Regulation of Chromosome Segregation by Shugoshin and Protein Phosphatase 2A in Budding Yeast

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Declaration

I declare that this thesis was composed by myself and the research presented is my own except where otherwise stated. This work has not been submitted for any other degree or professional qualification except as specified.

Dean Clift

2010
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Thanks
Abbreviations

Amp - Ampicillin
APC – Anaphase Promoting Complex
APS – Ammonium Persulfate
ATP – Adenosine triphosphate
bp – Base pairs
BSA – Bovine Serum Albumin
ChIP – Chromatin Immunoprecipitation
DAPI - 4',6-diamidino-2-phenylindol
DMSO – Dimethyl Sulfoxide
DNA – Deoxyribonucleic Acid
dNTPs – Deoxyribonucleotides
DTT - Dithiothreitol
ECL – Enhanced Chemiluminescence
EDTA – Ethylenediaminetetraacetic acid
FAA - 5-Fluoroanthranillic acid
FEAR – Cdc14 Early Anaphase Release
FOA - 5-fluoroorotic acid
FRET – Fluorescence Resonance Energy Transfer
GFP – Green Fluorescent Protein
HA – Hemagglutinin
HU – Hydroxyurea
IF - Immunofluorescence
IP – Immunoprecipitation
kb – kilo base pairs
kDa – kilo Daltons
LB – Luria-Bertani media
MEN – Mitotic Exit Network
OD – Optical Density
PAGE – Polyacrylamide Gel Electrophoresis
PBS – Phosphate Buffered Saline
PCR – Polymerase Chain Reaction
PEG – Polyethylene Glycol
PP2A – Protein Phosphatase 2A
SCF – Skp-Cullin-F-box ubiquitin ligase
SDS – Sodium Dodecyl Sulfate
SPB – Spindle pole body
TCA – Trichloroacetic acid
TEMED - Tetramethylethylenediamine
Abstract

The accurate distribution of genetic information (chromosomes) during cell division is essential for the growth and proliferation of all living organisms. Errors in chromosome segregation in humans have been linked to cancer progression, infertility and developmental diseases. In my PhD I study how chromosome segregation is regulated in the genetically amenable budding yeast *Saccharomyces cerevisiae*. Since the mechanisms of chromosome segregation are highly conserved amongst eukaryotes, studies in yeast will provide a fundamental understanding of this process. Sgo1 is the budding yeast member of a highly conserved family of shugoshin proteins, which play a key role in chromosome segregation. My work characterizes a previously unidentified role of Sgo1 in inhibiting separase; an enzyme that triggers chromosome segregation by cleaving the cohesin protein complex that holds replicated chromosomes together. I demonstrate that this novel function of Sgo1 requires a specific form of Protein Phosphatase 2A (PP2A<sup>Cdc55</sup>), an enzyme that itself is highly conserved amongst eukaryotes. I propose that PP2A<sup>Cdc55</sup> is a separase inhibitor that is employed by Sgo1 when sister chromatids are not under tension. Finally, I go on to initiate preliminary studies into the mechanism whereby PP2A<sup>Cdc55</sup> inhibits separase. In sum, this study uncovers an additional layer of separase regulation mediated by Sgo1 and PP2A<sup>Cdc55</sup> and therefore makes a significant contribution to our understanding of the all-or-nothing nature of chromosome segregation.
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Chapter 1
Introduction
Chapter 1 - Introduction

1.1 The cell cycle

The growth and proliferation of all living organisms depends on cell division. During every cell division, the cell must ensure the accurate duplication of cell contents followed by an equal distribution of those contents into two daughter cells. The regulated sequence of events that leads to cell division is called the cell cycle.

The eukaryotic cell cycle is typically characterised by four phases: G₁, S, G₂ and M (Figure 1.1). During S (synthesis) phase, the DNA is replicated resulting in duplication of the genome. The cell can then enter M (mitosis) phase, during which replicated chromosomes are divided equally between two daughter cells. Most cell cycles contain gap phases between S and M phases. The first gap phase, G₁, occurs before S phase. During G₁, the cell monitors its size and the environment before deciding whether to enter the cell cycle or to enter a prolonged non-dividing phase (G₀), which is the final differentiated state for most cells in multicellular organisms. The second gap phase, G₂, occurs before M phase and allows time for the cell to prepare for the upcoming mitosis (Figure 1.1).

![Diagram of the eukaryotic cell cycle](image)

Figure 1.1 Summary of the eukaryotic cell cycle and the fate of a single chromosome.
A family of enzymes called cyclin-dependent kinases (Cdks) play a central role in regulating progression through the cell cycle. Cdks are activated by binding to regulatory proteins called cyclins and therefore their activity depends on the levels of cyclins available in the cell at any time. Phosphorylation and the binding of Cdk inhibitors also regulate cyclin-Cdk complex activity (Morgan 1997; Murray 2004).

There are three major classes of cyclin-Cdk complexes that control passage through the cell cycle: G₁, S phase and M phase cyclin-Cdk complexes (Figure 1.2). After the previous mitosis and during G₁, overall Cdk activity is low due to the combined action of a ubiquitin ligase that targets cyclins for destruction, the inhibition of cyclin gene transcription and the high concentration of Cdk inhibitors (Sullivan and Morgan 2007). This allows the pre-replicative complex to assemble at replication origins on the DNA, a process that is inhibited by Cdk activity (Diffley 2004). Entry into the cell cycle begins only when the cell has achieved a sufficient size and environmental conditions are favourable. These conditions trigger a signal transduction cascade that leads to the expression of G₁ cyclins. G₁-Cdk activity rises rapidly, and functions mainly to activate S-Cdks by stimulating the transcription of S phase cyclins, targeting S-Cdk inhibitors for destruction and inactivating the ubiquitin ligase that mediates S phase cyclin degradation (Bloom and Cross 2007). Once activated, S-Cdks phosphorylate proteins in the pre-replicative complex. This process initiates DNA replication by promoting the formation of a pre-initiation complex that unwinds the DNA at replication origins and loads the replication machinery. Importantly, because Cdk activity remains high until late mitosis, new pre-replicative complexes cannot be assembled at origins until the next G₁, ensuring that DNA is replicated only once per cell cycle (Diffley 2004).

M phase cyclin-Cdk complexes are synthesised during S phase and G₂, but are kept inactive by inhibitory phosphorylation of the Cdk subunit at one or two sites (depending on the organism) by the Wee1 family of kinases (Nurse 1990). Entry into M phase is triggered by the rapid dephosphorylation of the Cdk subunit by the Cdc25 family of phosphatases, resulting in the activation of M-Cdks, which promote early mitotic events such as chromosome condensation, nuclear envelope breakdown and mitotic spindle assembly. M-Cdks also stimulate the activity of the anaphase-
promoting complex (APC), a ubiquitin ligase that targets key cell cycle regulatory proteins for degradation by the proteosomal machinery. The APC triggers chromosome segregation and, at the same time, initiates the inactivation of cyclin-Cdk complexes by targeting cyclins for destruction (Peters 2006). Further cyclin-Cdk inactivation, which is required for the transition from M phase to the next G1, is achieved with the help of reduced cyclin expression and the accumulation of Cdk inhibitors. Low levels of Cdk activity allow the dephosphorylation of Cdk substrates, which is necessary for spindle disassembly, decondensation of the segregated chromosomes, nuclear envelope reformation and ultimately the generation of two separate daughter cells (Sullivan and Morgan 2007).

![Cell Cycle Control Diagram](image)

Figure 1.2 An overview of cell cycle control by Cdks
1.2 Mitosis

After DNA replication in S phase, replicated chromosomes remain in tightly associated pairs called sister chromatids. This pairing is mediated by a combination of sister DNA catenation and protein complexes called cohesins. M phase, or mitosis, is the period of the cell cycle when sister chromatids are segregated equally between two daughter cells. Cytologically, mitosis can be divided into distinct stages: prophase, prometaphase, metaphase, anaphase and telophase. During prophase, the replicated chromosomes undergo condensation to form compact structures that are more easily moved by the mitotic spindle. In addition, sister chromatid cohesion is partially loosened, at least in higher eukaryotes, by the removal of DNA catenations and the loss of some, but not all, cohesin complexes. Also during prophase, duplicated centrosomes (spindle pole bodies in yeast) separate and migrate to opposite poles of the cell, where they nucleate the microtubules that form the bi-polar mitotic spindle. In higher eukaryotes, the nuclear envelope breaks down at the end of prophase. In yeast, on the other hand, mitosis occurs within an intact nucleus. The plus-ends of spindle microtubules attach to specialised protein complexes assembled at sister centromeres called kinetochores during prometaphase. Accurate chromosome segregation requires that sister kinetochores attach to microtubules emanating from opposite poles, known as bi-polar attachment or bi-orientation. Metaphase is the period during which attached chromosomes are aligned at the centre of the mitotic spindle where a tug-of-war occurs between the pulling forces of microtubules and sister chromatid cohesion. Anaphase is defined by the abrupt destruction of cohesin complexes which allows sister chromatids to be pulled apart to opposite poles of the spindle. Later in anaphase, the spindle poles also move further apart to complete the segregation of sister chromatids to opposite ends of the dividing cell. Mitosis comes to an end in telophase when chromosomes decondense, the mitotic spindle disassembles and segregated chromosomes are separated into two distinct daughter cells by cytokinesis.
1.3 Sister chromatid cohesion

The equal distribution of chromosomes into daughter cells requires that sister chromatids are tightly associated from their synthesis in S phase until their final separation in anaphase. This sister chromatid cohesion facilitates the attachment of sister kinetochores to microtubules emanating from opposite spindle poles (Tanaka et al. 2000; Dewar et al. 2004; Tanaka 2005), thereby ensuring that sister chromatids are segregated away from each other by the mitotic spindle once cohesion is destroyed in anaphase. Defects in sister chromatid cohesion invariably lead to chromosome segregation errors.

Two different mechanisms contribute to sister chromatid cohesion. During DNA replication, sister chromatids become intertwined when two adjacent replication forks meet (Sundin and Varshavsky 1980). The resulting DNA catenations must be resolved by Topoisomerase II (Topo II) for chromosomes to segregate (DiNardo et al. 1984). It was therefore initially proposed that catenations hold sister chromatids together until decatenation by Topo II promotes sister chromatid separation in anaphase (Murray and Szostak 1985). However, catenation cannot be sufficient for sister chromatid cohesion because plasmid DNA within cells arrested in metaphase is not catenated, presumably because Topo II activity removes catenations earlier in the cell cycle (Koshland and Hartwell 1987). This conclusion was supported by the findings from genetic screens that several proteins are required for sister chromatid cohesion, which are not involved in DNA catenation (Guacci et al. 1997; Michaelis et al. 1997). Subsequent studies proved that a number of conserved proteins function to hold sister chromatids together, these proteins are collectively known as cohesins (reviewed in Nasmyth 2005; Nasmyth and Haering 2009).

1.3.1 The cohesin complex

Cohesins form a core complex that consists of four subunits: Smc1, Smc3, Scc1 and Scc3. The cohesin complex is highly conserved amongst all eukaryotes tested to date, although some organisms contain multiple homologues of one or more subunits (reviewed in Nasmyth 2005; Nasmyth and Haering 2009). Furthermore, Rec8 mostly replaces the Scc1 subunit for the meiotic cell divisions, which is critical for
specialized stepwise loss of cohesion that occurs during meiosis (Marston and Amon 2004). A summary of the genes encoding subunits in different eukaryotes in mitosis and meiosis is given in Table 1.1. However, as the structure and function of the cohesin complex has been best characterized in budding yeast (*Saccharomyces cerevisiae*), I will use the budding yeast terminology unless otherwise stated.

Table 1.1 Genes encoding cohesin subunits

<table>
<thead>
<tr>
<th>Saccharomyces cerevisiae</th>
<th>Schizosaccharomyces pombe</th>
<th>Caenorhabditis elegans</th>
<th>Drosophila melanogaster</th>
<th>Xenopus laevis</th>
<th>Homo sapiens</th>
</tr>
</thead>
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<tr>
<td>mitosis</td>
<td>meiosis</td>
<td>mitosis</td>
<td>meiosis</td>
<td>mitosis</td>
<td>meiosis</td>
</tr>
<tr>
<td>SMC1</td>
<td>psm1&lt;sup&gt;+&lt;/sup&gt;</td>
<td>him-1</td>
<td>SMC1</td>
<td>smc1a</td>
<td>smc1b</td>
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<tr>
<td>SMC3</td>
<td>psm3&lt;sup&gt;+&lt;/sup&gt;</td>
<td>smc-3</td>
<td>Cap</td>
<td>smc3</td>
<td>SMC3</td>
</tr>
<tr>
<td>SCC1</td>
<td>REC8</td>
<td>rad21&lt;sup&gt;+&lt;/sup&gt;</td>
<td>rec8&lt;sup&gt;+&lt;/sup&gt;</td>
<td>rad21</td>
<td>RAD21</td>
</tr>
<tr>
<td>SCC3</td>
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<td>rec11&lt;sup&gt;+&lt;/sup&gt;</td>
<td>scc-3</td>
<td>SA</td>
<td>stag1</td>
</tr>
<tr>
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<td></td>
<td>STAG3</td>
<td></td>
</tr>
</tbody>
</table>

Nomenclature according to *Saccharomyces* Genome Database (www.yeastgenome.org), *S. pombe* GeneDB (www.genedb.org), WormBase (www.wormbase.org), FlyBase (www.flybase.org), XenBase (www.xenbase.org) and HGNC (www.genenames.org).

Two of the core cohesin subunits, Smc1 and Smc3, are members of the structural maintenance of chromosomes (SMC) family. Members of this family are also found in prokaryotes and have evolutionary conserved roles in chromosome structure and dynamics. Other SMC family members include Smc2 and Smc4, which are subunits of the condensin complex that functions in chromosome condensation. An Smc5-Smc6 complex also plays a role in DNA damage repair (for reviews on SMC proteins see Losada and Hirano 2005; Hirano 2006). All SMC proteins, including Smc1 and Smc3 of the cohesin complex, are composed of a long antiparallel coiled-coil flanked by globular hinge domain at one end and an ATPase head domain at the
other end (Melby et al. 1998; Haering et al. 2002; Hirano and Hirano 2002; Hirano and Hirano 2006). The hinge domains of Smc1 and Smc3 interact, forming a V-shaped Smc1/Smc3 heterodimer (Figure 1.3; Haering et al. 2002). Scc1 is a member of the Kleisin protein family, which function to bridge the ATPase heads in different SMC complexes (Schleiffer et al. 2003). The N-terminus of Scc1 (and Rec8) binds to the Smc3 head domain, and the C-terminus binds to the Smc1 head, forming a ~50nm tripartite ring-like structure (Figure 1.3; Haering et al. 2002; Gruber et al. 2003; Haering et al. 2004). The existence of such a ring-like formation is confirmed by electron microscopy of purified Xenopus cohesin complexes (Anderson et al. 2002). The structure of the fourth subunit of the cohesin complex, Scc3, has yet to be determined, however, its interaction with the cohesin ring is mediated by Scc1 (Figure 1.3; Haering et al. 2002). Two Scc3 homologues exist in vertebrate somatic cells (STAG1 and STAG2), and cohesin complexes in these cells contain either STAG1 or STAG2, but never both (Losada et al. 2000; Sumara et al. 2000). Exactly how the cohesin complex holds sister chromatids together is not completely understood. The fact that cohesins form a ring-shaped structure led to the proposal that the cohesin complex embraces sister DNA molecules in this ring (Haering et al. 2002; Gruber et al. 2003). However, variations on the ring model and alternative models have also been proposed (Huang et al. 2005; Nasmyth 2005).

Figure 1.3 Structure of SMC heterodimers and the cohesin complex (Xiong and Gerton 2010)
1.3.2 Establishment of sister chromatid cohesion

Cohesin associates with DNA during G1 phase in budding yeast (Guacci et al. 1997; Michaelis et al. 1997), whilst vertebrate cohesin is loaded onto DNA as early as telophase of the previous mitosis (Gerlich et al. 2006) (Figure 1.4). In all organisms studied so far, the association of cohesin with chromatin depends on a conserved Scc2/Scc4 complex (Ciosk et al. 2000; Gillespie and Hirano 2004; Takahashi et al. 2004; Bernard et al. 2006; Seitan et al. 2006; Watrin et al. 2006). However, cohesin association with chromosomes likely involves additional factors such as heterochromatin, kinetochores, pre-replicative complexes, the DNA replication machinery and tRNA transcription apparatus (reviewed in Nasmyth and Haering 2009). The precise mechanisms by which the cohesin ring is loaded onto DNA are not known, and it may also involve ATP hydrolysis by the Smc1 and Smc3 ATPase head domains (Arumugam et al. 2003; Weitzer et al. 2003), opening of the ring via the SMC hinge domain (Gruber et al. 2006), and the binding of this hinge domain to DNA (Hirano and Hirano 2006). Once bound to chromatin, the sister chromatid cohesion function of cohesins can only be established during DNA replication as cohesin expressed after S phase, although capable of associating with chromatin, cannot function to hold sister chromatids together (Uhlmann and Nasmyth 1998). An exception to this rule is cohesin that is loaded onto chromosomes post-replication in response to DNA double strand breaks (Strom et al. 2007; Unal et al. 2007). In both cases, the establishment of sister chromatid cohesion (but not cohesin loading onto DNA) requires activity of an acetyl-transferase known as Eco1 (Skibbens et al. 1999; Toth et al. 1999), which promotes cohesion establishment by acetyllating Smc3, in yeast and in humans (Rolef Ben-Shahar et al. 2008; Unal et al. 2008; Zhang et al. 2008; Rowland et al. 2009).
1.4 Sister chromatid separation

Sister chromatid separation in anaphase requires the complete loss of sister chromatid cohesion (Figure 1.5). This process must be tightly regulated, as loss of sister chromatid cohesion before chromosomes achieve bi-orientation on the mitotic spindle would lead to chromosome missegregation and aneuploidy. Early in the cell cycle, cohesion mediated by DNA catenations are removed by Topo II to prepare sister chromatids for separation (DiNardo et al. 1984). In vertebrate cells, the bulk of cohesin is removed from chromosome arms in prophase, whereas cohesin remains bound to centromeres until the onset of anaphase (Sumara et al. 2000; Waizenegger et al. 2000). Centromeres are the site where sister chromatids attach to spindle microtubules via their kinetochores, therefore centromeric cohesins play a key role in holding sister chromatids together and ensuring bi-orientation (Tanaka et al. 2000; Eckert et al. 2007). Budding yeast cells lack the prophase pathway of cohesin removal, nevertheless, cohesin is enriched in the vicinity of the centromeres in these cells (Weber et al. 2004; Kiburz et al. 2005) and reducing the level of cohesin in this region causes chromosome segregation errors (Eckert et al. 2007; Fernius and Marston 2009; Ng et al. 2009). At the onset of anaphase in all eukaryotes, sister chromatid separation requires the cleavage of the cohesin ring to destroy all remaining sister chromatid cohesion (Nasmyth and Haering 2009).
1.4.1 Prophase pathway of cohesin removal

Unlike cohesin removal at the onset of anaphase (see section 1.4.3), the prophase pathway does not involve the APC or cohesin cleavage (Sumara et al. 2000; Waizenegger et al. 2000). Instead, this process is facilitated by the Aurora B and polo-like (Plk) kinases, condensin I, and a cohesin-associated protein known as Wapl (Losada et al. 2002; Sumara et al. 2002; Gimenez-Abian et al. 2004; Hirota et al. 2004; Gandhi et al. 2006; Kueng et al. 2006; Lenart et al. 2007). Plk1 contributes to cohesin dissociation by phosphorylating the C-terminal domain of the Scc3 (STAG1/STAG2) subunit of cohesin (Hauf et al. 2005). Aurora B does not phosphorylate cohesin subunits and may promote cohesin dissociation indirectly (Losada et al. 2002; Gimenez-Abian et al. 2004), possibly by regulating condensin I binding to chromatin (Lipp et al. 2007). Wapl appears to be more crucial for removing cohesin in prophase as depletion of Wapl causes a much more dramatic reduction in cohesin dissociation than inactivation of Plk, Aurora B or condensin I (Gandhi et al. 2006; Kueng et al. 2006). However, how all these proteins cooperate to remove cohesin from the chromatin in prophase is not known. Similarly, the physiological significance of the prophase pathway of cohesin removal is unclear. In its absence (due to Wapl depletion), sister chromatid separation still proceeds at the onset of anaphase (Kueng et al. 2006), suggesting that the prophase pathway of cohesin removal may not be essential for chromosome segregation. Indeed, the prophase pathway is absent during yeast mitosis, where most, if not all, cohesin remains associated with chromatin until its cleavage at the onset of anaphase (Michaelis et al. 1997; Uhlmann et al. 1999).

1.4.2 Transient separation of centromeres before anaphase

During metaphase, sister chromatids attach (via their kinetochores) to microtubules emanating from opposite poles of the spindle in a process known as bi-orientation. Bi-orientation requires that sister chromatids are linked by cohesin, which provides resistance to the pulling force of the bipolar spindle to generate tension across attached kinetochores (Tanaka et al. 2000). As discussed in section 1.7.2, tension at kinetochores is required for anaphase to proceed. From yeast to humans, tension
across bi-oriented kinetochores is apparent as a visible increase in distance between kinetochores, or sister centromere sequences, prior to anaphase (Shelby et al. 1996; Waters et al. 1996; Nabeshima et al. 1998; Goshima and Yanagida 2000; He et al. 2000; Tanaka et al. 2000). This pre-anaphase centromere separation is often followed by re-association of sister centromeres, and therefore is a transient process sometimes referred to as ‘centromere breathing’. Centromere breathing seems to be odds with the fact that cohesin is enriched in the vicinity of the centromere (Waizenegger et al. 2000; Bernard et al. 2001; Weber et al. 2004; Kiburz et al. 2005), however, recent work in budding yeast demonstrated that centromeric cohesins dissociate from chromatin during centromere separation, and then re-accumulate on centromeric chromatin when centromeres re-associate (Eckert et al. 2007; Ocampo-Hafalla et al. 2007). Cohesin dissociation during centromere breathing does not involve cohesin cleavage, as centromere breathing still occurs in a temperature-sensitive separase mutant (He et al. 2000). Both the re-association of centromeres and the re-accumulation of centromeric cohesins during centromere breathing partially depends on the cohesion establishment factor Eco1, suggesting that the re-association of centromeres might be facilitated by the establishment of sister chromatid cohesion (Ocampo-Hafalla et al. 2007).

1.4.3 Cohesin cleavage by separase triggers chromosome segregation in anaphase

Chromosome segregation in anaphase requires the abrupt destruction of all sister chromatid cohesion (Figure 1.5). Seminal experiments in budding yeast showed that the loss of sister chromatid cohesion in anaphase is triggered by the cleavage of cohesins Scc1 subunit by an enzyme known as separase. By monitoring the dissociation of Scc1 from chromatin in vitro, Uhlmann et al (1999) made the key observation that Scc1 induced to dissociate from chromatin appeared in the supernatant as a cleaved product. Two cleavage sites were subsequently mapped in Scc1, and mutations at both these sites together completely blocked Scc1 cleavage, cohesin dissociation from chromatin and sister chromatid separation in anaphase (Uhlmann et al. 1999). Scc1 cleavage required the protease activity of separase, and either ectopic separase activation in metaphase, or artificial cleavage of Scc1 in
metaphase, was sufficient to trigger sister chromatid separation and anaphase onset (Uhlmann et al. 2000). Therefore, cohesin cleavage by separase is both necessary and sufficient for sister chromatid separation in budding yeast. Similarly, defects in chromosome segregation are caused by non-cleavable Scc1 expression or separase inactivation in fission yeast, *Drosophila* and mammalian cells (Tomonaga et al. 2000; Hauf et al. 2001; Jager et al. 2001; Kumada et al. 2006; Wirth et al. 2006). The recent finding that, like in budding yeast, artificial cleavage of Scc1 in metaphase-arrested *Drosophila* embryonic cells is sufficient for sister chromatid separation (Oliveira et al. 2010), suggests that cohesin cleavage by separase might be a universal trigger for chromosome segregation in anaphase.

![Diagram of chromosome segregation](image)

**Figure 1.5** Loss of sister chromatid cohesion during mitosis
1.5 The regulation of separase

The decision to separate sister chromatids in anaphase is irreversible; once sister chromatid cohesion is destroyed, it cannot be established again until the next round of DNA replication (Uhlmann and Nasmyth 1998). Consequently, the cell must ensure that cohesin cleavage by separase occurs at the proper time during the cell cycle. Separase activation before all sister chromatids have achieved bi-orientation on the mitotic spindle would lead to chromosome missegregation.

Before anaphase, separase is inhibited through its association with an inhibitory chaperone known as securin (Ciosk et al. 1998; Hornig et al. 2002; Waizenegger et al. 2002). Anaphase is initiated by the degradation of securin, which is mediated by the anaphase-promoting complex (APC) bound to its activator Cdc20. Securin degradation is necessary for separase activation, as expression of a non-degradable securin mutant, or inactivation of the APC$_{Cdc20}$, effectively prevents sister chromatid separation (Cohen-Fix et al. 1996; Funabiki et al. 1996a; Zou et al. 1999; Hagting et al. 2002). In budding yeast, deletion of securin allows sister chromatid separation when APC$_{Cdc20}$ is inactive, suggesting that securin is the only substrate of the APC$_{Cdc20}$ whose destruction is essential for anaphase in this organism (Yamamoto et al. 1996b).

1.5.1 Separase regulation by securin

Separases are found in all eukaryotes (Table 1.2), however only the C-terminal separase domain shows any sequence conservation. Sequence alignments revealed that the conserved C-terminus of separase contains a caspase-like protease domain (Uhlmann et al. 2000). The catalytic activity of this domain is essential for cohesin cleavage (Uhlmann et al. 2000). The bulk of the separase protein, however, consists of a large N-terminus, which shows very little sequence similarities between species. This N-terminal domain is predicted to adopt an alpha-alpha superhelical structure similar to ARM (Armidillo) repeat-containing proteins (Jager et al. 2004) or TPR (tetratricopeptide) repeat proteins (Katis et al. 2010). An unstructured region separates the N- and C-terminal domains (Viadiu et al. 2005). In Drosophila, the N-
and C-terminal domains of separase are found in separate proteins, THR and SSE1 (Jager et al. 2001; Jager et al. 2004). The N- and C-termini of separase (and also THR and SSE1 in *Drosophila*) interact, and this interaction appears to be important for the proteolytic activity of separase (Jager et al. 2001; Hornig et al. 2002; Jager et al. 2004). In fact, random mutagenesis of budding yeast separase, Esp1, revealed that the entire separase protein participates in its proteolytic function (Baskerville et al. 2008).

Table 1.2 Genes encoding separase and securin proteins

<table>
<thead>
<tr>
<th>Generic name</th>
<th><em>Saccharomyces cerevisiae</em></th>
<th><em>Schizosaccharomyces pombe</em></th>
<th><em>Caenorhabditis elegans</em></th>
<th><em>Drosophila melanogaster</em></th>
<th><em>Xenopus laevis</em></th>
<th><em>Homo sapiens</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Separase</td>
<td>ESP1</td>
<td>cut1’</td>
<td>sep-1</td>
<td>Sse1</td>
<td>espl1</td>
<td>ESPL1</td>
</tr>
<tr>
<td>Securin</td>
<td>PDS1</td>
<td>cut2’</td>
<td>ify-1</td>
<td>pim</td>
<td>securin</td>
<td>PTTG1</td>
</tr>
</tbody>
</table>

Nomenclature according to *Saccharomyces* Genome Database (www.yeastgenome.org), *S.pombe* GeneDB (www.genedb.org), WormBase (www.wormbase.org), FlyBase (www.flybase.org), XenBase (www.xenbase.org) and HGNC (www.genenames.org).

Securins are functionally conserved as separase inhibitors in all eukaryotes (Table 1.2) yet their primary sequences are extremely divergent in different organisms. Biochemical studies with yeast and human proteins found that the binding of securin to separase prevents the access of substrates to the active site of the separase protease domain (Hornig et al. 2002; Waizenegger et al. 2002). In budding yeast, securin interacts with both the C- and N-terminal regions of separase and disrupts the intramolecular interaction between these two regions (Hornig et al. 2002). Similarly, the *Drosophila* securin, PIM, binds to both SSE1 and THR, and an interaction between SSE1 and THR only occurs in the absence of PIM (Jager et al. 2001). Securin may therefore inhibit separase by a conserved mechanism that blocks access to the active site and involves the disruption of intramolecular interactions within separase that are important for its catalytic activity (Jager et al. 2001; Hornig et al. 2002; Jager et al. 2004).
The inhibitory interaction of securin with separase may also be stimulated by phosphorylation of securin by Cdk1, at least in budding yeast (Agarwal and Cohen-Fix 2002).

Paradoxically, securin also has a positive role in regulating separase activity. Mutations in the Drosophila securin are lethal, not due to premature separase activation, but due to a block to sister chromatid separation, reminiscent of a separase mutant (Stratmann and Lehner 1996; Leismann et al. 2000). Similarly, fission yeast cells lacking securin (Cut2) are inviable due to an inability to separate sister chromatids (Funabiki et al. 1996b), this may be because Cut2 is required for the nuclear localisation of the fission yeast separase, Cut1 (Kumada et al. 1998). Conversely, securin-deficient budding yeast, mammalian cells and mice are viable and able to separate sister chromatids (Yamamoto et al. 1996a; Jallepalli et al. 2001; Wang et al. 2001), however separase activity is also reduced in these organisms (Jallepalli et al. 2001; Hornig et al. 2002; Wirth et al. 2006). Human securin+/− cells have a reduction in the overall amount of separase (Jallepalli et al. 2001), whilst budding yeast cells lacking securin are defective in the nuclear localization of separase and also show slightly reduced separase activity in vitro (Jensen et al. 2001; Hornig et al. 2002). Securin therefore contributes to separase activation by regulating the stability of separase protein, its localization, and proteolytic activity. In vitro studies of budding yeast proteins suggest that securin may facilitate the active folding of the separase protein. By bridging the N- and C-termini of separase, securin may bring these domains into close proximity, preparing them for interaction and activation of separase upon securin degradation (Figure 1.6; Hornig et al. 2002).

![Figure 1.6 The regulation of separase by securin (adapted from Hornig et al. 2002)](image-url)
1.5.2 Additional mechanisms of separase regulation

Although securin destruction is necessary for anaphase, real-time measurements in human cells revealed that there is a delay between securin degradation and sister chromatid separation (Hagting et al. 2002), suggesting that securin degradation might not be sufficient to initiate separase-dependent cohesin cleavage. This idea is supported by the findings that, in many eukaryotes, securin is not essential for viability. Accordingly, securin-deficient mice are apparently normal and mammalian securin−/− cells undergo a largely normal anaphase (Jallepalli et al. 2001; Mei et al. 2001; Wang et al. 2001; Pfleghaar et al. 2005; Wirth et al. 2006). Furthermore, budding yeast cells lacking securin are viable (Yamamoto et al. 1996a) and initiate cohesin cleavage and sister chromatid separation with similar timing to wild type cells during an unperturbed mitosis (Alexandru et al. 1999; Alexandru et al. 2001). Therefore, it is likely that securin-independent mechanisms exist to prevent cohesin cleavage before anaphase.

1.5.2.1 Regulation of cohesin cleavage by substrate modification

Cohesin cleavage by separase can also be regulated at the level of the substrate. In budding yeast, Scc1 is phosphorylated prior to anaphase by the polo-like kinase Cdc5 and the phosphorylation of Scc1 by recombinant human polo-like kinase 1 (Plk1) enhances its rate of cleavage by separase in vitro (Alexandru et al. 2001). Similarly, human Scc1 is phosphorylated in mitosis, and the in vitro cleavage of human Scc1 is enhanced by the presence of Plk1 (Hauf et al. 2005). However, Scc1 phosphorylation appears to play only a minor role in regulating cohesin cleavage in vivo. Budding yeast cells expressing a non-phosphorylatable Scc1, or cells depleted of Cdc5, show only a slight defect in defect in sister chromatid separation and are still able to cleave cohesin (Alexandru et al. 2001; Hornig and Uhlmann 2004). Also human cells expressing a non-phosphorylatable Scc1 have no abnormalities in mitosis or overall proliferation (Hauf et al. 2005). Therefore, cohesin cleavage by separase is enhanced by, but not dependent on, phosphorylation of Scc1 by Cdc5/Plk1. However, there may be additional phosphorylation sites on Scc1 that are important for cohesin
cleavage that have yet to be identified. During meiosis, the Scc1 subunit of cohesin is replaced by Rec8 (Table 1.1) and, at least in yeast, Rec8 phosphorylation is essential for cohesin cleavage by separase in meiosis I (Brar et al. 2006; Ishiguro et al. 2010; Katis et al. 2010). The control of cohesin cleavage during meiosis is discussed later in section 1.8. Additionally, human separase depends on the presence of chromosomal DNA to cleave cohesin in vitro (Sun et al. 2009), whereas yeast separase (Esp1) can cleave cohesin in the absence of chromatin (Hornig and Uhlmann 2004).

1.5.2.2 Multiple modes of separase inhibition

By re-examining the effect of CDK activity on sister chromatid separation in *Xenopus* egg extracts, Stemmann et al (2001) found that high levels of the mitotic cyclin B1 caused a block to sister chromatid separation, even when securin was degraded. This phenotype is rescued by inhibition of Cdk1, and also by mutating a single phosphorylation site at serine 1126 of separase, suggesting that Cdk1-mediated phosphorylation of serine 1126 is able to inhibit separase. Subsequent experiments found that serine 1126 phosphorylation is necessary for the binding of the Cdk1-cyclin B1 complex to separase, which is sufficient for separase inhibition (Gorr et al. 2005). This mode of separase inhibition is likely to occur in vivo, as separase-Cdk1-cyclin B1 complexes are found in metaphase arrested human cells (Gorr et al. 2005; Holland and Taylor 2006) and expression of a non-degradable cyclin B1 mutant in human cells also prevents sister chromatid separation in the absence of securin (Hagting et al. 2002; Chang et al. 2003; Wolf et al. 2006). Furthermore, separase phosphorylation at an analogous serine residue (serine 1121) appears to be particularly important in regulating separase activity in mouse embryonic germ cell development (Huang et al. 2008; Huang et al. 2009). Securin and cyclin B1 interact with separase in a mutually exclusive manner, suggesting that they may be redundant separase inhibitors (Gorr et al. 2005; Holland and Taylor 2006). Cyclin B1 remains bound to, and inhibits, separase that has been dephosphorylated at serine 1126, therefore it is unlikely that separase dephosphorylation activates separase (Holland and Taylor 2006). As both securin
and cyclin B1 are substrates of APC^{Cdc20}, anaphase onset in vertebrates might be triggered by the simultaneous degradation of two separase inhibitors. However, there is still a delay between APC^{Cdc20} activation and sister chromatid separation in human cells (Hagting et al. 2002), suggesting that additional mechanisms may cooperate to regulate separase-dependent cohesin cleavage. This idea is supported by the findings that in mitotically-arrested human cells depleted for the kinetochore protein Astrin, securin and cyclin B1 proteins are stable, yet sister chromatids separate in a separase dependent manner (Thein et al. 2007).

The role of Cdk1-mediated phosphorylation of separase in lower eukaryotes is less clear. The fact that budding yeast cells are able to initiate sister chromatid separation in the presence of high Cdk1 activity (Surana et al. 1993) suggests that Cdk1-cyclin may not inhibit separase in this organism. However, other mechanisms may still play a role in separase regulation in budding yeast, as cells lacking the S phase cyclins Clb5 and Clb6 are able to enter anaphase in the presence of non-degradable securin (Meyn and Holloway 2000).

1.6 Cohesin cleavage-independent roles of separase

At the onset of anaphase in higher eukaryotes, separase, in addition to cleaving Scc1, also cleaves itself at three sites just upstream of the C-terminal domain (Waizenegger et al. 2002; Zou et al. 2002). The separase cleavage products remain associated and are still capable of cleaving cohesin in vitro, and a non-cleavable separase has no proteolytic defect (Waizenegger et al. 2002; Zou et al. 2002). The C-terminal part of cleaved separase, however, is unstable and this instability may facilitate the downregulation of separase after anaphase (Waizenegger et al. 2002; Zou et al. 2002). Indeed, Drosophila THR is cleaved by SSE1, which contributes to the inactivation of the SSE1 proteolytic activity (Herzig et al. 2002). In human cells, separase auto-cleavage in anaphase appears to promote entry into mitosis during the next cell cycle by a poorly understood mechanism (Papi et al. 2005). There is no evidence of separase auto-cleavage in budding yeast (Hornig et al. 2002).
In addition to triggering sister chromatid separation, separase has an additional role in regulating centriole separation in human cells (Tsou and Stearns 2006; Tsou et al. 2009). Human cells depleted of separase are defective in the disengagement of centrioles during mitotic exit (Tsou et al. 2009). Centriole disengagement requires the proteolytic activity of separase, but not Scc1 cleavage, suggesting that separase cleaves other substrates in this role (Tsou et al. 2009). By promoting centriole separation, separase functions to re-license centriole duplication in the next cell cycle (Tsou et al. 2009).

The proteolytic activity of separase is not only restricted to Scc1 in budding yeast. At the onset of anaphase in this organism, separase also cleaves a kinetochore protein called Slk19 (Sullivan et al. 2001). Slk19 cleavage is not necessary for anaphase onset, as expression of a non-cleavable Slk19 mutant has little affect on the cell cycle (Sullivan et al. 2001). However, the stable C-terminal cleavage product of Slk19 localizes to the spindle midzone in anaphase where it plays a role in maintaining spindle integrity (Sullivan et al. 2001). Separase also localizes to the spindle midzone (Jensen et al. 2001), and the spindle midzone localization of separase and Slk19 are dependent on each other (Sullivan et al. 2001; Khmelinskii et al. 2007). However, the precise function of separase at the spindle midzone is unclear (Khmelinskii and Schiebel 2008). The spindle localization of separase is also observed in fission yeast (Kumada et al. 1998) and C.elegans (Bembenek et al. 2007; Bembenek et al. 2010) so its function at the spindle may be conserved.
1.6.1 Separase initiates mitotic exit in budding yeast

Mitotic exit involves a coordinated sequence of events that follow sister chromatid separation and culminate in the completion of cell division by cytokinesis. Following sister chromatid separation, the transition from the mitotic state to G1 phase of the cell cycle requires the inactivation of Cdks and the dephosphorylation of mitotic Cdk substrates. Indeed, persistent Cdk activity causes a cell cycle arrest in telophase with an elongated mitotic spindle and a divided nucleus (Surana et al. 1993). Cdk inactivation begins at the onset of anaphase, when the APC$^{Cdc20}$ targets S phase cyclins Clb5 and Clb6 for destruction (Shirayama et al. 1999) and triggers the destruction of some, but not all, of the major mitotic cyclin Clb2 (Yeong et al. 2000). However, mitotic exit requires the complete inactivation of Cdks and dephosphorylation of Cdk substrates, all of which is controlled by Cdc14, an essential phosphatase (Stegmeier and Amon 2004). Cdc14 drives Cdk inactivation by two mechanisms. Firstly, Cdc14 dephosphorylates the APC activator protein Cdh1, thereby activating APC$^{Cdh1}$, which completes the degradation of Clb2 that was initiated by APC$^{Cdc20}$ (Visintin et al. 1997; Visintin et al. 1998; Yeong et al. 2000). Secondly, Cdc14 dephosphorylates the Cdk inhibitor Sic1 (Visintin et al. 1998), resulting in the stabilization of Sic1 whose phosphorylated form is targeted for destruction by the constitutively active SCF (Skp1-cullin-F box) ubiquitin ligase (Feldman et al. 1997). Dephosphorylation of the transcription factor Swi5 by Cdc14 also contributes to Sic1 accumulation due to activation of $SIC1$ transcription (Visintin et al. 1998). Therefore, the activation of Cdc14 is essential for exit from mitosis.

Cdc14 activation is controlled by its localization. Before anaphase, Cdc14 is sequestered in the nucleolus by its association with its nucleolar-localized inhibitor Net1 (Shou et al. 1999; Visintin et al. 1999). Cdc14 is activated in anaphase by its dissociation from Net1 and subsequent release from the nucleolus into the nucleus and cytoplasm where it can dephosphorylate its substrates (Shou et al. 1999; Visintin et al. 1999). The activation and release of Cdc14 depends on two regulatory pathways. First, at the onset of anaphase, the FEAR (cdc fourteen early anaphase release) network initiates the transient Cdc14 dissociation from Net1 and release
from the nucleolus (Stegmeier et al. 2002). Second, during the later stages of anaphase, the MEN (mitotic exit network) maintains Cdc14 in its released state (Stegmeier et al. 2002). How the FEAR and MEN proteins cooperate to regulate Cdc14 release is not understood, and has been the subject of several reviews (Dumitrescu and Saunders 2002; D'Amours and Amon 2004; Stegmeier and Amon 2004; Bosl and Li 2005).

Separase was found to be part of the FEAR network based on the inability of a separase temperature sensitive mutant (esp1-1) to transiently release Cdc14 in the absence of MEN (Stegmeier et al. 2002). Subsequently it was shown that complete inactivation of separase, by a more stringent allele (esp1-2^td) or by overexpression of non-degradable securin, causes a block to Cdc14 release and cells arrest with high Cdk activity (Queralt et al. 2006). These results suggest that Esp1 is essential for Cdc14 release. However, under conditions where spindle elongation is forced by artificial cleavage of cohesin, Esp1 activity may not be required for mitotic exit (Lu and Cross 2009). Nevertheless, the ectopic activation of separase in metaphase-arrested cells is sufficient to induce Cdc14 release and interestingly, this function of separase does not require its protease activity (Sullivan and Uhlmann 2003). Therefore, separase activation at the onset of anaphase triggers cohesin cleavage through its proteolytic activity and Cdc14 release through a non-proteolytic mechanism. The key role of separase in Cdc14 release might ensure that mitotic exit does not occur prior to sister chromatid separation (Figure 1.7).

Separase appears to trigger Cdc14 release by promoting Net1 phosphorylation (Queralt et al. 2006), which reduces the affinity of Net1 for Cdc14 in vitro (Shou et al. 2002; Yoshida and Toh-e 2002). During metaphase, the inhibitory interaction between Net1 and Cdc14 is maintained by dephosphorylation of Net1 by protein phosphatase 2A (PP2A) bound to its Cdc55 regulatory subunit (Queralt et al. 2006). At the onset of anaphase, PP2A^{Cdc55} phosphatase activity is reduced in a separase-dependent manner, allowing Net1 phosphorylation and subsequent Cdc14 release (Queralt et al. 2006). Separase may also stimulate Cdc14 release by modulating the activity of the kinases responsible for Net1 phosphorylation (Visintin et al. 2003; Visintin et al. 2008). The mechanisms by which the non-proteolytic function of
separase promotes PP2A$^{Cdc55}$ downregulation and Cdc14 release are not completely understood. However, the alpha-alpha superhelical structure of the N-terminus of separase is reminiscent of proteins that assume scaffold or adaptor roles (Jager et al. 2004), therefore the non-proteolytic function of separase may involve multiple protein-protein interactions. In support of this view, fission yeast separase is known to exist as part of a large protein complex (Funabiki et al. 1996) and separase-dependent Cdc14 release involves the cooperation of Zds1 and Zds2, two proteins that interact with separase and may function as general PP2A regulators (Queralt and Uhlmann 2008).

![Diagram](image)

Figure 1.7 Separase couples sister chromatid separation with mitotic exit in budding yeast
1.7 Regulation of the metaphase to anaphase transition

The metaphase to anaphase transition is defined by the initiation of sister chromatid separation, which is triggered by the cleavage of cohesin linkages by separase. Separase activation at the onset of anaphase requires the destruction of securin (and cyclin B in vertebrates) to release an active separase enzyme. Securin destruction depends on its ubiquitination by the APC in association with its activating subunit Cdc20. Therefore, APC$^{Cdc20}$ activation and securin degradation play a central role in regulating the metaphase to anaphase transition (Figure 1.8).

