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GLASGOW**

Plo1p regulation of M/G1 transcription in  
*Schizosaccharomyces pombe*

A thesis submitted for the degree of  
Doctor of Philosophy at the University of Glasgow

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***Declaration***

The research reported in this work is my own work  
except where otherwise stated, and has not  
been submitted for any other degree.

*Szu Shien Ng*

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## ***Abstract***

Cell cycle events are the basis of cell duplication and division. Control of the cell cycle involves various mechanisms, which appear conserved throughout eukaryotic evolution. Among them, transcription plays a major part. Studies on gene expression in two yeast model organisms, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, identified waves of transcription through the cell cycle, which require specific transcription factor complexes binding to promoter sequences. These periodic expression patterns are clustered corresponding to the four main cell cycle transitions, suggesting that their serial regulation is interdependent.

Cytokinesis occurs at the end of the cell cycle, and includes formation of the actomyosin ring, synthesis of a membrane barrier and the eventual splitting of mother-daughter cells. In *S. pombe*, genes that are transcriptionally regulated during the M/G1 transition encode many of the components required for cytokinesis. Previously, we identified a transcription factor complex, which we named the PBF, and a promoter motif, the PCB, that together control M/G1 gene expression in *S. pombe*.

In this thesis, we provide evidence for three transcription factors, Fkh2p, Sep1p and Mbx1p, as components of PBF and describe the isolation a Fkh2p-interacting protein, which we named Lyn1p. We also show that Fkh2p and Mbx1p are both phosphoproteins and present data that a polo-like kinase, Plo1p, phosphorylates Mbx1p *in vitro*. Furthermore, we show that Plo1p controls M/G1 gene expression, and is itself regulated by the PBF-PCB complex. Recent reports identified a glycolytic enzyme, Tdh1p, as a component of the Mediator complex, which bridges the transcription activating proteins to the RNA polymerase II (RNAP II) machinery that controls RNA production. Here, we present evidence that Sep1p interacts with Tdh1p, therefore suggesting a mechanism by which PBF regulates M/G1-specific gene expression, through direct interaction with the RNAP II machinery.

In summary, we have characterised the molecular mechanism that controls M/G1 transcription in *S. pombe*. This involves Plo1p phosphorylating and controlling the PBF transcription factor complex, which in turn interacts with RNAP II machinery to control specific gene expression, and eventually cytokinesis and cell separation.

## ***Abbreviations***

A	absorbance
ACS	ARS consensus sequence
Amp <sup>R</sup>	ampicillin resistant
ARS	autonomous replication sequence
ATP	adenosine triphosphate
cDNA	complementary deoxyribonucleic acid
ChIP	Chromatin Immunoprecipitation
CIP	calf intestinal alkaline phosphatase
DAPI	4'-6-Diamidono-2-phenylindole
DPE	downstream promoter element
DTT	dithiothreitol
ECB	Early cell cycle box
ECL	Enhanced Chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EMM	Edinburgh minimal media
EMSA	electrophoretic mobility shift assay
g	gravity (relative centrifugal force; rcf)
GST	glutathione-S-transferase
GTF	general transcription factor
HA	hemagglutinin
Hepes buffer	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) buffer
HNF	hepatocyte nuclear factor
HRP	horseradish peroxidase
IgG	immunoglobulin
Inr	initiator element
IPTG	isopropyl $\beta$ -thiogalactopyranoside

Kan <sup>R</sup>	kanamycin resistant
kb	kilo basepair
kV	kilo-Volt
LB	Luria Broth
MBF	<i>MluI</i> cell cycle box binding factor
MCB	<i>MluI</i> cell cycle box
MCM	minichromosome maintenance
mcs	mitotic catastrophe suppressor
min	minute
MNE	MOPS/NaOAc/EDTA
MOPS buffer	3-(N-Morpholino)-propanesulfonic acid buffer
N <sub>6</sub>	random hexanucleotide
NIMA	never in mitosis A
nmt	no message in thiamine
NP-40	Nonidet-P40
Nrk	NIMA-related kinase
OD	optical density
ORC	Origin Replication Complex
ORF	open reading frame
PBD	polo-box domain
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
Plk	polo-like kinase
PMSF	phenylmethanesulfonylfluoride
PNK	polynucleotide kinase
pre-RC	pre-replication complex
psi	pounds per square inch

RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolution per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
SBF	Swi4 cell cycle box binding factor
SCB	Swi4 cell cycle box
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoresis
sec	second
SWI/SNF	mating-type switching/sucrose non-fermenting
TAF	TATA-binding protein-associated factor
TAP	tandem affinity purification
TBE	Tris/Borate/EDTA buffer
TBP	TATA-binding protein
TCB	TEV cleavage buffer
TE	Tris/EDTA
TEMED	N, N, N <sup>1</sup> , N <sup>1</sup> -tetramethylethylen diamine
TEV	tobacco-etch-virus
THESIT	polyoxyethylene-9-lauryl ether
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Triton X-100	octyl phenol ethoxylate or polyoxyethylene Octyl phenyl ether
U	units
UTR	untranslated region
w/v	weight for volume
YE	yeast extract
YEP	yeast extract with peptone
YPD	yeast extract with peptone and dextrose

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## *Chapter 1: Introduction*

### **1.1 Introduction**

The cell is the basic working unit of all living organisms. In a cell, instructions for functional activities are contained within a chemical code known as DNA. Typically, all living organisms are made of similar chemical and physical properties. Despite these similarities, every living system has unique traits due to the various sequential arrangements of the DNA molecules. It is the different blocks of DNA sequences that encode various gene products necessary for conducting distinct functions within the cell. Although the gene is fundamental for function it is the product, protein, which is responsible for most of the biological activities. Thus, understanding the molecular arrangements and control mechanisms of proteins form the basis towards understanding cell behavior.

Today much scientific research focuses on characterising regulatory pathways and mechanisms responsible for cell integrity, especially growth and division. Of relevance to modern medicine, defects in control of growth and division is the basis of many diseases. In such diseased cells, the normal control mechanisms for growth and division are altered. Perhaps the most striking example of this is cancer where cells share a common feature of uncontrolled and disorganized cell growth and division. In general, a normal cell divides for a certain number of times before becoming terminally differentiated, but cancer cells continue to divide and reproduce without arrest. Often, the production of cancer cells is due to changes in genes, so-called mutations. Therefore, to investigate this uncontrolled and disorganized growth and division of cells, we are particularly interested to study genes and their products that regulate the fundamental processes of cell division during the mitotic cell cycle.

In this thesis we describe experiments to better understand cell division using the classic model organism, *Schizosaccharomyces pombe*. In the introduction, we begin with a brief description of the life cycles of two distantly related yeasts, *S. pombe* and *Saccharomyces cerevisiae*, and outline the advantages of unicellular eukaryotes for experimentation. We also describe the relevance of transcription and gene expression in cell cycle controls, as it is on this area that our research focuses.

### **1.2 Life Cycles of Yeasts**

The term yeast is broadly used for unicellular fungi. They have simple nutritional needs and undergo aerobic respiration that requires carbon and nitrogen sources. The two species discussed in this thesis are *S. pombe* and *S. cerevisiae*.

*S. pombe* and *S. cerevisiae* are both ascomycete fungi characterised by the formation of ascii after sporulation. One obvious difference between the two is that *S. pombe* divides by fission while *S. cerevisiae* undergoes budding division. There have been controversies on the phylogeny of both yeasts relative to higher eukaryotes, mainly due to the use of various molecular factors as comparison. However, all phylogeneticists share a unified view that the two yeasts are highly diverged, therefore making them ideal as comparative experimental models to study eukaryotic processes (Sipiczki, 2000).

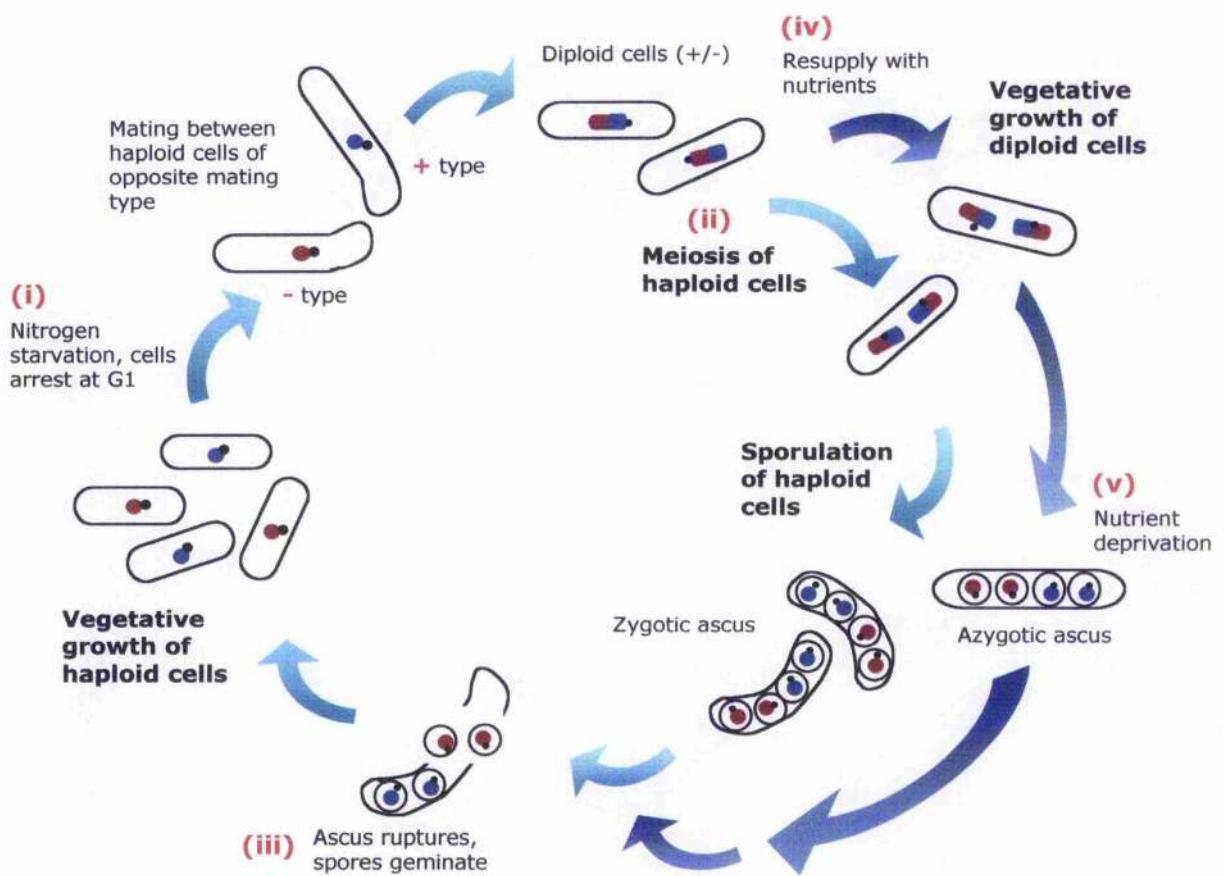
The genomes of both yeast species have been fully sequenced. *S. pombe* contains three chromosomes of 5.7, 4.7 and 3.5 Mb, which make up a 14 Mb genome (Wood *et al*, 2002). In contrast, *S. cerevisiae*'s 14 Mb genome is distributed within 16 chromosomes, which range from 230 kb to over 1 Mb in size (Goffeau *et al*, 1996).

### **1.2.1 *S. pombe***

*S. pombe* haploid cells can multiply logarithmically by mitotic division when supplied with sufficient nutrients (Leupold, 1956). The mother cell grows by tip extension and the daughter cells arise by medial fission of the mother. When nutrients are limiting and only one mating type exists, cells enter a quiescent phase. In contrast, if both mating types are present, conjugation preceding the sexual cycle is entered with cell and nuclear fusion, giving rise to diploid zygotes (Fig. 1.1). These cells immediately undergo meiosis and sporulation, to produce an ascus containing four haploid spores.

Ascospores are each enclosed in an ascus wall and these are encapsulated in a cell wall originated from the two initial haploids. Interestingly, the diploid phase is normally brief or missing. However, if diploid zygotes are transferred to nutrient-rich media before meiosis, they can enter mitosis as diploids. Alternatively, stable diploid cells can be constructed experimentally by selecting for complementation of auxotrophic markers before the zygotic cells commit to meiosis. These cells are maintained in nutrient-rich media, which inhibits meiosis and sporulation.

Diploid cells are morphologically larger as DNA and RNA content, protein and cell volume are proportional to the degree of ploidy (Ciferri *et al*, 1969; Schweizer and Halvorson, 1969; Fraser and Nurse, 1979). If diploids are nutritionally deprived, the cells enter meiosis and sporulate without requiring prior conjugation. The resulting spores are dormant, having thick walls that are resistant to heat and desiccation. However, when resupplied with nutrients they germinate to form mitotic haploids. Both haploid and diploid cells that undergo meiosis and sporulation can be morphologically distinguished. Unruptured zygotic ascii are recognized as "banana-shape", while azygotic ascii appear "rod-shape", with rounded ends.



**Figure 1.1.** Life cycles of *S. pombe*. **(i)** Under nitrogen starvation, two haploid cells of opposite mating types (+ and -) arrest in G1, can then mate to form a diploid (+/-) and multiplies by fission. **(ii)** Diploid cells are unstable and under starvation conditions, undergo meiosis forming zygotic asci containing haploid spores. **(iii)** Rupture of an ascus releases four haploid spores which then germinate into haploid cells. **(iv)** However, if diploid cells are given nutrients directly after mating, they can undergo diploid vegetative growth. **(v)** When nutrients are limiting again, these cells undergo meiosis forming an azygotic ascus, resulting in four haploid spores.

Lindner first describes *S. pombe* in 1893 (Lindner, 1893). He named the yeast after the Swahili word for beer, pombe, as it was isolated from an East African beer. Initially, the isolated haploid strain was homothallic containing both mating type, termed + and -. Subsequently, heterothallic strains with only one mating type, either + or -, were isolated and today, the standard haploid laboratory strain was derived from Leupold's 972 isolate used for his genetic analyses in the early 1950s (Leupold, 1970).

### **1.2.2 *S. cerevisiae***

The life cycles of *S. cerevisiae* are similar to *S. pombe* except that it can grow and divide in both haploid and diploid states. Most haploid laboratory strains express one of the two mating types, termed  $\alpha$  and  $\alpha$ .

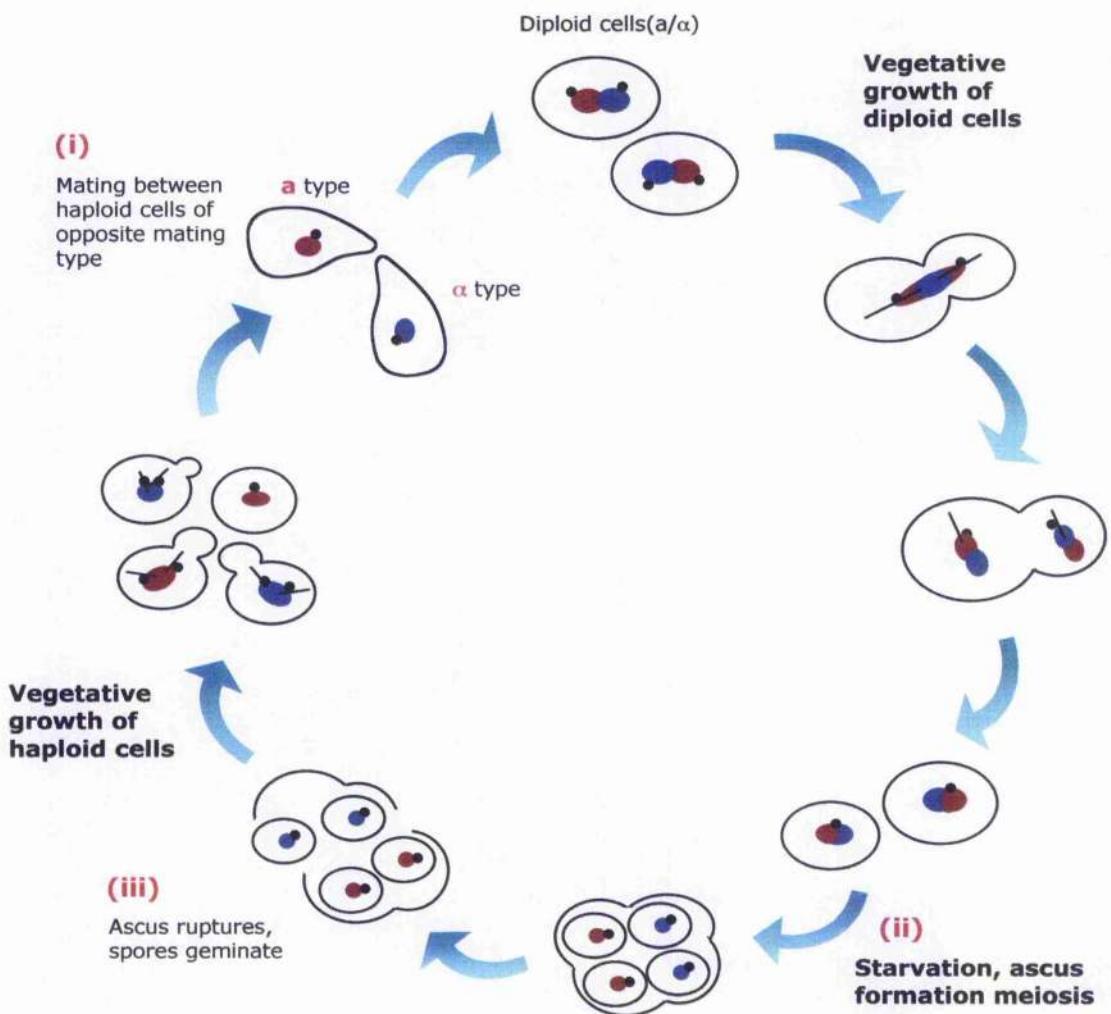
In the absence of a mating partner, haploid cells divide mitotically by budding (Fig. 1.2). However, if haploids of opposite mating types are present, they fuse to form diploid cells ( $\alpha/\alpha$ ). Unlike *S. pombe*, diploid cells of *S. cerevisiae* are stable. When starved for nutrients they undergo meiosis and sporulation to yield ascospores, enclosed by a cell wall of the original diploid cell, containing four meiotic products.

Conjugation is composed of series of events initiated by extracellular peptides, the mating pheromones, produced by each haploid cell. When haploids of opposite mating types meet, they form multicellular aggregates and arrest in the G1 phase (Pringle and Hartwell, 1981). Upon contact, pairs of  $\alpha/\alpha$  cells undergo directional cell growth, which result in cytoplasmic fusion (plasmogamy) followed by nuclear fusion (karyogamy) to form a diploid cell with single nucleus (Zaborowska et al, 1975). The resulting cells then choose to enter mitosis or meiosis depending on nutrient conditions. These ordered events are also observed in *S. pombe* (Miyata et al, 1997).

### **1.2.3 Advantage of *S. pombe* as an experimental model**

A good model organism for the analysis of eukaryotic functions should have several properties. It should have a small amount of genetic material but similar to those in higher eukaryotes, be simple for genetic manipulations and easily analysed using standard methods.

*S. pombe* and *S. cerevisiae* both fulfil these criteria as they have proven to be easy to work under laboratory conditions. Their simple life cycle with rapid growth (2 to 4 hours in *S. pombe* and 1.5 to 2 hours in *S. cerevisiae*), powerful genetics and biochemical accessibility make them ideal experimental models for eukaryotic studies, in particular cell cycle regulation.



**Figure 1.2.** Life cycles of *S. cerevisiae*. **(i)** Two haploid cells that differ in mating type (*a* and  $\alpha$ ) mate to form a diploid (*a/α*), which then multiplies by budding. **(ii)** Under starvation conditions, diploid cells undergo meiosis forming haploid spores. **(iii)** Rupture of an ascus releases four haploid spores which then germinate into haploid cells.

The biological explanations for some complex functions in higher eukaryotes can be found in lower eukaryotes. Therefore, both *S. pombe* and *S. cerevisiae* could provide a means to identify conserved key molecules and control mechanisms.

#### **1.2.4 History of *S. pombe* and *S. cerevisiae* as cell cycle organisms**

Horowitz and Leupold (1951) first suggested that the isolation of temperature sensitive mutants might provide a technique for obtaining mutations in essential genes to allow access to gene function. In 1967, Hartwell reported the isolation of temperature sensitive mutants in *S. cerevisiae*, in which 10% were cell division cycle (*cdc*) mutants (Hartwell, 1971). Temperature sensitive *cdc* mutants were defined as mutants that arrested division at a unique stage of the cell cycle, regardless of their stage at the time when they were shifted from permissive to restrictive temperatures. The crucial observation was that all cells with the same *cdc* mutation arrested division with a uniform morphology at the restrictive temperature.

Hartwell's observations revealed fundamental information about the control of the cell cycle (Hartwell et al, 1974). He proposed that late cell cycle events could not occur until after the completion of preceding events. This aided the classification of *cdc* mutants according to the stage-specific arrest phenotypes. In the 1950s, Mitchison was studying the cell cycle in *S. pombe* and devised methods to synchronise cells to permit physiological studies (Mitchison, 1957). By the 1970s, Leupold had established techniques required for genetic analyses in *S. pombe* (Leupold, 1970). Following this, Nurse replicated Hartwell's experiments in *S. pombe* and identified a number of *cdc* genes, including *cdc2* (Nurse et al, 1976).

Hartwell's work demonstrated that cells must reach a minimum size before bud formation and entry into S phase, and this event is regulated at a specific point which he named "start" (Hartwell et al, 1974). He went on to show that "start" is controlled by *CDC28*. Nurse and Bissett (1981) observed that *S. pombe* cells accumulated before "start" when nutritionally-deprived and when "start" genes are mutated, the cells showed phenotype similar to "start" mutants of *S. cerevisiae* (Nurse and Bissett, 1981). The wild type *cdc2<sup>+</sup>* gene was then isolated by complementing the temperature sensitive *cdc2-33* mutant with an *S. pombe* DNA library (Beach et al, 1982). Next they asked whether the molecular control of "start" was similar to *S. cerevisiae*. Using complementation, a gene isolated from a *S. cerevisiae* DNA library was shown to be functionally homologous to *S. pombe cdc2<sup>+</sup>*, which was identical to *S. cerevisiae CDC28*. When the same strategy was applied using a human DNA library a gene was identified, and that gene, named *CDC2*, functionally complemented *S. pombe cdc2-33* (Lee and Nurse, 1988). Further

characterisation of peptide sequences revealed high degree of homology between *S. cerevisiae* *CDC28*, *S. pombe* *cdc2<sup>+</sup>* with human *CDC2*, later shown to be cyclin-dependent kinases, CDKs. Taken together, these observations revealed that key regulators of the cell cycle are conserved among eukaryotes, and that *S. pombe* proved to be an excellent model organism for cell cycle studies.

Today, researchers have identified many similarities between *S. pombe* and higher eukaryotes in processes such as cytoskeletal organisation, cell compartmentation, transcription initiation, proteolysis, protein phosphorylation and RNA splicing (Kaufer *et al*, 1985; Fikes *et al*, 1990; Iggo *et al*, 1991; Spataro *et al*, 1997; Machesky *et al*, 1997; Hashemzadeh-Bonehi *et al*, 2003). This confirmed that complex mechanisms in higher eukaryotes could be studied in miniature systems such as *S. pombe*.

### **1.3 The Eukaryotic Cell Cycle**

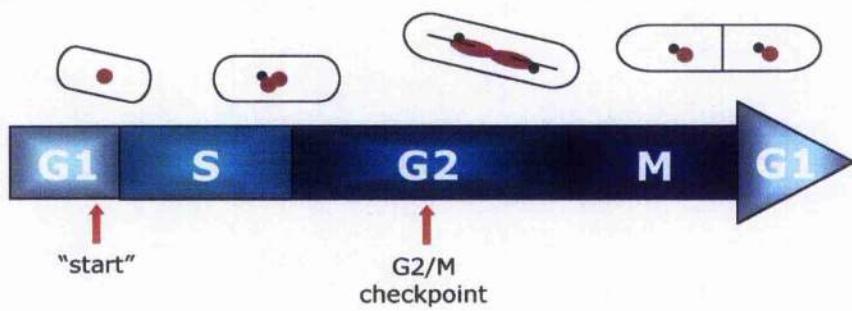
The finding of temperature sensitive *cdc* mutants in *S. cerevisiae* and *S. pombe* revolutionalised study of the cell division cycle. These mutants allowed morphological identification of genes, as mutant cells arrest at specific cell cycle stages (Lew *et al*, 1997; MacNeill and Nurse, 1997). Such *cdc* mutants in *S. cerevisiae* show various bud phenotypes, while *S. pombe cdc* mutants are elongated.

Today, we know that the cell division cycles of *S. pombe*, *S. cerevisiae* and higher eukaryotes share a universal cascade of events, each following the completion of the former (Hartwell *et al*, 1974; Nurse *et al*, 1976). Furthermore, the discovery of cyclins and their periodic regulation provides a general basis towards cell cycle control (Evans *et al*, 1983).

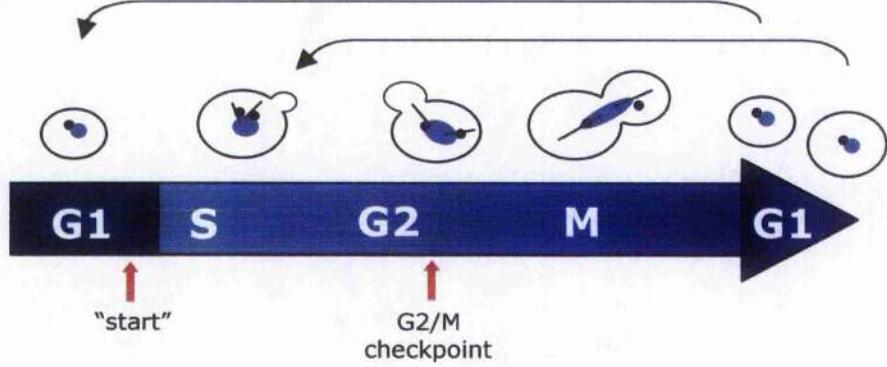
The term cell division cycle, used interchangeably with cell cycle, defines the series of events that a cell undergoes to produce a daughter cell with similar genetic content and cellular components as the parent. These events yield two daughter cells. A successful cell cycle requires properly co-ordinated order of temporal and spatial processes. Three major events describe a cell cycle: genome replication, chromosome segregation, and cell division. In all eukaryotic cells the cell cycle is composed of four discrete phases namely G1, S, G2 and M (Mitchison, 1971).

G1 is the interval or gap 1 between mitosis and DNA synthesis, characterised by RNA and protein synthesis, and when DNA is primed for replication. Towards the end of G1 there is a checkpoint known as "start" in *S. pombe* and *S. cerevisiae* and "restriction point" (R) in higher eukaryotes (Fig. 1.3) (Hartwell *et al*, 1974; Pardee, 1974). In response to extracellular mitogens, growth factors or nutrients cells pass through G1 and commit to mitosis and proceed with DNA synthesis, named S phase. However, if nutrients are limiting,

A)



B)



**Figure 1.3.** Cell cycles of *S. pombe* and *S. cerevisiae*. **A)** Haploid cells of *S. pombe* and their position relative to the various stages of the cell cycle. **B)** Haploid cells of *S. cerevisiae* and their position relative to the various stages of the cell cycle. The mitotic spindle in *S. cerevisiae* forms early in M phase and S, G2 and M phases overlap. Arrows in red indicate checkpoints.

cells can either proceed into the quiescence phase or undergo the sexual cycle. In general, the G1 phase contributes a large proportion of the cell cycle, however, *S. pombe* contains a relatively short G1 phase.

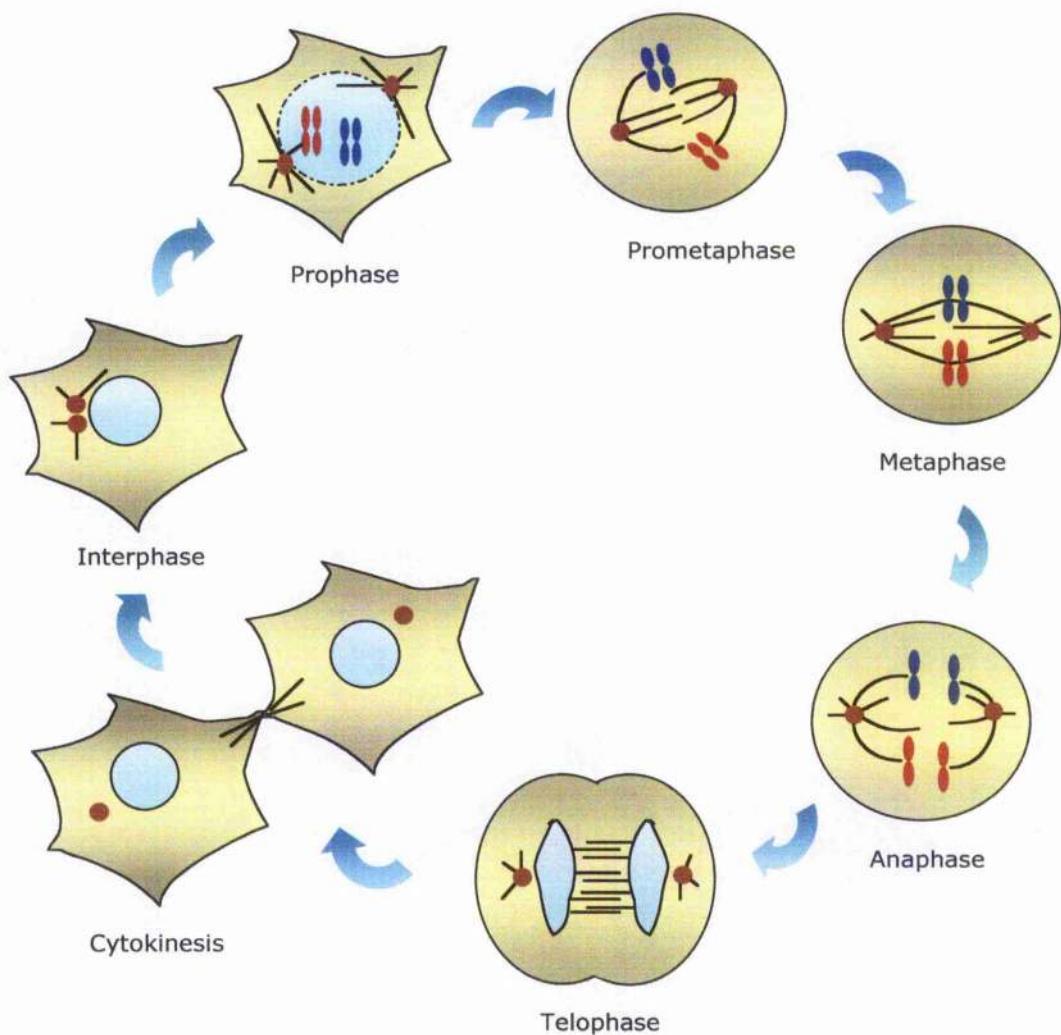
S phase begins with DNA replication and when completed cells enter a new cell cycle phase termed gap 2 (G2). G2 is the interval between the completion of DNA synthesis and M phase (mitosis). Cells continue to grow in size until the end of G2 when a second checkpoint ensures that cells have reached the minimum size required for entry into M phase.

Typical of eukaryotic cells, the start of M phase is marked by the generation of bipolar mitotic spindles and is subdivided into five stages: prophase, prometaphase, metaphase, anaphase and telophase (Fig. 1.4) (Ritzi and Knippers, 2000). Prophase begins when chromosomes condense and is characterised by thick and short threadlike replicated sister chromatids, often seen moving towards the equator along mitotic spindles. Metaphase then follows a short prometaphase and is characterised by chromosome alignment at the cell equator. During anaphase, sister centromeres divide and chromosomes separate towards opposite poles of the cell. Telophase then follows and is marked by the completion of equal chromosomal distribution in daughter nuclei. Once mitosis is completed cytokinesis occurs, characterised by physical division of the cell.

The progression from one cell cycle stage to the next consists of highly regulated events. Stage-specific key controllers are limited to the times when they are required, resulting in periodic activities throughout the cell cycle. An example of this is the cyclins (Evans *et al*, 1983). These proteins accumulate until they reach a critical level, at which point they set off a series of event ensuring a regulated transition between each cell cycle stage. Cyclins function by direct association with CDKs. Since cyclins are transcribed at specific stages of the cell cycle, their expression levels play critical roles during cell cycle progression and are rate limiting for specific processes. Cyclin genes are transcribed when they are required, and constitutive expression is often lethal. Thus, understanding the function and how cyclins are regulated is key to understanding control of the cell cycle.

#### **1.4 *Schizosaccharomyces pombe***

When nutrients are plentiful, *S. pombe* completes a cell division cycle in approximately three hours (Forsburg and Nurse, 1991). The cell cycle is unusual in that the G1 phase is short and often masked, because cells achieve the minimum size of "start" before cell division takes place. However, if the G2 phase is shortened the "start" interval becomes apparent because the cell cycle pauses till the minimum size is achieved (MacNeill and Nurse, 1997). Under



**Figure 1.4.** The five phases during mitosis (M) in animal cells. During prophase, interphase chromatin condenses into well-defined chromosomes, and duplicated centrosomes separate. Then prometaphase microtubules are captured by kinetochores. Chromosomes align at equatorial plane at metaphase. During anaphase, sister chromatids are pulled apart towards the poles. The nuclear envelope then reforms around daughter chromosomes and chromatin condensation begins at telophase. Finally, an actomyosin-based contractile ring forms and cytokinesis is completed.

optimal conditions, the time of each phase has been estimated as approximately 0.1, 0.1, 0.7 and 0.1 of the cell cycle, corresponding to G1, S, G2 and M phases (Fig. 1.3.A) (Nasmyth *et al.*, 1979).

In growing cells, G1 and S phases follow immediately after nuclear division (Fig. 1.5). At the time of cell division, the daughter cells are already at early G2. A second checkpoint (G2/M checkpoint) exists in late G2, which controls entry into mitosis (Nurse, 1975). This is dependent on three events: the cell reaching a minimum size, the completion of S phase and the repair of DNA damaged. G2 in *S. pombe* occupies a large proportion (70%) of the cell cycle unlike in *S. cerevisiae* and higher eukaryotes, which only occupy 10%. This is possibly due to the fact that *S. pombe* exists naturally as a haploid, thus cells spend most time after DNA replication with two copies of genome, facilitating DNA repair.

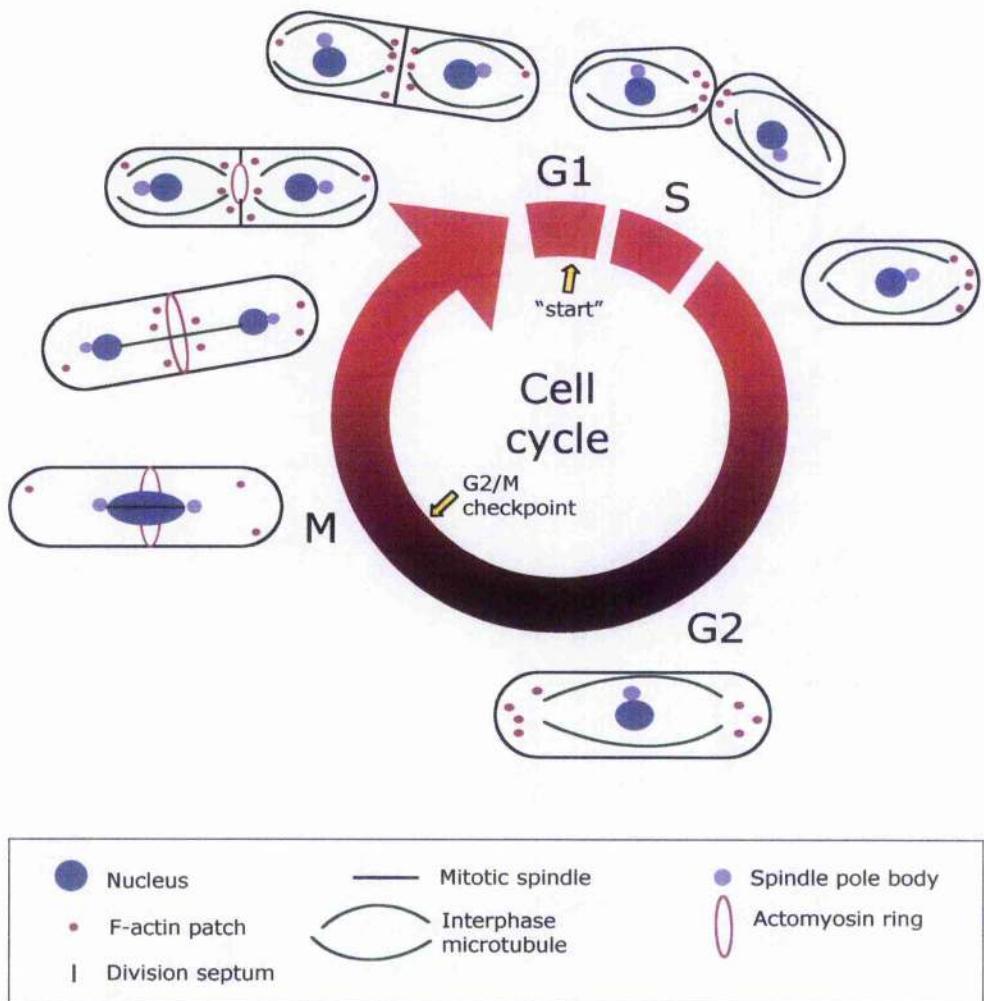
Following a lengthy G2, mitosis follows without nuclear membrane breakdown (McCully and Robinow, 1971). Entry into M phase is marked by chromosomal condensation and cytoskeletal reorganization, and is characterised by interphase cytoplasmic microtubules being replaced by intranuclear mitotic spindles (Robinow, 1977; Hagan and Hyams, 1988).

#### **1.4.1 The role of cyclin-dependent kinases (CDK) and cyclin partners**

During the cell cycle, CDKs bind to specific cyclins at distinct intervals. When they exist as a monomer, CDKs are catalytically inactive (Morgan, 1995). *cdc2<sup>+</sup>* is the only CDK essential for cell cycle progression in *S. pombe*. It encodes a 34 kD serine/threonine kinase that binds to three interphase and one mitotic cyclins: Cig1p, Cig2p, Puc1p and Cdc13p, respectively (Moser and Russell, 2000). Though *cdc2<sup>+</sup>* mRNA and protein levels remain constant through the cell cycle, its kinase activity is highly regulated and controls cell cycle progression. In contrast to CDKs, cyclins are periodically expressed and degraded, and the correct temporal association with Cdc2p is essential for maintaining cell cycle integrity.

Each of the four cyclins has specific roles. Cdc13p is the single essential cyclin of *S. pombe* and is a member of cyclin B family (Moser and Russell, 2000). Its abundance rises during G2 and falls as cells progress through mitosis and G1 (Creanor and Mitchison, 1996). Cdc13p is required for entry into mitosis and prevents reinitiation of S phase through ubiquitin-mediated proteolysis (Yamano *et al.*, 1996).

The two other B-type cyclins of *S. pombe* are Cig1p and Cig2p, which also form complexes with Cdc2p (Fisher and Nurse, 1996). However, the precise function of Cig1p is unclear (Bueno and Russell, 1993; Moser and Russell, 2000). Cig2p is the major S phase B-type cyclin. Similar to Cdc13p, it is targeted for proteolytic degradation. In addition, *cig2<sup>+</sup>*



**Figure 1.5.** Cytoskeletal rearrangements in *S. pombe*. Changes in cell size, cytoplasmic distribution of F-actin, spindle pole body (SPB) and microtubules, from birth to cell separation. Arrows in yellow indicate checkpoints for cell size controls and timing into mitosis.

expression is controlled by the MBF-MCB (*MluI* cell cycle box binding factor-*MluI* cell cycle box) transcription system, and passage into "start" and S phase are dependent on its transcript accumulation during late G1 (Yamano *et al.*, 2000; Ayte *et al.*, 2001).

Another G1 cyclin in *S. pombe* is Puc1p. It was identified through complementation of *S. cerevisiae* G1 cyclins, *cln* mutants (Forsburg and Nurse, 1991). Sequence analysis of Puc1p identified a high degree of homology to Clnps but not to B-type cyclins. Similar to Cig1p, the role of Puc1p is not clear.

#### **1.4.2 G1/S control**

Before S phase, cells pass through "start" and this is the point when they become committed and initiate the mitotic cell cycle. Therefore, the molecular mechanisms of "start" are crucial for cell cycle progression and will be discussed, beginning with transcriptional control, followed by CDK and Rum1p activities.

##### **1.4.2.1 Transcriptional activity**

Cell cycle-regulated transcription has been shown as a major form of control during cell cycle progression (Struhl, 1989). In *S. pombe*, a large number of genes have been identified whose mRNA accumulates during late G1. Many of these encode functions required for the onset of and progression through S phase. They share a common upstream-activating sequence (UAS) known as an MCB in their promoter region (McIntosh, 1993). MCB elements are necessary for periodic expression and are recognized by a DNA-binding factor named MBF (Lowndes *et al.*, 1992). A similar MBF-MCB system controls the expression of a group of genes at the start of S phase in *S. cerevisiae* (Dirick *et al.*, 1992).

In *S. pombe* at least eleven genes have been identified that are expressed at G1/S. These include *cdc22*<sup>+</sup>, encoding the large subunit of ribonucleotide reductase, *cdc18*<sup>+</sup>, required for DNA replication initiation, *cig2*<sup>+</sup>, encoding B-type cyclin, *cdt1*<sup>+</sup>, essential for DNA synthesis and the "start" control, and *cdt2*<sup>+</sup>, required for DNA replication (McInerny, 2004).

Four proteins have been identified as components of MBF in *S. pombe* and these include *cdc10*<sup>+</sup>, *res1*<sup>+</sup>, *res2*<sup>+</sup> and *rep2*<sup>+</sup> (Lowndes *et al.*, 1992; Miyamoto *et al.*, 1994; Sugiyama *et al.*, 1994; Nakashima *et al.*, 1995).

*S. pombe* possesses two functionally redundant MBF complexes, Res1p-Cdc10p and Res2p-Cdc10p-Rep2p, with Rep2p acting as a coactivator that drives S phase progression. No coactivator subunits have been identified for the Res1p-Cdc10p complex. It is believed that Res1p-Cdc10p has a predominant role in mitosis, whereas Res2p-Cdc10p-Rep2p is more important in meiosis. Resp subunits are all required for correct G1/S transcription (Reymond *et al.*, 1993; Baum *et al.*, 1997).

#### **1.4.2.2 CDK activity**

In *S. pombe* the role of the CDK, Cdc2p, at "start" has long been studied (Nurse and Bissett, 1981). However, the precise molecular control mechanisms are still incomplete. At "start" Cdc2p is predominantly associated with Cig2p to promote the G1/S transition (Fisher and Nurse, 1996; Moser and Russell, 2000).

Some *cdc2* mutants arrest in late G1 contained a reduced MCB activity (Reymond *et al*, 1993). There is also evidence that a Cdc2p kinase complex phosphorylates Cdc10p, to induce formation of the Res1p-Cdc10p complex (Connolly *et al*, 1997). However, these results are controversial as the timing of the phosphorylation activity corresponded with the inactivated state of MBF-MCB transcription (Baum *et al*, 1997). Nevertheless, since the level of *cig2<sup>+</sup>* mRNA is itself regulated by the MBF-MCB complex, it is possible that Cig2p maintains G1/S gene expression through an autofeedback regulation (Ayte *et al*, 2001). But whether this control mechanism is coupled to the Res-Cdc10/Rep complex is still unclear.

Other work has identified a novel CDK-G1 cyclin-like complex named Pef1p-Pas1p. Pas1p is an orthologue of Pcl-like family found in *S. cerevisiae* (Tanaka and Okayama, 2000). Despite the structural homology to Pcl-like proteins, Pas1p shares functional homology to Cln1p/2p/3p of *S. cerevisiae* and promotes "start" by activating Res2p-Cdc10p complex, in combination with Pef1p.

The inactivation of CDKs requires two mechanisms: enzymatic proteolysis through the degradation of B-type cyclins, and direct inhibition by the cyclin-dependent kinase inhibitor (CKI), Rumi1p (Correa-Bordes *et al*, 1997; Kitamura *et al*, 1998; Benito *et al*, 1998).

The ubiquitin-dependent proteolysis, catalysed by the proteosome, of cell cycle proteins have been studied in both yeasts and humans (Irniger *et al*, 1995; King *et al*, 1995; Yu *et al*, 1998; Yamano *et al*, 2000). Ubiquitin, a 76-residue ubiquitous protein acts as a recognition tag for proteosome-dependent proteolysis (Hershko, 1997). Ubiquitin is transferred to its substrate in a multiubiquitinated form through a series of enzymatic reaction comprising E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin ligase). Mitotic cyclins containing "destruction" boxes were the first substrates identified for the anaphase promoting complex/Cyclosome (APC/C) proteolysis (Glotzer *et al*, 1991; King *et al*, 1996).

During early G1 in *S. pombe*, Ste9p associates with the APC/C, therefore promoting the ubiquitination of Cig1p and Cdc13p (Kitamura *et al*, 1998; Yamaguchi *et al*, 2000). In G1 arrested cells, under nutrient-deprived conditions, Ste9p is seen as essential for the degradation of Cig1p and Cdc13p, possibly to allow sexual differentiation (Kominami *et al*, 1998). When Cdc2p activity is low, Ste9p is dephosphorylated resulting in Cig1p and Cdc13p degradation through the APC/C system (Kitamura *et al*, 1998). However, under normal

condition, Ste9p does not appear to have a role for Cig1p and Cdc13p degradation (Blanco et al, 2000).

#### **1.4.2.3 *rum1<sup>+</sup>* mRNA and Rum1p activity**

The single CKI in *S. pombe*, Rum1p, is controlled at the level of mRNA and protein, and simultaneous stabilisation of both products is lethal to cells (Daga et al, 2003). Rum1p has three functions during G1.

The first function of Rum1p is to prevent mitosis occurring in G1. This is observed in *rum1A* cells when S phase is blocked, which attempt to enter mitosis (Moreno and Nurse, 1994; Correa-Bordes and Nurse, 1995; Jallepalli and Kelly, 1996). Furthermore, high levels of Rum1p initiate re-replication of DNA without undergoing mitosis. Second, in response to mating and conjugation factors, Rum1p becomes critical whereby it inhibits Cdc2p-Cig2p activation and cells are arrested in G1. The third function is to determine the timing of "start" through direct inhibition of Cdc2p-Cig2p during G1. When this occurs, cells arrest until they achieve the minimal mass required for S phase entry (Martin-Castellanos et al, 2000). Therefore the correct balance and degradation of CKI is important for a precise co-ordinated entry from one cell cycle phase to the other.

Two pathways have been proposed that control *rum1<sup>+</sup>* mRNA and Rum1p degradation. These mechanisms can be divided into transcriptional and translational levels and both *rum1<sup>+</sup>* mRNA and protein levels demonstrate periodic fluctuation during the cell cycle, both peaking in late M/G1 (Moreno and Nurse, 1994; Benito et al, 1998).

#### **1.4.3 S phase control**

The replication of chromosomal DNA in eukaryotes is initiated at specific regions known as autonomous replication sequence (ARS) elements. ARS elements were first identified for their ability to promote high frequency transformation in *S. cerevisiae* (Hsiao and Carbon, 1979).

The *S. cerevisiae* ARSs are composed of adenine-thymine (A-T) rich consensus sequence of 12 bp in length. These short sequences are distributed over a region of 100-200 bp. However, subsequent DNA sequence comparisons of ARS-containing fragments led to two generalisations. First, not all DNA fragments with ARS functions contain copies of consensus sequence, but they all contain A-T rich regions (Kelly and Brown, 2000). Second, short DNA fragments with ARS activity have a higher A-T content (73-82%) than any coding regions in genomic DNA (60%). In general, ARS elements bind initiator proteins that promote the assembly of initiation replication complex. These steps include the local

unwinding of the DNA duplex and the recruitment of additional components to the replication machinery.

