suggests that the Sfi1p mutants cause stabilization of microtubules, or they have some previously undiscovered defect that benefits from a microtubule de-stabilising drug. At this stage no great conclusions can be drawn from this result, but it should be kept in mind during further analysis.

The sf1-CT mutants show temperature sensitivity, and grow as well as wild type at 23 °C. However at this ‘permissive’ temperature they still show synthetic lethality with mad1Δ so must still have mitotic defects. The presence of mitotic defects at 23 °C is also demonstrated by the increased rate of chromosome loss shown in the sf1-CT mutants. These strains have a functional checkpoint, but the increased level of chromosome loss indicates that not all of the mitotic defects are being recognised or resolved by the checkpoint. Division is continuing aberrantly in the presence of the defect causing mis-segregation of the chromosomes. These defects do not appear to be affecting growth at 23 °C, at the level of sensitivity of the crude spotting method. More subtle differences in growth rate may be detected by measuring growth of liquid culture by O.D., especially in competition with wild type strains.

A wide range of factors from all aspects of mitotic spindle function can affect chromosome loss. For example, in the large synthetic lethality screen performed by Daniel et al, 61% of all mutants identified showed elevated chromosome loss. This means that the chromosome loss phenotype alone does not yield much information on the function of Sfi1p. It adds weight to the conclusion from the other data in this chapter that the mutants have a mitotic defect that impinges on the function of the mitotic spindle.
Very little is known about Sfilp wild type protein at this point in the study. It is essential and preliminary reports suggest a G2/M arrest when depleted (Ma et al., 1999). This would fit with a spindle checkpoint mediated arrest. In the next chapter I aim to discover some basic facts about the wild type protein. Firstly, its localisation will help to narrow down where on the mitotic spindle it is acting. Raising antibodies to the protein will yield useful tools for further investigation.
Chapter 4 - Sfl1p is a spindle pole body protein with cell cycle regulated phosphorylation

4.1 Introduction

In chapter 3, four novel alleles of sfI1 were identified. At this stage very little is known of wild type Sfilp. The eukaryotic cell is a complex compartmentalised system. Knowing where in this complicated system a protein is localized can give valuable information and considerably narrow down the possibilities in the search for its function. This approach is particularly effective in S. cerevisiae, where the sole genomic copy of a protein can be tagged with a fluorescent protein, and kept under control of its endogenous promoter. This eliminates artefacts from overexpression and additional untagged copies of the gene. In the last few years the advent of PCR based tagging mechanisms in yeast has simplified this process. Several genome wide or specific screens have recognised the value of this information (Huh et al., 2003; Sundin et al., 2004). In light of this, the localisation of Sfil1p is investigated in this chapter.

A second basic piece of information about a protein is its behaviour on a Western blot. This can yield information on protein stability and post-translational modification and is a useful basic way of assessing the behaviour of mutant proteins. Many proteins are subject to post-translational modification (most commonly phosphorylation) that regulates their function. The sfI1-CT mutants were identified due to their mitotic defect, and in the case of many proteins involved in mitosis, post-translational modification changes through the cell cycle. In order to look at Sfi1p protein, antibodies were required. In second part of this chapter antibodies were created and used to investigate stability and post-translational modification of both wild type and mutants Sfi1p.

4.2 Localisation of Sfi1p

A simple way of visualising the localisation of a protein is to tag it with a fluorescent protein. Different fluorescent proteins with different emission wavelengths can be
visualised in the same cell. Fluorescent microscopes can distinguish the signals using filters. To this end reagents were prepared to tag the C-terminus of Sfi1p with Yellow Fluorescent protein (YFP). This allows later distinction from Cyan Fluorescent protein (CFP).

4.2.1 Sfi1p-YFP localises to discreet foci.
Sfi1p was tagged with YFP using the PCR based method described in (Longtine et al., 1998). Briefly, this method involves amplifying the tag and marker from a plasmid cassette by PCR. The primers are approximately 100 bases and include the junctions either side of the tag – in this case the 5’ primer contained the end of SF11 in frame with the start of the YFP tagging cassette, and the 3’ primer contained the end of the cassette with the section of the 3’ UTR of SF11. This PCR product can be transformed directly into the yeast cells using the lithium acetate transformation method. The yeast recombination system recognises the two regions of homology with the genomic sequence and recombines the PCR product complete with tag into the genome, at the locus of the gene in question. This results in a single tagged genomic copy of the gene in haploid cells, under the endogenous promoter. This tagging strategy is illustrated in figure 4.1A. In this case, a YFP tag with the HIS3 marker was used. After transformation the cells were grown on –HIS media to select for transformants. Correct transformants were verified first by colony PCR using a 5’ primer in SF11 (SiA) and a 3’ reverse primer in the YFP (VEA4). PCR positive strains were sequenced over both the 5’ and 3’ junctions of the tagging strain to confirm that YFP gene was in frame. Whole cell extract of strains with the correct sequence were then checked by Western blot analysis with anti-Sfi1p antibody (see section 4.3). The Western blot is shown in figure 4.1B. A band of the expected size 140 kDa was observed, which was absent in the untagged wild type strains, and the wild type band (112 kDa) was absent in the transformants. This combination of PCR, sequencing and western blotting, clearly demonstrates that the SF11 gene has been successfully tagged. The tagged strain (VEA1) grew as wild type on YPDA plates. Strains successfully tagged and expressing Sfi1-YFP were grown to mid-log phase in CSM media. The cells were observed live, mounted in low melting temperature agarose, by fluorescence microscopy. The Sfi1-YFP appeared to
localise to one or two foci in the cells, dependant on cell cycle stage. Representative cells of each cell cycle stage (based on bud size) are shown in figure 4.1C. The Sfl1-YFP signal appeared to be duplicated early in the cycle and then segregated one to each daughter cell. This pattern of segregation is most likely to be either kinetochores (which appear as two clusters in *S. cerevisiae* (He et al., 2000)) or the spindle pole body.

### 4.2.2 Sfl1p-YFP co-localises with the spindle pole body

In order to distinguish between kinetochores and spindle pole bodies, further co-localisation studies were performed. First a plasmid (pOK–MTW1-CFP, see materials and methods) coding a tagged kinetochore protein (Mtw1p-CFP) was transformed into Sfl1-YFP cells, to create strain VEA2. To retain the plasmids the cells were grown in selective media (CSM-LEU). Live cells were imaged as above. The Mtw1p-CFP displayed a typical kinetochore pattern (in green in figure 4.2B). In *S. cerevisiae* the kinetochores of sister chromatids are separated, even in metaphase, and oscillate back and forth (see section 1.4.4). Although *S. cerevisiae* has 16 chromosomes, therefore 16 kinetochores on each side of the spindle, the kinetochores cluster so the 16 spots appear by light microscopy as one patch of fluorescence (He et al., 2000). This is represented schematically in figure 4.2A. A representative cell is shown in figure 4.2B. One can clearly see the Sfl1-YFP spots (red) as distinct from the Mtw1p-CFP cluster (green). This shows the Sfl1-YFP spots are not kinetochores, and their position relative to the kinetochore spots suggests spindle pole body localisation.

To confirm spindle pole body localisation, another co-localisation experiments was performed. An integrating plasmid that inserts a tagged SPB protein (Spc42p-CFP) at the *TRP* locus was also transformed into *SFI1-YFP* cells (strain VEA3). Cells were grown in CSM-TRP and imaged as above and a representative cell shown in figure 4.2C. The Spc42-CFP spots (green) appear as discreet spots smaller and tighter than the Mtw1-CFP spots in 4.2B. The Sfl1-YFP spots co-localised with Spc42-CFP in all cells seen, signifying that the Sfl1-YFP spots are spindle pole bodies.
Figure 4.1. Tagging of Sfi1p with YFP. A) Schematic of PCR tagging strategy
Figure 4.1. Tagging Sfi1p with YFP  
B) Successful endogenous tagging of Sfi1p with YFP. Cells were lysed in samples buffer and run on a 10% SDS PAGE gel and western blotted with either anti-Sfi1-N or tubulin antibody.  
C) Sfi1p-YFP localises to discrete spots which segregate during the cell cycle. Live cells expressing Sfi1-YFP were grown in CSM media. Representative cells of each cell cycle stage are shown.
Figure 4.2. Sfi1-YFP localises to the spindle pole bodies. A) Model showing spindle pole body and kinetochores in pre-anaphase spindle. B) Sfi1p does not co-localise with the kinetochores. C) Sfi1p localises to spindle pole bodies. For B and C, cells expressing Sfi1-YFP plus either Mtw1-CFP (kinetochore) or Spc42-CFP (spindle pole body) were grown to log phase in CSM and visualised live by fluorescence microscopy. Representative cells are shown.
Figure 4.3. *Sfi1-120-cherry is correctly localised*. Cells were grown to log phase and then shifted to 37 °C for 3 hours. Cells were fixed in formaldehyde for 10 minutes. *sfi1-120* untagged, SPC42-GFP cells are shown as a control for bleed through. Maximum intensity projections of 5 planes 0.5 μm apart are shown.
4.2.3 Sfi1p-CT mutant proteins are correctly localised

One possibility for the phenotype seen in *sfi1-CT* mutants is that the mutant proteins have no or reduced recruitment to the SPB, leaving them compromised in or unable to perform a vital SPB function. To investigate this I attempted to make fluorescently tagged versions of Sfi1p-CT mutant proteins. Transformation efficiency was very low as the mutants are temperature sensitive and do not tolerate heat shock, however this proved a more effective method than no heat shock at all. Attempts with the YFP-HIS cassette as for the wild type were unsuccessful. A new tag and marker cassette was selected. The cassette contained the same flanking sequences as the YFP cassette used for the wild type, hence the same primers and method was used. The tag used was ‘mCherry’ a new form of RFP (Shaner et al., 2004) and a drug resistance marker NAT, allowing resistance to ClonNAT (Snaith et al., 2005). This was used to tag Sfi1p in cells already containing Spc42-GFP to mark the spindle pole bodies. Transformation and confirmation of tagging were done as for *SFII-YFP* above. In live cells there was some bleed-through of the strong GFP signal into the RFP channel. This was abolished by fixing cells (3% formaldehyde, 10 min). A strain expressing Spc42-GFP alone is shown as a control for bleed-through. Cells were grown to log phase at 23°C, and then shifted to either 37 °C or kept at 23 °C for 3 hours before fixation and imaging. Representative cells from the 37 °C sample are shown in figure 4.3. The Sfi1-120-mCherry is localised to the SPB and there is no mCherry signal above background anywhere else in the cell. Thus, Sfi1p localisation is not affected by the *sfi1-120* mutation.

4.3 Sfi1p antibodies

From the experiment above Sfi1-120-mCherry is stable enough to be seen by microscopy, but direct comparison between wild type and mutant proteins cannot be made, as they have different fluorescent tags. If the mutants are correctly localised then what defects may they have? Many spindle pole body proteins are phosphorylated. Often the post-translational modification of proteins is used to regulate their activity. In the case of cell cycle related proteins including some SPB components, this modification often changes through the cell cycle (Donaldson and
A common way to look at protein stability and post-translational modification is by Western blot. As the mutant proteins proved hard to tag, it would be very useful to have antibodies that recognise the Sfi1p protein.

### 4.3.1 Creation and testing of antibodies

Two sets of Sfi1p antibodies were made, one to the N-terminal half (J.Blyth) and one to the C-terminal 200 amino acids (section 5.3). Constructs were made to express fragments of Sfi1p fused to GST in *E. coli*. The appropriate section of Sfi1p was gained by PCR (see 5.2) and cloned into the pGEX vector.

These GST tagged constructs were expressed and purified from bacteria (see section 5.3) and used to inoculate rabbits (Diagnostics Scotland). Antibodies were purified from serum harvested from the rabbits against columns of the original antigen (see 2.5.6) to give antibodies Sfi1-N4 and Sfi1-C. These two antibodies were used in western blots against whole cell extracts of wild type, *sfi1-120*, and *sfi1-229* cells (western blotting method in 2.5.2). A variety of antibody dilutions and NaCl concentrations in wash buffer were tested. Changing conditions did not significantly change the signal seen with the C-terminal antibody. The N-terminal antibody is cleaner, with an optimum dilution of 1 in 2000. Differing wash buffers made little difference. The N-terminal antibody often displays non-specific spots of background, these are reduced by pre-incubating the antibody/BLOTTO mix with (pre-blocked) blank nitrocellulose. Also the antibody/BLOTTO mix is mixed for about 10-30 min immediately after addition of antibody. A comparison of the signals seen under optimum conditions for both antibodies is seen in figure 4.413. The N-terminal antibody is both cleaner and recognises the mutant proteins better. As Sfi1-229p has part of the C-terminus missing it is not surprising the C-terminal antibody does not recognise Sfi1p in this strain as efficiently. Due to these reasons the Sfi1-N antibody was used in the rest of this study.
Figure 4.4. Sfi1p antibodies. A) Schematic of Sfi1p showing fragments used for antibody production. B) Western blots using affinity purified antibodies against whole cell extracts. Blots using the optimum conditions for each antibody are shown.
4.4 Sfi1p behaviour through the cell cycle

4.4.1 Comparison of wild type and mutant proteins in cycling cells
I first set out to establish that the wild type and mutant proteins were the predicted size and stable. The Sfi1p in wild type and sfi1-CT strains were compared. Another mutant strain, sfi1-3 (Kilmartin, 2003), was also used. Whole cell extract samples from cells grown overnight at 23 °C were run on a 10 % SDS-PAGE gel, blotted and probed with the Sfi1-N antibody. The blot in figure 4.5A shows the antibody recognises a band of the predicted 112 kDa in all strains except sfi1-229. This has a band of predicted 97 kDa. The Sfi1p band is not sharp, but 'fuzzy', and a second fainter higher band can be seen – this is most likely the result of some post-translational modification. This is investigated later.

As shown in figure 3.3, the sfi1-CT mutants are temperature sensitive and die at higher temperatures. One possible explanation for this is that the mutant proteins become unstable above a certain temperature. Strains as above were grown to log phase at 23 °C and then shifted to 37 °C or kept at 23 °C for a further 3 hours. Whole cell extract were made and analysed as above. The blot was also probed for tubulin, which acts as a loading control as levels of tubulin remain constant in cells. As can be seen in figure 4.5B the levels of Sfi1p look the same in all the strains at both temperatures. It is unlikely then, that the phenotype in sfi1-CT strains results from protein instability. The Sfi1-3p mutant protein is also temperature stable. The pattern of post-translational modification also appears unaffected in the sfi1-CT mutants at 37 °C.

4.4.2 Post-translational modification through cell cycle
Changes in gel-mobility of proteins are due to post-translational modification, most often phosphorylation. This ‘fuzzy’ pattern of post-translational modification shown by Sfi1p was investigated further. When extra phosphatase inhibitors are added to the protein sample buffer, the Sfi1p signal can be resolved into up to 3 separate bands. It is most often seen as two broad lower bands with some hazy signal above (see figure 4.6, and later).
Figure 4.5. Post-translational modification and protein stability

A) The Sfil N-terminal antibody recognises a band of the predicted size. Cycling Cells were lysed in SDS sample buffer and run on 10% SDS PAGE gel followed by western blotting with either α-Sfi1N (J Blyth, K. Hardwick) or α-tubulin (loading control). The antibody recognises a band of the predicted size (113 kDa) except in sfi1-229 a predicted size of 97 kDa.