1.7.1 The anaphase promoting complex

The APC is a highly conserved multisubunit E3 ubiquitin ligase that functions by assembling polyubiquitin chains on substrate proteins, which then act as a degradation signal for the 26S proteosome (reviewed in Peters 2006). The APC was first described as a 20S complex essential for the destruction of mitotic cyclins in yeast and vertebrates (Irniger et al. 1995; King et al. 1995; Sudakin et al. 1995; Tugendreich et al. 1995), which is necessary for exit from mitosis (Shirayama et al. 1999; Wasch and Cross 2002). The APC gains its name from a second essential function in targeting securin for destruction and thereby triggering anaphase onset (Cohen-Fix et al. 1996; Shirayama et al. 1999). Many other substrates are targeted for destruction by the APC (Reed 2003), however in budding yeast the only essential function of the APC appears to be the degradation of securin and mitotic cyclins, as cells lacking both securin (Pds1) and mitotic cyclins (Clb5) can proliferate when the APC is inactive (Thornton and Toczyski 2003).

The substrates of the APC contain amino-acid sequence motifs that are required for their ubiquitination. The two best described motifs are the destruction box (D-box), which contains the sequence R-X-X-L-X-X-X-N (Glotzer et al. 1991) and the KEN box (K-E-N-X-X-X-E/D/N), where X is any amino acid (Pfleger and Kirschner 2000). Mutations in these motifs generally prevent the ubiquitination and destruction of the protein in vivo; for example, deletion or mutation of the conserved D-box motif in the securin protein inhibits its degradation, causing cells to arrest in
metaphase (Cohen-Fix et al. 1996; Funabiki et al. 1996a; Zou et al. 1999; Hagting et al. 2002).

Ubiquitination of substrates by the APC is controlled by activator subunits that bind the APC at different stages of the cell cycle. Two activators, Cdc20 and Cdh1, control APC activity throughout the vegetative cell cycle (Visintin et al. 1997). A third meiosis-specific activator of the APC, called Ama1, is also found in budding yeast (Cooper et al. 2000). The APC activators contain WD40 repeats situated towards the C-terminus, which likely play a role in substrate binding (Kraft et al. 2005). These proteins also carry an N-terminal C-box motif and a C-terminal IR motif, both required for binding to the core APC (Schwab et al. 2001; Passmore et al. 2003; Vodermaier et al. 2003).

As the cell reaches metaphase, increasing Cdk activity promotes the binding of Cdc20 to the APC by phosphorylating several core APC subunits (Rudner and Murray 2000; Kraft et al. 2003). By contrast, the interaction of Cdh1 with the APC is prevented at this stage of the cell cycle due to inhibitory Cdh1 phosphorylation by mitotic Cdns (Zachariae et al. 1998; Jaspersen et al. 1999; Lukas et al. 1999; Kramer et al. 2000). At the onset of anaphase, APC\textsuperscript{Cdc20} specifically targets securin and most mitotic cyclins for destruction, thereby triggering sister chromatid separation and initiating Cdk inactivation (Visintin et al. 1997; Shirayama et al. 1999). The resulting decrease in Cdk activity allows the formation of APC\textsuperscript{Cdh1} in anaphase, which completes the degradation of mitotic cyclins and other substrates not recognised by APC\textsuperscript{Cdc20} to promote mitotic exit (Visintin et al. 1997; Visintin et al. 1998; Yeong et al. 2000). The APC\textsuperscript{Cdc20} is also inactivated at this stage, presumably because APC dephosphorylation leads to the dissociation of Cdc20 from the APC and also because the APC\textsuperscript{Cdh1} targets Cdc20 for degradation (Prinz et al. 1998; Shirayama et al. 1998).
1.7.2 Cell cycle checkpoints inhibit the metaphase to anaphase transition

The concept of cell cycle checkpoints, as control mechanisms that ensure entry into the next stage of the cell cycle is dependent on completion of earlier events, was initially introduced in 1989 by Leland Hartwell and Ted Weinert (Hartwell and Weinert 1989). Two evolutionary conserved checkpoints, the DNA damage checkpoint and the spindle checkpoint, regulate the metaphase to anaphase transition (Figure 1.8). Cells respond to DNA damage, or defects in mitotic spindle attachment to chromosomes, by arresting the cell cycle to allow sufficient time for repairs before the cell cycle can resume. These checkpoints are critical for the accuracy of cell division. Sister chromatid separation before DNA damage can be repaired may lead to the segregation of fragmented chromosomes. Similarly, the spindle checkpoint prevents sister chromatid separation until all chromosomes have attached to the mitotic spindle in a bipolar manner, and therefore ensures the equal distribution of chromosomes between daughter cells.

![Figure 1.8 Regulation of the metaphase to anaphase transition in budding yeast](image-url)
In fission yeast and mammalian cells, DNA damage induces a cell cycle arrest at the G2/M transition due to inhibition of the Cdk1 (Jin et al. 1996; Blasina et al. 1997; Rhind et al. 1997), thus preventing the onset of events leading to chromosome segregation. However, budding yeast cells arrest at the metaphase to anaphase transition in response to DNA damage. In these cells, DNA damage triggers a signal transduction cascade leading to the phosphorylation of securin by the checkpoint kinase Chk1/ATR (Cohen-Fix and Koshland 1997). Chk1-phosphorylated securin is resistant to APC\textsuperscript{Cdc20}-dependent ubiquitination \textit{in vitro} (Agarwal et al. 2003), and mutation of the Chk1 phosphorylation sites in securin impairs the DNA damage checkpoint response (Wang et al. 2001). The interaction between securin and Cdc20 is also prevented in response to DNA damage (Agarwal et al. 2003). Therefore, the DNA damage checkpoint stabilizes securin to prevent inappropriate sister chromatid separation until chromosomal repairs can be made.

The spindle checkpoint also functions to stabilize securin but via a different mechanism to the DNA damage checkpoint. Spindle checkpoint activation leads to the inhibition of APC\textsuperscript{Cdc20} (Hwang et al. 1998; Kim et al. 1998), thus preventing securin ubiquitination and destruction and anaphase onset. The conserved components that mediate the spindle checkpoint response were initially identified in budding yeast from two independent screens to identify mutants that could no longer arrest the cell cycle in the presence of spindle poisons (Hoyt et al. 1991; Li and Murray 1991). These screens identified the \textit{MAD} (mitotic-arrest deficient) genes \textit{MAD1}, \textit{MAD2} and \textit{MAD3} and the \textit{BUB} (budding uninhibited by benzimidazole) genes \textit{BUB1} and \textit{BUB3} (Hoyt et al. 1991; Li and Murray 1991). These genes encode the proteins that are considered as the core spindle checkpoint components and are conserved in all eukaryotes. Several other components of the spindle checkpoint have also been identified, however most of these additional factors are found only in higher eukaryotes, the notable exceptions being the Mps1 and Aurora B kinases, which are conserved from yeast to humans (for reviews see May and Hardwick 2006; Musacchio and Salmon 2007; Kang and Yu 2009; Zich and Hardwick 2009). The spindle checkpoint components perform many different functions that somehow
cooperate to inhibit Cdc20 (Hwang et al. 1998; Kim et al. 1998), the APC activator protein required for the ubiquitin-mediated destruction of securin (Peters 2006).

1.7.2.1 Activation of the spindle checkpoint

Accurate chromosome segregation requires that sister chromatids attach to microtubules emanating from opposite poles of the mitotic spindle (bi-orientation) before they are separated. The spindle checkpoint monitors the attachment of sister kinetochores to the spindle and prevents anaphase onset until tension-generating bi-orientation has been achieved. In animal somatic cells this is an essential task during every cell division, as inactivation of the spindle checkpoint (by inhibitory Mad2 antibody injection or expression of a dominant-negative Bub1 mutant) results in premature sister chromatid separation before chromosomes have aligned on the metaphase plate (Taylor and McKeon 1997; Gorbsky et al. 1998). Similarly, mouse knockouts of Mad2 and Bub3 accumulate chromosome segregation errors and die in the embryonic stage (Dobles et al. 2000; Kalitsis et al. 2000). In contrast, budding yeast cells lacking spindle checkpoint components are viable and initiate anaphase with normal timing. The spindle checkpoint in budding yeast only becomes essential in response to mitotic defects.

Although it is clear that the spindle checkpoint prevents anaphase by inhibiting APC^Cdc20 (Hwang et al. 1998; Kim et al. 1998), the primary defect that is sensed by the spindle checkpoint remains unclear. In 1991 McIntosh proposed that “chromosome attachment and the application of tension are the factors that distinguish chromosomes that are appropriate for mitotic progression from those that are not” (McIntosh 1991, p.617). Since then, experimental evidence from several different organisms suggests that the spindle checkpoint does indeed delay anaphase onset in response to both a lack of kinetochore-microtubule attachment and a lack of tension normally generated by the opposing pulling forces of microtubules attached to sister kinetochores linked by cohesion (reviewed in Zhou et al. 2002; Pinsky and Biggins 2005; Maresca and Salmon 2010).
The importance of microtubule attachment in spindle checkpoint function was revealed by Rieder et al. who showed that a single unattached kinetochore is sufficient to prevent anaphase onset in rat kangaroo kidney epithelial (PtK1) cells (Rieder et al. 1994). Laser ablation of the last unattached kinetochore triggered anaphase onset in these cells, indicating that the spindle checkpoint is mediated by a signal generated at the unattached kinetochore (Rieder et al. 1995). However, a role for tension sensing in the spindle checkpoint was clearly demonstrated by a classic study of praying mantid spermatocytes undergoing meiosis I (Li and Nicklas 1995). Proper spermatogenesis in the praying mantid requires that the two genetically different X chromosomes segregate away from the single Y chromosome in meiosis I (Li and Nicklas 1995). In some cells, the three sex chromosomes fail to remain connected in a trivalent, resulting in an X-Y bivalent and a free X chromosome; a situation that delays the onset of anaphase (Li and Nicklas 1995). Remarkably, anaphase proceeds rapidly after tension is applied to the free X chromosome by pulling that chromosome with a microneedle (Li and Nicklas 1995), suggesting that the spindle checkpoint prevents anaphase onset in response to lack of tension.

Clearly both lack of attachment and lack of tension are sensed by the spindle checkpoint, however, it is unclear whether these signals are separate or interdependent given the intimate relationship between attachment and tension: the generation of tension at kinetochores requires kinetochore-microtubule attachment, yet the application of tension to kinetochores (of grasshopper spermatocytes) by micromanipulation also increases the number of kinetochore-microtubule attachments (King and Nicklas 2000). This relationship between tension and attachment is simplified by using budding yeast as a model system to study the spindle checkpoint. Unlike metaphase kinetochores from animal cells, such as praying mantid spermatocytes and PtK1 cells, which bind an average of 23-24 microtubules (McEwen et al. 1997; King and Nicklas 2000), budding yeast kinetochores attach to only a single microtubule (Winey et al. 1995). Therefore, budding yeast kinetochores are either attached or unattached, eliminating any complications involving partial kinetochore occupancy by microtubules. By performing a series of elegant experiments in budding yeast, Murray and colleagues solidified the role of tension in the spindle checkpoint (Shonn et al. 2000; Biggins
and Murray 2001; Stern and Murray 2001)(Figure 1.9). During meiosis I, sister chromatids move to the same spindle pole but segregate away from the homologous chromosome. Linkages between homologous chromosomes are made by reciprocal recombination events, and it is these linkages that generate tension during meiosis I by counteracting the force of spindle microtubules that pull homologous chromosomes towards opposite poles. Shonn et al. (2000) showed that by preventing homologous recombination, and therefore causing a loss of tension, budding yeast cells exhibited spindle checkpoint-dependent delay in entering anaphase I as judged by a delay to securin destruction. Because under these conditions homologous chromosomes are still pulled to spindle poles, presumably by attachment to microtubules, this experiment suggests that the spindle checkpoint can delay anaphase onset specifically in response to a lack of tension independently of a lack of attachment (Shonn et al. 2000). During mitosis, tension is generated by the opposing forces of the bipolar spindle and the cohesin linkages between sister chromatids. Murray and colleagues generated a tension-less mitosis in budding yeast by either depleting cells of cohesin or by preventing DNA replication so cells entered mitosis with only single chromatids (Biggins and Murray 2001; Stern and Murray 2001). They found that a lack of tension in mitosis caused by these different manipulations is sufficient to cause a spindle checkpoint-dependent delay to securin destruction (Biggins and Murray 2001; Stern and Murray 2001). Importantly, in both situations, the chromosomes are pulled to the spindle poles (Tanaka et al. 2000; Stern and Murray 2001), indicating that kinetochore-microtubule attachments are intact and that the spindle checkpoint can respond to lack of tension during mitosis. Of course, in budding yeast the spindle checkpoint also responds to a lack of attachment, as the depolymerization of microtubules causes a robust delay to anaphase onset that requires the core spindle checkpoint components (Hoyt et al. 1991; Li and Murray 1991).
Figure 1.9 The spindle checkpoint responds to both lack of attachment and lack of tension to prevent anaphase onset in budding yeast.
1.7.2.1.1 **Proteins that distinguish between tension and attachment defects and ensure bi-orientation in budding yeast**

All of the core spindle checkpoint components, Mad1-3, Bub1, Bub3 and Mps1, are essential for the spindle checkpoint response to both lack of attachment and lack of tension. However, in budding yeast, the spindle checkpoint response to lack of tension between sister chromatids requires two additional proteins, Shugoshin (Sgo1) and Aurora B (Ipl1), which are dispensable for delaying anaphase onset when kinetochores lack attachment (Biggins and Murray 2001; Indjeian et al. 2005).

Ipl1 is the kinase component of the chromosomal passenger complex, which also includes the non-catalytic components Sli15 and Bir1. This complex is highly conserved in all eukaryotes and exhibits a characteristic dynamic localization during the cell cycle, appearing at the inner centromere in metaphase and then the spindle midzone in anaphase (reviewed in Ruchaud et al. 2007). An essential role for Ipl1 in sensing tension became apparent from the initial analysis of a temperature-sensitive *ipl1-321* allele (Biggins et al. 1999; Biggins and Murray 2001). In *ipl1-321* cells at the restrictive temperature, sister chromatids are frequently attached to microtubules emanating from the same pole (mono-orientation or syntelic attachment), resulting in massive chromosome missegregation and lethality (Biggins et al. 1999). However, despite the presence of mono-oriented chromosomes that cannot be under tension, *ipl1-321* cells progress through the cell cycle without a delay to anaphase onset (Biggins and Murray 2001). This result suggested that Ipl1 is required for the spindle checkpoint response to lack of tension, a conclusion that was confirmed by the findings that *ipl1-321* mutants cannot stabilize securin in response to tension defects induced by conditional inactivation of sister chromatid cohesion or DNA replication (Biggins and Murray 2001). By contrast, Ipl1 is not required to delay anaphase onset in response to lack of attachment induced by the microtubule depolymerising drug nocodazole (Biggins and Murray 2001). In several kinetochore mutants that have unattached kinetochores, inactivation of Ipl1 both restored kinetochore-microtubule attachments at these defective kinetochores and abolished the spindle checkpoint
arrest (Pinsky et al. 2003; Pinsky et al. 2006). This led to the proposal that Ipl1 activates the spindle checkpoint when there is lack of tension indirectly by creating unattached kinetochores (Pinsky et al. 2006). However, Ipl1 may also directly activate the spindle checkpoint directly by phosphorylating the core checkpoint component Mad3 (King et al. 2007).

*IPL1* is an essential gene, and the ability of Ipl1 to generate unattached kinetochores appears to be essential in ensuring chromosome bi-orientation during every cell cycle. In budding yeast, centromeres are replicated early during S phase (McCarroll and Fangman 1988), before the newly formed spindle pole body (SPB) has fully matured as a microtubule organising centre (Segal and Bloom 2001). The old SPB remains intact from the previous cell cycle and is destined for the daughter cell in 98% of cells (Pereira et al. 2001). Temperature sensitive *ipl1-321* mutants progressing through mitosis at the restrictive temperature contain mono-oriented sister chromatids that are preferentially co-segregated to the daughter cell (Tanaka et al. 2002). It was therefore suggested that Ipl1 is required to destabilize any initial kinetochore-spindle pole microtubule connections that do not generate tension, allowing the chance of new attachments that result in bi-orientation and accurate chromosome segregation (Tanaka et al. 2002). The kinetochore protein Dam1, which contributes to the kinetochore-microtubule binding interface, is a key substrate for Ipl1 kinase in promoting bi-orientation. Mutations in Dam1 that mimic phosphorylation by Ipl1 cause bi-orientation defects *in vivo* (Cheeseman et al. 2002) and perturb Dam1 complex formation *in vitro* (Wang et al. 2007), likely affecting its interaction with microtubules. The core spindle checkpoint kinase Mps1 plays a similar role to Ipl1 in promoting bi-orientation, however, given that Ipl1 and Mps1 appear not to regulate each other, the two kinases likely function independently (Maure et al. 2007), possibly by phosphorylating different residues in Dam1 (Cheeseman et al. 2002; Shimogawa et al. 2006).

Sgo1 was originally discovered as a protector of centromeric cohesion during meiosis (section 1.8), however, Sgo1 was also independently identified in a screen for budding yeast mutants that could not arrest the cell cycle in response to the presence of tension-less linear mini-chromosomes (Indjeian et al. 2005). Subsequent
experiments found that \textit{sgol} mutants fail to stabilize securin when mitotic sister chromatids lack tension due to loss of sister chromatid cohesion, yet the spindle checkpoint response to lack of attachment is maintained in \textit{sgol} mutant cells (Indjeian et al. 2005). These results demonstrate that Sgo1 is required for the checkpoint response to lack of tension but not lack of attachment. This phenotype is remarkably similar to Ipl1, however, the mechanism by which Sgo1 functions in the spindle checkpoint is not known. Unlike Ipl1, inactivation of Sgo1 does not restore kinetochore-microtubule attachments and cell cycle progression in kinetochore mutants such as \textit{ndc80-1} (Pinsky et al. 2006), suggesting that Sgo1 may not be required to generate unattached kinetochores when sister chromatids lack tension due to defective kinetochores.

Budding yeast cells lacking \textit{SGO1} are viable and do not show any defects in bi-orienting sister chromatids during an unperturbed cell cycle (Indjeian et al. 2005). However, Sgo1 becomes essential for chromosome bi-orientation when microtubules repolymerise after cells are released from a nocodazole-induced metaphase arrest (Indjeian et al. 2005; Indjeian and Murray 2007). Despite executing a normal metaphase arrest when released from \textit{G\textsubscript{i}} in presence of microtubule depolymerising drugs, \textit{sgol} mutants die rapidly after the drugs are removed (Indjeian et al. 2005). This puzzling phenotype is likely due to the presence of mono-oriented chromosomes, as sister chromatids are often pulled to the same spindle pole once the mitotic spindle reforms (Indjeian et al. 2005). Therefore, \textit{sgol} mutants are defective in bi-orienting sister chromatids only when kinetochore-microtubule attachments are initiated after the cell has progressed from \textit{G\textsubscript{i}} to metaphase without microtubules. Interestingly, under these conditions the spindle pole bodies (SPBs) are yet to be separated (Indjeian and Murray 2007) and subsequently sister chromatids might initially attach to microtubules that emanate from the old SPB, leading to mono-orientation. Unlike wild type cells, \textit{sgol} mutants appear to be unable to re-orient those kinetochore-spindle pole microtubule connections occurring in metaphase that do not generate tension (Indjeian and Murray 2007). The mechanism whereby Sgo1 contributes to bi-orientation is not known. However as the process probably requires the destabilization of mono-oriented attachments, Sgo1 might influence Ipl1 activity somehow. Fission yeast contains two shugoshin proteins, Sgo1 and Sgo2 (Kitajima et
al. 2004; Rabitsch et al. 2004). Sgo1 is meiosis-specific and contributes to centromeric cohesion protection during meiosis (see section 1.8), whereas Sgo2 is expressed during both meiosis and mitosis (Kitajima et al. 2004; Rabitsch et al. 2004). Fission yeast Sgo2 is also required for the spindle checkpoint response to lack of tension induced by cohesion defects, and sgo2 mutants also co-segregate sister chromatids after release from a metaphase arrest induced by microtubule depolymerization (Kawashima et al. 2007; Vanoosthuyse et al. 2007). In this organism, sgo2 mutants have reduced levels of chromosomal passenger proteins, including the Ipl1 homologue Ark1, at centromeres, which likely explains their bi-orientation defects (Kawashima et al. 2007; Vanoosthuyse et al. 2007). However, a role for budding yeast Sgo1 in Ipl1 localization is less clear. One study suggested that Ipl1 localization to the centromere is dependent on Sgo1, albeit in meiotic cells (Yu and Koshland 2007), whereas a subsequent study found that centromeric Ipl1 is maintained in meiotic cells depleted for Sgo1 (Kiburz et al. 2008). Similarly, the centromeric localization of Ipl1 is not affected in budding yeast mitotic cells where Sgo1 is absent from the centromere (Fernius and Hardwick 2007). Sgo1 might be involved in the fine-tuning of Ipl1 localization, perhaps explaining the different phenotypes of ipl1 and sgo1 mutants during an unperturbed cell cycle and when kinetochores are defective. Alternatively, Sgo1 might directly affect kinetochore microtubule attachment, as a vertebrate Sgo1 homologue has been shown to bind to microtubules in vitro and regulate kinetochore microtubule stability in vivo (Salic et al. 2004).

1.7.2.1.2 Proteins that promote bi-orientation in higher eukaryotes

Similar to yeast, inactivation of the Aurora B kinase in mammalian cells causes sister chromatids to be pulled to the same pole in anaphase due to mono-oriented (syntelically attached) chromosomes (Hauf et al. 2003; Lampson et al. 2004), suggesting a conserved role for Aurora B in destabilizing kinetochore-microtubule attachments that do not generate tension. A key target for Aurora B in mammalian cells is Ndc80/HEC1, as phosphorylation of Ndc80/HEC1 by Aurora B reduces its
affinity for microtubules (Cheeseman et al. 2006; Ciferri et al. 2008), and mutations in Ndc80/HEC1 that mimic phosphorylation by Aurora B cause bi-orientation defects in vivo (DeLuca et al. 2006). A more recent study showed that, in addition to Ndc80/HEC1, the microtubule binding domain of KNL-1 and the Dsn1/KNL-3 subunit of the Mis12 complex are key targets of Aurora B in destabilizing kinetochore-microtubule attachments (Welburn et al. 2010). Human cells depleted of the microtubule depolymerase MCAK (mitotic centromere-associated kinesin) also have bi-orientation defects that lead to chromosome missegregation (Kline-Smith et al. 2004). Aurora B phosphorylates MCAK, and might direct MCAK to depolymerise incorrectly oriented kinetochore microtubules (Andrews et al. 2004; Lan et al. 2004). One of the two human shugoshin proteins, Sgo2, is also required to destabilize inappropriate kinetochore-microtubule attachments (Huang et al. 2007).

Similar to budding yeast Sgo1, the centromeric localization of Aurora B does not depend on Sgo2 in human cells (Huang et al. 2007). Instead, human Sgo2 is required for the centromeric localization of MCAK, likely explaining the bi-orientation defects of Sgo2-depleted human cells (Huang et al. 2007). MCAK function is also regulated indirectly by the kinetochore protein Bod1 (Biorientation Defective 1). Human cells depleted of Bod1 are unable to re-orient syntelic chromosome attachment, and this is not due to defective Aurora B localization (Porter et al. 2007). Instead, Bod1 depletion causes a reduction in phosphorylated MCAK at centromeres, which might explain the observed bi-orientation defects (Porter et al. 2007).

Once tension-generating bi-orientation is established, the turnover of kinetochore-microtubule attachments must stop in order for bi-orientation to be maintained. It was recently found in budding yeast that tension between sister kinetochores reduces Ipl1-dependent phosphorylation of Dam1 (Keating et al. 2009), indicating that either Ipl1/Aurora B activity or the access of Aurora B to its substrates is inhibited upon bi-orientation. Ipl1/Aurora B kinase activity is regulated by the other chromosomal passenger proteins Bir1/Survivin and Sli15/INCENP (Ruchaud et al. 2007). In budding yeast, a Bir1-Sli15 complex bridges centromeres to microtubules, and it was proposed that this complex may no longer be able to directly activate Ipl1 once tension is applied (Sandall et al. 2006). Alternatively, tension might sequester kinetochore substrates away from constitutively active Aurora B that localizes to the
inner centromere. A recent study in human cells provides strong evidence in support of this model. By using a phosphorylation sensor based on FRET, it was found that an Aurora B substrate peptide specifically targeted to the kinetochore is phosphorylated only when tension is low and dephosphorylated when tension is applied (Liu et al. 2009). Furthermore, a centromere-targeted substrate is constitutively phosphorylated, independent of tension (Liu et al. 2009). Finally, repositioning Aurora B from the inner centromere to the kinetochore causes the destabilization of kinetochore-microtubule attachments (Liu et al. 2009). Taken together, these findings suggest that establishing bi-orientation increases the distance between kinetochore substrates and inner centromeric Aurora B, resulting in the stabilization of kinetochore-microtubule attachments that generate tension. Recent evidence from budding and fission yeast suggests that kinetochore-localised protein phosphatase 1 (PP1) might contribute to the dephosphorylation of Aurora B substrates upon bi-orientation (Pinsky et al. 2009; Vanoosthuyse and Hardwick 2009).

1.8 The regulation of centromeric cohesion

1.8.1 Sister chromatid cohesion is lost in a stepwise manner during meiosis

Meiosis is a specialized cell division programme that produces four daughter cells with a complete haploid genome from a diploid precursor cell. This process requires that a single round of DNA replication be followed by two successive chromosome segregation events. During meiosis I, maternally and paternally derived homologous chromosomes pair up and recombine, resulting the formation of at least one chiasmata (the cytological appearance of the cross-over point between two homologues) in which one sister chromatid from one homologue is covalently linked to a sister chromatid from the other homologue. Sister chromatid cohesion distal to the chiasmata stabilizes the homologue interactions, allowing the bi-orientation of homologous chromosomes on the meiosis I spindle. The segregation of homologous chromosomes at anaphase of meiosis I requires that sister chromatid cohesion is released along chromosome arms to resolve chiasmata. However, sister chromatid
cohesion is retained at the centromere, where it plays a critical role in ensuring the bi-orientation of sister chromatids on the meiosis II spindle. Analogous to mitosis, the complete loss of sister chromatid cohesion at anaphase of meiosis II triggers the segregation of sister chromatids segregate to opposite poles. Therefore, sister chromatid cohesion is lost in a step-wise manner during meiosis (reviewed in Marston and Amon 2004; Watanabe 2004) (Figure 1.10).

As in mitosis, sister chromatid cohesion during meiosis is provided by the multisubunit cohesin complex, which also must be loaded onto chromosomes during pre-meiotic S phase to be functional (Watanabe et al. 2001). However, the Scc1 cohesin subunit is largely replaced by a meiosis-specific Rec8 subunit (Klein et al. 1999; Parisi et al. 1999; Watanabe and Nurse 1999; Pasierbek et al. 2001; Eijpe et al. 2003). Rec8 plays an essential role in ensuring the step-wise loss of sister chromatid cohesion, as the replacement of Rec8 with Scc1 results in the complete loss of sister chromatid cohesion at the onset of anaphase I (Toth et al. 2000; Yokobayashi et al. 2003). Both the segregation of homologous pairs in anaphase I, and the segregation of sister chromatids in anaphase II, requires cleavage of Rec8 by separase (Buonomo
et al. 2000; Kitajima et al. 2003; Kudo et al. 2006). Therefore, Rec8 must be specifically protected from separase activity at the centromere during meiosis I, so that centromeric cohesion is retained until the onset of anaphase II.

1.8.2 Shugoshin protects centromeric cohesion at meiosis I

An ingenious genetic screen in fission yeast led to the identification of a conserved protein responsible for Rec8 protection (Kitajima et al. 2004). When Rec8 replaces Scc1 in fission yeast mitotic cells viability is not affected, suggesting that factors other than Rec8 itself are responsible for the protection of centromeric Rec8 (Watanabe and Nurse 1999). Kitajima et al. searched for genes that when co-overexpressed with Rec8 in mitotic cells allowed Rec8 to persist on chromosomes, resulting in a failure to separate sister chromatids and lethality (Kitajima et al. 2004). They isolated one such gene and named the product of this gene shugoshin (Sgo1), which means ‘guardian spirit’ in Japanese (Kitajima et al. 2004). Independent screens for deletion mutants that exhibit an abnormal chromosome segregation pattern during meiosis also identified the sgo1/SGO1 gene in fission yeast and budding yeast (Marston et al. 2004; Rabitsch et al. 2004). Sequence analysis revealed that shugoshin shares a limited homology to proteins in most eukaryotes from yeast to humans; however, this homology is based only on a coiled-coil region at the N-terminus and a cluster of basic residues at the C-terminus (Kitajima et al. 2004; Rabitsch et al. 2004). Importantly, such homology searches identified the Drosophila MEI-S332 protein, which had previously been shown to be required for the persistence of centromeric cohesion during meiosis I (Kerrebrock et al. 1992; Kerrebrock et al. 1995). Budding yeast, Drosophila and Xenopus have a single shugoshin protein; on the other hand, fission yeast, plants, mice and humans contain two shugoshin-like proteins, which are referred to as Sgo1 and Sgo2. In budding yeast, fission yeast and plants Sgo1 functions in the protection of centromeric cohesion, whereas in mice Sgo2 carries out this function. For simplicity, I will refer to these proteins collectively as shugoshin unless otherwise stated. Shugoshin-deficient cells of mice, Drosophila, maize, fission yeast and budding yeast, prematurely lose centromeric cohesion during meiosis (Kerrebrock et al. 1995; Katis
et al. 2004; Kitajima et al. 2004; Marston et al. 2004; Rabitsch et al. 2004; Hamant et al. 2005; Lee et al. 2008; Llano et al. 2008), suggesting that shugoshin constitutes a conserved protein family that protects centromeric cohesion during meiosis, presumably by protecting centromeric Rec8 from cleavage by separase.

1.8.3 Shugoshin cooperates with protein phosphatase 2A to protect centromeric cohesion at meiosis I

Rec8 cleavage by separase is required for cohesin dissociation from chromosomes and loss of sister chromatid cohesion in meiosis, as expression of a non-cleavable Rec8 or inactivation of separase blocks both these processes (Buonomo et al. 2000; Kitajima et al. 2003; Kudo et al. 2006). It has been demonstrated in budding yeast, fission yeast and mice that shugoshin-deficient cells fail to retain Rec8 on centromeric chromatin beyond meiosis I, suggesting that shugoshin functions to prevent separase cleavage of Rec8 specifically at the centromere (Katis et al. 2004; Kitajima et al. 2004; Marston et al. 2004; Rabitsch et al. 2004; Lee et al. 2008; Llano et al. 2008). How does shugoshin prevent Rec8 cleavage at the centromere during meiosis I? An important insight came from the findings that protein phosphatase 2A (PP2A) is a major binding partner of yeast meiotic shugoshin (Kitajima et al. 2006; Riedel et al. 2006). PP2A is a serine/threonine phosphatase that is composed of a heterotrimeric complex of scaffold, regulatory and catalytic subunits. Although several sub-families of PP2A regulatory subunits exist (B, B’, B” or B”’)(Janssens and Goris 2001; Lechward et al. 2001), meiotic shugoshin interacts only with the subset of PP2A that contain B’ regulatory subunits (Kitajima et al. 2006; Riedel et al. 2006). PP2A complexes are found in many places in the cell, however, PP2A-B’ was found to preferentially co-localize with shugoshin at meiotic centromeres (Kitajima et al. 2006; Riedel et al. 2006). Shugoshin is required for the centromeric localization of PP2A-B’ in meiosis and, similar to sgo1 mutants, inactivation of PP2A-B’ (by deletion of the catalytic subunit or the B’ subunit) causes premature loss of centromeric Rec8 (Kitajima et al. 2006; Riedel et al. 2006). Similarly in mouse oocytes, the localization of PP2A to centromeres depends on shugoshin (Lee et al. 2008) and the treatment of oocytes with okadaic acid (a phosphatase inhibitor)
induces premature separation of sister chromatids during meiosis I (Mailhes et al. 2003), suggesting that the protection of centromeric Rec8 by shugoshin and PP2A is a conserved mechanism. The artificial tethering of PP2A-B’ to yeast chromosome is sufficient to protect Rec8 from separase even in the absence of shugoshin (Kitajima et al. 2006; Riedel et al. 2006), therefore the main role of shugoshin in Rec8 protection at meiotic centromeres might be to recruit PP2A-B’ to that region. This idea is supported by a recent structural study of the shugoshin-PP2A interaction (Xu et al. 2009). Here the authors demonstrate that the conserved coiled-coil region of human shugoshin directly interacts with PP2A-B’ in vitro, but does not influence the enzyme activity of PP2A, arguing that shugoshin does not directly activate PP2A (Xu et al. 2009). The crystal structure of this complex revealed that a shugoshin coiled-coil homodimer interacts with both the catalytic subunit and the B’ subunit of a single PP2A enzyme (Xu et al. 2009). This structure aided the identification of residues on shugoshin that are critical for its interaction with PP2A, allowing the significance of the shugoshin-PP2A interaction to be directly tested in vivo (Xu et al. 2009). Mutation to alanine of three surface residues of budding yeast shugoshin predicted to contact PP2A (sgo1-3A) completely abolished the localization of PP2A-B’ to the centromeres of meiotic cells, without affecting the centromeric localization of shugoshin itself (Xu et al. 2009). The sgo1-3A mutant, although present at centromeres, failed to retain Rec8 at the centromere during meiosis I, thus providing strong evidence that the sole role of shugoshin in protecting centromeric Rec8 in meiosis is to recruit the phosphatase PP2A (Xu et al. 2009). Similar mutations in mouse shugoshin also abolished its ability to recruit PP2A and prevent Rec8 removal from chromosomes when overexpressed in oocytes (Xu et al. 2009).

If shugoshin is required for the recruitment of PP2A to meiotic centromeres to protect Rec8, what is the mechanism of PP2A action in this region? Until very recently, the widely accepted model was that shugoshin recruits PP2A-B’ to the centromere, where it locally protects Rec8 from separase by counteracting Rec8 phosphorylation by polo-like kinase Cdc5. This model was based on the following evidence from multiple organisms: First, budding yeast cells depleted of Cdc5 show reduced Rec8 phosphorylation and are defective in cleaving Rec8 in meiosis (Clyne
et al. 2003; Lee and Amon 2003). Second, mutating multiple serines and threonines in budding yeast Rec8 thought to be phosphorylated by Cdc5 causes a delay to Rec8 cleavage (Brar et al. 2006). Third, the artificial tethering of PP2A-B’ to chromosome arms in fission yeast not only prevents Rec8 removal from these regions, but also prevents Rec8 phosphorylation (Riedel et al. 2006). Fourth, the cleavage of mouse Rec8 by separase in vitro is dependent on recombinant human polo-like kinase Plk1 (Kudo et al. 2009). However, recent studies in yeast suggest that polo-like kinase might not be the kinase responsible Rec8 cleavage in meiosis I (Ishiguro et al. 2010; Katis et al. 2010). Live imaging of budding yeast cells depleted of Cdc5 suggest that the observed block to Rec8 cleavage is likely due to the stabilization of securin (Katis et al. 2010). Bypassing the requirement of Cdc5 for securin degradation (by deletion of the meiosis-specific APC activator Ama1) allowed timely Rec8 cleavage in budding yeast cells depleted of Cdc5 (Katis et al. 2010). In fact, centromeric Rec8 was lost prematurely in these cells, indicating that Cdc5 might actually contribute to the protection of Rec8 at centromeres (Katis et al. 2010). Furthermore, depletion of the fission yeast polo-like kinase Plo1 does not affect the timing of Rec8 cleavage in meiosis and Rec8 is inefficiently phosphorylated by Plo1 in vitro (Ishiguro et al. 2010).

Extensive phosphorylation site mapping of budding yeast Rec8 identified multiple phosphorylated serines and threonines that, when mutated to alanine, cause a complete block to Rec8 cleavage and removal from chromosomes despite separase activation (Katis et al. 2010). Such a non-phosphorylatable Rec8 mutant is also resistant to cleavage by separase in vitro (Katis et al. 2010). Crucially, mutating these sites to aspartic acids to mimic constitutive phosphorylation results in the loss of centromeric Rec8 in meiosis I, reminiscent of shugoshin- or PP2A-B’-deficient cells (Katis et al. 2010). This provides strong evidence that the shugoshin and PP2A-B’ protect centromeric Rec8 from separase by counteracting Rec8 phosphorylation that is essential for its cleavage. In support of this view, phospho-specific Rec8 antibodies recognises Rec8 on chromosome arms but not the centromeric Rec8 that is retained beyond meiosis I (Brar et al. 2006).
Rec8 cleavage in meiosis I appears to be dependent on a combination of casein kinase 1 (CK1) and Cdc7-Db4 kinase (DDK) (Ishiguro et al. 2010; Katis et al. 2010). Budding yeast Rec8 interacts with both CK1 and DDK from meiotic prophase onwards, and the combined inactivation of both kinases causes a reduction in Rec8 phosphorylation and a block to Rec8 cleavage similar to the non-phosphorylatable Rec8 mutant (Katis et al. 2010). Fission yeast cells depleted of CK1 also show a significant delay to Rec8 cleavage in meiosis (Ishiguro et al. 2010). These cells show a reduction in Rec8 phosphorylation and, furthermore, Rec8 is efficiently phosphorylated by CK1 in vitro (Ishiguro et al. 2010). Mutation of the putative CK1 phosphorylation sites on Rec8 causes a block to Rec8 cleavage (Ishiguro et al. 2010). Experiments in fission yeast also provide key evidence that shugoshin-PP2A protects Rec8 from separase by removing the phosphate groups provided by CK1 (Ishiguro et al. 2010). CK1 localizes to pericentromeric chromatin in fission yeast meiotic cells, the region where Rec8 is enriched and protected by shugoshin (Ishiguro et al. 2010). Significantly, artificial tethering of excess CK1 to the pericentromere causes premature loss of centromeric Rec8 despite the presence of shugoshin and PP2A (Ishiguro et al. 2010). This is consistent with the notion that shugoshin-PP2A complexes protect Rec8 by removing phosphate groups produced by CK1 (Figure 1.11), a view supported by the finding that immunoprecipitated PP2A-B’ can dephosphorylate a Rec8 fragment that was previously phosphorylated by CK1 in vitro (Ishiguro et al. 2010).

Figure 1.11 Model for regulation of cohesin cleavage in meiosis I
1.8.4 Shugoshin-PP2A protects centromeric cohesion during mitosis in vertebrates

In vertebrate cells, the bulk of cohesin is removed from chromosome arms in prophase, however, cohesin at the centromere is maintained where it ensures the bi-orientation of sister chromatids on the metaphase spindle (Sumara et al. 2000; Waizenegger et al. 2000)(see section 1.4.1). When shugoshin is depleted in human cells, cohesin dissociates prematurely from centromeres, resulting in premature sister chromatid separation before separase activation, indicating that shugoshin is required to protect centromeric cohesins from the prophase pathway of cohesin removal (Salic et al. 2004; Tang et al. 2004; Kitajima et al. 2005; McGuinness et al. 2005). As in meiotic cells of budding and fission yeast, human shugoshin also interacts with PP2A-B’ and inactivation of PP2A, by RNAi or okadaic acid treatment, results in premature cohesin dissociation from centromeres in prophase (Kitajima et al. 2006; Tang et al. 2006). However, unlike in yeast, it is unclear whether human shugoshin protects centromeric cohesion in mitosis by recruiting PP2A to centromere. Human cells contain two shugoshin-like proteins, Sgo1 and Sgo2, both of which are required for protection of centromeric cohesin from dissociation in prophase (Kitajima et al. 2006). In contrast to yeast meiotic cells and mouse oocytes, PP2A is required for the centromeric localization Sgo1 in human cells, and not vice versa (Kitajima et al. 2006; Tang et al. 2006). The centromeric localization of PP2A depends on Sgo2, yet neither PP2A nor Sgo1 are required for Sgo2 localization (Kitajima et al. 2006). At first sight it appears that, in human cells, centromeric Sgo2 recruits PP2A, which in turn recruits Sgo1 to the centromere. However, it is puzzling that depletion of Sgo2 does not affect Sgo1 localization, despite the absence of PP2A from centromeres (Kitajima et al. 2006), therefore the interplay between shugoshin and PP2A might be more complex in human cells.

It appears that shugoshin-PP2A prevents centromeric cohesin dissociation during prophase by reversing the phosphorylation of the STAG2 cohesin subunit, as a non-phosphorylatable STAG2 mutant rescues the premature sister centromere separation phenotype of human cells depleted of Sgo1 (McGuinness et al. 2005). Also the STAG2 that dissociates from chromosome arms in prophase is hyperphosphorylated compared to the STAG2 that remains bound to centromeres and, furthermore,
immunoprecipitated Sgo1 can dephosphorylate STAG2 in vitro (Kitajima et al. 2006). Because phosphorylation of STAG2 by Plk1 plays a role in cohesin dissociation by the prophase pathway (Hauf et al. 2005), shugoshin-PP2A might counteract this kinase at the centromere. However, a number of proteins cooperate to promote cohesin dissociation in the prophase pathway (see section 1.4.1) and depletion of Plk1 does not suppress the premature sister centromere separation phenotype of Sgo1-depleted cells (McGuinness et al. 2005; Tang et al. 2006), therefore the precise mechanism whereby shugoshin-PP2A protects centromere cohesion in vertebrate mitotic cells remains unclear.

1.8.4.1 Shugoshin regulates centriole cohesion in vertebrates

In human cells there exists a smaller splice variant of Sgo1 (sSgo1) that, unlike the full length protein, does not localize to kinetochores (Wang et al. 2006). Instead, sSgo1 localizes to centrosomes (Wang et al. 2008). Sgo1 depletion by RNAi in human cells effectively depletes both Sgo1 isoforms and, in addition to causing premature sister chromatid separation, also causes premature centriole disengagement (Wang et al. 2008), which is a separase-dependent process (Tsou et al. 2009). Introduction of sSgo1 into Sgo1-depleted cells rescues premature centriole disengagement, but not sister chromatid separation, indicating that sSgo1 plays a specific role in regulating centriole cohesion (Wang et al. 2008). A role for Sgo1 at centrosomes is conserved in vertebrates because embryonic fibroblast cells derived from haploinsufficient Sgo1+/- mice have a centriole cohesion defect that can be suppressed by expression of sSgo1 (Wang et al. 2008). Therefore shugoshin has dual roles in protecting cohesion between sister chromatids and sister centrioles.
1.8.5 The regulation of shugoshin

1.8.5.1 Positive regulators of shugoshin localization

In budding and fission yeast, the spindle checkpoint component Bub1 is required for the protection of centromeric cohesion at meiosis I (Bernard et al. 2001; Riedel et al. 2006). Because the centromeric localization of shugoshin is abolished in meiotic cells lacking Bub1 (Kitajima et al. 2004; Kiburz et al. 2005; Riedel et al. 2006), it is likely that the meiotic defect of bub1Δ mutants is due to the loss of centromeric shugoshin. Similarly, shugoshin localization to mitotic centromeres in budding and fission yeast depends on Bub1 (Kitajima et al. 2004; Fernius and Hardwick 2007). The role of Bub1 in localizing shugoshin appears to be separate from its role as a core spindle checkpoint component, as inactivation of the Bub1 kinase domain does not affect the spindle response to lack of attachment, yet centromeric shugoshin is lost (Fernius and Hardwick 2007; Kawashima et al. 2010). Accordingly, Bub1 kinase mutants show mitotic and meiotic defects identical to shugoshin mutants (Fernius and Hardwick 2007; Kawashima et al. 2010). The centromeric localization of human shugoshin also depends on Bub1 (Tang et al. 2004; Kitajima et al. 2005), indicating that the dependency of shugoshin on Bub1 is conserved. In humans cells depleted of Bub1, shugoshin relocates from the centromere to chromosome arms where it ectopically prevents cohesin dissociation during prophase, indicating that Bub1 plays a role in human cells to restrict the protection of centromeric cohesion by shugoshin to the centromere (Kitajima et al. 2005). Recently, the mechanism whereby Bub1 controls shugoshin localization has been uncovered (Kawashima et al. 2010). Kawashima et al. found that Bub1 phosphorylates a conserved residue at position 121 of histone H2A in fission yeast (Kawashima et al. 2010). This single phosphorylation event is essential for the centromeric localization and function of shugoshin in fission yeast mitosis and meiosis (Kawashima et al. 2010). Furthermore, Bub1 phosphorylation of H2A is crucial for the centromeric localization of shugoshin in budding yeast and human cells, indicating that Bub1 controls shugoshin function by a highly conserved mechanism (Kawashima et al. 2010).
Several other factors are also involved in promoting shugoshin localization at the centromere. In fission yeast, *Drosophila* and human cells, shugoshin fails to accumulate at centromeres when components of the chromosomal passenger complex (CPC) are inactivated (Resnick et al. 2006; Kawashima et al. 2007; Vanoosthuyse et al. 2007). However, depletion of Ipl1 (a CPC component) causes only a mild defect in shugoshin localization in budding yeast (Monje-Casas et al. 2007; Yu and Koshland 2007). The localization of fission yeast shugoshin to pericentromeric heterochromatin in meiosis is dependent on the heterochromatin protein HP1 (Yamagishi et al. 2008). Human shugoshin also depends on HP1 to localize to the centromere, suggesting a conserved role for heterochromatin in shugoshin recruitment (Yamagishi et al. 2008). Although budding yeast cells lack pericentromeric heterochromatin, the recruitment of shugoshin to the pericentromere in budding yeast meiosis appears to be at least partially dependent on two kinetochore proteins (Iml3 and Chl4), a meiosis-specific factor Spo13 and on the cohesin Rec8 itself (Kiburz et al. 2005). The pericentromeric recruitment of shugoshin might also involve an interaction with histone H3, at least in mitotic cells (Luo et al. 2010). How all these factors cooperate with Bub1 to ensure proper shugoshin localization and function is not known. However, it was recently found that Bub1 and HP1 control shugoshin localization to centromeres and heterochromatin respectively in mouse embryonic fibroblasts (MEFs) (Perera and Taylor 2010). Intriguingly in *BUB1*-null MEFs, which fail to localize shugoshin to the centromere, centromeric cohesion is maintained until anaphase (Perera et al. 2007). It was suggested that the localization of shugoshin to heterochromatin, which is Bub1-independent but HP1-dependent, is sufficient for the protection of centromeric cohesion, at least in MEFs (Perera and Taylor 2010).