Similar to higher eukaryotes, DNA replication in *S. pombe* occurs from multiple ARS elements consisting of discrete tracts of A-T rich residues spanning between 0.5 - 1 kb (Okuno *et al*, 1999; Takahashi *et al*, 2003). Furthermore, ARS elements in *S. pombe* and most eukaryotes do not contain the 12 bp consensus sequence identified in *S. cerevisiae*. However, the mechanisms and factors involved in replication initiation appear to be highly conserved (Cvetic and Walter, 2005).

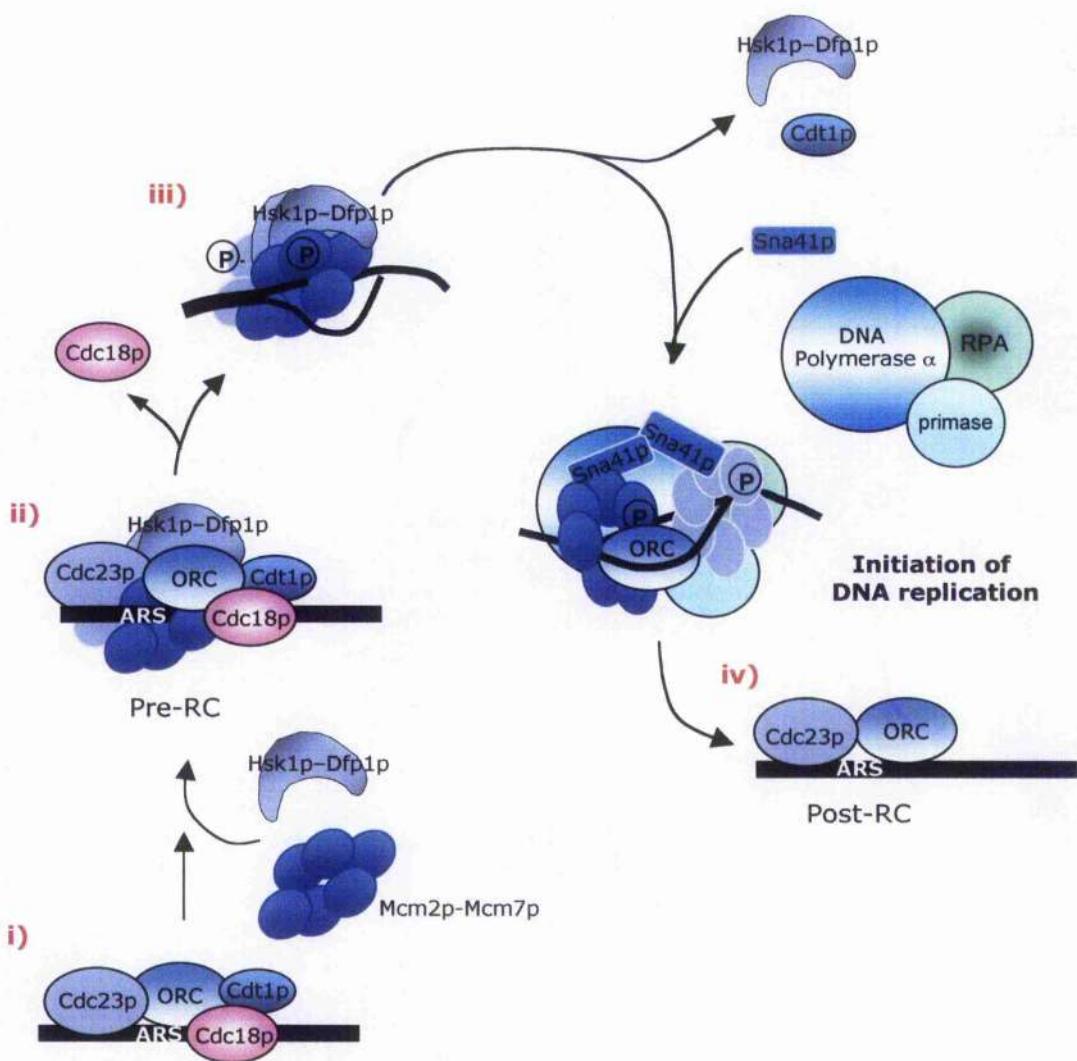
Three features form the basis of ARS elements. First, they must be discretely located at intervals to allow timely completion of DNA replication. Second, initiation events are confined to the parent template such that replication occurs only once per cell cycle. Third, DNA replication initiation must undergo timely co-ordination to ensure that replication is completed within a certain time frame.

The flanking sequences of ARS elements are recognised by the Origin Recognition Complex (ORC). Initiation of DNA replication is a multistep process and begins with the binding of the ORC to the recognition sites. In *S. pombe* the ORC remains associated to the origins throughout the cell cycle (Chuang *et al*, 2002). Unlike other organisms, the specificity of ORC-DNA is unique to *S. pombe*, where only a single subunit, Orc4p, shows direct contact with DNA (Chuang and Kelly, 1999). Orc4p contains an N-terminal domain consisting of nine A-T hook motifs. This site binds A-T tracts and serves to tether the ORC complex to ARS elements.

#### ***1.4.3.1 Assembly of initiation complexes at replication origins***

Once the ORC is bound to an ARS element, it serves as a platform for the assembly of the pre-replicative complex (pre-RC), during late M and early G1 phases (Fig. 1.6) (Nishitani *et al*, 2000). The components of pre-RC include ORC subunits, Cdc18p, Cdt1p, and the hexameric minichromosome maintenance (MCM) complex (Mcm2p-Mcm7p).

Initially, the ORC interacts with Cdc18p to result in a change of conformation followed by the association to ARS element (Chuang *et al*, 2002). Cdc18p then interacts directly with Cdt1p and the co-operative activities are essential for the loading of the MCM helicase complex, thereby completing the pre-RC assembly (Nishitani *et al*, 2000). The assembly of pre-RC to ARS elements in *S. pombe* is an ordered process dependent on ATPase activity (Takahashi *et al*, 2003).



**Figure 1.6.** Assembly of pre-replication complex (pre-RC) in *S. pombe*. **(i-ii)** During assembly the Origin Replication Complex (ORC) binds to autonomous replication sequence (ARS) elements, which serve as a platform for the recruitment of other protein factors to form pre-RC during G1 phase. **(iii)** During the G1/S interval, Hsk1p-Dfp1p activates pre-RC resulting in a replication fork. **(iv)** The degradation of Cdc18p and Cdt1p inhibits further re-replication within one cell cycle.

#### **1.4.3.1.1 *Cdc18p***

*Cdc18p* of *S. pombe* is the functional homologue of *Cdc6p* in *S. cerevisiae* (Sanchez *et al.*, 1999). Both products are required during the initiation of DNA synthesis and form a component of the pre-RC (Nishitani and Nurse, 1997). *S. pombe* *Cdc18p* possesses a nucleotide binding site and is a member of the large AAA<sup>+</sup> family of ATPases involved in ATP-dependent protein complex assembly, similar to ORC and MCM proteins (Liu *et al.*, 2000).

The expression of *cdc18<sup>+</sup>* mRNA is controlled by MBF and is expressed during the G1/S interval (Nishitani and Nurse, 1997). In addition, its protein levels vary periodically and are regulated at the level of protein degradation by a mechanism that depends on CDK activity (Kelly *et al.*, 1993; Jallepalli and Kelly, 1996; Baum *et al.*, 1998). *Cdc18p* becomes hyperphosphorylated as cells progress from G1 to S and is dependent on CDK-cyclin activity. Mutants of *cdc18* that do not interact with CDK cannot rescue the viability in cells depleted of *Cdc18p*. Furthermore, mutation in any of the putative CDK phosphorylation sites found on *Cdc18p* abolishes CDK-dependent phosphorylation both *in vitro* and *in vivo*. This suggests that CDK-dependent phosphorylation functions to prevent inappropriate DNA replication.

#### **1.4.3.1.2 *Cdt1p***

Similar to *Cdc18p*, *cdt1<sup>+</sup>* mRNA and protein levels fluctuate as cells progress through the cell cycle (Nishitani *et al.*, 2000). *cdt1<sup>+</sup>* transcription is regulated by MBF, peaking at the G1/S interval.

*Cdt1p* binds directly to *Cdc18p* and together they recruit MCM proteins, consistent with the proposal that *Cdt1p* is required for pre-RC assembly. Upon DNA replication initiation, *Cdt1p* is released from the chromatin and degraded, possibly in a CDK-dependent manner. Down regulation of *Cdt1p* is required to inhibit the recruitment of pre-RC factors, thereby preventing re-replication (Yanow *et al.*, 2001).

#### **1.4.3.1.3 Minichromosome maintenance (MCM) proteins**

The *MCM* genes, members of a family of six related genes *MCM2-MCM7*, were initially identified in *S. cerevisiae* from mutants that were unable to maintain plasmids (Tye and Sawyer, 2000). Purification of the MCM complex in *S. pombe* demonstrated that it contains six heterosubunits similar to *S. cerevisiae*: *Mcm2p/Cdc19p/Nda1p*, *Mcm3p*, *Mcm4/Cdc21p*, *Mcm5p/Nda4p*, *Mcm6p/Mls5p* and *Mcm7p* (Coxon *et al.*, 1992; Miyake *et al.*, 1993; Forsburg and Nurse, 1994).

In *S. pombe*, each of these subunit shares a common central region containing an ATPase motif essential for activity, thought to bind or modify chromatin to allow DNA

replication. However, only the trimeric complex composed of Mcm4p/Cdc21p, Mcm6p/Mis5p and Mcm7p possesses ATP-dependent helicase activity required to unwind the parental DNA strand during replication (Lee and Hurwitz, 2001). Similar to other eukaryotes, the six MCM proteins are expressed constitutively and localise to the nucleus throughout the cell cycle (Forsburg and Nurse, 1994).

Together, these observations support the hypothesis that the MCM complex functions as the replicative helicase during DNA elongation.

#### **1.4.3.2 Activation of initiation complex**

The initiation of DNA replication requires the formation of pre-RCs at ARS elements (Kelly and Brown, 2000). The co-operative recruitment of pre-RCs requires chromatin association of ORC and MCM proteins, and this is dependent on Cdc18p and Cdt1p, possibly through the phosphorylation of Mcm4p/Cdc21p (Nishitani *et al*, 2000). However, formation of the pre-RC is separated from DNA replication activation to prevent re-replication within one cell cycle (Diffley, 2001). Its activation requires at least two protein kinases; Hsk1p also known as DDK (Dfp1p-dependent kinase and a homologue of *S. cerevisiae* Cdc7p), and Cdc2p (CDK), which drive the onset of S phase (Kelly and Brown, 2000; Bell and Dutta, 2002).

Both of these kinases are heterodimers, consisting of catalytic and regulatory subunits. The catalytic subunits are present throughout the cell cycle, but the regulatory subunits demonstrate periodic expression (Kelly and Brown, 2000; Yamano *et al*, 2000). Therefore, the association of Hsk1p-cyclin and Cdc2p-cyclin are central to controlling the timing of S phase.

#### **1.4.3.2.1 CDK phosphorylation**

It is now known that active CDK-cyclins are essential for the initiation of DNA replication. In *S. pombe* Cdc2p associates with Cig2p and plays a major role in promoting the initiation of DNA replication. This is observed through the levels of Cig2p and Cdc2p-Cig2p kinase activities, which increase during G1/S and peak during S phase.

One target of Cdc2p-Cig2p is Drc1p (Slc2p in *S. cerevisiae*) (Noguchi *et al*, 2002). Drc1p is essential for DNA replication and interacts with components that function after pre-RC assembly. Mutation of the potential CDK sites in Drc1p reduces CDK phosphorylation resulting in protein inactivation. Whether or not the phosphorylation of Drc1p is essential for DNA replication has yet to be established.

#### **1.4.3.2.2 DDK phosphorylation**

Hsk1p DDK is a member of the Cdc7p family of protein kinases required for initiation of DNA replication in eukaryotes (Masai *et al*, 1995). It is activated when associated with Dfp1p, the regulatory subunit, which targets the kinase to specific substrates (Kelly and Brown, 2000). Dfp1p is regulated both at the level of transcription and protein degradation. The protein is absent in G1 but levels increase as cells traverse through G1/S, coincident with the onset of DNA synthesis, and persist until late M phase. Some evidence supports the idea that Hsk1p-Dfp1p phosphorylates the MCM complex through the Mcm2p subunit, as observed with human Cdc7p.

#### **1.4.3.2.3 DNA polymerase complex**

Chromosomal DNA replication requires three DNA polymerases, named Pol  $\alpha$ , Pol  $\delta$  and Pol  $\epsilon$ . Pol  $\alpha$  is required for replication initiation while Pol  $\delta$  and Pol  $\epsilon$  are both involved in elongation processes (Stillman, 1994). The catalytic subunit of Pol  $\alpha$  is Pol1p/Swi7p/Cdc29p and is required for DNA replication and mating-type switching (Singh and Klar, 1993).

A general property of Pol  $\alpha$  from studies in viral DNA and in human cells suggests that *S. pombe* Pol  $\alpha$ , together with a RNA polymerase complex named primase, forms a short RNA-DNA primer at the replication fork (Bullock, 1997). The helicase activity of MCM proteins, separates the DNA into single-stranded templates, which then recruit replication protein A (RPA). RPA maintains the template in a conformation optimal for polymerisation. Elongation then follows, aided by Pol  $\delta$  and Pol  $\epsilon$ . The DNA polymerase-primase-RPA complex then forms a higher ordered complex with replication factor C (Rfc) and proliferating cell nuclear antigen (PCNA). Primase-Pol  $\alpha$  is then removed from the complex and elongation continues.

#### **1.4.3.2.4 Other protein activators**

*S. pombe* Sna41p (*S. cerevisiae* Cdc45p) interacts with the MCM complex and is required to recruit Pol  $\alpha$  to the replication fork (Uchiyama *et al*, 2001). A number of other proteins required for initiation of DNA synthesis include Cdc23p (*S. cerevisiae* Dna43p/Mcm10p), Sld3p, Rad4p/Cut5p and Drc1p (Bell and Dutta, 2002). The precise role of Cdc23p during DNA replication is unclear. However, there is evidence that it interacts with Mcm2p/Cdc19p/Nda1p, Mcm4p/Cdc21p, Mcm5p/Nda1p and Mcm6p/Mls5p and several of ORC subunits; Orc1p, Orc2p, Orc5p and Orc6p (Hart *et al*, 2002).

#### **1.4.4 G2/M control**

When cells pass "start" Cdc2p associates with the Cdc13p cyclin and the complex remains inactive by phosphorylation on tyrosine-15 (Tyr-15) of Cdc2p (Gould, 2003). The phosphorylation activity oscillates throughout the cell cycle beginning in S phase and peaks at late G2, just before the initiation of mitosis (Atherton-Fessler, 1993). At the end of G2 when the cell size requirement is reached, M phase occurs.

In *S. pombe*, the level of Cdc2p-Cdc13p activity is essential in promoting M phase entry. Activity is kept low by two control mechanisms: activation of phosphatases, Cdc25p and Pyp3p, and inactivation of two kinases, Wee1p and Mik1p. Thus, Cdc25p, Pyp3p and Wee1p, Mik1p act in opposition to the reversible Tyr-15 residue of Cdc2p (Russell and Nurse, 1987; Lundgren *et al*, 1991; Parker *et al*, 1991).

The activation of Cdc2p-Cdc13p to allow entry into M phase requires the dephosphorylation of Tyr-15 on Cdc2p by Cdc25p. Cdc25p is a universal regulator of Cdc2p, with functional homologues identified in *S. cerevisiae* (Mih1p), humans (Cdc25), *Drosophila melanogaster* (string, twine), *Xenopus laevis*, mouse and *Aspergillus nidulans* (nimT) (Russell *et al*, 1989; Kakizuka *et al*, 1992; Alphey *et al*, 1992; O'Connell *et al*, 1992; Okazaki *et al*, 1996; Mata *et al*, 2000; Lemaire *et al*, 2004).

##### **1.4.4.1 *cdc25<sup>+</sup>* mRNA, Cdc25p and Pyp3p**

The levels of *cdc25<sup>+</sup>* mRNA and Cdc25p vary throughout the cell cycle, both peaking at G2/M. Evidence suggests that besides inhibition at the level of transcription, Cdc25p is also translationally regulated under the control of a translation factor eIF4A, *tif1<sup>+</sup>* (Daga and Jimenez, 1999).

##### **1.4.4.2 Wee1p, mik1<sup>+</sup> mRNA and Mik1p**

Wee1p directly inhibits the Cdc2p-Cdc13p complex by phosphorylating Cdc2p on Tyr-15 (Parker *et al*, 1991). *wee1<sup>+</sup>* was identified as a dose-dependent inhibitor of mitosis (Fantes, 1983; Russell and Nurse, 1987). In the absence of functional Wee1p, cells enter mitosis prematurely, while overexpression of *wee1<sup>+</sup>* leads to G2 delay and cell elongation. Genetic analysis in *S. pombe* identified Cdr1p/Nim1p as a regulator of Wee1p (Atherton-Fessler *et al*, 1993). Furthermore, phosphorylation by Cdr1p/Nim1p inhibits the activity of Wee1p, thereby inducing mitotic entry.

Another mitotic regulator is the Mik1p kinase. Initially, Wee1p and Mik1p were thought to be redundant because the *wee1Δmik1Δ* double mutant results in a lethal mitotic catastrophic phenotype, while the single *mik1Δ* mutant is non-essential for viability.

However, subsequent analyses identified independent roles for each protein. While *wee1*<sup>+</sup> mRNA and protein levels remain constant throughout the cell cycle, *mik1*<sup>+</sup> mRNA and Mik1p oscillate and increase during S phase (Christensen *et al*, 2000; Ng *et al*, 2001). It was proposed that Mik1p functions to block premature entry into mitosis by maintaining low levels of Cdc2p-Cdc13p activity during S phase. When S phase is completed, Mik1p undergoes proteolytic degradation and Wee1p functions to regulate the activity of the Cdc2p-Cdc13p complex (Baber-Furnari *et al*, 2000).

#### **1.4.5 M phase control**

In a typical mitotic cell cycle, M phase is composed of mitosis (chromosome segregation and nuclear division) and cytokinesis. Mitosis is initiated by spindle pole body (SPB) duplication and separation, which functions as microtubule organising centre during late G2, when rapid chromosome condensation and microtubular reorganization occurs (Robinow, 1977; Hagan and Hyams, 1988). The newly formed mitotic spindle separates sister chromatids into two new cells.

Sister chromatid separation during M phase requires successful DNA replication, sister chromatid cohesion and centrosome duplication. Therefore, the timing and order of changes in chromosomal morphology and topology must be tightly controlled to coincide with cytoskeletal changes and progression of genome distribution. The three transitions of chromosomal (sister chromatids) morphology include cohesion, condensation and separation (Takahashi and Yanagida, 2003). Chromosome cohesion occurs from S to M phases, ensuring that sister chromatids are properly attached to centromere on spindles before anaphase. Chromosome condensation initiates the onset of M phase, a prerequisite for mitotic spindles to hold sister chromatids effectively and separate them appropriately. Cohesion between sister chromatids is then dissolved and chromosome separation proceeds during the onset of anaphase, to ensure that each set of sister chromatids is properly distributed to the opposite poles.

The regulation of M phase entry and progression relies mainly on two interdependent control mechanisms: phosphorylation and proteolysis (Moser and Russell, 2000). In *S. pombe*, the most prominent mitotic kinase is Cdc2p. However, much evidence suggests roles for additional mitotic kinases including members of Polo, aurora and never-in-mitosis A (NIMA) families.

##### **1.4.5.1 CDK, Cdc2p**

Activation of the mitotic Cdc2p-Cdc13p complex depended on three residues: threonine-14 (Thr-14), Tyr-15 and Thr-167 (Gould, 2003). The phosphorylation of Cdc2p on all residues

occurs during G2/M when the activity of the Cdc25p phosphatase exceeds the opposing kinases, Wee1p and Mik1p. However, only low levels of Tyr-14 Cdc2p have been detected when *wee1*<sup>+</sup> is overexpressed (Den Haese et al, 1995). Furthermore, Tyr-14 phosphorylation has not been detected when *mik1*<sup>+</sup> is overexpressed, presumably having a cryptic role during the cell cycle (MacNeill and Nurse, 1993).

Cdc2p Thr-167 is likely to be phosphorylated by the CDK-activating kinase (CAK), Mcs6p-Mcs2p, similar to vertebrate cells, although no formal proof has been provided (Molz et al, 1989; Gould, 2003). Mcs6p kinase and its cyclin partner Mcs2p are essential proteins with mutant cells arresting with similar phenotypes. Roles for Mcs6p/Mcs2p in phosphorylating the C-terminal domain of RNA polymerase II (RNAP II-CTD) were identified, suggesting a link between the activity of CDK and RNAP II machinery (Lee et al, 2005).

#### **1.4.5.2 Polo-like kinase, *Plo1p***

In human cells, when Cdc2-Cyclin B controls mitotic entry, further activation of Cdc25 through feedback regulation amplifies mitotic entry (Hoffmann et al, 1993). In addition, polo-like kinases (Plks) are thought to act in this feedback regulation as *X. laevis* Plk, Plx, was purified as a Cdc25-activating kinase (Kumagai and Dunphy, 1996).

In *S. pombe*, several lines of evidence suggest that the association of Plk, Plo1p, to the SPB plays a role in mitotic entry through Cdc2p-Cdc13p (Grallert and Hagan, 2002). Although Plo1p can regulate SPBs separation, evidence is emerging for its control on other mitotic events such as ring constriction and mitotic spindle formation, cytokinesis and gene expression (Bahler et al, 1998; Anderson et al, 2002; Reynolds and Ohkura, 2003).

The fact that Mid1p, an early component of the actomyosin ring, interacts with Plo1p, and the inability of Mid1p to leave the nucleus in *plo1* mutant cells, suggests that Plo1p might phosphorylate Mid1p or substrates that in return regulate the ring formation. Consistent with this suggestion, some recessive mutants of *plo1* do not form the constriction ring and others form highly disorganized and misplaced rings (Ohkura et al, 1995, Anderson et al, 2002). In addition, the constriction of the actomyosin ring is co-ordinated with septum formation at the end of anaphase, and certain *plo1* mutants do not form septa, while *plo1*<sup>+</sup> overexpression in interphase cells drives inappropriate septation (Ohkura et al, 1995).

Therefore, Plo1p appears to act as a key kinase that contributes to both nuclear and cell division.

#### **1.4.5.3 Never-in-Mitosis A (NIMA)-related kinase, Fin1p**

A mitotic kinase family termed NIMA-related kinase (Nrk) was identified in *A. nidulans* by mutation. These mutants were "never in mitosis" due to an interphase arrest (Morris, 1976).

Subsequent work identified sequence homologues in *S. pombe* and *S. cerevisiae*, *fin1<sup>+</sup>* and *kin3<sup>+</sup>*, respectively. NIMA activity in *A. nidulans* is tightly cell cycle regulated by phosphorylation and proteolysis and at the levels of transcription and post-translation during mitotic entry (Pu and Osmani, 1995).

*S. pombe* Fin1p shares many functional similarities to NIMA of *A. nidulans*. Its overexpression induces a Cdc2p-dependent premature condensation of chromosomes (Krien et al, 1998). Furthermore, Fin1p activity is cell cycle regulated and contains a PEST (proline, glutamine, serine and threonine-rich regions) sequence, potentially required for proteolytic degradation (Krien et al, 2002). *fin1<sup>+</sup>* expression is under the control of the PBF (Pombe cell cycle-box binding factor) transcription factor complex that binds to a specific promoter sequence known as pombe cell cycle box (PCB) (Anderson et al, 2002). Fin1p is low during interphase and localised to the SPBs throughout mitosis, with a peak of activity similar to its abundance levels.

Mutant *fin1* cells exhibit monopolar spindles, while overexpression of *fin1<sup>+</sup>* results in chromosome condensation in interphase cells. Cells lacking *fin1* exhibit a cell cycle delay in G2 and the phenotypes of several *fin1* mutants suggest that it is either directly or indirectly regulating the association of Plo1p with SPBs.

#### **1.4.5.4 Aurora-related kinase, Ark1p**

Aurora related kinases (Arks) regulate several mitotic events including spindle formation and constriction of the cytokinetic ring. Metazoans contain three distinct Arks, namely aurora-A, aurora-B and aurora-C (Giet and Prigent, 1999; Bischoff and Plowman, 1999; Kimura et al, 1999). Each controls separate events during mitosis. For example, aurora-A regulates centrosomal separation and spindle formation, and aurora-B controls chromosome condensation and cytokinesis. Consistent with the proposed roles, mutation in *D. melanogaster* aurora-A fails to form bipolar mitotic spindles while aurora-B mutant cells loose their ability to associate with the chromatin and the spindle mid-zone, resulting in the loss of chromosome condensation and an inorganise central spindle during cytokinesis (Glover et al, 1995; Giet and Glover, 2001). Therefore, the general function of Arks involves co-ordinating chromosome segregation with cell division.

*S. pombe* contains a single Ark termed Ark1p, which controls a combination of functions regulated by aurora-A and aurora-B in higher systems, demonstrated by the phenotypes of *ark1Δ* (Petersen et al, 2001). Although Ark1p levels remain constant during the cell cycle, its cellular localisation varies between nucleoplasm, chromatin and kinetochores.

Unlike Plo1p and Fin1p, Ark1p does not form a complex with the SPBs (Petersen et al, 2001). However, Ark1p can phosphorylate serine-10 of histone H3 similarly to higher eukaryotes, possibly required for chromosome condensation linked to cytokinesis events.

#### ***1.4.5.5 Anaphase-promoting complex/Cyclosome (APC/C)***

In *S. pombe*, like other eukaryotes, several forms of control mechanisms have been implicated for chromosome segregation including the APC/C, the securin-separase and the cohesion and condensin complexes. At least 7 subunits of the APC/C have been characterised and shown to form a 20S APC/C (Takahashi and Yanagida, 2003). The function of APC/C is to recognise and tag target substrates with multiple ubiquitin residues, which in return are localised to and degraded by the 26S proteasome.

At the metaphase-anaphase transition APC/C activates the separase protease by ubiquitinating its inhibitor securin (Cut2p). The separase then destroys the cohesion between sister chromatids and enables the mitotic spindles to pull sister chromatids to opposite poles, thus allowing spindles to elongate. The APC/C is a cell cycle regulated ubiquitin ligase, which controls progression through anaphase and is also known to target mitotic cyclins for polyubiquitination and subsequent degradation, thereby inactivating mitotic Cdc2p-Cdc13p required for mitotic exit and cytokinesis.

The proper timing of chromosome segregation co-ordinated with other cell cycle events is monitored by the spindle assembly checkpoint, which ensures that anaphase only begins when a stable chromatid-spindle assembly is achieved. It is believed that components of the kinetochores confer a "waiting" signal to the APC/C until the kinetochores establish an accurate bioriented attachment to the kinetochore microtubules. When all chromosomes are ready for separation, the "waiting" signal is inactivated and the ubiquitin ligase activity of APC/C is free to polyubiquitinate downstream substrates. These substrates include several key cell cycle regulators, for example Cut2p and Plo1p and Cdc13p, required for the progression of distinct post-metaphase events (Yanagida, 2000; May et al, 2002).

#### ***1.4.6 Regulation of cytokinesis***

During mitosis, the interphase cytoskeleton undergoes a series of rearrangements, including formation of the mitotic spindle and initiation of cytokinesis to cause cell separation. Cytokinesis is the stage when a barrier between mother and daughter cells is established, eventually leading to cell separation. Since yeasts such as *S. pombe* and *S. cerevisiae* have a rigid cell wall, two events are necessary following nuclear division: membrane separation (cytokinesis) and cell wall separation (cell separation), with these processes collectively known as cell division (Hartwell, 1971).

The processes of spindle formation and cytokinesis are two independent but highly co-ordinated events, which ensure high fidelity in newly born cells. Although cell division has been studied for over half a century, and great progress towards understanding of the nuclear cycle has been made, the regulation of cytokinesis and how it is coupled to nuclear cycle are still poorly understood (Le Goff *et al*, 1999; McCollum, 2004).

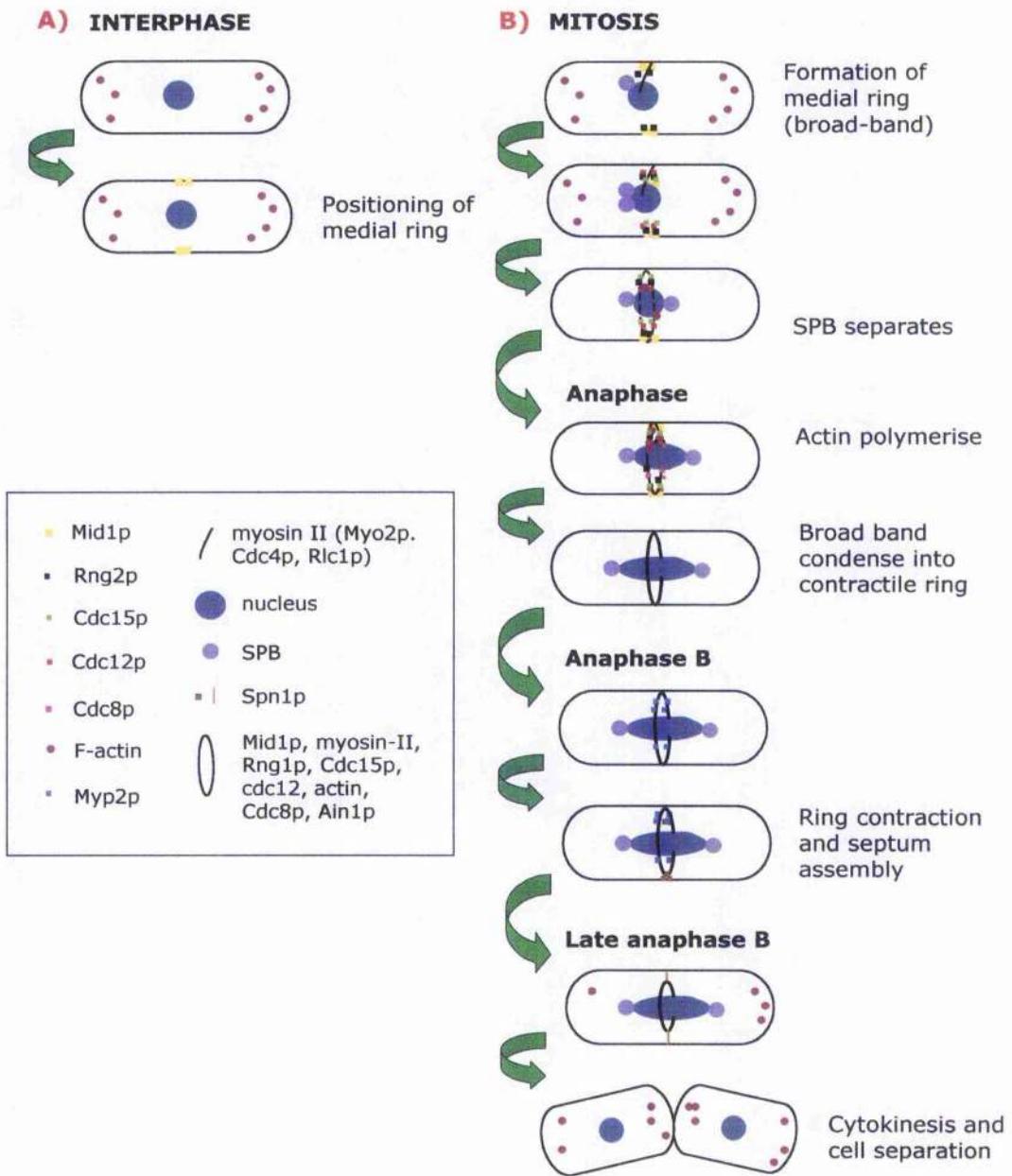
In the last few years many advances have been made in isolating, defining and characterising genes and their products required for septation, cytokinesis and cell wall separation in *S. pombe* (Fankhauser and Simanis, 1994). Various mechanisms are used to determine the divisional plane in different organisms. For example, animal cells co-ordinate cell division according to the position of mitotic spindle (Rappaport, 1986). Instead in *S. cerevisiae* a cortical marker locates the site of nuclear division and *S. pombe* determines the division site by receiving signals from the nucleus (Chant and Herskowitz, 1991; Chang and Nurse, 1996). Following the determination of the medial division site, components essential for actomyosin ring formation assemble.

#### **1.4.6.1 Co-ordination of actomyosin ring and septum assembly**

As in higher eukaryotes, the cytoskeleton of *S. pombe* undergoes a series of sequential reorganisation events during mitosis and cytokinesis (Hagan and Hyams, 1988). During interphase, F-actin is mainly observed as actin patches at the growing ends of the cell (Fig. 1.7.A) (Balasubramanian *et al*, 1998). During early mitosis before chromosome separation, a medial ring forms in the center of the cell, overlying the nucleus but perpendicular to the mitotic spindle. The position of this ring anticipates the site of septum formation. Although medial ring formation is independent of septum formation, the two processes are tightly coupled (Fankhauser *et al*, 1995; Ohkura *et al*, 1995).

As cells progress into mitosis, F-actin patches relocate to the medial ring (Balasubramanian and McCollum, 2003). At the end of anaphase, when the spindle begins to break down, biosynthesis of septal material is initiated and the primary septum grows inward from the cell cortex. At the end of anaphase, the actomyosin ring constricts while the primary septum, initially formed at cell cortex, moves inward along with the constricting ring. Underlying cytokinesis is the contraction of the ring itself. This is based on the sliding of actin filaments caused by type II myosin.

Upon completion of the primary septum, the arrangement of F-actin changes from a ring to clusters of dots. Once the primary septum is generated, secondary septa are formed



**Figure 1.7.** Events of cytokinesis and septum formation in *S. pombe*. **A)** During the G2/M interval, Mid1p leaves the nucleus and marks the division site. **B)** When cells enter mitosis, myosin II and Rng2p accumulate into a broad medial ring that subsequently contribute to actin polymerization, aided by Cdc15p and Cdc12p. Once the SPBs are fully separated, Cdc8p joins the ring resulting in the compaction of medial ring. Myp2p then binds to the ring during anaphase B followed by the localization of septin, Spn1p. When septum formation is completed, fission begins with cell wall degradation to bring about cytokinesis and cell separation.

on either side. The completed septa have a three-layered structure composed of an inner primary septum, flanked by two secondary septa. Cell separation begins by degradation of the primary septum and subsequent rupture of the pre-existing cell wall, thus liberating the two new cells.

#### **1.4.6.2 Contractile ring**

In *S. pombe*, the formation and positioning of the equatorial contractile ring is controlled by the nucleus (Chang and Nurse, 1996). The orderly events begin when the Mid1p exits the nucleus during interphase (Fig. 1.7) (Bahler et al, 1998). This process marks the cell cortical overlying the nucleus, thus, forming a broad equatorial band. Just before SPB separation at the G2/M interval, myosin II consisting of heavy chain (Myo2p) and two light-chain subunits (Cdc4p and Rlc1p), IQGAP protein (Rng2p), PH protein (Cdc15p), and formin (Cdc12p), joins Mid1p in a step-wise manner (Wu et al, 2003).

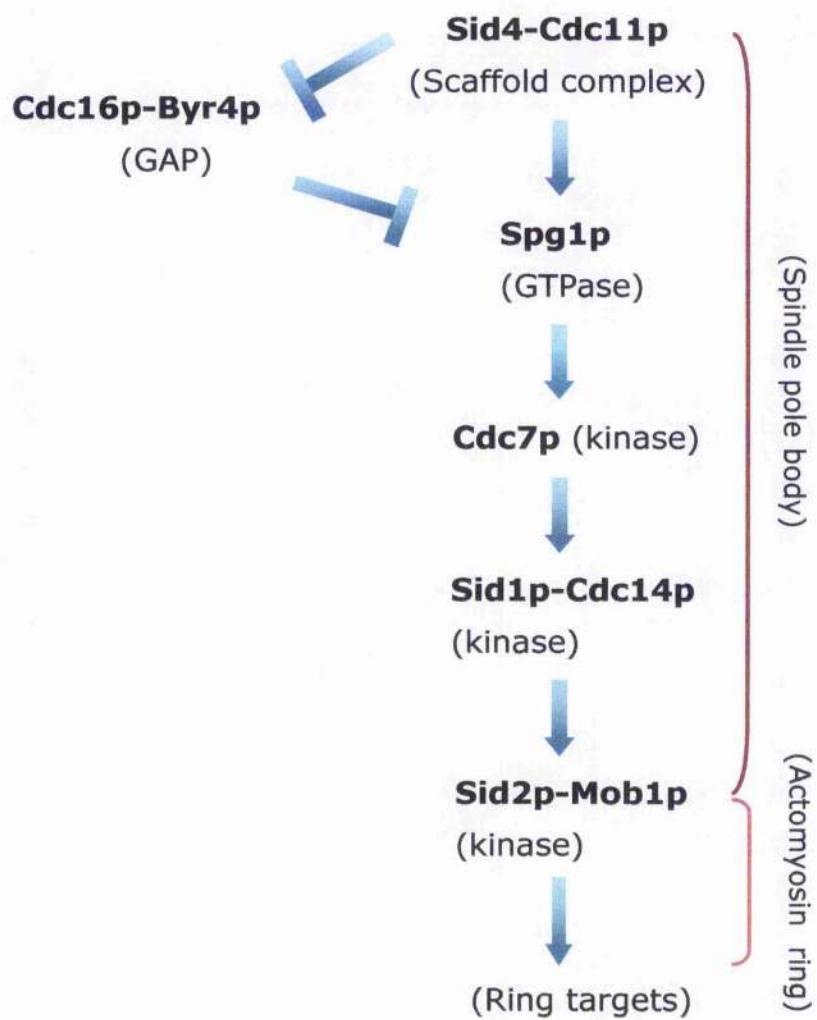
During anaphase, actin polymerisation begins with formin, Cdc12p, interacting with profilin, Cdc3p which both then localise to the division site. This process initiates tropomyosin, Cdc8p, which binds to polymerize actin, aided by the crosslinking protein  $\alpha$ -actinin, Ain1p, to assemble the broad equatorial band into a compact contractile ring, during anaphase B. Upon completion, the ring maintains and matures by the addition of a subclass myosin II, Myp2p. When cells enter late anaphase B, actin patches and the capping protein, Acp2p, join the ring. The septin, Spn1p, then concentrates near the ring.

When the actomyosin ring has formed and the mitotic spindle breaks down, a pathway named Septation Initiation Network (SIN) controls the initiation of ring constriction and septum formation (Balasubramaniam et al, 2000; McCollum and Gould, 2001).

#### **1.4.6.3 Septation initiation network**

The SIN is a signalling cascade that consists of a small GTPase, Spg1p, and three protein kinases, Cdc7p, Sid1p and Sid2p (Fig. 1.8) (McCollum and Gould, 2001). In addition, Cdc14p and Mob1p form a regulatory complex with Sid1p and Sid2p. SIN was suggested as important for stabilising the actomyosin ring during anaphase, since SIN mutants can form the ring but fail to initiate constriction and septation. These mutants are grouped into two forms: elongated cells with multiple nuclei that eventually lyse, and multiseptated cells that fail to undergo cell separation.

Activity of the SIN requires the recruitment of SIN proteins to the SPB during M phase. A scaffold complex forms and is comprised of Sid4p and Cdc11p, which localises to the SPB throughout the cell cycle and functions to recruit SIN components in a sequential order



**Figure 1.8.** Septation initiation network (SIN) signalling cascade in *S. pombe*. The SIN pathway regulates actomyosin ring constriction and septum assembly in response to signals originating from spindle pole body (SPB).

(McCollum and Gould, 2001; Morrell *et al*, 2004). Spg1p controls the SIN signaling since overexpression of *sgp1<sup>+</sup>* triggers septation at all stages of the cell cycle. Both Cdc16p-Byr4p and Spg1p-GDP localise to the SPBs during interphase. During interphase, Cdc16p-Byr4p (a GTPase activating protein (GAP)) negatively regulates Spg1p (a GTPase). Mutation in either Cdc16p or Byr4p results in multiple septation without undergoing nuclear division (Minet *et al*, 1979; Song *et al*, 1996).

When cells enter mitosis, Spg1p is activated (GTP-form) on both poles and Cdc16p-Byr4p leaves the SPBs; in return Cdc7p is recruited to the poles. During anaphase B, Cdc16p-Byr4p returns to one of the two SPBs and Spg1p-GTP is hydrolyzed into the inactive Spg1p-GDP form. Therefore, Cdc7p dissociates from the inactive pole, containing Spg1p-GDP, resulting in asymmetrical SPBs.

Sid1p and Sid2p kinases, with their regulatory subunits, Cdc14p and Mob1p, act in a linear pathway (McCollum and Gould, 2001). During anaphase, Sid1p-Cdc14p localise to the single Cdc7p-containing SPB, which then activate Sid2p-Mob1p. This causes Sid2p-Mob1p to translocate to the actomyosin ring to trigger ring constriction and septation. This is the first evidence of a SIN component linked to the division site.

The Sid1p-Cdc14p complex is detected throughout the cell cycle, but only associates to the SPB during anaphase when mitotic CDK is low and Cdc16p and Byr4p are inactive. There is evidence that Cdc16p-Byr4p plays a critical role to ensure that cells do not initiate cytokinesis during interphase when CDK is also low (Minet *et al*, 1979; Cerutti and Simanis, 1999; Li *et al*, 2000). Furthermore, it is believed that mitotic CDK functions to inhibit SIN activation by preventing the localisation of the SIN kinase, Sid1p, to the SPB. As a result, Sid2p, which functions downstream of Sid1p, fail to translocate from the SPB to the site of septum formation, so inhibiting cytokinesis.

## 1.5 *Saccharomyces cerevisiae*

In *S. cerevisiae*, bud size is indicative of cell cycle progression with bud initiation marking the beginning of a new cell cycle. Cells grown in rich medium complete one cell cycle in approximately 90-120 minutes. Daughter cells are generally smaller than mother cells at the time of cell division and undergo a longer G1 (Fig. 1.3.B) (Pringle and Hartwell, 1981).

Once past G1, cells commit to S phase whereby DNA replication occurs after the appearance of a new bud. During this phase, cells occupy 25% of the cell cycle, in contrast to the observation in *S. pombe*. However, similar for both organisms, their G1 phases are dependent on nutritional status for commitment into the S phase.

Following the completion of DNA replication and G2 events, during which RNAs and proteins required for nuclear and cell division are made, nuclear division (M phase) begins at

approximately halfway through the cell cycle, occupying approximately 10-20% (Williamson, 1966). Prior to M phase, the nucleus migrates to the neck between mother-daughter cells and undergoes mitosis. Bud formation and nuclear migration require SPB duplication and mitotic spindle formation relatively early in the cell cycle, precluding a clear comparison of a distinct S, G2 and M phases. Finally, cell separation occurs during late M phase coincident with the next round of budding (Pringle and Hartwell, 1981).

### **1.5.1 Overview of interphase control**

The mitotic cell cycle has been extensively studied in *S. pombe*, *S. cerevisiae* and many other eukaryotic organisms. The first three initial phases include G1, S, and G2, collectively known as interphase, which precede nuclear and cell division phase (M phase).

In *S. cerevisiae*, mitotically dividing cells require a single Cdc28p CDK for cells to transit G1/S and initiate a new cell cycle (Reed, 1980). Cdc28p is a serine/threonine protein kinase homologue to Cdc2p of *S. pombe*. During G1, Cdc28p activity is regulated by three cyclin subunits, Cln1-3p (Hadwiger et al, 1989). There are two groups of cyclins identified in *S. cerevisiae*: G1 cyclins (*CLNs*) and B-type cyclins (*CLBs*). Besides three G1 cyclins (*CLN1-CLN3*), there are six cyclins during S phase (*CLB5-CLB6*), G2 phase (*CLB1-CLB2* and *CLB5-CLB6*) and M phase (*CLB1-CLB4*).

In a normal cell cycle the abundance of these cyclins fluctuates, regulated at transcriptional and post-translational levels. Two transcription factors, Swi4p and Swi6p, are necessary for the expression of *CLN1* and *CLN2*. These cyclins contain a *SWI4/6 cell cycle box* (SCB) UAS at their promoter regions (Nasmyth and Dirick, 1991; Ogas et al, 1991). It was proposed that phosphorylation of Swi4p and Swi6p by protein kinases, potentially Cdc28p/Cln3p, leads to activation of SCB transcription, suggesting these cyclins regulate their own expression through an autofeedback loop.

When two cells of opposite mating types are present, they secrete hormones ( $\alpha$  and  $\alpha$  factors) that bind to the opposing cell's hormone receptor. These events result in an arrest at "start" through Cdc28p-Cln1p/Cln2p inactivation. In addition, the single CKI of *S. cerevisiae*, Sic1p, binds to complexes of Cdc28p with Clb5p and Clb6p therefore maintaining them in an inactive form causing cell cycle arrest.

When cells re-enter the mitotic cell cycle, they pass "start" and enter S phase whereby preparations for replication have to be made, such as the formation and maintenance of the pre-RC. B-type cyclins that are present at high concentration during mitosis must be degraded because they inhibit pre-RC formation. Their destruction by proteosome-mediated proteolysis during anaphase allows the formation of pre-RCs during late G1 (Amon et al, 1994). In addition, Sic1p functions to keep G1 CDK/Cyclin B activity

low. At the end of G1, Sic1p is degraded resulting in CDK-Clb5p/Clb6p activity to rise, triggering entry into S phase. High levels of CDK-Cyclin B are then maintained throughout S, G2 and M phases to prepare and initiate mitotic entry, and to prevent DNA re-replication.

### **1.5.2 Overview of mitotic control**

In *S. cerevisiae*, Cdc28p is also required for the entry into M phase and its activity is controlled by its interaction with B-type cyclins (Clb1p-Clb4p) (Surana *et al*, 1991; Fitch *et al*, 1992). *CLB* genes are expressed periodically: *CLB3* and *CLB4* peak during S phase while *CLB1* and *CLB2* peak during M phase. Each cyclin pair drives distinct cell cycle events that coincide with the time of expression, such as spindle morphogenesis (Clb3p-Clb4p) and mitosis (Clb1p-Clb2p).

Swe1p (the homologue to *S. pombe* Wee1p) negatively regulates mitotic Cdc28p-Clbps by phosphorylating the Tyr-19 residue, the equivalent of *S. pombe* Cdc2p Tyr-15 (Booher *et al*, 1993). This phosphorylation is repressed and reversed by Mih1p (the counterpart of *S. pombe* Cdc25p) (Russell *et al*, 1989). Similar to Wee1p of *S. pombe*, the timely phosphorylation and degradation of *S. cerevisiae* Swe1p is critical for mitotic entry. Swe1p accumulates during S phase and becomes hyperphosphorylated as cells proceed through the cell cycle (Shulewitz *et al*, 1999). When Swe1p is not required, the hyperphosphorylated form is targeted for ubiquitin-mediated proteolysis through APC/C degradation process (Kaiser *et al*, 1998; Thornton and Toczyki, 2003).

Hsl1p (the homologue of *S. pombe* Nim1p/Cdr1p) and Hsl7p are both required for efficient recruitment of Swe1p (McMillan *et al*, 1999). They are also the primary negative regulators of Swe1p. Studies of the *S. cerevisiae* Plk, Cdc5p, showed that it plays a role in phosphorylation and degradation of Swe1p prior to G2/M transition to promote M phase entry (Sakchaisri *et al*, 2004). Cdc5p is an unstable protein whose proteolysis is regulated by the APC/C (Shirayama *et al*, 1998). Furthermore, Cdc5p protein levels accumulate during late G2/M phase and disappear at late anaphase, and this facilitates the activation of Clb2p proteolysis.

To exit mitosis, cells must complete chromosome segregation and inactivate mitotic kinase-cyclins complexes. Similar to *S. pombe*, both steps require the activity of APC/C (Zachariae and Nasmyth, 1999; Yanagida, 2000). During mitosis, the activity of APC/C relies predominantly by two substrates: Cdc20p and Hct1p (Sethi *et al*, 1991; Schwab *et al*, 1997). Cdc20p initiates the transition between metaphase to anaphase by inducing the ubiquitination of anaphase inhibitor, Pds1p (Cohen-Fix *et al*, 1996). Down-regulation of Pds1p is required for sister chromatids separation, while Hct1p-dependent APC/C activity is

necessary for the ubiquitination and degradation of mitotic cyclin Clb2p, required to exit mitosis (Zachariae *et al*, 1998).