B) The sfi1-CT mutants are stable and show no clear change in post-translational modification at the restrictive temperature. As (A) except samples were taken after incubation for 3 hours at 23 and 37°C for each strain.
A preliminary study of samples from different cell cycle arrests suggested a relative enrichment of the middle band in an alpha factor arrest (not shown). This suggested cell cycle regulated modification of Sfi1p, which was investigated further. Strains were transformed with an integrating plasmid that tagged endogenous form of Pds1p with an HA tag. Pds1 (securin) is expressed in S-phase and degraded upon anaphase onset (Cohen-Fix et al., 1996), and is often used in this manner to monitor cell cycle progress.

These tagged strains were used for all cell cycle time course experiments. Log phase cells were arrested with α-factor at 30°C for 3 hours. They were then released from this arrest into fresh media at 30 °C. Samples were taken in the alpha factor arrest and at 10 minute intervals thereafter. Cells were pelleted and frozen in liquid nitrogen. Sample buffer was added to these pellets and whole cell extract prepared as 2.5. The samples were run on a 10% SDS Page gel, blotted, and different sections probed with several antibodies. In addition to anti -Sfi1, Pds1-HA, a cell cycle indicator and Madlp as a loading control were blotted for in parallel. The experiment was performed 3 times with wild type and sJuI-120 strains in parallel, and a representative set of blots from the same experiment shown in figure 4.6.

The Pds1 blot shows the cells were cycling, with Pds1 appearing at 20 minutes and being degraded at about 60-70 minutes. The Sfi1p signal show an increase in the intensity of the middle band relative to the others in the alpha factor arrest, and the lowest band (presumably un-modified) is at its weakest here. The strength of the middle band decreases at about 20 minutes after release. The band does not disappear completely though, for the majority of the cycle Sfi1p appears as one stronger lower band with fainter, ‘fuzzy’ higher bands. Sfi1p therefore exists in many different forms, and there is a specific modification event resulting in a clear sharp band in the middle of the Sfi1p signal at around G1/S transition, or ‘start’. This is the time of spindle pole body duplication, in particular satellite assembly that requires function of the half bridge.
The *sfl-1-120* mutation is a serine substitution within a potential Cdc28 phosphorylation site so the mutant most likely to show changes in post-translational modification. The time course was performed in parallel with this mutant. The pattern of modification is the same in the *sfl-1-120* mutant.

### 4.4.3 The modification is phosphorylation

In order to test whether this post-translational modification is phosphorylation, the samples can be treated with lambda phosphatase to remove all phosphorylation. This treatment is difficult on whole cell extracts, instead purer samples of Sfi1p protein are needed. To this end an Sfi1-TAP strain (J.Blyth) was used. The TAP (Tandem affinity tag) consists of a calmodulin binding domain and an IgG binding domain separated by a site for the TEV protease (Rigaut et al., 1999). This allows two-step protein purification under mild conditions. In this case I used IgG sepharose beads to pull down Sfi1p-TAP via the IgG binding domain (see 2.5.8). Cell extracts were made from cells arrested with alpha factor at 23 °C. After pulling down the Sfi1p the beads were washed and split into two. Half of the sample was treated with lambda phosphatase. Samples were run on a Western blot and probed with Sfi1-N4 antibody, shown in figure 4.6B. The TAP tagged protein shows similar patterns of phosphorylation as the wild type proteins under the same conditions. The TAP tagged protein is much larger, so even running the gel much longer the bands are closer together. It can be clearly seen that upon phosphatase treatment the upper bands disappear and the protein runs as one sharp lower band. The post-translational modification seen in an alpha factor arrest is therefore phosphorylation.
Figure 4.6. Sfi1p phosphorylation changes though the cell cycle. A) Sfi1p modification changes through the cell cycle and is unchanged in the sfi1-120 mutant. Cells were grown to log phase in YPDA at 30 °C and then arrested with alpha factor for 3 hours. The cells were then released into fresh YPDA and samples taken for Western blotting at 10 min intervals. Times indicates min from alpha factor release and α indicates alpha factor arrest.

B) This modification is phosphorylation. Cells expressing Sfi1-TAP either in an alpha factor arrest were lysed and Sfi1p-TAP pulled down with IgG sepharose. Half the sepharose beads for each sample were then treated with lambda phosphatase (+ p'tase). The samples were then used in a Western blot as above.
Figure 4.7. Sfi1p phosphorylation in alpha factor is not affected by mutants in cdc28 or mps1 kinases. A) Cdc28 and Mps1 are not required for maintenance of Sfi1p phosphorylation in alpha factor. Cells were arrested in alpha factor at 23 °C (α) for 3 hours and then either released into fresh media (rel) with or without 1'NMPP1 (wild type and cdc28-as) or temperature shift (mpsl-1), or kept in alpha factor with addition of drug or temperature shift. After 2 hours cells were harvested and frozen and samples prepared and used in Western blot as in figure 4.5. B) Cdc28 and Mps1 are not required for the establishment of Sfi1p phosphorylation in alpha factor. Cells were arrested in mitosis with nocodazole (noc) at 23 °C and then released into alpha factor with and without 1’NMPP1 (wild type and cdc28-as) or temperature shift (mpsl-1) or kept in nocodazole with 1’NMPP1 or temperature shift. After 2 hours cells were processed as above.
4.4.4 What is phosphorylating Sfi1p?

It is shown above that Sfi1p is phosphorylated, and at least some of this phosphorylation is confined to G1/S phase. The two most common kinases for spindle pole body proteins, especially at the G1/S border are Cdc28p and Mps1p.

Cdc28p is the cyclin dependent kinase in budding yeast. It has a role in SPB duplication (Haase et al., 2001) and identified substrates include Spc42p, Spc29p, Mps2p and Bbp1p (Ubersax et al., 2003). Sfi1p contains 9 potential Cdc28 sites, 7 of which are in the C-terminus and 2 in the N-terminus. Bishop et al created a mutant strain, cdc28-as that is sensitive to a specific inhibitor (1'NMPP1) (Bishop et al., 2000). When the drug is added at high concentrations (5 μM) cells arrest at G1 and mitosis.

Another cell cycle kinase is Mps1 (Lauze et al., 1995; Winey et al., 1991b). It has no tight consensus target sequence, but is involved in the cell cycle, and specifically spindle pole body duplication. It also has been implicated in phosphorylation of Spc42p, Spc110p and Spc98p (Friedman et al., 2001; Jaspersen et al., 2004; Pereira et al., 1998). A temperature sensitive strain, mps1-1, loses Mps1p kinase activity upon shifting to 37 °C (Schutz and Winey, 1998).

The most noticeable feature of Sfi1p phosphorylation is the stronger middle band that can be seen in an alpha factor arrest. The conditions that affect this band can be investigated using an alpha factor arrest to reliably gain this phosphorylation pattern.

Phosphorylation can be dynamic such that constant activity of a kinase is required to maintain the phosphorylation state. The effect of cdc28-as and mps1-1 on maintenance of the alpha factor phosphorylation band was investigated first. Cells were grown to log phase at 23°C and then arrested with alpha factor for 3 hours also at 23 °C. Each culture was then divided into four: Two samples remained in alpha factor, a control at 23 °C, while the test culture was subject to restrictive conditions - 1'NMPP1 (wt, cdc28-as) or 37 °C (mps1-1). The remaining two samples were released from the alpha factor arrest into fresh media at either the permissive or
restrictive conditions (as above). After 2 hours, the cells were harvested and frozen, and whole cell protein samples prepared for western blot as above. The results are shown in figure 4.7A. The controls show that all cultures arrested and released correctly. The cdc28-as mutant shows the same pattern of phosphorylation as wild type – it can maintain the alpha factor phosphorylation band even under restrictive conditions. The effect of the mps1-1 mutant is less clear; this strain does indeed maintain the phosphorylation in an alpha factor arrest but maintains more of the phosphorylation upon release than the wild type strain. This may be an effect of the mutation on cell cycle dynamics, even at 23°C, or another indirect effect. The experiment in figure 4.7A shows neither Cdc28p nor Mps1p are responsible for maintenance of this phosphorylation, or that the phosphorylation is very stable in an alpha factor arrest.

Neither Cdc28p nor Mps1p activity is required for the maintenance of phosphorylation in an alpha factor arrest, but they may be required for the initial establishment of phosphorylation in this arrest. The cdc28-as strain arrests before mitosis under restrictive conditions, so a reversible mitotic arrest using nocodazole was used to synchronise the cells, they could then be released into alpha factor arrest under the restrictive conditions. Cells were grown to log phase at 23 °C and then arrested with nocodazole for 3 hours at 23 °C. The culture was divided into four, two control samples were kept in nocodazole, one in permissive and one in restrictive (1'NMPP1 or 37 °C) conditions. The other two samples were released from the nocodazole arrest into alpha factor, again one in permissive and one in restrictive conditions. The cells were left in these conditions for a further 2 hours and then spun down and frozen and samples processed as before. The results are shown in figure 4.7B. Both mutant strains under the restrictive conditions still show the middle band of phosphorylation in an alpha factor arrest, indicating that neither Cdc28p nor Mps1p kinase activity is required for this particular Sfl1p phosphorylation event. There remains the possibility that despite the drug causing arrest at these levels, or the temperature sensitivity of mps1-1, that the kinases were not fully inactivated in these assays.
4.5 Discussion

4.5.1 Localisation

I have shown that Sfi1-YFP is present on the spindle poles. The YFP signal is fairly weak, so it is possible that Sfi1p is also in additional localisations in small amounts. Since this part of the work was undertaken, Kilmartin further localised Sfi1-GFP to the half bridge of the spindle pole body by immuno-electron microscopy (Kilmartin, 2003). Therefore Sfi1p is a half bridge protein but may have additional localisations not yet detected. The Sfi1-120-mCherry protein is also seen at spindle poles, so I conclude that the sfi1-CT mutations do not affect protein localisation to the SPB. It is possible that SPB binding is reduced but as at present the wild type and mutant proteins have different fluorescent tags it was not possible to quantitate this.

Figure 3.2 shows that the amino acid repeats of Sfi1p are conserved in a wide range of organisms. This is interesting because very few spindle pole body proteins are conserved to the centrosome. The only other spindle pole body proteins conserved are Cdc31 (centrin), Cmd1 (calmodulin), Spc110/kendrin and components of the gamma tubulin complex. Two other forms of Sfi1, Schizosaccharomyces pombe Sfi1p and Homo sapiens HsSfi1p are also localised to the spindle pole body and centrosome respectively (Kilmartin, 2003). Thus, Sfi1p is a new addition to the short list of conserved spindle pole body/centrosome proteins. This suggests it may be playing an important role in microtubule organising centres.

Knowledge of the localisation can throw new light on previous results. Sfi1p is essential (Ma et al., 1999), as are the majority of spindle pole body proteins. All other half-bridge proteins are essential. Conditional mutants in spindle pole body proteins are usually defective in some aspect of spindle pole body duplication. Most lethal phenotypes either fail to assemble a daughter SPB at all or the daughter is not correctly inserted into the membrane and so unable to nucleate microtubules on the nuclear side. In either case this leads to a monopolar spindle organised by the functional mother spindle pole body. This phenotype is recognised by the spindle assembly checkpoint. If the defect cannot be fully corrected, after a delay the cells
progress through mitosis resulting in mis-segregation of the genetic material, which results in inviability within a few generations. This scenario fits well with the checkpoint synthetic lethality and chromosome loss seen in the sf1l-CT mutants in chapter 3. The sf1l-CT mutants are inviable without a functional spindle checkpoint (section 3.4), which would be expected for a spindle pole body mutant unable to assemble a functional bipolar spindle. Anaphase resulting in missegregation of chromosomes in these mutants would agree with the high levels of chromosome loss seen in these mutants (section 3.6). How benomyl resistance fits into the function of a half bridge protein is unclear.

4.5.2 Phosphorylation

Sfi1p is phosphorylated throughout the cell cycle. In cycling cells it runs as a stronger lower band at the expected molecular weight with additional fainter slower migrating broad bands. In an alpha factor arrest a sharp strong band can be seen in the middle of the broad signal. This cell cycle phosphorylation is unaffected by the sf1l-120 mutation. The phosphorylation seen in cycling cells is unaffected in the other sf1l-CT mutants.

Many spindle pole body proteins are highly phosphorylated (Donaldson and Kilmartin, 1996; Ficarro et al., 2002; Friedman et al., 1996; Gruneberg et al., 2000; Pereira et al., 1998; Schaerer et al., 2001; Vogel et al., 2001; Wigge et al., 1998). In the case of Spc42p, this promotes assembly of the central plaque (Jaspersen et al., 2004). Phosphorylation of Spc110p and Spc98p regulates nuclear microtubule nucleation (Friedman et al., 2001; Pereira et al., 1998). Both Cdc28p and Mps1p kinases are known to phosphorylate spindle pole body proteins and are the most likely candidate kinases at the stage at which the specific band occurs.

Sfi1p contains 9 potential Cdc28p consensus sites, 5 of which fit the full consensus. 7 of these, including all 5 full consensus sequences are in the C-terminus and the other 2 are in the N-terminal region (outwith the amino acid repeats). In a large-scale screen of Cdc28p substrates, Sfi1p scored highly (Ubersax et al., 2003). The authors used an analogue sensitive Cdc28p that added a radioactive phosphate in cell
lysates containing individual GST tagged proteins that contained Cdc28p consensus sites. Sfi1p was the best substrates of all SPB proteins by a large margin, in the top 9% of the ~700 potential substrates tested. This was higher than well-documented SPB substrates such as Spc110p (within top 25%) or Spc42p (top 45%). There is no doubt that Sfi1p is a Cdc28p substrate in these cell lysate experiments. However, the cdc28-as mutant does not affect the Sfi1p phosphorylation pattern seen on a 1D gel. Often with highly phosphorylated proteins the many different phospho-forms can only be distinguished by 2D gel electrophoresis (Donaldson and Kilmartin, 1996). It may be that cdc28-as does affect some of the phosphorylation, but that the difference can only be observed by 2D gel. The sfi1-120 mutant has lost one serine residue from a full Cdc28p consensus site, and this mutation also does not affect the phosphorylation pattern. This adds to the argument that Sfi1p may exist in so many phospho forms that they can only be distinguished by 2D gel.

Another kinase also documented to have a role at the spindle pole body is Mpslp (Castillo et al., 2002; Schutz et al., 1997; Schutz and Winey, 1998). Mps1 does yet not have a consensus target sequence identified as very few substrates/sites have been identified. The mpsl-1 mutant, which loses kinase activity at the restrictive temperature, does not have any visible effect on the pattern of Sfi1p phosphorylation in an alpha factor arrest. Again, the mpsl-1 may affect Sfi1p phosphorylation subtly which might only be detected on a 2D gel. Also, there is always a chance with conditional mutants that the protein has not been inactivated fully. In this case there may still be some residual Mpslp kinase activity. In the future this experiment could be repeated with an analogue sensitive mpsl mutant.

This still leaves the question of which kinase is responsible for the specific phospho band seen in an alpha factor arrest. This band does disappear upon treatment with lambda phosphatase, so it is due to phosphorylation. One other kinase that is localised to the SPB and has been implicated in Spc42 function is Cdc5 (polo) (Song et al., 2000). Sfi1p does contain several sites conforming to the consensus sequence determined for mammalian polo (Nakajima et al., 2003), which seems to apply in yeast too (Alexandru et al., 2001). Cdc5p is at the spindle pole body, recruited by
Bbp1p (Park et al., 2004). Other kinases at the spindle pole body are Dbf1p and Cdc15p. Future experiments could investigate the role of these three other kinases in Sfi1p phosphorylation.