### 1.8.5.2 Inactivation of shugoshin

In order for sister chromatids to separate in meiosis II and in mitosis, the protection of centromere cohesion by shugoshin must be inactivated. Furthermore, shugoshin mediates a delay to anaphase onset in response to lack of tension (Indjeian et al. 2005; Kawashima et al. 2007; Vanoosthuyse et al. 2007), but this function of
shugoshin must be inactivated for anaphase to proceed once bi-orientation occurs. In fission yeast meiosis, shugoshin is lost from centromeres in anaphase I due to its APC-dependent degradation (Kitajima et al. 2004), however, budding yeast and *Drosophila* shugoshin/MEI-S332 is present at centromeres in meiosis II and does not leave this region until the onset of anaphase II (Kerrebrock et al. 1995; Katis et al. 2004; Marston et al. 2004). In mitotic cells of most organisms studied, shugoshin is degraded in anaphase, likely due to ubiquitination by the APC (Salic et al. 2004; Karamysheva et al. 2009). In contrast, the dissociation of *Drosophila* MEI-S332 from centromeres in meiosis II and mitosis does not involve the APC, but instead requires phosphorylation of MEI-S332 by the polo-like kinase POLO (Clarke et al. 2005). Nevertheless, despite the active degradation of shugoshin and/or dissociation of shugoshin from centromeres in various organisms, it appears that these mechanisms are not the trigger for shugoshin inactivation. First, the ectopic expression and centromeric localization of fission yeast shugoshin in meiosis II does not block the separation of sister chromatids (Rabitsch et al. 2004; Gregan et al. 2008). Second, preventing MEI-S332 dissociation from centromeres does not prevent sister chromatid separation in *Drosophila* meiosis II and mitosis (Clarke et al. 2005). Third, expression of a non-degradable shugoshin does not interfere with sister chromatid separation or mitotic progression in human cells (Karamysheva et al. 2009).

Recent work suggests that tension between sister chromatids might be required to inactivate shugoshin in mitosis and meiosis II (Lee et al. 2008). Analysis of mouse oocytes revealed that shugoshin co-localizes with Rec8 at the inner centromere in metaphase I, when centromeric Rec8 is protected by shugoshin (Lee et al. 2008). In metaphase II, when sister chromatids become bi-oriented and under tension, shugoshin relocates toward the kinetochores away from Rec8, which remains at the inner centromere (Lee et al. 2008). A similar tension-dependent re-distribution of shugoshin occurs in mouse spermatocytes in metaphase II (Gomez et al. 2007). As Rec8 is no longer protected in meiosis II, these observations suggest that shugoshin relocation away from Rec8 upon tension might contribute to the deprotection of Rec8. In support of this view, fission yeast mutants that bi-orient sister chromatids in meiosis I prematurely lose centromeric cohesion (Vaur et al. 2005). On the contrary,
however, sister chromatids that are forced to bi-orient during meiosis I in budding yeast retain centromere cohesion until meiosis II in a shugoshin-dependent manner (Petronczki et al. 2006). Budding yeast might employ a different mechanism to inactivate shugoshin, although it is possible that the forced bi-orientation of sister chromatids in meiosis I might not reflect the proper tension-generating bi-orientation that would normally occur in meiosis II.

In human cells undergoing mitosis, shugoshin also relocates from centromeres towards the kinetochores upon sister chromatid bi-orientation (Lee et al. 2008). When these cells are arrested in metaphase by inhibition of the proteosome, tension-dependent shugoshin relocation towards kinetochores is accompanied by the loss of centromere cohesion, presumably because the prophase pathway is able to promote the dissociation of the now exposed centromeric cohesin (Lee et al. 2008). This implies that tension between sister chromatids is sufficient to inactivate the protective function of shugoshin. Crucially, there is also evidence to suggest that tension between kinetochores is necessary for the inactivation of shugoshin. Human cells treated with the microtubule depolymerizing drug nocodazole arrest in metaphase due to spindle checkpoint activation. Inhibition of the spindle checkpoint in these nocodazole-treated cells (by depletion of Mad2) allows entry into anaphase without microtubules and therefore without tension between sister chromatids (Lee et al. 2008). Remarkably, in such a ‘tension-less’ anaphase, both shugoshin and cohesin are retained at the centromere and co-localize with each other, indicating that tension-dependent re-distribution of shugoshin away from cohesin is necessary to inactivate shugoshin function (Lee et al. 2008). Intriguingly, this result hints that shugoshin is also capable of counteracting separase-dependent cleavage of cohesin during mitosis and not just during meiosis.
1.9  Aim of this study

In this study, I will use the budding yeast *Saccharomyces cerevisiae* as a model system to attempt to gain a greater understand of the mechanism by which shugoshin regulates chromosome segregation. Since its discovery, much research has focused on the mechanisms of shugoshin function during meiosis; however, exactly how shugoshin regulates chromosome segregation during mitotic cell division has remained elusive. I aim to utilize the genetic amenability of budding yeast to identify novel factors that are involved in shugoshin function during mitosis. Elucidation of the shugoshin pathway should make a significant contribution to our understanding of how cells ensure the accurate distribution of genetic information between daughter cells during cell division. Because aneuploidy is a hallmark of cancer, I hope that this work will also provide an insight into possible causes of human disease.
Chapter 2

Materials and Methods
2 Chapter 2 – Materials and Methods

2.1 GENERAL INFORMATION

2.1.1 Supplier information

Chemicals used in this work were purchased from: BDH, Boehringer Mannheim, Fisher, Gibco NRL, Melford and Sigma, unless stated otherwise. Formedium, Difco and Sigma supplied the growth medium reagents.

2.1.2 Sterilization

Solutions were sterilized by filtration using 0.45 µm filters (Nalgene). Growth media were sterilized by autoclaving at 120°C and 15 pounds/inch² for 15 minutes. All glassware was sterilized by baking at 250°C for 16 hours.

2.2 E.coli METHODS

2.2.1 E.coli strains

Table 2.1 E.coli strains used in this study

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Genotype</th>
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<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>
</tr>
<tr>
<td>XL10-gold</td>
<td>F′ TetΔ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F′ proAB lacF′ZΔM15 Tn10 (Tet) Amy Cam’]</td>
</tr>
<tr>
<td>XL1-red</td>
<td>F′ endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutΔ5 mutS mutT Tn10 (Tet)³</td>
</tr>
</tbody>
</table>

DH5α electrocompetent cells were used for cloning and propagation of plasmids. XL10-gold competent cells (Stratagene) were used for site-directed mutagenesis. XL1-red competent cells (Stratagene) were used for random mutagenesis of plasmids.
2.2.2 *E.coli* media

For solid media 2% (w/v) agarose was added prior to autoclaving

**LB:**
- 1% (w/v) Bacto-tryptone
- 0.5% (w/v) Bacto-yeast extract
- 0.5% (w/v) NaCl
- pH adjusted to 7.2 with NaOH

**NZY+:**
- 1% (w/v) NZ amine
- 0.5% (w/v) Bacto-yeast extract
- 0.5% (w/v) NaCl
- pH adjusted to 7.5 with NaOH
- 12.5 mM MgCl$_2$
- 12.5 mM MgSO$_4$
- 10 mM Glucose

**SOC:**
- 2% (w/v) Bacto-tryptone
- 0.5% (w/v) Bacto-yeast extract
- 0.5% (w/v) NaCl
- 20 mM Glucose
- 10 mM MgCl$_2$
- 10 mM MgSO$_4$
- 10 mM KCl

2.2.3 *E.coli* growth conditions

Bacteria were typically grown on solid LB medium at 37°C or in liquid LB at 37°C with shaking. 100 µg/ml Ampicillin was added to media for plasmid selection.
2.2.4 Storage of E.coli

Bacterial strains were kept on solid medium at 4°C for 1-2 weeks. For frozen stocks, bacterial strains were resuspended in 20% glycerol in cryotubes and stored long-term at -80°C.

2.2.5 Preparation of electrocompetent DH5α E.coli

DH5α cells from frozen glycerol stocks were inoculated into 5 ml of LB and grown at 37°C for 24 hours. 1 ml of this starter culture was inoculated into 50ml of LB and grown at 37°C overnight. The next morning 20 ml of this second culture was inoculated into 2 litres of LB and grown at 37°C for ~3.5 hours until OD_{600} = 0.5-0.7. Subsequent steps were performed in a 4°C room using pre-chilled equipment. Cells were harvested by chilling on ice for 30 minutes and then spun at 5000 rpm for 15 minutes at 4°C in Beckman Avanti J-25 centrifuge. The cell pellet was washed with 2 litres of cold sterile water, then 1 litre of cold sterile water and then with 40 ml of cold 10% glycerol. The cell pellet was resuspended in 10% glycerol in a final volume of 4-6 ml, distributed in 100 µl aliquots into cold 1.5 ml tubes and frozen in liquid nitrogen. Electrocompetent cells were stored long-term at -80°C.

2.2.6 DH5α E.coli transformation by electroporation

DH5α electrocompetent cells were thawed slowly on ice and 40 µl aliquoted into pre-chilled 2 mm gap electroporation cuvettes (Cell Projects) together with 0.5 – 2 µl of DNA. Electroporation was done using Biorad Gene Pulser II at 2.5 volts, 200 ohms and 2.5 µF. Immediately after electroporation, 1 ml of pre-warmed LB was added, the reaction mix transferred to glass tubes and incubated at 37°C for 1 hour. Cultures were then transferred to 1.5 ml tubes and spun at 3000 rpm for 3 minutes. Most of the supernatant was discarded to leave ~100 µl volume and the pellet resuspended and plated onto pre-warmed LB-Amp. Plates were incubated at 37°C overnight.
2.2.7 XL10-gold (stratagene) *E.coli* transformation

Transformation was performed according to the manufacturer’s protocol. NZY+ broth was prepared as described in section 2.2.2.

2.2.8 XL1-red (stratagene) *E.coli* transformation

Transformation was performed according to the manufacturer’s protocol. SOC was prepared as described in section 2.2.2.
2.3 YEAST METHODS

2.3.1 Yeast strains

All strains used in this study are derivatives of W303 (AM1176) and mating type MATa unless otherwise stated. Details of strain construction and origin are given in section 2.3.1.2.

Table 2.2 *Saccharomyces cerevisiae* strains used in this study

<table>
<thead>
<tr>
<th>Strain Number</th>
<th>Genotype</th>
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</thead>
<tbody>
<tr>
<td>AM826</td>
<td>sgo1Δ::KanMX6</td>
</tr>
<tr>
<td>AM870</td>
<td>ura3::GAL-SGO1::URA3</td>
</tr>
<tr>
<td>AM914</td>
<td>MET-CDC20::URA3, promURA3::tetR-GFP::LEU2, cenIV::tetOx448::URA3</td>
</tr>
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2.3.1.1 Yeast strain construction and origin

The *GAL-SGO1* strain was generated by integration of plasmid AMp37, in which *SGO1* is cloned downstream of the *GAL1-10* promoter, at the EcoRV site in the *URA3* locus (Adele Marston). The *GAL-CDC55::TRP* strain was generated by integration of plasmid AMp649, in which *CDC55* is cloned downstream of the *GAL1-10* promoter, at the Bsu36I site in the *TRP1* locus. The *GAL-CDC55::URA* strain was generated by integration of plasmid AMp684, in which *CDC55* is cloned downstream of the *GAL1-10* promoter, at the NcoI site in the *URA3* locus. The copy number of *GAL-SGO1* and *GAL-CDC55* integrations are not known. The *scc1-tev* *GAL-TEV* strain was described previously (Uhlmann et al. 2000). The *SCC1-6HA* tag was described in (Megee et al. 1999). The *SLK19-13MYC* tag was described in (Lee et al. 2002). The *PDS1-3HA* tag was a gift from Angelika Amon. The *IPL1-6HA* tag was generated by the PCR-based method described in Knop et al. (1998) using the PCR product from primers 297 and 298 on plasmid AMp470 (Adele Marston). The *PDS1-18MYC* tag was described previously (Shirayama et al. 1998). The *3HA-CDC14* tag was described previously (Taylor et al. 1997). The *ESP1-18MYC* tag was described previously (Ciosk et al. 1998). The *3HA-CDC55* tag was generated by the PCR-based method described in Knop et al. (1999) using the PCR product from primers 656 and 657 on plasmid AMp650 (Adele Marston). The *CEN4-GFP* chromosome label was described previously (He et al. 2000). The *URA3-GFP* chromosome label was described previously (Michaelis et al. 1997). The *pds1A* allele was a gift from Breck Byers. The *mad1A* allele was gift from David Pellman. The *bub1A* allele was a gift from Brian Lee. The *ipl1-321* allele was described previously (Biggins et al. 1999). The *cdc55A* allele was generated by the PCR-based method described in Longtine *et al.* (1998) using the PCR product from primers 132 and 133 on plasmid AMp195 (Adele Marston). The *rts1A* allele was generated by transformation with DNA amplified from the genome of the *rts1A* deletion collection strain (Giaever *et al.* 2002) with primers 138 and 139 (Jin Shin). The *pph21-L369A* allele was generated by the PCR-based method described in Longtine *et al.* (1998) using the PCR product from primers 802 and 803 on plasmid AMp191 (Adele Marston). The *pph22-172*, *pph21A* and *pph3A* alleles were described previously.
The \textit{pph21}\Delta allele was generated by transformation with DNA amplified from the genome of the \textit{pph21}\Delta deletion collection strain (Giaever et al. 2002) with primers 1120 and 1121. The \textit{pph22}\Delta allele was generated by transformation with DNA amplified from the genome of the \textit{pph22}\Delta deletion collection strain (Giaever et al. 2002) with primers 808 and 809 (Adele Marston). The \textit{sgo1}\Delta allele was generated by the PCR-based method described in Longtine \textit{et al.} (1998) using the PCR product from primers 18 and 2 on plasmid AMp195 (Adele Marston). The \textit{ubr1}\Delta allele was a gift from Frank Stegmeier. The \textit{sgo1-3A} allele was described previously (Xu et al. 2009). The \textit{esp1-1} allele was described previously (Baum et al. 1988). The \textit{ite1}\Delta and \textit{spo12}\Delta alleles were described previously (Stegmeier et al. 2002). The \textit{pph21-148} and \textit{pph22-156} alleles are described in Appendix section A.3. The \textit{SCC1-NC} allele was described previously (Uhlmann et al. 1999). The \textit{PDS1-db} allele was described previously (Cohen-Fix et al. 1996). The \textit{cdc28-as1} allele was described previously (Bishop et al. 2000). The \textit{cdc28^{Y19F}} allele was a gift from Angelika Amon. The \textit{mih1}\Delta and \textit{swe1}\Delta alleles were described previously (Pal et al. 2008). The \textit{esp1}\Delta allele was generated by the PCR-based method described in Longtine \textit{et al.} (1998) using the PCR product from primers 494 and 495 on plasmid AMp195 (Adele Marston). The \textit{MET-PDS1} allele was generated by the PCR-based method described in Longtine \textit{et al.} (1998) using the PCR product from primers 7 and AA777 on plasmid AMp409 (Adele Marston). The \textit{MET-CDC20} allele was described previously (Ferrius and Marston 2009). The \textit{MET-SCC1} allele was generated by the PCR-based method described in Longtine \textit{et al.} (1998) using the PCR product from primers 107 and 108 on plasmid AMp295 (Adele Marston).
2.3.2 Yeast media

For solid media 2% (w/v) agarose was added prior to autoclaving

YEPD:
- 2% (w/v) Bacto-peptone
- 1% (w/v) Bacto-yeast extract
- 2% (w/v) Glucose
- 0.3 mM Adenine

YEPR:
- 2% (w/v) Bacto-peptone
- 1% (w/v) Bacto-yeast extract
- 2% (w/v) Raffinose
- 0.3 mM Adenine

YEPRG:
- 2% (w/v) Bacto-peptone
- 1% (w/v) Bacto-yeast extract
- 2% (w/v) Raffinose
- 2% (w/v) Galactose
- 0.3 mM Adenine

For dropout media, yeast nitrogen base without amino acids was combined with synthetic complete (SC) lacking the appropriate amino acid (aaa).

SC/-aaa/D:
- 1% (w/v) Yeast nitrogen base without amino acids
- 1x Formedium SC –aaa
- 2% Glucose
- 0.3 mM Adenine
2.3.2.1 Drugs

2.3.2.1.1 G418

Used for KanMX6 marker selection in solid media. G418 powder was dissolved in sterile water, filter sterilized and added to cooled melted agar-media at a final concentration of 300 µg/ml.

2.3.2.1.2 Hygromycin

Used for HphMX marker selection in solid media. Hygromycin solution (50 mg/ml) was added to cooled melted agar-media at a final concentration of 300 µg/ml.

2.3.2.1.3 Clonat

Used for NatMX4 selection in solid media. A 200 mg/ml stock solution was prepared in DMSO and stored at -20°C. Clonat stock solution was added to cooled melted agar-media at a final concentration of 100 µg/ml.

2.3.2.1.4 FOA

5-fluoroorotic acid (FOA) was used for URA counterselection. FOA powder was dissolved in synthetic complete media at a final concentration of 0.7g/l.

2.3.2.1.5 FAA

5-Fluoroanthranillic acid (FAA) was used for TRP counterselection. FAA powder was dissolved in synthetic complete media at a final concentration of 1g/l.

2.3.2.1.6 Benomyl

A 30 mg/ml stock solution was prepared in DMSO and stored at -20°C. Benomyl stock solution was added to boiling media to ensure it dissolves. For liquid media, a concentration of 30 µg/ml benomyl was used. For plates, concentrations of between 4 µg/ml and 20 µg/ml were prepared.
2.3.2.1.7  **Nocodazole**

A 1.5 mg/ml stock solution was prepared in DMSO and stored at -20°C. Nocodazole was added to liquid media at a final concentration of 15 µg/ml.

2.3.2.1.8  **1NM-PP1**

A 300 mM stock solution was prepared in DMSO and stored at -20°C. 1NM-PP1 was added to liquid media at a final concentration of 5 µg/ml.

2.3.2.1.9  **Phloxine B**

Used to stain dead/sick cells. Phloxine B powder was dissolved in sterile water, filter sterilized and added to melted agar-media at a final concentration of 1 µg/ml.

2.3.3  **Storage of yeast strains**

For frozen stocks, yeast were resuspended in 20% glycerol in cryotubes and stored long-term at -80°C.

2.3.4  **Yeast growth conditions**

Strains were taken from frozen stocks onto solid media 1-2 days prior to use. Cells were inoculated into flasks containing 1/5 volume media and grown with aeration on a shaking platform.

2.3.5  **Yeast transformations**

2.3.5.1  **Preparation of carrier DNA**

Carrier DNA from salmon testes was used to improve transformation efficiency. 1 gram of DNA sodium salt from salmon testes (Sigma) was hydrated in 100 ml of sterile water overnight to give a final concentration of 10 mg/ml. The solution was then sonicated using Branson Sonifier 450 sonicator at output 6, duty cycle 100% for 7 minutes on ice with 30 second intervals, then autoclaved at 120°C and 15 pounds/inch² for 15 minutes. 1 ml aliquots were stored long term at -20°C.
2.3.5.2 High efficiency yeast transformation

Transformation mix:

- 40% (w/v) PEG 4000
- 0.1 M LiAc
- 280 µg/ml carrier DNA

50 ml cultures were grown overnight to OD\textsubscript{600} = 0.8 – 3.0. The next morning, cultures were diluted to OD\textsubscript{600} = 0.2 and grown until OD\textsubscript{600} = 0.7-1.0. Cells were spun down in 50 ml falcon tubes at 3600 rpm for 3 minutes and washed twice with sterile distilled water and transferred to 1.5 ml tubes. Cells were washed once with 1 ml of 0.1 M LiAc and resuspended in 250 µl of 0.1M LiAc. For each transformation 50 µl of yeast suspension, 5-10 µl of DNA and 300 µl of transformation mix were added to 1.5 ml tubes. Tubes were incubated at 30°C for 30 minutes and then at 42°C for 15 minutes. Tubes were spun at 3000 rpm for 3 minutes, cell pellets resuspended in 200 µl of water and plated out.

2.3.6 Crossing strains

Sporulation plates:

- 1% (w/v) Potassium acetate
- 2% Agarose
- 1x Synthetic complete

To cross strains, haploid \textit{MATa} and \textit{MATa} cells were mated, and resulting diploids selected for and sporulated. For mating and selecting for diploids, a small amount of one strain containing a unique selectable marker was mixed with excess of another strain of the opposite mating type on solid media and left to mate overnight. The mating was streaked onto a selection plate and incubated until single colonies appear. The high efficiency of mating means that most colonies were diploids. From one mating, 6 or more diploid colonies were picked and plated onto sporulation plates and incubated for 2-3 days until tetrads form.
2.3.7 Tetrad dissection

Matings plated onto sporulation plates were checked for tetrads using a light microscope. A tiny amount of tetrads (< 1mm³) were resuspended in 20 µl of 1 mg/ml zymolase 100T (AMS biotechnology) in 1M sorbitol and incubated for 8 minutes. 1 ml of sterile water was added to dilute the zymolase and tetrads and then 20 µl of tetrads were spread on solid media 10 cm plates and allowed to dry. Tetrads were then dissected using a Nikon Eclipse 50i microscope equipped with micromanipulator and incubated until spores proliferate to form colonies.

2.3.8 Yeast cell cycle analysis

Details of individual experiments are given in the figure legends. The α-factor peptide (WHWLQLKPGQPMY) was synthesised by Peptide Protein Research. A 5 mg/ml stock of α-factor was prepared in DMSO and stored at -20°C.

2.3.8.1 α-factor arrest in G₁ and release

Typically cultures were grown overnight until OD₆₀₀ = 0.8 – 3.0. The next morning cultures were diluted to OD₆₀₀ = 0.2 and grown for ~3 hours until OD₆₀₀ = 0.4. Cultures were then diluted back to OD₆₀₀ = 0.2 and 5 µg/ml of α-factor added to begin arrest. After 1.5 hours, 2.5 µg/ml of α-factor was re-added. A good G₁ arrest was judged as > 95% of cells unbudded, typically after 2.5 – 3 hours in α-factor. For a synchronous release from G₁, cells were by collected onto 0.45 µm membrane filters (Whatman) using a Konte filtration system (Thistle Scientific), washed with 10x culture volume of media and resuspended in the appropriate release media. For some experiments, 5 µg/ml of α-factor was added after release when cells had small buds (and therefore were no longer in G₁) to limit analysis to one cell cycle.
2.3.8.2 Temperature sensitive alleles

Cells were G₁-arrested at the permissive temperature and released at the non-permissive temperature. Release media and wash media were pre-warmed to the non-permissive temperature before release from G₁. Cultures at the non-permissive temperature were grown on shaking platform in a water bath to ensure a constant temperature.

2.3.8.3 Expression from galactose promoter

Cells were G₁-arrested in YEPR, and 2% galactose added 30 minutes prior to release from G₁ into YEPRG.

2.3.8.4 Repression using methionine promoter

For MET-SCC1 experiments, cells were grown in SC/-met/D before arresting with α-factor in YEPD + 8mM methionine. Cells were released from G₁ into YEPD + 8mM methionine and 4mM methionine re-added every hour.

For MET-PDS1 and MET-CDC20 experiments, cells were grown, then G₁-arrested in, SC/-met/D and released from G₁ into YEPD + 8mM methionine 4mM methionine re-added every hour. For Cdc20 re-addition, cells were collected onto membrane filters, washed with SC/-met/D and resuspended into SC/-met/D.

2.3.8.5 Microtubule depolymerization

For experiments requiring microtubule depolymerization, 30 µg/ml of benomyl was added to boiling media to ensure it dissolves, and the media allowed to cool before use. Cells were released from G₁ into media containing 30 µg/ml benomyl and 15 µg/ml nocodazole. Cells were typically exposed to benomyl/nocodazole for 3 hours, with 7.5 µg/ml of nocodazole re-added every hour. Cells were released from benomyl/nocodazole by collection onto membrane filters using a Konte filtration system, washing with 10x culture volume of media and resuspended in the appropriate release media.
2.4 MICROSCOPY METHODS

2.4.1 Antibodies used for immunofluorescence

Table 2.3 Antibodies used for immunofluorescence in this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Origin</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mono Tubulin-α</td>
<td>Rat</td>
<td>AbD serotec</td>
<td>1 mg/ml</td>
</tr>
<tr>
<td>Mono 9E10 (c-myc)</td>
<td>Mouse</td>
<td>Covance</td>
<td>3-5 mg/ml</td>
</tr>
<tr>
<td>Mono HA.11</td>
<td>Mouse</td>
<td>Covance</td>
<td>2-3 mg/ml</td>
</tr>
<tr>
<td>anti-rat FITC</td>
<td>Donkey</td>
<td>Jackson immuno re</td>
<td>1.25 mg/ml</td>
</tr>
<tr>
<td>anti-mouse Cy3</td>
<td>Donkey</td>
<td>Jackson immuno re</td>
<td>1.25 mg/ml</td>
</tr>
</tbody>
</table>

2.4.2 Pre-absorbing antibodies for immunofluorescence

Antibodies were aliquoted into 200 µl aliquots in 1.5 ml tubes. 200 µl of fixed wildtype yeast cells (fixed and washed as is section 2.4.3) were added and tubes incubated on rotating wheel at room temperature for 20 minutes. Tubes were spun at 3000 rpm for 3 minutes and the supernatant transferred to a new tube. 200 µl of fixed cells were added and the procedure repeated 5 more times to achieve a 6-fold dilution of the antibody. After pre-absorbing, 0.01% NaN₃ was added, the antibody dilution distributed in 50 µl aliquots and stored long-term at -20°C. Once pre-absorbed antibodies were thawed they were stored at 4°C.
2.4.3 Whole cell Immunofluorescence

Sorbitol-citrate:
1.2 M Sorbitol
0.1 M K$_2$HPO$_4$
36 mM Citric acid

PBS-BSA:
1% (w/v) BSA
0.04 M K$_2$HPO$_4$
0.01 M KH$_2$PO$_4$
0.15 M NaCl
0.1% NaN$_3$

DAPI-mount:
9 mM p-phenylenediamine
0.04 M K$_2$HPO$_4$
0.01 M KH$_2$PO$_4$
0.15 M NaCl
0.1% NaN$_3$
50 ng/ml DAPI
90% (w/v) Glycerol

1 ml of culture from cell cycle timecourse was spun at 13,000 rpm for 1 minute and the cell pellet resuspended in 1 ml of 3.7% formaldehyde in 0.1M K Phosphate buffer pH 6.4. Cells were fixed in formaldehyde for between 10 minutes and overnight depending on the protein of interest (Table 2.4). Fixed cells were washed 3 times with 1 ml of 0.1M K Phosphate buffer pH 6.4 and resuspended in 1 ml of sorbitol-citrate. Cells were spun down, resuspended in 200 µl sorbitol citrate with 100 µg/ml zymolase 100T and 10 % glusolase (Perkin Elmer) and incubated at 30°C for 15-45 minutes, digested cells were judged as phase-dark under the light microscope. Digested cells were spun down slowly at 3000 rpm for 3 minutes, washed twice with 1 ml of sorbitol-citrate and resuspended in ~ 50 µl of sorbitol-citrate. 5 µl of cells per well were placed on multi-well polylysine slides and left for
10 minutes. Wells were aspirated and slides then fixed in methanol for 3 minutes, then immediately in acetone for 10 seconds and allowed to dry. 5 µl of primary antibody solution (Table 2.4) was added per well and the slides incubated in a moisture chamber for 2 hours at room temperature. Wells were washed 5 times with PBS-BSA, then 5 µl of secondary antibody solution (Table 2.4) added per well and slides incubated in moisture chamber in dark for 2 hours. Wells were washed 5 times with 5 µl of PBS-BSA and then 3 µl of DAPI-mount added per well. Slides were topped with glass coverslips, sealed with nail polish and used for microscopy or stored long-term at -20°C.

Table 2.4 Fixation times and antibody dilutions for whole-cell immunofluorescence

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fixation time</th>
<th>Primary antibody</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ipl1-6HA</td>
<td>10 minutes</td>
<td>ms α-HA 1:250</td>
<td>α-ms CY3 1:1000</td>
</tr>
<tr>
<td>Tubulin</td>
<td>Overnight (4°C)</td>
<td>rat α-TUB 1:50</td>
<td>α-rat FITC 1:100</td>
</tr>
<tr>
<td>Cdc14-3HA</td>
<td>10 minutes</td>
<td>ms α-HA 1:150</td>
<td>α-ms CY3 1:500</td>
</tr>
<tr>
<td>Pds1-18MYC</td>
<td>10 minutes</td>
<td>ms α-MYC 1:250</td>
<td>α-ms CY3 1:1000</td>
</tr>
<tr>
<td>Pds1-3HA</td>
<td>10 minutes</td>
<td>ms α-HA 1:250</td>
<td>α-ms CY3 1:500</td>
</tr>
</tbody>
</table>

Pre-absorbed antibodies were diluted in PBS-BSA.
2.4.4 Chromosome spreads

Sol1:

200 mM Potassium acetate
0.8% (w/v) Sorbitol

Sol2:

0.1 M MES
1 mM EDTA
0.5 mM MgCl$_2$
1 M Sorbitol
pH to 6.4 with NaOH

Fixative:

4% (w/v) Paraformaldehyde
3.4% (w/v) Sucrose

7 ml of culture from cell cycle timecourse was spun down in 15 ml falcon tubes at 3600 rpm for 2 minutes, most of the supernatant was discarded and cells were transferred to 2 ml tubes using the residual supernatant. Cells were spun down, resuspended in 200 µl of Sol1 + 2 µl of 1M DTT + 2.6 µl of 10 mg/ml zymolase and incubated at 37°C for 10-20 minutes until cells were digested. Tubes were filled with ice-cold Sol2 to stop digestion, spun slowly at 2000 rpm for 4 minutes and the cell pellet resuspended in 200 µl of ice-cold Sol2 and stored on ice for up to 3 hours. Spreads were performed by adding 15 µl of cells, then 30 µl of fixative, then 60 µl of lipsol (Western Laboratory Science) and then 60 µl of fixative onto a glass slide and the mixture spread evenly across the slide using a glass rod. Slides were allowed to dry overnight before immunofluorescence.
2.4.5 Immunofluorescence of chromosome spreads

PBS:
- 13.7 mM NaCl
- 270 µM KCl
- 1 mM Na₂PO₄
- 176 µM KH₂PO₄

Blocking buffer:
- 0.2% (w/v) Gelatin
- 0.5% (w/v) BSA
- Made in PBS

Dried spread slides were incubated in PBS for 10 minutes at room temperature. The 60 µl of blocking buffer was added to the slides and incubated under coverslips for 30 minutes. Coverslips were washed off using PBS and 60 µl of primary antibody solution were added and incubated under coverslips for overnight at 4°C in a moisture chamber. Coverslips were washed off using PBS and slides were incubated in PBS for 10 minutes at room temperature. 60 µl of secondary antibody solution were added and incubated under coverslips for 4 hours at 4°C in a moisture chamber. Coverslips were rinsed off with PBS. Several drops of Vectashield DAPI mounting media were added and covered with a coverslip. Slides were stored long term at -80°C.

Table 2.5 Antibody dilutions for immunofluorescence of chromosome spreads

<table>
<thead>
<tr>
<th>Protein</th>
<th>Primary antibody</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scc1-6HA</td>
<td>ms α-HA 1:1000</td>
<td>α-ms CY3 1:1000</td>
</tr>
</tbody>
</table>

Pre-absorbed antibodies were diluted in PBS-BSA.
2.4.6 Visualizing GFP-labelled chromosomes

1 ml of culture from a cell cycle timecourse was added directly to tubes containing 100 µl of 37% formaldehyde and cells fixed for 5-10 minutes at room temperature. Tubes were spun in a microcentrifuge at 13,000 rpm for 1 minute, washed with 1 ml of 80% ethanol and the cell pellet resuspended in 20 µl of 1 µg/ml DAPI. Cells were briefly sonicated (1 pulse with micro tip at 40 amp) before use. Typically 5 µl of cells were placed onto superfrost slides (BDH) and covered with a glass coverslip before microscopy.

2.4.7 Microscopy

For fluorescence microscopy, samples were analyzed on a Zeiss Axioplan 2 microscope and images were grabbed using a Hamamatsu camera operated through Axiovision software.

2.4.7.1 Spindle morphology analysis

To monitor cell cycle progression, the morphology of the mitotic spindle was analysed after tubulin immunofluorescence. Cells with a short, bar-like, spindle within the mother cell were scored as metaphase. Cells with an elongated spindle spanning the bud neck were scored as anaphase. At least 200 cells were scored.

2.4.7.2 Analysis of Scc1 on chromosome spreads

To determine the association of Scc1 with chromatin, Scc1-6HA was analysed by anti-HA immunofluorescence of chromosome spreads. Scc1 was judged to be associated with chromatin if the Scc1-6HA signal colocalized with the DAPI signal. At least 100 cells were scored.

2.4.7.3 Cdc14 localization

Cdc14 localization was determined after whole cell immunofluorescence. Cells with a discrete 3HA-Cdc14 signal adjacent to the DAPI signal were scored as having Cdc14 sequestered in the nucleolus. Cells with a diffuse 3HA-Cdc14 signal that
either co-localised with the DAPI signal, or co-localised with the DAPI signal and spread throughout the cytoplasm were scored as having released Cdc14 from the nucleolus. At least 200 cells were scored.

2.4.7.4 Analysis of Pds1 staining

To identify Pds1-positive cells, either Pds1-18MYC or Pds1-3HA localization was analysed by whole cell immunofluorescence. Pds1-positive cells were scored on the bases of a Pds1-18MYC or Pds1-3HA signal that colocalized with the DAPI signal. At least 200 cells were scored.

2.4.7.5 GFP-labelled sister chromosome analysis

Sister chromatids were scored as separated if two GFP dots could be resolved within a single cell. At least 200 cells were scored.
2.5 PROTEIN ANALYSIS

2.5.1 TCA protein extract preparation

Trichloroacetic acid (TCA) protein extraction method was used for protein analysis from cell cycle cultures for rapid protein precipitation and preservation of protein phosphorylation.

TE (pH 7.5):

10 mM Tris-HCl
1 mM EDTA

3x SDS sample buffer:

187 mM Tris (pH 6.8)
6% (w/v) β-mercaptoethanol
30% (w/v) Glycerol
9% (w/v) SDS
0.05% (w/v) Bromophenol blue

5 ml of culture from cell cycle timecourse was spun down in 15 ml falcon tubes at 3600 rpm for 2 minutes. The supernatant was discarded and cell pellets resuspended in 5 ml of 5% TCA and incubated in ice for 10 minutes. Tubes were spun at 3600 rpm for 2 minutes at 4°C, most of the supernatant was removed and pellets were transferred to 2 ml Fastprep tubes (MP Biomedicals) with residual liquid. Tubes were spun briefly at 13000 rpm, the supernatent removed and tubes stored at -80°C. Pellets were then resuspended in 1 ml of acetone by vortexing, spun down at 14000 rpm for 7 minutes and the acetone removed. Pellets were left to dry in a fume hood for 2-3 hours. Acetone washed and dried pellets were resuspended in 100 µl of ice-cold TE plus 2.75 µl of 1M DTT and 1x Roche EDTA-free protease inhibitors and 100 µl of acid-washed glass beads (Sigma) were added. Tubes were subjected to 3 x 45 seconds on Fastprep Bio-pulverizer FP120 at speed 6.0, 50 µl of 3x SDS sample buffer was added and the extracts incubated at 95°C for 5 minutes before loading on a gel. Bradford assay was not used to determine protein concentrations of TCA.
extracts. Instead, western blotting against either Pgk1 or Kar2, whose levels do not alter during the cell cycle, was used to control for equal loading.

2.5.2 Protein extract preparation without TCA

When rapid protein extraction and preservation of protein phosphorylation were not essential, protein extract preparation without TCA was used. 10 ml cultures were grown overnight to saturation. Cultures were spun at 3600 rpm for 3 minutes, cell pellets were resuspended in 1 ml of ice-cold 10 mM Tris pH 7.5 and transferred to Fastprep tubes. Tubes were spun quickly at 4°C, the supernatent aspirated and tubes snap-frozen in liquid nitrogen and stored at -80°C. Pellets were then resuspended in 100 µl of ice-cold TE plus 2.75 µl of 1M DTT and 1x Roche EDTA-free protease inhibitors and 100 µl of glass beads (sigma) were added. Tubes were then subjected to 3 times 45 seconds on Fastprep Bio-pulverizer FP120 at speed 6.0 and 10 µl of extract taken for Bradford assays (see section 2.5.3). To the remaining extract 50 µl of 3x SDS sample buffer was added and the extracts incubated at 95°C for 5 minutes before loading on a gel.

2.5.3 Bradford assay

Solubilizing solution:

50 mM Tris-HCl pH 7.5
0.3 M NaCl

Before adding sample buffer, 10 µl of protein extracts were added to tubes containing 20 µl of solubilizing solution. Tubes were vortexed and spun at 13000 rpm for 3 minutes. 3 µl of spun solubilized extracts were added to 1 ml cuvettes containing 1 ml of Bradford reagent (Bio-Rad). Cuvettes were mixed well and OD_{595} measured. Protein concentration was calculated against a standard BSA curve
2.5.4 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

4x Resolving buffer
1.5 M Tris
0.4% (w/v) SDS
pH adjusted to 8.8 with glacial acetic acid

2x Stacking buffer
0.25 M Tris
0.2% (w/v) SDS
pH adjusted to 8.8 with glacial acetic acid

Table 2.6 Polyacrylamide gel recipe (amounts per one gel):

<table>
<thead>
<tr>
<th></th>
<th>10% resolving gel</th>
<th>8% resolving gel</th>
<th>6% resolving gel</th>
<th>4% stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% acrylamide : 0.8% bis-acrylamide (National Diagnostics)</td>
<td>10 ml</td>
<td>8 ml</td>
<td>6 ml</td>
<td>2 ml</td>
</tr>
<tr>
<td>4x Resolving buffer</td>
<td>7.5 ml</td>
<td>7.5 ml</td>
<td>7.5 ml</td>
<td>-</td>
</tr>
<tr>
<td>2x stacking buffer</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>Water</td>
<td>12.5 ml</td>
<td>14.5 ml</td>
<td>16.5 ml</td>
<td>5.3 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>450 µl</td>
<td>450 µl</td>
<td>450 µl</td>
<td>150 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>30 µl</td>
<td>30 µl</td>
<td>30 µl</td>
<td>15 µl</td>
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</tbody>
</table>

Two glass plates were assembled with a 1.5mm gap and the edges sealed with 1% agarose. Polyacrylamide resolving gels prepared according to Table 2.5 were poured between the two glass plates, topped with a layer of isopropanol and allowed to polymerise. The isopropanol was then removed, the stacking gel poured and a comb of 20 wells inserted into the stacking gel. After polymerization, the comb was removed and the wells rinsed with water. Gels were run using Biometra V15.17 electrophoresis apparatus in 1x running buffer at 65 mA until samples reached the resolving gel and then at 12mA overnight. A pre-stained protein marker (NEB) was loaded alongside samples.
2.5.4.1 Mn$^{2+}$-Phos-Tag SDS-PAGE

Gels were prepared and run according to section 2.5.4 except that 150 µl of 10 mM MnCl$_2$ and 150 µl of 5 mM Phos-tag were added prior to the addition of APS and TEMED. In addition, gels were overlayed with water instead of isopropanol and pre-stained protein marker was loaded at least 2 wells away from samples.

2.5.5 Western blotting

Transfer buffer:
- 25 mM Tris
- 1.5% (w/v) Glycine
- 0.02% (w/v) SDS
- 10% (v/v) Methanol

Ponceau S:
- 0.47 % (w/v) Ponceau S
- 3 % (w/v) Trichloroacetic Acid
- 1% (v/v) Acetic Acid

PBS-T:
- 0.1% Tween-20 in PBS

Antibody buffer:
- 1% milk and 1% BSA in PBS-T

Following electrophoresis, gels were transferred onto protran BA 85 nitocellulose membrane (Whatman) using Amersham TE70 semi-dry transfer unit according to manufacturers instructions. The membrane and blotting paper (Amersham) were soaked in transfer buffer prior to transfer at 1 mA/cm$^2$ for 2.5 hours. After transfer, membranes were stained with Ponceau S and then blocked by incubating in 3% milk in PBS-T. Membranes were then incubated with primary antibody (diluted in antibody buffer) overnight at 4°C. Membranes were then washed 3x for 15 minutes with PBS-T. Membranes incubated with the Clb2 antibody were washed with PBS-T + 500 mM NaCl. After washes, membranes were incubated with secondary antibody (diluted in antibody buffer) for 1 hour at room temperature. Membranes were then washed 3x for 5 minutes with PBS-T and subjected to chemiluminescence using the
SuperSignal West Pico chemiluminescent kit (Thermo scientific) according to manufacturers instructions. Membranes incubated with the Phospho-Cdc2 (Y15) antibody were subjected to chemiluminescence using the SuperSignal West Femto chemiluminescent kit (Thermo scientific) according to manufacturers instructions. Membranes were wrapped in Saran wrap and exposed to Kodak Bio-Max light film. Films were developed using Konica-Minolta SRX-101A developer.