### **1.5.3 Mitotic exit network (MEN)**

In *S. cerevisiae*, the analogous signalling pathway to the SIN in *S. pombe*, regulating the initiation of cytokinesis, is termed the mitotic exit network (MEN). *sin* mutants do not initiate ring constriction and septum formation but still undergo nuclear divisions, and so morphologically are long and contain multiple nuclei. In contrast, *MEN* mutants arrest cells in late anaphase, and contains elongated spindles with segregated chromosomes, and do not initiate cytokinesis (Bardin and Amon, 2001).

In common with the SIN, the MEN also functions in a GTPase-regulated protein kinase-signalling cascade (Fig. 1.9). The GTPase that controls septation in *S. cerevisiae* is Tem1p, which triggers the onset of signalling through four protein kinases: Cdc5p, Cdc15p, Dbf2p and Dbf20p (Schweitzer and Phillipson, 1991; Toyn *et al*, 1991; Shirayama *et al*, 1994).

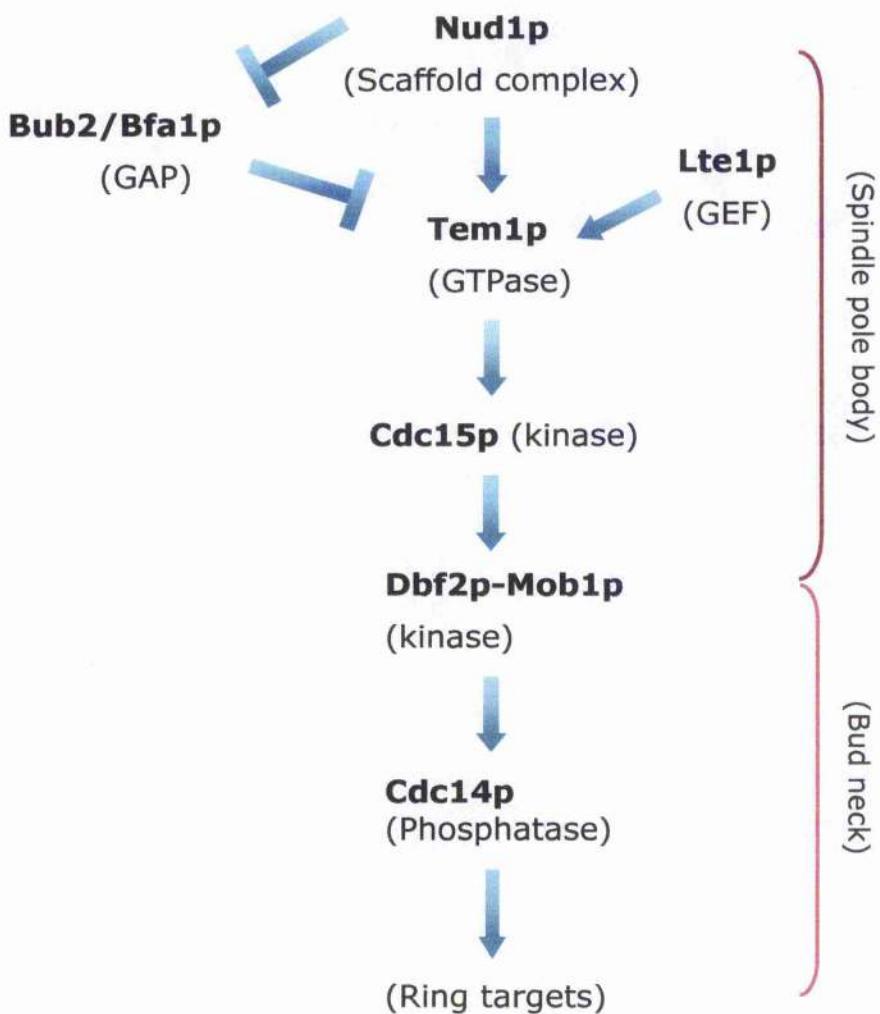
The nucleoplasm cycling of Cdc14p is required for MEN activation (Shou *et al*, 1999). During most of the cell cycle Cdc14p binds to Net1p/Cfi1p and remains in the nucleus. Once anaphase is activated, through APC signalling, Cdc14p is released and translocates into the cytoplasm. The free Cdc14p then reverses CDK-phosphorylation activity to promote mitotic exit and cytokinesis.

Bfa1p, a component of the GAP, is a target of Cdc14p. Cdc14p is also thought to activate the Cdc15p protein kinase, which when not required is inhibited by CDK phosphorylation. When Cdc15p is in its active form it stimulates the Dbf2p-Mob1p kinase-regulator complex, the effector that links mitotic exit and cytokinesis.

The GEF (GDP-GTP exchange factor) for Tem1p GTPase in *S. cerevisiae* is Lte1p, which is lacking in *S. pombe*. Lte1p localises at the bud while Bfa1p, Tem1p and Cdc15p localise to the cytoplasmic face of the SPB (Xu *et al*, 2000; Bardin *et al*, 2001). Therefore, it is believed that one of the SPBs carrying Tem1p activates MEN signalling when in contact with Lte1p at the bud, which then bind to Cdc15p and recruits Dbf2p-Mob1p kinase.

## **1.6 Transcription in Eukaryotic Cells**

All forms of RNAs are synthesized by RNA polymerases from DNA templates through a process known as transcription. In eukaryotes, three types of RNA polymerases (RNAP) termed RNAP I, RNAP II and RNAP III, are required for the transcription of different genes. The three species of polymerases transcribe different sets of nuclear DNA genes: RNAP I



**Figure 1.9.** Mitotic exit network (MEN) signalling cascade in *S. cerevisiae*. The MEN pathway regulates actomyosin ring constriction and septum assembly in response to signals originating from spindle pole body (SPB).

transcribes ribosomal RNA encoding genes, RNAP II transcribes protein-encoding genes and RNAP III transcribes transfer RNA encoding genes. The synthesis of protein by RNAP II is directed from messenger RNAs (mRNA) that functions as an intermediary between DNA and protein synthesis.

The regulation of gene expression (the process from transcription to protein synthesis) is fundamental for both cell growth and division. Transcription processes are highly conserved being similar in simple eukaryotes, such as *S. pombe* and *S. cerevisiae*, to higher eukaryotes (Struhl, 1999).

### **1.6.1 RNA Polymerase II (RNAP II)**

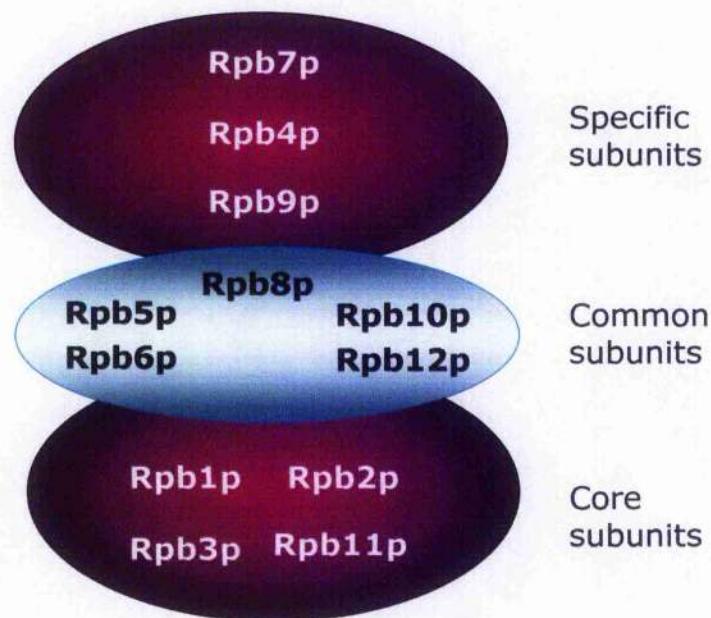
Transcription directed by RNAP II is the initial step of protein synthesis and their products are required for cell regulation processes, such as cell growth and division. In *S. pombe*, RNAP II is composed of 12 subunits named Rpb1p-12p (Ishihama et al, 1998). Similar to higher eukaryotes, RNAP II can be divided into three subunits: core, common and specific (Fig. 1.10).

The four core subunits of *S. pombe*; Rpb1p, Rpb2p, Rpb3p and Rpb11p, form the catalytic core (Ishihama et al, 1998). While five subunits (Rpb5p, Rpb6p, Rpb8p, Rpb10p and Rpb12p) are shared in all RNAPs (RNAP I-III), three other subunits (Rpb7p, Rpb4p and Rpb9p) are specific to all RNAPs, in *S. pombe* (Hampsey, 1998). Not only is there high conservation in structure and function of RNAP IIs among eukaryotes, but several subunits also appear to have homologues in bacteria (McKune et al, 1995).

The function of RNAP II in *S. pombe* is modulated by phosphorylation at the C-terminal domain (CTD) of Rpb1p, consisting of 29 repeats of conserved heptapeptide residues (Hampsey, 1998). Rpb1p exists in two forms, the hypophosphorylated and hyperphosphorylated forms (Cadena and Dahmus, 1987). While only the hypophosphorylated form can associate with the general transcription factors (GTFS), the hyperphosphorylated form is involved in elongation processes. It is believed that this is an important step, allowing shift from the preinitiation to elongation steps (Svejstrup et al, 1997).

### **1.6.2 Initiation of transcription**

Promoters contain a stretch of core DNA sequences to which transcription factor proteins bind. The most well characterised RNAP II promoter element is the TATA-box. However, not all RNAP II genes contain TATA-box elements, with those without TATA elements referred to as TATA-less. TATA-less promoters, which are often found in higher eukaryotes, contain initiator element (Inr) functionally similar to TATA-box (Smale and Kadonaga, 2003).



**Figure 1.10.** The subunit composition of RNA polymerase II (RNAP II) in *S. pombe*. The core subunits form the catalytic core, the common subunits are common among RNAP I, II and III, while the specific subunits are only found in RNAP II.

Another element associated to TATA-less promoters is the downstream promoter element (DPE) situated downstream of transcription start site and often act in combination with Inr.

In eukaryotes, mRNAs synthesis by RNAP II is dependent on several factors: the GTFs and specific transcription factors, which are linked by the Mediators (Roeder, 1996).

#### **1.6.2.1 General transcription factors (GTFs)**

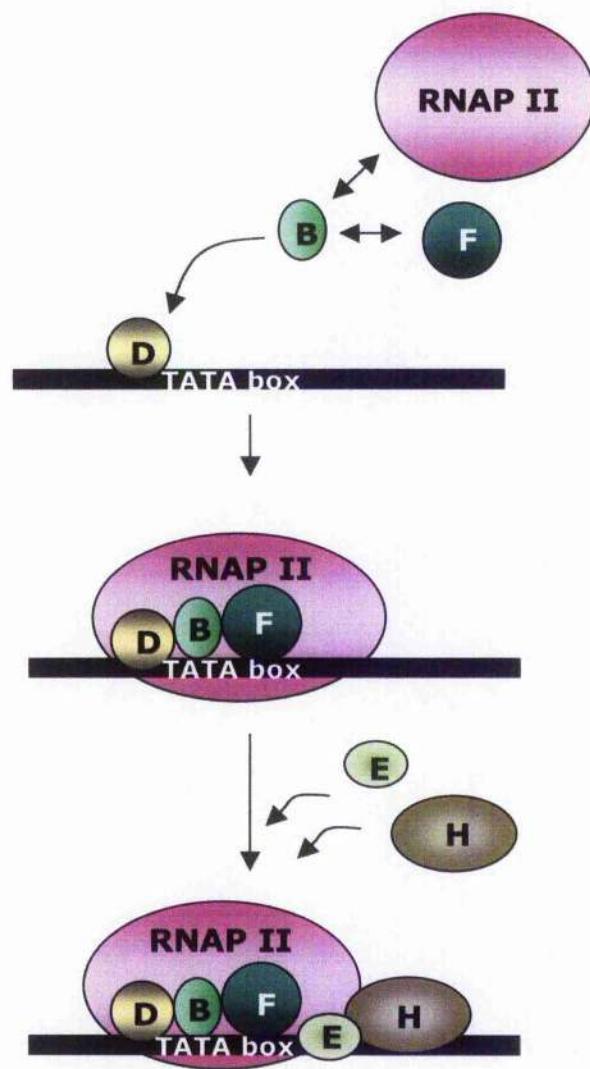
Five GTFs have been identified including TFIIB, TFIID, TFIIE, TFIIF and TFIIH (Weil *et al*, 1979). Transcription begins when the TATA binding protein (TBP), a component of TFIID, recognizes and binds to TATA-box, thus resulting in DNA bending (Fig. 1.11) (Buratowski, 1994). Subsequent binding of TFIIB results in the association of RNAP II and TFIIF. Once TFIID-B-F-RNAP II complex forms, TFIIE is recruited followed by TFIIH's association so forming a pre-initiation complex.

TFIID is a large multi subunit complex containing the TBP and TBP-associated factors (TAFs). It functions to recognize and bind the TATA-box, and provides a platform for the assembly of other GTFs (Nikolov and Burley, 1997). TFIIB, a single polypeptide, bridges RNAP II to the TATA-box (Buratowski *et al*, 1994). In yeast, TFIIF contains three subunits and is tightly associated with RNAP II (Greenblatt, 1991; Conaway and Conaway, 1993). Furthermore, it assists in stabilizing the pre-initiation complex.

TFIIE is the least well understood and is thought to function as a bridge between RNAP II and TFIIH (Van Mullem *et al*, 2002). TFIIH is the largest subunit and contains enzymatic activities (Tirode *et al*, 1999). It can be divided into two subcomplexes: core TFIIH and the CDK/cyclin containing TFIIK. In *S. pombe*, the CDK/cyclin (Mcs6p/Mcs2p) complex phosphorylates the CTD of RNAP II, required for the initiation of elongation process during transcription (Lee *et al*, 2005).

#### **1.6.2.2 Specific transcription factors**

Genetic analyses in yeast and higher eukaryotes revealed that cis-acting elements upstream of TATA boxes, called either enhancers or UASs, activate transcription of most protein-encoding genes. They are bound by structurally similar and functionally analogous trans-acting elements called transcription factor proteins (Angel *et al*, 1988; Struhl, 1989). A large number of specialised transcription factors have been identified which although having significant variation in structure, all share certain common features, such as a DNA binding domain and transactivation domain. These domain-specific transcription factors interact with coactivator proteins and subunits of RNAP II through a Mediator complex to stimulate gene expression (Roeder, 1996).



**Figure 1.11.** The step-wise assembly of RNA polymerase machinery in *S. pombe*. DNA transcription factors (TFs) including RNA polymerase II (RNAP II), TFIIB, D, E, F and H bind sequentially to TATA-box to initiate basal transcription.

Structural studies have allowed the identification and classification of specific protein domains according to their regular peptide sequences and secondary structural properties. In general, specific transcription factors can be classified into five types, according to the structure of their DNA binding domain: helix-turn-helix motif, zinc finger, POU domain, helix-loop-helix and leucine zippers.

Relevant to this thesis, we describe two transcription factor families important for cell cycle regulated transcription in *S. pombe*, namely the HNF-3 (hepatocyte nuclear factor-3)/forkhead and MADS-box families.

#### ***1.6.2.2.1 Forkhead/Hepatocyte nuclear factor-3 (HNF-3) protein family***

The HNF-3/forkhead was initially identified in *D. melanogaster* and is essential for the proper formation of terminal structures of the embryo (Kaufmann and Knochel, 1996). Mutation of *D. melanogaster* forkhead gene causes a homeotic transformation of gut structures into head-derived element and localisation studies revealed that the protein functions as a transcription regulator (Weigel and Jackle, 1990). Thus, it is required for proper formation of organs and tissues where the genes are expressed. Shortly after the description of the forkhead gene, a small family of HNF-enriched DNA-binding transcription regulators was identified in rodents (Lai et al, 1990). Therefore, forkhead and HNF-3 are the founding members of a family of transcription factors derived from the evolutionarily conserved helix-turn-helix family.

When the amino acid sequences of forkhead and HNF-3 were compared, high sequence identity was detected within their DNA-binding domains that encompass a region of 110 amino acid residues (Weigel and Jackle, 1990). The structure of HNF-3/forkhead DNA binding domain has been solved by X-ray crystallographic analysis. It contains clusters of  $\alpha$ -helices and  $\beta$ -sheets, flanked by two non-ordered wing like regions, therefore these proteins are also called "winged helix" (Clark et al, 1993).

Since the discovery of the forkhead and HNF-3 proteins, many more containing the conserved domain have been identified. In addition to *D. melanogaster* forkhead, seven other forkhead-encoding genes (FD1-FD7) were found in *D. melanogaster* expressed during embryogenesis (Hacker et al, 1992). Forkhead proteins are not only involved in morphogenesis but also in other processes best exemplified in three main HNF-3 proteins ( $\alpha$ ,  $\beta$  and  $\gamma$ ) of rodents, each found in separate organs (Lai et al, 1990). However, *S. cerevisiae* forkhead proteins, Hcm1p, Fkh1p, Fkh2p and Fhl1p, do not show function specificity as described in higher eukaryotes, but have wider roles, such as energy-dependent and rRNA processing, and cell cycle regulation (Zhu et al, 1993; Hermann-Le Denmat et al, 1994; Kumar et al, 2000).

#### **1.6.2.2.2 MADS-box protein family**

The MADS-box motif was initially identified from its four founding members and the name is derived from the initial of these members: *S. cerevisiae MCM1*, *Arabidopsis thaliana AG*, *Antirrhinum majus DEFA* and human *SRF* (Shore and Sharrocks, 1995). Since its discovery, many proteins containing the recognition domain have been identified. These are involved in a diverse range of biological activities, such as muscle-specific and mating-type-specific transcription, tracheal development, pheromone response and cell cycle regulation.

The MADS-box motif encompasses a region of 56 amino acid residues within the DNA-binding domain. Alignment analyses of various MADS-box proteins suggest that the overall fold of this motif is strongly conserved among family members (Shore and Sharrocks, 1995). A similar analysis also suggests that the majority of MADS-box proteins contain MADS-box motif at the N-terminus. Deletion of a 90 amino acid polypeptide that encompasses the 56 MADS-box residues abolishes sequence-specific DNA binding, implying this region is required for DNA contact. The MADS-box proteins often bind DNA as dimers, suggested by the symmetry of their DNA-binding sites (Norman *et al*, 1988).

A common feature of MADS-box proteins and other transcription factors is the protein-protein interactions that result in a transcriptional regulatory complex. Evidence has indicated that a number of MADS-box proteins bind to members of other classes of DNA-binding proteins (Sprague, 1990). MADS-box proteins do not appear to exert their function alone, but require other transcription factors for their regulatory specificity. One example of this is *Mcm1p* of *S. cerevisiae*.

*Mcm1p* is required for the transcription of mating-type-specific and cell cycle-regulated genes (Shore and Sharrocks, 1995). It forms various complexes in which each determines the expression of a particular set of genes. These complexes can act either to increase or decrease the expression of target genes.

#### **1.7 Cell Cycle Regulated Transcription in *S. pombe* and *S. cerevisiae***

The completion of the genome sequencing projects of *S. cerevisiae* and *S. pombe* allowed a better understanding of the genomic characters of both yeasts (Goffeau *et al*, 1996; Wood *et al*, 2002). Although the genome sizes are similar, their contents are different. *S. pombe* contains approximately 4900 predicted ORFs while *S. cerevisiae* contains nearly 5800 ORFs. This is due to larger intergenic region between genes and the abundance of introns found in *S. pombe*.

High-throughput technologies allowed a comprehensive functional study of the *S. pombe* and *S. cerevisiae* genomes, permitting valuable complementary analyses to conventional methods. Genome-wide studies on gene expression identified more periodically

transcribed genes (400 - 800) in *S. cerevisiae* than in *S. pombe*, 400 genes (Cho *et al.*, 1998; Spellman *et al.*, 1998; Rustici *et al.*, 2004). It seems that periodic transcription is important for controlling cell cycle progression possibly to ensure orderly progression. But important questions are how are these genes regulated to confer mRNA periodicity, and are the control mechanisms conserved through evolution?

The first genes to be identified whose mRNA show periodicity were the histones, with peak levels during S phase (Hereford *et al.*, 1981). Subsequent studies identified many more periodically transcribed genes and transcription factor complexes that control their expression. It now appears that the phase-specific transcription is a universal theme in cell division cycle (Fig. 1.12).

The combination of studies from genome-wide and single gene analyses, created a paradigm that describes waves of transcriptional activity coincident with the four main cell cycle transitions: G1 and S phases, initiation of DNA replication; G2/M transition, entry into mitosis; and M/G1 transition, exit from mitosis.

### **1.7.1 G1-phase gene cluster and regulation**

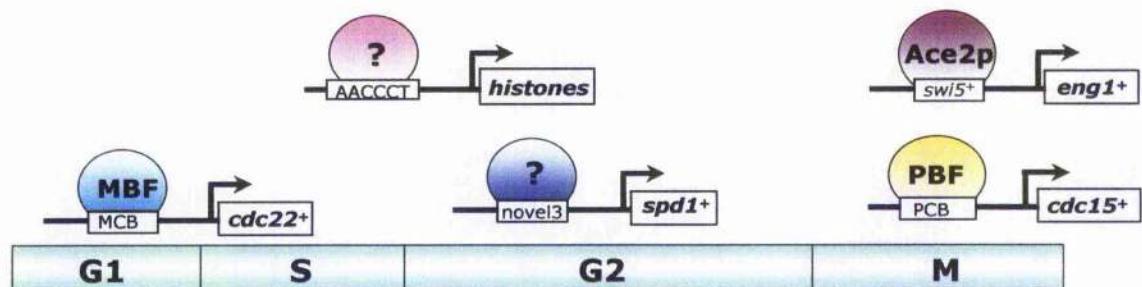
In *S. pombe* and *S. cerevisiae*, the genes whose mRNA levels peak during late G1 are required for the initiation of DNA replication and other events that promote S phase. This is consistent with the suggestion that serial regulation of gene expression by transcription factor complexes during one cell cycle phase is required for the next phase.

In *S. cerevisiae*, two transcription factor complexes, MBF and SBF (Swi4 cell cycle box binding factor), are necessary for the periodic expression of G1/S genes. The MBF complex consists of a DNA-binding component Mbp1p and a regulatory component, Swi6p. Both proteins bind to an UAS named MCB (Koch *et al.*, 1993; Breeden, 1996). In contrast, the SBF complex consists of a unique DNA-binding component Swi4p, and the common regulatory component, Swi6p, which bind to the SCB UAS (Breeden and Nasmyth, 1987; Andrews and Herskowitz, 1989).

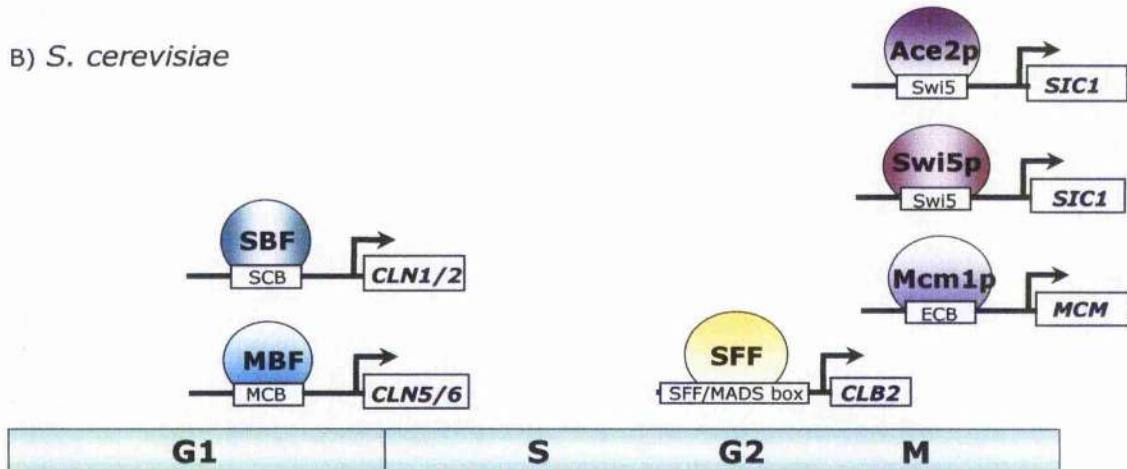
Data from both single gene and microarray analyses have identified many target genes of MBF and SBF. Although MBF and SBF-dependent genes share partially redundant functions and sequences in their promoters, they both also play distinct roles. MBF-controlled genes encode proteins required for DNA replication and repair (*POL1/2*, *CDC6*, *RNR1*, *CLB5/6*) while SBF-regulated genes are involved in cell morphogenesis, SPB duplication and other growth-related functions (*CLN1/2*, *PCL1/2*, *GIN1*, *FKS1/2*) (Iyer *et al.*, 2001; Simon *et al.*, 2001).

During G1, MBF and SBF-mediated transcription is switched on when the complexes are recruited to their UASs and activated through CDK-Cln3p activity with Whi5p acting as

A) *S. pombe*



B) *S. cerevisiae*



**Figure 1.12.** Schematic diagram of cell cycle promoter motifs and transcription factors in *S. pombe* and *S. cerevisiae*. In both organisms, phase-specific gene transcription is regulated corresponding to the mitotic cell cycle phase. A) Five waves of expression have been identified in *S. pombe*. The G1 wave contains the MBF/MCB group. The histones group is expressed during S phase. A small group of genes is expressed during G2. During M/G1, the PBF/PCB and Ace2p/swi5<sup>+</sup> groups are expressed. B) In *S. cerevisiae*, there are six known waves. At G1, two functionally redundant groups are expressed: MBF/MCB and SBF/SCB, followed by SFF group at G2/M. During M/G1 phase, three groups are expressed: Mcm1p/ECB, Swi5p/Swi5 and Ace2p/Swi5 groups.

an intermediary factor between Cln3p and subunits of MBF and SBF (Costanzo *et al*, 2004; de Bruin *et al*, 2004). The binding of Whi5p inhibits G1/S transcription. However, during late G1 this process is reversed when Whi5p leaves the nucleus due to CDK-dependent phosphorylation. Other regulators such as Bck2p and Stb1p have also been identified for G1/S transcription and shown to interact with Cln3p, but their precise role is not clear (Wijnen and Futcher, 1999; Costanzo *et al*, 2003).

In *S. pombe*, a related MBF transcription factor complex also operates during the G1/S transition by binding to MCB UAS. MBF, also known as DSC1 (DNA synthesis control 1), binds to the UAS site throughout the cell cycle stimulating transcription during late G1 (Lowndes *et al*, 1992). MBF in *S. pombe* consists of two functionally redundant heterocomplexes consisting of a common component, Cdc10p, two ankyrin-repeat proteins, Res1p and Res2p, and an additional transactivator, Rep2p, shown to bind specifically to Cdc10-Res2p complex (McInerny, 2004). The target genes for MBF include those involved in the control and execution of DNA replication such as *cdc18<sup>+</sup>*, *cdc22<sup>+</sup>*, *cdt1<sup>+</sup>*, *cdt2<sup>+</sup>* and *dfp1<sup>+</sup>*.

It has been suggested that the transcription of G1/S genes is activated by Cdc2p/G1-associated cyclin complex while others proposed that a pcl-like cyclin Pas1p when associated to its kinase partner Pef1p, may control MBF during G1/S (Reymond *et al*, 1993; Nakashima *et al*, 1995). It is also thought that protein degradation by the APC/C and SCF proteasomes are involved in maintaining the specificity of MBF-specific transcription, such that timely activation occurs (Yamano *et al*, 2000).

### **1.7.2 S-phase gene cluster and regulation**

In *S. cerevisiae*, two waves of transcriptional activity occur during S phase and these are encoded by histone and MET genes (Cho *et al*, 1998; Spellman *et al*, 1998). The expression of histone genes requires the SBF complex, consistent with the presence of Swi4-binding motifs in their UASs (Osley *et al*, 1986; Iyer *et al*, 2001; Simon *et al*, 2001; Kato *et al*, 2004).

Several lines of evidence suggest that transcriptional repressors encoded by *hir1<sup>+</sup>* and *hir2<sup>+</sup>* bind to the promoter of histone genes but the control mechanisms involved are not known (Sherwood *et al*, 1993). It is possible that Cdc2p/G1-cyclins are somehow involved in these regulations, however neither of the *hir* genes contained putative phosphorylation sites of Cdc2p. Therefore, an alternative regulator may be involved.

The transcription factor complex and control mechanisms required for histone gene expression are not known in *S. pombe*. Furthermore, the histone UAS sites are not conserved between *S. cerevisiae* and *S. pombe*, though the 10 most highly regulated genes in this cluster all encode histone genes in both yeasts (Spellman *et al*, 1998; Cho *et al*,

1998; Rustici *et al*, 2004). *S. pombe* Hip1p (the homologue of *S. cerevisiae* Hir1p and Hir2p) is similarly required for the repression of histone gene expression although recent evidence suggests that these genes are regulated at the level of mRNA stability (Blackwell *et al*, 2004).

### **1.7.3 G2/M-phase gene cluster**

Genes of this M-phase cluster are transcribed from the end of S until late M phase. In *S. cerevisiae*, this cluster has been named the “*CLB2* cluster” based on the mitotic cyclin gene *CLB2*, whose mRNA showed periodicity across the cell cycle (Surana *et al*, 1991). Members of this family include *CLB1/2*, *SWI5*, *CDC5*, *CDC20* and *ACE2*. A protein complex known as the *SWI5* factor (SFF) binds to a UAS of *SWI5*, and it was through studying the *SWI5* and *CLB2* UASs that insight into the components and control mechanisms of this gene cluster was first obtained (Lydall *et al*, 1991; Pic *et al*, 2000; Kumar *et al*, 2000).

These studies revealed that Mcm1p, a MADS-box transcription factor protein, binds to the UAS as a dimer in combination with a ternary SFF complex. SFF-binding sites consist of a forkhead-binding motif in addition to a MADS-box motif (Pic *et al*, 2000; Kumar *et al*, 2000). Subsequent experiments identified two forkhead factors, Fkh1p and Fkh2p, which recognise the forkhead/SFF-binding sites. Furthermore, these binding sites are often located next to Mcm1p sequence in the UASs of *CLB2*-dependent genes. Both Fkh1p and Fkh2p have redundant roles, yet distinct functions. Fkh2p mainly forms a complex with Mcm1p while Fkh1p can bind UAS alone. However, single mutant *FKH1Δ* or *FKH2Δ* cells do not disrupt the periodic accumulation of *CLB2*-genes, whereas deletion of both does (Hollenhorst *et al*, 2000; 2001).

The Fkh2p-Mcm1p complex binds to its UAS throughout the cell cycle, therefore an additional component is required to promote cell cycle periodicity (Koranda *et al*, 2000). Ndd1p is periodically expressed during S phase and acts as a transcriptional coactivator to Fkh2p-Mcm1p complex, when associated with the forkhead-homologue associated (FHA) domain of Fkh2p (Loy *et al*, 1999).

CDK-Clb1/2p mediates the phosphorylation of Ndd1p to induce the transcription of *CLB2*-genes (Reynolds *et al*, 2003). It is thought that basal levels of Clb2p are sufficient to activate *CLB2*-dependent gene transcription, which in return amplifies expression through an autofeedback loop (Amon *et al*, 1994). Recent evidence suggests that CDK/Clb5p phosphorylates Fkh2p and in return recruits Ndd1p to the Fkh2p/Mcm1p complex (Pic *et al*, 2004). The Ndd1p-Fkh2p association triggers Ndd1p phosphorylation so activating *CLB2*-dependent transcription.

#### **1.7.4 M/G1-phase gene cluster**

In *S. cerevisiae*, Mcm1p also controls M/G1 expression of *SWI4* and *CLN3* (McInerny *et al.*, 1997). Unlike the *CLB2* gene cluster, this *MCM* group of genes does not contain forkhead-binding sites in their promoters but instead, contains a UAS named the ECB (Early cell cycle box). The ECB contains a Mcm1p-binding site and an upstream homeodomain-binding site. Yox1p and Yhp1p directly bind to Mcm1p and repress *MCM1*-mediated transcription (Pramila *et al.*, 2002). Since Mcm1p occupies ECBS throughout the cell cycle, both Yox1p and Yhp1p act as a mechanical switch that determines the gene-specific periodicity (Mai *et al.*, 2002).

The second class of M/G1 genes in *S. cerevisiae* contains a Swi5p-binding site in their UAS, also referred to as the *SIC1* cluster. This cluster is controlled by the Swi5p and Ace2p transcription factors that have overlapping yet distinct functions (McBride *et al.*, 1999; Simon *et al.*, 2001; Doolin *et al.*, 2001). A striking feature of Ace2p is that it accumulates asymmetrically between mother-daughter cells, and so has been proposed for a role to mark mother-daughter cells (Bobola *et al.*, 1996).

Swi5p cycles in the nucleoplasm and is negatively regulated by CDK-Clb2p (Nasmyth *et al.*, 1990; Moll *et al.*, 1991). The proteolysis of Clb2p during anaphase leads to dephosphorylation of Swi5p, which then accumulates in the nucleus to activate transcription.

In *S. pombe*, we identified an UAS element in the promoter region of *cdc15<sup>+</sup>* that we named the PCB (Anderson *et al.*, 2002). PCB controls the transcription of a group of genes at the M/G1 boundary. Similar sequences are also present in the UAS of at least eight other genes including *fkh2<sup>+</sup>*, *plo1<sup>+</sup>*, *spo12<sup>+</sup>*, *cdc19<sup>+</sup>*, *sid2<sup>+</sup>*, *fin1<sup>+</sup>*, *ppb1<sup>+</sup>* and *mid1/dmfi<sup>+</sup>*. We also identified a transcription factor complex that binds to the PCB sequence, which we named PBF. PBF is likely to contain more than one gene product, none of which has been identified. Genome database search in *S. pombe* identified four forkhead-like and three putative MADS-like transcription factor proteins (Wood *et al.*, 2002). It is the intention of this thesis to test whether these forkhead and MADS-box proteins have a role in PBF-PCB regulated transcription in *S. pombe*.

In addition to PBF-PCB complex in *S. pombe*, an orthologue of *S. cerevisiae ACE2* has been identified and given the same name (Martin-Cuadrado *et al.*, 2003). Ace2p of *S. pombe* controls the expression of a subset of genes, required for degradation of cell wall, whose transcript levels also peak at M/G1. The UAS of these genes are enriched for a binding site similar to the Swi5p binding site (Rustici *et al.*, 2004; Alonso-Nunez *et al.*, 2005). In addition, *ace2<sup>+</sup>* mRNA of *S. pombe* is itself regulated at M/G1, as it is in *S. cerevisiae* (Buck *et al.*, 2004).

### **1.7.5 Conservation of cell cycle-regulated transcription**

Much has been learnt about cell cycle regulated genes and their control mechanisms, through genome wide and single gene analyses in *S. pombe* and *S. cerevisiae*. Genome wide studies complemented initial single gene studies showing that genes expressed during one stage of the cell cycle are required during the next stage, resulting in a continuous cycle of interdependence (Breedon, 2000; McInerny, 2004).

Genome wide studies, through microarray analyses, identified more cell cycle regulated genes in *S. cerevisiae* than in *S. pombe*. However, a higher number of conserved genes were observed in *S. pombe* than *S. cerevisiae* when compared to higher eukaryotes, consistent with the notion that human transcriptional control mechanisms and gene products are closer to *S. pombe* than to *S. cerevisiae* (Bahler and Wood, 2003; Bahler, 2005).

The overall conservation of cell cycle transcription factors and their regulatory networks between *S. pombe* and *S. cerevisiae* are possibly a general feature of cell cycle function among eukaryotes. For example, the regulation of MBF in *S. pombe* and MBF/SBF in *S. cerevisiae* show functional similarities to the regulation of metazoan E2F/Rb, required during the G1/S transition (Dyson, 1998; Nevins, 2001; Constanzo *et al*, 2003; de Bruin *et al*, 2004; McInerny, 2004). In addition, the transcription mechanism required for late mitotic events identified in *S. pombe* and *S. cerevisiae*, PBF-PCB and Fkh2p-Mcm1p complexes, exhibits sequence similarities to the mammalian TCF-SRF (ternary complex factor-serum response factor) complex (Treisman, 1994; Sharrocks, 2002).

A separate forkhead homologue-dependent transcription factor (FKH-TF) complex in humans has been identified (Alvarez *et al*, 2001). Strikingly, it controls late mitotic events and regulates the expression of *Ptk* similar to *plo1<sup>+</sup>* and *CDC5* in *S. pombe* and *S. cerevisiae*. This suggests that transcription might function as a universal control mechanism required for eukaryotic cell cycle progression.

## **Chapter 2: Materials and Methods**

### **2.1 Materials**

#### **2.1.1 Chemicals**

All chemicals used were of analytical research quality. The chemicals were obtained commercially and prepared with distilled water of Millipore-Q quality. Commonly used antibiotics (ampicillin, kanamycin, chloramphenicol and G418), protease inhibitors (chymostatin, pepstatin, antipain, leupeptin, aprotinin and PMSF) and detergents (NP-40 and SDS) were obtained from Sigma. Triton X-100 was obtained from Fiscons.

The basic chemicals used for *S. pombe* and bacterial maintenance were obtained from BD Biosciences while NZ amine was obtained from Sigma.

Sephadex G-50, cold ATP, [ $\gamma$ -P<sup>32</sup>] ATP (10  $\mu$ Ci/ $\mu$ l), [ $\alpha$ -P<sup>32</sup>] dCTP (3000 Ci/mmol) and dIdC (1 mg/ml) were obtained from Amersham Pharmacia Biotech. N<sub>6</sub>, Klenow enzyme and glass beads (425 – 600  $\mu$ m) were obtained from Sigma.

The DNA Molecular Weight Markers X (0.07 – 12.2 kb) was obtained from Roche. The protein Full-Range Rainbow™ Molecular Weight Markers (10-250 kD) was obtained from Amersham Pharmacia Biotech, while the BenchMark™ Protein Ladder (10-220 kD) was obtained from Invitrogen. The Bradford reagent was obtained from Bio-Rad.

Electrophoretic chemicals were obtained from Sigma: acrylamide/bis-acrylamide 40% solution (37.5:1), TEMED and ammonium persulphate crystals. The Novex pre-cast Tris-glycine 4-20% gradient gel was obtained from Invitrogen.

All enzymes used including T4 DNA ligase, calf intestinal alkaline phosphatase (CIP), Taq DNA polymerase, *Pfu* DNA polymerase and T4 polynucleotide kinase, and dNTPs used for PCR amplification were obtained from Promega. Expand High Fidelity PCR system was obtained from Roche. *Tobacco Etch Virus* (TEV) protease used during tandem affinity purification (TAP) was obtained from Invitrogen. The yeast wall digesting enzyme,  $\beta$ -glucuronidase, was obtained from Sigma. QIAprep® Spin Miniprep Kit and the QIAquick Gel Extraction Kit were obtained from QIAGEN and Superscript® II Reverse transcriptase kit was obtained from New England BioLabs Inc. The TA Cloning® Kit obtained from Invitrogen was used to clone blunt-ended PCR products as described in the manufacturer's instructions, while X-gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside) was obtained from Sigma.

The DNA staining dye DAPI, chitin staining dye Calcofluor Fluorescence Brightener, and routinely used protein staining dyes, Ponceau S solution, Brilliant Blue R-250 and Coomassie Blue G-250 colloidal stain, were all obtained from Sigma. The PlusOne Silver staining kit was obtained from Invitrogen.

Primary antibodies: Y-11 rabbit polyclonal anti-HA and 9E10 mouse monoclonal anti-Myc were obtained from Santa Cruz Biotech. Mouse monoclonal anti-His antibody was obtained from Sigma and rabbit polyclonal anti-GST antibody was obtained from Amersham Pharmacia Biotech. Peroxidase-conjugated rabbit anti-mouse immunoglobulins and peroxidase-conjugated swine anti-rabbit immunoglobulins were both obtained from DAKO.

The protein expression inducer IPTG and thiol-reducing agent DTT were obtained from Melford Lab. Ltd, while thiamine was obtained from Sigma. The GST-tagged protein eluent agent, reduced glutathione, was obtained from Sigma, and the His-tagged purification agents, imidazole and zinc chloride, were obtained from MERCK. Glutathione Sepharose 4B, Chelating Sepharose™ Fast Flow slurry and IgG Sepharose™ 6 Fast Flow beads were obtained by Amersham Pharmacia Biotech, while Calmodulin Affinity Resin was obtained from Stratagene. The Poly-prep Chromatography Column (0.8x4 cm) and Econo column funnel were obtained from Bio-Rad.

### **2.1.2 DNA**

All oligonucleotides used were custom synthesized by MWG-AG Biotech and DNA Technology A/S. DNA fragments required for sequencing were prepared according to manufacturer's protocol and sequenced by MWG-AG Biotech.

Oligonucleotides used in this thesis are listed in Appendix IV, with a specific GO annotation that indicates the Glasgow Lab. oligonucleotide collection number.

### **2.1.3 Bacteria strains and plasmid vectors**

One Shot™ ultracompetent InVaF *E.coli* cells were obtained from Invitrogen. The *E. coli* strains BL21 CodonPlus (DE3)-RIL (GB445) a general purpose strain and for high level protein expression and *E. coli* DH5 $\alpha$  (GB4), a recombinant deficient strain used for the propagation and storage of plasmid DNA, are described in Appendix IV and were obtained from the laboratory collection.

The pET vector, pET-28c(+), for expression of His-tagged recombinant proteins, where the six histidine residues are linked to the N-terminus of the cloned gene were obtained from Novagen. The pGEX-KG vector for expression of GST-tagged recombinant proteins, where the GST tag is linked to the N-terminus of the cloned gene and pCR2.1 for one step cloning of a polymerase chain reaction product with polydeoxyadenosine 3' overhangs were obtained from Invitrogen.

All bacteria, and plasmids maintained in bacteria used in this thesis are listed in Appendix IV, with a specific GB annotation that indicates the Glasgow Lab. oligonucleotide collection number.

#### **2.1.4 *S. pombe* strains and plasmid vectors**

The pREP1, pREP3X, pREP41X and pREP81X vectors, of various expression strength, were used for overexpressing gene under the control of the *nmt1<sup>+</sup>* (*no message in thiamine*) promoter. pREP3X, pREP41X and pREP81X are derivatives of pREP1 vector, but contain different restriction sites in their polylinkers.

All *S. pombe* strains used in this thesis are listed in Appendix IV, with a specific GG annotation that indicates the Glasgow Lab. oligonucleotide collection number.

#### **2.1.5 Media**

All strains of *E. coli* were grown in Luria Broth (LB; 10 g Bacto-tryptone, 5 g Bacto yeast extract and 10 g NaCl/litre, pH 7.5) or NZY Broth (5 g NaCl, 2 g MgSO<sub>4</sub>.7H<sub>2</sub>O, 5 g yeast extract, 10 g NZ amine/litre, pH 7.5). Solid LB was made by adding 7.5 g Bacto-agar to 500 ml LB. All media were autoclaved before usage and supplemented with ampicillin (100 µg/ml), kanamycin (70 µg/ml) or chloramphenicol (34 µg/ml), where appropriate.

All *S. pombe* strains were propagated and maintained in either liquid or on solid YE (Appendix I; Moreno *et al*, 1991). Strains containing plasmids or used for physiological study were propagated in EMM supplemented with the appropriate concentration of amino acids (Appendix II). Freezer stocks were maintained in 15-20% glycerol and YE at -70°C.

All *S. cerevisiae* strains were cultivated and maintained in YPD liquid (2% Bacto-peptone, 1% yeast extract, 2% dextrose) or YPD + 2% Bacto-agar to make solid media. Strains used for gene expression assays were maintained in YEP liquid (2% Bacto-peptone and 1% yeast extract) supplemented with appropriate carbon source.

#### **2.1.6 Equipment**

The Bio-Rad *E. coli* pulser was used for electro-transformation with 0.2 cm electroporation cuvettes. Bacterial cultures were grown in an orbital incubator obtained from Sanyo-Gallenkamp and yeast cultures were grown in shaking water-bath obtained from Grant – OLS200. Synchronisation of *S. pombe* by centrifugal elutriation was performed using Beckman J-6M/E Centrifuge with JE-6B rotor, and cell number per ml of liquid culture was determined electronically with a Z2 Coulter Counter. Bacteria and yeast cells were routinely collected in 50 ml centrifuge tubes in chilled benchtop Eppendorf Centrifuge 5810R.

Bacterial cells were commonly lysed using French Pressure cell at either 750 or 950 psi depending on the culture volume. Large scale *S. pombe* cultures were disrupted using Constant Cell Disruption System obtained from International Labmate Ltd, while small scale extraction utilised the Ribolyser from Hybaid Ltd. Disrupted yeast and bacterial cells were pelleted and the supernatants clarified using a Beckman J2-21 centrifuge with a JA-14 rotor, Beckman L8M Ultracentrifuge using a T865 rotor, Hettich Zentrifugation EBA12RT benchtop microcentrifuge, or temperature adjustable Helena Biosciences Centrifuge 5415R benchtop microcentrifuge. To concentrate protein samples, a Microcon YM-50 centrifugal concentrator was used, obtained from Millipore Corporation. Quantification of cell density, protein and nucleic acid samples were completed using a spectrophotometer adjusted to an appropriate absorbance wavelength.

Electrophoretic assays were performed using the Bio-Rad Mini-Protean gel kit system. All PCR reactions were carried out in a Primus Thermal Cycler with 0.5 ml thin walled PCR tubes obtained from Anachem. Hybridisation of DNA probes on RNA blots was carried out in a Techne Hybridizer HB-1D. PAGE gels were routinely dried using a Bio-Rad Gel Dryer Model 583. Autoradiography film was obtained from Fujifilm, while the processing machine – X-Omat 100 – was obtained from Kodak.

The *S. pombe* DIC and fluorescence microscopic images were captured using a Sony DS-75 digital camera attached to Zeiss AxioStar R-grade microscope. The Singer Micromanipulator System Series 200 was routinely used for tetrad dissection in *S. pombe*.

### **2.1.7 Computational databases**

All genomic sequences were obtained from the Wellcome Trust Sanger Institute webpage: <http://www.sanger.ac.uk>. Sequence alignments and domain studies were performed using web tools at <http://www.ebi.ac.uk/ciustalw/index.html>, [/emboss/allgn/index.html](http://emboss.allgn/index.html) and [/InterproScan/](http://www.ncbi.nlm.nih.gov/InterproScan/), and <http://www.ncbi.nlm.nih.gov/BLAST/>.

## **2.2 Methods**

### **2.2.1 Maintenance and manipulation of bacteria and *S. pombe* cells**

Basic bacterial culture maintenance was carried out as described in Sambrook et al (2001).

### **2.2.2 Preparation of competent bacteria cells**

500 ml of LB were inoculated with 1/100 volume of 16 h bacterial culture. This culture was grown to an OD<sub>600</sub> 0.5 – 0.6 at 37°C. The cells were harvested by centrifugation at 2500 g for 15 min. Supernatant was then removed and cells subjected to a brief centrifugation to

remove the remaining supernatant. Cells were then resuspended in a total of 10 ml of ice-cold 10% glycerol. The process was repeated twice but instead with 20 ml ice-cold 10% glycerol. Finally, the cells were centrifuged and resuspended in 1-2 ml of ice-cold 10% glycerol, depending on the size of the cell pellet. Aliquots of 50–100 µl cell suspension were prepared and used immediately, or stored for later usage at -70°C.

#### **2.2.3 Preparation of *S. pombe* competent cells**

500 ml of EMM with the appropriate supplements was inoculated with a 10 ml clonal inoculation prepared the previous evening. The culture was grown at 30°C to a density of  $5 \times 10^7$  cells/ml. The cells were harvested by centrifugation at 1250 g for 5 min. The supernatant was discarded and the cells resuspended in a total of 50 ml ice-cold dH<sub>2</sub>O. The cells were centrifuged again and resuspended in 20 ml ice-cold 1 M sorbitol. The process was repeated with the cells again resuspended in 20 ml ice-cold 1 M sorbitol. Once the cells were pelleted, the supernatant was discarded and the cells resuspended in residual supernatant. Aliquots of 40-60 µl were prepared and stored at -70°C.