A few other SPB proteins are phosphorylated specifically in G1/S, for example Spc110 (Stirling and Stark, 1996). The Sfi1p band seems to be pretty specific to the G1/S transition phase – it is reduced within 20 minutes of alpha factor release. This is the time in the cycle the satellite is assembled on the half bridge, as a satellite is already built in an alpha factor arrested cell (Byers and Goetsch, 1974; Byers and Goetsch, 1975). It is possible therefore, that this particular phosphorylation is controlling the function of the half bridge during SPB duplication. Another possibility is that the phosphorylation is preparing the half bridge for mating, and the phosphorylation is karyogamy specific. The half bridge and satellite are the site of SPB fusion during mating (Byers and Goetsch, 1975) and cells exposed to alpha factor are preparing to mate. Using conditional mutant that arrests cells at start, or following cells released from a mitotic arrest release instead of an alpha factor arrest could distinguish these possibilities. If the phosphorylation is not seen in this event, then it is karyogamy specific.

The *sfi1-120* mutation does not have an apparent affect on either protein localisation or phosphorylation in the assays in this chapter. The mutations in the C-terminus do not affect protein stability or post-translational modification. This leaves the question of what exactly is happening in these mutants to cause chromosome loss and activation of the spindle assembly checkpoint. What function is this C-terminal portion performing that is important in the mitotic spindle? The next chapter looks at the C-terminal portion of Sfi1p in more detail.
Chapter 5 – The C-terminus of Sfi1p

5.1 Introduction

5.1.1 The C-terminus within the Sfi1p protein
As established in chapter 3, all the sfi1 mutations gained in the mad1Δ synthetic lethal screen are in the C-terminal tail. Other published mutants, sfi1-3 and sfi1-7, which were created by random PCR mutagenesis (Kilmartin, 2003), have several mutations within the conserved repeats of Sfi1p. Kilmartin showed these mutants are lethal at 37 °C and are defective for spindle pole body duplication. He also showed that the Sfi1p repeats bind Cdc31p, as much as one Cdc31p per Sfi1p repeat.

The C-terminus of Sfi1p is not conserved outside the budding yeasts, however it is the most conserved area within the budding yeasts (25% identity vs 16% identity within the repeats). A short section containing both the sfi1-65 and sfi1-120 mutations has 69% identity. The % identity of different regions of Sfi1p within budding yeasts is shown in figure 5.1A. An alignment of the C-terminus and last repeats is shown in 5.1B. This all suggests that this C-terminal portion of Sfi1p has an important role in budding yeasts. In this chapter the C-terminus of Sfi1p is investigated further.

5.1.2 Definition of the C-terminus
From the work in section 3.3 the last amino acid repeat in Sfi1p ends at residue 769. The fragment from 770 – end (946) is used as the ‘C-terminus’ from now on. This fragment was used in the physical interactions studies (5.3 and 5.4). Since creation of the constructs for those studies, another lab published a differing view of the repeats, with them ending at residue 800 (Kilmartin, 2003). This was taken into consideration in creation of the truncation strains in section 5.2.
Figure 5.1. The C-terminus of Sfi1p. A) Schematic of Sfi1p showing positions of all mutations. Percentages show % identity amongst budding yeasts for each region B) Alignment of the C-termini of Sfi1p from budding yeasts. Positions of sfil-CT mutants are shown in red, the potential last extra repeat in green, and positions of two truncations shown in blue.
5.2 Truncations of C-terminus

In order to establish the importance of the C-terminus, sf11 mutants were created with the C-terminus removed. Two truncations were created, one removing the C-terminal fragment from 770 as explained above (sf11-Δ770). Using Kilmartin’s definition of the repeats this Δ770 truncation also removes a final repeat. To ensure a truncated protein containing all potential repeats a second truncation was made from residue 801 (sf11-Δ801).

5.2.1 Creation of strains truncated in the C-terminus of Sfi1p

Primers were constructed to create the two truncations in S. cerevisiae using the PCR method (Longtine et al., 1998). This method uses the same principle of homologous recombination as the PCR tagging method used in chapter 4 (and figure 4.1A). The difference is the forward primer contains sequence immediately 5' of the site of truncation instead of the end of the gene. In this experiment I created a fusion protein of the first (769 or 800) amino acids of Sfi1p with the fluorescent tag. The ‘mCherry’ red fluorescent protein (Shaner et al., 2004) with a ClonNat marker was chosen, as experience had shown ClonNAT to be a good selectable marker and mCherry a bright fluorescent tag allowing co-localisation with GFP.

The PCR product was transformed into wild type diploids carrying a CEN SFII, URA3 marked plasmid. Transformed cells were grown on YPDA plates for a day, then replica plated onto YPDA supplemented with ClonNAT. NAT+ transformant strains were checked for the truncation by western blot. Strains expressing the truncated, tagged Sfi1p were grown on 5’FOA to select against URA3 plasmid. Those that were still NAT+ after plasmid loss were those that had been tagged in the genomic copy rather than the plasmid copy of SFII. The original strains with the URA3 plasmid were also used for sporulation.

These diploid strains, with the SFII URA3 plasmid, were sporulated, and tetrads of haploid spores dissected onto –URA CSM plates. Haploid strains that were NAT+ were checked for the sf11 truncation by western blot. The truncations remove 20 or 27kDa of Sfi1p, and mCherry is 45 kDa, so the predicted size of the truncated and
tagged proteins is approximately 20 kDa larger than the wild type protein. The four strains from one tetrad of each truncation are shown on the blot in figure 5.2A.

Interestingly, the bands corresponding to truncated proteins on the blot show the same band pattern of phosphorylation as the wild type protein (see section 4.4). This suggests that the majority of the phosphorylation that causes the band pattern is not in the C-terminus. The protein levels of truncated proteins and endogenous wild type proteins are similar, however the stability or levels of wild type protein expressed from the \textit{CEN} plasmids appear to be lower.

5.2.2 The C-terminus of Sfi1p is essential

The reason the wild type \textit{SFII} plasmid with a \textit{URA3} marker was used is that 5'FOA can be used to select against it (see section 3.4.2). The haploid strains confirmed to contain the truncated and tagged \textit{sfi1} gene were replica spotted onto YPDA, NAT and 5'FOA plates and grown for 3 days at 23°C. The same selection of strains as for figure 5.2A above is shown in figure 5.2B. All strains carrying the truncation (NAT+) were unable to grow on 5'FOA and all NAT- strains could grow on 5'FOA. Therefore I conclude that loss of either C-terminal fragment is lethal, as the strains containing these truncations cannot grow without the \textit{SFII} plasmid. The lower levels of wild type protein expressed from the \textit{CEN} plasmid appear to be sufficient for growth.

5.2.3 The truncated proteins are mislocalised

The truncations are also tagged with mCherry, which allows their localisation to be visualised. Truncation strains along with \textit{sfi1-120}-cherry containing Spc42-GFP and \textit{sfi1-120} untagged with Spc42-GFP alone were grown to log phase in CSM in parallel then formaldehyde fixed as section 4.2.3. In order to confirm that the strong Cherry signal in the \textit{sfi1-120} cells was not due to bleed through from Spc42-GFP, the strain carrying Spc42-GFP only was imaged under the same conditions. No-bleed through was seen under the fixation conditions used.
Figure 5.2. C-terminal truncations of Sfi1p are mislocalised.  A) Truncations on a Western blot Western blot showing samples spores from sporulation of a SFI1/sfi1-ΔCT diploid.  B) Truncations strains cannot grow on 5'FOA. Serial dilutions of above strains spotted out on YPDA, Nat and 5'FOA plates and grown at 23 °C for 3 days.  C) (overleaf) Localisation of Cherry tagged versions of Sfi1p. Truncation strains were grown in parallel with sfi1-120 cherry strain containing Spc42-GFP. Cells growing at 23 °C in log phase were fixed. Images show maximum intensity projection of 7 sections 0.5 μm apart.
C

GFP  Cherry  DIC

Control
sfi1-120, SPC42-GFP

Mixed
• sfi1-120-Cherry, SPC42-GFP
• sfi1-Δ770-Cherry, p(SFI1)

Mixed
• sfi1-120-Cherry, SPC42-GFP
• sfi1-Δ801-Cherry, p(SFI1)
The truncation strains were mixed with *sfi1-120-cherry* *SPC42-GFP* during slide preparation so the strains could be observed side-by-side in the same field of view. *sfi1-120-cherry* strains could be identified by the GFP signal. Representative cells are shown in figure 5.2C. The truncation strains showed no spots of mCherry signal when compared to the Sfi1p-120-mCherry and a small increase in background signal. This shows that the C-terminus is necessary for enrichment of Sfi1p at the SPB. However the *sfi1-120* mutation does not appear to affect localisation.

5.3 GST fusion proteins

I have shown above that the C-terminus of Sfi1p is required for its recruitment to the spindle pole body. The *sfi1-120* mutation, which is in the C-terminus, does not appear to affect localisation, however. What else might it be affecting?

Most proteins do not act alone in the cell. Investigating the binding partners of a protein or domain can yield more clues to its function. In the case of Sfi1p, there is the question of the identity/s of the essential interaction of the C-terminus that brings it to the spindle pole body? Is there an interaction affected in the *sfi1-CT* mutants? If any potential interactors can be found the interaction could also be tested in the *sfi1-CT* mutants. Two complementary approaches were taken to investigate the physical interactions of the C-terminus, affinity chromatography and a yeast 2-hybrid screen. These two methods are discussed in 5.4.

5.3.1 Expression and purification of GST fusion proteins

For affinity chromatography experiments purified GST tagged protein was required. The C-terminus of the *SFI1* gene from both wild type and *sfi1-120* was made by colony PCR, and cloned into the pGEX-6P vector. This allowed expression of GST-Sfi1-CT fusion proteins in BL21 cells (Strategene). Both wild type and Sfi1-120p C-termini proteins were purified from 4 litres of bacteria using method in section 2.5.4. Samples were taken at each stage and analysed by SDS-PAGE and coomassie blue staining, shown in figure 5.3. The expected size of the Sfi1p fragment was 20 kDa, with GST tag of 25 kDa. The final purified sample from the wild type fragment shows a band of 45 kDa, the expected size of the fusion protein.
Figure 5.3. Purification of GST-Sf11-CT. GST-Sf11CT, both wild type (wt) and containing the sfi1-120 mutation was purified from 4l BL21 E. coli. Samples were taken at various stages of purification - Supernatant (Sup) and Pellet (Pel) from cell lysate, GST column flow through (FT) and eluate from column post-dialysis (Sf1). These samples were run on a 10% SDS-PAGE gel and stained with Coomassie Blue. The predicted size for GST-Sf11-CT is 45 kDa.
This sample was used both for injection of rabbits to make antibodies, (see section 4.3) and for affinity chromatography described below.

In the purification of the GST-Sfl1-120-CT the only protein recovered ran at a size of 25 kDa and is most likely to be GST alone. A possible explanation is that the C-terminus of Sfl1-120 is unstable without the rest of the Sfl1 protein, whereas the wild type is stable. It would be interesting to see if the other C-terminal mutant fragments are also unstable in E.Coli.

5.4 Physical interactions of C-terminus
Two approaches were taken to search for binding partners of the Sfl1p C-terminus. Binding partners were searched for directly by running yeast extract through a column with immobilised GST-Sfl1-CTp. This identifies strong stable interactions under the buffer conditions used in vitro. It will not identify proteins that are insoluble in the cell extract. In parallel a yeast 2-hybrid screen was performed. This is indirect method that assays in vivo protein interaction through activation of reporter genes. It is prone to false positives such as general transcriptional activating proteins. Both methods screen the entire genome/proteome.

5.4.1 Affinity Chromatography
The Sfl1-CT protein fragment created in 5.3 was immobilized (covalently cross-linked) on an affigel column (Bio-rad). Yeast extract was prepared using the method in 2.5.7 from 4 litres of yeast. The extract was first run through a BSA column to remove non-specific binding and clear the lysate of aggregates that would clog the smaller Sfl1p column. The extract was then slowly run through the GST-Sfl1-CTp column, or a GST only control column, using a pump. Any proteins that bind the Sfl1 C-terminus would bind the Sfl1-CTp column specifically. The GST control column is to identify any proteins that are binding the GST tag and not the Sfl1-CTp.

The column was washed extensively with wash buffer (more details in 2.5.7) to remove non-bound proteins. After washing, bound proteins were eluted in fractions with increasing concentrations of KCl. Eluate samples were TCA precipitated then
Figure 5.4. GST-Sfi1CT binds to many proteins A) The supernatant (S) from an extract from (JB811) was run through a BSA control column then either a GST control column or a column containing GST-Sfi1CT immobilised on an Affigel. The column was washed and then fractions of bound protein eluted in increasing concentrations of KCl from 50 mM to 1M. Samples of lysate pellet (P) and supernatant (S), BSA column flow through (BSA FT), column flow through (FT) and elutions were run on a 10% SDS PAGE gel and stained with Gel code blue. Bands present or enriched in Sfi1-CT elutions were cut out and analysed by Mass spectrometry. B) Results from Mass Spectrometry
run on an SDS-PAGE gel. Bands were stained with GelCode blue. As can be seen in figure 5.4A many proteins were eluted from the Sfi1-CT column. Under illumination of the gel and careful examination, some bands that were enriched in the column eluate compared to the GST eluate and column flow through were identified. These bands were cut out of the gel, and gel slices sent for mass spectrometry analysis.

The bands that were identifiable by this method are shown in figure 5.4B. None of the proteins found are likely to be biologically significant. None have a role in mitosis, or are localised to the nuclear membrane or spindle. Chaperones such as Atp11p are common in physical interaction studies as they may interact with the protein in order to help it fold correctly. In a pull down experiment with full length Sfi1p-PrA Kilmartin also found Tef2p (Kilmartin, 2003). It is a translation elongation factor so probably unspecific. The large amount of proteins bound to the Sfi1p-CT column suggest this domain may be unstructured in isolation from the rest of the protein, and consequently very ‘sticky’ with many exposed hydrophobic residues.

5.4.2 Yeast 2-hybrid screen
In parallel a yeast 2-hybrid screen was performed, using the same C-terminal fragment as used in the affinity chromatography. A yeast 2-hybrid screen also tests for protein-protein interactions. It uses the Gal4 protein, a transcriptional activator that binds the GAL promoter. This protein is made up of two main domains, a DNA binding domain and a transcriptional activation domain. A useful property of this protein is that the two domains do not have to be in the same protein in order to activate transcription, as long as they are brought into close enough proximity by other factors. In this screen, a ‘bait’ protein (in this case Sfi1-CT) is fused to the Gal4 DNA binding domain. ‘Prey’ proteins are fused to the DNA activation domain. The prey can either be a specific protein of interest, or a library of potential binding partners. The assay is carried out in a strain that has the Gal4 promoter upstream of several reporter genes. If the bait and prey interact the two Gal4 domains will be brought together and activate transcription of the reporter genes.
In this study, the library and strain used were as described previously (James et al., 1996). The library is a genomic DNA library. It consists of 3 pools of plasmids containing the same set of DNA fragments cloned into different reading frames relative to the GAL4 activation domain. The strain, PJ69-4A contains 3 independent reporters (HIS, ADE and Lac) with different GAL promoters. This helps to eliminate any promoter specific false positives. The C-terminal fragment of Sfi1 was cloned into the pAS2 plasmid. This results in a fusion protein of the GAL4 DNA binding domain and Sfi1 C-terminus. Cloning was confirmed by sequencing.

Test transformations were performed to investigate optimum transformation conditions. Co-transformation of both library and bait plasmid together was just as efficient as sequential transformation of the plasmids, and less time-consuming. The bait alone did not auto-activate, however there were a large number of colonies on the –LWH test plates when the library was used. The background on –His selection was reduced by growing the initial transformants on (-LWH) plates containing 50 mM 3-aminotriazole (3-AT).