Table 2.7 Antibodies used for western blots in this study

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<th>Antibody</th>
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<th>Concentration</th>
<th>Dilution used</th>
<th>Origin</th>
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<td>Mouse</td>
<td>3-5 mg/ml</td>
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<td>Covance</td>
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<tr>
<td>Mono HA.11</td>
<td>Mouse</td>
<td>2-3 mg/ml</td>
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<td>Clb2</td>
<td>Rabbit</td>
<td>-</td>
<td>1:10000</td>
<td>Fred Cross (gift)</td>
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<tr>
<td>Phospho-Cdc2 (Y15)</td>
<td>Rabbit</td>
<td>-</td>
<td>1:1000</td>
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<td>Invitrogen</td>
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<td>Kar2</td>
<td>Rabbit</td>
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<td>Rabbit-HRP</td>
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### 2.6 NUCLEIC ACIDS

#### 2.6.1 Plasmids

Table 2.8 Plasmids used in this study

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<td>AMp37</td>
<td><em>Yiplac211-pGAL-SGO1</em></td>
<td><em>SGO1</em> ORF cloned from wild type (AM1176) yeast genomic DNA with primers 14 and 15 into BamHI and Sall sites downstream of <em>GAL1-10</em> promoter in plasmid AMp73</td>
<td>Adele Marston</td>
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<tr>
<td>AMp58</td>
<td><em>Yiplac211</em></td>
<td>Described previously (Gietz and Sugino 1988)</td>
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<td>AMp73</td>
<td><em>Yiplac211-pGAL</em></td>
<td><em>GAL-10</em> promoter cloned into EcoR1 and BamH1 sites of plasmid AMp58</td>
<td>Angelika Amon</td>
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<td>AMp77</td>
<td><em>Yiplac204</em></td>
<td>Described previously (Gietz and Sugino 1988)</td>
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<td>Amp168</td>
<td><em>YCp50-LTE1</em></td>
<td>Plasmid <em>YCp50</em> carrying <em>LTE1</em>. Isolated from Sau3A partial digest genomic DNA library</td>
<td>Frank Stegmeier</td>
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<td>AMp181</td>
<td><em>Yiplac204-pGAL</em></td>
<td><em>GAL-10</em> promoter cloned into EcoR1 and BamH1 sites of plasmid AMp77</td>
<td>Rosella Visintin</td>
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<td><em>pFA6a-His3MX6</em></td>
<td>Described previously (Longtine et al. 1998)</td>
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<td>AMp193</td>
<td><em>pFA6a-kanMX6-pGal1-3HA</em></td>
<td>Described previously (Longtine et al. 1998)</td>
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<td><em>pFA6a-kanMX6</em></td>
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<td>Described previously (Longtine et al. 1998)</td>
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<td><em>MET3</em> promoter cloned into plasmid AMp58</td>
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<td>AMp404</td>
<td><em>pFA6a-ESP1-18MYC-TRP1</em></td>
<td>ESP1 promoter sequence and ORF upstream of 18MYC sequence cloned into plasmid AMp196</td>
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<td><em>MET3</em> promoter cloned into plasmid p195</td>
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<td><em>Yiplac204-pGAL-CDC55</em></td>
<td><em>CDC55</em> ORF cloned from wild type (AM1176) yeast genomic DNA with primers 659 and 660 into Sall and BglIII sites downstream of <em>GAL1-10</em> promoter in plasmid AMp181</td>
<td>Adele Marston</td>
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<td>AMp650</td>
<td>pFA6a-kanMX6-pCDC55-3HA</td>
<td>CDC55 promoter cloned from wild type (AM1176) yeast genomic DNA with primers 664 and 665 into BglII and PacI sites in plasmid AMp193</td>
<td>Adele Marston</td>
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<td>AMp684</td>
<td>YIplac211-pGAL-CDC55</td>
<td>CDC55 ORF cloned from wild type (AM1176) yeast genomic DNA with primers 659 and 660 into BamHI and SalI sites downstream of GAL1-10 promoter in plasmid AMp73</td>
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<td>AMp691</td>
<td>pRS314</td>
<td>Described previously (Sikorski and Hieter 1989)</td>
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<td>pRS313</td>
<td>Described previously (Sikorski and Hieter 1989)</td>
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<td>AMp693</td>
<td>pRS415-ESPl</td>
<td>ESPl promoter sequence and ORF cloned into SalI and ScaI site of pRS415. Described previously (Jensen et al. 2001)</td>
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<td>AMp694</td>
<td>YIplac204-PPH21</td>
<td>PPH21 promoter sequence and ORF cloned from wild type (AM1176) yeast genomic DNA with primers 1116 and 1117 into BamHI and PstI sites in plasmid AMp77</td>
<td>This study</td>
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<tr>
<td>AMp695</td>
<td>YIplac204-PPH22</td>
<td>PPH22 promoter sequence and ORF cloned from wild type (AM1176) yeast genomic DNA with primers 1118 and 1119 into BamHI and PstI sites in plasmid AMp77</td>
<td>This study</td>
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<td>AMp696</td>
<td>YIplac204-pph21-148</td>
<td>Site-directed mutagenesis performed on plasmid AMp694 using primers 1143 and 1144 with Quikchange II XL kit. G422 to A mutation corresponding to D148 to N in protein.</td>
<td>This study</td>
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<td>AMp697</td>
<td>YIplac204-pph22-156</td>
<td>Site-directed mutagenesis performed on plasmid AMp696 using primers 1143 and 1144 with Quikchange II XL kit. G466 to A mutation corresponding to D156 to N in protein.</td>
<td>This study</td>
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<td>AMp698</td>
<td>YIplac211-PPH21</td>
<td>BamH1/PstI fragment containing PPH21 promoter sequence and ORF was cloned from plasmid AMp694 into BamH1/PstI sites of plasmid AMp58.</td>
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<td>AMp699</td>
<td>YIplac211-PPH22</td>
<td>BamH1/PstI fragment containing PPH22 promoter sequence and ORF was cloned from plasmid AMp695 into BamH1/PstI sites of plasmid AMp58.</td>
<td>This study</td>
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<td>AMp701</td>
<td>YIplac211-pph22-156</td>
<td>BamH1/PstI fragment containing pph22-156 promoter sequence and ORF was cloned from plasmid AMp697 into BamH1/PstI sites of plasmid AMp58.</td>
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<td>AMp707</td>
<td>pRS314-ESP1</td>
<td>Sall/SacI fragment containing ESP1 promoter sequence and ORF cloned from AMP693 into Sall and SacI site of plasmid AMP691</td>
<td>This study</td>
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<tr>
<td>AMp708</td>
<td>pRS313-ESP1</td>
<td>Sall/SacI fragment containing ESP1 promoter sequence and ORF cloned from plasmid AMP693 into Sall and SacI site of plasmid AMP691</td>
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<td>AMp713</td>
<td>pRS313-esp1(T1014A)</td>
<td>Site-directed mutagenesis performed on plasmid AMp708 using primers 714 and 715 with Quikchange II XL kit. A3040 to G mutation corresponding to T1014 to A in protein.</td>
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<td>AMp714</td>
<td>pRS313-esp1(S1027A)</td>
<td>Site-directed mutagenesis performed on plasmid AMp708 using primers 716 and 717 with Quikchange II XL kit. T3079 to G mutation corresponding to S1027 to A in protein.</td>
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<td>AMp715</td>
<td>pRS313-esp1(S1027D)</td>
<td>Site-directed mutagenesis performed on plasmid AMp708 using primers 722 and 723 with Quikchange II XL kit. T3079 to G, C3080 to A and A3081 to T mutations corresponding to S1027 to D in protein.</td>
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<td>AMp716</td>
<td>pRS313-esp1(T1014D)</td>
<td>Site-directed mutagenesis performed on plasmid AMp708 using primers 720 and 721 with Quikchange II XL kit. A3040 to G and C3041 to A mutations corresponding to T1014 to D in protein.</td>
<td>This study</td>
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<td>AMp717</td>
<td>pRS313-esp1(T1014D;S1027D)</td>
<td>Site-directed mutagenesis performed on plasmid AMp716 using primers 722 and 723 with Quikchange II XL kit. A3040 to G and C3041 to A mutations corresponding to T1014 to D in protein. T3079 to G, C3080 to A and A3081 to T mutations corresponding to S1027 to D in protein.</td>
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<tr>
<td>AMp719</td>
<td>pRS313-esp1(S13A)</td>
<td>Site-directed mutagenesis performed on plasmid AMp708 using primers 1245 and 1246 with Quikchange II XL kit. A37 to G and G38 to C mutations corresponding to S13 to A in protein.</td>
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<td>AMp720</td>
<td>pRS313-esp1(S13D)</td>
<td>Site-directed mutagenesis performed on plasmid AMp708 using primers 1247 and 1248 with Quikchange II XL kit. A37 to G and G38 to A mutations corresponding to S13 to D in protein.</td>
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<td>AMp721</td>
<td>pRS313-esp1(T16D)</td>
<td>Site-directed mutagenesis performed on plasmid AMp708 using primers 1251 and 1252 with Quikchange II XL kit. A46 to G and C47 to A mutations corresponding to T16 to D in protein.</td>
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<tr>
<td>AMp724</td>
<td>pRS313-esp1(T16A)</td>
<td>Site-directed mutagenesis performed on plasmid AMp708 using primers 1249 and 1250 with Quickchange II XL kit. A46 to G mutation corresponding to T16 to A in protein.</td>
<td>This study</td>
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<td>AMp735</td>
<td>pRS313-esp1*50</td>
<td>Plasmid AMp708 propagated through XL1-red E.coli for 50 generations</td>
<td>This study</td>
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<tr>
<td>AMp740</td>
<td>pRS313-esp1*100</td>
<td>Plasmid AMp708 propagated through XL1-red E.coli for 100 generations</td>
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<td>AMp742</td>
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<td>T4676 to C mutation within ESP1 ORF. Isolated from screen for temperature-sensitive esp1 alleles using plasmid AMp735</td>
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<td>AMp744</td>
<td>pRS313-esp1(E715K)</td>
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<td>AMp745</td>
<td>pRS313-esp1(Y648C)</td>
<td>A1943 to G mutation within ESP1 ORF. Isolated from screen for temperature-sensitive esp1 alleles using plasmid AMp735</td>
<td>This study</td>
</tr>
<tr>
<td>AMp750</td>
<td>pFA6a-esp1(T1014A)-18MYC-TRP1</td>
<td>Sph1 fragment from plasmid AMP404 (wildtype ESP1) was replaced with Sph1 fragment from plasmid AMp713 (ESP1 containing T1014A mutation)</td>
<td>This study</td>
</tr>
</tbody>
</table>
2.6.2 *E.coli* plasmid mini-prep

GTE:

- 50 mM Glucose
- 10 mM EDTA
- 25 mM Tris
- pH adjusted to 7.5 with HCl

Alkaline SDS:

- 200 mM NaOH
- 1% (w/v) SDS

High salt buffer:

- 2.5 M Potassium acetate
- pH adjusted to 4.8 with glacial acetic acid

TE: (see section 2.5.1)

*E.coli* colonies were inoculated into 2 ml of LB-Amp and incubated at 37°C overnight. 1.5 ml of the cultures were transferred to 1.5 ml tubes and spun at 13000 rpm for 1 minute. The supernatants were discarded and the cell pellets resuspended in 100 µl GTE by vortexing. 200 µl of alkaline SDS and 100 µl of high salt buffer were added, mixed by vortexing briefly, and incubated on ice for 15 minutes. Tubes were spun at 13000 rpm for 5 minutes and the supernatants transferred to new 1.5 ml tubes containing 1 ml of cold ethanol. Tubes were spun at 13000 rpm for 5 minutes and the supernatants discarded. Cell pellets were washed with 100 µl of 70% ethanol, air-dried, and resuspended in 50 µl of TE. Mini-preps were stored long-term at -20°C.
2.6.3 Yeast plasmid mini-prep

Plasmid DNA was extracted from yeast using the Zymoprep I kit (Zymo Research) according to manufacturers instructions.

2.6.4 Genomic DNA extraction from yeast

DNA breakage buffer:
   2% (v/v) Triton X-100
   1% (w/v) SDS
   100 mM NaCl
   10 mM Tris-HCl pH 8.0
   1 mM EDTA

TE: (see section 2.5.1)

Approximately 5 mm$^3$ of yeast cells were scraped from solid media and resuspended in 200 µl of DNA breakage buffer in 1.5 ml tubes. 100 µl of glass beads and 200 µl of phenol chloroform were added and the mixture vortexed for 4 minutes. Tubes were spun down at 14000 rpm for 5 minutes and the upper aqueous phase (~130 µl) transferred to new tubes containing 1 ml of cold ethanol. Tubes were mixed by inversion then spun at 14000 rpm for 5 minutes, the supernatant discarded and the pellets air-dried. Pellets were resuspended in 50 µl of TE
2.6.5 Agarose gel electrophoresis

10x TAE:
0.4M Tris
10mM EDTA
1.1% (v/v) Glacial acetic acid

10x DNA loading buffer:
1mM EDTA
0.25% (w/v) Bromophenolblue
0.25% (w/v) Xylencyanoblu
50% (v/v) Glycerol

DNA was analysed using agarose gel electrophoresis with ethidium bromide staining. Typically 0.6 – 1.5 % gels were prepared by dissolving Sea-Kem agarose in 1x TAE buffer using a microwave. A final concentration of 0.5 µg/ml ethidium bromide was added to cooled agarose prior to pouring gel. DNA samples were loaded with 1x DNA loading buffer and run at a constant voltage of 90-120 V depending on the gel size. 1kb DNA ladder was used as a band size marker. Bands were visualised using UV transilluminator-based gel-doc system (Syngene).

2.6.6 DNA extraction from agarose gel

Bands were visualized using a UV transilluminator and excised with a scalpel. To purify DNA fragments the Qiagen Gel Extraction Kit was used following the manufacturer’s protocol.
## 2.6.7 PCR

### 2.6.7.1 Oligonucleotides

Table 2.9 Oligonucleotides used in this study

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GAATTTCAGCTCGTTTAAAAC

AATTTGGGAAAAGAGTCGTTGG

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<tr>
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</tr>
<tr>
<td>AA777</td>
<td>CGGCAGAAGGCAAGTAACCGGTTGCTAGCGGTTTTCATTCACATACTCATTTAAAC</td>
</tr>
</tbody>
</table>
2.6.7.2 Polymerase chain reaction

10x PCR buffer:
   100 mM Tris-HCl pH 8.3
   500 mM KCl
   20 mM MgCl₂
   0.1% (w/v)

PCR reaction:
   10-500 ng template DNA
   1 µM each oligonucleotide primer
   0.2 mM dNTPs
   1x PCR buffer
   DNA polymerase*
   Sterile distilled water

*For most applications, Taq polymerase purified in the lab was used. When high fidelity was essential or large PCR products expected ExTaq (TaKaRa) was used with dNTPs and buffer supplied by the manufacturer.

PCR programmes varied depending on the size of the expected product and the annealing temperature of primers used.

Table 2.10 Template PCR programme

<table>
<thead>
<tr>
<th>Segment</th>
<th>Cycles</th>
<th>Temperature</th>
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<tbody>
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<td>95°C</td>
<td>5 minutes</td>
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<tr>
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<td>55°C</td>
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<td></td>
<td></td>
<td>72°C</td>
<td>1 minute/kb</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>72°C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>4°C</td>
<td>forever</td>
</tr>
</tbody>
</table>
2.6.7.2.1 **Yeast colony PCR**

A fraction of a colony (<1mm³) were added to 0.2 ml PCR tubes (Axygen). Tubes were microwaved at full power for 1 minute and afterwards placed immediately on ice. Microwaved cells were used as template DNA for PCR reaction (section 2.6.7.2) using the PCR programme described below.

Table 2.11 Colony PCR programme

<table>
<thead>
<tr>
<th>Segment</th>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
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<tbody>
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<tr>
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<td>1</td>
<td>72°C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>4°C</td>
<td>forever</td>
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</tbody>
</table>

2.6.7.3 **Purification of PCR products**

To purify PCR products, the Qiagen PCR purification kit was used according to manufacturers instructions.

2.6.8 **Site directed mutagenesis**

For site-directed mutagenesis of *Ylplac204-PPH21* (AMp694) and *Ylplac211-PPH22* (AMp699) plasmids, the Quikchange II XL kit (Stratagene) was used following the manufacturers protocol. For site-directed mutagenesis of plasmids larger that 10kb (*ESP1*-containing plasmids) the Quikchange II XL kit was used following the manufacturers protocol except that the PCR extension time was increased to 2 min/kb. In addition, DNA was precipitated and resuspended in 3 µl TE after *DpnI* digestion and all DNA was transformed.
2.6.9 Cloning

2.6.9.1 Restriction digest

Restriction digests were performed using NEB restriction enzymes and buffers according to manufacturers instructions. Digests were subjected to agarose gel electrophoresis followed by band extraction and DNA purification (section 2.6.6)

2.6.9.2 Ligation

DNA ligations were performed in a total volume of 30 µl using 2 µl of 400,000 U/ml T4 DNA ligase (NEB) and 1x buffer supplied by the manufacturer. Typically a 3:1 ratio of insert DNA to vector DNA was used and distilled water added to 30 µl. Reactions were incubated at 18°C overnight.

2.6.9.3 Ligation precipitation for transformation

To 30 µl ligations 10 µl of 10 mg/ml tRNA (sigma), 4 µl of 3M NaOAc and 100 µl of ethanol were added and tubes incubated at -20°C for 1-2 hours. Tubes were then spun at 13000 rpm for 5 minutes at 4°C, the supernatant discarded and the pellets washed with 200 µl of 80% ethanol. The pellets were air-dried and resuspended in 6 µl of TE. 2 µl were used for E.coli transformation by electroporation and the remainder stored long-term at -20°C.

2.6.9.4 Sequencing

DNA samples were sequenced using the Big Dye Terminator kit v3.1 (Applied Biosystems)

Sequencing reaction (10 µl):

1 µl mini-prep DNA
2 µl Big Dye v3.1
0.5 µl primer (5 µM)
2 µl 5x Big Dye buffer
4.5 µl water
Table 2.12 Sequencing PCR programme

<table>
<thead>
<tr>
<th>Segment</th>
<th>Cycles</th>
<th>Temperature</th>
<th>Time</th>
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<tbody>
<tr>
<td>1</td>
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<td>95°C</td>
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<td>55°C</td>
<td>15 seconds</td>
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<td></td>
<td>60°C</td>
<td>4 minute</td>
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<tr>
<td>3</td>
<td>1</td>
<td>72°C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>4°C</td>
<td>forever</td>
</tr>
</tbody>
</table>

Samples were analysed by the SBS sequencing service, Ashworth Laboratories, University of Edinburgh, on an ABI 3730 DNA analyser (Applied Biosystems)
2.6.10 Southern blotting

DNA breakage buffer: (see section 2.6.4)

TE: (see section 2.5.1)

Denaturing solution:
1.5M NaCl
0.5M NaOH

Church buffer:
0.5M Na Phosphate buffer pH 7.2
1mM EDTA
7% (w/v) SDS
1% BSA

Wash buffer:
40mM Na Phosphate buffer pH 7.2
1mM EDTA
1% (w/v) SDS

2.6.10.1 Genomic DNA extraction from yeast for southern analysis

Yeast were grown overnight in 10 ml YPDA and harvested by centrifugation at 3600 rpm for 2 minutes. Cell pellets were transferred to 1.5 ml tubes with 500 µl of sterile water and spun down, supernatant discarded and the pellet resuspended in 200 µl of DNA breakage buffer. 100 µl of glass beads and 200 µl of phenol:chloroform were added and the mixture vortexed for 4 minutes. Tubes were spun down at 14000 rpm for 5 minutes and the upper aqueous phase (~130 µl) transferred to new tubes containing 1 ml of cold ethanol. Tubes were mixed by inversion then spun at 14000 rpm for 5 minutes, the supernatant discarded and the pellets air-dried. Pellets were then resuspended in 400 µl of TE, then 1.5 µl of 20 mg/ml RNase was added and incubated at 37°C for 5 minutes. After incubation, 10 µl of 4M ammonium acetate and 1 ml of cold ethanol were added and mixed by inversion. Tubes were spun at
14000 rpm for 5 minutes, the pellet was then air-dried and resuspended in 50 µl of TE. 10 µl was used for digests for southern analysis.

2.6.10.2 Digestion and agarose gel electrophoresis of genomic DNA for southern analysis

10 µl of genomic DNA was digested overnight in a 100 µl volume reaction using 20 units of restriction enzyme. DNA was precipitated by adding 20 µl of 3M NaOAc and 500 µl of ethanol and incubating at -20°C for 1-2 hours. Tubes were spun at 13000 rpm for 5 minutes, the supernatant discarded and DNA pellets air dried and resuspended in 15 µl of TE. DNA loading buffer was added and run on 0.7% agarose gels without ethidium bromide.

2.6.10.3 Capillary transfer to membrane and cross-linking

Gels were soaked in 0.25 M HCl for 10 minutes until DNA loading dye turned yellow, then rinsed twice with water and soaked in denaturing solution for 2 x 15 minutes. Gels were then transferred onto GeneScreen membrane (Perkin Elmer) by capillary transfer overnight. Denaturing solution was used for the reservoir and to soak the GeneScreen membrane and 3 MM Whatmann paper. After transfer membranes were soaked in 50 mM Na phosphate pH7.2 for 2 x 10 minutes, blotted dry and then crosslinked twice using UV crosslinker (Stratagene) at 12000 Joules.

2.6.10.4 Radiolabelled probe manufacture

25 ng of probe DNA fragments generated by PCR using plasmid DNA as a template were diluted to 13 µl with sterile distilled water and denatured by incubating at 95°C for 10 minutes then placed on ice. To label, the denatured probes were incubated with 3 µl of 30 µCi P³² (Perkin Elmer) and 4 µl of High Prime DNA labelling solution (Roche) and incubated at 37°C for 1 hour. The radiolabelled probes were denatured at 95°C for 5 minutes immediately before hybridization.
2.6.10.5 Hybridization and exposure of membrane

Membranes were incubated in pre-warmed church buffer at 65°C for 1 hour, followed by incubation with pre-warmed church buffer plus radiolabelled probe at 65°C overnight. Membranes were then washed twice for 10 minutes with wash buffer at 65°C, then exposed to Kodak Bio-Max light film with intensifying screen at -80°C overnight. Films were developed using Konica-Minolta SRX-101A developer.
Chapter 3
Dissecting the Sgo1 pathway in mitosis
3 Chapter 3 – Dissecting the Sgo1 pathway in mitosis

3.1 Introduction

Shugoshin proteins constitute a conserved family in eukaryotes that play a role in regulating chromosome segregation during the cell divisions of mitosis and meiosis. Budding yeast contain a single shugoshin protein, Sgo1, which performs both the mitotic and meiotic functions of the shugoshin proteins. During meiosis, Sgo1 is required to protect centromeric Rec8 from cleavage by separase at meiosis I, thereby ensuring that centromere cohesion is retained to allow the accurate segregation of sister chromatids in meiosis II (Katis et al. 2004; Marston et al. 2004). During mitosis, Sgo1 is required to prevent anaphase onset in response to lack of tension between sister kinetochores and ensure the bi-orientation of sister chromatids in metaphase (Indjeian et al. 2005). At the onset this work, the mechanisms by which Sgo1 performs these functions were completely unknown. Intriguingly, however, both the meiotic and mitotic functions of Sgo1 appear to share a common theme, in that they culminate in the inhibition of cohesin cleavage. I initiated this study to understand more about how Sgo1 functions in mitosis and meiosis, with the aim of uncovering how Sgo1 prevents cohesin cleavage.

Fission yeast contain two shugoshin proteins, Sgo1 and Sgo2 (Kitajima et al. 2004; Rabitsch et al. 2004). Sgo2 is expressed ubiquitously in mitosis and meiosis; Sgo1 on the other hand is meiosis-specific and carries out the conserved function of shugoshin proteins in protecting centromere cohesion during meiosis (Kitajima et al. 2004; Rabitsch et al. 2004). The cohesin protection function of Sgo1 can be ectopically reconstituted by overexpressing sgo1+ and rec8+ in fission yeast mitotic cells, resulting in defective nuclear division (Kitajima et al. 2004). I sought to develop a similar assay for Sgo1 function in budding yeast based on this observation. In this chapter, I show that SGO1 overexpression in mitotic cells causes a cell cycle delay in metaphase, and that this is due to a failure to cleave cohesin. I use this observation to dissect the mechanism Sgo1 function. I find that SGO1 overexpression prevents cohesin cleavage by inhibiting separase independently of any known upstream regulators. I reveal that protein phosphatase 2A specifically
coupled to its Cdc55 regulatory subunit (PP2A$^{\text{Cdc55}}$) is required downstream of $SGO1$ overexpression for the inhibition of separase. My results uncover a previously unidentified mechanism of Sgo1 function, which involves PP2A$^{\text{Cdc55}}$ and separase inhibition.

3.2 Results

3.2.1 Overexpression of $SGO1$ causes slow growth

With the aim of determining the mechanisms by which Sgo1 functions, I developed an assay based on its overproduction. To study the effects of high levels of Sgo1 protein on the cell, I used the $GAL1-10$ bidirectional promoter to overexpress the $SGO1$ gene. Growth in the presence of galactose induces a high level of transcription from the $GAL1-10$ promoter, whereas growth in glucose medium represses this transcription (St John and Davis 1981). I integrated into the genome of otherwise wild type cells multiple copies of a construct containing the $SGO1$ gene under the control of the $GAL1-10$ promoter ($GAL\text{-}SGO1$) at the $URA3$ locus. Note that these cells also contain $SGO1$ at its endogenous locus. I first tested if $SGO1$ overexpression had an effect on cell growth. Wild type and $GAL\text{-}SGO1$ cells were streaked onto medium containing either glucose or galactose. On glucose medium, $GAL\text{-}SGO1$ cells showed similar growth to wild type cells (Figure 3.1A), consistent with the notion that expression from the $GAL\text{-}SGO1$ construct is repressed. Interestingly, however, $GAL\text{-}SGO1$ cells grew very slowly compared to wild type on galactose medium (Figure 3.1B), indicating that overexpression of $SGO1$ somehow inhibits cell growth. Overexpression of gene can cause a reduced growth rate for many different reasons, however genes that show a cell-cycle regulated pattern of expression are more likely to cause a cell cycle arrest when overexpressed (Sopko et al. 2006). Sgo1 protein levels are cell cycle regulated, peaking when cells are in metaphase and then declining as cells enter anaphase (Marston et al. 2004; Indjeian et al. 2005). I therefore considered it likely that the reduced growth of $SGO1$-overexpressing cells is due to a defect in cell cycle progression.
3.2.2 Overexpression of SGO1 causes a metaphase delay

To study the consequences of SGO1 overexpression on mitotic cell cycle progression, I utilized a method of cell synchronisation using the budding yeast mating pheromone, α-factor (Breeden 1997). Alpha-factor is an oligopeptide produced by mating type α (MATα) cells which specifically acts on cells of mating type a (MATa), arresting them in the G1 stage of the cell cycle (Duntze et al. 1970). This arrest is reversible, as removing α-factor from the growth medium of MATa cells results in the rapid and synchronous progression of the cell population into the cell cycle (Breeden 1997). In this study, I used the α-factor synchronization and release technique to analyse cell cycle progression and, consequently, all strains used were MATa (Table 2.2).

Wild type and GAL-SGO1 cells were arrested in G1 with α-factor, and released synchronously into the cell cycle in the presence of galactose to induce high levels of SGO1 expression. To monitor cell cycle progression, the morphology of the mitotic spindle was analysed after tubulin immunofluorescence (Figure 3.2A,B). Wild type cells progressed synchronously through the cell cycle as judged by the appearance of short metaphase spindles followed by anaphase spindle elongation (Figure 3.2A). Strikingly, however, cells overexpressing SGO1 accumulated as large budded cells with short metaphase spindles (Figure 3.2A), suggesting that high levels of Sgo1 delay entry into anaphase. To aid interpretation of the spindle morphology data, the ratio of metaphase to anaphase cells was calculated for this and subsequent experiments. For wild type, this value is usually close to 1.0 and increases in GAL- SGO1 cells, indicative of a metaphase delay (numbers above graphs). I concluded from this result that the reduced growth of SGO1 overexpressing cells is most likely due to a cell cycle delay in metaphase.
Figure 3.1 Overexpression of SGO1 causes slow growth. Wild type (AM1176) and GAL-SGO1 (AM870) cells were streaked onto either (A) glucose medium (YEPD) or (B) galactose medium (YEPRG) and incubated at 30°C until significant growth was seen for the wild type control.

Figure 3.2 Overexpression of SGO1 causes a metaphase delay. Wild type (AM1176) and GAL-SGO1 (AM870) cells were grown at room temperature in YEPR, arrested in G₁ using α-factor then released into YEPRG at room temperature. When cells had budded, α-factor was re-added to limit analysis to a single cell cycle. Samples were taken at the indicated timepoints after release from G₁. (A) The percentage of cells in metaphase and anaphase was determined by spindle morphology analysis after tubulin immunofluorescence (at least 200 cells were counted at each timepoint). The numbers above the graphs correspond to the overall ratio of metaphase to anaphase cells for each strain. (B) Images are representative of the spindle morphology of metaphase and anaphase cells.
3.2.3 Overexpression of SGO1 inhibits cohesin cleavage

High levels of Sgo1 could delay anaphase onset by either inhibiting sister chromatid separation, or by somehow interfering with mitotic spindle elongation. To distinguish between these possibilities, I wanted to test whether overexpression of SGO1 could cause a metaphase delay in the absence of sister chromatid cohesion. The cohesin complex holds sister chromatids together until the onset of anaphase when cleavage of the cohesin subunit Scc1 by separase (Esp1 in budding yeast) triggers loss of sister chromatid cohesion (Uhlmann et al. 1999; Uhlmann et al. 2000). To inactivate cohesion between sister chromatids, I used an SCCI allele with one of its two Esp1 cleavage sites exchanged for the recognition sequence for TEV protease (scc1-tev). The presence of one remaining Esp1 cleavage site allows for normal growth in scc1-tev cells. However, expression of the gene encoding TEV protease from the GAL1-10 promoter (GAL-TEV) is sufficient to induce cleavage of scc1-tev and loss of sister chromatid cohesion in these cells (Uhlmann et al. 2000). The scc1-tev allele and GAL-TEV construct were introduced into cells carrying GAL-SGO1 so that ectopic cohesin cleavage could be induced simultaneously with SGO1 overexpression by galactose addition. Cells were released from a G1 arrest into galactose-containing media and cell cycle progression monitored by spindle morphology analysis. Loss of sister chromatid cohesion by ectopic cohesin cleavage allowed GAL-SGO1 cells to enter anaphase with similar timing to wild type cells (Figure 3.3). This result demonstrates that high levels of Sgo1 do not prevent mitotic spindle elongation, but rather prevent sister chromatid separation, thereby delaying anaphase onset. This conclusion predicts that cohesin cleavage is inhibited by SGO1 overexpression. To directly address whether this was the case, I analysed the kinetics of cohesin cleavage in GAL-SGO1 cells by western blot analysis of Scc1 cohesin protein. In wild type cells, full-length Scc1 levels declined and the Scc1 cleavage product, which is known to be unstable (Rao et al. 2001), transiently accumulated as the cells entered anaphase (Figure 3.4). In contrast, full length Scc1 protein persisted in GAL-SGO1 cells and very little Scc1 cleavage product was observed (Figure 3.4), indicating that overexpression of SGO1 prevents cohesin cleavage.
Figure 3.3 Cohesin cleavage is sufficient to trigger anaphase in SGO1-overexpressing cells. Wild type (AM1176), GAL-SGO1 (AM870), scc1-tev GAL-TEV (AM4786) and scc1-TEV GAL-TEV GAL-SGO1 (AM4784) cells were grown and treated as in figure 3.2 except that α-factor was not re-added. Samples were taken at the indicated timepoints for spindle morphology analysis.
Figure 3.4 Overexpression of SGO1 prevents cohesin cleavage. Wild type (AM1145) and GAL-SGO1 (AM1126) cells carrying SCC1-6HA were grown and treated as in figure 3.2. Samples were taken at the indicated timepoints for (A) spindle morphology analysis and (B) anti-HA western blot analysis of TCA protein extracts separated on a 10% SDS polyacrylamide gel. Pgk1 is shown as a loading control.
3.2.4 Overexpression of SGO1 inhibits Esp1 activity

Cohesin is cleaved by the proteolytic activity of Esp1 (Uhlmann et al. 2000). High levels of Sgo1 could therefore prevent cohesin cleavage by either inhibiting Esp1 activity, or by rendering its substrate, Scc1, resistant to Esp1-dependent proteolysis. In addition to Scc1, Esp1 is known to cleave the kinetochore protein, Slk19, at anaphase onset (Sullivan et al. 2001). I reasoned that if high levels of Sgo1 inhibited Esp1, rather than specifically protecting cohesin from cleavage, Slk19 should also fail to be cleaved upon SGO1 overexpression. Western blot analysis of C-terminally tagged Slk19 detected three bands (Figure 3.5.B). The slower migrating band corresponds to full-length Slk19 that is maximally phosphorylated in metaphase. The two faster migrating bands represent phosphorylated and non-phosphorylated isoforms of cleaved Slk19, which appear in anaphase and are stable throughout the next cell cycle (Sullivan et al. 2001). In wild type cells, full length Slk19 disappeared concomitantly with the appearance of Slk19 cleavage product as the cells entered anaphase (Figure 3.5). Interestingly, the decline in full length Slk19 was delayed in GAL-SGO1 cells (Figure 3.5), suggesting that Slk19 cleavage is prevented by SGO1 overexpression. Taken together with the Scc1 western blot data (Figure 3.4), this result suggests that high levels of Sgo1 cause Esp1 inhibition. Furthermore, it is likely that Esp1 inhibition is responsible for the GAL-SGO1 metaphase delay, because GAL-SGO1 cells cannot delay in metaphase when ESP1 is also overexpressed (Clift et al. 2009).
Figure 3.5 Overexpression of SGO1 prevents Slk19 cleavage. Wild type (AM2753) and GAL-SGO1 (AM1066) cells carrying SLK19-13MYC were grown and treated as in figure 3.2 except at 30°C. Samples were taken at the indicated timepoints for (A) spindle morphology analysis and (B) anti-MYC western blot analysis of TCA protein extracts separated on a 6% SDS polyacrylamide gel. Kar2 is shown as a loading control.
3.2.5 Dissecting the Sgo1 pathway

3.2.5.1 High copy suppressors of GAL-SGO1

To uncover genes that may play a role in the Sgo1 pathway to inhibit separase, I performed a screen to hunt for suppressors of GAL-SGO1. The basis of the screen was to identify genes that, when present on high-copy number plasmids, suppressed the poor growth of SGO1-overexpressing cells. For an outline of the screen procedure see appendix section A.1. In total 70 suppressor plasmids were recovered and the identity of the DNA inserts revealed by sequencing (Table A.1). The majority of the isolated plasmids contained independent DNA fragments, and no single gene appeared more than twice. The variety of suppressor plasmids isolated suggested that suppression of GAL-SGO1 could be achieved by perturbing the Sgo1 pathway at many different levels (Figure A.3). With the help of an undergraduate, Tara Mills, I performed secondary screens with the aim of understanding the function of the suppressor plasmids (see appendix section A.1.1). Interestingly, we found that six of the plasmids showed genetic interactions with either Scc1 or Esp1 (Table A.1), indicating a possible role in the regulation of Esp1 activity or sister chromatid cohesion. Further study of the genes contained in these suppressor plasmids may shed light into the mechanisms of Sgo1 function. However, due to the success of the candidate-based approach described below, I decided not to follow up the high-copy suppressor screen any further.
3.2.5.2 A candidate approach to dissecting the Sgo1 pathway

In parallel with the high-copy suppressor screen, I identified potential candidates from the literature and investigated their role in preventing anaphase onset when SGO1 is overexpressed.

3.2.5.2.1 Pds1 is stabilized during, but dispensable for, the GAL-SGO1 metaphase delay

3.2.5.2.1.1 Pds1 is stabilized upon SGO1 overexpression

I have shown that Esp1 activity is inhibited upon SGO1 overexpression (section 3.2.4). Securin (Pds1 in budding yeast) is an Esp1 inhibitor, which is degraded at the onset of anaphase to liberate Esp1 to initiate sister chromatid separation (Ciosk et al. 1998). It may be that SGO1 overexpression leads to Pds1 stabilization, which in turn causes Esp1 inhibition. To explore this possibility, I determined if Pds1 was stabilized by analysing Pds1 protein levels in GAL-SGO1 cells. In wildtype cells, Pds1 protein accumulated as cells entered metaphase and then was degraded at the onset of anaphase (Figure 3.6). In contrast, the degradation of Pds1 was delayed in SGO1-overexpressing cells (Figure 3.6), suggesting that Pds1 could be responsible for the inhibition of Esp1 when SGO1 is overexpressed.

3.2.5.2.1.2 The GAL-SGO1 metaphase delay and Pds1 stabilization does not require the spindle checkpoint.

The spindle checkpoint monitors the attachment of chromosomes to the mitotic spindle and, in response to defects in this process, brings about a cell cycle arrest in metaphase through inhibition of APC$^{Cdc20}$, thereby preventing Pds1 degradation (reviewed in May and Hardwick 2006). I therefore considered it possible that the metaphase delay and Pds1 stabilization caused by SGO1 overexpression was due to activation of the spindle checkpoint. If this were the case, inactivation of the spindle checkpoint, by deleting a critical component of the checkpoint, MAD1, should alleviate the GAL-SGO1 metaphase delay. Interestingly, mad1Δ GAL-SGO1 cells were still able delay anaphase onset and delay Pds1 degradation to a similar extent as
GAL-\textit{SGO1} cells (Figure 3.6), indicating that spindle checkpoint activation is not responsible for the metaphase delay caused by high levels of Sgo1.

### 3.2.5.2.1.3 Pds1 is dispensable for the GAL-\textit{SGO1} metaphase delay

The spindle checkpoint ultimately functions to stabilize Pds1 (reviewed in May and Hardwick 2006). The finding that spindle checkpoint activation is not required for \textit{SGO1}-overexpressing cells to delay anaphase onset (Figure 3.6) raised the possibility that Pds1 itself may be dispensable for the \textit{GAL-SGO1} metaphase delay. Cells lacking \textit{PDS1} are viable (Yamamoto et al. 1996a), making it possible to test whether high levels of Sgo1 can cause a metaphase delay in the absence of \textit{PDS1}. Indeed, it was found that deletion of \textit{PDS1} did not alleviate the \textit{GAL-SGO1} metaphase delay (Clift et al. 2009). Furthermore, both Scc1 and Slk19 cleavage were inhibited in \textit{pds1\Delta GAL-SGO1} cells (Clift et al. 2009), indicating that high levels of Sgo1 cause Esp1 inhibition by a mechanism independent of Pds1.
Figure 3.6 Overexpression of SGO1 prevents Pds1 destruction independently of the spindle checkpoint. Wild type (AM1290), GAL-SGO1 (AM3917), mad1Δ (AM4162) and mad1Δ GAL-SGO1 (AM4160) cells carrying PDS1-3HA were grown and treated as in figure 3.2. Samples were taken at the indicated timepoints for (A) spindle morphology analysis and (B) anti-HA western blot analysis of TCA protein extracts separated on an 8% SDS polyacrylamide gel. Pgk1 is shown as a loading control.
3.2.5.2.2  Bub1 is required for the GAL-SGO1 metaphase delay

I also wanted to use the GAL-SGO1 assay to understand the mechanisms functioning upstream of Sgo1 in the prevention of cohesin cleavage. During metaphase, endogenous Sgo1 localizes to both the centromere and an approximately 50kb cohesin-enriched region surrounding the centromere (pericentromere; Kiburz et al. 2005). This localization is critical for Sgo1 function because in cells lacking BUB1, Sgo1 is absent from the pericentromere and the phenotype closely resembles that of an SGO1 deletion (Kiburz et al. 2005; Riedel et al. 2006; Fernius and Hardwick 2007). I reasoned that if SGO1 overexpression represented the ectopic activation of Sgo1 activity, Bub1 should also be required for the correct localization and function of overproduced Sgo1. I tested if Bub1 was required for the function of overproduced Sgo1. I found that overexpression of SGO1 in cells lacking BUB1 could no longer cause a metaphase delay (Figure 3.7.A). Because Pds1 protein is stabilized in SGO1-overexpressing cells that delay in metaphase (Figure 3.6), I also monitored Pds1 destruction as a marker for anaphase onset. In support of the spindle morphology data, the timing of Pds1 destruction was similar in bub1Δ compared to bub1Δ GAL-SGO1 cells (Figure 3.7.B), demonstrating that BUB1 is required for SGO1-overexpressing cells to delay in metaphase. Importantly, the amount of overproduced Sgo1 associated with chromatin, but not overall Sgo1 protein levels, was reduced in cells lacking BUB1 (Clift et al. 2009). Taken together, this data suggests that high levels of Sgo1 require efficient targeting to the chromatin by Bub1 in order to delay anaphase onset.
Figure 3.7 Overexpression of SGO1 causes a metaphase delay dependent on Bub1. Wild type (AM1290), GAL-SGO1 (AM3917), bub1Δ (AM5131) and bub1Δ GAL-SGO1 (AM5132) cells carrying PDS1-3HA were grown and treated as in figure 3.2. Samples were taken at the indicated timepoints for (A) spindle morphology analysis and (B) anti-HA western blot analysis of TCA protein extracts separated on an 8% SDS polyacrylamide gel. Pgk1 is shown as a loading control.
3.2.5.2.3 Ipl1 localization is affected by SGO1 overexpression, but its activity is not required for the metaphase delay

3.2.5.2.3.1 Overexpression of SGO1 affects Ipl1 localization

Previous studies in fission yeast and budding yeast have suggested that Shugoshin functions through controlling the localization of the Aurora B kinase (Kawashima et al. 2007; Vanoosthuyse et al. 2007; Yu and Koshland 2007). I therefore sought to determine whether high levels of Sgo1 affect the localization of Aurora B (Ipl1 in budding yeast). Ipl1 is part of the chromosomal passenger complex that exhibits a characteristic localization pattern during the cell cycle, localizing to the inner centromere in metaphase and the spindle midzone in anaphase (reviewed in Ruchaud et al. 2007). I analysed the localization of Ipl1 by whole cell immunofluorescence, which has previously been shown to be sufficient to visualise nuclear Ipl1 in metaphase cells and the spindle midzone localization of Ipl1 in anaphase (Biggins et al. 1999). As expected, in a wild type population, the vast majority of metaphase cells exhibited a nuclear Ipl1 signal, which then re-located to the spindle midzone in anaphase (Figure 3.8C,D). Strikingly, however, in 85% of GAL-SGO1 metaphase cells, Ipl1 strongly co-localized with the metaphase spindle (Figure 3.8C,D). SGO1 overexpression did not affect Ipl1 spindle midzone localization in those cells that escaped into anaphase (Figure 3.8C,D) or the overall levels of Ipl1 protein itself (Figure 3.8A,B). This result seems to suggest that Ipl1 localization in metaphase is affected by SGO1 overexpression. However, it remains a possibility that the colocalization of Ipl1 with the metaphase spindle in GAL-SGO1 cells may just be a consequence of delaying cells in metaphase, rather than as a direct result of Sgo1 activity. Further analysis of Ipl1 localization in cells arrested in metaphase by means other than SGO1 overexpression, such as by microtubule depolymerization or APC inhibition, may resolve this issue. Nevertheless, the difference in localization pattern between wild type and GAL-SGO1 cells raised the possibility that Ipl1 function may be responsible for the GAL-SGO1 metaphase delay.
Figure 3.8 Overexpression of SGO1 affects Ipl1 localization. Wild type (AM3513) and GAL-SGO1 (AM3860) cells carrying IPL1-6HA were grown and treated as in figure 3.2 except that α-factor was not re-added. (A) The percentage of cells in metaphase and anaphase was determined by spindle morphology analysis and (B) Ipl1-6HA levels were analysed by anti-HA western blot analysis of TCA protein extracts separated on an 8% SDS polyacrylamide gel. Kar2 is shown as a loading control. Ipl1-6HA localization was determined after immunofluorescence. (C) Images are representative of Ipl1-6HA localization in metaphase and anaphase cells. (D) Ipl1-6HA localization was scored in metaphase and anaphase cells for each strain (at least 200 cells were counted for each strain).
3.2.5.2.3.2 Ipl1 is not required for the GAL-SGO1 metaphase delay

If Ipl1 functions downstream of overproduced Sgo1, inactivation of Ipl1 should alleviate the GAL-SGO1 metaphase delay. *IPL1* is an essential gene (Chan and Botstein 1993), I therefore used the *ipl1-321* temperature sensitive mutant allele (Biggins et al. 1999) to study the effects of Ipl1 inactivation. Wild type, GAL-SGO1, *ipl1-321* and *ipl1-321 GAL-SGO1* cells were arrested in G1 with α-factor and then released synchronously into the cell cycle at the non-permissive temperature (37°C), in the presence of galactose. At 37°C, the GAL-SGO1 metaphase delay was less pronounced, nevertheless, spindle elongation and Pds1 destruction was clearly delayed compared to wild type (Figure 3.9). Importantly, however, inactivation of Ipl1 in GAL-SGO1 cells did not alleviate the metaphase delay, as metaphase spindles persisted for longer and Pds1 destruction, a marker for anaphase onset, was delayed in *ipl1-321 GAL-SGO1* compared to *ipl1-321* cells (Figure 3.9). This result indicates that Ipl1 function is not required for the GAL-SGO1 metaphase delay. However, due to the apparently altered localization of Ipl1 in SGO1-overexpressing cells, I cannot rule out the possibility that Ipl1 functions in other roles downstream of Sgo1 that do not cause a delay to anaphase onset.
Figure 3.9 Overexpression of SGO1 causes a metaphase delay independently of Ipl1. Wild type (AM1290), GAL-SGO1 (AM3917), ipl1-321 (AM4416) and ipl1-321 GAL-SGO1 (AM4414) cells carrying PDS1-3HA were grown and treated as in figure 3.2 except that cells were released at at 37°C. Samples were taken at the indicated timepoints for (A) spindle morphology analysis and (B) anti-HA western blot analysis of TCA protein extracts separated on an 8% SDS polyacrylamide gel. Pgk1 is shown as a loading control.
3.2.5.2.4 Sister chromatids are bioriented in GAL-SGO1 cells

During a normal mitosis, sister kinetochores attach to microtubules emanating from opposite poles (bi-orientation), the poleward forces of microtubules, in turn, generate tension across the kinetochores (reviewed in Tanaka 2005). Ipl1 functions to destabilize inappropriate kinetochore-microtubule attachments that do not generate tension, thereby activating the spindle checkpoint to delay anaphase onset (Pinsky et al. 2006). The findings that Ipl1 (Figure 3.9) and the spindle checkpoint (Figure 3.6) are dispensable for the GAL-SGO1 metaphase delay is consistent with the hypothesis that SGO1 overexpressing cells delay in metaphase with bi-oriented chromosomes.