#### **2.2.4 Growth of bacterial for protein induction**

Cells from single colonies selection, from appropriately transformed BL21 CodonPlus strains, were grown in 10 ml NZY medium containing appropriate antibiotic. The cell culture was propagated for not more than 16 h at 37°C, shaking. The following day, a 1 ml subculture was inoculated into a 50 ml NZY medium containing appropriate antibiotic. The culture was then incubated at 37°C, until an OD<sub>600</sub> of 0.4-0.6 was obtained. At this point, a final concentration of 0.1–0.5 mM IPTG (0.1 mM for 15°C induction and 0.5 mM for 30°C) was added to the culture to induce gene expression. Induction was carried out at 30°C for 4 h or 15°C for 16 h. During the course of induction, 1 ml of cell culture was harvested at hourly intervals. As a control, a 1 ml culture was harvested prior to the addition of IPTG. The 1 ml control culture was then subjected to high-speed centrifugation at 18500 g for 1 min and the pellet resuspended in Laemmli sample buffer (2% w/v SDS, 10% w/v sucrose, 62.5 mM Tris/HCl and Pyronin Y dye, pH 6.8) using 10 µl per 0.1 OD<sub>600</sub> units as a reference. After induction, the cell culture was harvested by at 2500 g for 15 min and the supernatant discarded. The cell pellet was then resuspended in 3 ml of the appropriate lysis buffer.

#### **2.2.5 Growth of *S. cerevisiae* for protein induction**

Single cells grown on YPD were picked and inoculated into a 10 ml starter culture containing YEP medium supplemented with 1-2% glucose. At 4 pm the following day, a 1:1500-2000

dilution from the starter culture was inoculated into 25 ml of YEP plus 1% sucrose and 0.05% glucose, grown overnight at 30°C, shaking at 180 rpm. At 9 am the next day, cell number per ml of liquid culture was counted with a concentration of  $5 \times 10^7$  cells/ml typically obtained, indicating an exponential phase of growth. Cells were then diluted 2-3 fold in 50 ml YEP with 1% galactose, so that the final cell number at the end of induction would reach late-exponential phase growth. The culture induction was made between 6-9 h and harvested at 2500 g for 10 min. Finally, the pellet was washed once in ice-cold dH<sub>2</sub>O and snap frozen on dry ice. The cells were stored at -70°C.

### **2.2.6 Growth of *S. pombe* for gene overexpression**

*S. pombe* strains containing the pREP1 vector series fused to gene of interest were used for overexpression analyses. The pREP vectors utilize the *nmt1*<sup>+</sup> promoter to control the expression of the inserted gene. The desired strain was streaked onto solid EMM supplemented with (*nmt1*<sup>+</sup> promoter "off") or without (*nmt1*<sup>+</sup> promoter "on") 5 µg/ml thiamine.

To overexpress a gene of interest, a strain was grown from a starter culture grown in 10 ml EMM supplemented with appropriate amino acid and thiamine. The cell culture was grown to a density of  $1-2 \times 10^6$  cells/ml (mid-log phase) at 30°C. The culture was then washed 3 times with dH<sub>2</sub>O before inoculation into fresh supplemented EMM and allowed to grow for another 3.5 generations (16-24 h). The cells were harvested at 1000 g for 5 min.

### **2.2.7 Synchronisation of *S. pombe* cells by centrifugal elutriation**

1 litre of culture was grown in YE at 25°C to a density of  $2 \times 10^6$  cells/ml. The cells were then subjected to centrifugal elutriation using a Beckman JM/E with JE-6B rotor. When the centrifugal chamber was filled with cells, small cells in early G2 were selected by a gradual reduction in centrifugal pump speed. The isolated cells were then allowed to grow at an appropriate temperature for subsequent manipulations. In parallel, a small volume of cells was collected to monitor the success of cell synchrony.

### **2.2.8 DNA Manipulation**

#### **2.2.8.1 Plasmid DNA isolation from bacteria**

Small scale rapid plasmid DNA preparation was performed from clonal 10 ml cultures grown for 16 h. 5 ml of culture were harvested using a benchtop microcentrifuge. The supernatant was removed and the pellet resuspended in 125 µl of Buffer P1 (50 mM Tris/HCl pH 8.0, 10

mM EDTA, RNase A 10 mg/ml). 125 µl of Buffer P2 (0.2 M NaOH, 1% SDS) was added and the tube inverted several times to form a uniform mixture. 125 µl of Buffer P3 (2.55 M KOAc pH 4.8) was then added and the tube immediately inverted several times and pelleted at 18500 g for 10 min. The supernatant was carefully transferred into a 1.5 ml microfuge tube containing 1 ml of 100% ethanol. This was then centrifuged for a further 10 min at 18500 g. The supernatant was discarded and the pellet was washed in 100 µl 70% ethanol. The pellet was then allowed to air-dry before being resuspended in 50 µl of dH<sub>2</sub>O.

When pure DNA was required, such as for DNA sequencing, a Qiaprep Spin Miniprep kit was used following the manufacturer's instructions.

#### **2.2.8.2 Genomic DNA preparation from *S. pombe***

10 ml cultures were grown to saturation for 2-5 days in centrifuge tubes. Cells were harvested by centrifugation for 5 min at 1000 g. The supernatant was removed and the cells were resuspended in 500 µl dH<sub>2</sub>O and transferred into screw-top microfuge tubes and subjected to a brief centrifugation, to pellet the cells. The supernatant was removed and the tube was vortexed briefly to resuspend the cells in the residual liquid. 200 µl solution A (2% Triton X-100, 1% SDS, 100 mM NaCl, 100 mM Tris pH 8.0, 1 mM EDTA), 200 µl of 1:1 phenol/chloroform and acid-washed glass beads were added into the tube. The tube was then agitated in a Ribolyser for 3 times 40 sec at setting 4.0.

400 µl TE was added and the tube was centrifuged for 5 min at 18500 g. Only the aqueous layer was removed to a fresh 1.5 ml microfuge tube, leaving behind the protein interface. 1 ml 100% ethanol was added and the tube was recentrifuged for 2 min at 18500 g to precipitate the nucleic acid. The ethanol was carefully removed and the pellet resuspended in 400 µl TE and 0.3 µg/µl RNase A. The mixture was incubated for 5 min at 37°C to remove the RNA from the pellet. 8 µl of 5M NH<sub>4</sub>Ac and 1 ml 100% ethanol were added before freezing at -70°C for 30-180 min or at -20°C overnight. The following day, the precipitate was centrifuged for 10 min at 18500 g and the supernatant removed. The DNA pellet was air-dried and resuspended in 30-100 µl dH<sub>2</sub>O.

#### **2.2.8.3 Quantification of nucleic acids by spectrophotometry**

DNA and RNA samples were diluted 100 fold in dH<sub>2</sub>O and the concentration estimated by measuring the absorbance at 260 nm with a spectrophotometer. The following formulae were used to calculate nucleic acid concentrations:

$$A_{260} \text{ of } 1.0 = 50 \mu\text{g/ml DNA or } 40 \mu\text{g/ml RNA}$$

#### **2.2.8.4 Electro-transformation of bacteria**

Aliquots of competent DH5 $\alpha$  cells were thawed on ice for 10-15 min. 1-5  $\mu$ l of appropriate plasmid DNA was added to the cell suspension, gently mixed and incubated on ice for 1 min. The cell-DNA suspension was transferred to a prechilled 0.2 cm electroporation cuvette and electro-transformed at 2.4 kV in a Bio-Rad *E. coli* pulser. 1 ml of SOC (2% Bacto-tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgSO<sub>4</sub>, 20 mM glucose) was added immediately and the cells transferred to a 1.5 ml microfuge tube, and incubated at 37°C for 45-60 min before growth on selective medium.

#### **2.2.8.5 Heat-shock transformation of bacteria**

OneShot™ InVoF and BL21 CodonPlus (DE3)-RIL *E. coli* cells were commonly used for the propagation of recombinant plasmid DNA using the heat-shock method. Aliquots of competent cells were thawed on ice for 10-15 min. 1-5  $\mu$ l of appropriate plasmid DNA was added to the thawed suspension, and mixed in a prechilled 1.5 ml microfuge tube. The cell-DNA suspension was incubated on ice for 15 min and subjected to heat-shock for 90 sec at 42°C, and returned to ice for 2 min. 450  $\mu$ l of SOC was then added and the mixture was left to shake at 37°C for 45-60 min. Finally, the cells were transferred onto LB plates containing appropriate antibiotic and incubated at 37°C for 16 h.

#### **2.2.8.6 Electro-transformation of *S. pombe***

Aliquots of competent cells were thawed on ice for 15 min. 1-3  $\mu$ l of miniprep DNA was added to the cell suspension, mixed and incubated on ice for 5 min. The cell-DNA suspension was transferred to a prechilled sterile 0.2 cm electroporation cuvette and pulsed at 1.5 kV. 900  $\mu$ l of 1 M sorbitol was added immediately, and the cells plated out on selective medium.

#### **2.2.8.7 Lithium acetate-transformation of *S. pombe***

The lithium acetate transformation was adapted from Bahler *et al* (1998). *S. pombe* strains were grown to ~10<sup>7</sup> cells/ml in 500 ml YE medium, and were harvested by centrifugation for 5 min at 1000 g. They were washed once with 200 ml ice-cold dH<sub>2</sub>O and reharvested at 800 g for 1 min. The pellet was transferred to a 1.5 ml microfuge tube and resuspended in 1 ml LiAc/TE (prepared from 10x filter-sterilised stocks of 1M LiAc pH 7.5 and TE: 0.1 M Tris/HCl, 0.01 M EDTA pH 7.5). The cells were centrifuged for a further 1 min at 800 g and

resuspended at an approximate density of  $2 \times 10^9$  cells/ml. 100  $\mu$ l of concentrated cells were mixed with 2  $\mu$ l 10 mg/ml salmon sperm that had been denatured at 95°C for 5 min, and incubated on ice for 4 min. The mix was added 10  $\mu$ l of transforming DNA and incubated at 25°C for 10 min. 260  $\mu$ l of PEG/LiAc/TE was added (8 g PEG-3650 MW 3650, 2 ml 10x LiAc, 2 ml 10x TE and 9.75 ml dH<sub>2</sub>O), and the mixture was agitated and incubated at 25°C for 40-60 min, with 43  $\mu$ l DMSO added prior to heat-shocked for 5 min at 42°C. Finally, the cells were washed once in 1 ml dH<sub>2</sub>O and resuspended in 400  $\mu$ l dH<sub>2</sub>O before plating out onto selective medium.

#### **2.2.8.7.1 Confirmation of stable DNA integration**

Transformed cells were replica plated onto selective medium after incubation for 18 h. Stable colonies were grown for 2-5 days at 30°C, and were picked and streaked onto selective medium. Genomic DNA was extracted and subjected to colony PCR screening.

#### **2.2.8.8 Polymerase chain reaction (PCR)**

All oligonucleotides used in this thesis for DNA amplifications are compiled in Appendix IV. Specific GO annotation has been given according to Glasgow Lab. collection number.

Standard PCR reactions were carried out in a Primus Thermal Cycler using *Taq* DNA polymerase. PCR reactions were carried out in 0.5 ml thin wall PCR tubes. Each reaction mixture contained a final volume of 50 or 100  $\mu$ l with 10x THESIT buffer (300 mM Tris-HCl pH 8.5, 20 mM MgCl<sub>2</sub>, 50 mM  $\beta$ -mercaptoethanol, 1% THESIT), 500  $\mu$ M of each dNTPs, 100 pmole of each primer, 50 ng of plasmid-DNA template or 1  $\mu$ g of genomic-DNA template and 2.5 U of *Taq* DNA polymerase.

The following was the general PCR programme adapted for the standard PCR reaction:

94°C for 5 min	denaturation
30 cycles	-
94°C for 30 sec	denaturation
50°C for 1 min	annealing
68°C for 1 min	extension
68°C for 4 min	further extension

The temperature, number of cycles and enzymes used varied according to oligonucleotides, the type of DNA template used and the purpose of the PCR product generated.

#### **2.2.8.8.1 Expand High Fidelity PCR**

DNA amplification was carried out as described for the standard PCR method (Section 2.2.8.8). However, 2.6 U Expand High Fidelity enzyme was used instead, with 100 pmole of oligonucleotides, 50 ng plasmid DNA or 1 µg genomic DNA templates in a 24 cycle reaction.

#### **2.2.8.8.2 Taq:Pfu (15:1) PCR**

DNA amplification was carried out as described for the standard PCR method except for using 40°C for 30 sec as annealing temperature. Also, 5 U/µl *Taq* DNA polymerase and 0.45 U *Pfu* DNA polymerase were used in a 24 cycle reaction.

#### **2.2.8.8.3 cDNA synthesis and PCR amplification from cDNA**

Following the total RNA extraction (Section 2.2.9.1), appropriate volume of RNA was used in a 20 µl nuclease-free reaction to synthesize cDNA of a specific gene as described in New England Biolabs's manual.

10% of the cDNA from first strand reaction was used for gene amplification by PCR. Specific primers were employed in the PCR reaction to amplify a specific gene. The following were used for 34 cycle amplifications:

10x High Fidelity enzyme (Buffer 2)	5 µl
2 mM dNTP Mix	1 µl
Forward primer (100 pmol/µl)	0.5 µl
Reverse primer (100 pmol/µl)	0.5 µl
High Fidelity enzyme (2.6 U/µl)	0.75 µl
cDNA from first-strand reaction	2 µl
PCR water	to 50 µl

#### **2.2.8.9 Agarose-ethidium bromide gel electrophoresis**

1% agarose (1 g/100 ml) was dissolved in 1x TBE buffer (45 mM Tris-borate, 1 mM EDTA) with 1 µl ethidium bromide (0.5 µg/ml). Prior to running the nucleic acid an appropriate volume of 6x loading dye (0.25% w/v bromophenol blue, 0.25 w/v xylene cyanol FF, 0.15% w/v Ficoll) was added to each sample, to give 1x final concentration. Gels were run in 1x

TBE buffer at 100 V for 40 min, visualized on a UV transilluminator and photographed using E.A.S.Y imaging software.

#### **2.2.8.9.1 DNA fragment purification**

DNA fragments used in ligations and as DNA probes were purified with 0.8-1.2% agarose gels. Fragments were excised and subjected to gel extraction according to QIAquick manual.

Alternatively, DNA fragments generated from PCR reactions were purified by extraction with equal volumes of phenol, phenol/chloroform (1:1) and chloroform, and ethanol precipitated in 2 times volume 100% ethanol and 1/10 volume 3M NaOAc. The precipitate was frozen at -70°C for 2-5 h or -20°C overnight. The following day the DNA was centrifuged for 10 min at 18500 g and resuspended in 70% ethanol. The pellets were air-dried and resuspended in appropriate volume of dH<sub>2</sub>O.

#### **2.2.8.10 Restriction digestion**

DNA fragments were routinely digested as follows:

DNA (~200 ng)	2-3 µl
Appropriate buffer (10x)	2 µl
Appropriate enzyme (10 U)	1 µl
dH <sub>2</sub> O	to 20 µl

Reactions were incubated for 1 h at 37°C or 25°C depending on the restriction enzyme.

Blunt-ended DNA fragments were sometimes used if targeted site/s of the DNA insert does not complement the multiple cloning site/s of the vector. To generate a blunt-ended DNA fragment from a 3' overhang, the 5' end of DNA fragment was cut with an appropriate restriction enzyme. The remaining 3' overhang was removed using 3' to 5' exonuclease activity of T4 DNA polymerase. However, to generate the blunt-ended copy of DNA fragment produced by a 5' overhang, the overhang was repaired using the polymerase activity of the Klenow enzyme. Immediately after completing the digestion reaction, 0.5 µl of 500 mM of dNTPs were added into the reaction mix along with 1 µl of Klenow enzyme and incubated at 30°C for 15 min. Finally the reaction was heat-inactivated at 75°C for 10 min.

#### **2.2.8.10.1 Ligation of DNA**

Ligation of DNA was performed using 1 U of T4 DNA ligase with the recommended buffer provided by the manufacturer. DNA insert to vector ratios of 1:1, 2:1 and 5:1 were used in 16 h incubations at 4°C.

#### **2.2.8.10.2 TA Cloning of blunt ended PCR products**

The TA Cloning Kit was used to clone blunt-ended PCR products. TA Cloning exploits the terminal transferase activity of *Taq* polymerase. The enzyme adds a single, 3'-A overhang to each end of the PCR product. This makes it possible to clone the PCR product directly into a linearised cloning vector with single, 3'-T overhangs. This method used followed the manufacturer's protocol.

#### **2.2.8.11 Purification of radiolabelled DNA probes**

Sephadex G-50 was prepared by adding two volumes of TE and autoclaved. A column made by removing the plunger from a 1 ml syringe (Plastipak) and a small wad of siliconised glass wool was used as a plug. Sephadex G-50 was added to fill the entire syringe and centrifuged for 2 min at 1000 g to remove excessive TE. The procedure was repeated until only 700 µl of Sephadex G-50 remained in the syringe. Finally, the radiolabelled probe was added and centrifuged at 1000 g for 2 min, collected in a microfuge tube.

#### **2.2.8.11.1 Preparation of DNA probe for Northern blotting analysis**

DNA probes were made by PCR amplifications, corresponding to short specific sites or the full length ORF of the gene of interest. The DNA fragments were labelled with [ $\alpha$ -<sup>32</sup>P] dCTP using the random hexanucleotide primed labelling (N<sub>6</sub> labelling) procedure of Feinberg and Volgelstein (1983).

#### **2.2.8.11.2 Preparation of DNA probe for EMSA assay**

DNA fragments from PCR amplifications were 5' end-labelled using T4 PNK. An approximate amount of 5-10 µg DNA in 5 µl of dH<sub>2</sub>O was added to 10 U of T4 PNK in 1x T4 PNK buffer, supplied by manufacturer. Then 10 µCi of [ $\gamma$ -<sup>32</sup>P] ATP was added to the reaction tube to give a total volume of 8 µl. Finally, the reaction was incubated at 37°C for 1 h and the DNA was purified using a Sephadex G-50 column (Section 2.2.8.11).

### **2.2.8.12 Random spore analysis in *S. pombe***

Strains of opposite mating type required for mating were streaked onto a fresh YE plate and grown overnight at an appropriate temperature. A loopful of each was taken and spread onto a small and concentrated area on an ME plate. The two strains were mixed with the aid of a few drops of dH<sub>2</sub>O, and incubated at 25°C for 2-3 days.

A loopful of mated cells was added into 100 µl dH<sub>2</sub>O with 0.5% β-glucuronidase and incubated overnight at 36°C. The mixture was then plated onto solid YE and incubated at 25°C until stable colonies formed – usually 5-7 days. 100 colonies were picked and transferred to another YE plate and incubated at 25°C. The master plate was replica plated onto selective EMM to determine genotypes. Cells with the correct genotype were then streaked onto fresh YE plate to obtain single colonies and the genotype was checked again.

### **2.2.8.13 Tetrad analysis in *S. pombe***

*S. pombe* strains were mated as described for random spore analysis (Section 2.2.8.12). A loopful of mated cells was taken and spread onto a YE plate. Ascospores were picked using a Singer micromanipulator. The cells were incubated at 37°C for 2-5 h until the ascus walls were observed to have broken down. Each ascus was then dissected forming four isolated spores. The spores were incubated until colonies formed. Colonies were then replica plated onto appropriate selective medium to distinguish the genotype of each progeny.

## **2.2.9 RNA Manipulation in *S. pombe***

### **2.2.9.1 Total RNA isolation**

Total RNA was extracted according to method adopted from Kaufer *et al* (1985). Mid-exponentially growing cells were harvested by centrifugation at 1000 g for 5 min from a 20-50 ml experimental culture. Cells were washed once in 1 ml dH<sub>2</sub>O and transferred to a 1.5 ml microfuge tube, and were given a brief centrifugation and the supernatant removed. Cells were stored at -70°C.

To extract RNA, cells were washed once in 200 µl STE (0.32 M sucrose, 20 mM Tris/HCl pH 7.5 and 10 mM EDTA pH 8.0) then resuspended in 200 µl of STE and 600 µl NTES (100 mM NaCl, 5 mM EDTA, 50 mM Tris/HCl pH 7.5 and 1% w/v SDS). The cells were transferred to the preheated (65°C) screw-top tube containing 500 µl of hot phenol and acid-washed glass beads, and then agitated in a Ribolyser for 3 times 40 sec at setting 4.0. Broken cells were centrifuged for 5 min at 18500 g, and the aqueous and protein interface were transferred into a second tube containing 500 µl hot phenol. The tube was again

agitated in the Ribolyser with the same settings before centrifugation. The aqueous phase was removed and transferred into 400 µl of phenol, Ribolysed once 40 sec at setting 4.0, and centrifuged as before.

The aqueous phase was removed to a screw-top tube containing 1:1 phenol/chloroform, mixed and centrifuged. The aqueous phase was then transferred to 300 µl of chloroform, mixed and centrifuged. The aqueous phase was removed to a microfuge tube, 3M NaOAc and 100% ethanol added. The RNA was frozen at -70°C.

The RNA was centrifuged at 18500 g for 10 min and the supernatant discarded. The pellet was then washed in a 150 µl 70% ethanol with RNase-free water. The pellet was left to air-dry and dissolved in 50-100 µl dH<sub>2</sub>O. RNA was stored at -70°C.

#### **2.2.9.2 Formaldehyde gel electrophoresis of RNA**

1% agarose formaldehyde gels were routinely used to separate RNA samples. 1 g agarose was dissolved in 63 ml dH<sub>2</sub>O and 20 ml 5x MNE (120 mM MOPS, 25 mM NaOAc, 5 mM EDTA pH 7.0). The mixture was cooled to 60°C and with 17 ml formaldehyde added (38% aqueous solution). The solution was mixed and kept for 1 min before pouring onto a horizontal casting mould.

RNA samples containing 10 µg of RNA were prepared in 20 µl of RNA buffer (600 µl formaldehyde, 200 µl formamide, 240 µl 5x MNE buffer and 160 µl dH<sub>2</sub>O). 1 µl of 1:50 diluted ethidium bromide was added and each sample incubated at 65°C for 5 min prior to loading onto the gel. Gels were run in 1x MNE buffer at 50 V for 4-5 h, visualized on a UV transilluminator and photographed using E.A.S.Y imaging software.

#### **2.2.9.2.1 RNA transfer onto nitrocellulose membrane**

Prior to RNA transfer, the RNA gel was soaked in 50 mM NaOH for 15 min to facilitate transfer of larger transcripts. The gel was next soaked in 10 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> pH 6.5 for 15 min, to remove residual formaldehyde. The nylon membrane, Genescreen, was also briefly soaked, first in dH<sub>2</sub>O then in 10 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> pH 6.5 to equilibrate the membrane, before blotting. The transfer of RNA from the gel onto the membrane was facilitated by capillary action according to Sambrook *et al* (2001). Transferred RNA was fixed to the membrane by crosslinking in a UV oven at 1200 X 100 J/CM<sup>2</sup> or baked at 80°C for 3 h.

#### **2.2.9.2.2 Membrane probing with radiolabelled DNA fragment**

The RNA membrane was prepared for hybridisation by preincubating at 42°C, 1-2 h in 18 ml prehybridisation buffer (10 ml formamide, 4 ml P buffer, 4 ml 33% w/v Dextran sulphate, 1.16 g NaCl, 200 µl 10 mg/ml denatured salmon sperm DNA) in Techne oven. [P buffer: 1% BSA, 1% pyrrolidine, 1% Ficoll, 250 mM Tris/HCl, 0.5% Na Pyrophosphate, 5% SDS]. The denatured radiolabelled DNA probes were then added and left to hybridize overnight.

The following day, membrane washed twice at 5 min intervals in 2x SSPE (20x stock: 3 M NaCl, 20 mM NaHPO<sub>4</sub>, 200 mM EDTA pH 7.4) at 42°C. The membrane was then washed in 0.2x SSPE + 0.5% SDS at 65°C for 15 min. Finally, the membrane was rinsed in 0.1x SSPE, wrapped in Saran wrap before exposure to an autoradiography film at -70°C, overnight.

If required for rehybridisation, the membrane probe was removed in stripping solution (5 mM Tris/HCl pH 8.0, 0.2 mM EDTA, 0.05% Na pyrophosphate, 0.002% polyvinyl pyrrolidine, 0.002% BSA and 0.002% Ficoll) at 70°C for up to 2 h.

#### **2.2.9.2.3 Quantification of Northern blots**

Intensity of radioactivity signals was quantified using the Image J programme, which was normalised against the control signal at the corresponding rRNA bands. Triplicate measurements were made for each sample and the results from at least two separate experiments were averaged to give the results shown.

### **2.2.10 Protein Manipulation**

#### **2.2.10.1 Small scale protein extraction in *S. pombe***

200 ml mid-exponentially growing cultures were harvested by centrifugation at 1000 g for 5 min. The cells were washed once with ice-cold dH<sub>2</sub>O and transferred to 1.5 ml microfuge tubes. The cells were then resuspended in 200 µl of ice-cold lysis buffer (50 mM KCl, 50 mM Tris/HCl pH 8.0, 25% Glycerol, 2 mM DTT, 0.1% Triton X-100, 25 µg/ml each of protease inhibitors from a stock of 5 mg/ml: chymostatin, pepstatin, antipain, leupeptin, aprotinin, and 0.2 mM PMSF) in screw-top microfuge tubes. The cells were pelleted at 18500 g for 1 min with the supernatant discarded and resuspended in 200 µl lysis buffer. Acid washed glass beads were added and the tubes were chilled on ice for 2-3 min before being disrupted for 3 times 40 sec using a Ribolyser at setting 4.0. During the disruption process, the cells were place on ice for 1 min between each agitation. Finally, the cell debris was pelleted by centrifugation at 18500 g for 10 min and the supernatants transferred to a fresh chilled 1.5

ml microfuge tube. To clarify the supernatants, the tubes were re-centrifuged at 18500 g for a further 30 min and transferred to another microfuge tube. 1 µl of supernatant was withdrawn from the sample to determine protein concentration, which was estimated using Bradford reagent, prepared according to the manufacturer's instructions. The remaining protein samples were stored at -70°C.

### **2.2.10.2 Large scale protein extraction in *S. pombe***

4 litres of late-exponentially growing culture ( $2\text{-}5 \times 10^7$  cells/ml or  $\text{OD}_{595}$  1-1.4) were harvested by centrifugation at 10000 rpm, JA 14 rotor, for 10 min. The cells were washed once with ice-cold dH<sub>2</sub>O, pooled and transferred to centrifuge tubes. The cell pellet was then washed in 300 ml of ice-cold Buffer E (150 ml NaCl or 260 mM NaCl or 350 mM NaCl, 20 mM Na-Hepes pH 8.0, 0.1% Tween 20 and 10% glycerol) and stored at -70°C.

Cells were resuspended in 20 ml ice-cold Buffer E and disrupted using a Cell Combustion Breaker at 30 kpsi for 3 passes. It was important that the tubes used to collect the cells were kept chilled at all times. The cells were collected in a chilled centrifuge tube and protease inhibitors added (from a stock of 5 mg/ml each: 1 µg/ml chymostatin, 1 µg/ml pepstatin, 0.5 µg/ml antipain, 0.5 µg/ml leupeptin, 2 µg/ml aprotinin, 1 mM PMSF) to a final volume of 30 ml. The cell debris was centrifuged at 1500 g for 10 min. The supernatant was then subjected to clarification at 45000 rpm, T865 rotor, for 45 min and stored at -70°C.

#### **2.2.10.2.1 TAP purification**

All procedures were carried out at 4°C. The column volumes of beads to buffers were 1:1, unless otherwise stated, and incubations performed on a rotation platform. The method was pioneered by Rigaut *et al* (1999) and adapted in *S. pombe* by Tasto *et al* (2001).

A Poly-Prep Chromatography column containing 500 µl IgG Sepharose™ 6 Fast Flow beads was equilibrated in a total of 25 ml Buffer E (350 mM NaCl, 20 mM Na-Hepes pH 8.0, 0.1% Tween 20 and 10% glycerol) in a 5 ml increment. Aliquots of pre-equilibrated IgG Sepharose beads incubated with clarified supernatant and allowed to rotate for 2 h. The supernatant-beads mixtures were then transferred into the initial chromatography column and allowed to pack by gravity. The column was washed 3 times and incubated for 15 min in 10 ml Buffer E.

5 ml of freshly prepared TCB (TEV Cleavage Buffer) (10 mM Tris pH 8.0, 150 mM NaCl, 0.5 mM EDTA and 0.1% Tween 20) was used to equilibrate the beads for 3 times at 15 min incubation intervals. The beads were then drained in 25 ml TCB at 5 ml increments. 300-500 U of TEV proteases and 2 ml TCB was then added, and incubated at 16°C for 2 h.

A fresh column containing 300 µl of calmodulin resin was prepared by equilibrating with 10 ml CBB (calmodulin binding buffer: 10 mM Tris pH 8.0, 150 mM NaCl, 1 mM MgAc, 1 mM imidazole, 2 mM CaCl<sub>2</sub>, 0.1% Tween 20, 10 mM β-mercaptoethanol and 10% glycerol). The column was sealed and the beads immersed in 7 ml CBB and 7 µl 1M CaCl<sub>2</sub>, which neutralises the EDTA from TCB. Then, the cleavage mix was drained into the CBB column. 500 µl TCB was next loaded onto the cleavage mix column to elute residual mix. The second CBB column was incubated for 1 h. The beads containing bound protein were washed 3 times with 1 ml CBB and the procedure was repeated twice in 10 ml CBB with 15 min incubation intervals. Finally the beads were eluted in 5 fractions of 300 µl CEB (CBB elution buffer: 10 mM Tris pH 8.0, 150 mM NaCl, 1 mM MgAc, 1 mM imidazole, 2 mM EGTA, 0.1% Tween 20, 10 mM β-mercaptoethanol and 10% glycerol). All fractions were pooled and concentrated using Microcon concentrator and processed using precast Tris/glycine-PAGE 4-20% gradient gel according to the Laemmli *et al* (1970) method (Section 2.2.10.6).

#### **2.2.10.3 Quantification of protein by Bradford assay**

The method of Bradford (1976) was routinely applied. A standard curve was produced using known BSA concentrations. The absorbance of samples was measured at 595 nm and the concentrations extrapolated from the standard curve.

Using the Bradford method, 1 in 5 dilution of Bradford reagent was used to dilute 1 in 1000 of protein sample. The protein mixes were measured using spectrophotometer at A<sub>595</sub>. The OD<sub>595</sub> reading was calculated as follow:

$$X \mu\text{g}/\mu\text{l} = \text{OD}_{595} + 0.0735$$

#### **2.2.10.4 Protein extraction in *S. cerevisiae***

Cell pellets were transferred to 1.5 ml screw-top microfuge tubes and resuspended in 500 µl of ice-cold breakage buffer (GST-tagged fusion protein: 50 mM Tris pH 7.5, 250 mM NaCl, 4 mM DTT, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10% glycerol, 1% NP-40 and protease inhibitors from a stock of 5 mg/ml each: 1 µg/ml chymostatin, 1 µg/ml pepstatin, 0.5 µg/ml antipain, 0.5 µg/ml leupeptin, 2 µg/ml aprotinin and 1 mM PMSF) (His-tagged fusion protein: 50 mM Tris pH 7.5, 500 mM NaCl, 0.1% NP-40, 20 mM imidazole, 5 mM β-mercaptoethanol and protease inhibitors from a stock of 5 mg/ml each: 1 µg/ml chymostatin, 1 µg/ml pepstatin, 0.5 µg/ml antipain, 0.5 µg/ml leupeptin, 2 µg/ml aprotinin and 1 mM PMSF). Acid-washed glass beads were added and the cells disrupted using a Ribolyser, 3 times 10 sec at setting 4.0. The cells were placed on ice between every

agitation for 1 min. The cell lysate was collected by inserting the screw-top microfuge tube (pierced at the bottom with a hot needle) containing disrupted cells into a 15 ml centrifuge tube and briefly centrifuged at 500 g, 4°C. The cell lysate was subjected to a similar second round of disruption, with fresh acid-washed glass beads. The supernatant was clarified in two rounds of centrifugation at 18500 g for 10 min and 15 min.

#### ***2.2.10.4.1 Column purification of GST-tagged proteins***

100 µl of Glutathione Sepharose 4B beads were prepared by equilibrating in 2 times 5 column volumes of GST wash buffer (50 mM Tris pH 7.5, 250 mM NaCl and 1 mM DTT). The GST-tagged beads were then equilibrated in breakage buffer at 5 column volumes. The clarified supernatant was loaded onto the pre-equilibrated column and incubated at 4°C, 2 h.

The supernatant was drained and the column was given 5 washes in 5 column volumes of wash buffer. A 15 µl elution buffer (50 mM Tris pH 7.5, 250 mM NaCl, 0.1% Triton X-100 and 20 mM reduced glutathione) was added and incubated for 15 min and finally the elution fraction was collected and stored at -70°C.

To analyse the eluted sample, a small aliquot of 5-10 µl of elution sample was added to an appropriate volume of Laemmli sample buffer and boiled at 95°C for 5 min. The sample was then processed using SDS-PAGE.

#### ***2.2.10.4.2 Column purification of His-tagged proteins***

100 µl Chelating Fast Flow slurry was washed with 10 column volumes of His wash buffer (50 mM Tris pH 7.5, 500 mM NaCl, 0.1% NP-40 and 20 mM imidazole) to remove excess ethanol. The column-containing beads was then loaded with 10 column volumes of zinc ions (0.1 M ZnCl<sub>2</sub> pH 4.5-5.0) to the imidodiacetate binding sites, and incubated at 4°C for 45-60 min on a rotating platform. The zinc ions were removed with 3 times washes in 10 column volumes dH<sub>2</sub>O followed by 5-10 column volumes of 0.5 M NaCl. The beads were then equilibrated in 2 times 5 column volumes of His wash buffer. Finally His-tagged beads were equilibrated in breakage buffer, 5 column volumes. Clarified supernatant was loaded onto pre-equilibrated column and incubated at 4°C on a rotating platform for 2 h.

The column was washed 5 times in 5 column volumes of wash buffer. A 15 µl elution buffer (50 mM Tris pH 7.5, 500 mM NaCl, 0.1% Triton X-100 and 250 mM imidazole) was added and incubated for 15 min and finally, the elution fraction was collected and stored at -70°C.

To analyse the eluted sample, a small aliquot of 5-10 µl elution sample was processed using SDS-PAGE.

### **2.2.10.5 Preparation of tagged protein from bacteria**

The pellet from a GST-protein culture (50-200 ml) was resuspended in 3-10 ml 1x PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub> and pH to 7.4) while the pellet from His-protein culture (50-200 ml) was resuspended in 3-10 ml Buffer A (100 mM NaCl, 5 mM imidazole, 20 mM KH<sub>2</sub>PO<sub>4</sub> and pH to 7.5). The cells were disrupted under high pressure using French Pressure at 750 psi for 50-100 ml cell culture or 950 psi for 200 ml cell culture. Typically, 3 passes were made before adding protease inhibitors. The supernatant was clarified by centrifugation at 18500 g for 15 min and subsequently used.

#### **2.2.10.5.1 Column purification of GST-tagged proteins**

The column containing 100-200 µl Glutathione Sepharose 4B beads was prepared by equilibrating with 2x 5 column volumes of 1x PBS. Freshly clarified supernatant was loaded onto the column and incubated at 4°C, for 45-60 min. The column was given 3 washes in 5-10 column volumes of 1x PBS. Finally the beads were incubated for 10 min in elution buffer (10 mM reduced glutathione in 50 mM Tris/HCl at pH 8.0). Several 400 µl of elution fractions were collected and stored in -70°C.

To analyse the sample, a 10 µl aliquot was added to an appropriate volume of Laemmli sample buffer and processed using SDS-PAGE.

#### **2.2.10.5.2 Column purification of His-tagged proteins**

The column was prepared by washing the Chelating Fast Flow slurry with 25 column volumes of Buffer A, to remove excess ethanol. The column was then loaded with 10 column volumes of zinc ions (0.1 M ZnCl<sub>2</sub>, pH 4.5-5.0), to bind the imidodiacetate binding sites, and incubated at 4°C for 45-60 min on a rotating platform. The zinc ions were removed by 3 washes in 10 column volumes dH<sub>2</sub>O followed by 5-10 column volumes of 0.5 M NaCl. The column was next washed with 5 column volumes of Buffer B (100 mM NaCl, 500 mM imidazole and 20 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.5). Finally the column-containing beads were equilibrated in 5 column volumes of Buffer A.

The clarified supernatant was transferred into the column and incubated at 4°C, rotating, for 45-60 min. The column was given 2 washes in 5-10 column volumes of Buffer A and finally incubated for 10 min in Buffer B. Several 400 µl of elution fractions were collected and stored at -70°C.

To analyse the sample, a 10 µl aliquot was added to an appropriate volume of Laemmli sample buffer and processed using SDS-PAGE.

## **2.2.10.6 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)**

Standard Laemmli *et al* (1970) (Appendix III) methods were used with slight adaptation according to the proteins used. Proteins were resolved under denaturing conditions. Samples for analyses on SDS-PAGE were resuspended in 2x Laemmli sample buffer (62.5 mM Tris, 2% SDS, 10% sucrose, Pyronin Y dye, pH 6.8) to a final concentration of 1x, to which 150 mM DTT was added prior to boiling for 5 min at 95°C. 25 µl of protein sample was loaded onto the gel along with an appropriate volume of molecular weight markers (10-15 µl of Full-Range Rainbow marker or 5 µl when using BenchMark marker). Gel was run using the Bio-Rad Mini Protean gel kit system at 120 V for 1 h 30 min in 1x tank buffer (10x tank buffer: 250 mM Tris/HCl, 1.92 M glycine 3.5% SDS and pH adjusted to 8.3). Gels were stained either in Coomassie Brilliant stain (0.1% w/v Brilliant Blue R250, 10% v/v acetic acid and 50% v/v methanol) for 1 h or processed for transfer onto nitrocellulose membrane. Coomassie Blue stained proteins were destained in 10% v/v acetic acid and 10% v/v methanol for 2 h, or overnight.

### **2.2.10.6.1 Immunoblotting using Enhanced Chemiluminescence (ECL)**

The Western blotting method was adapted from Towbin *et al* (1979). Proteins were transferred from the gel to a nitrocellulose membrane using Bio-Rad gel kit system at 52 V for 2 h in 1x transfer buffer (10x transfer buffer: 250 mM Tris/HCl pH 7.2, 1.92 M glycine and 0.3% w/v SDS). Prior to use, 1x transfer buffer was added to 20% v/v methanol. Once transfer was completed, staining of the nitrocellulose membrane with the non-fixative dye Ponceau S solution was employed to ensure efficiency of protein transfer. Ponceau S was removed by rigorous rinsing in dH<sub>2</sub>O and the protein non-specific binding sites blocked by immersing the nitrocellulose membrane in 1x wash buffer (20x wash buffer: 20 mM Tris/HCl pH 7.2, 15 mM NaCl, 0.1% v/v Tween-20 and pH adjusted to 7.0; with the addition of 1-5% final concentration of non-fat milk) for 1 h. The membrane was washed twice for 10-15 min with 1x wash buffer, and incubated in primary antibody according to manufacturer's recommendation. The blot was incubated for 2-4 h or 4°C overnight.

The membrane was next washed 3 times at 15 min intervals in wash buffer, then incubated in secondary antibody for 2 h and washed for 15 min, 3 times.

The detection step was carried out as described in the Amersham protocol for ECL detection. In the dark room, autoradiography film was placed onto the membrane and exposed for an appropriate time according to the required intensity of detection. The autoradiography film was then processed.

### **2.2.10.6.2 Silver staining of protein gels**

The silver staining procedure was adapted from Heukeshoven and Dernick (1988), and processed according to Invitrogen's manual. However, slight modifications were made to complement mass-spectrometry analysis. Based on the protocol, the gel was fixed twice at 15 min intervals each; or once overnight and another at 15 min. Formaldehyde was omitted from Silver solution while it was included at double the recommended concentration in developing solution. Finally, glutaraldehyde was removed from the sensitising solution.

### **2.2.10.7 Mass-spectrometry analysis**

Mass spectra were determined in collaboration with Dr. Burchmore from Sir Henry Wellcome Functional Genomics Facility, University of Glasgow.

Gel bands were broken into approximately 1 mm cubes, destained and dehydrated by sequential washing with 300 µl of 25 mM ammonium bicarbonate, 50% 25 mM ammonium bicarbonate in acetonitrile and 100% acetonitrile, followed by drying in a centrifugal evaporator (Univap, Uniscience). Gel slices were rehydrated in 20 µl of 20 ng/ml trypsin in 25 mM ammonium bicarbonate for 15 min. Additional 25 mM bicarbonate was then added to ensure the gel slice was covered and the digest incubated at 37°C overnight. The supernatant was removed, acidified by the addition of 1 µl 5% formic acid and used directly for mass spectrometry or frozen at -20°C until required. 10 µl aliquots of the samples were subjected to LC-MS on a QStar Pulser I electrospray mass spectrometer fitted with a nanospray source (Protana), using a 20 µ i.d., 10 µ orifice distally coated electrospray tip mounted in a ProADP2 adaptor (New Objective) connected to a nanoflow LC system (LC Packings) with the minimum length of 20 µ i.d. fused silica capillary possible. NLC was performed using a 0.3x5 mm reversed phase trap (PepMap C18, Dionex) and a 75 µ x 15 cm PepMap C18 reversed phase column (Dionex) using standard methodology, with a loading pump flow of 30 µl/min and main flow of 200 nl/min. Peptides were trapped on the C18 trap and desalted for 5 min before being separated using a 5-40% acetonitrile gradient over 15 min. All solutions contained 0.5% formic acid. Mass spectrometric analysis was performed in IDA mode (AnalystQS software, Applied Biosystems), selecting the 4 most intense ions for MSMS scans of 50-2000 Da using the standard rolling collision energy settings. IDA was triggered for ions with charge states 2-4 above a threshold of 10 counts. Masses were then added to the exclusion list for 3 min. Peaks were extracted using the Mascot script (BioAnalyst, Applied Biosystems) and automatically exported to the mascot (Matrix Science) search engine. Data was searched against a database using the MASCOT Daemon, with a peptide tolerance of 1.0 Da, an MSMS ion tolerance of 0.5 Da allowing for 1 missed cleave and variable methionine oxidation.

## **2.2.11 In-vitro Assay**

### **2.2.11.1 Electro-mobility shift assay (EMSA)**

EMSA was performed by adding 10 µl of sample buffer (1 M Tris/HCl pH 7.5, 1 M KCl, 50% Glycerol, 100 mM DTT, 100 mM protease inhibitor: chymostatin, pepstatin, antipain, leupeptin, aprotinin, 100 mM PMSF, 1 M MgCl<sub>2</sub>) to 20 µg of protein, 1 µl of dIdC (1 mg/ml) and 1-2 µl of labelled probe (Section 2.2.8.11.2). If required, non-specific or specific DNA was also added. Samples were incubated on ice for 5 min before the addition of each reagent. Analysis of the formation of protein-DNA complexes was achieved by electrophoresis on a 10% acrylamide gel (5 ml 40% solution acrylamide, 45 ml 0.5x TBE, 400 µl 10% APS and 100 µl TEMED) in 1x TBE buffer (45 mM Tris-acetate, 1 mM EDTA pH 8.0) for 1.5-2 h at 180 V. The gel was dried 1 h at 80°C and exposed to an autoradiography film at -70°C.

### **2.2.11.2 Kinase assay**

Following the purification of recombinant enzyme (Section 2.2.10.4.1 and 2.2.10.4.2), a reaction mix containing 10 µl of 25 mM Tris/HCl pH 7.5, 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 1 mM DTT, 30 µM cold ATP, 2 µCi [ $\gamma$ -<sup>32</sup>P] ATP, 1 µg/µl recombinant enzyme and dH<sub>2</sub>O were added to 5 µg/µl of the recombinant substrate (Sections 2.2.10.5.1 and 2.2.10.5.2) and made up to 30 µl. The enzyme-substrate mixture was incubated at 30°C for 20-30 min and the reaction stopped by the addition of Laemmli sample buffer. The mixture was subjected to SDS-PAGE analysis. The gel was stained in colloidal Coomassie Brilliant Blue and destained according to the adopted protocol from Neuhoff *et al* (1988). The gel was fixed in fixing solution (10% v/v acetic acid and 50% v/v methanol) for 30 min, then stained in colloidal Coomassie Blue solution and incubated for at least 3 h. The gel was rinsed with destain (25% v/v methanol) 3 times, 1 min each.

The gel was dried for 1 h at 80°C and exposed to autoradiography film.

## **2.2.12 Light and Fluorescence Microscopic Observations in *S. pombe* Cells**

### **2.2.12.1 Differential interference contrast (DIC) microscopy**

To visualise cells, mutant and wild-type asynchronous cultures were grown to a density of  $5 \times 10^6$  cells/ml. Cells were concentrated by centrifugation and 5-7 µl of cell suspension was placed on a microscope slide. DIC microscopy study was performed with Sony DS-75 digital camera attached to Zeiss Axiostar R-grade microscope integrated with 40x and 100x DIC filter set.

### **2.2.12.2 4'-6-Diamidono-2-phenylindole (DAPI)-dye staining**

To visualize DNA, cells were grown to mid-logarithmic phase. The cells were concentrated by centrifugation and 5-7 µl of cell suspension were combined with 1 µl of 1 µg/ml DAPI and placed on a microscope slide. DNA was observed by excitation at ~365 nm and photographed with Sony DS-75 digital camera.

### **2.2.12.3 Calcofluor-dye staining**

To visualise septum, mid-logarithmic growing cells were concentrated by centrifugation and a concentration of 1 mg/ml of Calcofluor Fluorescence Brightener was added to 50-100 µl of cell suspension and incubated for 5 min in the dark. The cells were briefly centrifuged at 10000 g and the supernatant removed. The cells were washed twice with 1x PBS and 5-7 µl of cell suspension was then spotted onto a microscope slide.

## **Chapter 3: Components of PBF**

### **3.1 Introduction**

In eukaryotes, the developmental control of a cell occurs during the G1 interval – termed “start” in yeasts and restriction point (R) in higher eukaryotes. Depending on physiological cues, cells decide whether to enter a new mitotic cycle, advance into a quiescent phase or alternatively, differentiate into the meiotic cell cycle. Once committed, cells must complete the cycle of choice before making the decision again.

Cell development processes such as growth and division consist of highly complicated mechanisms, which require precisely co-ordinated gene expression. This prompted the study of molecular processes towards understanding how gene expression occurs.

To date, many molecular forms of control of gene regulation have been identified, with transcription being found to play a major role (Struhl, 1989). Furthermore, a consensus has emerged that many of the control mechanisms in gene transcription are fundamentally conserved among eukaryotes (Struhl, 2001).

The basic rationale behind understanding gene regulation was pioneered by Jacob and Monod in the 1960s (Jacob and Monod, 1961). Subsequently, scientists discovered three highly associated but distinct DNA units that are necessary for transcription to occur: promoters, operators and enhancers (Struhl, 1999). Promoters confer transcription expression at basal levels and are recognized by RNA polymerase machineries, containing all the necessary information for an accurate transcription initiation (Roeder, 1996). Operators consist of inhibitory elements that block transcription which otherwise would occur from the promoter. Enhancers, also known as upstream activating sequences (UAS) in yeasts, are short DNA sequences found either upstream or downstream of the promoter, required for maximal level of transcription (Roeder, 1998).

Basal levels of transcription for protein-encoding genes are controlled by the RNA polymerase II transcription machinery (RNAP II), composed mainly of two elementary components, TFIID and the RNAP II holoenzyme. TFIID has high affinity for specific TATA box-containing promoter sequences, while the RNAP II holoenzyme consists of RNA polymerase II (RNAP II), general transcription factors (GTF) and other associated proteins. *In vitro*, the basic RNAP II machinery is sufficient to generate a modest but accurate transcription initiation on model promoters (Ptashne and Gann, 1997). Nevertheless, the key problem in gene regulation is to understand how, in eukaryotic organisms, such diverse range of genes can be activated precisely, while maintaining the regulatory balance within the cell environment. It is believed that the answer lies in the enhancers, which have been

shown to form an essential component for all temporal-spatio regulated genes (Struhl, 1999; 2001).