Once optimal conditions were established, large-scale transformations were performed in batches. For each batch a twentieth of the transformations was plated on –LW media (selective for both plasmids but not 2-hybrid interaction). This allowed estimation of the total number transformants in that batch. Over the whole screen, an average of 80 000 colonies (estimated) per pool were screened, a total of 250 000. At an average insert size of 750 bases, the genome (~14 Mb) was covered a minimum of 12 times. This should be enough to identify any important interactors. Colonies growing on the –LWH + 3AT plates after a few days were then replica plated onto –LWA for the more stringent adenine selection. Colony PCR was performed on any ADE colonies using primers to either side of the cloning site of the library plasmid. The original library was created from fragments between 500 bp and 3 kb (James et al., 1996), so any PCR product less than 500 bp was discounted. Any PCR products above 500 bp were purified and sequenced. The genes or gene fragments were then identified using BLAST program and the NCBI database.
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Figure 5.5. Results of the yeast 2-hybrid screen. No. hits = number individual colonies, no clones - number of independent clones
The results are summarised in figure 5.5. A wide variety of genes were pulled out of the screen, but few are likely to be of any significance. With the screen covering the genome about 12 times, any significant binding partners would be expected to appear multiple times. Only 5 genes came out more than once in this screen. The number of clones corresponds to how many individual clones of a particular gene were identified. For instance, upon sequencing it was clear that several of the YLR154c hits were from the same library plasmid (clone). YLR154c, the top hit is a gene for ribosomal RNA. None of the other top hits look likely to be involved in spindle function. Many of the genes pulled out are for nuclear membrane transporters or the nuclear pore. The half bridge of the spindle pole body is on the nuclear membrane, so these may represent general interactions of membrane proteins, or Sfi1p membrane interaction mediated by other factors. Poly(A) binding protein was identified in the column experiments as well. It binds the ends of messenger RNAs and is involved in translation initiation. Unfortunately the range and varied selection of genes pulled out furthers the argument that the Sfi1-CT fragment is likely to be sticky and bind many proteins non-specifically.

5.5 Genetic interactions of sfi1-CT mutants
I have already established sfi1-CT mutants are synthetic lethal with a range of checkpoint proteins (section 3.4.) Other genetic interactions of mutants can give clues to the pathways and processes the protein is involved in. While the assays above can only give information in direct physical interaction, genetic interactions can give much broader information. Synthetic lethality has already been explained in chapter 3. In the following section, positive genetic interactions are searched for — genes that can rescue or suppress the mutant phenotype of the sfi1-CT mutants.

5.5.1 Genetic interactions with CDC31
Cdc31p is the yeast homologue of centrin, one of the few conserved SPB proteins at the centrosome. It was the first half bridge protein discovered, and finding its binding partners has been a key question in the centrosome field. In a search for binding partners of Cdc31p, Kilmartin pulled down Sfi1p (Kilmartin, 2003). He showed that each of the amino acid repeats in Sfi1p can bind one molecule of
Cdc31p. The N- and C-terminal extensions cannot bind Cdc31p. He created the temperature sensitive mutants *sfi*-3 and *sfi*-7 by PCR mutagenesis. They contain mutations in the central amino acid repeat region.

*sfi*-3 and *sfi*-7 temperature sensitivity is rescued by a plasmid overexpressing Cdc31p (Kilmartin, 2003). This 2-micron plasmid overexpressing Cdc31p, or a control empty parent vector were transformed into wild type, *sfi*-3 and all *sfi*-CT mutant strains. The resultant strains were replica spotted onto plates selecting for the plasmid (URA3) and grown at either 37°C or 23°C for 2 days. The results are shown in figure 5.6. The plasmids have no effect on the growth of wild type. *sfi*-3 cannot grow at all at 37°C and the control plasmid has no effect on this. The *sfi*-3 strain is rescued to moderate growth rates by the *CDC31* plasmid, in agreement with previous data (Kilmartin, 2003). In contrast the *CDC31* plasmid does not rescue the temperature sensitivity of the *sfi*-CT mutants, in fact all except *sfi*-229 are slightly sicker with Cdc31p overexpression. A possible explanation is that the *sfi*-CT mutants are weakened in interaction with a half bridge protein other than Cdc31p. The excess Cdc31p titrates the Sfi1-CTp protein away from the SPB, but wild type Sfi1p interaction with the half bridge is strong enough not to be affected Cdc31p overexpression does not affect growth of wild type.

*sfi*-3 has mutations in the Cdc31p binding amino acid repeat section, excess Cdc31p most likely overcomes binding problems. The C-terminus of Sfi1p does not bind Cdc31p (Kilmartin, 2003). C-terminal mutations are not rescued by Cdc31p overexpression, which suggests that they do not affect Cdc31p binding. This adds weight to the theory that the C-terminus performs a function distinct from the Cdc31p binding of the central repeat section.
Figure 5.6. The temperature sensitivity of sfi1-CT mutants is not rescued by Cdc31 overexpression. Serial dilutions of strains containing control 2μ plasmid or Cdc31 overexpression (+) were spotted onto CSM-URA media and incubated at temperatures stated for 2 days. wt = wild type KH34.
Figure 5.7. The temperature sensitivity of *sfi1-3* is rescued in diploid strains by *sfi1-CT* mutants at 36 °C but not 37 °C, whereas diploid strains heterozygous with different *sfi1-CT* mutants are more temperature sensitive. Serial dilutions of strains were replica spotted onto YPDA media and grown at indicated temperatures for 1-2 days.
5.5.2 Genetic interactions between sfl1 mutants

Several lines of evidence suggest that the sfl1-CT and sfl1-3 mutations are very different (see chapter 6). Can they complement each other, i.e. can they each compensate for the function lost in the other? Is there a dominant/recessive relationship? This was tested in diploid strains created by mating the appropriate haploid strains. Their temperature sensitivity was assayed by the spotting out assay, shown in figure 5.7. Homozygous diploids grow much the same as haploid strains of the same mutation. In all cases heterozygous diploids containing one wild type copy and one mutant copy grew as wild type. All sfl1 mutants are recessive and one wild type copy in a diploid cell is enough for correct Sflp function. The sfl1-3 mutants cannot grow at either 36 °C or 37 °C. Heterozygous diploids with one sfl1-3 gene and one sfl1-CT gene can grow at 36 °C although not as well as the sfl1-CT mutants alone, which grow as well as wild type at 36 °C. The sfl1-CT alleles cannot compliment the sfl1-3 allele at 37 °C, the temperature at which the sfl1-CT mutants show some growth defects. In general the growth of sfl1-3/sfl1-CT strains is intermediate between that of the homozygous mutants. The heterozygous sfl1-65/sfl1-120 diploid grows the same as the sfl1-120 homozygous indicating no complementation between sfl1-CT alleles.

Overall these results suggest some semi-dominance of the sfl1-CT over the sfl1-3 allele at 36 °C, strengthening the theory that the mutations affect different functions of Sfl1p.

5.5.3 Search for other genetic interactions of sfl1-CT mutants

5.5.3.1 Synthetic lethality

Sfl1p is a spindle body protein at least in part on the half bridge (Kilmartin, 2003) and involved in spindle pole body function. Other sfl1 mutants have shown genetic interactions with CDC31. As with the original mad1Δ synthetic lethal screen, synthetic lethality may occur between two non-lethal mutants of proteins involved in the same processes. Three other half-bridge proteins, Kar1p, Mps3p and Cdc31p were tested.
sf1-CT mutants were crossed with either mps3-1, kar1-Δ17 or cdc31-2 containing a URA3 plasmid expressing the wild type gene. Synthetic lethality between the sf1 and other mutation could then be tested using the 5'FOA method to assess loss of wild type MPS3/KARI/CDC31 plasmid. None of the sf1-CT mutants are synthetic lethal with these mutants, as double mutant strains that could loose the plasmid were easily obtained (data not shown), many had lost the plasmid before 5'FOA sensitivity could be tested. Maybe sf1-CT mutants impact a new function of the half bridge that other components are not involved in. Alternatively Sfi1p may be at additional locations on the spindle pole body, as yet unidentified.

5.5.3.2 Sectoring assay
As established in the original screen, the sf1-CT mutants are synthetic lethal with mad1Δ. Overexpression of genes that in some way aid Sfi1p function or correct the mitotic defect in the sf1-CT mutants may rescue the synthetic lethality, i.e. restore sectoring in the original assay strains.

Sectoring strains from the original screen bearing the TRP MAD1 plasmid were transformed with a range of plasmids - a bank of spindle pole body proteins and selected others. All the plasmids were URA3 and the transformed colonies grown on -URA to select. Plates were incubated at 23 °C until colonies were formed and the red pigment had developed. An empty vector was used as a negative control and MAD1 and SF11 plasmids used as positive controls. Interestingly the SF11 plasmid (even overexpression) did not allow sectoring as well as a MAD1 plasmid. No genes were found that rescued as well as MAD1 but some showed a significant amount of sectoring. Positive results were repeated for verification, and any plasmids allowing sectoring in one sf1 mutant were re-tested in the others. Degrees of sectoring were categorised and representative colonies shown in figure 5.8A. Results are shown in 5.8B.

The sf1-CT mutants varied in their degree of rescue and genetic interactions. sf1-229 and sf1-273 were rescued by the most genes.
Figure 5.8. Genetic interactions of sfil-CT mutants. Search for suppressors of mad1 synthetic lethality. Using the sectoring assay described in figure 3.1. Original sfil-CT mutants containing the mad1 deletion and p(MAD1, ADE2, TRP) were transformed with URA marked plasmids containing CIN8, TUB1, SF11 and MAD1 from the original screen or a bank of spindle pole body proteins (S. Jaspersen). Sectoring was classified into 4 classes illustrated in (A). Results are show in (B).
sfi1-229 is the least temperature sensitive mutant so maybe the phenotype is less severe and more easily rescued. sfi1-120 is the most temperature sensitive mutant and hardest to rescue/suppress.

The two genes that allowed sectoring in all or most of the sfi1-CT mutants were NDC1 and BBP1. These have both been implicated in the process of inserting the newly formed SPB duplication plaque into the nuclear membrane. Bbp1p acts in a complex with Mps2p, which also allowed sectoring in sfi1-273. Ndc1p has roles in the nuclear pore (Chial et al., 1998), and Bbp1 has a role in mitotic exit by recruiting the Cdc5p kinase (Park et al., 2004). The fact that all three known membrane insertion factors rescued at least one sfi1-CT mutant each suggests that it could be the membrane insertion functions of these proteins that is rescuing the sfi1-CT phenotype. No physical interaction with these proteins were identified in the screen in section 5.4, however membrane proteins are notoriously hard to identify in these screens. In physical studies membrane proteins stay in the lipid instead of soluble fraction, and are hard to solubilise.

sfi1-229 and sfi1-273 also rescued by CIN8. Cin8p is a plus end directed microtubule motor required to separate duplicated spindle pole bodies by producing an outward force on the spindle.

These results together suggest a role for the Sfi1p C-terminus in either or both the membrane insertion step and SPB separation. The level of sectoring is quite modest so how significant these results are is not clear. It would be interesting to see if cin8/bbp1/ndc1 mutants are synthetic lethal with the sfi1-CT alleles.

5.6 Discussion
The sfi1-CT mutants all have mutations in the final 200 amino acid stretch at the C-terminus of Sfi1p. All of the mutations, except perhaps sfi1-273, occur outwith the amino acid repeats. This C-terminal portion is not conserved across organisms, but is highly conserved amongst budding yeasts, hinting it may have a distinct function in these organisms.
Truncating this C-terminal portion is lethal suggesting that this portion is important in budding yeast. The resulting truncated proteins are stable but not visible at the spindle pole body. This shows that the C-terminus is required for recruitment of Sfi1p to the SPB. This simplest explanation is that the C-terminus is a localisation domain. It could also be that loss of the C-terminus causes some structural change or weakens the interaction of the rest of the protein with its binding partners. If the protein is mis-localised then it is effectively a null allele for SPB function. We know Sfi1p is essential and this essential role seems to be at the SPB. Mis-localisation unlikely to be the cause of the phenotype in sfi1-120, as it localises correctly. This is not surprising as the sfi1-120 mutant is still viable, which would not be expected if an essential protein was mis-localised. In this study, it was not possible to quantitate relative amounts of wild type and Sfi1-120p protein at the spindle pole body, due to different fluorescent tags. It would be interesting to see if levels of the Sfi1-CTp mutant proteins were subtly reduced at the SPB.

Both Cdc31p (centrin) and the Sfi1p repeats it binds are conserved as far as humans. The C-terminus is only conserved amongst the budding yeast. The structure and life cycle of the S. cerevisiae SPB is also only conserved amongst budding yeasts. This suggests that the essential function of the C-terminus is specific to the budding yeast spindle pole body. Although a templating structure similar in function to the half bridge is theorised to exist in the centrosome, its interactions and the structure it has to promote via seeding are very different. Interestingly, a major difference between centrin-containing areas in the centrosome and budding yeast is that the budding yeast half bridge interacts with the nuclear membrane (discussed later).

An interesting point is that the truncated proteins show the same pattern of phosphorylation as the wild type on a Western blot. This suggests that despite the large number of consensus Cdc28p sites in the C-terminus that the phosphorylation causing the band shift is not in the C-terminus. The majority of phosphorylation is likely to be in the repeats section or the N-terminus. There are not many putative phosphorylation sites for either Cdc28p or Cdc5p in these sections, and mps1-1.
mutants seems to have no effect (section 4.4.3). This needs careful further investigation.

**Physical interactions**

It is known that only the repeats section of Sfi1p bind Cdc31 (Kilmartin, 2003). But does the C-terminus have any binding partners? To this end two complimentary screens for physical interactors of the C-terminus were performed. The results from truncating the C-terminus support a role in localisation. For this the C-terminus would need a binding partner/s at the spindle pole body and/or nuclear membrane. The C-terminal mutants do not appear to be mis-localised, so what function/interaction are these mutations potentially disrupting?

No meaningful protein interactions were discovered by either biochemical or 2-hybrid methods. This may suggest that the C-terminus does not act as a domain in isolation, that it only binds proteins in concert with other parts of the Sfi1p protein. This may be because it only assumes the correct structure when attached to the rest of the protein. Its correct binding partner may not recognise it in its isolated incorrectly structured form. Alternatively, it may be that this domain does not stably bind anything and has a function in regulating the rest of the protein. Experiments with the full length wild type versus a C-terminal truncation protein might prove more successful in this case.

A third option is that the correct interactors have not yet been identified. Evidence from the genetic interactions, and the fact the half bridge is a specialised part of the nuclear membrane suggest Sfi1p may interact with membrane bound proteins. Membrane proteins are by nature hydrophobic in order to interact with the hydrophobic lipid membrane. This makes them insoluble in cell lysates and unlikely to be in the correct part of the cell to give a positive result in a yeast 2-hybrid screen. Half bridge proteins in particular have been tricky to identify, both Mps3p (membrane) and Sfi1p are very recent additions (Jaspersen et al., 2002; Kilmartin, 2003). A system analogous to the 2-hybrid system does exist for membrane proteins.
Instead of the GAL transcription factor, it uses two domains of ubiquitin, which when brought in close proximity recruit a hydrolase that mediates the release of a transcription factor (Stagljar et al., 1998). It would be worth testing Sfi1p in this assay.

In both screens many, many weak false positives came out. This suggests the fragment is ‘sticky’ and displays hydrophobic surfaces that bind to other proteins in a non-specific way. This suggests that the C-terminus requires the rest of the protein to fold correctly, that it does not act as a separate domain in isolation. In the yeast 2-hybrid these would give a signal due to being overexpressed and in high concentrations in cell. In biochemistry it binds weakly to anything that comes its way.