To test this hypothesis, I monitored chromosome segregation in GAL-SGO1 cells, under the assumption that the segregation of sister chromatids to opposite poles in anaphase is indicative of properly bi-oriented chromosomes. To monitor chromosome segregation, I visualised chromosome IV by binding GFP-TetR to an array of TetO binding sites 2.4kb from centromere IV (CEN4-GFP; He et al. 2000). The accuracy of chromosome segregation was revealed by scoring the percentage of large-budded cells with divided nuclei that had segregated both copies of CEN4-GFP to the same nucleus (percentages inside graphs). As expected, in wild type cells, sister CEN4-GFP separation correlated with appearance of anaphase spindles and subsequently segregated to opposite poles, (Figure 3.10.A). Similarly, SGO1-overexpressing cells never segregated sister CEN4-GFP signals to the same pole (Figure 3.10.B), indicating that chromosomes attached to the mitotic spindle in a bipolar manner. In addition, a significant fraction of GAL-SGO1 cells separated CEN4-GFP during the metaphase delay (75 min; Figure 3.10.B), consistent with previous reports that pulling forces of the mitotic spindle cause bioriented sister centromeres to separate transiently during metaphase independently of cohesin cleavage (Goshima and Yanagida 2000; He et al. 2000; Tanaka et al. 2000). This analysis of centromere IV segregation provides strong evidence that sister kinetochores are attached to microtubules in a bipolar manner in SGO1-overexpressing cells.
Figure 3.10 SGO1-overexpressing cells bi-orient chromosomes. Wild type (AM2812) and GAL-SGO1 (AM3858) cells carrying CEN4-GFP were grown and treated as in figure 3.2 except at 30°C. Samples were taken at the indicated timepoints for spindle morphology analysis and the percentage of cells with separated GFP foci (at least 200 cells were counted at each timepoint). The percentage of large-budded cells with divided nuclei that had segregated both copies of CEN4-GFP to the same nucleus is shown inside each graph.
3.2.5.2.5 PP2A<sup>Cdc55</sup> is required for the GAL-SGO1 metaphase delay

My findings are consistent with the notion that SGO1 overexpression prevents anaphase onset independently of any known pathways that lead to Esp1 inhibition. Interestingly, during the course of this work, Sgo1 was shown to prevent cohesin cleavage during meiosis through its interaction with Protein Phosphatase 2A (Riedel et al. 2006), raising the possibility that overproduced Sgo1 may also function through a PP2A-dependent pathway in mitosis.

3.2.5.2.5.1 Rts1 is dispensable for the GAL-SGO1 metaphase delay

PP2A is a serine/threonine phosphatase consisting of a heterotrimeric complex of scaffold (A), regulatory (B, B’, B’’ and B’’’) and catalytic (C) subunits. The regulatory subunit is thought to dictate the subcellular localization and substrate specificity of the AC core catalytic enzyme (Janssens and Goris 2001; Lechward et al. 2001). In budding yeast there are two known regulatory subunits, Cdc55 (B) and Rts1 (B’), whose association with the AC core enzyme is mutually exclusive (PP2A<sup>Cdc55</sup> or PP2A<sup>Rts1</sup>) (Jiang 2006). Previously it was shown that Sgo1 recruits PP2A<sup>Rts1</sup> to centromeres during meiosis, where it protects cohesin from cleavage by Esp1 (Riedel et al. 2006). To determine whether overproduced Sgo1 requires PP2A<sup>Rts1</sup> to delay anaphase onset during mitosis, I deleted RTS1 in GAL-SGO1 cells. Deletion of RTS1 did not, however, alleviate the metaphase delay, as evident by a delay in spindle elongation and a higher metaphase to anaphase ratio in rts1Δ GAL-SGO1 cells compared to rts1Δ (Figure 3.11.C). This result suggests that PP2A<sup>Rts1</sup> may be less important for mediating Sgo1 function during mitosis than during meiosis.
Figure 3.11 PP2A\textsuperscript{Cdc55} is required to delay anaphase onset in \textit{SGO1}-overexpressing cells. (A,D) Wild type (AM1176), GAL-\textit{SGO1} (AM870); (B) \textit{cdc55}\textsuperscript{Δ} (AM3164), \textit{cdc55}\textsuperscript{Δ} GAL-\textit{SGO1} (AM3239); (C) \textit{rts1}\textsuperscript{Δ} (AM3209), \textit{rts1}\textsuperscript{Δ} GAL-\textit{SGO1} (AM3306); (E) \textit{pph21-L369}\textsuperscript{Δ} \textit{pph22}\textsuperscript{Δ} GAL-\textit{SGO1} (AM5271) and \textit{pph21-L369}\textsuperscript{Δ} \textit{pph22}\textsuperscript{Δ} GAL-\textit{SGO1} (AM5269) cells were grown and treated as in figure 3.2 except at 30°C and \textit{α}-factor was not re-added. Samples were taken at the indicated timepoints for spindle morphology analysis. Strains in D and E were analysed separately from A, B and C.
3.2.5.2.5.2 Cdc55 is required for the GAL-SGO1 metaphase delay

Unlike Rts1, Cdc55 was not found to interact with Sgo1 during meiosis (Riedel et al. 2006). Intriguingly, however, Cdc55 does interact with Esp1 during mitosis (Queralt et al. 2006) and, furthermore, overexpression of CDC55, like SGO1, causes a metaphase delay independently of Pds1 (Chiroli et al. 2007). I therefore tested whether overexpression of SGO1 can delay anaphase onset in the absence of CDC55. Strikingly, deletion of CDC55 almost completely abolished the metaphase delay of SGO1-overexpressing cells (Figure 3.11.B). The metaphase to anaphase ratio was only slightly increased, and the onset of anaphase spindle elongation only slightly delayed, in cdc55Δ GAL-SGO1 cells compared to cdc55Δ cells (Figure 3.11.B). Because CDC55 deletion does not affect the amount of overproduced Sgo1 protein or its association with chromatin (Clift et al. 2009), I conclude that SGO1 overexpression induces a mostly CDC55-dependent delay to anaphase onset.

3.2.5.2.5.3 Cdc55 bound to PP2A is required for the GAL-SGO1 metaphase delay

I wanted to know whether Cdc55 functions as an activator of PP2A to prevent anaphase onset upon SGO1 overexpression. If this were the case, preventing Cdc55 from binding to the PP2A core enzyme should also alleviate the GAL-SGO1 metaphase delay. Budding yeast cells contain two functionally redundant PP2A-C subunits, Pph21 and Pph22 (Sneddon et al. 1990), the carboxy-terminal leucine residues of which are essential for stable binding to Cdc55 (Evans and Hemmings 2000; Wei et al. 2001; Gentry et al. 2005). I removed the carboxy-terminal leucine of Pph21 and deleted PPH22 (pph21-L369Δ pph22Δ) such that Cdc55 cannot interact with the PP2A core enzyme (Wei et al. 2001; Gentry et al. 2005). Strikingly, the pph21-L369Δ pph22Δ mutations completely abolished the GAL-SGO1 metaphase delay (Figure 3.11.E), indicating that PP2A<sup>Cdc55</sup> is required for high levels of Sgo1 to delay entry into anaphase. The PP2A-C carboxy-terminal deletion, however, may also disrupt the formation of PP2A<sup>Rts1</sup> (Gentry et al. 2005). Because the pph21-
L369Δ pph22Δ mutation more completely eliminated the GAL-SGO1 metaphase delay than the cdc55Δ mutant, it is possible PP2A<sup>Rts1</sup> may play a minor role in preventing anaphase onset upon SGO1 overexpression.

### 3.2.6 CDC55 deletion alleviates Esp1 inhibition in SGO1-overexpressing cells

#### 3.2.6.1 Pds1 stabilization upon SGO1-overexpression requires Cdc55

In the preceding sections, I showed that high levels of Sgo1 delay cells in metaphase due to Esp1 inhibition, and that deletion of CDC55 alleviated this delay. This suggests that the loss of CDC55 may allow for Esp1 activation in SGO1-overexpressing cells. Esp1 activation requires the destruction of its inhibitor, Pds1 (Cohen-Fix et al. 1996; Ciosk et al. 1998). Despite the fact that Pds1 is dispensable for Esp1 inhibition when SGO1 is overexpressed, Pds1 protein levels are stabilized (Figure 3.6) and it is unlikely that Esp1 activation could occur without Pds1 destruction because non-degradable Pds1 blocks anaphase onset (Cohen-Fix et al. 1996). If Esp1 inhibition were indeed alleviated in SGO1-overexpressing cells lacking CDC55, I would predict that Pds1 is no longer stabilized in these cells. Deletion of CDC55 delayed Pds1 destruction compared to wild type, consistent with the slower cell cycle progression of cdc55Δ cells grown in raffinose/galactose media (Figure 3.12). Nevertheless, Pds1 destruction occurred with similar kinetics in cdc55Δ GAL-SGO1 compared to cdc55Δ (Figure 3.12), indicating that CDC55 is required for Pds1 stabilization in cells overexpressing SGO1.
Figure 3.12 Deletion of CDC55 advances Pds1 degradation in SGO1-overexpressing cells. Wild type (AM1290), GAL-SGO1 (AM3917), cdc55Δ (AM4332) and cdc55Δ GAL-SGO1 (AM4331) cells carrying PDS1-3HA were grown and treated as in figure 3.2 except at 30°C. Samples were taken at the indicated timepoints for (A) spindle morphology analysis and (B) anti-HA western blot analysis of TCA protein extracts separated on an 8% SDS polyacrylamide gel. Pgk1 is shown as a loading control.
3.2.6.2 Analysis of cohesin cleavage in SGO1-overexpressing cells lacking CDC55

Next I analysed the cohesin, Scc1, by western blot to try to determine if Esp1-dependent cleavage of cohesin coincided with anaphase onset in SGO1-overexpressing cells lacking CDC55. During anaphase, the carboxy-terminal Scc1 cleavage product is unstable owing to its amino-terminal arginine that targets it for destruction by the ubiquitin/proteasome-dependent N-end rule pathway (Rao et al. 2001). This inherent instability makes it difficult to visualise low levels of Scc1 cleavage product by western blot analysis of C-terminally-tagged Scc1. I therefore performed the following experiment in ubr1Δ cells, in which the N-end rule pathway is lacking and the Scc1 cleavage product is stabilized (Rao et al. 2001). In wild type cells, the appearance of anaphase spindles was coupled to accumulation of stabilized Scc1 cleavage product and the decline of full length Scc1 protein. The lack of UBR1 revealed the appearance of Scc1 cleavage product in GAL-SGO1 cells that was not seen previously (Figure 3.4; Figure 3.13), nevertheless, Scc1 cleavage product was significantly delayed in appearance compared to wild type (Figure 3.13), confirming that SGO1 overexpression delays cohesin cleavage. Similar to what was seen for Pds1 destruction, cohesin cleavage was slightly delayed in cells lacking CDC55 due to slower growth in raffinose/galactose media (Figure 3.13). Importantly, however, overexpression of SGO1 did not delay cohesin cleavage in cells lacking CDC55, as the majority of Scc1 cleavage product initially appeared between 90 and 120 minutes in both cdc55Δ and cdc55Δ GAL-SGO1 cells, coinciding with the time of anaphase onset judged by spindle elongation (Figure 3.13).

Intriguingly, despite the fact that Scc1 cleavage product and anaphase spindles appeared with similar timing in cdc55Δ and cdc55Δ GAL-SGO1 cells, some full length Scc1 persisted throughout the timecourse in the latter strain (Figure 3.13). I reasoned that this uncleaved Scc1 could represent a soluble pool of cohesin, which is cleaved less efficiently than the chromatin-bound pool (Hornig and Uhlmann 2004). It may be that high levels of Sgo1 prevent the cleavage of all cohesin, and that deletion of CDC55 allows a sub-set of cohesin, most likely chromatin-bound, to be cleaved and this is sufficient to allow anaphase onset. This interpretation predicts that
the uncleaved Scc1 in cdc55Δ GAL-SGO1 cells is not associated with chromatin. I was able to test this directly by analysing Scc1 localization in chromosome spreads. Scc1 association with chromatin is established in late G1 and lost abruptly at the onset of anaphase (Michaelis et al. 1997). Consistent with the cohesin cleavage data, Scc1 dissociated from chromosomes at the onset of anaphase in wild type cells, whereas SGO1 overexpression delayed this process (Figure 3.14A,B). Crucially, Scc1 dissociation from chromosomes coincided with anaphase onset in both cdc55Δ and cdc55Δ GAL-SGO1 cells (Figure 3.14C,D), indicating that the persistent uncleaved cohesin in SGO1-overexpressing cells lacking CDC55 is not bound to chromosomes. This result inferred that the uncleaved cohesin in cdc55Δ GAL-SGO1 cells cannot hold sister chromatids together. To test whether this was the case, I monitored the segregation of chromosome IV labelled with GFP close to the centromere (CEN4-GFP). Figure 3.15 shows that CEN4-GFP signals separated at the onset of anaphase in both cdc55Δ and cdc55Δ GAL-SGO1 cells, and were missegregated in only a small fraction of anaphase cells (Figure 3.15; percentages inside graphs), indicative of a normal anaphase.
Figure 3.13 Analysis of cohesin cleavage in SGO1-overexpressing cells lacking CDC55.

All strains used were ubr1Δ. Wild type (AM4576), GAL-SGO1 (AM4575), cdc55Δ (AM4564) and cdc55Δ GAL-SGO1 (AM4611) cells carrying SCC1-6HA were grown and treated as in figure 3.2 except at 30°C. Samples were taken at the indicated timepoints for (A) spindle morphology analysis and (B) anti-HA western blot analysis of TCA protein extracts separated on a 10% SDS polyacrylamide gel. Pgk1 is shown as a loading control. A short (upper) and long (lower) exposure is shown for Sccl western.
Figure 3.14 Analysis of cohesin localization in SGO1-overexpressing cells lacking CDC55. (A) Wild type (AM1145); (B) GAL-SGO1 (AM1126); (C) cdc55Δ (AM4757) and (D) cdc55Δ GAL-SGO1 (AM4758) cells carrying SCC1-6HA were grown and treated as in figure 3.2 except at 30°C. Samples were taken at the indicated timepoints for spindle morphology analysis and the percentage of cells with Scc1 on chromatin determined by immunofluorescence of Scc1-6HA on chromosome spreads (at least 100 cells were counted at each timepoint). (E) Images show examples of Scc1-6HA staining on chromosome spreads.
Figure 3.15 Sister chromatids separate to opposite poles in SGO1-overexpressing cells lacking CDC55, cdc55Δ (AM4330) and cdc55Δ GAL-SGO1 (AM4287) cells carrying CEN4-GFP were grown and treated as in figure 3.2 except at 30°C. Wild type and GAL-SGO1 control strains are shown in figure 3.10. Samples were taken at the indicated timepoints for spindle morphology analysis and the percentage of cells with separated GFP foci. The percentage of large-budded cells with divided nuclei that had segregated both copies of CEN4-GFP to the same nucleus is shown inside each graph.
3.2.6.3 Cohesin cleavage and Esp1 activity is required for anaphase in SGO1-overexpressing cells lacking CDC55

The data above showed that deletion of CDC55 in SGO1-overexpressing cells allowed anaphase onset, which coincided with the cleavage of chromosomally bound cohesin. However, because not all Scc1 was cleaved in SGO1-overexpressing cells lacking CDC55 (figure 3.13), I wanted to know whether cohesin cleavage is actually required for anaphase onset in these cells. I hypothesised that if cohesin cleavage is required for anaphase in cdc55Δ GAL-SGO1 cells, expression of a non-cleavable version of cohesin should block anaphase onset. Scc1 contains two Esp1 cleavage sites, each of which are sufficient to allow cohesin cleavage (Uhlmann et al. 1999). I therefore utilized a conditional dominant SCC1 allele with both Esp1 cleavage sites mutated (GAL-SCC1-NC) so that it cannot be cleaved (Uhlmann et al. 1999). Cells were released from a G1 arrest into galactose medium to induce SGO1-overexpression and SCC1-NC expression. Non-cleavable cohesin prevented anaphase onset in otherwise wild type, GAL-SGO1 or cdc55Δ cells (Figure 3.16A-C) and, significantly, SCC1-NC expression inhibited anaphase onset in SGO1-overexpressing cells lacking CDC55 (Figure 3.16D). I next used a temperature sensitive ESP1 allele (esp1-1) to test if Esp1 activity is required for anaphase in cdc55Δ GAL-SGO1 cells. Cells were arrested in G1 and released synchronously into the cell cycle at the non-permissive temperature. Similar to non-cleavable cohesin, inactivation of Esp1 prevented anaphase onset in otherwise wild type, GAL-SGO1 or cdc55Δ cells (Figure 3.17A-C) and, importantly, in cdc55Δ GAL-SGO1 cells (Figure 3.17D). Altogether, these findings demonstrate that deletion of CDC55 advances anaphase onset in SGO1-overexpressing cells by allowing cohesin to be cleaved by Esp1.
Figure 3.16 Cohesin cleavage is required for anaphase onset in SGO1-overexpressing cells lacking CDC55. (A) Wild type (AM1176), GAL-SCC1-NC (AM4564); (B) GAL-SGO1 (AM870), GAL-SGO1 GAL-SCC1-NC (AM5306); (C) cdc55Δ (AM3164), cdc55Δ GAL-SCC1-NC (AM5169); (D) cdc55Δ GAL-SGO1 (AM3239) and cdc55Δ GAL-SGO1 GAL-SCC1-NC (AM4620) cells were grown and treated as in figure 3.2 except at 30°C and α-factor was not re-added. Samples were taken at the indicated timepoints for spindle morphology analysis.
Figure 3.17 Esp1 activity is required for anaphase onset in SGO1-overexpressing cells lacking CDC55. (A) Wild type (AM1176), esp1-1 (AM5010); (B) GAL-SGO1 (AM870), GAL-SGO1 esp1-1 (AM4793); (C) cdc55Δ (AM3164), cdc55Δ esp1-1 (AM4794); (D) cdc55Δ GAL-SGO1 (AM3239) and cdc55Δ GAL-SGO1 esp1-1 (AM4792) cells were grown and treated as in figure 3.2 except cells were released from G1 at 37°C and α-factor was not re-added. Samples were taken at the indicated timepoints for spindle morphology analysis.
3.2.7 Overexpression of CDC55 prevents anaphase onset

I presented data above to suggest that CDC55 may function downstream of SGO1 in the inhibition of Esp1. In support of this, Chiroli et al. (2007) found that CDC55 overexpression delays the onset of anaphase independently of Pds1. To confirm this result and to study the consequences of CDC55 overexpression in more detail, I introduced into the genome of otherwise wild type cells multiple copies of the CDC55 gene under the control of the galactose inducible GAL1-10 promoter (GAL-CDC55) at the URA3 locus. Cells were released from a G1 block in the presence of galactose to induce high levels of CDC55 expression. Analogous to SGO1 overexpression, high levels of Cdc55 delayed entry into anaphase as evident by a delay in spindle elongation and Pds1 destruction compared to wild type (Figure 3.18). The GAL-CDC55 metaphase delay was less pronounced than that previously seen for GAL-SGO1, however, this is most likely due to fewer copies of the GAL-CDC55 construct in the genome. Figure 5.2 shows that a new isolate of the GAL-CDC55 strain causes a metaphase delay similar to GAL-SGO1, presumably due to a higher copy number.

Overexpression of CDC55 inhibits Esp1 activity independently of Pds1

To determine if high levels of Cdc55 (like Sgo1) cause Esp1 inhibition independently of Pds1, I analysed the Esp1 substrates, Scc1 and Slk19, by western blot. In wild type and pds1Δ cells, the levels of full-length Scc1 and Slk19 protein decreased concomitantly with the appearance of anaphase spindles (Figure 3.19). In CDC55-overexpressing cells, however, the decline in both full-length Scc1 and Slk19 protein levels was delayed, even in the absence of PDS1 (Figure 3.19). This result demonstrates that overexpression of CDC55 phenocopies SGO1 overexpression, that is, causes a Pds1-independent metaphase delay where cleavage of Esp1 substrates is prevented.
Figure 3.18 Overexpression of CDC55 causes a metaphase delay. Wild type (AM1290) and GAL-CDC55 (AM4503) cells carrying PDS1-3HA were grown and treated as in figure 3.2. Samples were taken at the indicated timepoints for (A) spindle morphology analysis and (B) anti-HA western blot analysis of TCA protein extracts separated on an 8% SDS polyacrylamide gel. Pgk1 is shown as a loading control.
Figure 3.19 Overexpression of \textit{CDC55} inhibits Esp1 activity independently of Pds1. Wild type (AM4639), \textit{GAL-CDC55} (AM4917), \textit{pds1Δ} (AM4631) and \textit{pds1Δ GAL-CDC55} (AM4915) cells carrying \textit{SCC1-6HA} and \textit{SLK19-13MYC} were grown and treated as in figure 3.2. Samples were taken at the indicated timepoints for (\textit{A}) spindle morphology analysis and (\textit{B}) anti-HA and anti-MYC western blot analysis of TCA protein extracts separated on a 10% SDS polyacrylamide gel. Pgk1 is shown as a loading control.
3.2.8 Sgo1 is dispensable for the GAL-CDC55 metaphase delay

Given that CDC55 is required to delay anaphase onset upon SGO1 overexpression, I tested the requirement for SGO1 for the GAL-CDC55 metaphase delay. Deletion of SGO1 should have no effect on the GAL-CDC55 metaphase delay if Cdc55 functions downstream of Sgo1. I found that, despite the fact that sgo1Δ cells were significantly delayed in cell cycle progression, CDC55 overexpression was still able to delay cells in metaphase in the absence of SGO1 (Figure 3.20). This result strongly indicates that Cdc55 functions downstream of Sgo1 in the inhibition of Esp1.
Figure 3.20 Overexpression of *CDC55* causes a metaphase delay independently of Sgo1.
Wild type (AM1176), *GAL-CDC55* (AM4394), *sgo1Δ* (AM826) and *sgo1Δ GAL-CDC55* (AM4708) cells were grown and treated as in figure 3.2 except that α-factor was not re-added. Samples were taken at the indicated timepoints for spindle morphology analysis.
3.3 Discussion

3.3.1 Overproduced Sgo1 prevents cohesin cleavage by causing separase inhibition

My results show that overexpression of *SGO1* during mitosis inhibits anaphase onset. I exclude the possibility that this inability to enter anaphase was due to defects in mitotic spindle elongation because artificial cleavage of cohesin was on its own sufficient to promote anaphase spindle elongation in *SGO1*-overexpressing cells (Figure 3.3). I conclude that the delay to anaphase onset caused by *SGO1* overexpression was due to a failure to cleave cohesin. The finding that a large proportion of Scc1 remained uncleaved in *GAL-SGO1* cells supports this conclusion (Figure 3.4). It has recently been demonstrated that Sgo1 prevents cohesin cleavage in meiosis I by counteracting Rec8 phosphorylation, thereby rendering Rec8 refractory to separase (Ishiguro et al. 2010; Katis et al. 2010). High levels of Sgo1 could therefore prevent cohesin cleavage by reversing Scc1 phosphorylation. However, the fact that preventing Scc1 phosphorylation causes only a minor defect to cohesin cleavage (Alexandru et al. 2001; Hornig and Uhlmann 2004), together with the observation that there is no change to the gel mobility shift of full length Scc1 in *GAL-SGO1* cells (Figure 3.4), argues against this possibility. Furthermore, *SGO1* overexpression can no longer prevent anaphase onset when separase is also present at high levels (Clift et al. 2009), indicating that Scc1 is accessible for separase-dependent cleavage in *GAL-SGO1* cells. Instead, I find that separase itself if inhibited when *SGO1* is overexpressed, as cleavage of another separase substrate, Slk19, is also prevented by *SGO1* overexpression (Figure 3.5).

How does overproduced Sgo1 inhibit separase? It is well established that securin plays a key role in preventing separase activation. Securin binds to and inhibits separase until the APC<sup>Cdc20</sup> targets it for destruction at onset of anaphase. However, the inhibition of separase brought about by high levels of Sgo1 does not require the budding yeast securin Pds1 (Clift et al. 2009). These results therefore identify a mode of separase inhibition that is independent of any known upstream regulators of separase. Surprisingly, overexpression of *SGO1* causes Pds1 stabilization (Figure
3.6). How can this apparent paradox be explained? The fact that Pds1 is stabilized indicates that the APC$^{Cdc20}$ might be inactive. Nonetheless, APC$^{Cdc20}$ inhibition cannot be solely responsible for the $GAL-SGO1$ metaphase delay as inhibition of APC$^{Cdc20}$ is unable to prevent anaphase onset when Pds1 is absent (Yamamoto et al. 1996b), yet high levels of Sgo1 can (Clift et al. 2009). Sgo1 could therefore act both upstream of the APC and downstream of Pds1, with the latter being more crucial in preventing anaphase onset. An alternative possibility is that $SGOI$ overexpression causes Pds1 to become refractory to APC$^{Cdc20}$-dependent degradation. Analysis of other APC$^{Cdc20}$ substrates, such as the S phase cyclin Clb5, in $GAL-SGO1$ cells may shed light on this possibility. Finally, it may be that Esp1 inhibition by Sgo1 itself is sufficient to prevent Pds1 degradation. However, the finding that Pds1 is degraded on time in $esp1$-I mutant cells suggests this is probably not the case (Tinker-Kulberg and Morgan 1999). Whatever the reason for Pds1 stabilization in $GAL-SGO1$ cells, the conclusion remains that high levels of Sgo1 inhibit separase and that this inhibition does not require Pds1.

3.3.2 Evidence that budding yeast Sgo1 does not function through Aurora B to inhibit separase

Endogenous budding yeast Sgo1 is known to function in mitosis to ensure the bi-orientation of sister chromatids that lack tension (Indjeian et al. 2005; Fernius and Hardwick 2007; Indjeian and Murray 2007). This role is shared by the fission yeast mitotic shugoshin, Sgo2, and studies on this protein revealed that this function can be attributed to the loading of Aurora B to centromeres (Kawashima et al. 2007; Vanoosthuyse et al. 2007). However, the role of Aurora B (Ipl1 in budding yeast) in the function of budding yeast Sgo1 is controversial. There are conflicting reports as to whether Sgo1 is required for the centromeric localization of Ipl1 in meiosis or not (Yu and Koshland 2007; Kiburz et al. 2008). Nevertheless, it is clear that in mitotic cells, the centromeric localization of Ipl1 is not affected in a Bub1 kinase mutant where Sgo1 is absent from the centromere (Fernius and Hardwick 2007). Intriguingly, I found that $SGO1$ overexpression in mitosis caused Ipl1 to strongly colocalize with the short metaphase spindle, a situation that was rarely seen in wild
type metaphase cells (Figure 3.8). However, it is conceivable that the apparent change in Ipl1 localization in \textit{GAL-SGO1} cells could just be a consequence of delaying cells in metaphase. Examination of Ipl1 localization in wild type cells arrested in metaphase could address this issue. Furthermore, I did not compare the localization of Ipl1 to another centromere protein; therefore it is difficult to interpret what this Ipl1 localization represents. In addition, I analysed Ipl1 localization by whole cell immunofluorescence, which was insufficient to detect the known centromere localization of Ipl1 even in wild type cells (Figure 3.8; Biggins et al. 1999). Analysis of Ipl1 localization on metaphase chromosome spreads or by chromatin immunoprecipitation would be required to truly address whether overproduced Sgo1 affects Ipl1 localization. These discrepancies mean that very little can be concluded from this result. Nevertheless, several lines of evidence lead me to conclude that Ipl1 activity is not required for the function of overproduced Sgo1. A major role of Ipl1 is to destabilize inappropriate kinetochore-microtubule attachments (Pinsky et al. 2006), yet \textit{GAL-SGO1} cells appear to establish and maintain proper kinetochore-microtubule attachments, resulting in bi-oriented chromosomes that are under tension (Figure 3.10). Ipl1 activity has been shown to cause spindle checkpoint activation by creating unattached kinetochores (Pinsky et al. 2006) and also by directly phosphorylating the core checkpoint component Mad3 (King et al. 2007), however, \textit{SGO1} overexpression causes a metaphase delay in cells lacking a functional spindle checkpoint (Figure 3.6). Crucially, \textit{SGO1} overexpression is capable of inhibiting anaphase onset even when Ipl1 is inactivated (Figure 3.9), thus providing direct evidence that Ipl1 activity is not required for the function of overproduced Sgo1.

3.3.3 Identification of PP2A\textsuperscript{Cdc55} as a novel effector of Sgo1 function

During meiosis in budding yeast and fission yeast, Sgo1 interacts with a specific form of protein phosphatase 2A containing the B' regulatory subunit. It has been demonstrated that the recruitment of PP2A-B' to centromeres by Sgo1 is required to
confer centromeric cohesin resistant to separase in meiosis I (Kitajima et al. 2006; Riedel et al. 2006). However, I find that the delay to anaphase onset caused by SGO1 overexpression in mitosis is largely independent of the PP2A B′ regulatory subunit Rts1 (Figure 3.11). This is similar to a previous report from fission yeast in which the ectopic protection of Rec8 by Sgo1 in mitosis can still occur in the absence of the PP2A B′ subunit Par1 (Kitajima et al. 2006). Therefore, not all aspects of Sgo1 function require PP2A-B′. Instead, I have demonstrated that PP2A containing the B regulatory subunit Cdc55 is a key mediator of the mitotic function of overproduced Sgo1. Deleting CDC55, or preventing the interaction of Cdc55 with the core PP2A enzyme, largely bypasses the metaphase delay caused by SGO1 overexpression (Figure 3.11). Both separase activity and cohesin cleavage are required for this bypass (Figure 3.16; Figure 3.17), suggesting that inactivation of PP2A<sup>Cdc55</sup> allows separase activation SGO1-overexpressing cells. Intriguingly, separase does not appear to be completely active in cdc55Δ GAL-SGO1 cells because a substantial fraction of full length Scc1 remains uncleaved (Figure 3.13). A possible explanation for this is that overproduced Sgo1 also targets PP2A<sup>Rts1</sup> to cohesin and prevents its phosphorylation, making it a poor substrate for separase (Riedel et al. 2006; Katis et al. 2010). This uncleaved cohesin is not, however, localized to chromosomes (Figure 3.13), explaining why it is unable to prevent sister chromatid separation (Figure 3.15). Therefore upon SGO1 overexpression, PP2A<sup>Cdc55</sup> is required to prevent separase-dependent cleavage of only the chromatin-bound cohesin, which is most functionally relevant in holding sister chromatids together.

My findings that high levels of Sgo1 require PP2A<sup>Cdc55</sup>, but not Pds1, to inhibit separase activity suggest that PP2A<sup>Cdc55</sup> might provide an additional mechanism of separase inhibition that is independent of Pds1. In support of this interpretation, Chiroli et al. (2007) found that high levels of Cdc55 are sufficient to inhibit anaphase onset independently of Pds1. I expanded this result, and found that overexpression of CDC55 prevents the cleavage of the separase substrates, Scc1 and Slk19, in the absence of Pds1 (Figure 3.19). Therefore, similar to SGO1 overexpression, the inhibition of separase might be responsible for the delay to anaphase onset in CDC55-overexpressing cells. High levels of Cdc55 do not, however, depend on Sgo1 to inhibit anaphase onset (Figure 3.20). My data is consistent with a model whereby
PP2A$^{\text{Cdc55}}$ acts downstream of Sgo1 in a pathway that is ectopically activated by overproduction of either protein, resulting in securin-independent separase inhibition (Figure 3.21).

I initiated this study with the aim of understanding the mechanism of Sgo1 function during meiosis and mitosis. In the mean time, it has been demonstrated elsewhere that Sgo1 functions to recruit PP2A$^{\text{Rts1}}$ to the centromere in meiosis, where it dephosphorylates Rec8, making it refractory to separase (Riedel et al. 2006; Katis et al. 2010). This suggests that separase inhibition is probably not important for the role of Sgo1 in preventing cohesin cleavage in meiosis. The mechanism of Sgo1 action during mitosis, however, has remained elusive. My findings suggest that Sgo1 and PP2A$^{\text{Cdc55}}$ may play an important role in controlling the metaphase to anaphase transition by regulating separase activity. Because Sgo1 is required to prevent anaphase onset when sister chromatids lack tension (Indjeian et al. 2005), separase inhibition might be an important aspect of Sgo1 function in mitosis. In the next chapter, I explore this possibility and analyse the role of PP2A$^{\text{Cdc55}}$ and its effect on separase downstream of Sgo1 in more detail.
Figure 3.21 A model for the mechanism of Sgo1 function in mitosis. PP2A<sup>Cdc55</sup> might function downstream of Sgo1 in a pathway that is ectopically activated by overproduction of either protein, resulting in securin-independent separase inhibition.
Chapter 4

$\text{PP2A}^{\text{Cdc55}}$ is a separase inhibitor that mediates the functions of Sgo1 in mitosis
Chapter 4 – PP2A<sup>Cdc55</sup> is a separase inhibitor that mediates the functions of Sgo1 in mitosis

4.1 Introduction

4.1.1 Regulation of separase

The decision to separate sister chromatids is irreversible; once sister chromatid cohesion is destroyed, it cannot be established again until the next round of DNA replication (Uhlmann and Nasmyth 1998). Consequently, the cell must have mechanisms in place to ensure that cohesin cleavage by separase occurs at the proper time during the cell cycle. For much of the cell cycle, separase is inhibited through its association with an inhibitory chaperone known as securin (Ciosk et al. 1998; Hornig et al. 2002; Waizenegger et al. 2002). Once chromosome bi-orientation occurs, the APC<sup>Cdc20</sup> ubiquitinates securin, targeting it for degradation by the proteosomal machinery (Peters 2006). Securin degradation is necessary for separase activation, as expression of a non-degradable securin mutant effectively prevents sister chromatid separation (Cohen-Fix et al. 1996; Funabiki et al. 1996a; Zou et al. 1999; Hagting et al. 2002). However, real-time measurements in human cells revealed that there is a delay between securin degradation and sister chromatid separation (Hagting et al. 2002), suggesting that securin degradation might not be sufficient to initiate separase-dependent cohesin cleavage. Therefore, it is likely that securin-independent mechanisms exist to prevent cohesin cleavage before anaphase.

Evidence for the existence of such a mechanism comes from the findings that, in many eukaryotes, securin is not essential for viability. Accordingly, securin-deficient mice are apparently normal and mammalian securin<sup>−/−</sup> cells undergo a largely normal anaphase (Jallepalli et al. 2001; Mei et al. 2001; Wang et al. 2001; Pfleghaar et al. 2005; Wirth et al. 2006). Furthermore, budding yeast cells lacking securin are viable (Yamamoto et al. 1996a) and initiate cohesin cleavage and sister chromatid separation with similar timing to wild type cells (Alexandru et al. 1999; Alexandru et al. 2001). It should be noted, however, that securin-deficient yeast, mammalian cells and mice have reduced separase activity (Ciosk et al. 1998; Jallepalli et al. 2001;
Hornig et al. 2002; Wirth et al. 2006), highlighting a positive role for securin in promoting separase activation. In fact, the lethality of fission yeast and Drosophila securin mutants is due to an inability to separate sister chromatids (Stratmann and Lehner 1996; Funabiki et al. 1996b).

Cohesin cleavage by separase can be additionally regulated at the level of the substrate. Phosphorylation of the budding yeast meiotic cohesin, Rec8, plays a key role in promoting cohesin cleavage during meiosis (Brar et al. 2006; Katis et al. 2010). The mitotic cohesin, Scc1, is also phosphorylated prior to anaphase and phosphorylation of Scc1 by human polo-like kinase 1 (Plk1) enhances its rate of cleavage by separase in vitro (Alexandru et al. 2001). However, preventing Scc1 phosphorylation by depleting Cdc5 or by mutating polo phosphorylation sites in Scc1 causes only a minor defect in cohesin cleavage and anaphase onset (Alexandru et al. 2001; Hornig and Uhlmann 2004). Similarly, the in vitro cleavage of human Scc1 is enhanced by phosphorylation by Plk1, yet a non-phosphorylatable mutant Scc1 does not cause any defects to mitosis in human cells (Hauf et al. 2005). It appears that Scc1 phosphorylation might only be necessary for cohesin cleavage in cells lacking securin (Alexandru et al. 2001), which have reduced separase activity (Jallepalli et al. 2001; Hornig et al. 2002).

Separase itself can also be inhibited independently of securin. In Xenopus cell-free extracts, phosphorylation-dependent binding of the Cdk1-cyclin B1 complex to separase is sufficient for securin-independent inhibition (Stemmann et al. 2001; Gorr et al. 2005). This mode of separase inhibition was initially uncovered by the finding that high levels of non-degradable cyclin B1 prevented anaphase onset despite securin degradation (Stemmann et al. 2001). Expression of a non-degradable cyclin B1 mutant in human cells also blocks sister chromatid separation in the absence of securin (Hagting et al. 2002; Chang et al. 2003; Wolf et al. 2006), suggesting that cyclin B1 can inhibit separase in vivo. Such a mechanism is unlikely to exist in budding yeast, however, as chromosome segregation can proceed in the presence of high Cdk1 activity (Surana et al. 1993).

In Chapter 3, I demonstrated that high levels of Sgo1 inhibit separase independently of securin, but dependent on PP2A Cdc55. These results suggested the existence of an
alternative separase regulatory pathway in budding yeast that requires Sgo1 and PP2A^{Cdc55}.

4.1.2 Preventing anaphase onset in response to bi-orientation defects

Accurate chromosome segregation requires that sister kinetochores attach to microtubules that emanate from opposite poles of the spindle (bi-orientation). The cohesin linkages between sister chromatids facilitate bi-orientation by counteracting the pulling forces of the bi-polar spindle, thereby ensuring that sister chromatids are under tension and primed to be segregated away from each other in anaphase (reviewed in Tanaka et al. 2005). Two types of defects in the bi-orientation process are recognised by the cell, which responds by preventing anaphase onset to allow sufficient time for defects to be repaired before chromosome segregation can initiate. First, kinetochores that are not attached to microtubules (Rieder et al. 1994; Rieder et al. 1995) and second, kinetochores that are not under tension (Li and Nicklas 1995; Shonn et al. 2000; Biggins and Murray 2001; Stern and Murray 2001). Key players in the response to both types of defect are the Mad and Bub proteins (and Mps1) of the spindle checkpoint, which work together to inhibit the APC^{Cdc20}, causing securin stabilization and thereby inhibiting separase (reviewed in Musacchio and Salmon 2007).

Budding yeast Sgo1 also prevents anaphase onset when there are defects in the bi-orientation process, but in this case, Sgo1 seems to respond only to tension defects. Unlike mad mutants, sgo1 mutants can still arrest in metaphase in the presence of microtubule depolymerising drugs, and therefore unattached kinetochores (Indjeian et al. 2005). However, Sgo1 is required to respond to a lack of tension between sister chromatids caused by depletion of the cohesin Scc1, as sgo1 mutants degrade securin under these conditions (Indjeian et al. 2005), albeit with a delay compared to mad mutants (Fernius and Hardwick 2007). The mechanism by which Sgo1 prevents anaphase onset in response to a lack of tension is completely unknown.
Despite executing a normal metaphase arrest when released from G₁ in presence of microtubule depolymerising drugs, *sgo1* mutants die rapidly after the drugs are removed (Indjeian et al. 2005). This puzzling phenotype is likely due to the presence of mono-oriented chromosomes, as sister chromatids are often pulled to the same spindle pole once the mitotic spindle reforms (Indjeian et al. 2005). However, *sgo1* mutants efficiently bi-orient chromosomes during an unperturbed cell cycle as these cells are viable (Indjeian et al. 2005). Therefore, *sgo1* mutants are defective in bi-orienting sister chromatids only when kinetochore-microtubule attachments are initiated after the cell has progressed from G₁ to metaphase without microtubules. Interestingly, under these conditions the spindle pole bodies (SPBs) are yet to be separated, subsequently sister chromatids might initially attach to microtubules that emanate from the old SPB, leading to mono-orientation (Indjeian and Murray 2007). Unlike wild type cells, *sgo1* mutants might be unable to re-orient sister chromatids and therefore missegregate chromosomes and lose viability (Indjeian and Murray 2007). Exactly how Sgo1 promotes the re-orientation of sister chromatids is unclear, however, this role of Sgo1 might be intimately linked to its role in preventing anaphase in response to a lack of tension between sister chromatids, as mono-oriented chromosomes would fail to generate tension.

In this chapter, I investigate the possibility that PP2A<sup>Cdc55</sup> contributes to separase inhibition during a normal cell cycle and ask whether this pathway could underlie the role of Sgo1 in mitosis. I show that Cdc55 works redundantly with securin to inhibit separase, uncovering a new mechanism for separase inhibition. I find that Cdc55 and Sgo1 play similar roles in sensing a lack of tension between sister chromatids and provide evidence that Sgo1 may perform its functions in bi-orientation through PP2A<sup>Cdc55</sup>. I propose that PP2A<sup>Cdc55</sup> is a separase inhibitor that is employed by Sgo1 when sister chromatids are not under tension.
4.2 Results

4.2.1 Cdc55 prevents precocious Esp1 activation in the absence of Pds1

4.2.1.1 Depletion of Pds1 in cdc55Δ cells causes inviability

In Chapter 3, I showed that Cdc55 is able to inhibit Esp1 activity when overproduced and that Cdc55 is required downstream of overproduced Sgo1 for Esp1 inhibition. Does Cdc55 contribute to Esp1 inhibition in a normal cell cycle? It is well established that Esp1 is inhibited by Pds1 (Ciosk et al. 1998), however, cells lacking PDS1 are viable (Yamamoto et al. 1996a), suggesting that there may be other modes of Esp1 inhibition, independent of Pds1. My overexpression data from Chapter 3 suggests that a pathway involving PP2A\textsuperscript{Cdc55}, downstream of Sgo1, may provide this additional level of Esp1 regulation. I reasoned that if inhibition of Esp1 by the Sgo1-PP2A\textsuperscript{Cdc55} pathway is physiologically relevant, rather than an artefact of overexpression, then simultaneous loss of this pathway and PDS1 should have additive effects.