### **3.1.1 Stage-specific cell cycle control**

Transcriptional regulation is a major form of control in gene expression during the cell cycle among eukaryotes, including yeasts such as *S. pombe* and *S. cerevisiae* (Breedon, 2000; McInerny, 2004). In the past, conventional methods, such as single gene analysis and specific mRNA measurements have identified many cell cycle regulated genes. However, the availability of the complete genome sequences of a large number of organisms, and the advent of large-scale array technology has accelerated the measurement of regulated transcript profiles. Microarray analyses have revealed that more than 10% of protein-encoding genes are periodic in *S. cerevisiae* with 8% in *S. pombe* (Spellman *et al*, 1998; Cho *et al*, 1998; Rustici *et al*, 2004).

Recent understanding of periodically transcribed genes throughout the cell cycle has created a paradigm described by Breedon (2000), based on pioneering work in *S. cerevisiae*. It outlines a gene clustering system characterised by consecutive waves of transcriptional activity. According to this model, periodic genes are grouped into four clusters corresponding to the four main cell cycle stages. In *S. pombe*, a similar approach was applied which identified related transcriptional waves (Anderson *et al*, 2002; Martin-Cuadrado *et al*, 2003; Rustici *et al*, 2004).

The next key questions are how and what facilitate these waves of gene expression?

Periodic gene expression is initiated by specific transcription factors. Several of these are conserved across the two yeasts. For example, the SBF and MBF complexes in *S. cerevisiae* are functional orthologues of the MBF/DSC1 complex in *S. pombe* (McInerny, 2004).

In this thesis, we describe the characterisation of the mechanisms that control M/G1-specific transcription in *S. pombe*.

### **3.1.2 A novel mechanism in transcriptional regulation**

A gene can have long distance contact between the core promoter and enhancer resulting in long haul transcriptional activation. This occurs by DNA looping controlled by protein-protein contacts between the RNAP II machinery and activators (Stefanovsky *et al*, 2001). Increasing evidence has suggested that an additional group of proteins are associated with the RNAP II machinery, named the Mediator complex (Malik and Roeder, 2000). Chromatin Immunoprecipitation (ChIP) experiments demonstrated that activator proteins can interact with many components of RNAP II machinery, but it is still poorly understood which

interactions are physiologically significant. Studies have shown that the link lies in the Mediator complex (Myers and Kornberg, 2000).

The identification of the Mediator as a target for transcriptional activators was initiated by biochemical analyses in *S. cerevisiae* (Flanagan *et al*, 1991; Kim *et al*, 1994). These studies suggested the existence of coactivating molecules besides transcription factor activators.

Although Mediator proteins do not initiate basal RNAP II transcription from core promoters, they are often considered as components of the basic RNAP II machinery (Malik and Roeder, 2000). In particular, several subunits are essential for general RNAP II transcription in yeast and form a stable complex with RNAP II, often called the RNAP II holoenzyme (Koleske and Young, 1994; Kim *et al*, 1994). Therefore, it appears that the Mediator serves as a bridge that connects the transcription activating proteins at their target sites to the basic RNAP II machinery at the core promoter site.

These studies were further enhanced by observations that metazoan Mediator coactivators also function in yeast (Malik and Roeder, 2000). Subunits of the Mediator are conserved from yeasts to humans, indicating a functional conservation between the transcriptional apparatus across the eukaryotic evolution (Gu *et al*, 1999; Sato *et al*, 2004).

### **3.2 Isolation and Characterisation of PBF Properties in *S. pombe***

#### **3.2.1 Introduction**

The *S. pombe* *cdc15<sup>+</sup>* gene was initially identified through a cell cycle mutant screen and subsequent characterisation showed that it encodes a key component of the actin contractile ring (Nurse *et al*, 1976; Fankhauser *et al*, 1995). Possibly relevant to its function, *cdc15<sup>+</sup>* mRNA levels oscillate with an accumulation during late mitosis.

To understand late mitotic processes in *S. pombe*, we identified a group of genes that undergoes M/G1 phase-specific transcription, which includes *cdc15<sup>+</sup>*. Our studies revealed a functional UAS in the promoter of *cdc15<sup>+</sup>* and subsequent studies on other M/G1 genes identified a related DNA sequence motif, which we named the PCB (Pombe cell cycle box) (Utzig *et al*, 2000; Anderson *et al*, 2002). In addition, we found a transcription factor complex that we named PBF (PCB-binding factor) that binds specifically to the PCB.

Comparative studies among model eukaryotic organisms have allowed the rapid identification of conserved mechanisms and properties. Our discovery of PBF complemented the G2/M-specific transcription factor complex of *S. cerevisiae*, Swi five factor (SFF), and the mammalian forkhead homologue transcription factors (FKH-TFs), also necessary for cell cycle completion (Kumar *et al*, 2000; Alvarez *et al*, 2001).

Therefore, to identify transcription factors required for M/G1 gene expression in *S. pombe*, we began by searching for potential forkhead homologues. Four genes encoding forkhead proteins were found, and it was first necessary to test whether these proteins are required for M/G1 transcription in *S. pombe*.

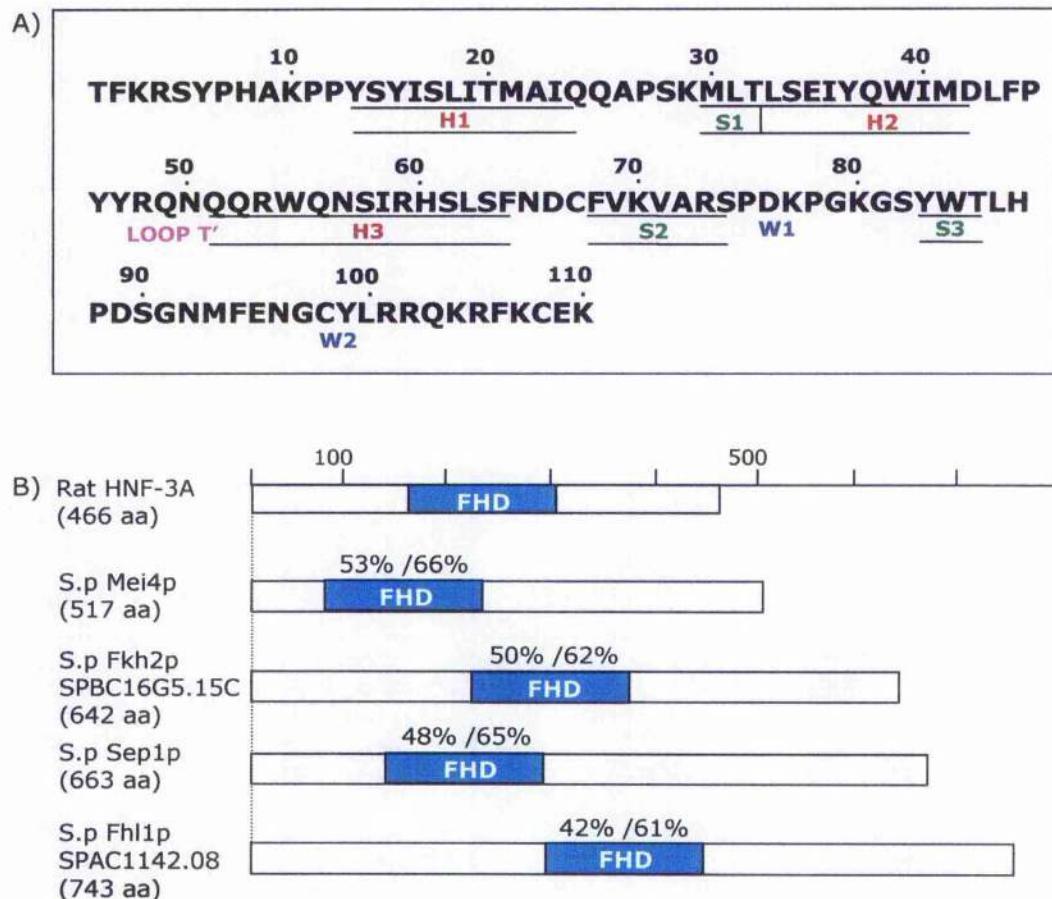
### **3.2.2 Sequence analysis of forkhead transcription factor proteins**

Searching the *S. pombe* protein database by peptide sequence homology revealed the presence of at least four closely related open reading frames (ORFs) that encode forkhead-like transcription factors: *sep1<sup>+</sup>*, *mei4<sup>+</sup>*, SPBC16G5.15c and SPAC1142.08 (Wood *et al.*, 2002; Buck *et al.*, 2004). The sequence alignment was completed using a 110 amino acid sequence initially identified in rat HNF-3A (hepatocyte nuclear factor 3A), against full length forkhead-like proteins of *S. pombe*, which identified the conserved regions, as illustrated in Figure 3.1.A (Kaufmann and Knochel, 1996). The conserved 110 amino acid sequence contained a subclass of DNA-binding motif characteristic of the helix-turn-helix motif, hereafter known as forkhead homologue DNA-binding domain (FHD). *S. pombe* Mei4p shares the highest degree of identity (53%) and similarity (66%) across its FHD region to the rat HNF-3A FHD (Fig. 3.1.B). Next was SPBC16G5.15c encoding a polypeptide with 50% identity and 62% similarity, Sep1p with 48% identity and 65% similarity, and SPAC1142.08 encoding a polypeptide with 42% identity and 61% similarity, between FHD regions.

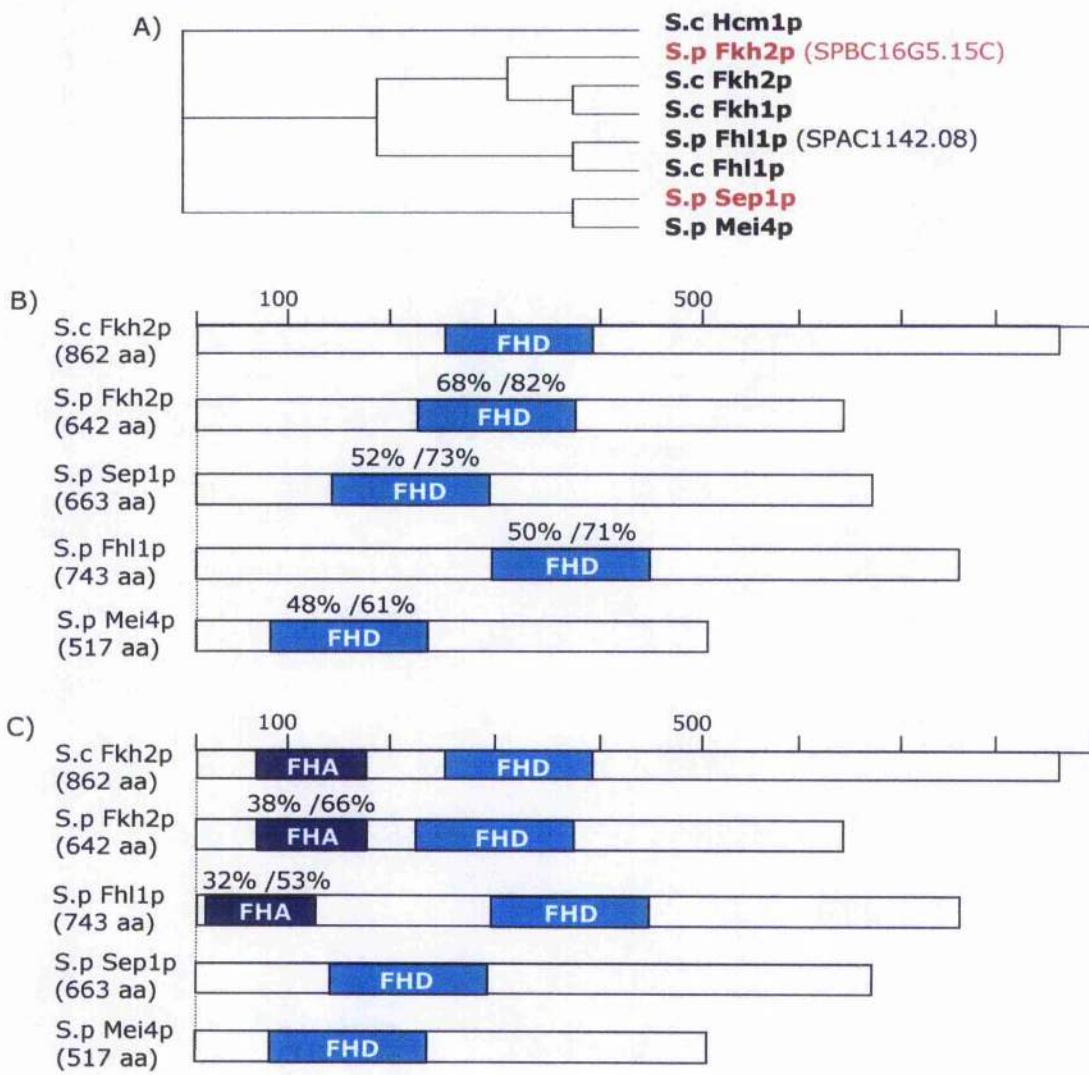
*sep1Δ* cells have previously been shown to exhibit cell cycle defects characteristic of division mutants (Ribar *et al.*, 1999). Further analysis revealed that they abolished the periodicity accumulation of *cdc15<sup>+</sup>* mRNA and so Sep1p was proposed to have a role in *cdc15<sup>+</sup>* expression (Zilahi *et al.*, 2000). Sequence analysis identified Sep1p as a potential DNA binding protein encoding a winged helix motif belonging to the HNF-3/FHD family (Ribar *et al.*, 1999). Subsequent immunofluorescence studies supported the DNA-binding property of Sep1p as it was found to be nuclear. Taken together, Sep1p is potentially a transcription factor required to control *cdc15<sup>+</sup>* M/G1 expression.

Using the ClustalW programme aligned across complete peptide sequences of *S. cerevisiae* and *S. pombe* FHD proteins, a phylogenetic tree was generated (Fig. 3.2.A). This showed that *S. pombe* SPAC1142.08 shares homology to *S. cerevisiae* Fhl1p, while *S. pombe* SPBC16G5.15c shares closest homology to *S. cerevisiae* Fkh2p. Therefore, we designated the former as *S. pombe* Fhl1p and latter as Fkh2p.

Sequence alignment against FHD region of *S. cerevisiae* Fkh2p showed 68% identical and 82% similar to the FHD region of *S. pombe* Fkh2p (Fig. 3.2.B). While the other *S. pombe* FHD proteins, Sep1p, Fhl1p and Mei4p, displayed a lower but similar degree of



**Figure 3.1** Forkhead-homologue domain (FHD) proteins in *S. pombe*. A) The DNA-binding domain of the rat HNF-3A transcription factor which encompasses a region of 110 amino acid (aa) residues. Structural elements of the domain are shown on top. **H** denotes  $\alpha$ -helix, **S** denotes  $\beta$ -sheet, **T** denotes loop region and **W** denotes wing-like structure. B) Amino acid sequence comparisons of FHD regions in *S. pombe* Mei4p, SPBC16G5.15C (Fkh2p), Sep1p and SPAC1142.08 (Fhl1p) against rat HNF-3A. Percent identity/percent similarity values are shown for FHD conserved regions.



**Figure 3.2** Forkhead-homologue domain (FHD) proteins. A) Phylogenetic tree showing the relationships between *S. cerevisiae* and *S. pombe* FHD proteins based on full-length peptide sequence alignments generated using ClustalW program. B) Amino acid sequence comparison of FHD region in *S. cerevisiae* Fkh2p with *S. pombe* Fkh2p, Sep1p, Fhl1p and Mei4p. C) Amino acid sequence comparison of forkhead homologue-associated domain (FHA) region in *S. cerevisiae* Fkh2p with *S. pombe* Fkh2p and Fhl1p. Sep1p and Mei4p do not contain FHA region of homology. Percent identity/percent similarity values are shown for FHD or FHA conserved regions.

homology: Sep1p 52% identical and 73% similar; Fhl1p 50% identical and 71% similar; and Mei4p 48% identical and 61% similar, between the FHD regions.

Further analysis among *S. cerevisiae* Fkh2p and all of *S. pombe* FHD proteins identified another conserved region known as forkhead homologue-associated (FHA) domain. The FHA domain was originally identified in FHD proteins, but was later found in a wide range of other types of transcriptional regulators (Durocher and Jackson, 2002). It has been implicated as a phosphopeptide-binding domain that mediates phosphoprotein interactions, with recent evidence indicating specificity for phosphothreonine and phosphoserine sites (Elia et al, 2003). *S. pombe* Fkh2p (38% identical and 66% similar between FHA regions) and Fhl1p (32% identical and 53% similar between FHA regions) contain FHA domains, while Sep1p and Mei4p do not contain the region of homology (Fig. 3.2.C).

In *S. pombe*, entry into mitosis is catalysed by the Cdc2p-Cdc13p complex (Fisher and Nurse, 1996). The inhibitory effect of Cdc2p-Cdc13p is maintained and regulated by the CDK inhibitor Rum1p during G1 through targeted proteolytic action on the Cdc13p subunit (Correa-Bordes and Nurse, 1995; Kitamura et al, 1998; Kominami et al, 1998). Cdc2p is itself controlled during S and G2 phases by inhibitory phosphorylation at the conserved Tyr-15 by two functionally overlapping kinases, Wee1p and Mik1p (Russell and Nurse, 1987; Lundgren et al, 1991). Prior to mitosis, Cdc25p receives a signal to dephosphorylate Tyr-15 of Cdc2p, thus driving the cells into mitosis (Millar and Russell, 1992). However, premature activation of Cdc2p-Cdc13p causes the cells to undergo cell death known as mitotic catastrophe. In a study to dissect the mechanisms governing Cdc2p-Cdc13p function, Samuel et al (2000) exploited a genetic approach to isolate mitotic catastrophic suppressors (*mcs*). Mutants of *mcs3* have been established to suppress synthetic lethal phenomenon of G2-arrested *cdc25-22 wee1-50* cells. Isolating suppressors of the synthetic lethal *msc3-12 wee1-50 cdc25-22* triple mutant, revealed a truncated genomic clone most similar in sequence to *S. pombe fkh2<sup>t</sup>* (Buck et al, 2004).

Full-length *S. pombe* Fkh2p contains 642 amino acids, with a mass of 71.1 kD, encoded by a gene containing one intron. The truncated Fkh2p clone, Fkh(t)2p, contains 132 amino acids with a total mass of 15.2 kD, missing its C-terminus, but still retaining part of the FHA region.

### **3.2.3 Role of forkhead proteins in PBF**

Electrophoretic mobility gel shift assay (EMSA), sometimes known as "band-shift", is an *in vitro* DNA binding method used to map the binding of transcription factors to DNA regulatory sites. The basic principle of EMSA is that interacting DNA-protein fragments move more

slowly in an electric field than free DNAs and proteins. Thus, it is possible to resolve free fragments and DNA-protein complexes by size.

To prove the specificity of DNA binding, an EMSA is performed with an increasing amount of unlabelled DNA fragment in a self-competing experiment. During incubation with protein sample, the radiolabelled and unlabelled DNA fragments compete for binding. After a fixed incubation time a stoichiometric reaction is achieved. The reaction mixture is then electrophoresed on a non-denaturing acrylamide gel. Specific binding activity can be identified when a decreasing amount of signal from radiolabelled DNA-protein complex is observed. Therefore, EMSA allows the mapping of specific DNA-protein interactions.

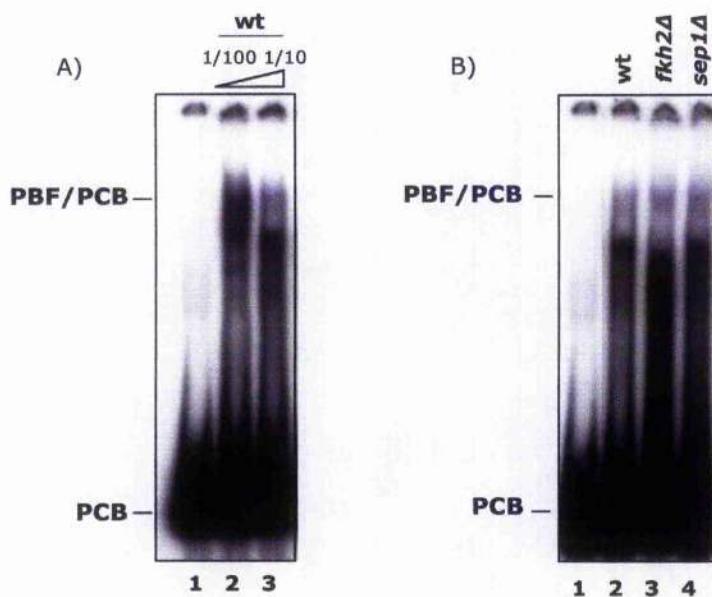
We have previously shown that PBF binds to a 100 bp *cdc15<sup>+</sup>* containing the PCB UAS using EMSA (Anderson *et al*, 2002; Fig. 3.3.A). Because the PCB UAS contains a conserved FHD DNA-binding motif and with the accumulating evidence for other late mitotic FHD-like transcription factor proteins in higher eukaryotes, we asked whether *S. pombe* FHD proteins, Sep1p, Fkh2p, Mei4p and Fhl1p are components of PBF. Although Mei4p contained a FHD, previous studies established that the gene has no role in mitosis, and so is irrelevant to our study (Horie *et al*, 1998). To discover if any of the other identified *S. pombe* FHD proteins are required for PBF activity, we obtained deletion mutants (as part of a collaborative effort with Dr. Millar, NIMR, London; and a gift from Dr. Sipiczki, Szeged University of Arts and Sciences, Hungary) and tested their effect on PBF binding *in vitro*.

### 3.2.3.1 *Deletion mutants*

To determine whether Fkh2p and Sep1p were required for PBF binding, we used the same 100 bp *cdc15<sup>+</sup>* promoter fragment containing the PCB UAS as DNA probe. The PCB fragment was labelled with radioactive [<sup>32</sup>P]-ATP by 5'-terminal labelling (Section 2.2.8.11.2). Asynchronous cultures of *fkh2Δ* (GG524), *sep1Δ* (GG515) and wild-type (GG217) cells were grown, protein extracts prepared and EMSA performed (Appendix IV; Sections 2.2.10.1 and 2.2.11.1) (Fig. 3.3.B).

The first lane in Figure 3.3.B shows an EMSA reaction containing radiolabelled PCB fragment alone, identified as "free probe" hereafter. The second lane shows the PBF-PCB complex. This reaction contains 20 µg of whole wild-type protein extract incubated with the radiolabelled PCB fragment. The subsequent lanes (three and four) were reactions containing protein extracts from *fkh2Δ* and *sep1Δ* cells, mixed with radiolabelled PCB.

The protein extracts from *fkh2Δ* and *sep1Δ* cells did not change the electrophoretic mobility of PBF (Fig. 3.3.B, lanes 3 and 4). The binding affinities were similar to wild-type extract (Fig. 3.3.B, lane 2). In a similar experiment, the electrophoretic mobility of PBF from



**Figure 3.3.** Pombe cell cycle box binding factor (PBF) binding to pombe cell cycle box (PCB) UAS DNA *in vitro*, as revealed by EMSA. Lane 1 in both panels shows the free radiolabelled PCB fragment used as DNA probe. A) Identification of PBF bound to the 100 bp *cdc15*<sup>+</sup> promoter fragment. Lanes 2 and 3, 20 µg wild-type protein extract with unlabelled PCB DNA fragment in 1/100 and 1/10 dilution of 250 ng *cdc15*<sup>+</sup> promoter fragment. B) Lack of effect of *fkh2*<sup>Δ</sup> and *sep1*<sup>Δ</sup> on PBF binding *in vitro*. Lanes contain 20 µg protein from *fkh2*<sup>Δ</sup> and *sep1*<sup>Δ</sup> cells with labelled 250 ng PCB DNA fragment.

*fkh1Δ* protein extracts was similarly unchanged (collaborative study with Dr. Millar; data not shown). These experiments showed that Fkh2p, Sep1p and Fhl1p are not required for PBF binding to the PCB UAS *in vitro* (Dr. Millar, personal communication).

### **3.2.3.2 C-terminus tagged proteins**

To investigate further the role of Sep1p, Fkh2p and Fhl1p, we constructed chromosomal C-termini HA-tagged and Myc-tagged strains (Section 2.1.7). The tagging strategy allowed us to perform a “super-shift” experiment using antibodies against the tags. If FHD proteins are components of PBF, the addition of an antibody specific to the tag should increase the molecular mass of the complex resulting in a slower mobility, known as a “super-shift”, detectable by electrophoresis.

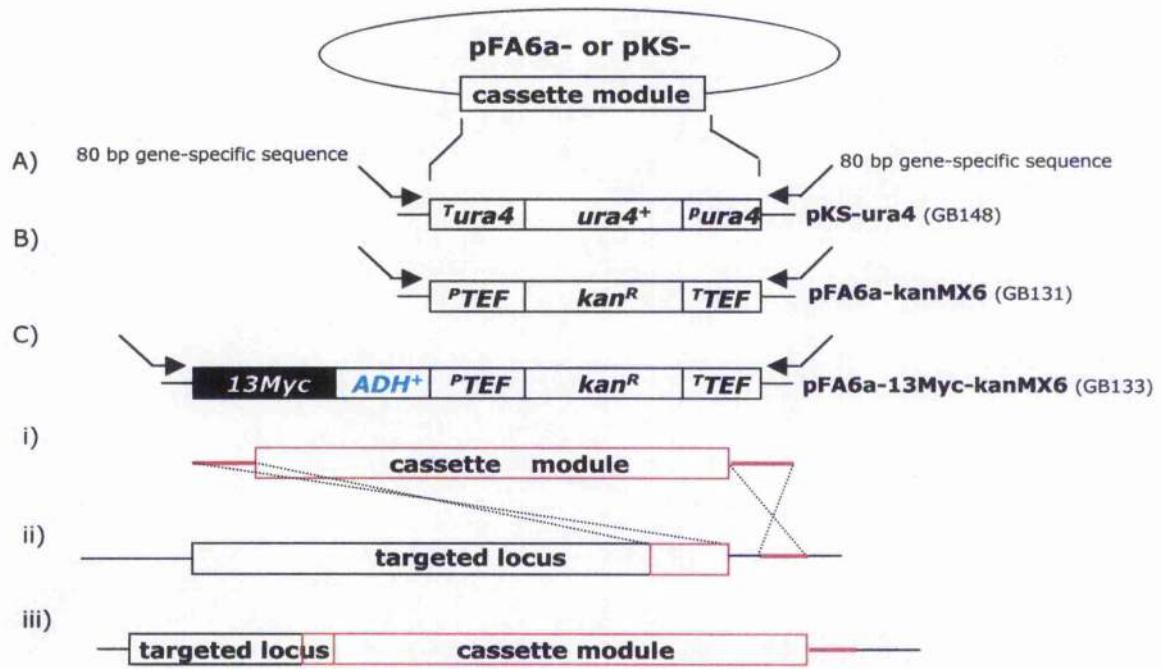
The *fkh2*-tagged strains (*HA*-tag: GG558 and *Myc*-tag: GG507) used in this thesis were obtained from Dr. Millar as part of a collaborative study.

To generate the *sep1*-Myc strain, we adopted the PCR-based one-step gene tagging method described by Bahler *et al* (1998) (Fig. 3.4). In this approach, engineered hybrid primers containing a flanking tail homologous to *sep1*<sup>+</sup>, and a short region corresponding to a plasmid module (pFA6a-13Myc-kanMX6; GB133) containing a heterologous selection marker, were employed to PCR amplify a flanking full-length *Myc* cassette (Sections 2.2.8.8 and 2.2.8.8.1). The *sep1*-Myc DNA was transformed into prototrophic and *leu1*-32 wild-type (GG214 and GG217) cells to generate strains in which the *sep1*<sup>+</sup> locus had been replaced by *sep1*-Myc (Section 2.2.8.7). GG214 contains an auxotrophic selection marker that allows extended manipulation of the tagged strain, while GG217 is a prototrophic strain.

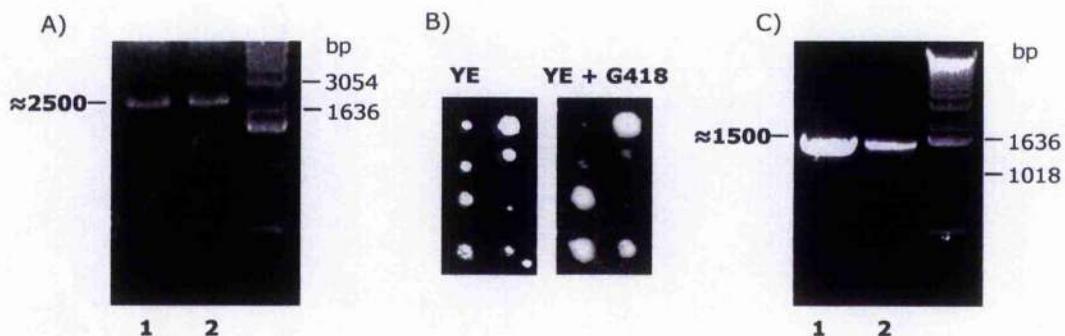
To validate integration, stable transformants were screened for G418-resistance and by colony PCR analysis, to eliminate non-homologous integration events (Section 2.2.8.7.1). The colony PCR strategy employs a pair of primers, with one recognizing the endogenous target gene while the other recognizes the heterologous selection marker, so an appropriate PCR fragment size can only be generated when the tag has integrated at the correct locus.

Figure 3.5.A shows a DNA fragment size of approximately 2500 bp containing 80 bp 5' and 3' *sep1*<sup>+</sup> in the hybrid primers (Appendix IV: GO183/184). Upon transformation, G418-resistant isolates were identified on a G418-containing YE plate (Fig. 3.5.B). When colony PCR analysis was performed on positive G418-resistant isolates, they revealed a single PCR fragment of 1500 bp, as predicted (GO195/196) (Fig. 3.5.C).

As the biological function of the FHD Fhl1p protein was unknown, we deleted *fhl1*<sup>+</sup> (*fhl1Δ*). This revealed that *fhl1*<sup>+</sup> is non-essential, as haploid *fhl1Δ* cells were viable (data obtained from collaborative study with Dr. Millar). Subsequent studies also demonstrated that *fhl1*<sup>+</sup> mRNA levels do not oscillate during mitotic cell cycle progression.



**Figure 3.4.** The use of heterologous modules for PCR-based one-step gene targeting strategy. The strategy places a C-terminus epitope-tag to the targeted locus. A, B and C are various pKS and pFA6a plasmid-template derivatives to generate in-frame tags. PCR primers contained approximately 80 bp nucleotide homology to the target locus and 20 bp nucleotide sequence homologous to epitope modules. Upon transformation, PCR-DNA fragment (i) align with targeted locus by sequence homology, depicted by (ii). Successful homologous recombination product should generate hybrid gene containing either pFA6akanMX6-tag or pKSura-tag cassettes, depicted by iii.



**Figure 3.5.** Verification of C-terminus *sep1-Myc*. A) Lanes 1 and 2 contained PCR fragment amplified from pKA6a-13Myc-kanMX6 module. B) G418 selection of integrated *sep1-Myc* into *sep1<sup>+</sup>* locus. C) Lanes 1 and 2, colony PCR selection of integrated *sep1-Myc* at *sep1<sup>+</sup>* locus. The approximate sizes of expected PCR fragments are indicated.

We constructed chromosomal C-terminal *fh1*-Myc (GG757) using an alternative two-step PCR method (Fig. 3.6) (Krawchuk and Wahls, 1999). Two pairs of primers were synthesized (GO574/575 and GO576/577): an independent primer from each pair contains specific homology to genomic *fh1*<sup>+</sup> loci, while the remaining are hybrid primers containing homology to the genomic *fh1*<sup>+</sup> loci and pFA6a-plasmid template (GB133). In the first round of PCR amplification, two fragments from separate reactions were made: one corresponds to the 5', and the other to the 3' of the genomic *fh1*<sup>+</sup> (Fig. 3.6.A-B) (Section 2.2.8.8.2). These generated PCR products of approximately 350 bp and 480 bp (Fig. 3.7.A).

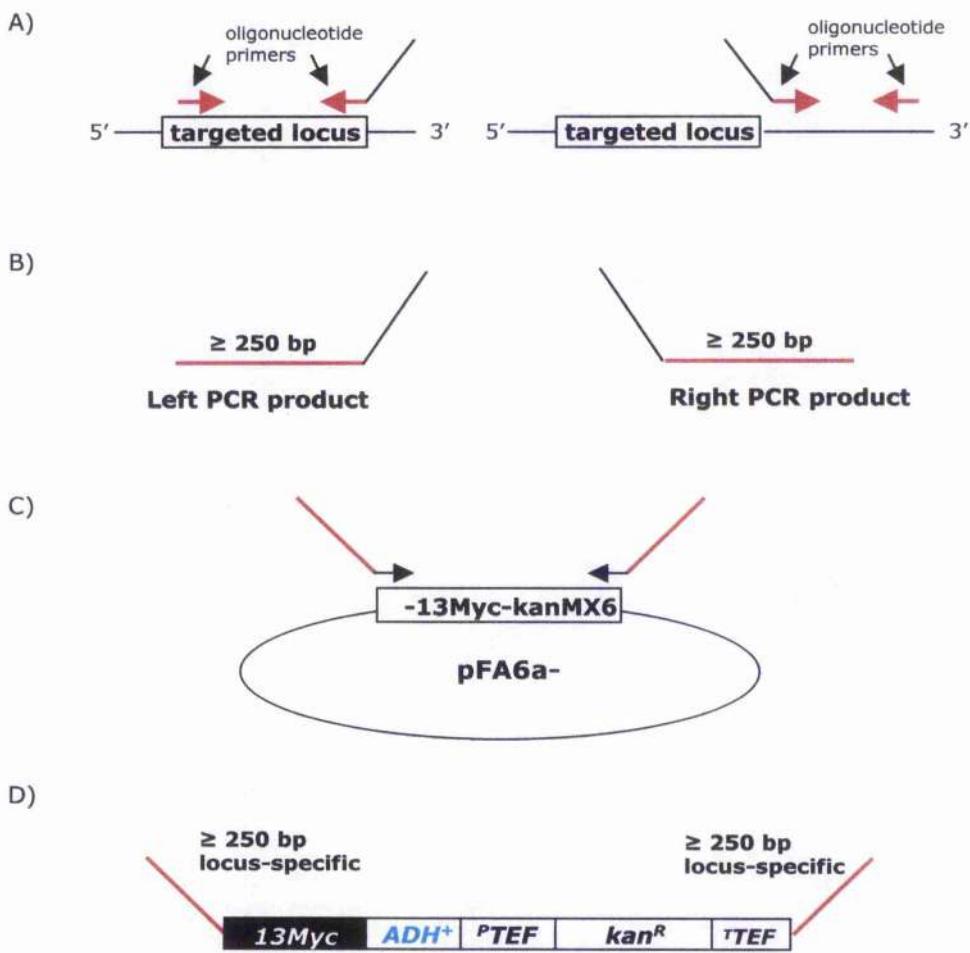
The second round of PCR amplification employed the 350 bp and 480 bp products as primers to pFA6a-13Myc-kanMX6 template. This amplification yielded a product of 3200 bp containing flanking *fh1* fragments attached to a full-length Myc-cassette (Fig. 3.7.B). Next, the *fh1*-Myc product was purified and transformed into prototrophic and *leu1*-32 wild-type cells (Section 2.2.8.9.1). Finally, stable G418 isolates were screened and validated by PCR to obtain a predicted fragment size of approximately 1600 bp (Fig. 3.7.C-D). In addition, Western blotting analysis revealed a gene product of a slower mobility (130 kD) than the calculated wild-type Fh1p, indicating that the clones contained the Myc-tag sequences (Fig. 3.7.E). Comparison between various *fh1*-Myc isolates further confirmed integrity of the product.

We then prepared crude protein extracts from cells containing HA-tagged and Myc-tagged Fkh2p, Sep1p and Fhl1p grown at 30°C. These were processed for EMSA using the PCB *cdc15*<sup>+</sup> fragment, but with one additional step: upon the incubation of radiolabelled probe, serial dilutions of antibodies (anti-HA or anti-Myc) were added to the binding reactions prior to electrophoresis.

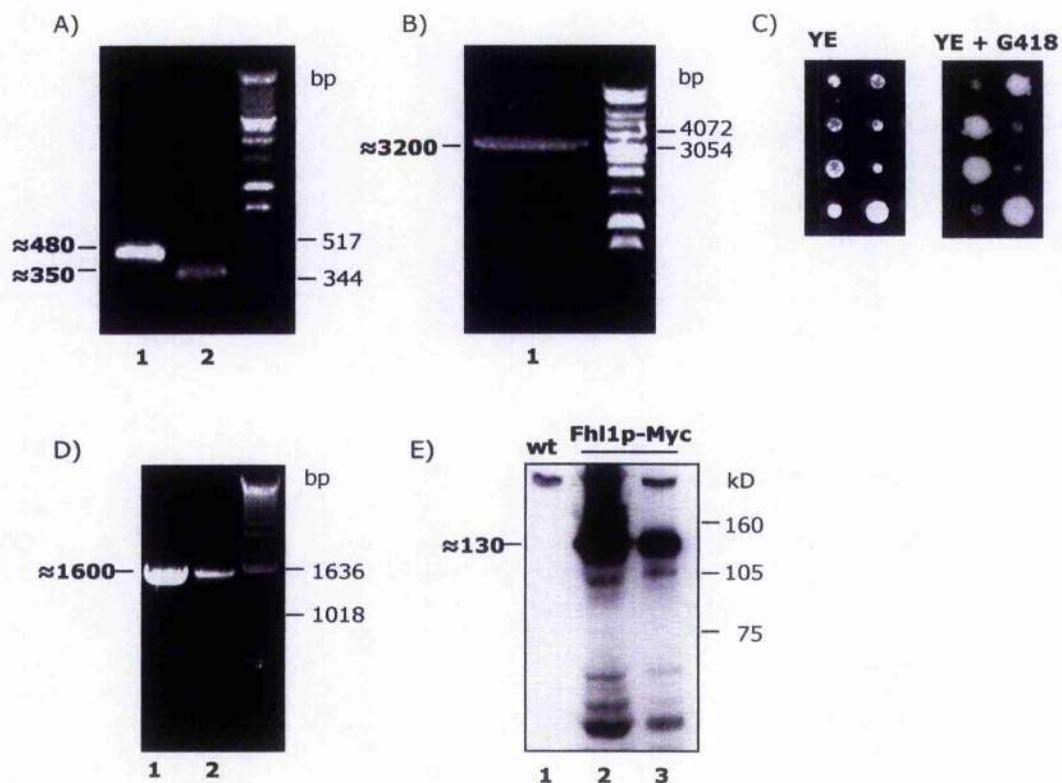
The autoradiogram shows that protein extracts from *sep1*-Myc and *fh1*-Myc cells did not abolish PBF-PCB binding. Furthermore, the addition anti-Myc antibody did not alter PBF mobility (Fig. 3.8.B-C). However, both Fkh2p-HA and Fkh2p-Myc extracts prevented PBF binding prior to the addition of antibodies (Fig. 3.8.A lanes 3-4). Because the binding activity remained in protein extracts from *fkh2Δ* cells, this argues for a functional consequence of the tag, therefore we examined this further. We introduced a multicopy plasmid carrying the native full-length *fkh2*<sup>+</sup>, "g", into the tagged strains. Interestingly, PBF binding was restored. In contrast, empty plasmid denoted "p", did not restore binding activity (Fig. 3.9, lanes 3-8).

These experiments suggest that Sep1p and Fhl1p are not directly responsible for PBF binding, although Fkh2p is possibly a component of PBF that requires a non-disrupted full length C-terminus for binding to the PCB UAS.

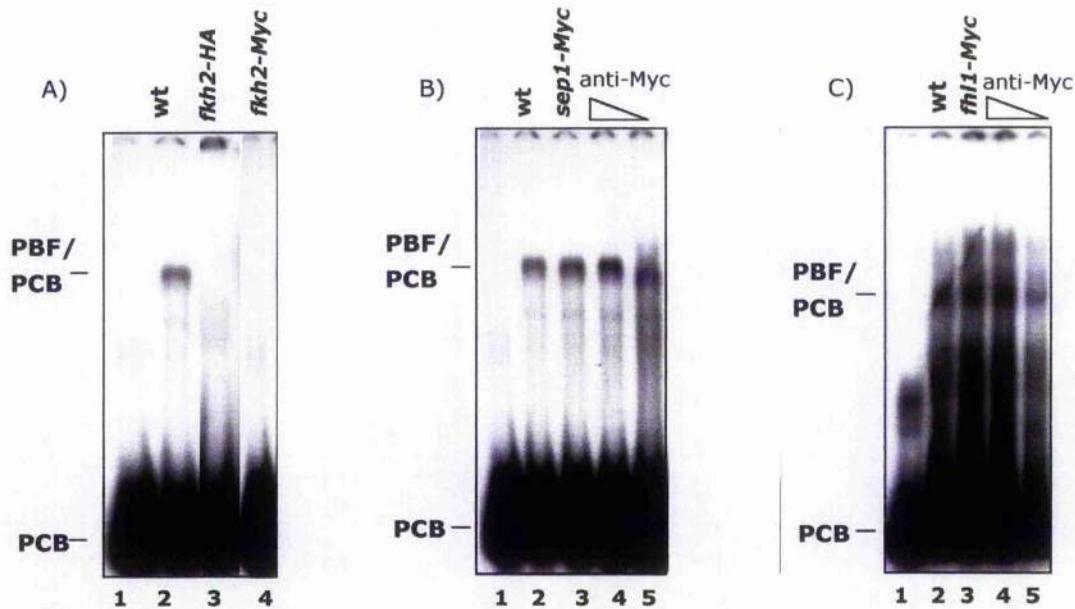
The eukaryotic genome, including *S. pombe*, contains multiple levels of functional redundancy, especially in multiprotein complexes. This observation could account for the



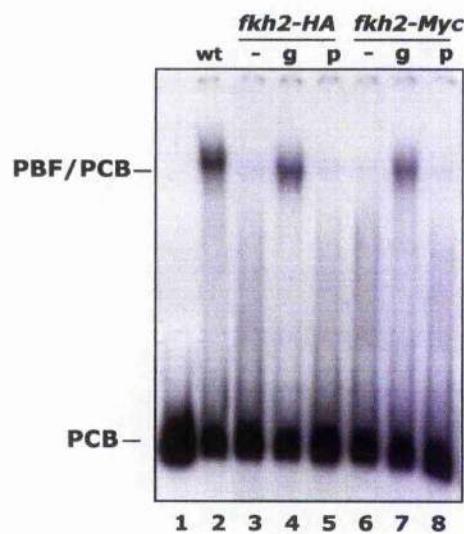
**Figure 3.6.** The use of pFA6a module for two-step PCR-based gene targeting strategy. A) Two PCR reactions are configured to amplify independently  $\geq 250$  bp of product containing targeted locus sequences. In both PCR reactions, either one or both of the oligonucleotide primers used are hybrids containing sequence-specific to locus of interest or template. B) The two independent PCR reactions generate  $\geq 250$  bp long DNA fragments product. C) The PCR products contain binding sites for pFA6a-template. D) The PCR amplification generates gene targeting fragment flanked on each side with sequences of homology to the targeted locus for efficient DNA recombination event.



**Figure 3.7.** Verification of C-terminus *fh1-Myc*. A) Lanes 1 and 2, first round PCR fragments amplified from genomic DNA. B) Lane 1, PCR product generated from pFA6a-13Myc-kanMX6 module. C) G418 resistance selection of integrated *fh1-Myc* into *fh1<sup>+</sup>* locus. D) Colony PCR selection of integrated *fh1-Myc* into *fh1<sup>+</sup>* locus. E) Protein extract isolated *fh1-Myc* cells separated on SDS-PAGE and processed for Western blot analysis. The approximate sizes of expected PCR fragments and peptide mass are indicated.



**Figure 3.8.** Effect of tagged FHD proteins on PBF binding activity *in vitro*. Lane 1 on every gel contained free radiolabelled PCB-probe. Lane 2, probe with wild-type protein extract. A) Lanes 3 and 4, protein extracts from *fkh2-HA* and *fkh2-Myc* cells. B) Lanes 4-5, addition of serial dilution of anti-Myc with protein extracts from *sep1-Myc* cells. C) Lane 3, protein extracts from *fh1-Myc* cells. Lanes 4-5, addition of a serial dilution of anti-Myc antibody with protein extracts from *fh1-Myc* cells.



**Figure 3.9.** The effect of tagged *fkh2*<sup>+</sup> on PBF binding activity *in vitro*. Lane 1, free PCB probe. Lane 2, probe mixed with wild-type protein extract. Lanes 3 and 6, protein extracts from *fkh2-HA* and *fkh2-Myc* cells. Lanes 4 and 7, protein extracts from cells with transformed genomic *fkh2*<sup>+</sup> "g". Lanes 5 and 8, protein extracts carrying empty plasmid "p".

presence of PBF in *sep1Δ*, *fkh2Δ* and *fhl1Δ* single deletion strains. To support this, Fkh1p and Fkh2p FHD proteins in *S. cerevisiae* share partially redundant roles in the SFF complex (Pic *et al*, 2000). The simple explanation is that in the absence of one, the alternative partner provides the same function.

In conclusion, the loss of DNA binding property observed in *fkh2*-tagged cells may represent a requirement for PBF binding to PCBs. In addition, the data suggest that the C-terminus of Fkh2p is required for this activity.

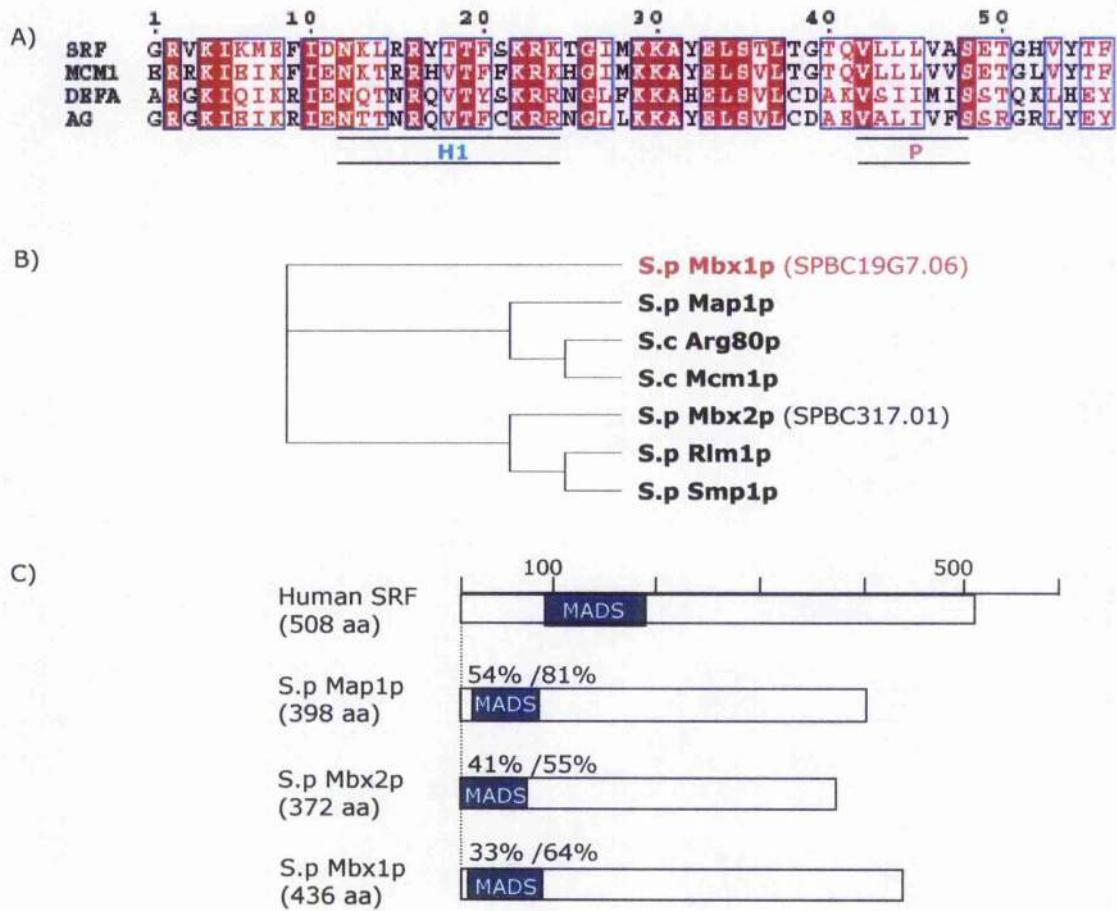
### **3.2.4 Sequence analysis of MADS-box transcription factor proteins**

In *S. cerevisiae* the SFF complex contains a MADS-box transcription factor Mcm1p in combination with FHD proteins. However, in the promoters of M/G1 transcribed genes in *S. pombe* the PCB UAS does not seem to be accompanied by a MADS-box binding motif (Anderson *et al*, 2002). Even so, we tested whether PBF also contains an associated MADS-box factor. We have identified three MADS-box proteins from the *S. pombe* genome database: Map1p, SPBC19G7.06 and SPBC317.01 (Wood *et al*, 2002). The sequence alignment was performed against the conserved 56 amino acids MADS-box motif identified from the four founding members – MCM1 in *S. cerevisiae*, AG in *A. thaliana*, DEFA in *A. majus* and SRF in human (Dodou and Treisman, 1997) (Fig. 3.10.A).