**Genetic interactions**

Kilmartin showed that the amino acid repeats bind Cdc31p, possibly as much as 1 molecule per repeat (Kilmartin, 2003). Temperature sensitive mutants in the repeat section (sfi1-3 and sfi1-7) are rescued by a plasmid overexpressing Cdc31p reflecting this interaction. The sfi1-CT mutants are not rescued by Cdc31p overexpression. Neither did they show synthetic lethality with mutants of other half bridge components, suggesting that the function disrupted in sfi1-CT mutants is different to usual half bridge function of satellite binding. This is supported by the partial complementation seen between sfi1-3 and sfi1-CT mutants.

The synthetic lethality with mad1Δ was partially relieved in the sfi1-CT mutants in varying amounts by a set of genes all implicated in insertion of the nascent SPB into the nuclear membrane. These included Mps2, Bbp1 and Ndc1. Mps2 and Bbp1 have other functions, but the fact all that three known membrane insertion proteins came out suggests that it is the SPB membrane insertion function of these proteins that is interacting with Sfi1p. Since this work was completed a new insertion protein, Nbp1, that physically links Ndc1 and the Mps2-Bbp1 complex has been identified (Araki et al., 2006). It would be interesting to see if overexpression of this
gene could also allow some sectoring in this assay. The mechanism by which these proteins achieve membrane insertion is not understood.

How could a half bridge function and membrane insertion/anchoring of SPBs be linked? There are several possibilities illustrated in figure 5.9. At this point it is useful to remember that the half bridge is a specialisation of the nuclear envelope, presumably continuous with it. Before insertion into the membrane the distal end of the half bridge fuses and is sometimes associated with a nuclear pore (Adams and Kilmartin, 1999). The half bridge retracts, exposing the duplication plaque to the nucleoplasm. The first possibility (5.9A) is that the sf1-CT mutants are slightly defective in this action of the half bridge, and extra membrane insertion factors help either with fusion of the distal end of the half bridge or with the retraction. Without knowing the mode of action of the membrane insertion factors it is hard to hypothesize further. Nbp1 and Bbp1 only have homologues in other budding yeasts (Araki et al., 2006). This is consistent with the idea that the function of the C-terminus, which genetically interacts with these proteins, is one unique to the budding yeast SPB. Higher eukaryotes go through open mitosis and the membrane breaks down so their centrosome never needs to be inserted.

A second idea is that the Sfl1-CT could be involved in anchoring the protein correctly to the membrane, or membrane bound factors (5.9B). The fission yeast SPB is shuttled in and out of the membrane fully formed (Ding et al., 1997). There is a S. pombe homologue of Ndc1p, (Cut11p (West et al., 1998)) that is associated with the SPB only when it is in the nuclear membrane, suggesting a membrane anchoring (rather than insertion per se) role for Ndc1p and Cut11p. Maybe the function of the Sfl1p C-terminus is involved in this membrane interaction, and the sf1-CT mutants, although recruited to the SPB have defective membrane interaction. As I could not quantitate levels of Sfl1-120-mCherry at the SPB it is possible that levels were slightly reduced also. Extra copies of membrane insertion/anchoring factors could help to restore correct membrane interactions, and thereby Sfl1p function.
Figure 5.9. Models for the interaction of Sfi1p and the Mps2p-Bbp1p complex and Ndc1p during SPB duplication. A) Sfi1p has a role in SPB insertion, possibly by affecting retraction of the half bridge or membrane fusion at the distal end of the bridge. B) The Mps2-Bbp1 complex and Ndc1 may anchor Sfi1p in the membrane via its C-terminus. C) The membrane factors and Sfi1p are both involved in bridge separation, possibly through mediating interaction of the half bridge and 'normal' membrane. D) Bbp1 recruits Cdc5p, which may phosphorylate Sfi1p.
A third point in spindle pole body duplication that may involve membrane and half bridge interaction is SPB separation. Cleavage between SPBs occurs at the centre of the bridge. As the half bridge is a specialisation of a membrane, after physical separation of the two half bridges, it is required for ‘normal’ membrane to insert itself in-between the half bridges as they separate. If we consider the half bridge in 3D, if will be surrounded be ‘normal’ membrane on all sides not contacting a spindle pole body so this process simply requires interaction between the two membrane types. It may be that the \textit{sfi1-CT} mutants have defects in bridge separation and the extra membrane insertion/anchoring factors assist. Maybe they help the new ‘ends’ of the half bridges interact with the envelope. This possibility is illustrated in figure 5.9C.

\textit{Bbp1p} has an additional role in recruiting the kinase \textit{Cdc5p} to the spindle pole body, which is important for the mitotic functions of \textit{Cdc5p} (Park et al., 2004). We know that \textit{Sfi1p} is phosphorylated, and \textit{Cdc5p} is a candidate kinase for this. \textit{BBP1} allows sectoring in three of the \textit{sfi1-CT} mutants, there is a possibility that this is due to extra \textit{Cdc5p} recruitment in addition to \textit{Bbp1p}’s membrane insertion role (5.9D). However, there is no clear change in phosphorylation pattern in the \textit{sfi1-CT} mutants compared to wild type.

Two of the \textit{sfi1-CT} alleles were also partially rescued with a \textit{CIN8} plasmid. Also during work on the other mutants isolated in the \textit{mad\textDelta} synthetic lethal screen, a \textit{SF11} plasmid rescued sectoring in one of the \textit{cin8} alleles. The function of \textit{Cin8p} is easier to relate to half bridge function. \textit{Cin8p} is required to separate spindle pole bodies, which requires cleavage and separation of the half bridge. If a mutant in a half bridge protein made it more stable or less sensitive to cleavage, extra force from additional motors could overcome this.

The results in this chapter suggest several possible functions for the C-terminus of \textit{Sfi1p} in half bridge function. Genetic interactions suggest a function in both insertion and half bridge cleavage. In the next chapter, I look at the spindle pole body cycle and where the execution point of the \textit{sfi1-CT} mutants is within this.
Chapter 6 – an Sfilp function during SPB separation?

6.1 Introduction

I have so far established that Sfilp is at the spindle pole body, and that *sfil*-CT mutants have significant mitotic defects. There are a number of spindle pole body defects that could impact on mitosis. For most spindle pole body mutants, the primary defect is in some aspect of spindle pole body duplication (Adams and Kilmartin, 2000). Proteins of the outer or inner plaques fail in the assembly of their respective plaque. Many mutants fail in insertion of the new SPB into the membrane. Most half-bridge mutants fail to assemble a satellite (Byers, 1981b; Jaspersen et al., 2002; Spang et al., 1995; Vallen et al., 1994).

In the case of most SPB mutants that cannot assemble a functional inner plaque a monopolar spindle is assembled from the functional mother SPB. The lack of tension across the kinetochores, and unattached kinetochores will activate the spindle checkpoint. Most monopolar spindle mutants eventually go through monopolar mitosis where all the DNA goes to one pole, resulting in diploidisation. The only exception is *mpsI*, which does not arrest at all due to having an important role in the spindle checkpoint (Weiss and Winey, 1996).

In the case of *sfil*-3 and *sfil*-7 (mutants in the Cdc31p binding repeats) the cells do not assemble the satellite as with other half bridge mutants (Kilmartin, 2003). Other work in this thesis has suggested that the *sfil*-CT mutants are defective in a different process to *sfil*-3. *sfil*-CT mutants can partially complement the *sfil*-3 mutant in diploid cells, and do not show the same genetic interactions with Cdc31p. Additionally they have shown genetic interactions with the set of genes known to be involved in inserting the new spindle pole body into the membrane.

In this chapter I aim to establish the precise spindle pole body and mitotic defect in *sfil*-CT mutants.
6.2 *sfil-CT* mutants show different mitotic defects to *sfil-3*

6.2.1 *sfil-CT* mutants arrest with duplicated spindle pole bodies close together

Mutants in the Sfi1p repeats (*sfil-3* and *sfil-7*) show defects in spindle pole body duplication such that when shifted to the restrictive temperature they arrest with an unduplicated spindle pole body (Kilmartin, 2003). This is the typical phenotype for half bridge mutants. Previous studies using an Sfi1p depletion method show that 90% of cells arrest with monopolar spindles and 10% with duplicated spindle pole bodies but a very short spindle as assessed by tubulin immunofluorescence (Ma et al., 1999).

In order to study the behaviour of the spindle pole bodies in *sfil-CT* mutants, strains also containing *SPC42-GFP::TRP* were obtained by mating. Cycling, log phase cultures of these strains grown at 23°C were shifted to 37°C for 3 hours and then fixed as in section 4.2.3. Representative cells are shown in figure 6.1. *sfil-3* cells arrest in this assay as large budded cells with only one SPB, in agreement with published data (Kilmartin, 2003). In contrast, many *sfil-CT* cells had duplicated their spindle pole bodies, but they were very close together. In wild type cells a short spindle of 1 - 1.5 µM is built early in S-phase, such that in a hydroxyurea induced (S-phase) arrest, cells have a uniform short spindle of this length. At the large bud size of *sfil-CT* cells, a spindle of at least 1 µM would be expected. This short spindle phenotype may be similar to the 10% of cells with a short spindle seen in the Sfi1p depletion experiments by Ma et al (1999).

cdc4 mutants arrest with multiple buds with spindle pole bodies that have duplicated but not separated to form a spindle (Byers and Goetsch, 1974). A *cdc4* strain expressing Spc42-GFP was analysed in parallel. The two side-by-side spindle pole bodies in *cdc4* can be clearly distinguished as 2 spots similar to the *sfil-CT* mutants. This is surprising as the resolution limit for the light microscope is about 200 nm and the bridge is only 150 nm (Byers, 1981a). The SPB phenotype of *sfil-CT* mutants cannot be distinguished from *cdc4* at the light microscope level.
Figure 6.1. *sfi1-CT* mutants arrest/delay at 37°C with a large bud and two SPBs abnormally close together. Cells were grown to early log phase (OD600 ~ 0.1) at 23°C in CSM then shifted to 37°C for 3 hours and fixed with formaldehyde for 10 min. Scale bars are 1.5 μm.
Figure 6.2. Spindle lengths in *sf11-CT* mutants A) Distribution of spindles lengths between 0.1-1.9 μm. Strains expressing Spc42-GFP were grown to log phase then arrested with alpha factor at 23 °C, they were then released into fresh media at 37 °C for 3 hours then fixed with formaldehyde. Spindle lengths in fixed cells were taken as the distance between Spc42-GFP spots. B) Distribution of spindle lengths in cycling cells at 23 °C or 37 °C. Strains as above were grown to log phase at 23 °C then shifted to 23 °C or 37 °C for 3 hours and fixed and assessed as above. C) Average spindle lengths from cells in (A).
6.2.2 Characterisation of short spindles

These close together spindle pole bodies are an important and striking phenotype of the \textit{sfi1-CT} mutants. A detailed investigation into their occurrence was undertaken.

Strains expressing Spc42-GFP were grown to log phase at 23°C, then arrested with alpha factor for 3 hours at 23°C followed by 1 hour at 37°C. Spindle pole body duplication happens soon after alpha factor release, so it was important that the cells were at 37°C prior to release. They were then released into fresh media at 37°C for three hours and then fixed with 3.7% formaldehyde for 10-15 minutes. After fixation cells were kept in PBS/Sorbitol. Large numbers of cell images of Spc42-GFP and DIC were taken within 24 hours of fixation. The distance between spindle pole bodies was measured using Slidebook computer software (n.b. the software only measures to the nearest 0.1\( \mu \)m), for at least 150 cells per sample.

The distribution of SPB distances between 0.1-2 \( \mu \)m is represented in figure 6.2a. The wild type strain shows an even distribution over all lengths in this range. The \textit{sfi1-CT} mutants all show a large peak in the 0.3-0.4 \( \mu \)m range and very few SPBs 0.6-1.3 \( \mu \)m apart (which is the range of normal ‘pre-metaphase’ spindles). The \textit{sfi1-CT} mutants also have much smaller peaks above 1.4 \( \mu \)m that correspond to normal metaphase spindles. The broad pattern is the same for all the mutants, although they vary in the proportion of abnormal to normal spindles. The \textit{cdc4} mutant also shows the same distribution pattern, also with a peak in 0.3-0.4 \( \mu \)m. The pattern and peak SPB distance according to slidebook looks the same for both the \textit{sfi1-CT} mutants and the \textit{cdc4} strain. This suggests that the close together SPBs in the \textit{sfi1-CT} arrest are in fact duplicated side-by-side SPBs that have not separated to form a spindle.

During the course of preliminary experiments, it was noted that the \textit{sfi1-CT} mutants had an abnormal number of close together spindle pole bodies even at 23 °C, which is the permissive temperature for colony formation. Next I carried out a study of the distribution of SPB separation at 23 °C and 37 °C. Strains as above were grown to log phase at 23 °C. The culture was split and half left at 23 °C and half shifted to 37
°C for 3 hours. The cells were then fixed and imaged as above. To simplify analysis, the spindles were categorised:
Those with only 1 SPB signal; 0.1-0.6 μm (which includes the very short sfI spindle and a stage wild type pass through very quickly; 0.7 – 1.9 μm (which encompasses all ‘normal’ pre-anaphase spindles); and 2.0 μm and over which would be expected to be anaphase and telophase spindles. The results are shown in figure 6.213. The distribution of spindle lengths in the wild type strain was unaffected by temperature, with the vast majority of cells having a spindle of at least 0.7 μm. This is expected as a short spindle is built in S-phase and remains until anaphase when it elongates. The most striking thing the graph shows is that all sfI-CT mutants show at least double the amount of spindles under 0.6 μm (red bars) than wild type, even at 23 °C. The number of very short spindles varies between mutants and is roughly correlated with severity of the temperature sensitivity on solid media (section 3.5).

The average length of ‘short’ spindles for the strains is shown in figure 6.2C. The spindle lengths were taken from the data used for 6.2A. The average length of all sfI-CT spindles is significantly shorter than wild type.

6.3 The sfI-CT temperature sensitive arrest
Where is this defect acting, i.e. where in the cell cycle do the cells arrest? Do they build a spindle that then collapse, or is one never built? Attempts were made to monitor spindle length and bud size in an alpha factor release time course by microscopy. It was apparent that some cells were arresting and some were completing mitosis (data not shown). The temperature sensitive arrest is not tight enough to draw many conclusions. Time-lapse microscopy of live cells shifted from 23 °C to 37 °C was also performed a number of times. Spindle collapse was never observed. As these methods did not yield clear results, I turned to biochemical markers of cell cycle progression.

The spindle pole bodies seem to arrest at early S phase but the cells are large budded which indicates a G2/M arrest. Although the SPB cycle may be in early S-phase, the rest of the cell most likely continued the cycle biochemically and has arrested at a
later point. The synthetic lethality with the spindle checkpoint suggests the defect may be sensed by the spindle checkpoint. When cells are arrested by the spindle checkpoint, they do so as large budded cells with a short metaphase spindle (1-1.5 μm). Many other spindle defects arrest at metaphase, including monopolar spindle mutants.

A simple way to investigate cell cycle progression is to look at levels of proteins that modulate through the cell cycle. To this end the PDS1-HA strains from section 4.4.2 were used. A time course from alpha factor arrest and release monitoring protein levels was performed similar to section 4.4.2. This time the final hour of alpha factor arrest and the time course were performed at 37 °C, and samples were taken at 20-minute intervals. The cycle of Pds1p was explained in 4.4.2; in this assay levels of Clb2 were also measured. Clb2 levels rise in late S phase, later than Pds1, and peak in mitosis. Clb2 is destroyed later in mitosis than Pds1. Levels of both remain high in a spindle checkpoint arrest. The experiment was performed 3 times with wild type and sfil-120 in parallel, and a representative set of blots from the same experiment show in figure 6.3.