Interestingly, cells lacking both CDC55 and PDS1 are inviable (Tang and Wang 2006; Chiroli et al. 2007), indicating that Cdc55 may indeed be important for Esp1 inhibition in a normal cell cycle. To determine the cause of the inviability of cdc55Δ pds1Δ cells, I generated a conditional PDS1 allele to allow its depletion in cdc55Δ cells. The endogenous PDS1 promoter was replaced with the MET3 promoter (MET-PDS1) such that PDS1 expression is repressed by addition of methionine to the growth medium (Cherest et al. 1985). Releasing MET-PDS1 cells from a G\textsubscript{1} arrest into methionine-containing medium efficiently repressed PDS1 expression, as sister chromatid separation occurred in the presence of benomyl and nocodazole (Figure 4.1A), similar to a pds1 mutant (Yamamoto et al. 1996b). As expected, combining cdc55Δ with MET-PDS1 resulted in lethality in the presence, but not the absence, of methionine (Figure 4.1B). I therefore used this conditionally lethal strain to analyse the consequences of loss of both Cdc55 and Pds1.
Figure 4.1 Depletion of Pds1 in cdc55Δ cells causes lethality. (A) Wild type (AM2812) and MET-PDS1 (AM3665) cells carrying CEN4-GFP were grown at room temperature in SC/-met/D, arrested in G1 with α-factor and released into YEPD + 8mM methionine to deplete PDS1 plus benomyl and nocodazole to depolymerize microtubules. The percentage of cells with separated GFP foci was determined at the indicated timepoints after release from G1 (at least 200 cells were counted at each timepoint). (B) Wildtype (AM1145), MET-PDS1 (AM4429), cdc55Δ (AM4757), cdc55Δ MET-PDS1 (AM4675), sgo1Δ (AM1474) and sgo1Δ MET-PDS1 (AM4422) cells carrying SCC1-6HA were streaked onto plates either lacking (SC/-met/D) or containing (YEPD+met) methionine and incubated at 30°C.
4.2.1.2 Cohesin is cleaved prematurely in cdc55Δ MET-PDS1 cells

I predicted that the lethality of cdc55Δ pds1Δ cells might be a result of unregulated Esp1 activity. If this were correct, one might expect precocious Esp1 activation during an unperturbed cell cycle. Wild type, MET-PDS1, cdc55Δ and cdc55Δ MET-PDS1 cells were released from a G1 arrest in the presence of methionine to repress PDS1 expression, and Esp1 activity was judged by monitoring Scc1 cleavage by western blot. In wild type, MET-PDS1 and cdc55Δ cells, Scc1 cleavage product appeared after 60 minutes, coinciding with a decline in full-length Scc1 and the onset of spindle elongation (Figure 4.2A-C). Strikingly, however, Scc1 cleavage product was detected after only 30 minutes in cdc55Δ MET-PDS1 cells, accompanied by a more rapid decline in full length Scc1 (Figure 4.2D), indicating that some Esp1-dependent cohesin cleavage occurred precociously in cells lacking both CDC55 and PDS1. Although sgo1Δ pds1Δ double mutants are viable (Figure 4.1B), I also analysed the effect of the combined loss of SGO1 and PDS1 on cohesin cleavage. The majority of Scc1 cleavage product appeared, and full-length Scc1 declined, after 90 minutes in both sgo1Δ and sgo1Δ MET-PDS1 cells, however, a low level of cleavage was seen at earlier time points in the latter (Figure 4.2E,F), suggesting that Sgo1 may play only a minor role in Esp1 inhibition in an unperturbed cell cycle. Perhaps in contrast to Cdc55, Sgo1 is not active in directing Esp1 inhibition during an unchallenged mitosis; therefore SGO1 deletion would be expected have little effect on Esp1 activity during an unperturbed cell cycle, even in the absence of PDS1.
Figure 4.2 Loss of both CDC55 and PDS1 causes precocious cohesin cleavage. (A) Wild type (AM1145); (B) MET-PDS1 (AM4429); (C) cdc55Δ (AM4757); (D) cdc55Δ MET-PDS1 (AM4675); (E) sgo1Δ (AM1474) and (F) sgo1Δ MET-PDS1 (AM4422) cells carrying SCC1-6HA were grown at 30°C in SC/-met/D, arrested in G1 with α-factor and released into YEPD + 8mM methionine to deplete PDS1. When cells had budded, α-factor was re-added to limit analysis to a single cell cycle. Samples were taken at the indicated timepoints for spindle morphology analysis and anti-HA western blot analysis of TCA protein extracts separated on a 10% SDS polyacrylamide gel. Pgk1 is shown as a loading control. A short (upper) and long (lower) exposure is shown for Scc1 western.
E

\[ sgo1 \Delta \]

\[ \text{metaphase} \]  \[ \text{anaphase} \]

Time (min) 0 30 45 60 75 90 105 120 150 165 180

Scc1 FL
Scc1 FL
Scc1 cleaved
Pgk1

F

\[ sgo1 \Delta \ M E T-P D S 1 \]

\[ \text{metaphase} \]  \[ \text{anaphase} \]

Time (min) 0 30 45 60 75 90 105 120 150 165 180

Scc1 FL
Scc1 FL
Scc1 cleaved
Pgk1

155
4.2.1.3 Precocious centromere separation and aneuploidy in

\textit{cdc55\Delta MET-PDS1} cells

To determine if premature cohesin cleavage in \textit{cdc55\Delta MET-PDS1} cells triggered premature sister chromatid separation, I monitored the segregation of chromosome IV labelled with GFP close to the centromere (\textit{CEN4-GFP}). In wild type, \textit{MET-PDS1} and \textit{cdc55\Delta} cells, \textit{CEN4-GFP} separation coincided with the appearance of anaphase spindles at 60 minutes (Figure 4.3A-C), consistent with the timely cohesin cleavage seen previously (Figure 4.2A-C). The separation of \textit{CEN4-GFP} signals coincided with anaphase onset and occurred with similar timing in \textit{sgo1\Delta} and \textit{sgo1\Delta MET-PDS1} cells (Figure 4.3E,F), underlining the minor role of Sgo1 in an unperturbed cell cycle. Interestingly, however, \textit{CEN4-GFP} signals began to separate after only 30 minutes in \textit{cdc55\Delta MET-PDS1} cells, before the appearance of anaphase spindles (Figure 4.3D), indicating that the precocious cohesin cleavage seen in these cells resulted in premature loss of centromere cohesion. The consequence of premature centromere separation on chromosome segregation was revealed by scoring the percentage of large-budded cells with divided nuclei that had segregated both copies of \textit{CEN4-GFP} to the same nucleus (Figure 4.3, percentages inside graphs). Importantly, chromosome IV missegregation occurred at a much higher frequency (10\% of cells) in \textit{cdc55\Delta MET-PDS1} cells compared to the control strains (0-2\% of cells; Figure 4.3), indicating that the production of aneuploid daughter cells may cause the inviability of \textit{cdc55\Delta MET-PDS1} cells.

I also examined the timing of sister chromatid separation outside the centromere, by visualizing a GFP-label at the \textit{URA3} locus (\textit{URA3-GFP}), 38.4kb from the centromere on chromosome V (Michaelis et al. 1997). As seen with \textit{CEN4-GFP}, \textit{URA3-GFP} signals separated as anaphase spindles appeared, and were missegregated at a low frequency, in wild type, \textit{MET-PDS1}, \textit{sgo1\Delta}, \textit{sgo1\Delta MET-PDS1} and \textit{cdc55\Delta} strains (Figure 4.4). Intriguingly, in \textit{cdc55\Delta MET-PDS1} cells precocious separation of \textit{URA3-GFP} signals (compared to \textit{CEN4-GFP}) was less prominent, however chromosome V was missegregated at a similar frequency (8.8\% of cells) as chromosome IV (Figure 4.4D). This result suggests that the premature loss of sister chromatid cohesion in cells lacking Cdc55 and Pds1 could be initiated at the
centromere, and that intact centromere cohesion is important for the accurate
distribution of chromosomes between daughter cells. Indeed, reduced centromeric
cohesin association decreases chromosome segregation fidelity (Eckert et al. 2007).
Furthermore, the finding that the \textit{cde55\Delta pds1\Delta} synthetic lethality is characterized by
precocious cohesin cleavage, precocious loss of centromere cohesion and
chromosome missegregation, indicates that Cdc55 and Pds1 function redundantly to
restrain Esp1 activity.
Figure 4.3 Loss of both CDC55 and PDS1 causes precocious centromere separation and chromosome missegregation. (A) Wild type (AM2812); (B) MET-PDS1 (AM3665); (C) cdc55Δ (AM4330); (D) cdc55Δ MET-PDS1 (AM4676); (E) sgo1Δ (AM962) and (F) sgo1Δ MET-PDS1 (AM3865) cells carrying CEN4-GFP were grown and treated as in figure 4.2. Samples were taken at the indicated timepoints for spindle morphology analysis and the percentage of cells with separated GFP foci (at least 200 cells were counted at each timepoint). The percentage of large-budded cells with divided nuclei that had segregated both copies of CEN4-GFP to the same nucleus is shown inside each graph.
Figure 4.4 Loss of both \( CDC55 \) and \( PDS1 \) leads to chromosome missegregation. (A) Wild type (AM2679); (B) \( MET-PDS1 \) (AM4980); (C) \( cdc55\Delta \) (AM5015); (D) \( cdc55\Delta MET-PDS1 \) (AM4979); (E) \( sgo1\Delta \) (AM1031) and (F) \( sgo1\Delta MET-PDS1 \) (AM5055) cells carrying \( URA3-GFP \) were grown and treated as in figure 4.2. Samples were taken at the indicated timepoints for spindle morphology analysis and the percentage of cells with separated GFP foci (at least 200 cells were counted at each timepoint). The percentage of large-budded cells with divided nuclei that had segregated both copies of \( URA3-GFP \) to the same nucleus is shown inside each graph.
4.2.2 The role of the Sgo1-PP2A<sup>Cdc55</sup> pathway in the spindle checkpoint

4.2.2.1 Sensitivity of sgo1∆ and cdc55∆ mutants to microtubule depolymerising drugs

My Pds1 depletion experiments suggested that Cdc55 and Pds1 might work redundantly to inhibit Esp1; furthermore, I found that high levels of Sgo1 required PP2A<sup>Cdc55</sup> to prevent Esp1 activation. This suggests that an Sgo1-PP2A<sup>Cdc55</sup> pathway plays a key role in regulating chromosome segregation. However, the relationship between Sgo1 and Cdc55 has only so far been established during non-physiological conditions, when SGO1 was highly overexpressed. I wanted to know whether Cdc55 is also required for the function of endogenous Sgo1. When sister chromatids lack tension, Sgo1 is required to arrest cells in metaphase, although it is not known whether Cdc55 also plays a similar role. However, it has been recognised that, unlike sgo1∆ mutants, cdc55∆ mutants are unable to prevent anaphase onset in response to unattached kinetochores induced by microtubule depolymerization (Minshull et al. 1996). Both sgo1∆ and cdc55∆ mutants exhibit growth sensitivity to the microtubule depolymerising drug benomyl (Minshull et al. 1996; Indjeian et al. 2005). Benomyl acts by destabilising microtubules and therefore disrupts kinetochore-microtubule attachments. Cells with a functional spindle checkpoint are able to recover kinetochore-microtubule attachments and continue to grow in the presence of benomyl. Checkpoint-deficient cells, however, die in the presence of benomyl because they continue to divide before proper kinetochore-microtubule attachments can be recovered, resulting in aneuploidy. I performed epistasis analysis of benomyl sensitivity with sgo1∆, cdc55∆ and mad1∆ mutants to help determine whether Sgo1 and Cdc55 function in a common pathway. I found that the cdc55∆ mutant is slightly more sensitive to benomyl than the sgo1∆ mutant, but is not significantly further compromised in combination with it, when taking into account the slower growth of cdc55∆ sgo1∆ in the absence of benomyl (Figure 4.5A). Furthermore, both sgo1∆ and cdc55∆ mutants were more benomyl sensitive than, and showed elevated benomyl sensitivity when combined with, the mad1∆ mutant (Figure 4.5A). An rts1∆
mutant, however, showed very weak benomyl sensitivity compared to the other mutants (Figure 4.5A). These results imply that Sgo1 and Cdc55 may function in the same spindle checkpoint pathway, but act in parallel to Mad1. It is important to note however, that Sgo1 and Cdc55 likely have distinct roles that are perhaps independent of the checkpoint, as demonstrated by the additive effects of the cdc55Δ sgo1Δ mutant in absence of microtubule-depolymerising drugs (Figure 4.5A).

4.2.2.2 Negative regulation of mitotic exit by Sgo1 and Cdc55

The role of Cdc55 in the spindle checkpoint response to unattached kinetochores has been attributed to its function in the inhibition of mitotic exit by preventing premature Cdc14 release from the nucleolus (Queralt et al. 2006; Yellman and Burke 2006). At the onset of anaphase, Cdc14 release from the nucleolus is initiated by Esp1, which in addition to its role in cleaving cohesin, is part of the Cdc14 early anaphase release (FEAR) network of proteins (Stegmeier et al. 2002; Sullivan and Uhlmann 2003). However, complete release of Cdc14 requires the activity of the mitotic exit network (MEN), which is also capable of inducing full Cdc14 release even in the absence of FEAR (reviewed in Stegmeier and Amon 2004). A spo12Δ lte1Δ double mutant is inviable because cells are defective in both FEAR (spo12Δ) and MEN (lte1Δ) pathways for mitotic exit (Stegmeier et al. 2002). Deletion of CDC55 suppresses the synthetic lethality of spo12Δ lte1Δ, indicating that Cdc55 is a negative regulator of mitotic exit (Figure 4.5B; Yellman and Burke 2006). I found that sgo1Δ spo12Δ lte1Δ triple mutants are also viable (Figure 4.5 B), demonstrating that Sgo1 also inhibits mitotic exit and supporting the notion that Sgo1 and Cdc55 function in a common pathway.
Figure 4.5 *sgo1Δ* and *cdc55Δ* mutants are benomyl sensitive and rescue the synthetic lethality of *lte1Δ spo12Δ* double mutants. (A) Ten-fold serial dilutions of wild type (AM5020), *mad1Δ* (AM5021), *cdc55Δ* (AM5022), *cdc55Δ mad1Δ* (AM5023), *sgo1Δ* (AM5041), *sgo1Δ mad1Δ* (AM5040), *cdc55Δ sgo1Δ* (AM5291) and *rts1Δ* (AM5039) cells were spotted onto YEPD medium containing benomyl at concentrations of 0 μg/ml, 4 μg/ml or 6 μg/ml and incubated at room temperature. (B) Ten-fold serial dilutions of *lte1Δ* (AM1600), *lte1Δ spo12Δ* (AM3814), *lte1Δ sgo1Δ* (AM3813), *lte1Δ spo12Δ sgo1Δ* (AM5095) and *lte1Δ spo12Δ cdc55Δ* (AM5127) cells carrying LTE1 on a CEN-URA plasmid were spotted onto medium lacking uracil (-URA; plasmid selection) or containing 5-fluoroorotic acid (FOA; plasmid counterselection) and incubated at 30°C.
4.2.2.3 Sgo1 and Cdc55 are required to respond to lack of tension between sister chromatids

The spindle checkpoint can respond to both unattached kinetochores and attached kinetochores that are not under tension by delaying anaphase, allowing time for all chromosomes to achieve tension-generating bi-orientation on the mitotic spindle. Tension at kinetochores is generated by the pulling force of the bipolar spindle on sister kinetochores that are linked by the cohesin complex (Tanaka et al. 2000). In the absence of cohesin, kinetochore-microtubule attachment still occurs as chromosomes are pulled towards the poles, however, tension cannot be generated at kinetochores because sister chromatids are no longer linked (Tanaka et al. 2000). By utilizing such conditions, Indjeian et al. demonstrated that Sgo1 is required for Pds1 stabilization in response to sister chromatids that lack tension (Indjeian et al. 2005).

If Cdc55 were to function downstream of endogenous Sgo1, I reasoned that Cdc55 should also be required for the response to lack of tension. To test this, I recapitulated a tension-less mitosis in which sister chromatid cohesion is absent, and tested the requirement of Sgo1 and Cdc55 for checkpoint activation. To monitor the checkpoint, I assayed for Pds1 destruction as well as indicators of mitotic exit, release of Cdc14 from the nucleolus and degradation of the mitotic cyclin Clb2; all of which should be inhibited if the checkpoint is functional.

For the experiment, I used cells with SCC1 expressed exclusively from the methionine-repressible MET3 promoter (MET-SCC1) such that cohesin is depleted by addition of methionine to the growth medium. Cells were arrested in G1 with α-factor in the presence of methionine to repress SCC1 expression, and then released synchronously into the cell cycle. Depletion of Scc1 in otherwise wild type cells resulted in robust checkpoint activation as Pds1 and Clb2 proteins were stabilized on western blots and immunofluorescence of Cdc14 revealed its sequestration in the nucleolus (Figure 4.6B,C). As expected, the spindle checkpoint mutant mad1Δ failed to arrest the cell cycle, as Pds1 and Clb2 destruction occurred and Cdc14 was released from the nucleolus (Figure 4.6B,C). Interestingly, both sgo1Δ and cdc55Δ mutants also failed to arrest the cell cycle, but in a manner that was distinct from the
\textit{mad1}\Delta\text{ mutant: Cdc14 was released from the nucleolus, yet Pds1 and Clb2 protein levels appeared to remain relatively stable on western blots (Figure 4.6B,C). Immunofluorescence analysis revealed, however, that Pds1 destruction was in fact advanced to a similar level in \textit{sgo1}\Delta and \textit{cdc55}\Delta cells compared to wild type, but was not completely destroyed as in the spindle checkpoint mutant \textit{mad1}\Delta (Figure 4.6A). This result is in contrast with the original report where \textit{sgo1}\Delta mutants were shown to respond to Scc1 depletion similarly to \textit{mad2}\Delta mutants (Indjeian et al. 2005). However, in support of my findings, a more recent study found that \textit{sgo1}\Delta mutants degrade Pds1 with a delay compared to \textit{mad2}\Delta mutants in response to lack of tension (Fernius and Hardwick 2007). Why do \textit{sgo1}\Delta and \textit{cdc55}\Delta mutants show a distinct phenotype to \textit{mad} mutants in response to lack of tension? The core spindle checkpoint proteins are required to inhibit the APC\textsubscript{Cdc20}, thus explaining why in \textit{mad1}\Delta mutants Pds1 protein is destroyed and mitotic exit proceeds. The partial degradation of Pds1 and Clb2 seen in \textit{sgo1}\Delta and \textit{cdc55}\Delta mutants, on the other hand, might be an indirect consequence of Cdc14 release, rather than due to an inability to inhibit the APC\textsubscript{Cdc20}. Cdc14 release promotes the activation of the Cdh1-dependent form of the APC by dephosphorylation of Cdh1 (Visintin et al. 1998; Jaspersen et al. 1999). APC\textsubscript{Cdh1} targets Clb2 for ubiquitin-dependent proteolysis, however, complete degradation of Clb2 requires both the Cdh1- and Cdc20-dependent forms of the APC (Yeong et al. 2000). Furthermore, APC\textsubscript{Cdh1} can promote the partial degradation of Pds1, at least when overexpressed, whereas complete Pds1 degradation requires APC\textsubscript{Cdc20} (Visintin et al. 1997). I suggest that Cdc14 release in \textit{cdc55}\Delta and \textit{sgo1}\Delta mutants in response to a lack of tension results in the activation of APC\textsubscript{Cdh1}, which in turn promotes the partial degradation of Pds1 and Clb2. Most importantly, my result establishes a relationship between endogenous Sgo1 and Cdc55 that was implicated by my overexpression studies, and suggests that these two proteins might indeed function in the same pathway, which is distinct from the canonical spindle checkpoint.
Figure 4.6 Sgo1 and Cdc55 are similarly required for the response to lack of tension between sister chromatids. Wild type (AM4772), mad1Δ (AM4856), sgo1Δ (AM4773) and cdc55Δ (AM4708) cells carrying PDS1-18MYC, 3HA-CDC14 and MET-SCC1 were grown in SC/-met/D at room temperature. Cells were transferred to YEPD + 8 mM methionine to deplete SCC1, arrested in G1 with α-factor and released into YEPD + 8 mM methionine at room temperature. Pds1-18MYC and 3HA-Cdc14 localization was determined after immunofluorescence. The percentage of cells with (A) Pds1 positive staining and (B) Cdc14 released from the nucleolus was determined at the indicated timepoints after release from G1 (at least 200 cells were counted at each timepoint). (C) Pds1-18MYC and Clb2 levels were analysed by anti-MYC and anti-Clb2 western blot analysis of TCA protein extracts separated on an 8% SDS polyacrylamide gel. Pgk1 is shown as a loading control.
4.2.2.4 Sgo1 and PP2A<sub>Cdc56</sub> ensure the bi-orientation of sister chromatids in metaphase

Sgo1 is not required for normal chromosomal bi-orientation during an unperturbed cell cycle; however, Sgo1 becomes essential for bi-orientation when the mitotic spindle reforms after transient exposure to the microtubule depolymerising drugs benomyl and nocodazole (Indjeian et al. 2005). More specifically, when cells are released from a benomyl:nocodazole arrest, sister kinetochores often attach to microtubules emanating from the same spindle pole body (SPB), and these defective attachments cannot be sensed and/or corrected in sgo1 mutants, resulting in mono-oriented chromosomes (Indjeian and Murray 2007). How Sgo1 responds to and/or corrects mono-oriented attachments is not known, I therefore asked if Cdc55, like Sgo1, is required for the bi-orientation after microtubule depolymerization.

To re-create the situation where Sgo1 is required for bi-orientation, I needed to release cells from G<sub>1</sub> into a metaphase arrest without microtubules, then repolymerise microtubules whilst maintaining the metaphase arrest. Sister chromatid bi-orientation could then be assayed directly by monitoring the separation of sister centromeres that occurs transiently upon chromosome bi-orientation in metaphase cells (Goshima and Yanagida 2000; He et al. 2000; Tanaka et al. 2000). To achieve the metaphase arrest, I used a strain with CDC20 expressed exclusively from the methionine-repressible MET3 promoter (<i>MET-CDC20</i>) such that Cdc20 is depleted, and therefore APC<sup>Cdc20</sup> is kept inactive, by addition of methionine to the growth medium. I monitored the bi-orientation of chromosome IV by visualizing the binding of GFP-TetR to an array of TetO binding sites 2.4kb from centromere (CEN4-GFP; He et al. 2000). Chromosome IV was scored as bi-oriented if two CEN4-GFP signals were seen, representing sister centromere separation, whereas for mono-oriented chromosome IV, sister CEN4-GFP signals were unresolved.

Cells were released from G<sub>1</sub> into a metaphase arrest by depleting CDC20 in the presence of the microtubule depolymerising drugs benomyl and nocodazole. The spindle checkpoint mutant mad1Δ, which fails to arrest in response to microtubule depolymerising drugs due to an inability to inhibit Cdc20 (Hwang et al. 1998; Kim et
al. 1998), did not separate sister chromatids anymore that wild type cells in benomyl:nocodazole (time 0; Figure 4.7B); therefore confirming the robustness of \textit{CDC20} depletion in this experiment. After 3 hours, the drugs were washed out to allow the mitotic spindle to reform whilst maintaining a metaphase arrest due to \textit{CDC20} depletion (Figure 4.7A). Chromosome bi-orientation occurred quickly in wild type cells and by 80 minutes after drug washout, \textasciitilde50\% of cells showed separated \textit{CEN4}-GFP signals, representing bi-orientation (Figure 4.7B,C). It should be noted, however, that bi-oriented centromeres oscillate between stages of separation and re-association in metaphase arrested cells (Indjeian and Murray 2007) and therefore a snap-shot of fixed cells will likely underestimate the percentage of cells with bi-oriented chromosome IV. As expected, \textit{sgo1}\textasciitilde\textit{A} mutants were defective in chromosome bi-orientation, as centromere IV separation occurred in no more that 20\% of cells (Figure 4.7B,C). Strikingly, \textit{cdc55}\textasciitilde\textit{A} mutants were similarly defective in chromosome bi-orientation as \textit{sgo1}\textasciitilde\textit{A} mutants (Figure 4.7C), whereas \textit{rts1}\textasciitilde\textit{A} and \textit{mad1}\textasciitilde\textit{A} mutants had no defect (Figure 4.7B). This data shows that Sgo1 shares a role with Cdc55 in ensuring proper chromosome bi-orientation upon spindle reformation after microtubule depolymerization, lending support to the idea that Cdc55 and Sgo1 function in a common pathway.

To determine whether Sgo1 functions through its interaction with PP2A for this role in bi-orientation, I analysed an \textit{sgo1} mutant that retains its centromere localization, but cannot bind to PP2A (\textit{sgo1-3A}; Xu et al. 2009). Interestingly, the \textit{sgo1-3A} mutant exhibited a bi-orientation defect identical to the \textit{sgo1}\textasciitilde\textit{A} and \textit{cdc55}\textasciitilde\textit{A} mutants (Figure 4.7C). Taken together with the finding that \textit{rts1}\textasciitilde\textit{A} cells do not have a bi-orientation defect, the similar phenotype of \textit{sgo1}\textasciitilde\textit{A}, \textit{cdc55}\textasciitilde\textit{A} and \textit{sgo1-3A} mutants suggest that Sgo1 ensures proper chromosome bi-orientation specifically through its interaction with PP2A\textasciitilde\textit{Cdc55}. However, it remains a possibility that the \textit{sgo1-3A} mutations disrupt the interaction of Sgo1 with additional proteins that might be important for bi-orientation.
Figure 4.7 Sgo1 and PP2A<sup>Cdc55</sup> ensure sister chromatid biorientation. (A) Schematic of the experiments conducted in B and C. Wild type (AM914), <i>mad1Δ</i> (AM5068), <i>sgo1Δ</i> (AM982), <i>cdc55Δ</i> (AM6486), <i>rts1Δ</i> (AM3667) and <i>sgo1-3A</i> (AM6478) cells carrying <i>MET-CDC20</i> and <i>CEN4-GFP</i> were grown at 30°C in SC/-met/D, arrested in G<sub>1</sub> with α-factor and released into YEPD + 8mM methionine to deplete Cdc20 plus benomyl and nocodazole to depolymerize microtubules. Cells were then released from benomyl and nocodazole into YEPD + 8mM methionine to re-polymerize microtubules whilst maintaining Cdc20 depletion. (B,C) The percentage of cells with separated GFP foci was determined in G<sub>1</sub> and then at the indicated timepoints after release from benomyl and nocodazole (at least 200 cells were counted at each timepoint). Strains in B and C were analysed in separate experiments.
4.3 Discussion

4.3.1 PP2A$^{Cdc55}$ contributes to separase inhibition

Cohesin cleavage by separase is an irreversible event that must be tightly regulated. If cohesin cleavage occurs before all chromosomes achieve bi-orientation, this could lead to chromosome missegregation and the production of aneuploid daughter cells. Securin plays a key role in regulating cohesin cleavage, by binding to and inhibiting separase until its destruction at the onset of anaphase. However, securin is not essential for proliferation in many systems, suggesting that other mechanisms exist to restrict cohesin cleavage to the appropriate window during the cell cycle. My overexpression data from Chapter 3 hinted that a pathway involving PP2A$^{Cdc55}$, downstream of Sgo1, might provide an additional level of separase regulation. Crucially, in this chapter, I have presented key evidence to suggest that PP2A$^{Cdc55}$ works to inhibit separase redundantly with securin during every cell cycle. I found that the combined loss of Cdc55 and Pds1 resulted in precocious cohesin cleavage, precocious sister chromatid separation and chromosome missegregation (Figure 4.2-4.4), all of which are indicative of unregulated Esp1 activation. Given that Esp1 activity is reduced when Pds1 is absent (Hornig et al. 2002), my Pds1 depletion experiments might even have undervalued the importance of Cdc55 in Esp1 inhibition. Interestingly, the precocious sister chromatid separation in $\textit{cdc55}^\Delta \textit{MET-PDS1}$ cells appeared to be initiated at centromere (Figure 4.3). Furthermore, it was previously shown that $\textit{cdc55}^\Delta$ mutants preferentially separate sister centromeres after DNA damage, independently of Pds1 degradation (Tang and Wang 2006). These observations raise the intriguing possibility that Cdc55 is particularly important in regulating Esp1 activity in the vicinity of the centromere. Because centromere cohesion is critical for accurate chromosome segregation (Eckert et al. 2007; Fernius and Marston 2009; Ng et al. 2009), an additional layer of Esp1 regulation by Cdc55 in this region might safeguard against aneuploidy. It is important to note that a reciprocal role for Esp1 in downregulating PP2A$^{Cdc55}$ activity in anaphase has previously been described (Queralt et al. 2006). I do not suggest that my results contradict those of Queralt et al. (2006), instead, I argue that PP2A$^{Cdc55}$ might
function both upstream and downstream of Esp1 as part of a regulatory feedback mechanism that ensures a switch-like activation of Esp1 at the onset of anaphase (Figure 4.8; Holt et al. 2008). However, I do not know whether PP2A<sup>Cdc55</sup> inhibits Esp1 directly and therefore it remains a possibility that other factors downstream of PP2A<sup>Cdc55</sup> may contribute to Esp1 regulation. Indeed, Esp1 might exist as part of a large protein complex, similar to fission yeast separase (Funabiki et al. 1996).

**4.3.2 PP2A<sup>Cdc55</sup> carries out the functions of Sgo1 in mitosis**

Previous studies showed that Sgo1 plays an important role in sensing lack of tension between sister chromatids and ensuring chromosome bi-orientation after spindle disruption, however the underlying mechanisms were unresolved (Indjeian et al. 2005; Indjeian and Murray 2007). In this chapter, I provide evidence that Sgo1 collaborates with PP2A<sup>Cdc55</sup> for both these functions. I find that <i>sgo1</i>∆ and <i>cdc55</i>∆ mutants have a similar defect in bi-orienting sister chromatids in metaphase after microtubule perturbation (Figure 4.7). Furthermore, this defect is shared by an <i>sgo1</i>-3<i>A</i> mutant that cannot bind PP2A, but not by an <i>rts1</i>∆ mutant, suggesting that Sgo1 promotes bi-orientation specifically through its interaction with PP2A<sup>Cdc55</sup>. I also show that Sgo1 and Cdc55 play a similar role in preventing cell cycle progression in response to lack of tension between sister chromatids (Figure 4.6). Taken together with the recent finding that <i>sgo1</i>-3<i>A</i> mutants cannot respond to lack of tension between sister chromatids (Xu et al. 2009), my results suggest that Sgo1 functions through PP2A<sup>Cdc55</sup> also for the tension checkpoint. However, I cannot rule out the possibility that PP2A<sup>Rts1</sup> also contributes to tension sensing, as this has yet to be tested. It is important to note that Sgo1 was found to co-purify with PP2A<sup>Rts1</sup> and not PP2A<sup>Cdc55</sup>, although this was only so far tested in meiotic cells (Riedel et al. 2006). It is therefore conceivable that Sgo1 might also bind PP2A<sup>Cdc55</sup> in mitotic cells, perhaps under conditions where there is a lack of tension between sister kinetochores. This possibility is supported by the observation that mouse Sgo2 can bind to PP2A complexes homologous to PP2A<sup>Cdc55</sup>, at least <i>in vitro</i> (Xu et al. 2009). However, experiments by myself and others have so far failed to detect an interaction between Sgo1 and PP2A<sup>Cdc55</sup> in mitotic cells (Dean Clift, Naoka Tamura and Adele Marston,
unpublished results). Nonetheless, my findings from Chapter 3 that PP2A\textsuperscript{Cdc55}, but not PP2A\textsuperscript{Rts1}, acts downstream of overproduced Sgo1, combined with the loss of function data in this chapter, suggest that PP2A\textsuperscript{Cdc55}, rather than PP2A\textsuperscript{Rts1}, might carry out the functions of Sgo1 in mitosis.

### 4.3.3 The role of the Sgo1-PP2A\textsuperscript{Cdc55} pathway in the spindle checkpoint

How do Sgo1 and PP2A\textsuperscript{Cdc55} respond to a lack of tension between sister chromatids? I found that when sister chromatids are not under tension due to cohesin depletion, the most striking phenotype of \textit{sgo1}\textsuperscript{Δ} and \textit{cdc55}\textsuperscript{Δ} mutants is Cdc14 release, and partial degradation of Pds1 and Clb2 likely a secondary consequence (Figure 4.6). This phenotype is distinct from \textit{mad1}\textsuperscript{Δ} mutants, which release Cdc14 and completely degrade both Pds1 and Clb2 due to an inability to inhibit APC\textsuperscript{Cdc20}. This data is consistent with the notion that Sgo1 and Cdc55 function in a common pathway in response to lack of tension that is separate from the canonical spindle checkpoint; an idea supported by the observation that the benomyl sensitivity of \textit{sgo1}\textsuperscript{Δ} and \textit{cdc55}\textsuperscript{Δ} mutants is exacerbated by \textit{MAD1} deletion (Figure 4.5A). Such a pathway might involve Esp1 inhibition, as my overexpression data from Chapter 3 indicated that PP2A\textsuperscript{Cdc55} is required downstream of Sgo1 to restrict Esp1 activity independently of the spindle checkpoint and Pds1. Furthermore, I have demonstrated in this chapter that Pds1 and Cdc55 act redundantly to inhibit Esp1 during every cell cycle. One possible model that incorporates all my data is that the absence of tension between sister chromatids stimulates Sgo1 to direct PP2A\textsuperscript{Cdc55}-mediated inhibition of Esp1 (Figure 4.9). Because Esp1 also promotes mitotic exit by triggering Cdc14 release (Stegmeier et al. 2002; Sullivan and Uhlmann 2003), a failure to inhibit Esp1 in \textit{sgo1}\textsuperscript{Δ} and \textit{cdc55}\textsuperscript{Δ} mutants in response to lack of tension might be the cause of the observed Cdc14 release. However, as PP2A\textsuperscript{Cdc55} has previously been reported to play a role in preventing Cdc14 release downstream of Esp1 (Queralt et al. 2006), an alternative possibility is that Cdc14 release in \textit{sgo1}\textsuperscript{Δ} and \textit{cdc55}\textsuperscript{Δ} mutants in response to lack of tension might be independent of Esp1 (Figure 4.9). In this case, the Sgo1-PP2A\textsuperscript{Cdc55} pathway might help maintain the inhibitory interaction of Net1 with
Cdc14 in the nucleolus (Queralt et al. 2006), thereby preventing inappropriate mitotic exit when sister chromatids are not under tension. In support of this view, deletion of *SGO1* or *CDC55* suppressed the synthetic lethality of a *spo12Δ lte1Δ* double mutant (Figure 4.5B), suggesting that both Sgo1 and Cdc55 are indeed negative regulators of mitotic exit.

What is the role of the Sgo1-PP2A<sub>Cdc55</sub> pathway in promoting bi-orientation? When the mitotic spindle is allowed to reform in metaphase after microtubule disruption, sister chromatids often attach to microtubules emanating from the same pole body (Indjeian and Murray 2007). My results suggest that, in this situation, Sgo1 functions through PP2A<sub>Cdc55</sub> to ensure that bi-orientation occurs (Figure 4.7). How this pathway contributes to the re-orientation of sister chromatids, however, remains unclear. Because mono-oriented sister chromatids cannot generate tension, Sgo1 may be required to sense the lack of tension in this situation, as it does when cohesin is depleted. In this case, Sgo1 might also respond by eliciting PP2A<sub>Cdc55</sub>-mediated Esp1 inhibition. An alternative possibility is that the Sgo1-PP2A<sub>Cdc55</sub> contributes to the detachment of kinetochore-microtubule attachments that do not generate tension, thereby allowing bi-polar tension-generating attachments to occur. The fact that the budding yeast Aurora B kinase, Ipl1, is required to detach microtubules from tension-less kinetochores (Pinsky et al. 2006), suggests that Ipl1 might be involved downstream of Sgo1 and PP2A<sub>Cdc55</sub>. Indeed, the fission yeast mitotic shugoshin, Sgo2, ensures bi-orientation by promoting the full centromeric localization of Aurora B (Ark1) kinase (Kawashima et al. 2007; Vanoosthuyse et al. 2007). However, a previous study found that Ipl1 localizes to mitotic centromere in the absence of Sgo1 in budding yeast (Fernius and Hardwick 2007), suggesting that any affect of the Sgo1-PP2A<sub>Cdc55</sub> pathway on Ipl1 function is probably not due to a major role in Ipl1 localization, although, Ipl1 could be regulated by other means. The Sgo1-PP2A<sub>Cdc55</sub> pathway might have other roles in promoting bi-orientation: perhaps by regulating kinetochore microtubule stability as has been shown for vertebrate Sgo1 (Salic et al. 2004) or by influencing additional proteins involved in the bi-orientation process. In support of this view, the role of human Sgo2 in chromosome bi-orientation appears to be through ensuring the proper localization of the microtubule depolymerase MCAK, rather than Aurora B (Huang et al. 2007). Additionally, given Cdc14 release
is the most obvious phenotype of $sgo1\Delta$ and $cdc55\Delta$ mutants when sister chromatids lack tension due to cohesin depletion; premature Cdc14 release could underlie the bi-orientation defect of $sgo1\Delta$, $sgo1\cdot3A$ and $cdc55\Delta$ mutants by an unknown mechanism.

4.3.4 Surveillance mechanisms converge on PP2A$^{Cdc55}$

My Pds1 depletion experiments indicate that, in contrast to Cdc55, Sgo1 plays only a minor role in regulating Esp1 activity in an unperturbed cell cycle. I interpret these results to suggest that Sgo1 is only important for Esp1 inhibition, through PP2A$^{Cdc55}$, when sister chromatids are not under tension. PP2A$^{Cdc55}$, on the other hand, might be more generally involved in restraining Esp1 activation. This mode of Esp1 regulation by PP2A$^{Cdc55}$ could be utilized by cell cycle surveillance mechanisms, one of which is lack of tension through Sgo1, to prevent anaphase onset until earlier cell cycle events have been successfully accomplished (Figure 4.9). In support of this view, PP2A$^{Cdc55}$ is also required to prevent sister chromatid separation, independently of Pds1, in response DNA damage (Tang and Wang 2006) or budding defects (Chiroli et al. 2007).

In conclusion, my results uncover an additional layer of Esp1 regulation, and provide an explanation for the viability of $pds1\Delta$ cells; in the absence of Pds1, PP2A$^{Cdc55}$ is sufficient to prevent Esp1 activation until the onset of anaphase. Furthermore, my data is consistent with the model whereby PP2A$^{Cdc55}$-mediated Esp1 inhibition is utilized by Sgo1 when sister chromatids lack tension. In the next chapter, I attempt elucidate the mechanism by which Esp1 is regulated by this newly uncovered Sgo1-PP2A$^{Cdc55}$ pathway.
Esp1 is inhibited by PP2A<sup>Cdc55</sup> and Pds1. PP2A<sup>Cdc55</sup> also negatively regulates mitotic exit by preserving the inhibitory interaction of Net1 with Cdc14 in the nucleolus (Queralt et al. 2006). At the onset of anaphase, APC<sup>Cdc20</sup>-dependent degradation of some Pds1 releases a small amount of Esp1. Esp1 then promotes Cdc14 release and downregulates PP2A<sup>Cdc55</sup> (Queralt et al. 2006), which in turn activates more Esp1 by relieving inhibition by PP2A<sup>Cdc55</sup>. Concomitantly, Cdc14 dephosphorylates Pds1 increasing its rate of ubiquitination by APC<sup>Cdc20</sup> (Holt et al. 2008), thereby releasing more Esp1. (Adapted from Holt et al. (2008))
Figure 4.9 A model for the mechanism of Sgo1 function in mitosis. PP2A<sup>Cdc55</sup> is an Esp1 inhibitor that is employed by Sgo1 when sister chromatids are not under tension. This pathway acts in parallel to the canonical spindle checkpoint which inhibits the APC<sup>Cdc20</sup>. Multiple surveillance mechanisms might converge on PP2A<sup>Cdc55</sup>, similar to Pds1, to prevent anaphase onset in response to cellular defects.
Chapter 5

Investigating the mechanism of separase regulation by the $\text{Sgo1-PP2A}^{\text{Cdc55}}$ pathway
5 Chapter 5 – Investigating the mechanism of separase regulation by the Sgo1-PP2A$^{\text{Cdc55}}$ pathway

5.1 Introduction

Separase proteins are found in all eukaryotes, and carry out a conserved function in initiating the separation of sister chromatids in anaphase. Surprisingly, however, separase shows very little sequence similarity between species. Only the C-terminal protease domain exhibits any sequence conservation (Uhlmann et al. 2000), whereas the bulk of the separase protein is made up of a large N-terminus, which is highly divergent in sequence amongst eukaryotes. Despite the lack of sequence conservation, the N-termini of separase proteins are predicted to adopt an alpha-alpha superhelical structure similar to ARM (Armidillo) repeat-containing proteins (Jager et al. 2004) or TPR (tetratricopeptide) repeat proteins (Katis et al. 2010). An unstructured region separates the N- and C-terminal domains (Viadiu et al. 2005) and might facilitate the interaction between the N- and C-termini of separase, which appears to be important for its proteolytic activity (Jager et al. 2001; Hornig et al. 2002; Jager et al. 2004).

Separase activity must be restricted until all sister chromatids have bi-oriented on the mitotic spindle to ensure the accuracy of chromosome segregation. Securins are functionally conserved as separase inhibitors in all eukaryotes, and biochemical evidence suggests that securin might inhibit separase by directly binding to the both the N- and C-termini of separase, thereby disrupting the intramolecular interactions within separase that are important for its catalytic activity (Jager et al. 2001; Hornig et al. 2002; Waizenegger et al. 2002). Although securin degradation mediated by the APC$^{\text{Cdc20}}$ is necessary to relieve separase inhibition (Cohen-Fix et al. 1996; Funabiki et al. 1996a; Zou et al. 1999; Hagting et al. 2002), it appears not to be sufficient: securin is not essential for viability in many eukaryotic organisms (Yamamoto et al. 1996a; Jallepalli et al. 2001; Mei et al. 2001; Wang et al. 2001; Pfleghaar et al. 2005; Wirth et al. 2006) and, at least in budding yeast, the cohesin cleavage activity of separase is initiated with proper timing in cells lacking securin (Alexandru et al. 2006).
1999; Alexandru et al. 2001). This suggests that additional securin-independent mechanism exist to regulate separase.

How is separase regulated independently of securin? In vertebrates, Cdk1-dependent phosphorylation of serine 1126 located within an unstructured central region of the separase protein mediates the binding of the Cdk1-cyclin B1 complex, which is sufficient for securin-independent inhibition (Stemmann et al. 2001; Gorr et al. 2005). Furthermore, securin and cyclin B1 interact with separase in a mutually exclusive manner, suggesting that they may be redundant separase inhibitors (Gorr et al. 2005; Holland and Taylor 2006). The serine 1126 site lies within a non-conserved region of separase, making it difficult to assess whether such a mechanism for separase inhibition also operates in other organisms. However, the fact that budding yeast cells are able to initiate sister chromatid separation in the presence of high Cdk1 activity (Surana et al. 1993) suggests that Cdk1-cyclin may not inhibit separase in this organism. In chapters 3 and 4 of this study, I provided evidence that a pathway involving PP2A<sub>Cdc55</sub>, downstream of Sgo1, contributes to securin-independent separase inhibition in budding yeast. My data are consistent with a model whereby PP2A<sub>Cdc55</sub> and securin act redundantly to inhibit separase.