Using ClustalW, a phylogenetic tree was generated based on full-length sequences of MADS-box proteins in both yeast species (Fig. 3.10.B). This computational alignment method confirmed that *S. pombe* Map1p shares the highest degree of homology to Mcm1p and Arg80p of *S. cerevisiae*, followed by *S. pombe* SPBC19G7.06. Therefore, we studied the function of Map1p and SPBC19G7.07, which we named Mbx1p, for MADS-box 1. The clustering method defined *S. pombe* SPBC317.01 as most similar to the *S. cerevisiae* Rim1p and Smp1p proteins, and we named it Mbx2p (MADS-box 2).

MADS-box region of *S. pombe* Map1p shares the highest degree of identity (54%) and similarity (81%) across the length of MADS-box region in human SRF, followed by Mbx2p with 41% identity and 55% similarity, and Mbx1p with 33% identity and 64% similarity, respectively (Fig. 3.10.C).

Comparative sequence analysis of MADS-box transcription factors, against the MADS-box region, between *S. cerevisiae* and *S. pombe* identified *S. pombe* Map1p as most closely related to *S. cerevisiae* Mcm1p (60% identical and 87% similar). Therefore, it is the most promising candidate for study. However, Map1p is not required for mitotic growth (Yabana and Yamamoto, 1996), which suggests it is not a component in PBF. Therefore we tested whether Mbx1p, whose MADS-box region sequence homology is 37% identical and 70% similar to the MADS-box region in *S. cerevisiae* Mcm1p, is part of the PBF complex.



**Figure 3.10.** MADS-box proteins. A) Sequence alignment of the 56 amino acids MADS-box motifs from four founding members: *S. cerevisiae* Mcm1p, *A. thaliana* AG, *A. majus* DEFA and human SRF. **H** denotes  $\alpha$ -helix and **P** denotes hydrophobic patch. B) Phylogenetic tree showing the relationships between *S. cerevisiae* and *S. pombe* MADS-box proteins based on full-length peptide sequence alignment generated using ClustalW. C) Amino acid sequence comparison of MADS-box region in human SRF with *S. pombe* Map1p, Mbx2p and Mbx1p. Percent identity/percent similarity values are shown for MADS-box conserved regions.

### **3.2.5 Role of MADS-box proteins in PBF**

The MADS-box proteins are ubiquitous transcription factors that often derive their functional specificity through combinatorial protein-protein interactions (Treisman, 1994; Shore and Sharrocks, 1995). To assess the role of Mbx1p in M/G1-specific gene expression, we obtained deletion mutant (*mbx1Δ*) and C-terminal tagged (*mbx1-Myc*) strains from Dr. Millar.

#### **3.2.5.1 Deletion mutant and COOH-terminus tagged proteins**

Figure 3.11 shows the results of EMSA experiments containing protein preparations with radiolabelled PCB fragments. Using *mbx1Δ* (GG503), we tested its effect on PBF binding *in vitro* and found it was abolished, suggesting that Mbx1p is required for PBF-PCB binding (Fig. 3.11.A, lane 3). To test whether Mbx1p is a component of PBF, we used an *mbx1-Myc* strain with the intention of carrying out supershift experiments (GG509). However, protein extracts from this strain prior to the addition of anti-Myc antibody resulted in loss of PBF binding (Fig. 3.11.B, lane 3). Expression of wild-type *mbx1<sup>+</sup>* from a multicopy vector "g" rescued binding, while an empty vector "p" did not (Fig. 3.11.C, lanes 4-5).

In conclusion, both *mbx1Δ* and *mbx1-Myc* cells resulted in the loss of binding of PBF to PCB DNA.

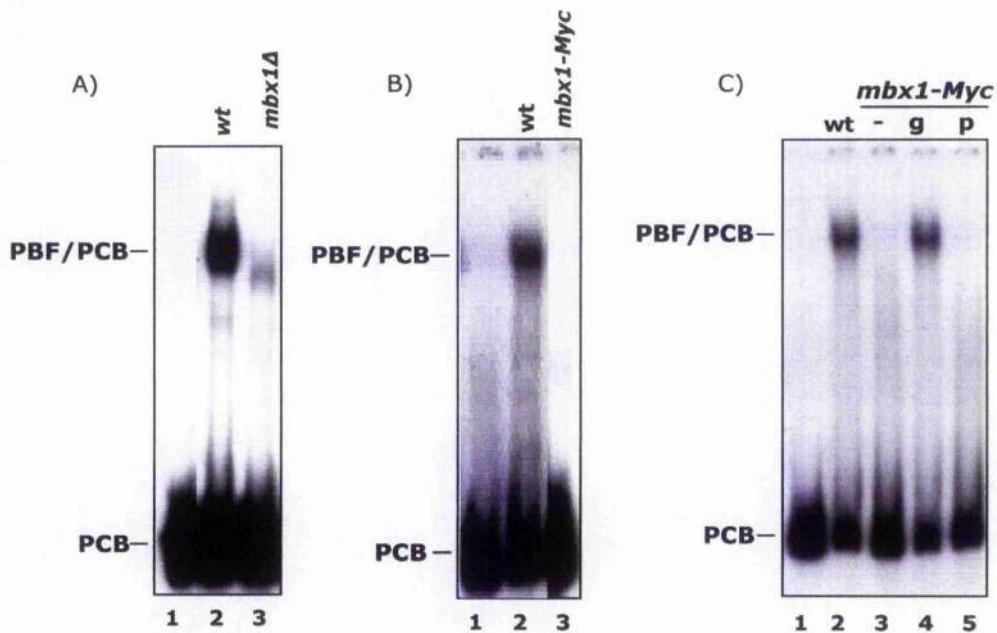
### **3.2.6 Genetic interactions among *fkh2<sup>+</sup>*, *sep1<sup>+</sup>* and *mbx1<sup>+</sup>***

To explore whether FHD and MADS-box proteins identified in *S. pombe* share functional redundancy or possible genetic interactions which might explain the loss of binding observed in both *fkh2-HA* and *fkh2-Myc* cells, we generated combinatorial deletion strains by tetrad analysis.

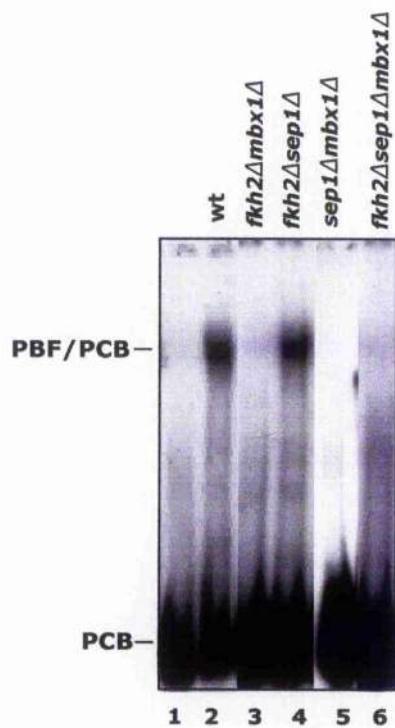
#### **3.2.6.1 PCB binding activity**

We have established that PBF binds specifically to the PCB UAS (Anderson *et al*, 2002; Fig. 3.3.A) and have subsequently identified two potential transcription factors required for PBF binding, which we named Fkh2p and Mbx1p. To provide further insight into their binding properties, we used protein extracts of *sep1Δ*, *fkh2Δ* and *mbx1Δ* combination deletion strains in EMSA experiments.

In all reactions containing protein from *mbx1Δ* cells, PBF-PCB-binding was lost (Fig. 3.12, lanes 3, 5 and 6). Unlike the *mbx1Δ*-containing reactions, the binding activities of



**Figure 3.11.** Lack of PBF binding in *mbx1Δ* and *mbx1-Myc* cells. Lanes 1 and 2 on each gel contained free PCB probe and probe mixed with wild-type protein extract. A) Lane 3, protein extract from *mbx1Δ* cells. B) Lane 3, protein extract from *mbx1-Myc* cells. C) Lane 3, protein extracts from *mbx1-Myc* cells. Lanes 4-5, protein extracts from *mbx1-Myc* cells transformed with genomic *mbx1*<sup>+</sup> "g" and empty plasmid "p".



**Figure 3.12.** Effect of combinatorial deletions of FHD and MADS-box proteins on PBF binding *in vitro*. Lane 1, free radiolabelled PCB probe. Lane 2, wild-type protein extract. Lane 3, protein extract from *fkh2Δmbx1Δ* cells. Lane 4, protein extract from *fkh2Δsep1Δ* cells. Lane 5, protein extract from *sep1Δmbx1Δ* cells. Lane 6, protein extracts from *fkh2Δsep1Δmbx1Δ* triple mutant.

PBF were detected in extracts isolated from mutants containing wild-type *mbx1*<sup>+</sup>, identical to wild-type cells (Fig. 3.12, lanes 2 and 4). Since PBF binding was still detectable in protein extracts from *fkh2Δsep1Δ* cells, this argues against redundant function for the two genes however, redundant roles have been reported for *S. cerevisiae FKH1* and *FKH2*.

Taken together these data suggest that Mbx1p is a component of PBF, but the nature of potential interactions with other proteins is unknown. We have provided some evidence that the C-terminal region of Fkh2p is required for PBF binding activity, and so possibly the two bind to the DNA in close proximity.

### **3.2.6.2 Morphological analyses**

Septation and cytokinesis in *S. pombe* begins with the formation of the actin ring, which in turn determines the position of the septum (Fankhauser *et al*, 1995; Ohkura *et al*, 1995). The physical division of cells starts with the erosion of the wall at the junction between mother and daughter cells, and ends with the dissolution of primary septa.

We further characterised functional and genetic links between potential components of PBF by monitoring microscopically septation and cytokinesis in single mutant and combinations of *fkh2Δ*, *sep1Δ* and *mbx1Δ* cells.

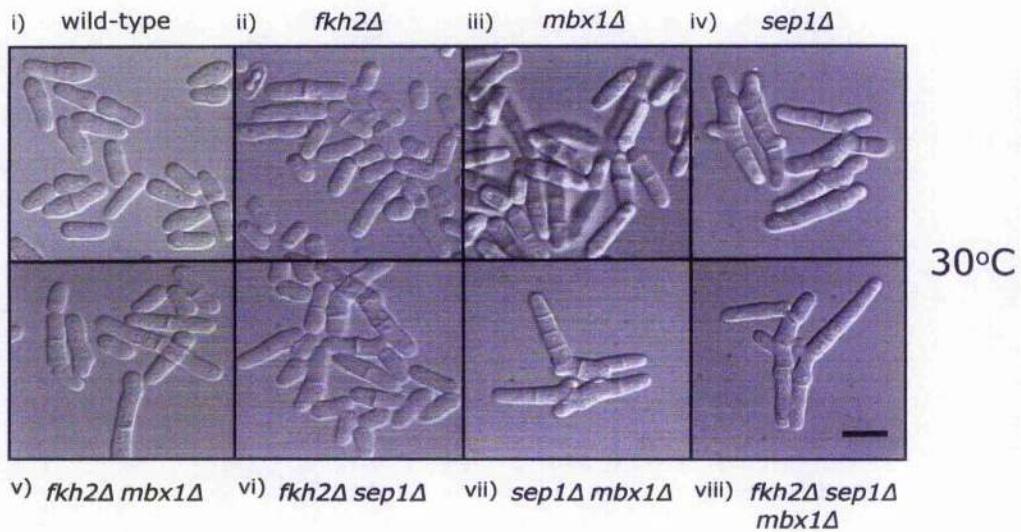
#### **3.2.6.2.1 Differential interference contrast (DIC) optic analyses**

To examine the phenotypes of deletion mutants, mutants grown in liquid culture were monitored using DIC optics (Fig. 3.13) (Section 2.2.12.1).

*fkh2Δ*, *sep1Δ* and *mbx1Δ* cells exhibited separation defects and increased lengths relative to wild-type (Fig. 3.13.i-iv). In addition, *fkh2Δ* cells displayed multiple levels of separation phenotypes, while *sep1Δ* and *mbx1Δ* cells demonstrated a homogenous septation phenotype. Whereas wild-type cells septated and divided at  $14.5 \pm 0.9$  nm, *fkh2Δ*, *sep1Δ* and *mbx1Δ* cells underwent division at  $16.7 \pm 3.6$  nm,  $12.8 \pm 1.8$  nm and  $13.4 \pm 1.8$  nm, respectively. In addition, *sep1Δ* cells showed a branched phenotype.

*fkh2Δsep1Δ*, *fkh2Δmbx1Δ* and *sep1Δmbx1Δ* double mutants displayed augmented separation defects when compared to single deletion mutants (Fig. 3.13.v-vii). Although viable, the *fkh2Δsep1Δmbx1Δ* triple mutant displayed the most severe defects (Fig. 3.13.viii).

In conclusion, combinatorial deletion cells exhibited various forms of defects, including multiseptated, misoriented and unconstricted septa. In addition to the separation phenotype, all mutant cells had growth defects. These observations suggest that Fkh2p, Sep1p and Mbx1p are required for normal cell separation in *S. pombe*.



**Figure 3.13.** Effect of FHD and MADS-box deletions on cell separation, cytokinesis and septation. i-viii) Various combinations of *fkh2Δ*, *sep1Δ* and *mbx1Δ* cells, grown at 30°C in liquid culture, viewed with DIC optics. Bar size  $\approx 5 \mu\text{m}$

### **3.2.6.2.2 Growth rate analyses**

Having observed that combining deletions of *fkh2Δ*, *sep1Δ* and *mbx1Δ* in liquid cultures lead to exaggerated septation and cell length phenotypes, this prompted us to examine whether similar phenotype were observed on solid media.

To evaluate growth rate, mutant and wild-type cells were diluted to single cells, streaked onto YE and EMM and incubated at 30°C for 3-5 days. However, it was difficult to restreak *fkh2Δ* cells, especially the *fkh2Δsep1Δmbx1Δ* triple mutant. Therefore, the experiment was repeated three times to achieve an average for scoring analyses.

Visual inspection revealed that although most cells were viable, they grew at differing rates (Fig. 3.14). Table 3.1 summarises the growth rates of all the tested strains, determined from time required to generate saturated cell colonies. In conclusion, the growth rates were reduced relative to wild-type. Also, the growth rate was exacerbated in all mutant cells containing *fkh2Δ*. Therefore, consistent with previous data, Fkh2p, Sep1p and Mbx1p co-operatively affect growth rates, potentially through defective septation.

### **3.2.6.2.3 Fluorescence optic analyses**

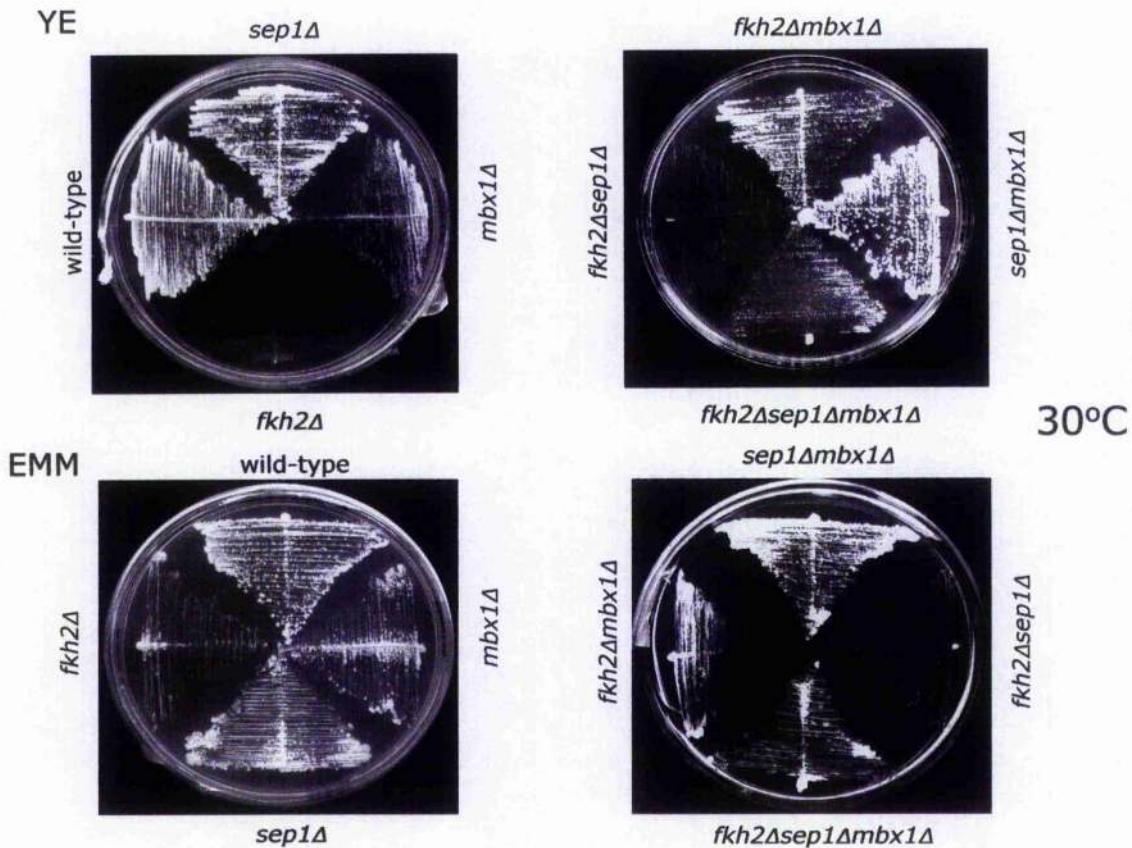
To examine whether the slower growth rate was associated with defective septation, we examined *fkh2Δ*, *sep1Δ* and *mbx1Δ* cells grown in liquid culture, after staining them with DAPI and Calcofluor, to allow visualisation of chromatin and septa (Sections 2.2.12.2 and 2.2.12.3).

*fkh2Δ* cells showed pleiotrophic separation defects. These phenotypes could be categorised into four groups: unconstricted, multiseptated, misplaced and misoriented septa (Fig. 3.15.ii; Table 3.2). The diverse septation defects suggest multiple roles for Fkh2p in cell separation.

Microscopic examination also revealed that a majority of branched *sep1Δ* cells contained segments of uninuclear cells connected by unseparated septa (Fig. 3.15.iii; Table 3.2). *mbx1Δ* cells, in comparison, exhibited milder separation phenotypes (Fig. 3.15.iv).

Double and triple mutants displayed highly elongated and mis-shaped cells that formed chains and clumps. DAPI and Calcofluor staining confirmed that these mutants had a higher percentage of septation phenotypes than single mutants (Fig. 3.15.v-viii; Table 3.2).

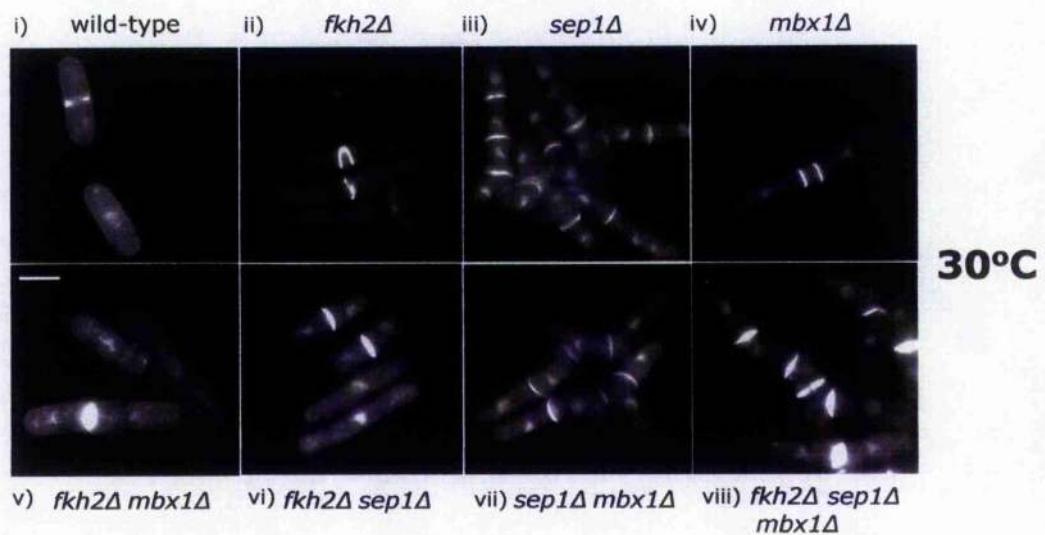
Overall, the deletion mutants had a greater proportion of septating cells relative to wild-type. Furthermore, these data revealed that slower cell growth which caused an increase in cell lengths was due to failure in cytokinesis, as there were many more septated cells than found in wild-type cells.



**Figure 3.14.** Growth rates effect by combinatorial deletions on solid YE and EMM, grown at 30°C. Cells contained either single *sep1Δ*, *fkh2Δ*, *mbx1Δ* cells or double and triple deletion mutants.

Cells	Growth rate	
	YE	EMM
wild-type	++	++
<i>fkh2Δ</i>	±	±
<i>sep1Δ</i>	++	++
<i>mbx1Δ</i>	+	+
<i>fkh2Δsep1Δ</i>	±	±
<i>fkh2Δmbx1Δ</i>	+	+
<i>sep1Δmbx1Δ</i>	++	++
<i>fkh2Δsep1Δmbx1Δ</i>	+	+

**Table 3.1.** Quantitation of growth rate among FHD and MADS-box deletion mutants. ++, Good growth comparable to wild-type or healthiest mutant strain. +, Poor growth with microcolony formation. ±, Very slow growth with many dead cells.



**Figure 3.15.** Cell separation defects in *fkh2Δ*, *sep1Δ* and *mbx1Δ* cells. Cells grown at 30°C, in liquid EMM were stained with DAPI and Calcofluor to reveal cell nuclei and septa and viewed with fluorescence optics. Bar size ≈ 5 μm

Cells	Septating cells (%)	Septating cells (%)		
		unconstricted	multiseptated	misplaced and misoriented
wild-type	15.2	0	0	0
<i>fkh2Δ</i>	27.7	3.2	13.5	4.4
<i>sep1Δ</i>	100	55.8	0	0
<i>mbx1Δ</i>	25.8	8.3	8.3	5.0
<i>fkh2Δsep1Δ</i>	24.7	6.2	1.8	6.2
<i>fkh2Δmbx1Δ</i>	24.2	3.8	4.8	4.8
<i>sep1Δmbx1Δ</i>	99.5	64.3	0	0
<i>fkh2Δsep1Δmbx1Δ</i>	100	60.5	4.7	9.3

**Table 3.2.** Quantitative analysis of cell separation defects in *fkh2Δ*, *sep1Δ* and *mbx1Δ* cells.

### **3.2.6.3 Overexpression analyses**

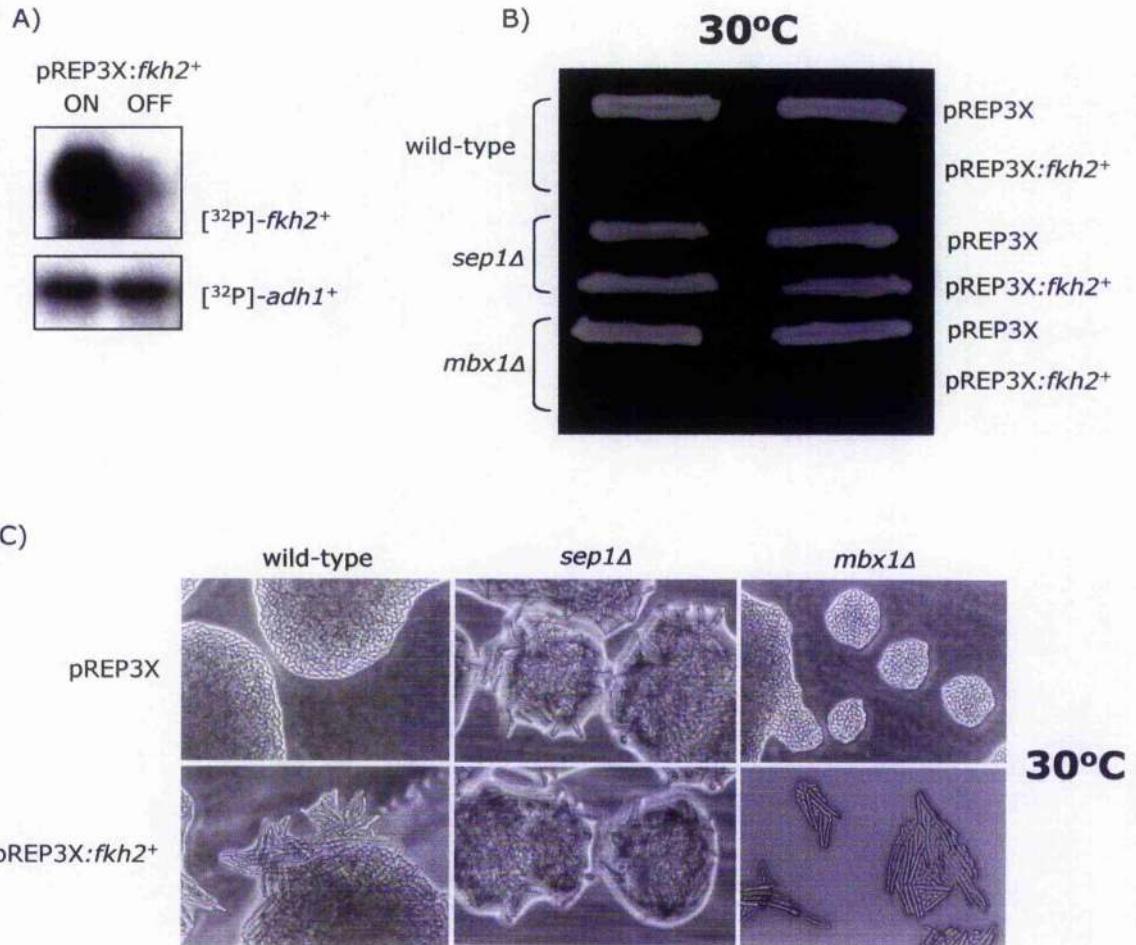
To further characterise potential genetic links between the FHD and MADS-box genes, overexpression of individual genes were examined in deletion mutants. Wild-type *fkh2<sup>+</sup>*, *sep1<sup>+</sup>* and *mbx1<sup>+</sup>* were placed under the control of the *nmt1*-repressible (no message in thiamine) pREP3X vector and introduced into *fkh2Δ*, *sep1Δ* and *mbx1Δ* cells (Section 2.2.6).

To amplify the full-length *fkh2<sup>+</sup>*, *sep1<sup>+</sup>* and *mbx1<sup>+</sup>* genes, oligonucleotides (GO507/508, *fkh2<sup>+</sup>*; GO515/516, *sep1<sup>+</sup>* and GO517/518, *mbx1<sup>+</sup>*) were engineered to include 5' *Bam*H I or *Xba*I sites and 3' *Pst*I, *Sa*I or *Sma*I sites. The amplified PCR products were digested and subcloned into the pREP3X vector (GB28), to create pREP3X:*fkh2<sup>+</sup>* (GB263), pREP3X:*sep1<sup>+</sup>* (GB290) and pREP3X:*mbx1<sup>+</sup>* (GB291), and each separately introduced into appropriate deletion strains (Section 2.2.8.10). To create control strains, the empty pREP3X vector was also transformed into wild-type and deletion mutants. Cell growth was maintained on a medium in the presence of thiamine (*nmt1* "off"). Cells containing expression vectors were confirmed by Northern blot analyses (Fig. 3.16.A, 3.17.A and 3.18.A).

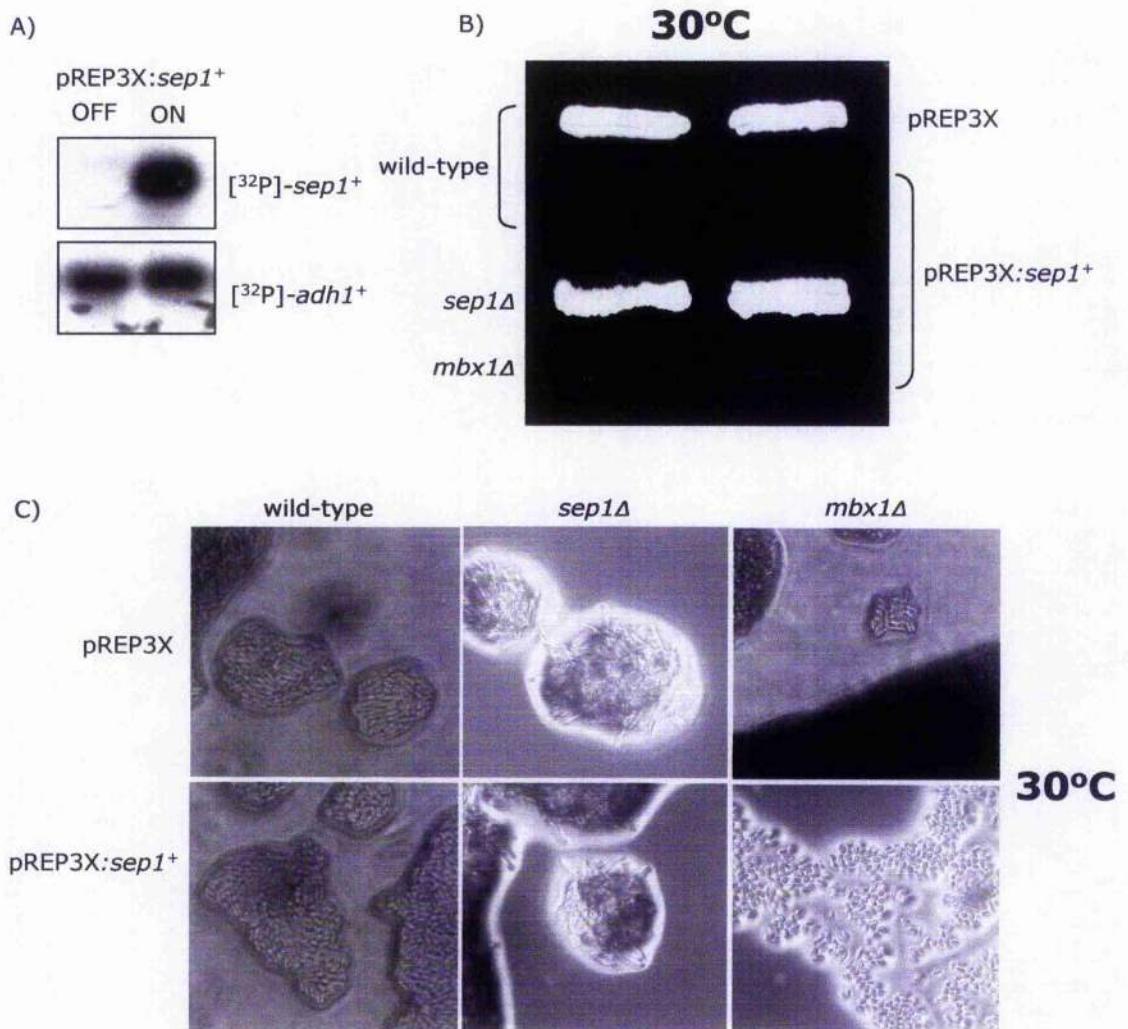
When *fkh2<sup>+</sup>* overexpression was induced in wild-type cells, they died. This loss of viability was accompanied by an elongated cell phenotype suggesting cell cycle arrest (Fig. 3.16.B-C). These cells also displayed missing and unconstricted septa. Interestingly, although overexpression of *fkh2<sup>+</sup>* caused cell death in wild-type, this lethal phenotype was not observed in *sep1Δ* cells (Fig. 3.16.B). This observation implies that Sep1p is required for Fkh2p function.

However, the reverse was not true: although overexpression of *sep1<sup>+</sup>* also killed wild-type cells, *fkh2Δ* did not rescue this phenotype (Fig. 3.17). Indeed, *fkh2Δ* cells died even in the non-induced pREP3X:*sep1<sup>+</sup>* strain, when *sep1<sup>+</sup>* was not overexpressed. A possible explanation for this observation is the fact that is known that the thiamine-repressible pREP3X plasmid confers very high overexpression (300-fold) in comparison to endogenous levels and that the *nmt1* promoter is known to be "leaky", resulting in lower overexpression even under non-inducing conditions. Therefore, we cloned *sep1<sup>+</sup>* into the weaker *nmt1*-expression vector, pREP81X (7-fold) (GG301), and tested their overexpression in *fkh2Δ* cells (Forsburg, 1993; Moreno *et al*, 2000). Nonetheless, despite being able to select for transformed clones, these cells also died.

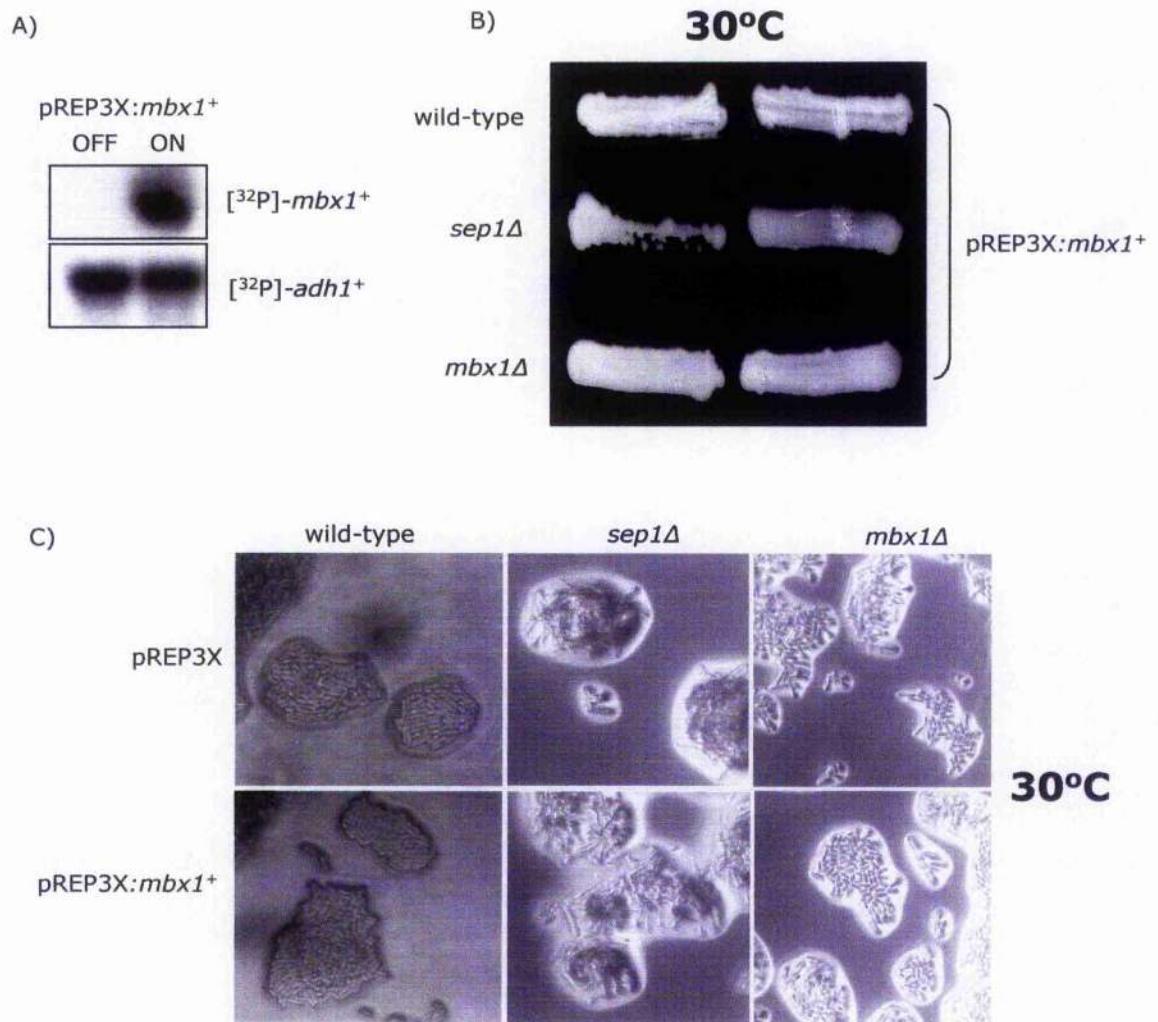
When *mbx1<sup>+</sup>* was overexpressed in wild-type and mutant cells, they survived. Furthermore, cell morphologies were indistinguishable between these cells (Fig. 3.18.B-C). Again, despite being able to select for cells containing pREP3X:*mbx1<sup>+</sup>* and pREP81X:*mbx1<sup>+</sup>* (GB293) in *fkh2Δ*, they subsequently died. The fact that we saw a similar effect with two



**Figure 3.16.** Effect of *fkh2*<sup>+</sup> when overexpressed. A) Verification of pREP3X:*fkh2*<sup>+</sup> strain in the presence and absence of thiamine by Northern blot analysis. B) Growth analysis in wild-type, *sep1* $\Delta$  and *mbx1* $\Delta$  cells, grown at 30°C on solid EMM. C) Phenotypes of wild-type, *sep1* $\Delta$  and *mbx1* $\Delta$  cells on solid EMM.



**Figure 3.17.** Effect of *sep1*<sup>+</sup> when overexpressed. A) Verification of pREP3X:*sep1*<sup>+</sup> strain in the presence and absence of thiamine by Northern blot analysis. B) Growth analysis in wild-type, *sep1*<sup>Δ</sup> and *mbx1*<sup>Δ</sup> cells, grown at 30°C on solid EMM. C) Phenotypes of wild-type, *sep1*<sup>Δ</sup> and *mbx1*<sup>Δ</sup> cells on solid EMM.



**Figure 3.18.** Effect of *mbx1*<sup>+</sup> when overexpressed. A) Verification of pREP3X:*mbx1*<sup>+</sup> strain in the presence and absence of thiamine by Northern blot analysis. B) Growth analysis in wild-type, *sep1* $\Delta$  and *mbx1* $\Delta$  cells, grown at 30°C on solid EMM. C) Phenotypes of wild-type, *sep1* $\Delta$  and *mbx1* $\Delta$  cells grown on solid EMM.

different plasmids in *fkh2Δ* cells implies a role for Fkh2p in plasmid maintenance, which may explain these observations.

To conclude, these overexpression experiments provide supportive evidence for genetic interaction between *sep1<sup>+</sup>* and *fkh2<sup>+</sup>*. Furthermore, these data suggest that *sep1<sup>+</sup>* is required for *fkh2<sup>+</sup>* function.

### **3.2.7 Isolation of Fkh2p and Sep1p-interacting proteins**

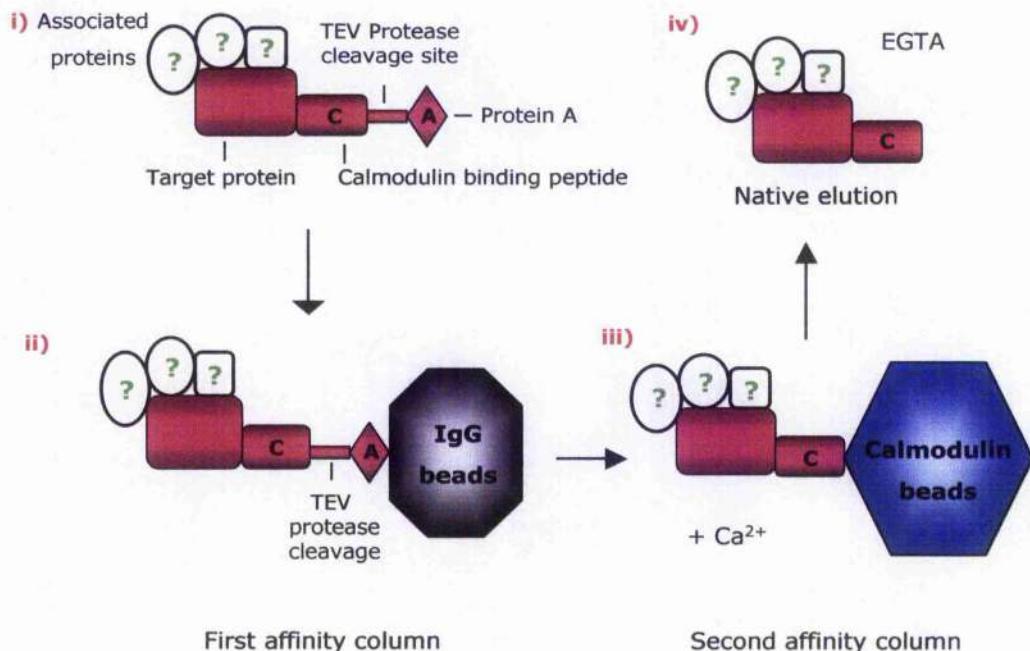
To identify other proteins that bind to Fkh2p and Sep1p, we employed a two round purification protocol, the TAP-tag method. The purification strategy was originally described by Rigaut *et al* (1999) for *S. cerevisiae*, with a similar system developed for *S. pombe* by Tasto *et al* (2001). The method uses the fusion of the TAP-tag (consisting of IgG-binding repeats from *Staphylococcus aureus* protein A and a Calmodulin binding peptide (CBP), separated by a cleavage site for *Tobacco Etch Virus* (TEV)) in-frame to the protein of interest (Fig. 3.19).

To facilitate the C-terminal TAP-tagging of *fkh2<sup>+</sup>* (GG886) and *sep1<sup>+</sup>* (GG878) at their genomic loci under the control of their own native promoters, we employed PCR-based gene tagging developed by Bahler *et al* (1998) for homologous Integration. The same oligonucleotides used for creating HA and Myc-tagged constructs were employed, but instead using plasmid templates (GB372 and GB374) containing the TAP-tag cassette (Section 3.2.3.2). An advantage of tagging each gene in their native locus for purification is that, each protein is present at physiological levels, enhancing the chances of identifying *bona fide* binding partners.

To isolate Fkh2p and Sep1p, and associated proteins, we adapted the method in which phosphate breakage buffer containing NP-40 was substituted with Hepes-Tween-20 breakage buffer, supplemented with salt (150, 260 or 350 mM NaCl). Combinations of these reagents are known to preserve protein-protein interactions but disrupt protein-DNA binding, which should facilitate the isolation of Fkh2p and Sep1p interacting proteins.

A large culture of each tagged strain was grown, and the harvested cell pellet disrupted using a Cell Combustion Breaker and subjected to two rounds of purification (Sections 2.2.10.2 and 2.2.10.2.1). Initially, the tagged protein and its binding proteins were recovered by affinity selection on an IgG-Sepharose matrix (Fig. 3.19.A). After washing, the TEV protease was added to release the bound proteins. The eluate was then incubated with calmodulin-coated beads in the presence of calcium, allowing the removal of remaining protease and contaminants after the first affinity selection. Finally, the bound material was eluted with EGTA and analysed by two methods: denaturing gradient gel (4–20%) and visualised by Silver staining, and tryptic digested for liquid chromatography with

A)



B)

Calmodulin Binding Peptide	Spacer	TEV cleavage site	Spacer
<b>SMEKRRWKKNFIAVSAANRFKKISSLGALDYDIPTTASEONLYFQGELKTAALAQHDEA</b>			
	IgG binding domain		
<b>VDNKFNKEQQNAFYEILHLPNLNEEQRNAFIQLKDDPSQSANLLAEAKKLNDQAQPK</b>			
IgG binding domain			
<b>VDNKFNKEQQNAFYEILHLPNLNEEQRNAFIQLKDDPSQSANLLAEAKKLNGAQAPKVDAKSAGKST</b>			

**Figure 3.19.** Schematic representation of tandem affinity purification (TAP) strategy. A) **i)** A TAP tagged version of the protein, along with unknown associated proteins, undergoes two rounds of purification. **ii)** The first round involves the Protein A portion of the tag binding to IgG beads. **iii)** This is cleaved from the beads by TEV protease treatment, which permits the second round of purification through the calmodulin binding peptide binding to calmodulin beads. **iv)** The highly purified protein is eluted in the presence of EGTA, and the products identified by SDS-PAGE and MALDI-TOF. B) Sequence of the TAP tag.

mass spectrometric analysis (LC-MS/MS), also known as MALDI-TOF (Matrix Assisted Laser Desorption/Ionization-Time Of Flight), which allow peptide identifications by mass (Sections 2.2.10.6.2 and 2.2.10.7).

MALDI-TOF analysis of isolated proteins from the Fkh2p-TAP purification identified two polypeptides, encoded by SPBC16G5.15C (Fkh2p) and a SPBC16A3.03C (a novel protein), while the Sep1p-TAP purification identified two other polypeptides encoded by the SPBC32F12.11 and SPBC839.15C genes (Table 3.3) (Wood *et al*, 2002).

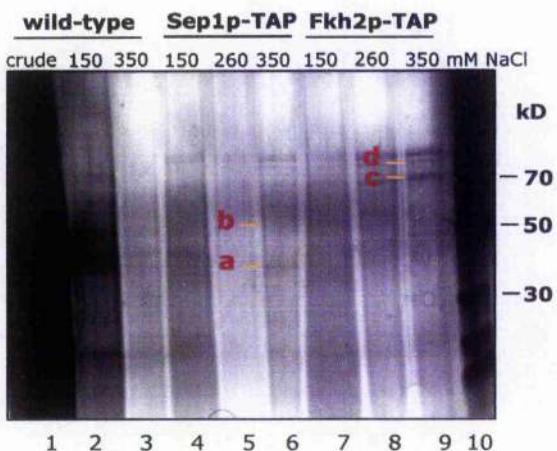
The Sep1p binding proteins recovered were glutaraldehyde 3-phosphate-dehydrogenase (GAPDH) and elongation factor 1 alpha-C, sharing highest degree of homology to polypeptides encoded by *S. pombe* *tdh1*<sup>+</sup> (SPBC32F12.11) and *ef1a-c*<sup>+</sup> (SPBC839.15C). All peptides identified by MALDI-TOF showed the correct predicted mobility when analysed by SDS-PAGE (Fig. 3.20, lanes 6 and 9). However, the sensitivity of Silver staining and MALDI-TOF analyses was limiting, as the Sep1p-bait was not detected.

### **3.2.7.1 Characterisation of *lyn1*<sup>+</sup>**

We named the novel interacting partner of Fkh2p, encoded by the SPBC16A3.03C gene, *Lyn1p*, for late cytokinesis 1 protein.