In the wild type strain, Pds1p expression is first seen at 20 min and peaks at about 40 min. The levels drop at 60-80 min indicating mitosis. The cells lose synchrony quickly at 37 °C, so the signal never disappears completely. The levels rise again for a second mitosis. The strong Clb2 signal is first seen at 40 min, and level peaks at 60 min, then falls and rises again. The Mad1p levels remain constant, so these level changes are due to levels of individual proteins in the cell, not uneven loading. Together the two markers indicate at 37 °C wild type cells go through mitosis at about 60-70 min, and a second at about 120 min.

Strain sfil-120 was chosen as a representative mutant as it is one of the most temperature sensitive and displays the highest number of close together SPBs at 37 °C. The arrest in e.g. sfil-229 would not be tight enough. Pds1-HA levels in sfil-120 rise from alpha factor with similar timing to the wild type. Levels drop slightly from the peak at 40-60 min but never drop as sharply as the wild type.
Figure 6.3. A proportion of *sfi1-120* cells arrest with high Clb2p and Pds1p at 37 °C. Cells were arrested with alpha factor for 3 hours at 23 °C and 1 hour at 37 °C. They were then released into fresh media at 37 °C. Cells samples were taken and frozen at 20 minute intervals (time, α = alpha factor arrest). Samples were used for Western blot.
Levels of Clb2p rise again with similar timing to the wild type, but never drop from the peak amount seen at 60 min. This suggests that the sfi1-120 cells enter mitosis with normal kinetics, but then become delayed and exit very slowly if at all. From the results of section 6.2 we know that even at 37 °C some normal spindles are built and mitosis is likely completed in these cells. The slight drop in Pds1p levels after 60 min probably indicates some cells going through mitosis at the normal time, and at times after, with a good proportion of the cells arrested in mitosis with the side-by-side SPBs.

We know that the sfi1-120 arrested cells also have large buds. This, along with the evidence above, suggests that the spindle defect causes a metaphase delay, most probably mediated by the spindle checkpoint. It would be interesting to see if this delay were still present without a functional checkpoint. This experiment would be difficult as the sfi1-CT mutants are synthetic lethal with checkpoint deletions, even at lower temperatures. A regulatable checkpoint strain, for example MAD3 under the Tet promoter could be used.

The cells are not tight temperature sensitive; there is still some growth at 37 °C (figure 3.4). This suggests that in some cells there is not an arrest, but rather a long delay, after which cells attempt mitosis. If the spindle pole defect were not overcome by this point, the high level of chromosome loss seen in these strains in section 3.6 would occur.

6.4 Electron microscopy
It has been established that in sfi1-CT mutants many cells delay in metaphase with the spindle pole bodes about 0.3 μm apart. The only thing that is clear from light microscopy is that the SPBs have duplicated the Spc42p signal. Are these side-by-side SPB signals really very short spindles, or side-by-side SPBs?

Both Tub1-GFP in live cells and tubulin immunofluorescence in fixed cells show a mass of tubulin associated with the paired spindle pole bodies (data not shown). No
linear spindle is seen with either method. The only way to determine exactly what is happening is to look at the SPBs at the level of the electron microscope.

Cells were released from an alpha factor arrest at 23 °C to fresh media at 37 °C for 3 hours, and then processed. They were gluteraldehyde fixed and stained with uranyl acetate and lead citrate (more details in materials and methods). Electron microscopy was performed in Edinburgh, Cambridge and Colorado. The images used for figure 6.4 were taken at the University of Colorado, Boulder, by T. Giddings.

sfil-65 and sfil-120 were used as representative strains. On a cell level many of the cells seen were large-budded, containing two spindle pole bodies in close vicinity, confirming light microscope results. As seen by fluorescence microscope, there were some cells with metaphase spindles, and these appeared normal. An example of a normal metaphase spindle from a wild type cell using these methods is shown in figure 6.4A.

Roughly 50% of cells had side-by-side spindle pole bodies. In these cells both spindle pole bodies look morphologically normal – the layered structure is clearly intact. It appears in both strains that the SPBs can organise microtubules, as nuclear microtubules are seen emanating from both SPBs in most cases. Some examples are shown in figure 6.4. Many of the paired SPBs in sfil-120 cell show a clear half bridge still connecting them.

The fact that both SPBs look normal and can nucleate nuclear microtubules suggests that the defect is in the SPB separation stage. Either the half bridge cannot physically cleave, or some aspect of the signal to cleave is defective. This side-by-side spindle pole body phenotype is shared with cdc4 at the electron microscope level too. cdc4 mutants are defective in the signal telling the SPBs to separate.
Figure 6.4. Electron microscopy of *sfi1-CT* mutants. A) Wild type large budded cell showing normal short spindle. B) Representative cells from *sfi1-65*. C) Representative cells from *sfi1-120*. Cells were released from alpha factor to 37 °C for 3 hours and then fixed and stained for electron microscopy. All images: T.Giddings.
Figure 6.4B sfi1-65
Figure 6.4C. sfil-120
6.5 Behaviour of chromosomes

The electron microscopy has established that *sfi1-CT* cells arrest with duplicated but unseparated spindle pole bodies. The electron microscopy reveals both spindle pole bodies nucleating microtubules that appear normal. Are the microtubules from the daughter SPB functional? Budding yeast kinetochores are attached to spindle pole bodies during most of the cell cycle. They are though to be shuffled around in S-phase, and re attached in late S-phase/G2. Are the chromosomes attached to the poles in an *sfi1-CT* arrest?

Recently, strains have become available that allow the monitoring just one of the 16 *S. cerevisiae* chromosomes. Strains are either labelled at the centromeres (Straight et al., 1997), which are not cohesed in metaphase and appear as one spot for each chromatid, or at distal sites that remain cohesed and appear as one spot until anaphase (Biggins et al., 2001). This section uses these strains to answer some of the above questions.

6.5.1 GFP chromosome strains

Primers were designed to tag Spc42p with mCherry using the previously described PCR method. This was transformed into cells already carrying a GFP marked chromosome IV. This works by having a tandem array of 256 lac operators (lacO, marked with TRP) plus a GFP tagged lac repressor (GFP-lacI, marked with LEU). The lacO array is integrated 12 kb from centromere IV, where sister chromatids remain cohesed until anaphase onset (Biggins et al., 2001).

These double-labelled strains with both GFP-Chr IV and Spc42-mCherry were crossed with the *sfi1-CT* mutants. The *sfi1-120* strain was used as an example.

Both wild type and *sfil-120* cells were grown to log phase at 23°C, then arrested with alpha factor at 23°C 3 hours then 37°C for 1 hour. A third of the cells were fixed at this point. The remaining cells were released from alpha factor into fresh media containing either 10 μg/ml hydroxyurea or 60 μg/ml benomyl and 15 μg/ml
nocodazole at 37 °C. Cells were fixed as before. Images of both mCherry and GFP were taken using the 'Slidebook' software as in section 6.2.

6.5.2 Attachment

The electron microscope pictures clearly show that in *sfl1-CT* mutants at the restrictive temperature, both spindle pole bodies can nucleate microtubules. Are the chromosomes attached to these microtubules?

Attachment was measured using data from the hydroxyurea arrest. Hydroxyurea (HU) halts DNA replication and induces the S-phase DNA damage checkpoint that arrests cell cycle progression. The bud continues to grow, so wild type HU arrested cells are large budded with a ~1-1.5 μm short spindle. This arrest does not affect the microtubules. It is hard to directly compare wild type and mutant strains. The wild type all have a short spindle with the GFP chromosome usually somewhere between. Unlike the Spc42-GFP, the Spc42-mCherry SPB cannot be distinguished as distinct side-by-side dots, but as a small dumbbell or line. The mutants have the GFP spot fairly close to the SPB doublet.

The distances between SPBs and the GFP chromosome were measured. The averages in μm are given on the graph in figure 6.5. For the wild type the distances from each spindle pole body was measured. The largest (lg) and smallest (sm) distances from each spindle were averaged. These are shown on the graph, along with an average of all the wild type distances (these are illustrated in figure 6.5). For the *sfl1-120* cells the distance between SPB dumbbell and the GFP spot was measured. The chromosomes in the *sfl1-120* strain are not significantly further away from the SPBs than in the wild type, this is consistent with them being attached to at least one of the SPBs.
Figure 6.5. Attachment of GFP-ChrIV to SPBs. Cells expressing both Spc42-mCherry and GFP-ChrIV were released from an alpha factors arrest to fresh media containing hydroxyurea, at 37 °C. After 3 hours cells were formaldehyde fixed and images taken within 24 hours. Representative cells and a schematic representation are shown in A. Distances between SPBs and chromosome were measured, for wild the the smallest and largest distances were measured (represented in diagram), for sfi1-120 the distance between GFP spot and SPB doublet was measured. The average lengths from at least 100 cells for each strain are shown in the graph in B.
This of course may mean that the attachment of one chromatid to the mother SPB is enough to keep the chromatid pair attached. Pereira et al (Pereira et al., 2001) created a strain in which mother and daughter SPB can be distinguished. The spindle pole bodies are labelled with both Spc110-GFP and Spc42-RFP. The RFP is slow to fold and therefore the daughter SPB does not give a red signal. This strain could be adapted, maybe with substitution of Spc110-CFP, to distinguish the SPB from the GFP chromosomes. It would still be hard to distinguish between monopolar attachment and attachment to both SPBs in the case of side-by-side SPBs. It may that in these strains the GFP chromosome is always nearer the mother SPB. An alternative method to investigate attachment is to utilise the recruitment of checkpoint proteins to unattached kinetochores. Any unattached kinetochores in a sfi1-CT arrest will recruit e.g. Mad2-GFP.

6.5.3 Sfi1p has no role in sister chromatid cohesion

Mps3p, a trans-membrane half bridge protein, shows defects in sister chromatid cohesion in addition to SPB defects (Antoniacci et al., 2004). An interaction with the cohesion factor Ctf7p was identified in a 2-hybrid screen. Mutants in the SUN (Sad1 UNC-84 homology) domain of Mps3p also show defects in sister chromatid cohesion in a nocodazole or hydroxyurea arrest (S. Jaspersen, personal communication, (Antoniacci et al., 2004)).

To assay cohesion the data from all three arrests were used. I chose the nocodazole arrest as nocodazole depolymerises microtubules. Lack of microtubules would allow diffusion of any separated sister chromatids making it easier to score. The downside of this is the arrest is not always sustained in all cells, and there is no easy way of distinguishing arrested and non-arrested cells. Antoniacci et al used Pds1-Myc immuno-fluorescence to select for cells in metaphase. A hydroxyurea arrest is tighter, but the microtubules are still intact and the spindle is small so there is less scope for diffusion of un-cohesed chromosomes. At the region of ChIV that is labelled, cohesion is maintained until anaphase, and the chromosome will appear as a single green spot. If there is no cohesion at this location, the sister chromatids will
dissociate and two green spots will be seen. The percent dissociation is the percent of total cells showing two rather than one GFP spot.

In my assay the wild type cells show 10.7% dissociation in the nocodazole arrest. Antoniacci et al published a figure of 3-4% but they were able to select for arrested cells using Pds1-HA immuno-fluoresence, discounting any dissociation due to not being in a mitotic arrest. In that assay mps3 showed 14.2% dissociation compared to 2.75% in wild type. In my assay sfi1-120 showed no significant difference relative to wild type, in fact was slightly lower (9.4% vs. 10.7%).

My results in hydroxyurea were 3.5% and 3.8% for wild type and sfi1-120 respectively, which agree better with published data. In a hydroxyurea arrest, S. Jaspersen saw under 5% dissociation in the wild type strain and 15-35% in an mps3 SUN domain mutant (personal communication). The small difference seen between wild type and sfi1-120 is unlikely to be significant.

Overall the data confirms that sfi1-120 shows no significant difference in sister chromatid cohesion.

6.6 Discussion
In this chapter I have established that sfi1-CT mutants at the restrictive temperature arrest with side-by-side SPBs that have not separated. This defect could be caused by physical defects involving Sfi1p in the half bridge, or a defect in some aspect of the signal telling the half bridge to separate. No clear defect in SPB membrane insertion was apparent, in conflict with some of the theories proposed in chapter 5.

When cell cycle progress is monitored by the levels of protein factors, a high proportion of the sfi1-CT cells arrested with high levels of securin and cyclin, suggesting a mitotic arrest. This is very likely a spindle checkpoint-mediated arrest, as all spindle pole body defects (except mps1) arrest here. The progress of the spindle cycle in co-ordination with the rest of the cell is not checked until the cells start to monitor the mitotic spindle in prometaphase/metaphase.
The side-by-side SPB arrest is a new phenotype both for Sfi1p and for spindle pole body mutants in general. Other mutants have been described that cannot or do not separate their duplicated spindle pole bodies.

The first class of mutants are those involved in the structure of the spindle. These include microtubule plus-end directed motors such as cin8 and kipl (Hoyt et al., 1992; Hoyt et al., 1993; Roof et al., 1992; Saunders and Hoyt, 1992) and midzone MAPs such as stul and ase1 (Pasqualone and Huffaker, 1994; Pellman et al., 1995). These factors are required to provide the force on interdigitated spindle microtubules to separate, or maintain spindle structure once it is built. Sfi1p, being on the half bridge, is unlikely to be affecting the microtubules in the way this first class of mutants do. However, there is also a possibility that Sfi1p is in additional locations on the spindle pole body and/or spindle.

The second class are mutants in cell cycle control factors such as the SCF (e.g. cdc4) and Cdc28/cyclins. The activity of Cdc28p/Clbs is required for the SPB separation signal (Haase et al., 2001). Mutants in the SCF^{Cdc4} also arrest with side-by-side unduplicated spindle pole bodies. A key target for destruction of the SCF^{Cdc4} is Sic1p, the Cdc28 inhibitor, so this phenotype is possibly due to low Cdc28p activity. Alternatively the SCF may be required for destruction of a protein that holds the two half bridges together in the bridge. It is possible that Sfi1p is one of the proteins at the effector end of one of these control pathways and this signal is not transduced or responded to in the sfl1-CT mutants. The most common form of signal is phosphorylation. The sfl1-CT mutants do not show any change in phosphorylation as assessed by 1D SDS PAGE, but it might be possible to detect more subtle changes in phosphorylation may be observed by 2D PAGE.

There is a third option - that the sfl1-CT mutants have a structural defect that renders the bridge unable to separate. Either the sfl1-CT mutants make the half bridge more stable or they fail to recruit factors required for its separation. In this first case it
may be that increased microtubule force is required to separate the bridge, which the cell manages to come up with after a long delay.

In a talk at the recent EMBO centrosome meeting (Heidelberg, 2005), Kilmartin shared some results that may shed light on this. He had performed immuno-electron microscopy on Sfi1p tagged at either the N or C terminus with GFP. When N-terminally tagged, the anti-GFP signal was at the side of the bridge nearest the spindle pole body. When C-terminally tagged however, the signal was at the centre of the bridge, or the distal end of the half bridge. This suggests that Sfi1p spans the half bridge. This was further supported by showing a fragment of 15 of the repeats is fibrous when viewed by E.M., having a length that fits with the length of the half bridge. The C-termini of Sfi1p from the two SPBs would then be in close proximity at the centre of the bridge. Assuming the centre of the bridge is where the physical separation happens (EM results show bridge seems to cleave evenly (Byers, 1981a; Byers and Goetsch, 1974; Byers and Goetsch, 1975)) then the C-termini of Sfi1p would be positioned to be involved in this separation. The sfi1-CT mutants may cause a stabilisation in the interaction of the two halves of the bridge, making separation harder, but not impossible. The cells delay in metaphase until sufficient force is gained to separate the SPBs. Alternatively the sfi1-CT mutants may fail to recruit a cleavage factor to the centre of the bridge.