In this chapter, I set out to gain an insight into the mechanism by which Sgo1 and PP2A<sub>Cdc55</sub> regulate separase. I describe a mutagenesis screen to attempt to identify sites within in separase that are important for securin-independent inhibition by Sgo1 and PP2A<sub>Cdc55</sub>, and provide preliminary evidence that the phosphatase activity of PP2A is required for its role in separase regulation. I find that separase phosphorylation is cell cycle regulated, and investigate the role of Cdk1 in separase regulation.
5.2 Results

5.2.1 Catalytic-dead Protein Phosphatase 2A phenocopies cdc55Δ

In Chapters 3 and 4, I describe a novel pathway for the regulation of Esp1. My data are consistent with a model whereby Pds1 and PP2A<sup>Cdc55</sup> act redundantly to inhibit Esp1. Pds1 is known to inhibit Esp1 by direct binding (Hornig et al. 2002), however, the mechanism by which PP2A<sup>Cdc55</sup> restrains Esp1 activity is not known. To try to determine whether the phosphatase activity of PP2A<sup>Cdc55</sup> is involved in Esp1 inhibition, I sought to construct a catalytically inactive PP2A enzyme. Mutation of a conserved aspartic acid residue to a neutral asparagine at position 88 (D88N) in the human PP2A catalytic subunit (PP2Ac) dramatically reduces its phosphatase activity (Myles et al. 2001). The primary sequence of PP2Ac is highly conserved between species (Barton et al. 1994), and an amino acid change in yeast PP2Ac analogous to D88N in human PP2Ac likely represents catalytically inactive phosphatase (Myles et al. 2001). Budding yeast cells contain two functionally redundant PP2Ac subunits, Pph21 and Pph22 (Sneddon et al. 1990), with the D88N-analogues substitutions being D148N and D156N in Pph21 and Pph22 respectively. To generate catalytically inactive yeast PP2A, <i>pph21-148</i> and <i>pph22-156</i> alleles (encoding the D148N and D156N substitutions) were generated and introduced into the genome of <i>pph21</i>Δ and <i>pph22</i>Δ deletion strains as single copies (for details, see appendix section A.3). The <i>pph21-148</i> and <i>pph22-156</i> single integrants were combined to generate a strain in which both the PP2Ac subunits are catalytically dead (<i>pp2a-cd</i>). Similar to a <i>pph21Δ pph22Δ</i> double mutant (Evans and Stark 1997), <i>pp2a-cd</i> cells are viable at 25°C, but exhibit a temperature-sensitive growth defect at 37°C (Figure 5.1A), indicating that inactivation of PP2A catalytic activity resembles a strain lacking both catalytic subunits. I next tested whether the <i>pp2a-cd</i> mutant phenocopies <i>cdc55Δ</i>. The <i>pp2a-cd</i> mutant displayed a high level of benomyl sensitivity, similar to the <i>cdc55Δ</i> mutant (Figure 5.1B). Furthermore, like <i>cdc55Δ</i>, combining <i>pp2a-cd</i> with MET-PDS1 resulted in lethality in the presence of methionine (Figure 5.1C). Taken together, these results suggest that the phosphatase activity of PP2A<sup>Cdc55</sup> may be required for its role in the spindle checkpoint and Esp1 inhibition.
Figure 5.1 Analysis of catalytic-dead PP2A. (A) 10-fold serial dilutions of Wild type (AM1176), *pph21Δ pph22-172 pph3Δ* (AM4629), *pph21Δ* (AM6088), *pph22Δ* (AM5212), *pph21-148* (AM6246), *pph22-156* (AM6267), *pph21Δ pph22-156* (AM6301), *pph21-148 pph22Δ* (AM6305) and *pp2a-cd* (AM6320) cells were spotted onto YEPD medium and incubated at 25°C, 30°C or 37°C. (B) 10-fold serial dilutions of Wild type (AM1176), *pph21-148* (AM6246), *pph22-156* (AM6267), *pp2a-cd* (AM6320) and *cdc55Δ* (AM3164) cells were spotted onto YEPD medium containing benomyl at concentrations of 0 µg/ml or 12 µg/ml and incubated at 30°C. (C) Wild type (AM1145), *MET-PDS1* (AM4429), *pp2a-cd* (AM6590) and *pp2a-cd MET-PDS1* (AM6591) cells carrying *SCC1-6HA* were streaked onto plates either lacking (SC/-met/D) or containing (YEPD+met) methionine and incubated at 30°C.
5.2.2 Screen for \textit{ESP1} alleles resistant to inhibition by the Sgo1-PP2A\textsuperscript{Cdc55} pathway

I reasoned that as Pds1 and PP2A\textsuperscript{Cdc55} appear to work redundantly to inhibit Esp1, it should be possible to identify mutations in Esp1 that separate the two modes of inhibition. I decided to address this possibility by performing a genetic screen for Esp1 mutants that cannot be inhibited by PP2A\textsuperscript{Cdc55}. Identification of such mutations will be invaluable in dissecting the mechanism and functional importance of Esp1 inhibition mediated by PP2A\textsuperscript{Cdc55}. In Chapter 3, I showed that \textit{SGO1} overexpression causes PP2A\textsuperscript{Cdc55}-dependent Esp1 inhibition, and that \textit{CDC55} overexpression also inhibits Esp1 activity independently of Pds1. I utilized the slow growth of \textit{GAL-SGO1} and \textit{GAL-CDC55} cells on medium containing galactose (Figure A.4) to isolate \textit{ESP1} alleles that rescued this phenotype. Details of the screen procedure are outlined in appendix section A.2. Briefly, I randomly mutagenized plasmid-borne \textit{ESP1} and introduced this \textit{ESP1} library into \textit{GAL-SGO1} or \textit{GAL-CDC55} cells as the sole copy of \textit{ESP1}. Approximately 15,000 colonies were screened, with 33 colonies exhibiting either complete, or partial suppression of the \textit{GAL-SGO1} or \textit{GAL-CDC55} slow growth on galactose. \textit{ESP1} plasmids were isolated from such positive colonies and re-transformed to confirm that the suppression was linked to the plasmid. Surprisingly, none of the plasmids were able to suppress the poor growth of \textit{GAL-SGO1} or \textit{GAL-CDC55} cells on galactose (for examples, see Figure A.8). Therefore, from this screen I was unable to isolate \textit{ESP1} alleles resistant to inhibition by Sgo1-PP2A\textsuperscript{Cdc55}. However, in a supplementary screen using the same library of transformants, I was able to isolate plasmids with single point mutations in \textit{ESP1} that were sufficient in rendering the cell temperature sensitive (Figure A.10). This demonstrates that the mutagenesis procedure works, but that mutations satisfying the screen criteria were not generated.
5.2.3 Analysis of Esp1 phosphorylation

In vertebrates, phosphorylation plays a key role in the regulation of separase activity (Stemmann et al. 2001). However, the role of phosphorylation in separase/Esp1 regulation in budding yeast is not known. My findings that the PP2A<sup>Cdc55</sup> phosphatase contributes to the inhibition of Esp1 raised the interesting possibility that Esp1 may also be regulated by phosphorylation and dephosphorylation events. In support of this notion, immunoprecipitated Esp1 protein exhibits a gel mobility shift that is lost by treatment with lambda phosphatase (Adele Marston, unpublished), indicating that Esp1 is indeed a phosphoprotein and that the gel mobility shift can be used as an indicator for its phosphorylation state.

5.2.3.1 Esp1 phosphorylation is cell cycle regulated

If Esp1 phosphorylation were to play a role in regulating its activity, I would expect that phosphorylation of Esp1 is cell cycle regulated. To test this, I released wild type cells synchronously into the cell cycle from a G<sub>1</sub> arrest and analysed the status of epitope-tagged Esp1 protein. The levels of Cdc55 protein do not change during the cell cycle and therefore serve as a loading control for this experiment (Figure 5.2B). In G<sub>1</sub> cells, Esp1 appeared as a fairly sharp single band (Time 0; Figure 5.2). Interestingly, however, the Esp1 band intensified and became smeared as the cells entered metaphase (Figure 5.2). This smear was similar to that seen for immunoprecipitated Esp1 (Adele Marston, unpublished), and therefore likely represents phosphorylation. The Esp1 band intensity then reduced, and the smear subsided, when the cells entered anaphase and returned to the next G<sub>1</sub> (Figure 5.1). This data suggests that Esp1 protein levels and phosphorylation are cell cycle regulated, and agrees with a previous observation that Esp1 protein levels are lower in G<sub>1</sub> compared to the rest of the cell cycle (Jensen et al. 2001).

To clarify the phosphorylation status of Esp1, I utilized a method of separating phosphorylated isoforms by phosphate affinity electrophoresis (Kinoshita et al. 2006). This method uses a phosphate binding compound (Mn<sup>2+</sup>-Phos-tag) that, when incorporated into polyacrylamide gels, causes increased mobility shift for
phosphorylated proteins (Kinoshita et al. 2006). Extracts from wild type cells released from a G₁ arrest were run on an Mn²⁺-Phos-tag gel and Esp1 protein analysed by western blot. As on a gel without Mn²⁺-Phos-tag, Esp1 appeared as a sharp single band in G₁ cells on the Mn²⁺-Phos-tag gel (Time 0; Figure 5.3). As the cells entered metaphase, the overall Esp1 protein levels increased and a small fraction of the total Esp1 protein converted to a slower migrating form that was clearly visible as a separate band (Figure 5.3). The timing of appearance of the slower migrating band was similar to the smear seen previously (Figure 5.2), indicating that this band likely represents a phosphorylated Esp1 isoform. The slower migrating band disappeared rapidly as the cells entered anaphase (Figure 5.2). This suggests that a fraction of Esp1 is phosphorylated prior to its activation at the onset of anaphase. Furthermore, this phosphorylated Esp1 isoform is absent in anaphase, suggesting that it is either rapidly dephosphorylated or degraded or both, at the onset of anaphase. Because the overall levels of Esp1 protein also decrease at the onset of anaphase, it is difficult to distinguish between these possibilities.

5.2.3.2 Overexpression of CDC55, but not SGO1, reduces Esp1 phosphorylation

It was previously shown that Esp1 and Cdc55 form a complex in vivo and that PP2ACdc55 phosphatase activity is maximal in metaphase, when Esp1 is inactive (Queralt et al. 2006). This opens the possibility that PP2ACdc55 might inhibit Esp1 by direct dephosphorylation. If this were the case, I would predict Esp1 to be dephosphorylated by high levels of Cdc55, which I previously showed to cause Esp1 inhibition (Chapter 3). I therefore analysed Esp1 protein by phosphate affinity electrophoresis upon CDC55 overexpression. Interestingly, the phosphorylated Esp1 isoform was largely absent in GAL-CDC55 cells, despite the fact that cells accumulated in metaphase where Esp1 phosphorylation is maximal in wild type cells (Figure 5.3). There was, however, some Esp1 phosphorylation, as shown by a slight smearing of the Esp1 band (Figure 5.3). This result suggests that Esp1 phosphorylation might be reversed by PP2ACdc55, at least when Cdc55 is present at high levels, although it is not possible to determine whether this effect is direct or
indirect. I also analysed Esp1 phosphorylation in \textit{SGO1}-overexpressing cells, which I have previously shown to inhibit Esp1 through a PP2A\textsuperscript{Cdc55}\textsuperscript{-dependent mechanism (Chapter 3). In contrast to \textit{CDC55} overexpression, cells overexpressing \textit{SGO1} delayed in metaphase with an Esp1 mobility shift similar to wild type metaphase cells (Figure 5.3). Therefore, Esp1 phosphorylation is reduced in cells overexpressing \textit{CDC55}, but not in cells overexpressing \textit{SGO1}.

\subsection*{5.2.3.3 Prediction of Esp1 phosphorylation sites}

To determine which sites of Esp1 are likely to be phosphorylated, I used a program to predict serine and threonine phosphorylation sites within yeast proteins (NetPhos Yeast; Ingrell et al. 2007). NetPhos Yeast identified 24 sites within the 1630 aa Esp1 primary sequence that were predicted to be phosphorylated (Figure 5.4A). Interestingly, 5 of these were putative cyclin dependent kinase (CDK) phosphorylation sites (Figure 5.4B). CDKs phosphorylate serine and threonine residues found within the consensus S/T-P-X-K/R where X represents any amino acid, although the K/R residue at position +3 relative to the serine or threonine is not essential (Songyang et al. 1994; Zhang et al. 1994; Srinivasan et al. 1995; Holmes and Solomon 1996). In total there are 6 CDK consensus sites within Esp1, which are conserved among related budding yeast species (Figure 5.4B,C). The CDK phosphorylation site at Thr\textsuperscript{1014} was not predicted to be phosphorylated according to NetPhos Yeast, however, this site lies within the full CDK consensus sequence (Figure 5.4C) and only just falls below the prediction threshold (Figure 5.4A). This analysis raises the intriguing possibility that CDKs (in budding yeast there is a single CDK, Cdc28) might phosphorylate Esp1.
Figure 5.2 Esp1 phosphorylation is cell cycle regulated. Wild type (AM5031) cells carrying ESP1-18MYC and 3HA-CDC55 were grown at room temperature in YEPD, arrested in G1 using α-factor and released into YEPD at room temperature. When cells had budded, α-factor was re-added to limit analysis to a single cell cycle. Samples were taken at the indicated timepoints for (A) spindle morphology analysis and (B) anti-MYC and anti-HA western blot analysis of TCA protein extracts separated on a 6% SDS polyacrylamide gel.
Figure 5.3 Esp1 phosphorylation is reduced by CDC55 overexpression. Wild type (AM1604), GAL-CDC55 (AM6407) and GAL-SGO1 (AM4329) cells carrying ESP1-18MYC were grown and treated as in figure 3.2. Samples were taken at the indicated timepoints for (A) spindle morphology analysis and (B) anti-MYC western blot analysis of TCA protein extracts separated on a 6% SDS polyacrylamide Mn\(^{2+}\)-Phos-tag gel.
Figure 5.4 Prediction of Esp1 phosphorylation sites. (A) Esp1 primary sequence was analysed using the NetPhos Yeast 1.0 server (www.cbs.dtu.dk/services/NetPhosYeast). Sites with a phosphorylation potential above 0.5 (grey line) are predicted to be phosphorylated. (B) Schematic of Cdc28 phosphorylation consensus sites within the Esp1. Consensus sequence for Cdc28 is S/T-P-X-K/R where X represents any amino acid. The K/R residue is not essential. *T1014 was not predicted to be phosphorylated by NetPhos Yeast 1.0. (C) Cdc28 phosphorylation consensus sites in Esp1 are conserved among related budding yeast species S.cerevisiae (Scer), S.bayanus (Sbay) and S.mikatae (Smik). Arrows show serine and threonine residues of consensus sites. Alignment performed using SGD (www.yeastgenome.org).
5.2.4 The role of Cdc28 in Esp1 regulation

5.2.4.1 Cdc28 activity at the onset of anaphase is not required for Esp1 activation

The presence of 6 CDK consensus sites in Esp1 (Figure 5.3B,C) suggests that Cdc28 might play a role in regulating Esp1. In support of this view, 3 of the CDK consensus sites (Thr\textsuperscript{1014}, Ser\textsuperscript{1027}, Thr\textsuperscript{1034}) are clustered close together towards the middle of the protein (Figure 5.4B), which is thought to be an unstructured regulatory region of separase (Viadiu et al. 2005). In vertebrates, Cdk1-mediated phosphorylation of serine 1126 within this region promotes separase inhibition through the binding of the Cdk1-cyclin B1 complex (Stemmann et al. 2001; Gorr et al. 2005). However, as budding yeast cells can enter anaphase in the presence of high Clb2-Cdc28 kinase activity (Surana et al. 1993), such a mechanism seems unlikely to inhibit Esp1. Nevertheless, I decided to take a different approach to test the possibility that Cdc28 might regulate Esp1 by phosphorylation. I reasoned that if Cdc28 kinase were to be involved in directly regulating Esp1, Cdc28 inactivation might affect Esp1 function at the onset of anaphase. To test this, I devised an experiment to arrest cells in metphase, then inhibit Cdc28 and release cells synchronously into anaphase whilst monitoring cohesin cleavage as a marker for Esp1 activation. For the metaphase arrest and release, I used a strain containing a methionine-repressible CDC20 allele (MET-CDC20). Cdc20 can be depleted, and therefore APC\textsuperscript{Cdc20} inactivated, by addition of methionine to the growth medium. Cdc20 can then be reintroduced by removal of methionine, leading to APC\textsuperscript{Cdc20} activation and entry into anaphase. To inactivate Cdc28 kinase, I utilized a CDC28 allele (cdc28-as1) that can be rapidly and specifically inhibited \textit{in vivo} by the addition of the ATP analogue 1NM-PP1 (Bishop et al. 2000). Wild type and cdc28-as1 cells were arrested in metaphase by Cdc20 depletion, 1NM-PP1 was added to inhibit cdc28-as1 and then the cells were released into a synchronous anaphase by Cdc20 re-addition in the presence of 1NM-PP1. In cdc28-as1 cells, the mitotic spindle collapsed after addition of 1NM-PP1, resulting in inefficient nuclear division (Figure 5.4B). This is consistent with previous reports that Cdc28 activity is required for maintenance of the mitotic
spindle (Richardson et al. 1992; Bishop et al. 2000), indicating that Cdc28 was effectively inhibited in my experiment. Both wild type and cdc28-as1 cells activated the APC\textsuperscript{Cdc20} after Cdc20 introduction because Pds1 was degraded in both strains (Figure 5.5C,D). Significantly, full length Scc1 disappeared in both wild type and cdc28-as1 cells (Figure 5.5C,D), providing evidence that Esp1 activation does not require Cdc28 kinase activity at the onset of anaphase. However, I cannot rule out the possibility that Cdc28 might influence Esp1 somehow earlier in the cell cycle.

I noticed that both the loss of full-length Scc1 and Pds1 degradation was advanced in cdc28-as1 cells compared to wild type (Figure 5.5C,D). I wanted to confirm this result, and so performed a similar experiment with different strains, this time monitoring Pds1 protein by immunofluorescence and the separation of GFP-labelled chromosome V (URA3-GFP). As above, wild type and cdc28-as1 cells were arrested in metaphase by Cdc20 depletion, 1NM-PP1 was added to inhibit cdc28-as1 and then the cells were released into a synchronous anaphase by Cdc20 re-addition in the presence of 1NM-PP1. Cdc28 was effectively inhibited in this experiment, as the mitotic spindle could not be maintained in cdc28-as1 cells (Figure 5.5F). Sister URA3-GFP signals separated in both strains, indicative of Esp1 activation, although the slower rate of separation in cdc28-as1 cells was likely due to a defective mitotic spindle (Figure 5.5E,F). Importantly, similar to the western blot data, immunofluorescence showed that Pds1 destruction occurred more rapidly in the cdc28-as1 culture compared to wild type (Figure 5.5E,F). These results confirm that Cdc28 inactivation in pre-anaphase cells results in a more rapid transition into anaphase.
Figure 5.5 Esp1 activation at anaphase onset does not require Cdc28. (A-D) Wild type (AM5910) and cdc28-as1 (AM5908) cells carrying MET-CDC20, SCC1-6HA and PDS1-18MYC were grown at room temperature in SC/-met/D, arrested in G1 using α-factor and released into YEPD + 8mM methionine to deplete Cdc20 and arrest cells in metaphase. Cells were then treated with 5µm 1NM-PP1 15 minutes before being released into anaphase by shifting cells to SC/-met/D to re-synthesize Cdc20. Samples were taken at the indicated timepoints after Cdc20 addition. (A,B) The percentage of cells with anaphase spindles and divided nuclei were determined by counting at least 200 cells at each timepoint. (C,D) Scc1-6HA and Pds1-18MYC levels were analysed by anti-HA and anti-MYC western blot analysis of TCA protein extracts separated on a 10% SDS polyacrylamide gel. Pgk1 is shown as a loading control. (E,F) Wild type (AM5987) and cdc28-as1 (AM5986) cells carrying MET-CDC20, PDS1-3HA and URA3-GFP were grown and treated as above. Pds1-3HA localization was determined after immunofluorescence. The percentage of cells with Pds1 positive staining, anaphase spindles and separated GFP foci were determined at the indicated timepoints after Cdc20 addition.
5.2.4.2 Mutagenic analysis of Cdc28 consensus sites in Esp1

I also decided to take a mutagenesis approach to address more directly the role of Cdc28-mediated phosphorylation on Esp1 activity. I set out to substitute the serines and threonines of the 6 CDK consensus sites in Esp1 for either an alanine or an aspartic acid residue. An alanine substitution prevents phosphorylation at that site, whereas an aspartic acid substitution mimics the incorporation of a negative charge that occurs upon phosphorylation. To date, 4 out of 6 Cdc28 consensus sites in Esp1 have been mutagenized (esp1-CDK mutants). Mutant alleles of ESP1 were then introduced into esp1Δ cells on an autonomously replicating plasmid to check viability (Table 5.1).

<table>
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<tr>
<th>Non-phosphorylatable</th>
<th>Phospho-mimic</th>
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<tr>
<td>S13A</td>
<td>S13D</td>
</tr>
<tr>
<td>T16A</td>
<td>T16D</td>
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<tr>
<td>T1014A (inviable)</td>
<td>T1014D</td>
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<tr>
<td>S1027A</td>
<td>S1027D</td>
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<td>T1014D; S1027D</td>
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I found all the esp1-CDK mutants apart from the T1014A mutation were viable (Figure 5.6A); raising the possibility that phosphorylation of Thr1014, possibly by Cdc28, is required for a functional Esp1. I first tested whether the T1014A mutation affected the stability of Esp1. A sequence encoding the 18MYC epitope was inserted downstream of either plasmid-borne ESP1 or esp1-T1014A and plasmids integrated into the genome of cells also containing wild type untagged Esp1. Steady-state levels of Esp1-18MYC and esp1-T1014A-18MYC were analysed by western blot using an anti-MYC antibody. Surprisingly, the non-phosphorylatable T1014A mutant was completely undetectable (Figure 5.6B), therefore explaining the inviability of cells producing esp1-T1014A as the sole source of Esp1. This could mean that the threonine residue at position 1014 is essential for the intrinsic stability of the Esp1.
protein, however, the fact that exchanging this residue for an aspartic acid does not cause inviability (Table 5.1) argues against this possibility. Alternatively, because the aspartic acid residue might mimic phosphorylation, it is conceivable that phosphorylation of Thr\textsuperscript{1014} is required for Esp1 stability. It will be necessary to map which Esp1 sites are phosphorylated \textit{in vivo} to validate any conclusions made from this mutagenic analysis.

PP2A is a serine/threonine phosphatase that I previously showed to contribute to the inhibition of Esp1 (Chapters 3 and 4). I considered the possibility that PP2A may regulate Esp1 through counteracting phosphorylation of serines and threonines of CDK consensus sites. Indeed, in human cells, PP2A binds to separase and appears to antagonise serine 1126 phosphorylation, although the functional consequence of this is not clear (Holland et al. 2007). I therefore tested whether the CDK consensus sites within Esp1 played a role in mediating inhibition by the Sgo1-PP2A\textsuperscript{Cdc55} pathway. I asked if any of the \textit{esp1-cdk} mutants I generated were able to suppress the slow growth of \textit{SGO1}- and \textit{CDC55}-overexpressing cells on galactose. Plasmid-borne wild type \textit{ESP1} and \textit{esp1-cdk} mutant genes were introduced into \textit{esp1}\textDelta cells containing either \textit{GAL-SGO1} or \textit{GAL-CDC55}. None of the \textit{esp1} alleles tested so far suppressed the sickness of \textit{SGO1}- or \textit{CDC55}-overexpressing cells (Figures A.13-16), suggesting that these sites alone are not involved in Esp1 inhibition by Sgo1-PP2A\textsuperscript{Cdc55}. However, mutants in all six Cdk phosphorylation sites, individually and combined, have yet to be tested.
Figure 5.6 The esp1-T1014A mutant is inviable and the mutant protein is undetectable. 
(A) esp1Δ cells carrying ESP1 on a CEN-TRP plasmid (AM6238) were transformed with a 
CEN-HIS plasmid containing either ESP1 (Wild type; AMp708) or esp1-T1014A (T1014A; 
AMp713). Four colonies from each transformation were streaked on SC-/his/D medium 
containing 5-fluoroantranillic acid (FAA) to counterselect against TRP. (B) ESP1-18MYC 
(Wild type; AMp404) and esp1-T1014A-18MYC (T1014A; AMp750) plasmids were integrated 
into the genome of wild type (AM1176) cells by transformation and homologous 
recombination at the TRP locus. Protein extracts from 7 colonies from each transformation 
were separated on a 6% SDS polyacrylamide gel and Esp1 levels analysed by anti-MYC 
western blot. Untransformed cells (No Tag; AM1176) and cells containing a single copy of 
ESP1-18MYC at the ESP1 locus (Esp1-18MYC; AM1604) are shown as controls.
5.2.4.3 Inhibitory phosphorylation of Cdc28 is partially responsible for the metaphase delay of CDC55-overexpressing cells

PP2A<sup>Cdc55</sup> has an established role in regulating Cdc28 activity. Deletion of CDC55 causes an increase in the inhibitory phosphorylation of Cdc28 on tyrosine 19, which is mediated by the Swe1 kinase (Minshull et al. 1996; Yang et al. 2000; Pal et al. 2008). Furthermore, PP2A<sup>Cdc55</sup> has been shown to dephosphorylate Mih1, the phosphatase responsible for reversing Cdc28-Tyr19 phosphorylation (Pal et al. 2008). I decided to investigate the possibility that PP2A<sup>Cdc55</sup> might contribute to Esp1 inhibition indirectly by affecting the phosphorylation status and activity of Cdc28. I found that either deletion of <i>SWE1</i>, or expression of a Cdc28 mutant that cannot be phosphorylated by Swe1 at tyrosine 19 (cdc28<sup>Y19F</sup>), partially rescued the slow growth of <i>CDC55</i>-overexpressing cells (Figure 5.7), suggesting that overproduced Cdc55 inhibits cell growth at least in part due to Swe1-mediated inhibitory Cdc28-Tyr19 phosphorylation.

It has previously been shown that overexpression of <i>CDC55</i> causes a metaphase delay independently of Pds1 (Chapter 3; Chiroli et al. 2007). I wanted to test whether this delay was instead dependent on Cdc28-Tyr19 phosphorylation, which might explain why <i>swe1Δ</i> and <i>cdc28<sup>Y19F</sup></i> partially rescued the slow growth of <i>CDC55</i>-overexpressing cells. To test this, I released wild type, <i>cdc28<i>Y19F</i></i>, <i>GAL-CDC55</i>, <i>cdc28<i>Y19F</i> GAL-CDC55</i>, <i>GAL-SGO1</i> and <i>cdc28<i>Y19F</i> GAL-SGO1</i> cells from a G<sub>1</sub> arrest synchronously into the cell cycle in the presence of galactose to induced high levels of expression from the <i>GAL</i> promoter. Cell cycle progression was monitored by analysing the morphology of the mitotic spindle together with Clb2 protein by western blot. Clb2 degradation is initiated at the onset of anaphase by the APC<sup>Cdc20</sup> and then completed as cells exit from mitosis due to the action of APC<sup>Cdh1</sup> (Yeong et al. 2000). As expected, <i>GAL-CDC55</i> and <i>GAL-SGO1</i> cells exhibited a prolonged metaphase delay compared to wild type, as judged by an accumulation of cells with short metaphase spindles (Figure 5.8A) stabilized Clb2 protein (Figure 5.8C). I also noticed that <i>CDC55</i>- and <i>SGO1</i>-overexpressing cells were slightly delayed in budding (Figure 5.8B), forming metaphase spindles (Figure 5.8A) and Clb2 accumulation (Figure 5.8C) in this experiment, however, this small delay in entering
mitosis cannot account for the observed delay in metaphase. The \textit{cdc28}^{Y19F} mutant did not rescue the mitotic entry defect of \textit{CDC55}- or \textit{SGO1}-overexpressing cells (Figure 5.8A,C), although it did advance the onset of budding in \textit{GAL-CDC55} cells (Figure 5.8B). Significantly, however, the major effect of the \textit{cdc28}^{Y19F} mutant on \textit{GAL-CDC55} cells was a partial alleviation of the delay to anaphase onset and Clb2 degradation; although, these cells still exhibited metaphase delay compared to the \textit{cdc28}^{Y19F} mutant alone (Figure 5.8A,C). The metaphase and Clb2 degradation delay in \textit{GAL-SGO1} cells was also slightly alleviated by the \textit{cdc28}^{Y19F} mutant; however, the effect was not as obvious as in \textit{GAL-CDC55} cells (Figure 5.8A,C). I conclude that inhibitory Cdc28-Tyr19 phosphorylation is partially responsible for the metaphase delay of \textit{CDC55}-overexpressing cells, but makes only a minor contribution to the metaphase delay of \textit{SGO1}-overexpressing cells.

These results predict that the phosphorylation status of Cdc28-Tyr19 should be affected by overexpression of \textit{CDC55} or \textit{SGO1}. To address this possibility, I analysed Cdc28-Tyr19 phosphorylation by western blot using an antibody specific for Cdk1 phosphotyrosine (see chapter 2). In wild type cells, Cdc28-Tyr19 phosphorylation appeared as the cells entered metaphase and then disappeared at the onset of anaphase (Figure 5.8C). This signal was absent in the \textit{cdc28}^{Y19F} strains, confirming that the observed band in wild type cells is specific to phosphorylated Cdc28-Tyr19. Overexpression of \textit{CDC55} delayed the initiation of Cdc28-Tyr19 phosphorylation to a similar extent as Clb2 accumulation. Significantly, however, Cdc28 inhibitory phosphorylation eventually accumulated to higher levels and persisted until the end of the timecourse. Cdc28-Tyr19 phosphorylation was similarly delayed in appearance, and increased to similar levels, when \textit{SGO1} was overexpressed; however, in contrast to \textit{GAL-CDC55}, the levels of phosphorylated Cdc28-Tyr19 reduced as these cells eventually entered anaphase (Figure 5.8C). Therefore, high levels of both Cdc55 and Sgo1 cause an increase in Cdc28 inhibitory phosphorylation, although the removal of this phosphorylation is more dramatically delayed by high levels of Cdc55 than Sgo1.
Figure 5.7 The slow growth of CDC55-overexpressing cells is partially dependent on Cdc28 tyrosine phosphorylation. Ten-fold serial dilutions of wild type (AM1176), GAL-CDC55 (AM6065), mih1Δ (AM6169), mih1Δ GAL-CDC55 (AM6198), swe1Δ (AM6168), swe1Δ GAL-CDC55 (AM6197), cdc28-Y19F (AM5227), cdc28-Y19F GAL-CDC55 (AM6176), GAL-SGO1 (AM870), mih1Δ GAL-SGO1 (AM6190) and cdc28-Y19F GAL-SGO1 (AM5228) cells were spotted onto plates containing glucose (YEPD) or galactose (YEPRG) and incubated at 30°C.
Figure 5.8 The metaphase delay of CDC55-overexpressing cells is partially dependent on Cdc28 tyrosine phosphorylation

Wild type (AM1176), cdc28Y19F (AM5227), GAL-CDC55 (AM6065), cdc28Y19F GAL-CDC55 (AM6176), GAL-SGO1 (AM870) and cdc28Y19F GAL-SGO1 (AM5228) cells were grown and treated as in figure 3.2. Samples were taken at the indicated timepoints for (A) spindle morphology analysis, (B) budding analysis and (C) anti-Clb2 and anti-Cdc28 tyrosine 19 western blot analysis of TCA protein extracts separated on an 10% SDS polyacrylamide gel. Pgk1 is shown as a loading control.
5.2.5 Discussion

5.2.6 Catalytic dead protein phosphatase 2A

In chapter 4, I provided evidence that PP2A\textsuperscript{Cdc55} mediates the mitotic functions of Sgo1, and also contributes to Esp1 inhibition in a normal cell cycle, based on the phenotype caused by removal of Cdc55, the B-type regulatory subunit of the trimeric PP2A. My conclusions were made under the assumption that deletion of \textit{CDC55} causes the loss of a Cdc55-specific activity of PP2A, however, I had no evidence to prove that this was truly the case. It is conceivable that deletion of \textit{CDC55} could alter PP2A activity in other ways, perhaps by causing an increase in total phosphatase activity or by causing some non-physiological phosphatase activity. Previously it was shown that the morphological defect at low temperature and elevated of Swe1 kinase protein levels in \textit{cdc55}\textsuperscript{Δ} mutants could be rescued by deletion of both PP2A catalytic subunits (Yang et al. 2000), suggesting that unregulated PP2A phosphatase activity is responsible for at least some of the phenotypes caused by \textit{CDC55} deletion. In this chapter, I have generated a strain containing single point mutations in both PP2A catalytic subunits that should inactivate the catalytic activity of PP2A (Myles et al. 2001). I found that this catalytic-dead PP2A mutant (\textit{pp2a-cd}) phenocopies \textit{cdc55}\textsuperscript{Δ} with respect to benomyl sensitivity and synthetic lethality with \textit{pds1}\textsuperscript{Δ} (Figure 5.1), indicating that a specific Cdc55-dependent PP2A phosphatase activity might indeed function in the spindle checkpoint and Esp1 regulation. However, the \textit{pp2a-cd} mutant should be subjected to the same tension checkpoint, bi-orientation and Pds1 depletion assays as the \textit{cdc55}\textsuperscript{Δ} mutant to confirm this conclusion. It will also be necessary to determine whether the phosphatase activity of \textit{pp2a-cd} is actually reduced compared to wild type PP2A, although, the similar temperature sensitive growth of the \textit{pp2a-cd} mutant compared to a \textit{pph21}\textsuperscript{Δ}\textit{pph22}\textsuperscript{Δ} double mutant hints that this is probably the case (Figure 5.1; Sneddon et al. 1990). Finally, it will be important to confirm that the catalytic site mutations do not interfere with PP2A holoenzyme assembly. If so, the \textit{pp2a-cd} mutant may prove to be very useful for analysing the effects of inactivating PP2A phosphatase activity without disputing PP2A complex assembly, and therefore presumably subcellular localization and substrate interactions.
5.2.7 How does PP2A\textsuperscript{Cdc55} inhibit separase?

In the previous two chapters I accumulated evidence that PP2A\textsuperscript{Cdc55} plays a key role in regulating Esp1 activation. My Pds1 depletion experiments from chapter 4 suggested that PP2A\textsuperscript{Cdc55} acts redundantly with Pds1 to inhibit Esp1 during every cell cycle. I also found that PP2A\textsuperscript{Cdc55} mediates Esp1 inhibition caused by high levels of Sgo1 (Chapter 3). How then does PP2A\textsuperscript{Cdc55} inhibit Esp1? In an attempt to answer this question, I took an unbiased mutagenesis approach to identify sites within Esp1 that are important for inhibition by PP2A\textsuperscript{Cdc55}. Unfortunately, this approach did not yield any Esp1 alleles resistant to PP2A\textsuperscript{Cdc55}-mediated inhibition. This might be due to a number of reasons. It could be that Esp1 is not actually a target for inhibition by high levels of Sgo1 or Cdc55. However, I consider this unlikely, as all the evidence so far indicates that Esp1 is inhibited by \textit{SGO1} or \textit{CDC55} overexpression (Chapter 3). Another possibility is that mutations that would render Esp1 resistant to PP2A\textsuperscript{Cdc55}-mediated inhibition are inviable, perhaps due to structural defects in the Esp1 protein. Alternatively the screen may not have been saturating, or the rate of \textit{ESP1} mutagenesis was not high enough to isolate the desired alleles. I generated a mutant \textit{esp1} library predicted to contain 1 to 2 point mutations per \textit{ESP1} molecule (Appendix section A.3.2). Conceivably, mutations affecting multiple residues in Esp1 might be required to resist inhibition by PP2A\textsuperscript{Cdc55}.

There are two possible ways in which PP2A\textsuperscript{Cdc55} could regulate Esp1; either PP2A\textsuperscript{Cdc55} acts directly on Esp1 to cause its inhibition or some other function of PP2A\textsuperscript{Cdc55} is indirectly required to prevent Esp1 activation. In support of a direct role, Esp1 and Cdc55 have been shown to form a complex \textit{in vivo} (Queralt et al. 2006). This interaction is present in metaphase, when Esp1 is inactive, consistent with the notion that PP2A\textsuperscript{Cdc55} might directly inhibit Esp1 (Queralt et al. 2006). However, unlike the Pds1-Esp1 interaction, which is abolished at the onset of anaphase due to Pds1 degradation, Cdc55 and Esp1 continue to co-immunoprecipitate from anaphase cells (Queralt et al. 2006). Therefore if PP2A\textsuperscript{Cdc55} does inhibit Esp1 directly, it is unlikely to be due to a mechanism involving direct binding as has been demonstrated for Pds1 (Hornig et al. 2002). Conceivably, this hypothesis could be tested with an \textit{in vitro} assay for Esp1 activity. The cohesin
cleavage activity of Esp1 can be reconstituted in vitro using Esp1 purified from yeast and recombinant Scc1 (Uhlmann et al. 2000), and addition of recombinant Pds1 to this assay inhibits Esp1 activity (Hornig et al. 2002). If PP2A<sup>Cdc55</sup> inhibits Esp1 directly, addition of PP2A<sup>Cdc55</sup> to this reaction should also inhibit Esp1 activity. However, as the proper assembly and substrate specificity of the PP2A<sup>Cdc55</sup> holoenzyme is dependent on complex protein-protein interactions and posttranslational modifications (Shi 2009), the purification of a functional PP2A<sup>Cdc55</sup> complex to use in such an in vitro assay may prove to be difficult.

The phosphatase activity of PP2A<sup>Cdc55</sup> is maximal at metaphase, and then declines as cells enter anaphase (Queralt et al. 2006). This raises the possibility that PP2A<sup>Cdc55</sup> might inhibit Esp1 by direct dephosphorylation in metaphase, which is then reversed by the downregulation of PP2A<sup>Cdc55</sup> phosphatase activity, and presumably by the action of a kinase, at the onset of anaphase. I attempted to address this possibility by analysing the phosphorylation status of Esp1. I found that Esp1 phosphorylation is markedly reduced when Cdc55 is present in high levels (Figure 5.3). Taken together with my finding that the phosphatase activity of PP2A is required for cell viability in the absence of Pds1 (Figure 5.1), this result lends support to the hypothesis that PP2A<sup>Cdc55</sup> might reverse Esp1 phosphorylation to cause its inhibition. However, my analysis of Esp1 phosphorylation during a synchronous cell cycle showed that a fraction of Esp1 is phosphorylated prior to anaphase in wild type cells (Figure 5.3), suggesting that inactive Esp1 is more likely to be phosphorylated rather than underphosphorylated. In addition, Esp1 phosphorylation is not reduced by high levels of Sgo1 (Figure 5.3), which I previously showed to cause PP2A<sup>Cdc55</sup>-dependent Esp1 inhibition (Chapter 3). These results argue against the idea that reduced Esp1 phosphorylation in GAL-CDC55 cells represents PP2A<sup>Cdc55</sup>-mediated inhibition. Perhaps, when overproduced, Cdc55 promotes the dephosphorylation of sites in Esp1 that are irrelevant for inhibition, thus causing the change in gel mobility shift. I also do not know whether the effect of overproduced Cdc55 on Esp1 phosphorylation is direct, as high levels of Cdc55 could indirectly affect Esp1 phosphorylation by influencing other kinases and/or phosphatase that target Esp1.
Does PP2A\textsuperscript{Cdc55} indirectly regulate Esp1? PP2A\textsuperscript{Cdc55} has multiple functions within the cell, ranging from a role in cell wall integrity (Evans and Stark 1997) and cytokinesis (Healy et al. 1991; van Zyl et al. 1992), to the regulation of Cdk1 activation (Minshull et al. 1996; Yang et al. 2000; Pal et al. 2008) and exit from mitosis (Queralt et al. 2006; Wang and Ng 2006; Yellman and Burke 2006). It is thus conceivable that the requirement for PP2A\textsuperscript{Cdc55} in restricting Esp1 activity that was uncovered from my experiments could be indirectly due to other functions of PP2A\textsuperscript{Cdc55}. The role of PP2A\textsuperscript{Cdc55} in mitotic exit is thought to be through maintaining the inhibitory interaction of Net1 with Cdc14 in the nucleolus, as inactivation of PP2A\textsuperscript{Cdc55} results in premature Cdc14 release from the nucleolus (Queralt et al. 2006; Wang and Ng 2006; Yellman and Burke 2006). One possibility is that premature Cdc14 release might be the cause of Esp1 activation when PP2A\textsuperscript{Cdc55} is inactivated in my \textit{SGO1} overexpression and Pds1 depletion assays. This idea could be addressed by testing whether Esp1 activation in these assays is dependent on Cdc14 (for example by using a temperature-sensitive Cdc14 allele). Also, given that PP2A\textsuperscript{Cdc55} has an established role in regulating the inhibitory phosphorylation of Cdc28 on tyrosine 19 (Minshull et al. 1996; Yang et al. 2000; Pal et al. 2008), another possibility is that PP2A\textsuperscript{Cdc55} might contribute to Esp1 inhibition indirectly by affecting the phosphorylation status and activity of Cdc28. I previously showed that overexpression of \textit{CDC55} causes a delay to the cleavage of Esp1 substrates independently of Pds1, resulting in a cell cycle delay in metaphase (Chapter 3). In this chapter, my results show that this metaphase delay is partially dependent on inhibitory Cdc28-Tyr19 phosphorylation (Figure 5.8), suggesting that PP2A\textsuperscript{Cdc55} might prevent Esp1 activation at least in part through an indirect mechanism involving Cdc28 phosphorylation. This mechanism is unlikely to involve delayed entry into mitosis, as the timing of mitotic spindle formation and Clb2 accumulation in \textit{GAL-CDC55} cells was not advanced by the \textit{cdc28-Y19F} mutant (Figure 5.8). Instead, phosphorylation of Cdc28-Tyr19 appears to play a minor role downstream of PP2A\textsuperscript{Cdc55} later in the cell cycle to prevent the onset of anaphase. However, as \textit{GAL-CDC55} cells are still delayed in metaphase even when Cdc28-Tyr19 cannot be phosphorylated, such a role is unlikely to be significant in mediating the inhibition of Esp1 by PP2A\textsuperscript{Cdc55}. Indeed, the \textit{cdc28-Y19F} mutant had only a minor effect on the
timing of anaphase onset in SGO1-overexpressing cells (Figure 5.8), and is neither benomyl sensitive nor lethal in combination with pds1Δ (Figure A.17).

5.2.8 Does phosphorylation regulate separase activity in budding yeast?

In Xenopus egg extracts, Cdk1-mediated phosphorylation of separase on serine 1126 promotes separase inhibition through the binding of the Cdk1-cyclin B1 complex (Stemmann et al. 2001; Gorr et al. 2005). Similarly, phosphorylation of separase at this site in mammalian cells appears to be important for negative regulation of separase activity (Huang et al. 2005; Holland and Taylor 2006; Huang et al. 2008; Huang et al. 2009). The role of phosphorylation in the regulation of separase in lower eukaryotes, however, is not known. In this chapter, my analysis of the budding yeast separase, Esp1, by phosphate-affinity electrophoresis shows that Esp1 phosphorylation is in fact cell cycle regulated (Figure 5.3). I find that a small proportion of Esp1 is phosphorylated as cells enter metaphase, and this phosphorylated form rapidly disappears at the onset of anaphase (Figure 5.3). Because the overall levels of Esp1 protein also decrease in anaphase, however, it is difficult to assess whether phosphorylated Esp1 is dephosphorylated at the onset of anaphase or degraded. Analysis of Esp1 phosphorylation during a synchronous cell cycle in the presence of a proteosome inhibitor such as MG132 may shed light into this issue. From my results it is also unclear as yet whether phosphorylated Esp1 represents an active or inactive form of the protein.

Esp1 contains six consensus sites for phosphorylation by CDKs, five of which are predicted to be phosphorylated using the NetPhosYeast phospho-site predictor (Figure 5.4). This raises the possibility that, similar to vertebrate separase, Esp1 might also be regulated by phosphorylation by Cdk1/Cdc28. For a long time it has been recognised that budding yeast cells can still enter anaphase in the presence of high Cdc28 activity (Surana et al. 1993), suggesting that Esp1 is unlikely to be inhibited by Cdc28-dependent phosphorylation. Does Cdc28 instead positively regulate Esp1? My results from this chapter provide evidence Cdc28 activity at the
onset of anaphase is not required for Esp1 activation (Figure 5.5), however, this finding does not rule out a role for Cdc28 in directly activating Esp1. Cdc28 activity is required for APC\textsuperscript{Cdc20} activation (Rudner et al. 2000; Rudner and Murray 2000; Rahal and Amon 2008), yet inhibition of Cdc28 in cells released into anaphase from a metaphase arrest did not prevent the activation of APC\textsuperscript{Cdc20} (Figure 5.5). This suggests that APC\textsuperscript{Cdc20} phosphorylation by Cdc28 might occur earlier in the cell cycle, possibly analogous to human cells (Kraft et al. 2003), priming it for activation by Cdc20. Perhaps Esp1 is similarly phosphorylated and primed for activation by Cdc28 well before Esp1 activity is required at the onset of anaphase. In this case, inhibition of Cdc28 just before the onset of anaphase (as in the experiment described in section 5.2.4.1) would be expected to have little effect on Esp1 activity. It is difficult to assess the consequences of Cdc28 inactivation earlier in the cell cycle on Esp1 activation, however, as Cdc28 activity is required for the completion of earlier events such as DNA replication (Diffley 2004).

5.2.9 Inactivation of Cdc28 results in a more rapid metaphase to anaphase transition

I noticed that full length Scc1 disappeared prematurely compared to wild type when Cdc28 was inactivated prior to anaphase onset (Figure 5.4C,D). Does this result suggest that Cdc28 plays a role in directly inhibiting Esp1, similar to vertebrate systems? This is a possibility, however, I consider it unlikely that this result provides evidence for such a mechanism. Firstly, Pds1 destruction also occurred earlier in cdc28\textsuperscript{-as1} cells, suggesting that early Esp1 activation was due to more rapid Pds1 degradation, rather than as a direct consequence of Cdc28 inhibition. Secondly, at least in vertebrates, Cdk1-mediated phosphorylation of separase is required to establish cyclin B1 binding and separase inhibition, but separase phosphorylation is not necessary to maintain this inhibition (Gorr et al. 2005; Holland and Taylor 2006). Therefore, inhibition of Cdc28 kinase activity in pre-anaphase cells is unlikely to relieve Esp1 inhibition if such a mechanism exists. One possible explanation for my results comes from the finding that phosphorylation of Pds1 by Cdc28 greatly inhibits Pds1 ubiquitination by the APC, at least \textit{in vitro} (Holt et al. 2008). Perhaps
by inhibiting Cdc28 in metaphase, Pds1 phosphorylation is prevented, thereby promoting more rapid ubiquitination and subsequent degradation of Pds1 upon APC\textsuperscript{Cdc20} activation. Alternatively, Cdc28 may negatively regulate anaphase onset by another unknown mechanism.