To initiate characterisation of *Lyn1p*, we compared *Lyn1p* with known components of the SFF complex in *S. cerevisiae*. Recent studies have revealed that activation of the Mcm1p-Fkh1p/Fkh2p involve a coactivator protein, Ndd1p (Pic *et al*, 2004). We therefore performed an alignment between Ndd1p and *Lyn1p* to see if the two polypeptides are related. However, the alignment revealed a low sequence similarity (6.6% identical and 11.1% similar). Other sequence analysis of *Lyn1p* revealed a conserved peptide motif, the pentatricopeptide repeat (PPR) at the C-terminus, thought to be involved in RNA binding and processing (Fig. 3.21).

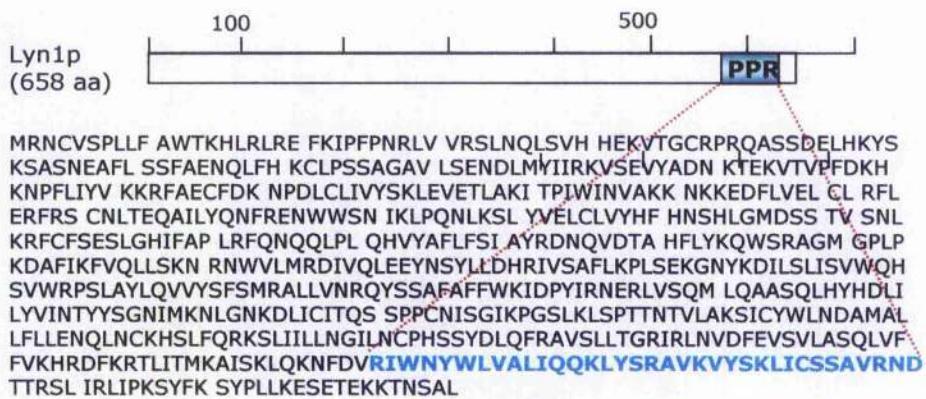
To investigate the functional properties of *Lyn1p*, we generated a chromosomal heterozygous deletion in a diploid strain (GG950). We constructed two different disruption mutants using the *kanMX* and *ura4*<sup>+</sup> cassettes described previously (Bahler *et al*, 1998) (Fig. 3.4.A-B) (Section 3.2.3.2). *lyn1::ura4*<sup>+</sup> and *lyn1::kan* PCR products were generated to amplify markers from pKSura4 (GB148) and pFA6a-kanMX6 template (GB131), which generated product sizes of 2300 and 1700 bp, respectively (Fig. 3.22.A). To assess whether *lyn1*<sup>+</sup> was essential for viability, purified PCR fragments were transformed into a wild-type diploid strain (GG908). All dissected tetrads produced viable haploid cells that segregated 2:2 with regards to the selection marker (Fig. 3.22.B). At least two *lyn1Δ* isolates were selected and confirmed by further PCR analysis (Fig. 3.22.C). This showed that *lyn1*<sup>+</sup> is a nonessential gene, unlike *S. cerevisiae* Ndd1p.



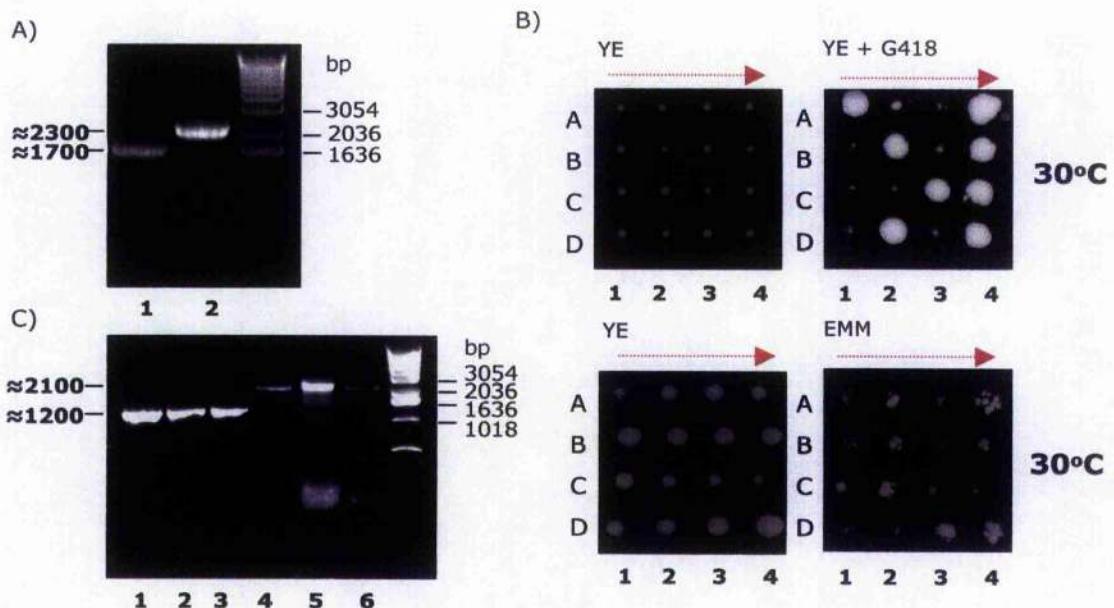
**Figure 3.20.** Purification of TAP-tagged Sep1p, Fkh2p and binding proteins. Lanes 1, non-purified wild-type protein extract. Lanes 2 and 3, wild-type protein extracts in 150 and 350 mM NaCl Hepes buffer. Lanes 4,5 and 6, Sep1p-TAP protein extracts in 150, 260 and 350 mM NaCl HEPES buffer. Lanes 7,8 and 9, Fkh2p-TAP protein extracts in 150, 260 and 350 mM NaCl HEPES buffer. Lane 10, molecular weight markers. Red letters indicated proteins identified by MALDI-TOF shown in Table 3.3.

Bands	NCBI accession number	Calculated size (D)	Protein species	
a	gi19112946	35897	SPBC32F12.11	Tdh1p:glyceraldehyde 3-phosphate dehydrogenase
b	gi1136787	49746	SPBC839.15C	eEF1A-cp:elongation factor 1 alpha-C
c	gi19113556	71130	SPBC16G5.15C	Fkh2p:forkhead protein
d	gi19113578	76669	SPBC16A3.03C	Lyn1p:hypothetical protein

**Table 3.3.** Identification of Fkh2p and Sep1p binding proteins. Biochemically purified proteins analysed by MALDI-TOF shown in Figure 3.20.



**Figure 3.21.** Amino acid sequence of Lyn1p. Lyn1p contains a potential PPR sequence (591-625 amino acids), highlighted in blue, thought to be important for RNA binding and processing.



**Figure 3.22.** Verification of *kan*<sup>R</sup> and *ura4*<sup>+</sup> *lyn1*<sup>Δ</sup> cells. A) Lane 1, PCR fragment amplified from pFA6a-kanMX6 cassette. Lane 2, PCR fragment amplified from pKSura4 cassette. B) Dissected tetrad selections for G418 and *ura4*<sup>+</sup> marker. Arrow in red indicated tetrad alignment. C) Lanes 1,2 and 3, colony PCR confirmation of G418-resistant isolates. Lanes 4,5 and 6, colony PCR confirmation of *ura4*<sup>+</sup> isolates. The approximate sizes of expected PCR fragments are indicated.

### **3.2.7.2 Morphogenetic analyses**

Preliminary visual inspection revealed that the two *lyn1Δ* strains (*lyn1::kan<sup>R</sup>* and *lyn1::ura4<sup>t</sup>*) germinated at the same rate as wild-type (Fig. 3.22.B). To avoid extragenic mutations, both *lyn1Δ* isolates were backcrossed to wild-type and verified progenies used for future manipulation (GG985 and GG989).

#### **3.2.7.2.1 DIC optic analyses**

To further characterise *lyn1Δ*, cells growing in liquid culture at 30°C were examined microscopically. As shown in Figure 3.23, mutant cells were longer relative to wild-type cells. This phenotype is typical of a cell cycle defective mutant in *S. pombe*. This could mean that *lyn1Δ* cells are delayed at entering mitosis or delayed in the formation of a division septum. Occasionally, septa were observed not forming at the centre, with cells dividing asymmetrically.

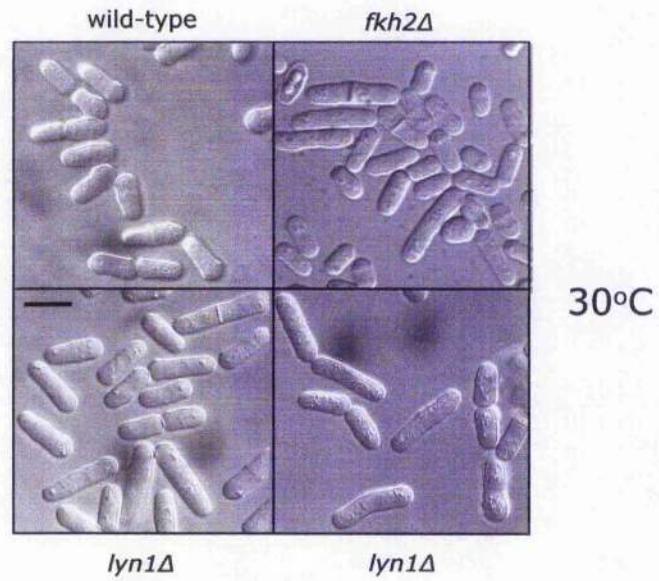
#### **3.2.7.2.2 Fluorescence optic analyses**

To examine septation defects in *lyn1Δ*, exponentially growing cells were stained with DAPI and Calcofluor (Fig. 3.24). Fluorescence microscopic examination revealed a higher percentage of septating cells, and that the elongated cells contained mild separation defects, with 2.8% population of cells showing misplaced and misoriented septa (Table 3.4). These observations are consistent with the hypothesis that *lyn1<sup>+</sup>* may be required for cell separation, and confirms previous data showing that *lyn1Δ* cells occasionally undergo asymmetric divisions.

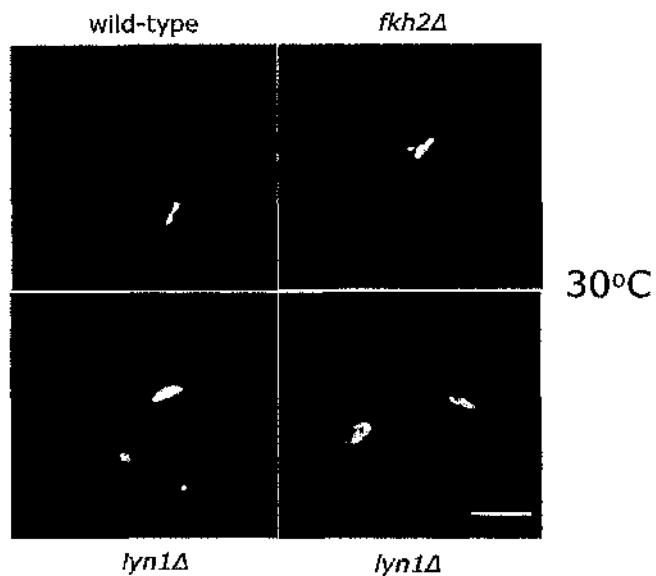
## **3.3 Discussion**

### **3.3.1 Characterisation of components required for PBF activity**

We identified a cell cycle transcription factor complex, PBF, bound to a DNA-regulatory element, PCB, which regulates the expression of genes during the M/G1 phase of the cell cycle (Anderson *et al*, 2002). In this thesis, we analysed PBF through both *in vitro* and *in vivo* experiments. We showed that two proteins required for PBF binding activity are the FHD transcription factor protein, Fkh2p, and a MADS-box-like DNA-binding protein, Mbx1p. A similar transcription factor was identified in *S. cerevisiae*, collectively known as the SFF complex. The identification of FHD proteins shared between these two species was initiated



**Figure 3.23.** *lyn1Δ* cells exhibited cell separation defects. Phenotypes of wild-type, *lyn1Δ* and *fkh2Δ* cells, grown in liquid medium at 30°C, viewed with DIC optics. Bar size  $\approx$ 5  $\mu\text{m}$



**Figure 3.24.** Cell separation defects in *lyn1Δ* cells. Wild-type, *lyn1Δ* and *fkh2Δ* cells grown in liquid medium at 30°C, stained with DAPI and Calcofluor to reveal cell nuclei and septa, and viewed with fluorescence optics. Bar size  $\approx 5 \mu\text{m}$

Cells	Septating cells (%)	Septating cells (%)		
		unconstricted	multiseptated	misplaced and misoriented
wild-type	15.2	0	0	0
<i>fkh2Δ</i>	27.7	3.2	13.5	4.4
<i>lyn1Δ</i>	19.6	0	0	2.8

**Table 3.4.** Quantitative analysis of cell separation defects in *lyn1Δ* cells.

through the computational analysis based on a conserved FHD region, first identified in HNF-3 protein family in rats (Lai et al, 1990).

Full-length amino acid analysis in *S. pombe* FHD proteins, Fkh2p, Sep1p, Mei4p and Fhl1p, revealed a significant degree of sequence identity to Fkh2p of *S. cerevisiae*. Subsequent analysis showed that *S. cerevisiae* Fkh2p shared highest degree of homology to *S. pombe* Fkh2p. Meanwhile, *S. cerevisiae* Fhl1p was the closest related to full-length Fhl1p in *S. pombe*, while Sep1p and Mei4p were shown to have cell cycle and meiotic-specific functions.

We have also found that *S. pombe* contains fewer MADS-box transcription factor proteins than *S. cerevisiae*. We reported a high degree of peptide conservation between *S. cerevisiae* Arg80p and Mcm1p to *S. pombe* Map1p and Mbx1p. *S. pombe* Mbx2p is closest in homology to *S. cerevisiae* Rlm1p and Smp1p. Our work has demonstrated that Map1p was not required for cell cycle function and that Mbx2p has roles for cell wall integrity (Buck et al, 2004). Therefore, Mbx1p is likely to be a component of PBF in partnership with Fkh2p and Sep1p.

Through EMSA analysis, we showed that PBF is absent in *mbx1Δ* cells, although PBF-binding remained unperturbed in *fkh2Δ* and *sep1Δ* cells. However, our tagged constructs argue that Fkh2p is potentially a component of PCB and its C-terminus tail is important for protein-DNA or protein-protein interactions. It is likely that Fkh2p recruits core component of PBF through specific base contact at the C-terminus region, perhaps through co-operative activity with Mbx1p. Furthermore, it is interesting that full-length *fkh2<sup>+</sup>* mRNA is periodically regulated during M/G1, coincidently to *plo1<sup>+</sup>* and *cdc15<sup>+</sup>* gene cluster, and could not rescue mutants of mitotic catastrophic suppressor *mcs3*, while the C-terminus truncated *fkh2<sup>+</sup>* allowed entry into mitosis (Anderson et al, 2002; Buck et al, 2004). In addition, *fkh2Δ* cells demonstrated constitutive expression of M/G1-specific cell cycle transcription, therefore supports the idea that Fkh2p may be the transcription factor required for M/G1 transcription. All these observations suggest that Fkh2p is a component of PBF and that there is some form of functional redundancy between Fkh2p and its interacting partners, and so do not abolish PBF-PCB binding using *fkh2Δ* protein extract, analysed through EMSA.

Given the reported redundancy between *S. cerevisiae* Fkh1p and Fkh2p, we have generated combinatorial deletions for *fkh2Δ*, *sep1Δ* and *mbx1Δ* in *S. pombe*. Despite all mutant cells being viable, they have decreased rates in cell growth, especially exacerbated in the *fkh2Δsep1Δmbx1Δ* triple mutant. Microscopic inspections revealed strong genetic interactions among the three genes. All three mutants exhibited septation defects and overexpression analysis found that increased levels of *fkh2<sup>+</sup>* were lethal in wild-type cells but not in *sep1Δ* cells. Despite the genes having roles in septation and cytokinesis, deleting all the three was not lethal. This suggests that there are other unidentified components in

PBF. Furthermore, MADS-box proteins often require combinatorial proteins to control regulatory specificity (Shore and Sharrocks, 1995). In agreement, studies in *S. cerevisiae* revealed that besides Fkh2p and Fkh1p, a non-DNA-binding protein Ndd1p acts as a coactivator to Mcm1p-regulated complex (Koranda *et al.*, 2000; Pic *et al.*, 2004).

To further explore the subunit properties of PBF, we generated chromosomal tagged *fkh2* and *sep1* for TAP-tag purifications. The Fkh2p-TAP purification recovered a Fkh2p-interacting protein, designated Lyn1p, along with Fkh2p. When *lyn1<sup>+</sup>* was deleted, the cells were viable showing it was non-essential. However, we observed a cell cycle phenotype in *lyn1Δ* cells, with cells long at division similar to that observed in *fkh2Δ*. Interestingly, we found that cell separation defects characteristic of *fkh2Δ* cells was also present in *lyn1Δ*. We therefore propose a shared function for Lyn1p and Fkh2p. Peptide analysis showed that Lyn1p does not share sequence homology to Ndd1p in *S. cerevisiae* but instead, contains a putative PPR at the C-terminus thought to be important for RNA ligand-binding.

To identify proteins that bind to Sep1p, components coeluted from Ca<sup>2+</sup>/calmodulin-Sepharose were identified by MALDI-TOF. This revealed GAPDH (Tdh1p) and a translational elongation factor (eEF1A-cp). Surprisingly, the recombinant bait (Sep1p) was not recovered when analysed on a denaturing gel or by MALDI-TOF analysis. This suggested that the stoichiometric amount of Sep1p was lower than its associate proteins and thus undetectable. In agreement, we and others (Dr. Millar, personal communication) were unable to detect Sep1p-Myc by Western blotting despite repeated efforts. Following this, two key questions need to be addressed: how do PBF-regulatory factors transfer their signal to the basic transcription machinery and which factor do they contact?

*tdh1<sup>+</sup>* encodes for a glycolytic enzyme abundant in the cytoplasm. Nevertheless, reports have shown evidence for a nuclear form whose function is involved in a diverse cellular events including cell cycle-regulated DNA replication (Ronai *et al.*, 1993). It has also been shown that Tdh1p interacts with the subunits of RNAP II – Rpb3p and Rpb7p (Kimura *et al.*, 2001; Mitsuzawa *et al.*, 2004). Tdh1p interacts with Rpb3p on an unphosphorylated CTD, therefore suggesting a role in transcription initiation. Another finding suggested that human GAPDH interacts with a POU-domain transcription factor protein shown to be necessary for the transcription of histone H2B (Zheng *et al.*, 2003). It was proposed that the glycolytic enzyme is a key component of the coactivator complex for H2B transcription. Interestingly, an alignment search against complete peptide sequences from human GAPDH revealed evolutionary conservation with *S. pombe* and *S. cerevisiae* GAPDHs – both named Tdh1p. Several lines of evidence have indicated that the Mediator complex directly interacts with the CTD of RNAP II (Myers *et al.*, 1998). Similarly, in a separate immunoaffinity assay and two-hybrid screen, when Rpb7p was used as bait, eEF1A-cp and Tdh1p were copurified and isolated (Mitsuzawa *et al.*, 2004).

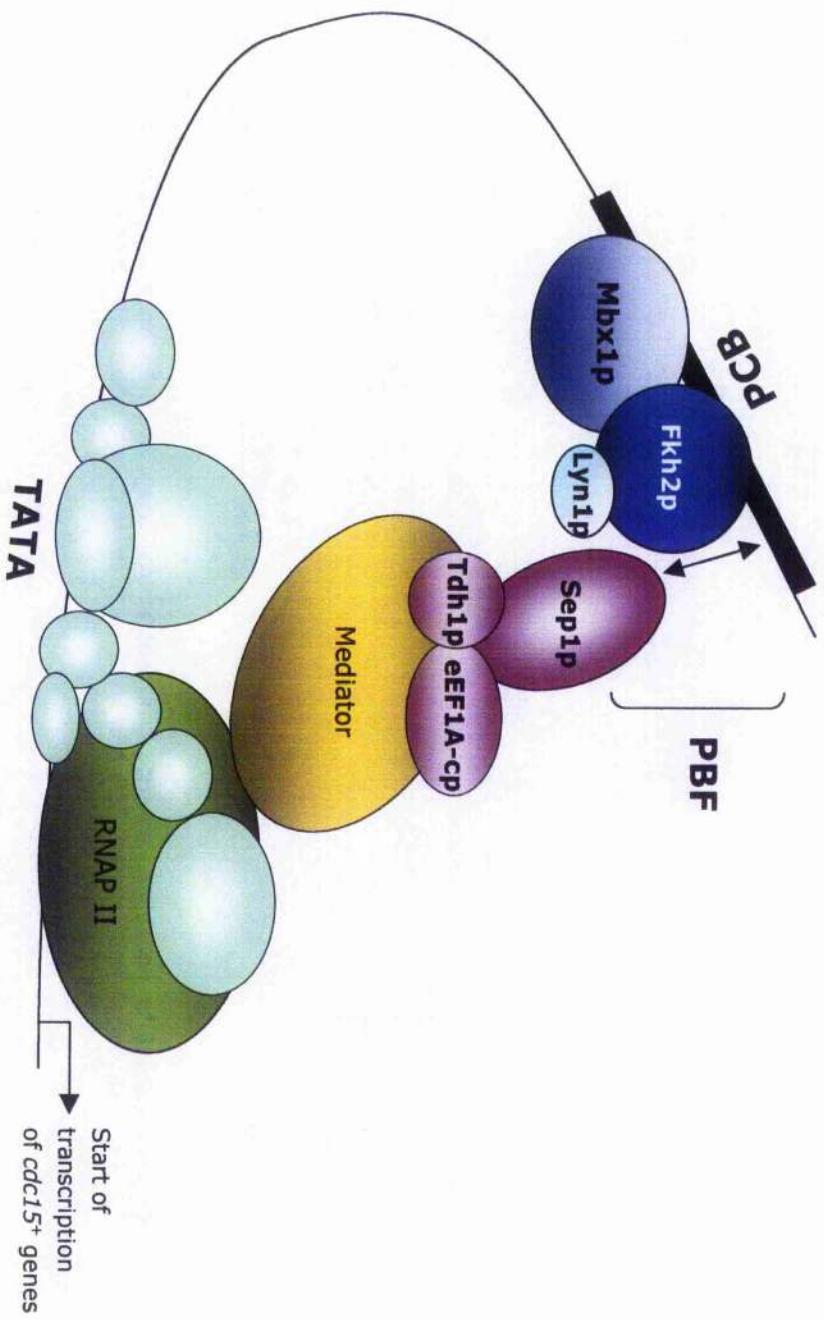
Based on these observations, we propose a model consisting of an Mbx1p-Fkh2p-Lyn1p activating complex, RNAP II machinery and a Sep1p-Tdh1p-eEF1A-cp complex that interacts with the Mediator (Fig. 3.25). Although we showed that *fkh2<sup>+</sup>* and *sep1<sup>+</sup>* are required for normal cell separation in *S. pombe* during M/G1, we have not formally proven that they are components of PBF. Nevertheless, since *mbx1Δ* cells result in the loss of PBF-PCB complex formation *in vitro*, and *mbx1<sup>+</sup>* genetically interacts with *sep1<sup>+</sup>* and *fkh2<sup>+</sup>*, this suggests that Mbx1p, Fkh2p and Sep1p are either directly or indirectly interacting through the PBF complex to regulate M/G1-specific transcription. Therefore, this led us to continue testing for other components that might regulate PBF-PCB gene transcription, and their control mechanisms.

### **3.3.2 Model for PBF regulation of M/G1 gene expression in *S. pombe***

For transcription activation to occur *in vivo*, the GTF complex, coactivators (Mediator complex) and regulatory activators must interact. It is thought that the Mediator complex is an essential element for basal and regulated expression of almost all RNAP II genes (Holstege *et al*, 1997) and forms a bridge to convey regulatory information between transcriptional activating proteins and RNAP II machinery (Kim *et al*, 1994).

We and others have shown that *sep1Δ* cells display a hyphal morphology suggesting Sep1p is required for cell separation. Three Mediator subunits (Pmc2p, Pmc3p and Pmc6p) exhibited similar cell separation and septation phenotypes to *sep1Δ* cells, when deleted (Spahr *et al*, 2001). In addition, *S. pombe* Sep15p/Mec8p, whose gene function was initially identified for cell separation, has also been implicated as a subunit of the Mediator complex (Spahr *et al*, 2001). It is also noteworthy that *sep15<sup>+</sup>* was shown to display genetic interaction with *sep1<sup>+</sup>* (Grallert *et al*, 1999). In addition, recent work demonstrated that *sep10<sup>+</sup>* and *sep15<sup>+</sup>*, both required for cell separation activity and are components of the Mediator complex, genetically interact with *sep1<sup>+</sup>* (Lee *et al*, 2005). Therefore, it is possible that PBF interacts with the Mediator complex through Sep1p. In addition, Spahr *et al* (2001) isolated thirteen subunits, thought to represent the complete *S. pombe* Mediator complex and from this, ten subunits contained homologues to *S. cerevisiae* Mediator while eight from the ten are essential for viability. They further hypothesized that the role of the non-conserved subunits are necessary for species and activator-specific, and have evolved due to evolutionary pressure.

*sep1Δ* cells abolish the periodic profile of genes expressed at the M/G1 interval including the *cdc15<sup>+</sup>* and *ace2<sup>+</sup>* groups (Buck *et al*, 2004; Rustici *et al*, 2004). However, while *sep1Δ* cells modestly affect the gene transcription of the M/G1 cluster, *ace2<sup>+</sup>*



**Figure 3.25.** Model for PBF regulation of M/G1 gene expression in *S. pombe*. Activation of transcription through interactions with the Mediator complex, basic transcription machinery and PBF transcription factor complex. RNAP II (green), general transcription factors (GTF) (light green) and the Mediator complex (yellow) are hypothetically located. Double headed arrow indicates interaction of Sep1p-Mediator complex and PBF-PCB elements. Three components of PBF are coloured in different shades of blue located at the PCB UAS (upstream activating sequence).

transcription factor, whose control selectively affects a small subset of *sep1*<sup>+</sup>-dependent genes, conferred control of > 2-fold difference between peak-to-trough levels (Rustici *et al*, 2004). Therefore, we propose that Sep1p could be the non-conserved species-specific Mediator subunit required for the M/G1-regulated transcription in *S. pombe*, which links the RNAP II machinery to PBF through its binding with Tdh1p and perhaps also eEF1A-cp.

Although most of the Mediator components in *S. cerevisiae* have been isolated, it was proposed that the Mediator exists in various forms and its function differ significantly. While some are necessary for the expression of nearly all genes, others are only required for a subset of genes (Holstege *et al*, 1997). For example, an essential subunit – Srb4p – is needed for 93% of all *S. cerevisiae* genes while Srb5p, a non-essential protein is only necessary for the expression of 16% of the genes (Spahr *et al*, 2001). Evidence in mice, *D. melanogaster*, *C. elegans* and human also supported the properties observed in *S. cerevisiae*. Therefore, it is likely that the same applies in *S. pombe*, consistent with our model that Sep1p acts as a species-specific Mediator subunit required for M/G1-regulated transcription.

## **Chapter 4: Upstream Regulation of PBF**

### **4.1 Introduction**

The results presented in the previous chapter identified at least three transcription factor proteins, Fkh2p, Sep1p and Mbx1p, which are important for PBF-PCB-mediated expression in *S. pombe*. We characterised the contribution of each towards DNA binding, gene expression, septation, cytokinesis and cell separation. Our next objective was to describe the control mechanisms required to execute PBF-PCB-mediated transcription. Since PBF appears to bind to the *cdc15<sup>+</sup>* UAS throughout the cell cycle (Anderson *et al.*, 2002), we propose that the expression of *cdc15<sup>+</sup>*-dependent genes require modification of either one or more of the PBF proteins.

Much research has revealed that M phase progression relies predominantly on two post-translational mechanisms: phosphorylation and proteolysis (Nigg, 2001). These are interdependent processes in that proteolytic machinery is sometimes controlled by phosphorylation, while several mitotic kinases are down-regulated by degradation. In *S. pombe*, the catalytic Cdc2p and cyclin Cdc13p are required to commit the cells into mitosis. However, when cells enter late anaphase Cdc13p level falls allowing them to exit mitosis.

Similarly, evidence in various organisms indicated that for septation to be initiated, prior inactivation of CDK activity through APC/C-mediated degradation of cyclin B has to occur. This is under the control of Polo-like kinases (Plks) (Brassac *et al.*, 2000; May *et al.*, 2002; Descombes and Nigg, 1998).

Since the discovery of Plks, much attention has turned towards them in the control of cell division. These serine/threonine kinases are conserved from yeast to humans, and were initially identified in *D. melanogaster* and *S. cerevisiae* as *polo* and *CDC5* (Sunkel and Glover, 1988; Llamazares *et al.*, 1991). Subsequent sequence searches identified at least three members of Plks in mammals and *X. laevis*, whereas *S. pombe* contains only one.

#### **4.1.1 Polo-like kinases (Plks)**

The discovery of CDKs had been valuable towards understanding the cell cycle. Indeed, they are the ultimate component controlling cells through cell division (Moser and Russell, 2000). However, if the hypothesis that important stages for cell division are controlled by phosphorylation-dependent mechanisms is true, then different families of kinases could also act at these key points, besides CDK-cyclin complexes.

The protein kinase Polo was identified in *D. melanogaster* when analysing mutant alleles that arrest at late mitosis (Glover, 1991). Sequence analyses revealed similarities between *D. melanogaster* Polo with *S. cerevisiae* Cdc5p and *S. pombe* Plo1p. While both

yeasts and *D. melanogaster* only contain a single Plk gene, higher vertebrates have multiple Plks, with mammals and *C. elegans* containing at least three, Plk1-3 and Plc1-3, respectively (Barr *et al.*, 2004).

All Plks have a similar kinase serine/threonine domain (KD) at the N-terminus and a C-terminus regulatory domain containing two signature motifs – known as the polo-box domain (PBD).

Plks play important roles in multiple stages of M phase such as entrance into mitosis, bipolar spindle formation, chromosome segregation and cytokinesis. In *D. melanogaster*, *polo* mutants cause the formation of abnormal spindle poles and the incorrect distribution of chromosomes (Sunkel and Glover, 1988). Similarly, in human cells and *X. laevis* embryo, PLK1 and Plx1 are required for centrosomal maturation and entry into mitosis. In *S. pombe*, the recruitment of Plo1p to the spindle pole body (SPB) promotes mitotic entry, through a feedback loop control mechanism (Grallert and Hagan, 2002), with a similar function identified for Cdc5p in *S. cerevisiae* (Bartholomew *et al.*, 2001).

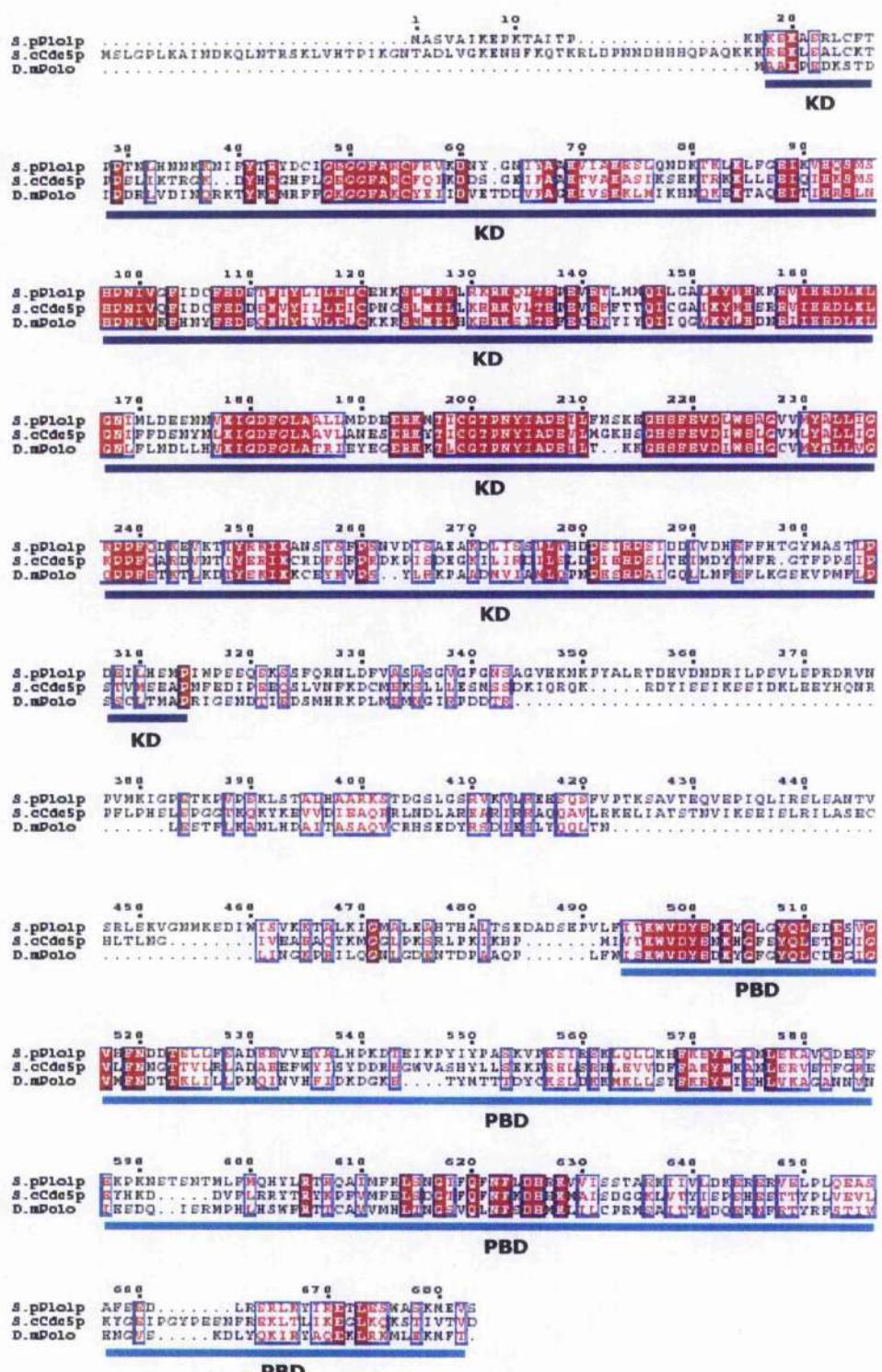
Early evidence for the role of Plks in cytokinesis came from studies with *S. pombe* Plo1p (Ohkura *et al.*, 1995; Bahler *et al.*, 1998; Mulvihill *et al.*, 1999). Later, mutant Plks in mammals and *D. melanogaster* demonstrated phenotypes consistent with impaired cytokinesis (Mundt *et al.*, 1997; Carmena *et al.*, 1998). Therefore, it appears that the role of Plks in cytokinesis is fundamentally conserved among eukaryotes.

#### **4.2 Characterisation of *S. pombe* Plk, Plo1p**

*S. pombe* *plo1*<sup>+</sup> was initially identified by sequence similarity with *S. cerevisiae* *CDC5*, *D. melanogaster* *polo* and the mammalian forms of Plks (Ohkura *et al.*, 1995). Subsequent analysis revealed that it is essential for viability and controls two important functions: the establishment of bipolar spindles and cytokinesis. In addition, when *S. pombe* *plo1*<sup>+</sup> was overexpressed it induced septation, even during interphase, showing that it controls this process. Characterisation of the KD and PBD regions of *plo1*<sup>+</sup> defined independent roles with the PBD required for mitotic spindle bipolarity, and the KD is responsible for septation (Reynolds and Ohkura, 2003).

In this chapter, we tested the role of Plo1p in controlling M/G1-specific transcription in *S. pombe*.

Sequence analysis confirmed the high amino acid sequence conservation of *S. pombe* Plo1p and was performed against the full-length peptide sequences from the founding members, Cdc5p in *S. cerevisiae* and Polo in *D. melanogaster* (Fig. 4.1) (Sunkel and Glover, 1988; Llamazares *et al.*, 1991). Subsequent analysis showed that *S. pombe*



**Figure 4.1.** Conserved amino acid sequences among polo kinase homologues. Shaded residues represent identical amino acids of *S. pombe* Plo1p (*S.p* Plo1p), *S. cerevisiae* Cdc5p (*S.c* Cdc5p) and *D. melanogaster* Polo (*D.m* Polo). Underlined residues at N-terminus represent kinase domain (KD) while underlined residues at C-terminus represent polo box domain (PBD).

Plo1p shares 45% identity and 65% similarity across the KD region, and 32% identity and 52% similarity across the PBD region with *D. melanogaster* Polo (Fig. 4.2).

We identified at least 11 genes that underwent M/G1-specific transcription, including *plo1<sup>+</sup>* (Anderson *et al.*, 2002). Our studies also showed that Plo1p function is required for correct cycle expression of M/G1 genes, and for PBF binding to the PCB UAS. Therefore, Plo1p was proposed to have a role in *cdc15<sup>+</sup>* expression. To better understand the role of Plo1p in regulating M/G1 genes, we tested its function using a conditional lethal double mutant, *plo1-ts35 cdc2-33* (Appendix IV: GG481). *plo1-ts35* contains a mutation in the KD and was a gift from Prof. Hagan (Paterson Institute, Manchester); we generated the double mutant by tetrad analysis (Section 2.2.8.13).

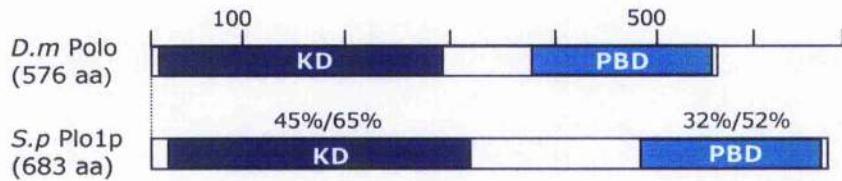
*cdc2-33* is a temperature sensitive mutant: at permissive temperature (25°C) the cell cycle progresses similarly to wild-type cells; however, when the temperature is increased to restrictive temperature (36°C) cells arrest at the G2/M boundary, when Cdc2p function is required. Importantly, when cells are returned to 25°C they re-enter the cell cycle synchronously.

To study the effect of Plo1p on PBF-dependent genes, G2 *plo1-ts35 cdc2-33* cells were initially selected by elutriation and immediately arrested at 36°C for 2 h (Section 2.2.7). This precludes the potential “leakiness” of *cdc2-33* when arrested at 36°C as the cells are held at this temperature for only two hours, and also creates higher cell synchrony. Once returned to 25°C, samples were collected at 10 min intervals and septation index was measured, to estimate cell synchronisation, as shown in Figure 4.3.A. For septum scoring, a small volume of cells was removed and mixed with 1/10 dilution of formaldehyde, with the remaining processed for Northern blot analysis (Section 2.2.9.1). Northern blotting showed that the *cdc15<sup>+</sup>* and *spo12<sup>+</sup>* mRNA fluctuated as cells progress through the cell cycle (Fig. 4.3.B). However, cell septation was delayed, approximately 45 min relative to the control, single *cdc2-33* mutant, and the periodic profiles of *cdc15<sup>+</sup>*-dependent genes were similarly retarded.

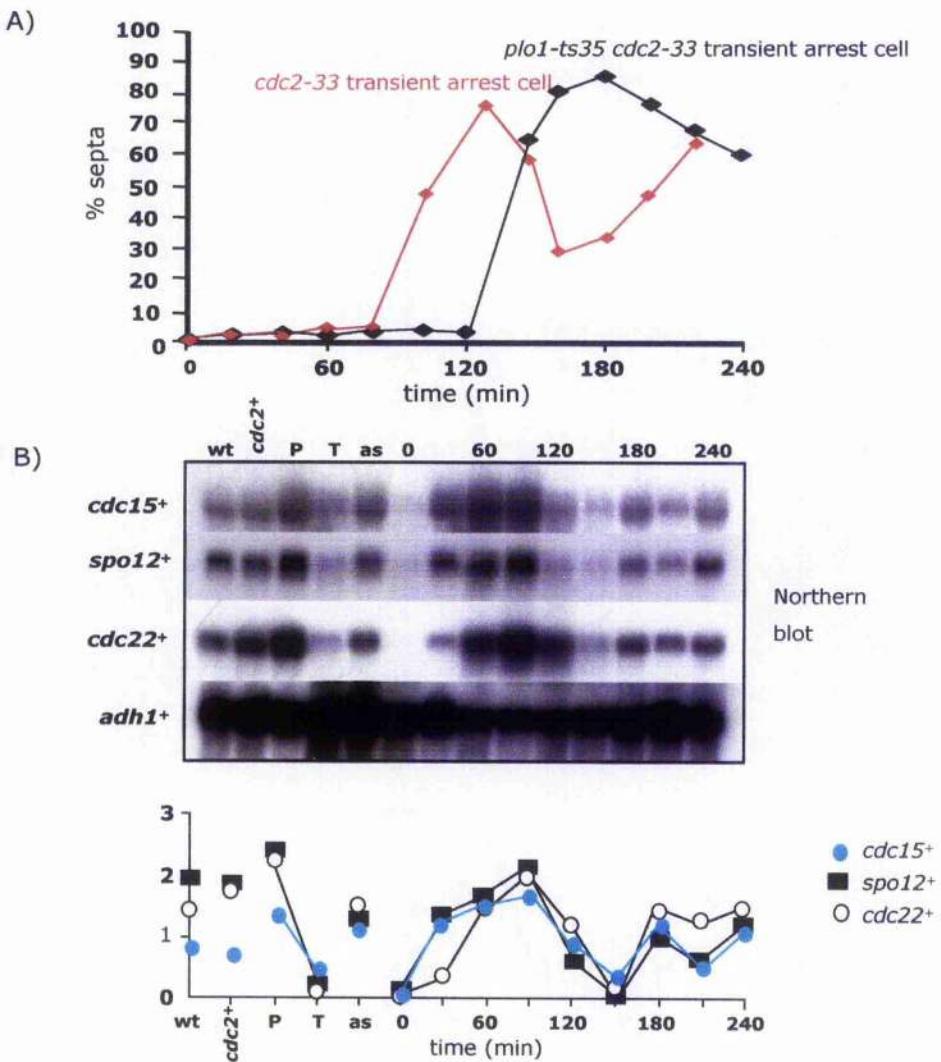
Thus, we propose that Plo1p is involved in the timing of cell separation potentially through controlling M/G1-specific transcription.

#### 4.2.1 Overexpression analyses

Previous work has shown that high expression of *plo1<sup>+</sup>* induces septation, regardless of the cell cycle phase (Ohkura *et al.*, 1995). Subsequently, two functional domains in *S. pombe* Plo1p were identified: the PBD and KD (Reynolds and Ohkura, 2003). Mutations in the PBD



**Figure 4.2.** Peptide sequence comparison of *D. melanogaster* Polo with *S. pombe* Plo1p. Percent identity/percent similarity values are shown for kinase domain (KD) and polo box domain (PBD) conserved regions.



**Figure 4.3.** Effect of *plo1-ts35* on PCB-regulated gene transcription *in vivo*. A) Septation index of *cdc2-33* and *plo1-ts35 cdc2-33* cells to indicate the degree of synchrony. B) Cell cycle transcription of *cdc15<sup>+</sup>* and *spo12<sup>+</sup>* in *plo1-ts35 cdc2-33* arrested cells. Cells were collected before temperature arrest (as), asynchronous wild-type cells (wt), asynchronous *cdc2-33* (*cdc2<sup>+</sup>*), peak (P) and trough (T). Quantification of each transcript against *adh1<sup>+</sup>* is shown.

specifically affect the polarity of mitotic spindle whereas mutation in the KD inhibits septation without affecting the former. Given that *plo1-ts35* cells affects separation timing and PBF-PCB binding (Anderson *et al*, 2002), we extended the genetic characterisation of *plo1<sup>+</sup>* through overexpression studies.

#### **4.2.1.1 *plo1<sup>+</sup>* overexpression**

To test whether overexpression of *plo1<sup>+</sup>* might affect mRNA levels of *cdc15<sup>+</sup>* and *spo12<sup>+</sup>*, we used an *nmt1:plo1<sup>+</sup>* overexpression plasmid (Appendix IV: GB297), a gift of Dr. Ohkura (University of Edinburgh). *nmt1:plo1<sup>+</sup>* was introduced into *leu1-32* wild-type cells (GG614) and maintained on media supplemented with thiamine (*nmt1* "off"). When *plo1<sup>+</sup>* was overexpressed by removal of thiamine (*nmt1* "on") elevated Plo1p conferred multiple rounds of septation, as previously reported (Fig. 4.4.A) (Ohkura *et al*, 1995).

To examine the transcript profiles of PCB genes when overexpressing *plo1<sup>+</sup>*, cells were harvested at hourly time points after 15 hr of induction. Interestingly, Northern blot analysis showed that *cdc15<sup>+</sup>* and *spo12<sup>+</sup>* mRNA were present at higher levels, relative to control cells (Fig. 4.4.B).

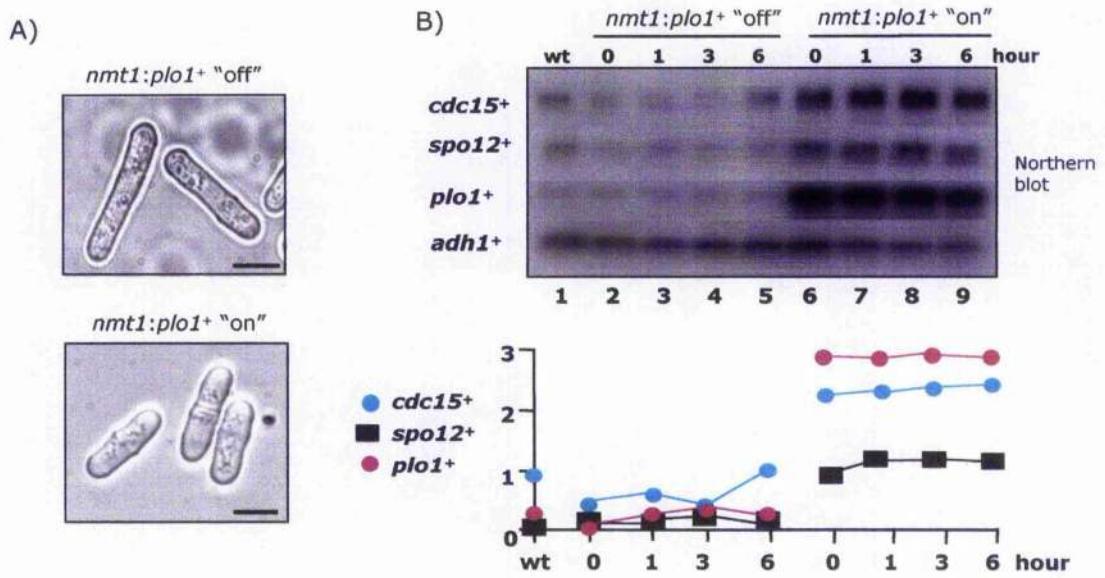
This experiment supports the proposal that Plo1p is a positive regulator of PCB-regulated transcription.

#### **4.2.1.2 Overexpression of *plo1* mutants**

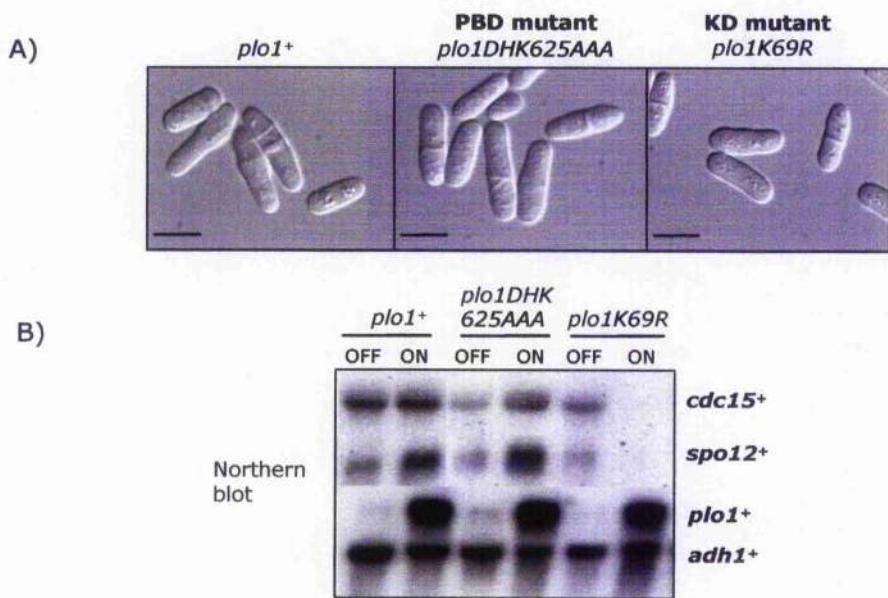
Next, we examined the *plo1<sup>+</sup>* domains required for PCB-specific transcription. Our attention was focused on the conserved KD and PBD, and so we obtained mutants in these domains from Dr. Ohkura (University of Edinburgh). We examined the effect of overexpressing a *plo1* PBD mutant (*plo1DHK625AAA*) and a KD mutant (*plo1K69R*) on septation and *cdc15<sup>+</sup>* transcript levels (Reynolds and Ohkura, 2003).