In section 5.5.3.2 we saw that overexpression of Cin8p can partially rescue the sfi1-229 and sfi1-273 mutants. Cin8p is a plus end sliding motors responsible for some of the outward forces in the spindle. This would suggest the first hypothesis, that the sfi1-CT makes the bridge more stable, requiring more force to separate the two halves.

As yet no dimerisation or self-interaction of Sfi1p has been seen. Sfi1p was not identified in the 2-hybrid screen in 5.4, but this screen was with only with a C-terminal Sfi1p fragment. There are many reasons an interaction may not have been identified in this case. The C-terminus may require the rest of the protein to be structured correctly for the interaction, or the rest of the protein may be involved in
the self-interaction also. Additional factors may be required to facilitate this selfinteraction. The interaction of the C-terminus with itself could be tested using a 2hybrid screen with the C-terminal fragment as both bait and prey.

In conclusion I have shown that Sfi1p has a role in SPB separation mediated at least in part by its C-terminus. This is an additional function to the role in satellite assembly reported by Kilmartin (Kilmartin, 2003) that is mediated by binding of Cdc31p to the central Sfi1p repeats.
Chapter 7 - Final discussion

7.1 Sfi1p is a conserved component of microtubule organising centres

I have established that Sfi1p is a spindle pole body protein. During the course of this study Kilmartin (2003) showed further using immuno-EM that Sfi1p is at the half bridge of the spindle pole body.

The most striking sequence feature of Sfi1p is a series of 21 internal amino acid repeats including a conserved tryptophan residue. Our work places the tryptophan at the centre of a 15 amino acid motif L-L-X$_3$-F/L-X$_2$-W-K/R-X$_2$-F/L. Kilmartin defines the repeats as a 23 amino acid sequence with the conserved tryptophan at the end A-X$_7$-L-L-X$_3$-F/L-X$_2$-W-K/R (Kilmartin, 2003). His rationale for placing the Trp residue at the end is similarity to the common WD repeat motif. WD repeats are ~40 amino acid motifs terminating in Trp-Asp (WD), present in 4-16 repeating units. They are present in diverse proteins but their common function is mediating protein-protein interactions in multi-protein complexes (Neer et al., 1994).

Sfi1p is conserved in a wide range of organisms including humans. There is one homologue in *S. pombe*, which was identified both in this study and by Kilmartin. In this study we also identified potential homologues in *Aspergillus* and *Neurospora* that contain 18 and 23 repeats respectively. This study identified one human homologue. Kilmartin (2003) identified several human proteins that have Sfi1p repeats, as well as a mouse homologue. Three of the human proteins had between 4 and 10 repeats, whereas another, now named hSfi had 23 repeats. hSfi1 exists in two forms with stop codons at either amino acid 968 or 1242. Since then John Kilmartin has found homologues in 23 different organisms (personal communication). The consensus for Sfi1p repeats in all organisms is the same as the Kilmartin one above. In budding yeast the gap between repeats can vary from 23 and 35 residues. In addition to the repeats, all homologues share a low proline content in repeats but no homology in the C and N terminal extensions outside the repeats.
Kilmartin showed that *S. pombe* and *H. sapiens* Sfi1p is at the spindle pole body and centrosome respectively. Therefore Sfi1p is one of only a few conserved spindle pole body/centrosome proteins, suggesting an important conserved role. This is an important discovery as very few spindle pole body proteins are conserved between budding yeast SPBs and the centrosome. Centrosome abnormalities have been implicated in cancer (see 1.7.4, and discussion later), and studying Sfi1p in yeast may yield information on centrosome function.

### 7.2 The C-terminus – function in budding yeast

**Orientation and spindle pole body separation**

The C-terminus of *S. cerevisiae* Sfi1p is conserved amongst budding yeasts (section 5.1). Experiments in section 5.2 show it is required for the spindle pole body localisation of Sfi1p, and is essential. Sfi1p is an essential protein (Ma et al., 1999), so these experiments suggest that the essential role is at the spindle pole body. The C-terminus is predicted to be unstructured. The *sfi1-CT* mutants have defects in SPB separation (chapter 6).

John Kilmartin has performed immuno-EM on Sfi1p tagged at either its N or C-terminus with GFP (personal communication, (Kilmartin, 2003)). When Sfi1p is tagged at the N-terminus with GFP, the anti-GFP signal is at the proximal end of the half bridge contacting the spindle pole body. When the GFP tag is C-terminal, the anti-GFP signal is seen at the distal end of the half bridge or the centre of the bridge. This suggests Sfi1p is tethered to the SPB directly or indirectly via its N-terminus, and extends the length of the half bridge with its C-terminus at the distal end, or centre of the bridge. This model is illustrated in figure 7.1. In agreement with this idea, John Kilmartin has expressed and purified a protein fragment of 15 Sfi1p repeats along with 15 centrin molecules (personal communication). EM shadowing on this purified complex reveals this fragment is fibrous with a length of 59 nm, which fits with the Sfi1p having a mostly α-helical structure. This fibrous helical structure would allow it to span the half bridge, which is a length of ~80 nm. Unlike the rest of the spindle pole body, the half bridge does not change in size with ploidy,
although it appears to expand and contract under the duplication plaque during SPB duplication (Adams and Kilmartin, 1999). This suggests that the Sfi1p fibre defines the length of the half bridge. As of September 2005, John Kilmartin had created strains expressing Sfi1p with both half and double the number of repeats. It will be interesting to see if half bridge length is affected in these strains.

This model fits with my data implicating the C-terminus in spindle pole body separation. Kilmartin’s data suggests an orientation of Sfi1p on the half bridge with the N-terminus at the spindle pole body, and the C-terminus at the distal end. This would suggest that in the case of the bridge between side-by-side SPBs the C-termini of Sfi1p molecules from opposing SPBs would be positioned together at the centre of the bridge. During spindle pole body separation the half bridge cleaves evenly, so each daughter SPB gets a full half bridge. If this is the case the C-terminus of Sfi1p is ideally placed to mediate bridge interactions. The *sfi1-CT* mutations may be inhibiting spindle pole body separation in a number of ways.

The first explanation is that in the *sfi1-CT* mutants the interaction at the half bridge is stabilised physically. The two C-termini may interact physically, holding the two half bridges together, or recruiting a complex responsible for this. The *sfi1-CT* mutations would stabilise the interaction, preventing cleavage of the bridge. This would require interaction of the C-terminus either with other factors or with another Sfi1p molecule. There has been no evidence presented so far for Sfi1p dimerisation. Neither John Kilmartin nor myself have yet done any such experiments with the full length protein. Most of Kilmartin’s work has been on small fragments of Sfi1p, except the initial pull down experiment, which would not distinguish between pulled down and associated Sfi1p. My search for physical interactions was confined to the C-terminal fragment. It may be that the C-terminus requires the rest of the protein to be structured correctly for dimerisation/oligomerisation, or that the self-interaction can happen only when other factors such as Cdc31p or others yet to be identified bring the protein into the correct orientation. Alternatively the C-terminus might recruit an additional ‘separation factor’ or stimulate microtubule motor function. Again, as yet no protein interactions with the C-terminus have yet been identified.
Figure 7.1. A) Model for orientation of Sfilp on the half bridge. Sfilp molecules are orientated with their N-termini at the spindle pole body and C-termini at the centre of the bridge. B) Model for C-terminus involvement in SPB separation. Sfilp C-termini interact at the centre of the bridge and respond to the signal to cleave by either dissociation of a physical CT-CT interaction or recruitment of additional proteins that cause cleavage of bridge.
A second explanation is that the *sfi1-CT* mutants cannot respond effectively to the signal telling the SPBs to separate. It is known the activity of Cdc28/Clbs is required for SPB separation (Haase et al., 2001). Although I have shown that Sfi1p is phosphorylated (section 4.4), the phosphorylation pattern is the same in the C-terminal truncated proteins, suggesting the majority of phosphorylation is not in the C-terminus. The *sfi1-CT* mutants could still fail to respond to the phosphorylation signal in a different part of the protein.

**Satellite assembly and phosphorylation**

The half bridge extends from 80 nm to 150 nm before satellite assembly (Byers, 1981b). If the orientation of Sfi1p above is correct, this would imply the recruitment of extra Sfi1p, end on end with the existing half bridge. The satellite assembles on a distal tip of the 150 nm bridge, according to the data above this would be an N-terminus of Sfi1p. Protein interactions of the N-terminus of Sfi1p have not yet been investigated, but presumably it must interact, directly or indirectly with core SPB components.

The timing of the extra phosphorylation seen in 4.4 corresponds more with satellite assembly than SPB separation. Correspondingly my data suggests that this phosphorylation is not on the C-terminus (both Sfi1-120p and c-terminal truncation retain this phosphorylation pattern, 4.4 and 5.2). The repeats do not contain any known consensus kinase sites, but the N-terminus contains two Cdc28p sites and two potential Cdc5p sites. Either N-terminus or repeats could be substrates for as yet unidentified kinases. The timing would fit with the phosphorylation of the N-terminus being part of the signal for satellite assembly.

**The half bridge and the nuclear membrane**

The C-terminus of budding yeast Sfi1p in not conserved to *S. pombe* Sfi1p. The localisation within the SPB of SpSfi1p is not known, although SpCdc31p is at the half bridge (Paoletti et al., 2003), so it is likely that at least some of the SpSfi1p is there too. This suggests the budding yeast Sfi1p C-terminus is mediating a half bridge function confined to budding yeast, or that its binding partners are not well
conserved. The major difference in half bridge function between the yeasts is that SPB duplication takes place in the cytoplasm in *S. pombe* (Ding et al., 1997), so membrane interactions of the half bridge are not necessarily required for SPB duplication. The *S. cerevisiae* half bridge is thought to be a specialisation of the nuclear membrane (O'Toole et al., 1999). This suggests a possibility that the C-terminus of ScSfi1p is involved in membrane interaction. A number of nuclear membrane proteins were identified in the yeast 2-hybrid screen with the Sfi1p C-terminus (section 5.4.2). This may reflect a domain for binding membrane proteins or a membrane interaction. Also, the *sfi1-CT* mutants are partially rescued/suppressed by additional Ndc1p and Bbp1p. Ndc1p is a membrane-anchoring factor common to SPBs and nuclear pores. Although Bbp1p is not a membrane protein, it is in a complex with Mps2p, which is. Both these proteins are involved in inserting or anchoring the SPB in the nuclear membrane. This all suggests an interaction of the C-terminus of Sfi1p with the membrane or membrane protein/s at the half bridge.

**Recruitment of Sfi1p to the SPB**
The C-terminus is essential for SPB localisation (5.2), but Sfi1-120p is still recruited to the SPB (4.2.3). The *sfi1-CT* mutants are rescued/suppressed by overexpression of membrane insertion/anchoring factors (5.5.3). The evidence presented above suggests that the C-terminus may mediate some as yet unidentified membrane factor interaction.

Overexpression of *CDC31*, which is known to bind the central repeats, rescues temperature sensitivity of *sfi1-3* and *sfi1-7* (Kilmartin, 2003). In contrast, *CDC31* overexpression makes *sfi1-CT* mutants more temperature sensitive (section 5.5.3). Cdc31p also binds Kar1p and Mps3p and both are partly responsible for recruitment of Cdc31p to the half bridge (Biggins and Rose, 1994; Jaspersen et al., 2002). Both *sfi1-3* and *sfi1-7* mutants were assumed, although not proved, to be unable to bind Cdc31p (Kilmartin, 2003).
Figure 7.2 Model for recruitment of Sfl1p to the half bridge. A) In wild type cells Sfl1p is recruited via Cdc31p interaction with repeats, and C-terminus interaction with protein(s) X. B) When the C terminus is removed the interaction with Cdc31p alone is not strong enough to retain Sfl1p at the half bridge. C) In the sfl1-CT mutants interaction with X is weakened, but additional membrane factors (D) have a positive effect. E) When Cdc31p is overexpressed in the sfl1-CT mutants it competes with Cdc31p on the half bridge and the weak interaction with X is not enough to retain Sfl1p at the half bridge.
In these mutants at the restrictive temperature, Cdc31p still localises to the spindle pole body as assessed by immuno-fluorescence (S. Jaspersen, personal communication). This suggests that the Cdc31p-Sfi1p interaction is not required for Cdc31p spindle pole body localisation.

These observations can be explained by the following model (illustrated in figure 7.2):

*Sfi1p requires interaction with both Cdc31p and additional (membrane?) protein(s) X for stable association with the half bridge* (figure 7.2A). In this model, in the absence of the C-terminus Sfi1p cannot interact with protein X at all, and cannot stably bind the half bridge, and is mis-localised as seen in 5.2 (figure 7.2B). In the *sfi1-CT* mutants the association with protein/s X is weakened but not abolished (figure 7.2C). It is possible the Sfi1-CTp mutant proteins are present at the half bridge at lower levels than wild type but this has not yet been investigated. In this case most of the Sfi1-CTp can still associate with the half bridge, but extra copies of membrane factors (which may in themselves be X) help strengthen the Sfi1p-X interaction, stabilising half bridge interaction (figure 7.2D). This is a possible explanation for the *sfi1-CT* rescue/suppression seen with *BBP1* and *NDC1* overexpression (section 5.5.3).

The *sfi1-CT* mutants can probably bind Cdc31p as well as wild type, as they have no mutations in the central repeats. When *CDC31* is overexpressed, there may be additional free cytoplasmic Cdc31p, as there is limited Kar1p and Mps3p to recruit it to the half bridge. In the model, this additional Cdc31p titrates the Sfi1-CTp away from the SPB (figure 7.2E). The interaction of the mutant C-terminus with X alone is not enough to keep Sfi1p at the half bridge. In wild type strains this does not happen, because of a stronger interaction of the C-terminus with protein(s) X, keeping the Sfi1p at the half bridge. Interestingly, expression of human centrin 3 in budding yeast disrupts SPB duplication, and this is thought to be due to titration of Cdc31p-binding factors away from the SPB (Middendorp et al., 2000). My model suggests this factor may be Sfi1p. Budding yeast Cdc31p can bind HsSfi1p, so the reverse, binding of centrin 3 to ScSfi1p is quite possible.
There are a number of issues raised by this model. We don’t yet know whether Sfi1p requires Cdc31p binding to associate with the half bridge, and no other Sfi1p interactors have yet been identified. If the sfil-CT mutants had problems stably associating with the half bridge a phenotype similar to sfil-3 an sfil-7 would be expected, or at least a delay in spindle pole body duplication. In an alpha factor release time course, the sfil-CT mutants appear to duplicate SPBs with normal kinetics (data not shown), there is no delay with unduplicated spindle pole bodies. It may be that when normal levels of Cdc31p are present, the half bridge association is strong enough for normal function. Alternatively maybe only stable association of Sfi1p with Cdc31p (providing Sfi1-CTp is at the half bridge, albeit with a weaker interaction) but not protein X is needed for the SPB duplication step. Stable anchoring of the C-terminus or perhaps recruitment of a cleavage factor by the C-terminus would then be required for separation.