### 5.2.10 Sgo1 and PP2A\textsuperscript{Cdc55} function in common and distinct pathways

I previously showed that overexpression of either \textit{SGO1} or \textit{CDC55} causes a similar delay to the cleavage of Esp1 substrates independently of Pds1, resulting in a cell cycle delay in metaphase (Chapter 3). This provided strong evidence for my model that Sgo1 and PP2A\textsuperscript{Cdc55} function in a common pathway. However, several lines of evidence from this chapter hint that the phenotypes caused by \textit{SGO1} and \textit{CDC55} overexpression are in fact slightly different: 1. \textit{CDC55}-overexpressing cells are delayed in the formation of mitotic spindles compared to wild type and \textit{SGO1}-overexpressing cells (Figure 5.3A). 2. Overexpression of \textit{CDC55}, but not \textit{SGO1}, reduces Esp1 phosphorylation (Figure 5.3B). 3. Preventing phosphorylation of Cdc28-Tyr19 partially alleviates the metaphase delay caused by \textit{CDC55} overexpression, but has only a minor effect on metaphase delay of \textit{SGO1}-overexpressing cells (Figure 5.8). 4. Cdc28-Tyr19 phosphorylation, although similarly increased by both \textit{CDC55} and \textit{SGO1} overexpression, fails to be removed in \textit{CDC55}-overexpressing cells but not in \textit{SGO1}-overexpressing cells (Figure 5.8). These observations highlight the need for caution when interpreting overexpression experiments; although both Sgo1 and Cdc55 do indeed prevent anaphase onset when overproduced, their overproduction appear also to activate distinct pathways. Nevertheless, despite exhibiting some distinct phenotypes, the major phenotype of both \textit{SGO1} and \textit{CDC55}-overexpressing cells is a delay in metaphase, suggesting that my initial conclusions remain valid. My loss of function data from chapter 4 also provides complimentary evidence to suggest that Sgo1 and PP2A\textsuperscript{Cdc55} function in a common pathway with respect to tension sensing and bi-orientation. However, it is important to note that Sgo1 and PP2A\textsuperscript{Cdc55} also have distinct cellular functions, as evident by the synthetic growth defect of \textit{sgo1Δ cdc55Δ} double mutants (Figure 4.5).
Chapter 6

Final Discussion
Chapter 6 – Final Discussion

The eukaryotic cell cycle requires a highly regulated sequence of events, which ultimately contribute to the accurate duplication of cell contents followed by an equal distribution of those contents, by division, into two daughter cells. The precise distribution of genetic information is particularly important, as errors in this process have been linked to cancer progression, infertility and birth defects such as Down syndrome (Hassold and Hunt 2001; Rajagopalan and Lengauer 2004). Accurate eukaryotic chromosome segregation absolutely requires that duplicated chromosomes remain physically linked from the moment they are synthesised in S-phase (Uhlmann and Nasmyth 1998). This linkage is mediated by the cohesin complex, which is thought to form a ring-shaped structure that encompasses sister chromatids (Nasmyth and Haering 2005). When the mitotic spindle forms in metaphase, the cohesin ring facilitates the attachment of sister chromatids to microtubules emanating from opposite poles by counteracting the pulling forces of microtubules, thereby ensuring that sister chromatids are under tension and primed to be segregated away from each other in anaphase (Tanaka 2005). Crucially, there is overwhelming evidence to suggest that the poleward movement of chromosomes in anaphase is triggered by the cleavage of the cohesin ring by a protease known as separase (Nasmyth and Haering 2009). Most notably, key experiments performed in budding yeast and more recently in flies demonstrated that cleavage of cohesin in metaphase-arrested cells is on its own sufficient to trigger the separation of sister chromatids (Uhlmann et al. 2000; Oliveira et al.).

There are two key events that need to be tightly controlled to ensure the equal distribution of the genome between daughter cells during cell division. First, sister chromatids must attach to microtubules emanating from opposite poles of the mitotic spindle in a process known as bi-orientation. This ensures that sister chromatids are in position to be segregated away from each other in anaphase. Second, separase activity must be restricted until all chromosomes have achieved bi-orientation. If chromosomes do not bi-orient, or if separase is allowed to cleave cohesin before bi-orientation occurs, chromosome missegregation is a likely consequence, leading to the production of daughter cells containing an abnormal number of chromosomes.
(aneuploidy). Because in somatic cells, aneuploidy likely contributes to oncogenesis (Rajagopalan and Lengauer 2004), and in gametes, aneuploidy can lead to birth defects and infertility (Hassold and Hunt 2001), understanding how the cell ensures the accuracy of chromosome segregation will provide an important insight into the causes of human disease.

In this study, I have used the budding yeast *Saccharomyces cerevisiae* as a model system to study chromosome segregation. Since the mechanisms of chromosome segregation are highly conserved amongst eukaryotes, studies in yeast will provide a fundamental understanding of this process in humans. I initiated this work by studying Sgo1, the budding yeast member of a highly conserved family of shugoshin proteins. Sgo1 had previously been shown to play a role in preventing cell cycle progression when sister chromatids are not under tension, and also in ensuring the proper bi-orientation of sister chromatids in metaphase (Indjeian et al. 2005), however, the underlying mechanism were completely unknown. By developing an in vivo assay for Sgo1 function based on its overproduction during mitosis, I found that high levels of Sgo1 cause a delay to anaphase onset due to separase inhibition. Does shugoshin also regulates separase activity in other organisms? Strong overexpression of Sgo1 interferes with mitotic growth in fission yeast, similar to what I showed for budding yeast Sgo1 (Kitajima et al. 2004). Moreover, mild mitotic expression of Sgo1 in fission yeast causes lethality to separase mutant (*cut1-206*), even at the permissive temperature for the *cut1-206* allele (Kitajima et al. 2004). Taken together, these results suggest that fission yeast Sgo1 may also be involved in the negative regulation of separase. There is also evidence from human cells that shugoshin can counteract separase activity in mitosis. If human cells are allowed to enter anaphase in the absence of tension between kinetochores, shugoshin persists at the inner centromere where it is sufficient to block separase-dependent sister chromatid separation (Lee et al. 2008). It is unclear as yet whether shugoshin is acting upon separase or cohesin itself in this situation, although as Scc1 phosphorylation is of little importance for cohesin cleavage in human cells (Hauf et al. 2005), a role in separase regulation is a possibility. Intriguingly, Sgo1 and separase appear also to have reciprocal roles in regulating centriole separation. Human cells depleted of separase are defective in separating centrioles during mitotic exit (Tsou et al. 2009);
whereas Sgo1 depleted cells have a premature centriole separation phenotype (Wang et al. 2008). Altogether these observations are consistent with the possibility that shugoshin might have a conserved role in regulating separase activity during mitosis.

It has previously been shown that an inhibitory chaperone, known as securin, plays a key role in preventing separase activation. Securin binds to and inhibits separase until the onset of anaphase when it is degraded due to its ubiquitination by the anaphase-promoting complex (Cohen-Fix et al. 1996; Funabiki et al. 1996a; Zou et al. 1999; Hagting et al. 2002). Given the importance of securin in separase inhibition, one would expect organisms lacking securin to be inviable due to premature separase activation and subsequent chromosome missegregation. Indeed, mathematical modelling of the budding yeast cell cycle predicts that securin-deficient cells should be inviable for such a reason (Chen et al. 2004). However, experimental evidence from several different organisms has proved that this is not the case: securin-deficient mice are apparently normal and mammalian securin^{-/-} cells undergo a largely normal anaphase (Jallepalli et al. 2001; Mei et al. 2001; Wang et al. 2001; Pfleghaar et al. 2005; Wirth et al. 2006). Furthermore, budding yeast cells lacking securin are viable (Yamamoto et al. 1996a) and initiate cohesin cleavage and sister chromatid separation with similar timing to wild type cells (Alexandru et al. 1999; Alexandru et al. 2001). This suggests that additional mechanisms must exist to restrict separase activation to the correct window of the cell cycle.

I found that high levels of Sgo1 cause separase inhibition independently of any known upstream regulators of separase, including securin; raising the possibility that Sgo1 might function in a novel pathway to restrict separase activity. By taking a candidate based approach, I identified protein phosphatase 2A specifically bound to its Cdc55 regulatory subunit (PP2A^{Cdc55}) to be required downstream of Sgo1 for separase inhibition. My data suggests that securin and PP2A^{Cdc55} act redundantly to restrict separase activity during every cell cycle, and that PP2A^{Cdc55} mediates the function of Sgo1 in sensing tension between sister chromatids and ensuring bi-orientation in metaphase. One possible model that explains my results is that PP2A^{Cdc55} is a separase inhibitor that is employed by Sgo1 when sister chromatids
are not under tension (Figure 4.9). This pathway appears to act in parallel to securin, thereby providing a possible explanation for the viability of securin-deficient cells.

Such a multi-layered regulation of separase could help safeguard against aneuploidy. Indeed, PP2A is thought to be a tumour suppressor, and the PP2A inhibitors okadaic acid and mycrocystin-LR are potent tumour promoters (Janssens et al. 2005; Eichhorn et al. 2009). Intriguingly, it has recently been shown that Sgo1 levels are reduced in human colorectal cancer tissues (Iwaizumi et al. 2009), suggesting that Sgo1 might also be tumour suppressor. I have demonstrated that Sgo1 and PP2A collaborate to control chromosome segregation by regulating separase activation and the bi-orientation process. Perhaps inactivation of Sgo1 or PP2A contributes to tumourogenesis by causing defects in chromosome segregation, possibly due to bi-orientation defects or hyperactive separase activity. Intriguingly, recent evidence indicates that separase is an oncogene. Overexpression of separase causes aneuploidy and tumourogenesis in immortalised mouse mammary epithelial cells with a p53 mutant background (Zhang et al. 2008). Furthermore, separase is found to be highly overexpressed in osteosarcoma, breast, and prostate tumour specimens (Meyer et al. 2009). Therefore, understanding how Sgo1 and PP2A regulate separase activity may prove to be valuable in understanding the mechanisms underlying human cancers.

6.1 Future plans

6.1.1 How does Sgo1 signal to PP2A\textsuperscript{Cdc55}?

My results provide strong evidence to suggest that PP2A\textsuperscript{Cdc55} carries out the functions of Sgo1 in budding yeast mitosis. First, PP2A\textsuperscript{Cdc55} mediates separase inhibition caused by high levels of Sgo1. Second, sgo1\textDelta and cdc55\textDelta mutants are similarly defective in sensing tension between sister chromatids and ensuring chromosome bi-orientation in metaphase. Finally, an sgo1-3A mutant that cannot bind to PP2A (Xu et al. 2009) shares a similar phenotype to sgo1\textDelta and cdc55\textDelta mutants. How then does Sgo1 signal to PP2A\textsuperscript{Cdc55}? The finding that an sgo1-3A mutant phenocopies cdc55\textDelta suggests that Sgo1 signals to PP2A\textsuperscript{Cdc55} by a direct interaction, an idea that is supported by the observation that mouse shugoshin can
bind to PP2A complexes homologous to PP2A<sup>Cdc55</sup>, at least <em>in vitro</em> (Xu et al. 2009). However, we have so far been unable to detect an interaction between Sgo1 and Cdc55. Immunoprecipitated Sgo1 and Cdc55 analysed by mass-spectrometry did not reveal Sgo1 and Cdc55 as potential interactors (Naoka Tamura, data not shown). Furthermore, Sgo1 and Cdc55 did not co-immunoprecipitate from cycling cell cultures, even when Sgo1 was overproduced (data not shown). Although these experiments suggest that Sgo1 and PP2A<sup>Cdc55</sup> do not interact, further work should be undertaken to address this possibility more rigorously. For example, an interaction between Sgo1 and PP2A<sup>Cdc55</sup> might be very weak or transient; repeating such immunoprecipitation experiments in the presence of a cross-linker might allow the detection of loosely associated proteins. Also, it could be that Sgo1 and PP2A<sup>Cdc55</sup> only form a complex under certain physiological conditions. It would be interesting to test if Sgo1 and Cdc55 co-immunoprecipitate when sister chromatids are not under tension due to microtubule depolymerization or cohesin depletion, when Sgo1 is presumably active. In <em>vitro</em> co-immunoprecipitation experiments might also provide an insight as to whether Sgo1 and PP2A<sup>Cdc55</sup> interact directly. For these co-immunoprecipitation studies, the <em>sgo1</em>-<em>3A</em> mutant, which should not bind to PP2A, would be a very useful control.

Co-localization studies can provide an insight as to whether two proteins function together in a complex. Cdc55 does not, however, co-localize with Sgo1 at the pericentromere, as judged by chromatin immunoprecipitation (ChIP) experiments (Adele Marston, data not shown). This suggests that Sgo1 and PP2A<sup>Cdc55</sup> might not interact close to the chromatin. Intriguingly, immunofluorescence analysis of Sgo1 shows that, in addition to localizing to the pericentromere, a significant proportion of Sgo1 protein is dispersed throughout the nucleus (Olga Nerusheva and Adele Marston, data not shown). Similarly, Cdc55 is localized to the nucleus throughout the cell cycle (Gentry and Hallberg 2002). Perhaps Sgo1 and PP2A<sup>Cdc55</sup> interact away from chromatin. If so, fluorescence resonance energy transfer (FRET) could be used to detect such an interaction. This method also has the advantage that it can be employed in live cells, therefore any protein-protein interactions that might occur transiently during the cell cycle may be detected (Truong and Ikura 2001).
An alternative possibility is that additional proteins mediate the connection between Sgo1 and PP2A\textsuperscript{Cdc55}. I performed a screen for high-copy suppressors of the GAL-\textit{SGO1} phenotype (Chapter 3 and Appendix). One of the suppressor plasmids isolated contained the \textit{ZDS1} gene, which, when introduced into cells alone on a high-copy plasmid, is able to bypass the metaphase delay caused by \textit{SGO1} overexpression, confirming that the screen was successful (Naoka Tamura, data not shown). It has recently been postulated that Zds1 (and a related protein Zds2) might function as a negative regulator of PP2A\textsuperscript{Cdc55} (Queralt and Uhlmann 2008). Perhaps Sgo1 influences PP2A\textsuperscript{Cdc55} indirectly by affecting the regulators of PP2A\textsuperscript{Cdc55}, such as Zds1. It is known that Cdc55 and Zds1 interact (Queralt and Uhlmann 2008); analysing whether Sgo1 also interacts with the Zds1 protein would be useful to test this hypothesis. An unbiased mutagenesis screen for mutants that suppress the GAL-\textit{SGO1} phenotype might also aid the identification of additional proteins that function downstream of Sgo1.

My data suggests that PP2A\textsuperscript{Cdc55} functions downstream of Sgo1 to inhibit separase. However, the securin-depletion experiments described in chapter 4 indicate that PP2A\textsuperscript{Cdc55}, but not Sgo1, plays a role in restricting separase activity during every cell cycle. I interpret these results to suggest that Sgo1 is only important for Esp1 inhibition, through PP2A\textsuperscript{Cdc55}, when sister chromatids are not under tension. How then does Sgo1 regulate the activity of PP2A\textsuperscript{Cdc55} as a separase inhibitor? Cdc55 and separase physically interact (Queralt et al. 2006), and my preliminary analysis of a catalytic-dead PP2A mutant suggests that phosphatase activity of PP2A\textsuperscript{Cdc55} might be required for its role in separase inhibition. Maybe Sgo1 functions to strengthen the interaction between Cdc55 and separase, or prevents the downregulation of PP2A\textsuperscript{Cdc55} phosphatase activity that normally occurs at the onset of anaphase (Queralt et al. 2006). These possibilities could be tested by comparing the phosphatase activity of, and the amount of separase protein pulled down by, immunoprecipitated Cdc55 when Sgo1 is present at wild type levels or overproduced.
6.1.2 Multilayered regulation of separase

Budding yeast cells lacking either securin or CDC55 are viable and initiate cohesin cleavage in a timely manner (Figure 4.2). Strikingly, however, I found that cells lacking both securin and CDC55 prematurely cleave cohesin, indicative of premature separase activation (Figure 4.2). As a consequence, these cells prematurely separate sister centromeres and missegregate chromosomes at a high frequency, resulting in the production of aneuploid daughter cells and subsequent inviability (Chapter 4). These observations suggest that PP2A<sup>Cdc55</sup> and securin act redundantly to inhibit separase and raise the intriguing possibility that PP2A<sup>Cdc55</sup> and securin might regulate different pools of separase. This idea is strengthened by the observation that deletion of CDC55 allows sister chromatid separation in cells expressing high levels of non-degradable securin (Figure A.18; Tang and Wang 2006). Securin inhibits separase by direct binding (Hornig et al. 2002; Waizenegger et al. 2002), and Cdc55 has also been found to physically interact with separase in vivo (Queralt et al. 2006). Are there two pools of separase, one associated with securin and the other with Cdc55? Performing reciprocal co-immunoprecipitation experiments with Cdc55, securin and separase would go some way to answering this interesting question.

By careful examination of separase protein by phosphate affinity electrophoresis, I found that a minor pool of separase is phosphorylated in a cell cycle-dependent manner (Chapter 5). This phosphorylation is reduced in cells overexpressing CDC55, however, the contribution of this phosphorylation pattern to separase activity remains unclear (Chapter 5 discussion). It would be interesting to map the phosphorylation sites within separase in vivo. This could be done by immunoprecipitating endogenous separase under denaturing conditions to preserve phosphorylation, with the immunoprecipitate then subjected to mass spectrometry-based phosphopeptide mapping (Shou et al. 2002). The identification of sites within separase that are phosphorylated in vivo would provide an insight into how separase is regulated by phosphorylation. For example, the phosphorylated residues might lie within protein kinases consensus sites, thus hinting which kinases might be responsible for phosphorylating separase. Also, the separase phosphopeptide mapping could be repeated using cells arrested in different stages of the cell cycle to judge which sites
are phosphorylated in a cell cycle-dependent manner. Phosphorylated residues could then be mutated and the phenotype of separase mutants analysed to determine whether separase phosphorylation plays a role in regulating its activity during the cell cycle.

In vertebrate cells, Cdk1-dependent separase phosphorylation at serine 1126 promotes the binding of the Cdk1-cyclin B1 complex, which is sufficient for securin-independent separase inhibition (Stemmann et al. 2001; Gorr et al. 2005). In this regard, it is important to note that budding yeast separase contains 6 potential CDK phosphorylation sites (Figure 5.4). Is budding yeast separase also regulated by Cdk1-dependent phosphorylation? Analysis of separase mutated in all potential CDK phosphorylation sites individually and combined might provide an insight into this question. It could also be tested whether Cdk1 is responsible for separase phosphorylation. Separase purified from yeast or after bacterial expression (Uhlmann et al. 2000) could be subjected to an in vitro kinase assay with immunoprecipitated cdc28-as1 from cells grown with or without 1NM-PP1. In vivo separase phosphorylation could also be analysed by phosphate affinity electrophoresis of extracts from metaphase arrested cdc28-as1 cells with or without 1NM-PP1.

6.1.3 How does the Sgo1-PP2A<sup>Cdc55</sup> pathway regulate chromosome bi-orientation?

It was previously shown that sgo1 mutants are defective in bi-orienting sister chromatids after the mitotic spindle is allowed to reform following a nocodazole arrest (Indjeian et al. 2005; Indjeian and Murray 2007). I have demonstrated that this phenotype is shared by both sgo1-3A and cdc55∆ mutants, suggesting that PP2A<sup>Cdc55</sup> functions downstream of Sgo1 in its role in bi-orientation. How does the Sgo1-PP2A<sup>Cdc55</sup> pathway regulate chromosome bi-orientation? My overexpression data suggests that PP2A<sup>Cdc55</sup> mediates separase inhibition by Sgo1. One possibility is that the Sgo1-PP2A<sup>Cdc55</sup> pathway ensures bi-orientation by restricting inappropriate separase activation. Given that intact sister chromatid cohesion is essential for the bi-orientation process (Tanaka et al. 2000), precocious separase activation and
subsequent cohesin cleavage might be responsible for the bi-orientation defect of sgo1Δ and cdc55Δ mutants. However, this possibility seems unlikely, as the expression of non-cleavable cohesin does not rescue the bi-orientation defect of sgo1Δ and cdc55Δ mutants (Appendix section A.4; Figure A.20). Nevertheless, it remains a possibility that another function of separase, such as Cdc14 activation, may be responsible for the bi-orientation defect. This possibility could be addressed by testing whether the bi-orientation defect of sgo1Δ and cdc55Δ mutants can be rescued by a temperature-sensitive separase mutant such as esp1-1 (Baum et al. 1988).

I found that deletion of SGO1 or CDC55 suppressed the synthetic lethality of a spo12Δ lte1Δ double mutant (Figure 4.5B), suggesting that both Sgo1 and Cdc55 are indeed negative regulators of mitotic exit. Furthermore, when sister chromatids are not under tension due to cohesin depletion, the most obvious phenotype of sgo1Δ and cdc55Δ mutants is a failure to sequester Cdc14 in the nucleolus (Figure 4.6). Does the Sgo1-PP2A-Cdc55 ensure bi-orientation in metaphase by preventing inappropriate Cdc14 release? In support of this view, cdc55Δ mutants fail to sequester Cdc14 in the nucleolus when arrested in metaphase by Cdc20 depletion or microtubule depolymerization (Queralt et al. 2006; Yellman and Burke 2006), exactly the conditions that I used for the bi-orientation assay. It would be interesting to test if sgo1Δ mutants also share this phenotype. If inappropriate Cdc14 activation is responsible for the bi-orientation defect of sgo1Δ and cdc55Δ mutants, inactivation of Cdc14 (by using a temperature-sensitive allele) should rescue this defect. Similarly, one could test whether the ectopic activation of Cdc14 in otherwise wild type cells causes a problem in bi-orienting sister chromatids. This could be done by repeating the bi-orientation experiment as described in Figure 4.7 with a strain containing the GAL-CDC14 construct (Visintin et al. 1998) and induce high levels of CDC14 expression with galactose prior to nocodazole wash-out.

The possibility that Cdc14 might inhibit bi-orientation in metaphase is intriguing. When activated at the onset of anaphase, Cdc14 dephosphorylates numerous substrates that were previously phosphorylated by mitotic kinases (Sullivan and Morgan 2007). One such substrate is Sli15, which is part of the chromosomal
passenger complex (CPC) that includes Bir1 and Ipl1 (Pereira and Schiebel 2003). Dephosphorylation of Sli15 by Cdc14 leads to the targeting of the CPC away from the inner centromere to the spindle midzone in anaphase (Pereira and Schiebel 2003). Inner centromeric Ipl1 is essential for the bi-orientation process, as it is required for the destabilization of kinetochore-microtubule attachments that do not generate tension, thereby allowing the generation of new attachments that lead to bi-orientation (Pinsky et al. 2006). A speculative model to explain the bi-orientation defect of sgo1Δ and cdc55Δ mutants could be that Cdc14 is prematurely activated, thereby leading to the premature delocalization of Ipl1 from the inner centromere where it can no longer destabilize inappropriate kinetochore-microtubule attachments. Such a model predicts that a sli15ΔA mutant, which is constitutively dephosphorylated, should exhibit a similar bi-orientation defect to sgo1Δ and cdc55Δ mutants; this prediction could easily be tested.

6.2 Conclusion

I initiated this study with the aim of understanding the mechanism by which the budding yeast shugoshin, Sgo1, functions to regulate chromosome segregation. My findings uncover an alternative separase regulatory pathway involving Sgo1 and PP2A<sup>Cdc55</sup>, and reveal that PP2A<sup>Cdc55</sup> is a novel downstream effector of Sgo1 function during mitosis. This work therefore makes a significant contribution to our understanding of the mechanisms controlling chromosome segregation.
Bibliography


A Appendix

A.1 Screen for high copy suppressors of GAL-SGO1

Figure A.1 provides a schematic of the high-copy suppressor screen procedure. A yeast 2µ genomic library (Nasmyth and Tatchell 1980) was transformed into a strain containing the GAL-SGO1 construct. Approximately 35,000 transformants were obtained, representing roughly 3-fold coverage of the yeast genome. Colonies were then replica-plated onto phloxine B plates containing either glucose or galactose. On plates containing the red dye phloxine B, colonies of cells with reduced viability appear dark pink whereas normal colonies appear practically white (Bonneu et al. 1991). Colonies were chosen that appeared white on phloxine B galactose plates, indicating that the GAL-SGO1 slow growth phenotype was suppressed in these cells. Plasmid DNA was isolated from candidate colonies and re-transformed into the GAL-SGO1 strain to verify the suppression phenotype is linked to the 2µ plasmids (Figure A.2). In total 70 suppressor plasmids were recovered and the identity of the DNA inserts revealed by sequencing (Table A.1). The majority of the isolated plasmids contained independent DNA fragments, and no single fragment appeared more than twice.

A.1.1 Secondary screening of suppressor plasmids

The variety of suppressor plasmids isolated suggested that suppression of GAL-SGO1 could be achieved by perturbing the Sgo1 pathway at many different levels (Figure A.3). With the help of an undergraduate, Tara Mills, I performed secondary screens with the aim of understanding the function of the suppressor plasmids.

A.1.1.1 Suppressors that reduce levels of overproduced Sgo1

We first aimed to identify whether some suppressor plasmids caused a reduction in the overall levels of overproduced Sgo1. Suppressor plasmids were transformed into a strain containing epitope-tagged SGO1 under control of the GAL1-10 promoter
(GAL-SGO1-9MYC). Transformants were grown in the presence of galactose to overexpress SGO1-9MYC and anti-MYC western blot analysis of protein extracts used to determine the levels of Sgo1-9MYC. Six out of seventy suppressor plasmids reduced the levels of Sgo1-9MYC to a similar level to Sgo1-9MYC synthesised from the endogenous SGO1 promoter (Table A.1). We reasoned that this might be due to either an increased rate of Sgo1 degradation, or alternatively, a reduction in the levels of expression from the GAL1-10 promoter.

A.1.1.2 Suppressors that genetically interact with ESP1 and/or SCC1

Because the slow growth of SGO1-overexpressing cells is likely due to a metaphase delay caused by an inability to cleave cohesin by Esp1 (Chapter 1), we predicted that the suppressor plasmids might function as positive regulators of Esp1, or negative regulators of cohesin. We therefore tested whether the suppressors showed genetic interactions with temperature sensitive ESP1 (esp1-1) and SCC1 (mcd1-1) alleles.

If the suppressor plasmids positively regulated Esp1, we reasoned that their presence might be able to suppress the temperature sensitivity of the esp1-1 allele. Suppressor plasmids were transformed into an esp1-1 strain, and transformants were tested for growth at 25°C, 30°C, 32°C, 34°C and 36°C. The esp1-1 strain transformed with an empty vector was inviable at temperatures above 32°C. Interestingly, 3 of the 70 plasmids suppressed the lethality of esp1-1 at 34°C (Table A.1).

If negative regulation of cohesin was the reason for suppression of GAL-SGO1, suppressor plasmids might reduce the viability of the mcd1-1 allele at lower temperatures. Suppressor plasmids were transformed into a mcd1-1 strain, and transformants were tested for growth at 25°C, 30°C, 32°C, 34°C and 36°C. Out of the 70 suppressor plasmids, 4 reduced the restrictive temperature of the mcd1-1 allele from 34°C to 30°C (Table A.1).
Figure A.1 Schematic of the screen for high-copy suppressor of *GAL-SGO1*. *GAL-SGO1* (AM870) cells were used. Screen described in detail in the text.
Figure A.2 An example of a GAL-SGO1 suppressor. (A) Original colony identified from screen, (B) GAL-SGO1 cells containing empty 2µ plasmid and (C) three independently isolated colonies containing GAL-SGO1 and the 2µ suppressor plasmid extracted from the original colony were streaked onto galactose medium lacking leucine (SC/-leu/RG) and incubated at room temperature for 3 days.

Figure A.3 How might suppressor plasmids perturb the Sgo1 pathway? A schematic of how high levels of Sgo1 prevent anaphase onset. Suppressor plasmids (SP) might perturb this pathway by acting at various levels within the pathway as indicated.
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Table A.1 Summary of GAL-SGO1 suppressor plasmids
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Table A.1 Summary of GAL-SGO1 suppressor plasmids
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A.2 Screen for Esp1 mutants resistant to inhibition by the Sgo1-PP2A\(^{Cdc55}\) pathway

A.2.1 ESP1 plasmid shuffle

ESP1 is an essential gene, therefore the plasmid shuffle technique (Sikorski and Boeke 1991) was used to introduce randomly mutagenized ESP1 into esp1\(^{\Delta}\) strains containing the GAL-SGO1 or GAL-CDC55 construct. The viability of the esp1\(^{\Delta}\) strain used depended on ESP1 encoded on an autonomously replicating plasmid containing the TRP1 marker (ESP1-CEN-TRP; AMp707), which can be counterselected for using the drug 5-fluoroanthranillic acid (Toyn et al. 2000). I first tested the efficiency of the plasmid shuffle technique using the TRP/FAA method. Figure A.5 shows that the ESP1-CEN-TRP plasmid can be effectively replaced by the ESP1-CEN-HIS plasmid (AMp708) in esp1\(^{\Delta}\) cells using plates containing FAA.

A.2.2 Random mutagenesis of ESP1

To introduce mutations randomly into the ESP1 gene, I mutagenized the ESP1-CEN-HIS plasmid using the E.coli mutator strain XL1-red (stratagene). This strain of E.coli almost completely lacks a DNA repair pathway; resulting in a high rate of spontaneous mutations (Greener et al. 1997). Based on a mutation rate of 1 mutation per 2kb per 120 generations for a pBR322-based plasmid (Greener et al. 1997), propagation of the ESP1-CEN-HIS plasmid in XL1-red E.coli for 50 to 100 generations was predicted to introduce 1 to 2 point mutations within the ESP1 coding sequence. As a test for mutagenesis efficiency, I transformed esp1\(^{\Delta}\) ESP1-CEN-TRP cells with ESP1-CEN-HIS that had been propagated in XL1-red E.coli for either 50 or 100 generations (esp1\(^{\ast}\)-CEN-HIS). After losing ESP1-CEN-TRP, up to 15% of these colonies failed to grow (Figure A.6), indicating that lethal mutations were introduced into the ESP1 coding sequence.
A.2.3 Screening the mutant esp1 library

Figure A.7 provides a schematic of the ESP1 allele screen procedure. For the screen, the ESP1-CEN-HIS plasmid was propagated in XL1-red E.coli for 50 and 100 generations, and the resulting plasmid preparations (esp1*-CEN-HIS) transformed into an esp1Δ strain containing the ESP1-CEN-TRP plasmid and either GAL-SGO1 or GAL-CDC55. A library size of approximately 15,000 transformants was obtained, which was then replicated onto plates containing FAA to lose the ESP1-CEN-TRP plasmid. The surviving colonies contained esp1Δ GAL-SGO1/CDC55 cells whose viability depended on mutagenized ESP1. These colonies were then subjected to a phloxine B-based screen as described above. Colonies were chosen that appeared white on galactose medium containing phloxine B, indicating that the mutagenized ESP1 in these cells might suppress the slow growth of SGO1- and CDC55-overexpressing cells.

Candidate colonies were then purified on FAA plates to ensure loss of wild type ESP1, and re-tested for growth on galactose. Of the original 55 colonies identified from the phloxine B screen, the majority (33/55) confirmed either complete, or partial suppression of the GAL-SGO1 or GAL-CDC55 slow growth on galactose. Plasmid DNA was then isolated from these colonies and re-transformed into the GAL-SGO1 or GAL-CDC55 strains to verify the suppression phenotype was linked to the mutagenized ESP1 plasmid. However, none of the 33 isolated plasmids confirmed suppression of GAL-SGO1 or GAL-CDC55 when re-transformed (for examples, see Figure A.8). This suggests that the apparent suppression of GAL-SGO1 and GAL-CDC55 was not linked to the plasmid and therefore not linked to mutant ESP1 alleles. So why were positive colonies identified? One possibility is that positive colonies had acquired mutations in the genome that caused suppression of GAL-SGO1/CDC55. To test this, I crossed one of the positive colonies to a wild type strain to generate diploids that should be heterozygous for any suppressor mutations. I sporulated these diploids with the aim of identifying GAL-SGO1 progeny that were either suppressed or not. Surprisingly, however, I found that none of the resulting spores actually contained the GAL-SGO1 construct (Figure A.9). I therefore inferred from this finding that positive colonies were identified from the
screen because they had either completely lost, or lost some copies of, the GAL-SGO1/CDC55 constructs from the genome.

**A.2.4 Isolation of temperature-sensitive esp1 mutants**

To determine whether this method of mutagenesis and screening was in fact capable of isolating ESP1 alleles, I screened ~500 of the library of transformants for temperature sensitivity using a phloxine B-based assay (Figure A.10A). Interestingly, 4 colonies were identified that showed temperature-sensitive growth at 37°C that was linked to the mutagenized ESP1 plasmids. Point mutations within the ESP1 coding sequence were identified in all 4 plasmids, each of which corresponded to an amino acid substitutions within the primary sequence of the Esp1 protein (Figure A.10B).
Figure A.4 Overexpression of *SGO1* or *CDC55* causes slow growth. 10-fold serial dilutions of Wild type (AM1176), *GAL-SGO1* (AM870) and *GAL-CDC55* (AM6065) cells were spotted onto medium containing glucose (YEPD) or galactose (YEPRG) and incubated at 30°C for 2 days.

Figure A.5 *ESP1* plasmid shuffle. *esp1Δ* cells carrying *ESP1* on a CEN-TRP plasmid (AM6238) were transformed with a CEN-HIS plasmid containing *ESP1* (AMp708). Colonies were replicated onto SC/-his/D medium containing 5-fluoroantranillic acid (FAA) to select for *HIS* and counterselect against *TRP*. Colonies were then replicated from FAA plates onto YEPD or SC/-trp/D. Colonies can grow on YEPD but not SC/-trp/D indicating that the *ESP1-CEN-TRP* plasmid has been lost and that the *ESP1-CEN-HIS* plasmid suffices to complement *esp1Δ*. 
Figure A.6 Random mutagenesis of ESP1. esp1Δ cells carrying ESP1 on a CEN-TRP plasmid (AM6238) were transformed with an ESP1-CEN-HIS that had been propagated through XL1-red E.coli for 50 (AMp735) or 100 (AMp740) generations (esp1*-CEN-HIS). Colonies were replicated onto FAA medium to lose ESP1-CEN-TRP plasmid and then replicated onto YEPD. White circles indicate colonies that failed to grow after losing ESP1-CEN-TRP, indicating that the mutagenized esp1*-CEN-HIS plasmid is not capable of complementing esp1Δ.
Figure A.7 Schematic of the screen for ESP1 alleles that suppress GAL-SGO1 or GAL-CDC55. GAL-SGO1 espΔ ESP1-CEN-TRP (AM6261) and GAL-CDC55 espΔ ESP1-CEN-TRP (AM6263) cells were used for the screen. Screen described in detail in the text.
Glucose

GAL-SGO1/CDC55
esp1Δ
esp1*-CEN-HIS3

Glucose

Galactose + Phloxine B

Purify colonies

Confirm growth on galactose

Recover plasmid

Retransform GAL-SGO1/CDC55 and check growth on galactose

Sequence ESP1
Figure A.8 Example candidates from ESP1 allele screen. 10-fold serial dilutions of cells spotted onto medium containing glucose (YEPD) or galactose (YEPRG). Examples of positive candidates from GAL-SGO1 screen (a1) and GAL-CDC55 screen (b1).
Figure A.9 Candidate colony had lost the \textit{GAL-SGO1} construct. An example candidate colony from the \textit{GAL-SGO1} screen \((a1)\) was crossed to wild type yeast \(\text{AM3440}\), diploids were sporulated and spores dissected and analysed for growth on YEPD + G418 (contains \textit{esp1}\textdelta{}), \text{SC/-his/D} (contains \textit{esp1-a1-CEN-HIS}) and \text{SC/-ura/D} (contains \textit{GAL-SGO1}). Note that \textit{esp1}\textdelta{} spores rely on \textit{esp1-a1-CEN-HIS} for viability.
Figure A.10 Screen for temperature sensitive ESP1 alleles. (A) Schematic of the screen procedure. Details are given in the text. (B) esp1Δ cells carrying a CEN-HIS plasmid containing either ESP1 (AM6261), esp1-V1559E (AM6599), esp1-E632G (AM6600), esp1-E715K (AM6601) or esp1-Y648C (AM6602) were streaked onto YEPD medium and incubated at either 25°C or 37°C.
A.3 Generation of catalytic-dead PP2A

To generate the *pph21-148* allele, site-directed mutagenesis was performed on plasmid-borne *PPH21* (AMp694) using primers 1143 and 1144. The *YIplac204-pph21-148* plasmid (AMp696) was linearized with *BsmI* restriction enzyme for integration by homologous recombination into the promoter region of the *PPH21* locus of a *pph21Δ* strain (AM6088). A diagram of possible homologous integration events is outlined in (Figure A.11A). To ensure transformants were chosen that contained a single copy of *pph21-148* in the genome, genomic DNA analysis by Southern blot was performed. Genomic DNA was digested with *Sphi* (S), which cut upstream and downstream of the *PPH21* locus and also within the *YIplac204* plasmid (Figure A.11A). Digested DNA was separated by agarose gel electrophoresis, blotted onto a membrane, and the membrane probed with a radiolabelled oligonucleotide designed to hybridize to the site of integration (PCR product using primers 1116 and 1197 on AMp698). For a single copy plasmid integrant, the probe was expected to hybridize to two fragments of 12.6kb and 7.8kb, whereas for transformants with more that one copy of the plasmid integrated in tandem, a fragment of 5.7kb was also expected (Figure A.11A). Lanes 8, 10 and 14 on the Southern blot show that single integrants of *pph21-148* were obtained (Figure A.11B). Similarly, a *YIplac211-pph22-156* plasmid (AMp701) was created by site-directed mutagenesis of *PPH22* (AMp699) using primers 1143 and 1144, integrated into the genome of a *pph22Δ* strain (AM5212) and a single *pph22-156* integrant confirmed by Southern blot using a PCR product from primers 1118 and 1198 on AMp699 as a probe (lane 8; Figure A.12B).
Figure A.11 Generation of the pph21-148 allele. (A) A diagram of possible homologous recombination events. Red bars indicate the extent of the probe used for Southern blot analysis. S indicates Sph1 restriction sites. Sph1 fragments expected to hybridize to the probe are bracketed and their expected sizes given. (B) Southern blot analysis of PPH21 and pph21-148 transformants together with untransformed wild type (AM1176), pph21Δ (AM6088) and pph22Δ (AM5212) controls.
Figure A.12 Generation of the *pph22-156* allele. (A) A diagram of possible homologous recombination events. Red bars indicate the extent of the probe used for Southern blot analysis. A indicates AatII restriction sites. AatII fragments expected to hybridize to the probe are bracketed and their expected sizes given. (B) Southern blot analysis of *PPH22* and *pph21-156* transformants together with untransformed wild type (AM1176), *pph21Δ* (AM6088) and *pph22Δ* (AM5212) controls.
Figure A.13 Analysis of esp1-ckd mutants. 10-fold serial dilutions of cells of the indicated genotypes were spotted onto medium containing glucose (YEPD) or galactose (YEPRG) and incubated at 30°C.
Figure A.14 Analysis of esp1-cdk mutants. 10-fold serial dilutions of cells of the indicated genotypes were spotted onto medium containing glucose (YEPD) or galactose (YEPRG) and incubated at 30°C.
Figure A.15 Analysis of esp1-ckd mutants. 10-fold serial dilutions of cells of the indicated genotypes were spotted onto medium containing glucose (YEPD) or galactose (YEPRG) and incubated at 30°C.
Figure A.16 Analysis of \textit{esp1-cdk} mutants. 10-fold serial dilutions of cells of the indicated genotypes were spotted onto medium containing glucose (YEPD) or galactose (YEPRG) and incubated at 30°C.
Figure A.17 Cdc28 tyrosine phosphorylation is not required for viability in the absence of Pds1 or in the presence of benomyl. (A) Ten-fold serial dilutions of wild type (AM1145), MET-PDS1 (AM4429), cdc28-Y19F (AM6666) and cdc28-Y19F MET-PDS1 (AM6665) cells carrying SCC1-6HA were spotted onto plates either lacking (SC/-met/D) or containing (YEPD+met) methionine and incubated at 30°C. (B) Ten-fold serial dilutions of wild type (AM1176), mad1Δ (AM2560), sgo1Δ (AM826), cdc55Δ (AM3164), rts1Δ (AM3209), mih1Δ (AM6169), swe1Δ (AM6168) and cdc28-Y19F (AM5227) cells were spotted onto YEPD medium containing benomyl at concentrations of 0 µg/ml or 15 µg/ml and incubated at 30°C.
Figure A.18 cdc55Δ mutants separate sister chromatids in the presence of stabilized Pds1. (A) Wild type (AM2812), GAL-PDS1Δdb (AM3838), cdc55Δ (AM4330), cdc55Δ GAL-PDS1Δdb (AM5335) cells carrying CEN4-GFP were grown at 30°C in YEPR, arrested in G1 with α-factor and released into YEPRG to induce GAL-PDS1Δdb plus benomyl and nocodazole to depolymerize microtubules. Samples were taken at the indicated timepoints for the percentage of cells with separated GFP foci (at least 200 cells were counted at each timepoint).
A.4 The bi-orientation defect of sgo1Δ and cdc55Δ mutants is not due to cohesin cleavage

It has recently been demonstrated that pericentromeric cohesion plays a key role in the bi-orientation process (Ng et al. 2009; Sakuno et al. 2009). I therefore considered the possibility that the bi-orientation defect of sgo1Δ and cdc55Δ mutants might be due to some small amount of premature cohesin cleavage during metaphase (i.e. not enough to trigger anaphase), possibly at the centromere and pericentromere. If this were the case, I reasoned that expression of non-cleavable cohesin should rescue the bi-orientation defect of sgo1Δ and cdc55Δ mutants. Wild type, sgo1Δ and cdc55Δ cells carrying a conditional dominant SCC1 allele with both Esp1 cleavage sites mutated (GAL-SCC1-NC) (Uhlmann et al. 1999) were released from G1 into media containing galactose to induce SCC1-NC expression and benomyl and nocodazole to depolymerise microtubules. After 3 hours, the drugs were washed out to allow the mitotic spindle to reform, however, anaphase cannot proceed because sister chromatids are linked by non-cleavable cohesin (Figure A.19A). Because the transient separation of sister centromeres upon bi-orientation does not require cohesin cleavage (He et al. 2000), I was able to score bi-orientation of chromosome IV by monitoring the separation of CEN4-GFP signals. By 75 minutes after drug washout, ~40% of wild type cells showed separated CEN4-GFP signals, representing bi-orientation (Figure A.19B). However, both sgo1Δ and cdc55Δ mutants were defective in chromosome bi-orientation, as centromere IV separation occurred in only ~20% of cells (Figure A.19B), similar to the previous experiment with wild type cohesin (Figure 4.7). I therefore conclude that sgo1Δ and cdc55Δ mutants have a similar defect in sister chromatid bi-orientation, which is not due to premature cohesin cleavage in metaphase.
Figure A.19 The bi-orientation defect of sgoΔ and cdc55Δ mutants is not due to cohesin cleavage (A) Schematic of the experiment conducted in B. Wild type (AM5368), sgoΔ (AM5451) and cdc55Δ (AM5452) cells carrying GAL-SCC1-NC and CEN4-GFP were grown at 30°C in YEPR, arrested in G1 with α-factor and released into YEPRG to induce GAL-SCC1-NC plus benomyl and nocodazole to depolymerize microtubules. Cells were then released from benomyl and nocodazole into YEPRG to re-polymerize microtubules whilst maintaining GAL-SCC1-NC expression. (B) The percentage of cells with separated GFP foci was determined at the indicated timepoints after release from benomyl and nocodazole (at least 200 cells were counted at each timepoint).
A.5 Appendix Bibliography


A.6 Publications