Further investigating the order and inter-dependence of half bridge recruitment of Sfi1p, Cdc31p, Kar1p and Mps3p would aid this model. Are sfil-3 and sfil-7 (which are compromised in Cdc31p binding) at the spindle pole body at the restrictive temperature?

The models in this section depend upon the C-terminus of Sfi1p interacting with other proteins and/or itself. As yet no interactors have been discovered, and my experiments suggest that the C-terminal fragment alone does not assume the correct structure when expressed in isolation. My work points towards interaction of the Sfi1p C-terminus with a membrane protein(s). Membrane proteins are hard to express and study in vitro due to their largely hydrophobic nature. Physical interaction studies of known trans membrane half bridge proteins Kar1p and Mps3p have relied on gel overlay assays (Biggins and Rose, 1994; Jaspersen et al., 2002). John Kilmartin’s work suggests that the N-terminus of Sfi1p is directly or indirectly bound to the SPB core. It would be interesting to discover what SPB protein(s) tether it to the spindle pole body.
7.3 Explaining the other sf11-CT phenotypes

I have established that the sf11-CT mutants are unable to or delayed in separating their spindle pole bodies. How does this relate to other phenotypes seen?

Synthetic lethality

The sf11-CT mutants were identified by virtue of their synthetic lethality with mad1Δ. In section 3.4 I showed that the sf11-CT mutants are also synthetic lethal with both mad3Δ and bub1Δ. As discussed in 3.7 this suggests that the sf11-CT defect is activating the spindle assembly checkpoint. We now know that this defect is in spindle pole body separation. Other mutants that result in side-by-side spindle pole bodies are those of plus end directed bimC motors such as cin8. cin8 mutants were also identified in the mad1Δ synthetic lethal screen in this study, as well as three other spindle checkpoint synthetic lethality studies (Daniel et al., 2006; Hardwick et al., 1996; Lee and Spencer, 2004). This shows that side-by-side spindle pole bodies are well documented to activate the spindle checkpoint. In the sf11-CT mutants and presumably in cin8 mutants also, both spindle pole bodies appear capable of nucleating spindle microtubules. This suggests that the kinetochores are all attached and that it is lack of tension across sister kinetochores that is activating the checkpoint.

As discussed in 3.7, mad3Δ has shown far fewer synthetic interactions than the other checkpoint mutants in several studies (Daniel et al., 2006; Hardwick et al., 1996; Lee and Spencer, 2004). There are opposing views as to whether this is due to Mad1p, Mad2p, Bub1p and Bub3p additional roles beyond those in the checkpoint, or as proposed by Lee and Spencer, that Mad3p is only required for a subset of the checkpoint response. Lee and Spencer suggest that Mad3p is not required for the checkpoint response to a lack of tension (Lee and Spencer, 2004). The synthetic lethality of both cin8 and sf11-CT mutants with mad3Δ would suggest otherwise, that the side-by-side phenotype does require Mad3p for a checkpoint response. Of course the possibility exists that there are also unattached kinetochores in these mutants, as even one unattached kinetochore can prompt a checkpoint arrest.
Chromosome Loss

Although a mitotic delay is seen, the sfil-CT strains still grow, even at 37 °C, so they are eventually attempting mitosis. There are two possible fates for the arrested side-by-side SPBs that result in mitosis rather than death. Firstly, the SPB defect could be overcome, through recruitment of extra bimC type motors, a required ‘cleavage factor’ or through cytoplasmic microtubules pulling the SPBs apart. A second option is that the side-by-side spindle pole bodies organise a monopolar spindle.

Most spindle pole body duplication defects result in only one functional spindle pole body. A haploid spindle pole body can only organise about 22 microtubules (O'Toole et al., 1999; Winey et al., 1995), usually one for each of 16 chromatids and a few interpolar microtubules. The one SPB can organise a monopolar spindle to which only about half of chromatids are attached. In this case the spindle checkpoint is activated by the unattached sister chromatids and/or the lack of tension. A mitotic delay is seen in cdc3l, karl, spc42, mps2 and ndc1 cells (Donaldson and Kilmartin, 1996; Hartwell et al., 1970; Rose and Fink, 1987; Winey et al., 1991b; Winey et al., 1993). Most of these mutants spontaneously diploidise, which is thought to be due to a monopolar mitosis, in which all DNA goes to one pole, resulting in one diploid and one aploid cell (reviewed in (Chial and Winey, 1999)). It is not known exactly how these cells exit or adapt to the spindle checkpoint delay. The single SPB could enlarge and become able to nucleate the 32 microtubules required to attach to all chromatids. Several SPB mutants which arrest with a single enlarged SPB, such as cdc3l (Byers, 1981b), support this. An alternative in the case of the sfil-CT mutants is that the paired side-by-side SPBs organise an effectively monopolar spindle between them. FACS (Fluorescence activated cell sorting) analysis of sfil-CT cultures could reveal whether a significant portion of them were diploid.

Another alternative fate for the sfil-CT mutants is that the mitotic delay allows them to correct or compensate for the SPB separation defect. The work in 6.3 suggested that although many sfil-120 cells are delayed in mitosis, some gradually exit over the last 2 hours of the time course, and this is likely to continue. The chromosome loss of sfil-CT mutants is in the middle of the range seen with spindle checkpoint
mutants. Although it occurs at much higher rate in sfi1-CT mutants than in wild type it is still only 1-2% of divisions in the mutants. Because at 23°C, 20-40% of sfi1-CT cells have side-by-side spindle pole bodies, this would suggest that many of these cells eventually manage to form a bipolar spindle and segregate DNA correctly. A subset of these spindles have abnormalities, or are not fully assembled before the spindle checkpoint arrest is adapted to and exited, and chromosome loss results.

7.4 Sfi1 and centrin
Cdc31p is a member of the centrin family, conserved from yeast to humans, including higher plants. Centrins are part of a larger family of small calcium binding ‘EF hand’ proteins that includes calmodulin. Calmodulin and centrin are made of two independent globular domains joined by a flexible linker. Each domain consists of two helix-loop-helix Ca$^{2+}$ binding EF hands. Calmodulin has four helix-loop-helix Ca$^{2+}$ binding motifs, whereas in centrins one or more of these is non-functional (e.g. Cdc31p only has two functional sites). On binding calcium these domains go from a ‘closed’ conformation to ‘open’, exposing hydrophobic surfaces for protein-protein interactions. Centrin has an unstructured N-terminus that is not conserved with calmodulin. This N-terminus has been shown to be involved in oligomerisation of human centrin 2 (Tourbez et al., 2004). In centrin, the two EF hand domains are not equal. Kar1p (or the 19 residue fragment that binds Cdc31p) binds only the C-terminal EF domain and this is calcium dependent in vitro. Structural studies on chlamydomonas centrin (CRC) showed that when bound to Kar1p the CRC C-domain adopts the ‘open’ conformation (Hu et al., 2004). The authors suggest that in vivo, there is an equilibrium between open and closed forms that is dependent on Ca$^{2+}$. Kar1p binds the open conformation and ‘locks’ it this way.

Centrins have been implicated in many fibrous structures with contractile and elastic properties. An example is the fibres associated with the centrioles of algal cells where centrins were first discovered. Centrin forms a major protein component of these fibres (Salisbury et al., 1984; Salisbury et al., 1987). These fibres are parallel linear arrays of fine fibres 200 nm long, 5-7 nm wide. Upon an increase in calcium levels, these fibres shorten by twisting and kinking (Salisbury, 1998). This
contraction is involved in position and orientation of centrosomal structures. In *Chlamydomonas*, centrin is present in nucleus-basal body connection fibres (Wright et al., 1985). These fibres contract in response to calcium. The 'spasmoneme' of vorticellid ciliates contracts in response to calcium, and the main component is spasmin, a small centrin related Ca$^{2+}$ binding protein (Routledge, 1978; Routledge et al., 1975). Therefore centrins are a conserved member of Ca$^{2+}$ dependent contractile fibres.

For a while, due to lack of identified binding partners, it was proposed that it was centrins alone that created these fibres. This has looked increasingly unlikely especially with the work placing them in the small EF hand family. Therefore there has been a search for conserved binding partners of centrins. In budding yeast both Kar1p (Biggins and Rose, 1994) and Mps3 (Jaspersen et al., 2002) have been revealed to be Cdc31p binding partners but neither are conserved in mammals, and their binding did not fit the fibre model. It is in a search for new binding partners of Cdc31p that Kilmartin identified Sfi1p. He pulled it down in an experiment under low calcium with Z-Cdc31p. Kilmartin also showed that the reciprocal is true; Cdc31p is pulled down with Sfi1p-protein A, confirming them as binding partners.

In a series of experiments with recombinant Cdc31p and various fragments of Sfi1p, Kilmartin showed that the central repeats, but not the C or N terminus, bind Cdc31p. As the repeats are the only conserved sequences in Sfi1p it makes sense that this is where centrins bind. Each Sfi1p protein fragment tested that contained multiple repeats bound more than one Cdc31p molecule. Kilmartin went on to test binding on smaller sections containing 1-3 repeats, and the protein ratios were consistent with one Cdc31p molecule bound per Sfi1p repeat. In agreement with this stoichiometry, mass spectrometry on the 15 repeat Sfi1p fragment created (see above) revealed that it does indeed bind 15 Cdc31p molecules (personal communication). Kilmartin also did binding experiments with human and mouse proteins. Protein fragments containing HsSfi1p repeats bound ScCdc31, human centrins 1 and 2, and mouse centrin 4. Binding of centrin 2 to Sfi1p was calcium dependent but that of Cdc31p wasn’t.
Kilmartin suggests the Sfi1p repeats bear similarity to the calmodulin binding IQ repeats of unconventional myosins. The IQ repeats show calcium independent calmodulin binding and are present in repeating arrays. Structure studies on Scallop myosin with two calmodulin domains suggests that repeats bind between the two calmodulin EF hand domains, and adjacent calmodulins can directly contact each other. The IQ repeats are closer together than Sfi1p repeats, allowing this direct contact between adjacent calmodulin molecules. Centrins have larger N terminal domains than calmodulin so may be able to contact each other across these larger distances. In support of this, the N-terminal extension is responsible for oligomerisation in hCen2 (Tourbez et al., 2004).

John Kilmartin has solved the crystal structure of two Sfi1p repeats with two molecules of Cdc31p (personal communication). The Sfi1p repeats form an alpha helix and the Cdc31p molecules are wrapped around, with the N-terminal extension not visible in the structure. The set distance between the A and LL residues within the Sfi1p repeats consensus forms the linker between Cdc31p binding domains. The Cdc31p molecules bind the most variable (in sequence and length) portion. The two centrins present in Kilmartin’s structure had the same tertiary structure, but were bound to the two Sfi1p repeats differently. There is interaction between the two centrins as predicted in the model. Interestingly, the orientation of Cdc31p on the Sfi1p repeats is the reverse to that seen of Chlamydomonas centrin on the Kar1p fragment (both contain K-X3-L-XX-W-X2-L-L-X2-D).

These discoveries allowed a re-evaluation of the model for centrin containing fibres. Kilmartin, elaborated by Salisbury ((Salisbury, 2004)) proposed a model for Sfi1/centrin fibres whereby Sfi1p is an elastic backbone onto which multiple centrins bind. On addition of calcium there is a conformational change in the centrin and it either self-associates or changes conformation, bending the Sfi1p. This causes the Sfi1p to ‘concertina’ up and shorten. The model proposed by Salisbury is shown in figure 7.3.
Figure 7.3. Model for Sfi1/centrin fibres. Multiple molecules of centrin (red) are bound onto Sfi1p (blue). The centrins are bound to one Sfi1p repeat each via their C terminal globular domain. On binding of calcium to the centrin N terminal domain there is a conformational change and/or binding of N terminal domain of centrin to other Sfi1p repeats, causing buckling/contraction of the Sfi1 fibre. Taken from Salisbury (2004).
Recently the structure of the *Chlamydomonas* centrin N term EF hand has been solved (Sheehan et al., 2006). The CRC-N domain can bind an Sfi1p repeat fragment, but not Kar1p, in agreement with previous Kar1p binding data. This Sfi1p fragment binding is Ca\textsuperscript{2+} sensitive, occurring only in the presence of calcium. Both binding of Sfi1p to Cdc31p in yeast and Kar1p to CRC-C in vitro can occur at the low free calcium levels present in a cell (Hu et al., 2004; Kilmartin, 2003). These findings elaborate on the model suggesting that the C-terminal EF hand of centrins are constitutively bound to binding partners, and the N-terminal domain responds and mediates changes in conformation or binding partners upon a calcium signal. In the case of Sfi1p and centrin this suggests that the C-terminal EF hand is always bound to Sfi1p and the N-terminal EF hand binds other distal Sfi1p sites upon calcium binding, causing crumpling/shortening of the Sfi1p fibre, in agreement with the model presented in figure 7.3.

Not all centrin containing contractile/elastic structures are subject to calcium signalling, for example there is no documented calcium fluctuation in yeast at the time of half bridge function. In *in vitro* binding studies, the binding of human centrin 2 to ScSfi1p was calcium-dependent but that of ScCdc31p wasn’t (Kilmartin, 2003). It may be that the calcium signalling is an extra layer of control in some organisms only. Budding yeast may have developed alternative methods of centrin regulation, for example Mps3p binds Cdc31p and is not conserved. Kar1p is present in algae but not vertebrates. Alternatively budding yeast may take advantage of the elastic properties of Sfi1p/centrin fibres, although the model in figure 7.1 suggests that Sfi1p is in the extended conformation under normal conditions, not able to extend any further.

### 7.5. Centrin, centrosomes and cancer

Roles of centrin-containing fibres have been mostly characterized in various protozoa. Roles include connecting basal bodies and nucleus, basal body positioning and flagella activity (reviewed in (Adams and Kilmartin, 2000; Salisbury, 2004; Schiebel and Bornens, 1995)). Less is known about the location and function of centrin containing fibres in mammals.
Mammals have four centrin forms, studied mostly in either humans or mice. Centrin 4 is only expressed in ciliated cells and therefore is likely to have a role in basal body/cilia (Gavet et al., 2003). Centrins 1-3 have either been localised to the centrosome or shown to have roles in centrosome duplication. Human centrins 1 and 2 are at the spindle poles (Errabolu et al., 1994), although centrin 1 may be specific for ciliated or flagellated cell types (Wolfrum and Salisbury, 1998) Centrin 3 bears the most sequence similarity to yeast Cdc31p, and is also most conserved between humans and mouse with 98% identity (Middendorp et al., 1997). Centrins 2 and 3 have a documented role in centrosome duplication (Middendorp et al., 2000; Middendorp et al., 1997; Salisbury et al., 2002).

HsSfilp is at the centrosomes and co-localises with centrin (Kilmartin, 2003) so we can predict that they are acting together at this location. Centrin is seen in fibres connecting centrioles to each other and to other components of the PCM. Injection of human centrins 2 or 3 into Xenopus blastomeres inhibits centrosome duplication (Middendorp et al., 2000; Paoletti et al., 1996). RNAi knock-down of human centrin 2 prevents centriole duplication, but mother and daughter centrioles do separate and form mono-centriolar spindle poles (Salisbury et al., 2002). This suggests the role is not as simple as separating centrioles, but may mediate the step that licenses centrioles to re-duplicate. A lot of work remains to be done on the role of Sfilp/centrin fibres in the centrosome. Whatever the involvement of Sfilp/centrin fibres in duplication, the duplication failure leads eventually to mono-centriolar and acentriolar cells. These may fail to organise proper bipolar spindles, leading to chromosome segregation defects and aneuploidy. Thus understanding centrin/Sfilp fibres may help in understanding the centrosome deregulation that leads to aneuploidy and cancer.
References


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