To test whether *plo1K69R* (GG670) and *plo1DHK625AAA* mutants (GG668) are functionally separable in the activation of PCB-regulated genes, we performed a similar experiment to wild-type *plo1<sup>+</sup>* overexpression. We found that high expression of *plo1DHK625AAA* induced untimely septation similarly to that seen with wild-type *plo1<sup>+</sup>*, whereas overexpression of *plo1K69R* abolished the untimely septation phenotype, as previously reported (Fig. 4.5.A) (Reynolds and Ohkura, 2003).

To examine the transcript profiles of PCB-regulated genes when overexpressing *plo1K69R* and *plo1DHK625AAA*, cells were harvested at time points after 15 hours of induction. Northern blot analysis of mRNA revealed that both *cdc15<sup>+</sup>* and *spo12<sup>+</sup>* transcript levels were comparable between *plo1DHK625AAA* and control cells (Fig. 4.5.B). In contrast,



**Figure 4.4.** Effect of overexpressing *plo1<sup>+</sup>* on PCB-regulated gene transcription. A) DIC images of cells grown in the presence of thiamine (*nmt1:plo1<sup>+</sup>* "off") and in the absence of thiamine (*nmt1:plo1<sup>+</sup>* "on"). B) Time course of *cdc15<sup>+</sup>* and *spo12<sup>+</sup>* mRNA levels when *plo1<sup>+</sup>* non-expressed and overexpressed in wild-type cells. Cell samples were harvested at time points after 15 hr induction, denoted as 0, 1, 3 and 6 hr. The blot was hybridised with *adh1<sup>+</sup>* as a loading control. Quantification of each transcript against *adh1<sup>+</sup>* is shown. Bar size  $\approx 5 \mu\text{m}$



**Figure 4.5.** Effect of overexpressing *plo1* mutants on PCB-regulated gene transcription. A) DIC images of cells when *plo1<sup>+</sup>*, *plo1K69R* (KD mutant) and *plo1DHK625AAA* (PBD mutant) overexpressed. B) The mRNA levels of *cdc15<sup>+</sup>* and *spo12<sup>+</sup>* when *plo1K69R* and *plo1DHK625AAA* non-expressed and overexpressed in wild-type cells. The blot was hybridized with *adh1<sup>+</sup>* as a loading control. Quantification of each transcript against *adh1<sup>+</sup>* is shown. Bar size  $\approx 5 \mu\text{m}$

*plo1K69R* overexpression abolished *cdc15<sup>+</sup>* and *spo12<sup>+</sup>* transcription. Therefore, we propose that M/G1-specific transcription requires a functional KD of *plo1<sup>+</sup>*.

To conclude, we confirmed that Plo1p KD and PBD are functionally separable, with the KD required for the activation of M/G1-specific transcription.

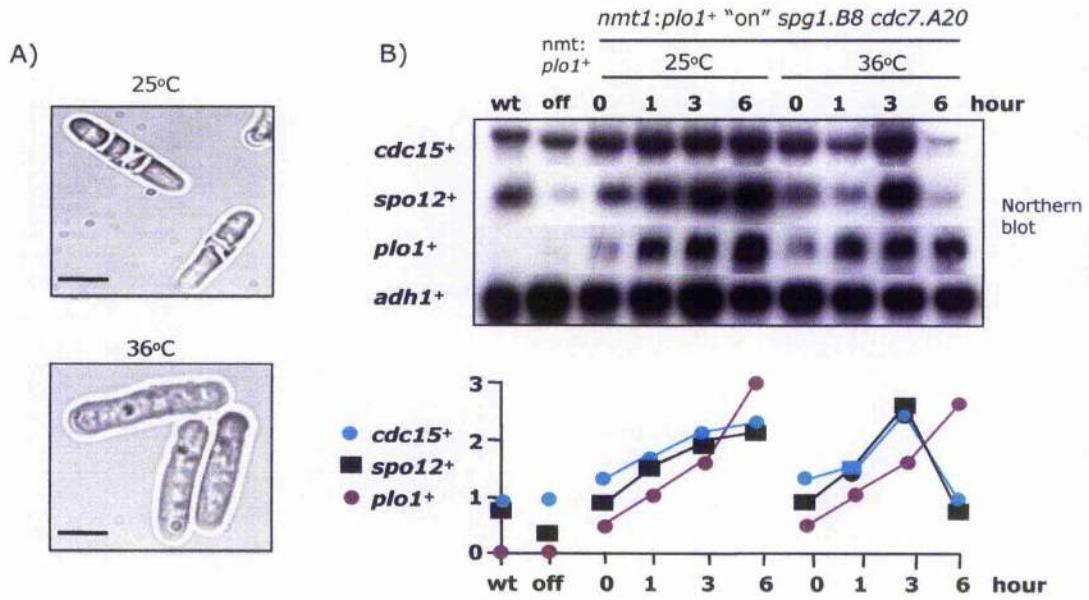
#### **4.2.2 Regulatory control of Plo1p, PCB-regulated genes and SIN pathway**

In *S. pombe*, the division of mitotic cells by cytokinesis occurs with the formation of a septum during late anaphase, initiated by the contraction of medial actomyosin ring. The current model proposes that the onset of septation is signaled through the septation initiation network (SIN) (McCollum and Gould, 2001). While *sin* mutants can assemble a normal contractile ring, they fail to initiate constriction and do not form a normal septum (Nurse *et al.*, 1976; Balasubramaniam *et al.*, 1998). SIN signalling requires a GTPase and several protein kinases, which include Sid2p, a target of PBF-PCB-mediated control (McCollum and Gould, 2001). Furthermore, activation of the SIN requires Plo1p (Tanaka *et al.*, 2001). This prompted us to ask whether PCB-dependent gene expression is dependent on the SIN pathway.

It is known that overexpression of *spg1<sup>+</sup>* or *cdc7<sup>+</sup>* can advance the onset of septation, while a *spg1.B8 cdc7.A20* temperature sensitive double mutant inhibits septation at 36°C (Fankhauser and Simanis, 1994; Schmidt *et al.*, 1997). Therefore, we examined the transcript levels of PCB genes when overexpressing wild-type *plo1<sup>+</sup>* in *spg1.B8 cdc7.A20* temperature sensitive cells (GG501). At 25°C, when supplemented with thiamine (*nmt1* "off") the cells grew and divided like wild-type. But when *plo1<sup>+</sup>* was overexpressed, *nmt* induction of 15 hr, at 25°C a high proportion of the cells showed cell cycle arrest. They elongated with multiple septa without physical separation (Fig. 4.6.A).

To evaluate the transcript profile of PCB-regulated genes under these conditions, samples were collected from various time points and assayed by Northern blot analysis. As both the *cdc7.A20* and *spg1.B8* gene products are active at 25°C, Plo1p can activate the SIN pathway. Our results indicated that *cdc15<sup>+</sup>* and *spo12<sup>+</sup>* mRNA levels were overexpressed under these conditions when compared to the control, non-induced *plo1<sup>+</sup>* (Fig. 4.6.B).

To test whether the up-regulation of PCB gene transcripts were dependent on the SIN pathway, the experiment was repeated at 36°C. Since Cdc7p and Spg1p are inactive at this temperature, *plo1<sup>+</sup>* overexpression should not initiate SIN to promote septation as shown by arrested cells with elongated phenotype that fail to form division septa (Fig. 4.6.A) (Fankhauser and Simanis, 1994; Schmidt *et al.*, 1997; Tanaka *et al.*, 2001). Under these conditions *cdc15<sup>+</sup>* and *spo12<sup>+</sup>* mRNAs were unaffected (Fig. 4.6.B).



**Figure 4.6.** Effect of *plo1+* overexpression in *spg1.B8 cdc7.A20* cells. A) DIC images of cells grown at permissive (25°C) and restrictive (36°C) temperatures when *plo1+* overexpressed. B) Time course of *cdc15+* and *spo12+* mRNA levels when *plo1+* overexpressed at 25°C and 36°C. Quantification of each transcript against *adh1+* is shown. Bar size  $\approx 5 \mu\text{m}$

Therefore, we propose that the induction of *cdc15<sup>+</sup>* and *spo12<sup>+</sup>* transcription by *plo1<sup>+</sup>* over-expression is independent of the SIN.

#### **4.2.3 Genetic interactions among *plo1<sup>+</sup>* and components of PBF**

We showed that *plo1<sup>+</sup>* when overexpressed, elevates transcript levels of PCB-dependent genes and that accumulation of *cdc15<sup>+</sup>* and *spo12<sup>+</sup>* mRNA is independent of the SIN pathway. To test whether *fkh2<sup>+</sup>*, *sep1<sup>+</sup>* and *mbx1<sup>+</sup>* genetically interact with *plo1<sup>+</sup>*, we made combination mutant strains to test for synthetic suppressor or lethality phenotypes.

To create these mutant strains, we crossed *plo1-ts35* with *fkh2Δ*, *sep1Δ* and *mbx1Δ* cells, and identified double mutants by tetrad analysis.

##### **4.2.3.1 Phenotypic analyses**

To examine the phenotype of the double mutants, asynchronous cells grown in liquid media were microscopically examined.

*fkh2Δ plo1-ts35* (GG763), *sep1Δ plo1-ts35* (GG761) and *mbx1Δ plo1-ts35* (GG760) cells, all displayed additive separation defects relative to single mutants at 25°C (Fig. 4.7.A). Although viable, these mutants were highly elongated and underwent repeated cell cycles without physical separation, providing genetic evidence for interactions between *plo1<sup>+</sup>* and the components of PBF.

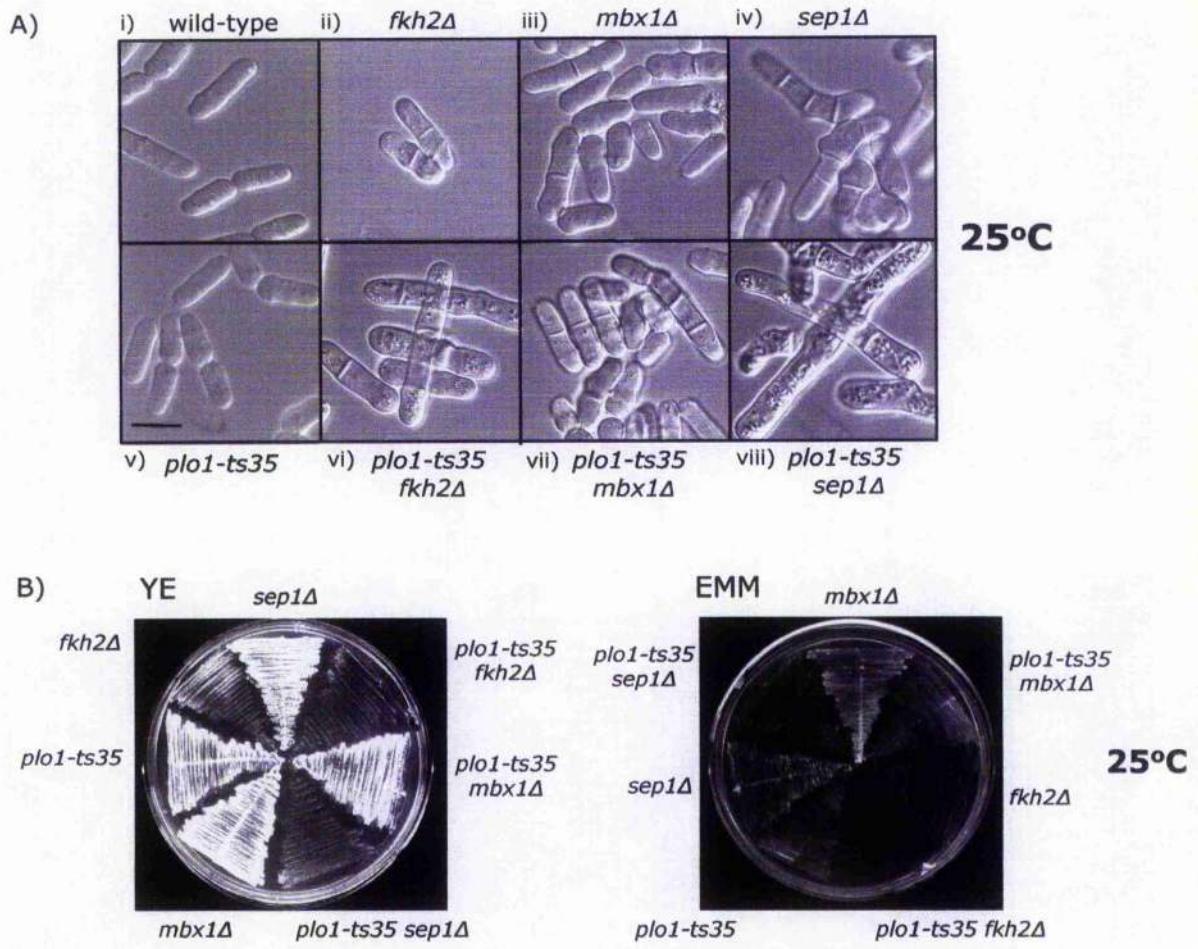
These data suggest that Plo1p acts in combination with the PBF transcription factor proteins in cell separation events, although the precise regulatory mechanism and pathway are unclear.

##### **4.2.3.2 Growth rate analyses**

Having established that the *fkh2Δ plo1-ts35*, *sep1Δ plo1-ts35* and *mbx1Δ plo1-ts35* cells demonstrated exaggerated separation and cell length phenotype in liquid growth, this prompted us to examine whether similar results were observed on solid media.

To score growth rate, mutants and wild-type cells were streaked onto solid YE and EMM and, incubated at 25°C. Visual observations indicated that the cells were viable, but grew at differing rates (Fig. 4.7.B). Table 4.1 summarises the growth rates of all mutant cells, both on YE and EMM.

In summary, the growth rates were all reduced relative to the parent cells but specifically exaggerated in cells containing *sep1Δ*. Therefore, we propose further genetic interaction between *plo1<sup>+</sup>* and *sep1<sup>+</sup>*.



**Figure 4.7.** Effect on cell separation and growth by combinatorial mutant cells, at 25°C. Cells contained combinatorial mutants of *fkh2Δ*, *sep1Δ*, *mbx1Δ* with *plo1-ts35* cells A) Cell separation defects of combinatorial mutants grown in liquid culture, viewed with DIC optics. B) Growth analysis of combinatorial mutants on solid YE and EMM. Bar size ≈5 μm

Cells	Growth rate	
	YE	EMM
<i>fkh2Δ</i>	+	+
<i>sep1Δ</i>	++	++
<i>mbx1Δ</i>	++	++
<i>plo1-ts35</i>	++	++
<i>plo1-ts35 fkh2Δ</i>	±	±
<i>plo1-ts35 sep1Δ</i>	±	±
<i>plo1-ts35 mbx1Δ</i>	+	+

**Table 4.1.** Quantitation of growth rate of *sep1Δ*, *fkh2Δ*, *mbx1Δ* with *plo1-ts35* cells. ++, Good growth comparable to parent strain. +, Poor growth with microcolony formation. ±, Very slow growth with many dead cells.

#### **4.2.3.3 Overexpression analyses**

Our studies provide evidence that PCB-dependent gene transcription is required for late events potentially under the regulation of PBF transcription factor proteins. We next showed that *Plo1p* through its KD regulates expression of the *cdc15<sup>+</sup>* group of genes. Further analysis revealed genetic interactions between *plo1<sup>+</sup>* and the components of PBF, especially *sep1<sup>+</sup>*. To extend the characterisation of genetic links between *plo1<sup>+</sup>* with *fkh2<sup>+</sup>*, *sep1<sup>+</sup>*, and *mbx1<sup>+</sup>* transcription factors, we explored the consequences of overexpressing components of PBF in *plo1-ts35* cells.

*plo1-ts35* cells were transformed with plasmids encoding wild-type *fkh2<sup>+</sup>* (GG699), *sep1<sup>+</sup>* (GG701) or *mbx1<sup>+</sup>* (GG697) under the control of the *nmt1* promoter, and their overexpression confirmed by Northern blotting (Fig. 4.8.A). As a control the empty pREP1 vector was also introduced into *plo1-ts35* cells (GG695). However, despite repeated efforts *sep1<sup>+</sup>* mRNA was undetected, though it was overexpressed when assayed in wild-type cells (Fig. 3.17). An explanation for this observation is not obvious, but it possibly suggests further genetic links between *sep1<sup>+</sup>* and *plo1<sup>+</sup>*.

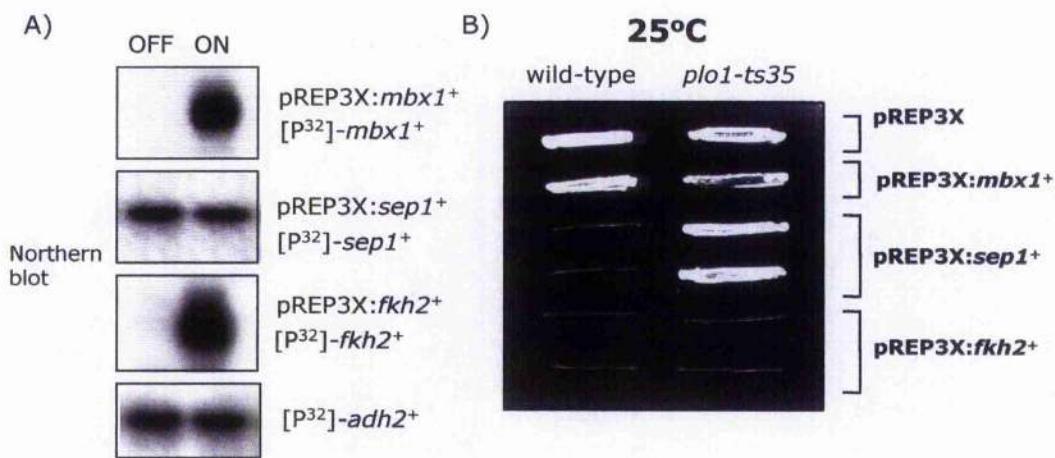
We have shown that overexpression of *fkh2<sup>+</sup>* and *sep1<sup>+</sup>* is lethal in wild-type cells, while overexpression of *mbx1<sup>+</sup>* was not (Fig. 3.16.B-18.B). When *fkh2<sup>+</sup>* was overexpressed in *plo1-ts35* cells, the cells also died (Fig. 4.8.B). Furthermore, *mbx1<sup>+</sup>* overexpression did not demonstrate genetic suppression or lethality in *plo1-ts35* cells. But when *sep1<sup>+</sup>* was overexpressed, *plo1-ts35* cells survived, because *sep1<sup>+</sup>* overexpression was inhibited.

In conclusion, analyses of the overexpression phenotypes of *fkh2<sup>+</sup>*, *sep1<sup>+</sup>* and *mbx1<sup>+</sup>* in *plo1-ts35* cells showed no suppression or synthetic lethality. However, *plo1-ts35* suppressed the lethal phenotype observed when overexpressing *sep1<sup>+</sup>*, suggesting a genetic interaction between the two genes.

### **4.3 Protein Biochemistry Study**

Protein phosphorylation has been shown to play a role during cytokinesis (Satterwhite and Pollard, 1992). For example, inactivation of the phosphatase 2A regulatory subunit leads to cytokinetic abnormalities in *S. cerevisiae*, and in *S. pombe* phosphorylation activity by Cdc7p is a key mechanism for controlling the onset of septum formation and cytokinesis (van Zyl et al, 1992; Fankhauser and Simanis, 1994). Potentially, phosphorylation is important for the PBF-PCB regulation of M/G1 gene expression.

To test this hypothesis, we examined the cell cycle-dependent phosphorylation status of PBF components. To do this we generated *fkh2-HA cdc25-22* (GG743) and *mbx1-Myc cdc25-22* (GG745) strains to allow arrest-release synchronisation in which we could monitor the Fkh2p and Mbx1p proteins by Western blot analysis.



**Figure 4.8.** Effect of *mbx1*<sup>+</sup>, *sep1*<sup>+</sup> and *fkh2*<sup>+</sup> when overexpressed in wild-type and *plo1-ts35* cells at permissive temperature, 25°C. A) Verification of pREP3X strains in the presence (*nmt1:plo1*<sup>+</sup> "off") and absence of thiamine (*nmt1:plo1*<sup>+</sup> "on") by Northern blot analysis. B) Growth analysis in wild-type and *plo1-ts35* mutant cells on solid EMM.

#### **4.3.1 *Fkh2p* cell cycle profile**

To analyse the pattern of Fkh2p phosphorylation during the cell cycle, exponentially growing *fkh2-HA cdc25-22* cells were incubated at 36°C for 3.5 h, to arrest cells at the G2/M interval. When the cells were returned to the 25°C, they entered mitosis synchronously and samples were taken at 5 min intervals throughout M phase, with septation indices monitored (Fig. 4.9). Then protein extracts were resolved by SDS-PAGE and processed for Western blot analysis with anti-HA antibody (Sections 2.2.10.6 and 2.2.10.6.1) (Appendix III).

Fkh2p-HA was found to be present throughout mitosis (Fig. 4.9.A). However, Fkh2p-HA migrated as multiple forms that changed during mitosis. At 10 min, Fkh2p-HA was present in a slower mobility form but returned to a faster mobility form as cells approached cytokinesis, after 40 min. To determine whether Fkh2p-HA periodicity was due to phosphorylation, we treated protein extract from the 20 min sample with calf-intestinal alkaline phosphatase (CIP), in the presence and absence of phosphatase inhibitor. Following treatment, the multiple forms of Fkh2p-HA collapsed to a single band, corresponding to the faster form, confirming that the slower forms were due to phosphorylation (Fig. 4.9.B).

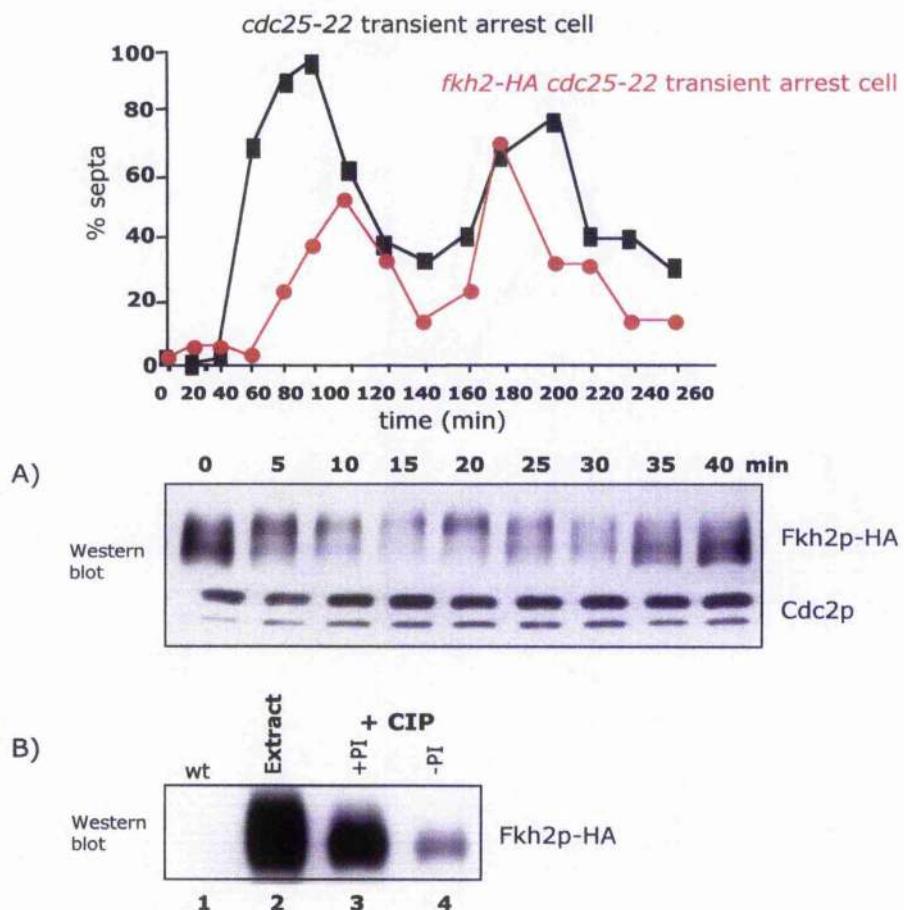
Combined, our data showed that Fkh2p is a phosphoprotein that is phosphorylated during mitosis. They echo observations in *S. cerevisiae* where Fkh2p is also transiently hyperphosphorylated during mitosis (Pic *et al.*, 2000).

#### **4.3.2 *Mbx1p* cell cycle profile**

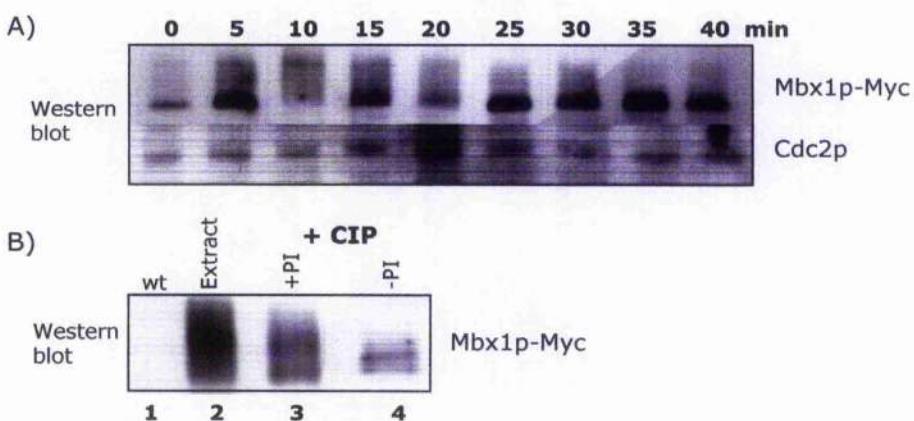
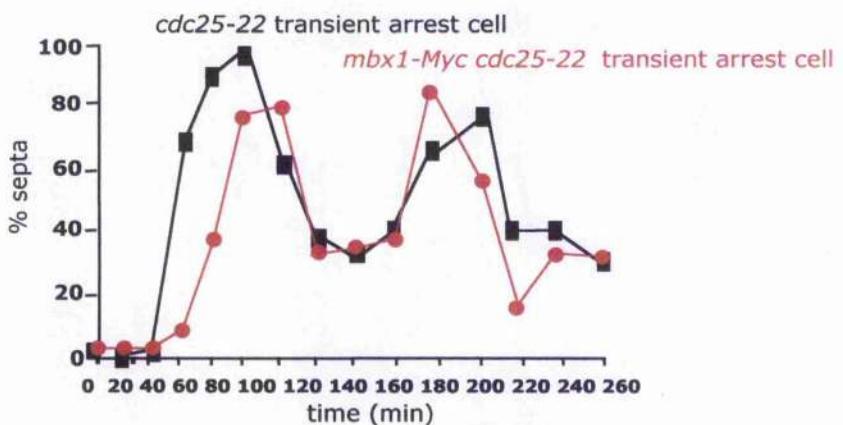
To detect whether Mbx1p also showed changes during mitosis, the same arrest-release synchronisation method was employed using *mbx1-Myc cdc25-22* cells (Section 4.3.1).

Interestingly, Mbx1p-Myc levels fluctuated as cells underwent mitosis, peaking when septation occurred. Mbx1p-Myc showed multiple mobilities that varied between the faster forms in late G2 and slower forms at 10 min after release into 25°C (Fig. 4.10.A). They then persisted for a short period, but gradually returned to the faster forms as cells underwent cytokinesis. Following treatment with CIP, in the absence of phosphatase inhibitor, the multiple forms returned to the faster form, indicating that the slower mobility was also due to phosphorylation (Fig. 4.10.B).

To conclude, this experiment provided evidence that Mbx1p is also phosphorylated in a cell cycle-dependent manner, peaking at the time when septation occurs (Tanaka *et al.*, 2001).



**Figure 4.9.** Fkh2p is phosphorylated during M phase. A) Fkh2p phosphorylation profile in *fkh2-HA cdc25-22* cells synchronised by transient temperature arrest. B) Lanes 1 and 2, immunoprecipitation of cell extracts prepared from wild-type and *fkh2-HA cdc25-22* cells, using anti-HA antibody. Lanes 3 and 4, immunoprecipitation of *fkh2p-HA cdc25-22* cell extracts, using anti-HA antibody, treated with calf intestinal phosphatase (CIP) in the presence (+PI) and absence (-PI) of phosphatase inhibitors (PI).



**Figure 4.10.** Mbx1p is phosphorylated during M phase. A) Mbx1p phosphorylation profile in *mbx1-Myc cdc25-22* cells synchronised by transient temperature arrest. B) Lanes 1 and 2, immunoprecipitation of cell extracts prepared from wild-type and *mbx1-Myc cdc25-22* cells, using anti-Myc antibody. Lanes 3 and 4, immunoprecipitation of *mbx1-Myc cdc25-22* cell extracts, using anti-Myc antibody, treated with calf intestinal phosphatase (CIP) in the presence (+PI) and absence (-PI) of phosphatase inhibitors (PI).

#### **4.3.2.1 Phosphorylation of Fkh2p and Mbx1p**

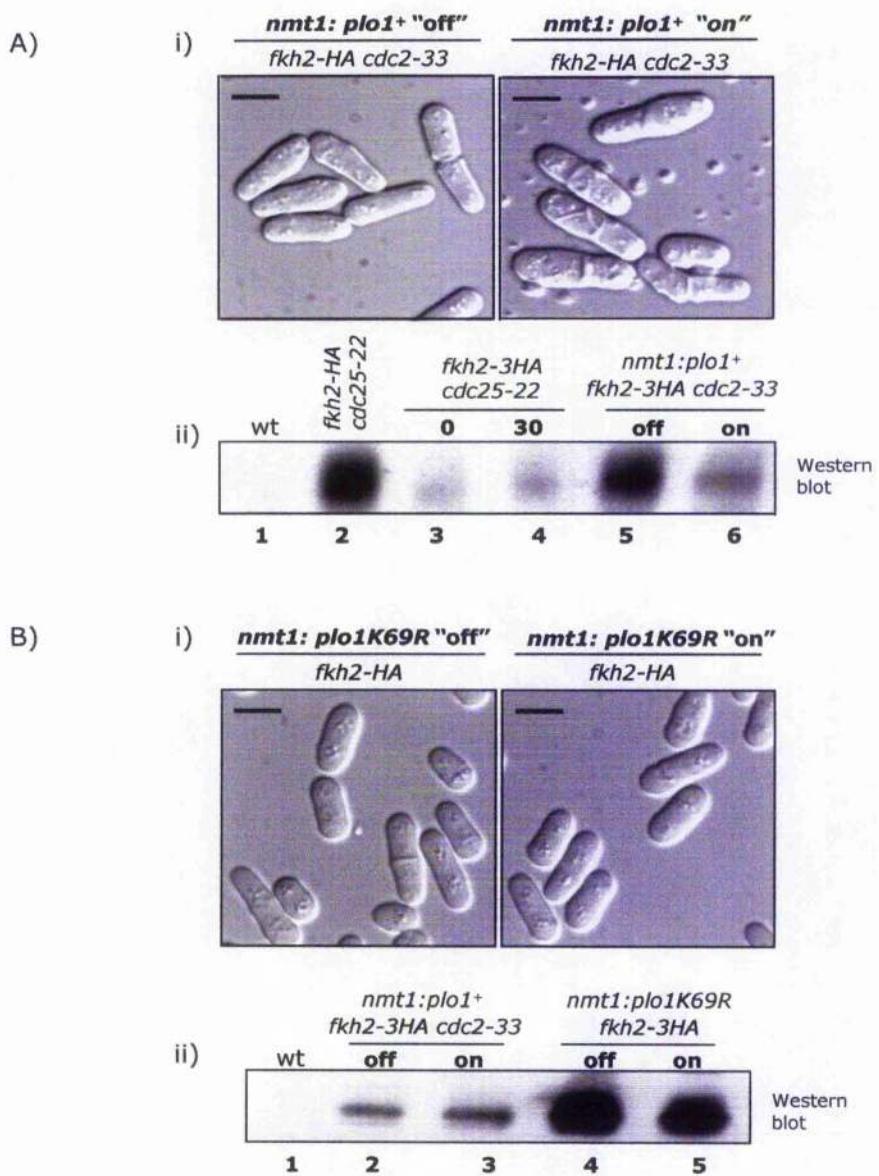
We have shown that Plo1p appears to play a role during septation through controlling M/G1-specific transcription, potentially by modulating PBF activity. We have discovered that mutation in the KD but not the PBD of *plo1<sup>+</sup>* prevented PCB-specific transcription, and that *fkh2Δ plo1-ts35*, *sep1Δ plo1-ts35* and *mbx1Δ plo1-ts35* cells exhibit augmented separation defects. Although cells overexpressing *fkh2<sup>+</sup>*, *mbx1<sup>+</sup>* and *sep1<sup>+</sup>* did not suppress the temperature sensitive phenotype of *plo1-ts35*, *sep1<sup>+</sup>* overexpression was blocked in *plo1-ts35* cells, suggesting genetic dependency between the two. Together, these results support the hypothesis that Plo1p is an upstream regulator of PBF-dependent transcription.

A common form of control in gene expression is through phosphorylation and we have shown that the phosphorylation status of Fkh2p and Mbx1p change through mitosis. Furthermore, other work identified two peaks of Plo1p-associated kinase activity during early and late mitosis, suggesting a Plo1p kinase-control mechanism during mitotic progression (Tanaka *et al*, 2001). As our genetic studies suggested a relationship between *plo1<sup>+</sup>* and PBF function, we next asked whether Plo1p regulates PBF activity through direct phosphorylation.

To analyse whether Fkh2p and Mbx1p are potential substrates of Plo1p, we tested their protein profiles when *plo1<sup>+</sup>* was overexpressed, in asynchronous cells. We transformed *nmt1:plo1<sup>+</sup>* into *fkh2-HA cdc2-33* and *mbx1-Myc cdc2-33* cells. Exponentially growing cells were incubated at 25°C for 13 h (3.5 cell cycle time), with samples harvested before and after induction of each gene caused by the removal of thiamine.

When *plo1<sup>+</sup>* was overexpressed in *fkh2-HA cdc2-33* (GG650), as expected, microscopic inspection revealed that the cells underwent untimely septation without the normal requirement for DNA synthesis and mitosis (Fig. 4.11.A.i). To investigate Fkh2p in these cells, samples were processed for Western blot analysis probed with anti-HA antibody. Fkh2p mobilities were similar in *plo1<sup>+</sup>* overexpressed and non-expressed conditions (Fig. 4.11.A.ii lanes 5-6). This suggests that overexpression of *plo1<sup>+</sup>* does not change the phosphorylation status of Fkh2p.

We have shown that overexpression of the *plo1<sup>+</sup>* KD mutant (*plo1K69R*) caused the absence of *cdc15<sup>+</sup>* mRNA. Therefore, we tested whether overexpression of the *plo1K69R* mutant changed the phosphorylation status of Fkh2p (GG753). When *plo1K69R* was overexpressed, microscopic inspection revealed correct septation, identical to control cells (Fig. 4.11.B.i) and Western blot analysis of Fkh2p showed that its mobility was unchanged



**Figure 4.11.** Effect of *plo1<sup>+</sup>* and *plo1K69R* overexpression on Fkh2p phosphorylation. A.i) DIC images of cells grown in the presence (*nmt1:plo1<sup>+</sup>* "off") and absence of thiamine (*nmt1:plo1<sup>+</sup>* "on"). ii) Lanes 1 and 2, immunoprecipitations of wild-type and *fkh2-HA cdc25-22* cells. Lanes 3 and 4, synchronous immunoprecipitation of *fkh2-HA cdc25-33* cells taken at 0 and 30 min interval upon release into permissive temperature (25°C). Lanes 5 and 6, immunoprecipitation of *fkh2-HA cdc2-33* cells carrying *nmt1:plo1<sup>+</sup>* plasmid in the presence and absence of thiamine. B.i) DIC images of cells grown in the presence (*nmt1:plo1K69R* "off") and absence of thiamine (*nmt1:plo1K69R* "on"). ii) Lane 1, immunoprecipitation of wild-type protein extract. Lanes 2 and 3, immunoprecipitation of *fkh2-HA cdc2-33* cells carrying *nmt1:plo1<sup>+</sup>* plasmid in the presence and absence of thiamine. Lanes 4 and 5, immunoprecipitation of *fkh2-HA cdc2-33* cells carrying *nmt1:plo1K69R* plasmid in the presence and absence of thiamine. Bar size  $\approx 5 \mu\text{m}$

(Fig. 4.11.B.ii lanes 2-5). These data suggest that Plo1p is not required for the phosphorylation of Fkh2p during mitosis.

Next, we examined the consequences of overexpressing either *plo1*<sup>+</sup> (GG651) or *plo1K69R* (GG749) in *mbx1-Myc* cells. First, we observed that *plo1*<sup>+</sup> overexpression conferred untimely septation while *plo1K69R* overexpression had no such effect (Fig. 4.12.A.i and 4.12.B.i). Second, we saw that neither overexpressing *plo1*<sup>+</sup> nor *plo1K69R* altered Mbx1p protein profiles (Fig. 4.12.A.ii lanes 5-6 and 4.12.B.ii lanes 4-5). These data indicate that *plo1*<sup>+</sup> and *plo1K69R* overexpressions do not affect the phosphorylation status of Mbx1p.

To conclude, we did not observe changes in the phosphorylation status of Fkh2p and Mbx1p when wild-type and a KD mutant of *plo1*<sup>+</sup> were overexpressed, suggesting Plo1p has no role in this phosphorylation process.

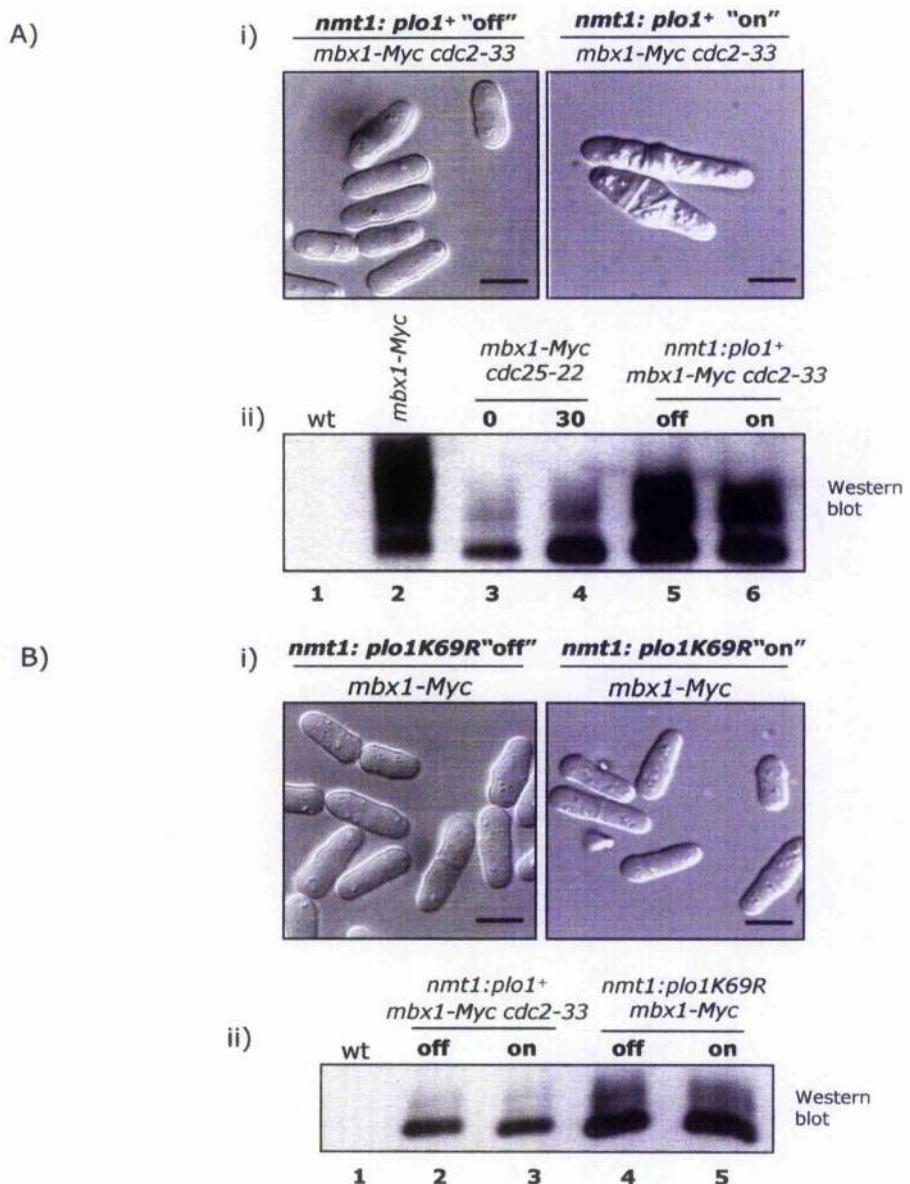
#### 4.3.3 Cloning of *fkh2*<sup>+</sup>, *sep1*<sup>+</sup> and *mbx1*<sup>+</sup> cDNA

We next studied the *in vitro* interactions of Plo1p with Fkh2p, Sep1p and Mbx1p. To do this we cloned *fkh2*<sup>+</sup>, *sep1*<sup>+</sup> and *mbx1*<sup>+</sup> to allow bacterial expression and purification. The genomic ORFs of *fkh2*<sup>+</sup>, *sep1*<sup>+</sup> and *mbx1*<sup>+</sup> were identified from the *S. pombe* database (Wood *et al.*, 2002). While *sep1*<sup>+</sup> does not contain an intron, *fkh2*<sup>+</sup> and *mbx1*<sup>+</sup> do, which required generating cDNA clones of these genes.

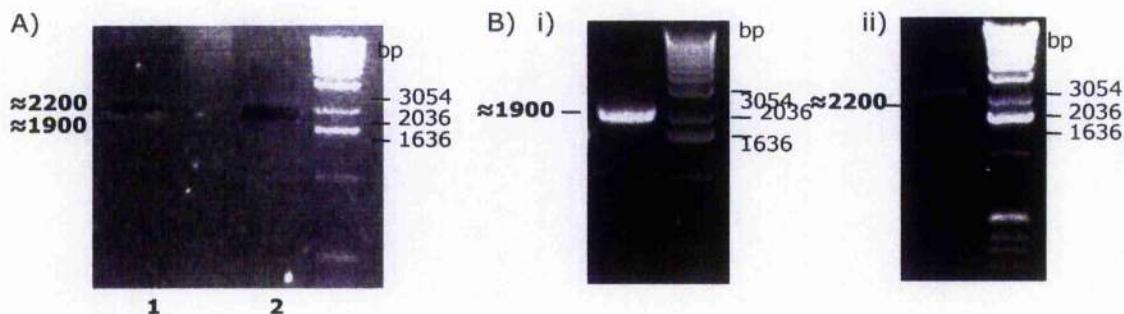
In order to clone *sep1*<sup>+</sup>, we isolated the gene from wild-type *S. pombe* genomic DNA by PCR amplification to include *Bam*HI and *Xba*I sites (Appendix IV: GO570/571). Amplified product was cloned into the pCR2.1 vector, sequenced, and a clone containing no mutations (GB444) was introduced into INVαF' cells (Sections 2.2.8.10.2).

To generate *S. pombe* *fkh2*<sup>+</sup> cDNA, RNA was extracted from wild-type cells (Section 2.2.9.1), and the corresponding cDNA synthesized using an appropriate antisense primer (GO508) (Section 2.2.8.8.3). This cDNA was next PCR amplified to generate a DNA product containing flanking 5' *Bam*HI and 3' *Xba*I sites (GO568/569). Figure 4.13.A shows DNA fragments of approximately 2200 and 1900 bp. We suspected that the slower-migrating band was an intron-containing genomic clone of *fkh2*<sup>+</sup> amplified from contaminating genomic DNA. However, both migrating products were excised, purified and cloned into pCR2.1 vector separately (Fig. 4.13.B.i-ii) (Sections 2.2.8.9.1).

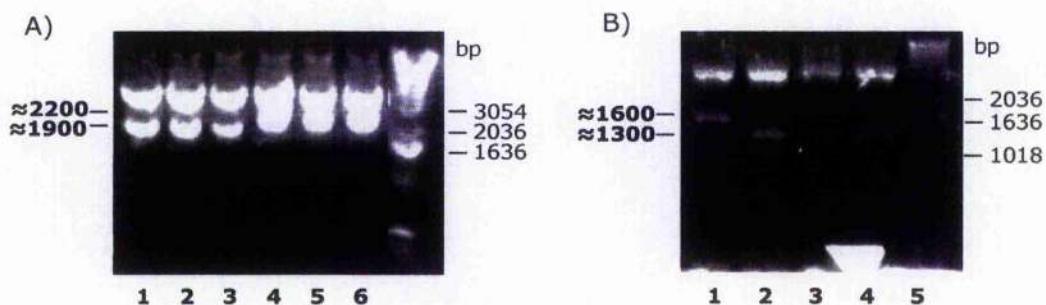
Cells were grown at 37°C on Amp-containing LB plates supplemented with X-gal, which allowed colour selection of positive clones. Several white colonies were picked and overnight cultures subjected to plasmid extraction followed by digestions. Figure 4.14.A shows digested product of approximately 1900 bp and 3900 bp from the putative spliced *fkh2*<sup>+</sup> cDNA, while the intron-containing DNA shows digested products of 2200 and 3900 bp.



**Figure 4.12.** The effect of *plo1<sup>+</sup>* and *plo1K69R* overexpression on Mbx1p phosphorylation. A.i) DIC images of cells grown in the presence (*nmt1:plo1<sup>+</sup> "off"*) and absence of thiamine (*nmt1:plo1<sup>+</sup> "on"*). ii) Lanes 1 and 2, immunoprecipitations from wild-type and *mbx1-Myc cdc25-22* cells. Lanes 3 and 4, synchronous immunoprecipitations of *mbx1-Myc cdc25-33* cells taken at 0 and 30 min interval upon release into permissive temperature (25°C). Lanes 5 and 6, immunoprecipitations of *mbx1-Myc cdc2-33* cells carrying *nmt1:plo1<sup>+</sup>* plasmid in the presence and absence of thiamine. B.i) DIC images of cells grown in the presence (*nmt1:plo1K69R "off"*) and absence of thiamine (*nmt1:plo1K69R "on"*). ii) Lane 1, immunoprecipitations of wild-type cells. Lanes 2 and 3, immunoprecipitations of *mbx1-Myc cdc2-33* cells carrying *nmt1:plo1<sup>+</sup>* plasmid in the presence and absence of thiamine. Lanes 4 and 5, immunoprecipitation of *mbx1-Myc cdc2-33* cells carrying *nmt1:plo1K69R* plasmid in the presence and absence of thiamine. Bar size ≈ 5 μm



**Figure 4.13.** *S. pombe* *fkh2<sup>+</sup>* cDNA. A) Lanes 1 and 2, PCR product amplified from *fkh2<sup>+</sup>* cDNA pool. B) Purified products of predicted *fkh2<sup>+</sup>* cDNA. The appropriate sizes of expected fragments are indicated.



**Figure 4.14.** Verification of *S. pombe* *fkh2<sup>+</sup>* cDNA. A) Lanes 1,2 and 3, *Bam*HI/ *Xba*I digested fragments of putative spliced *fkh2<sup>+</sup>* cDNA. Lanes 4,5 and 6, *Bam*HI/ *Xba*I digested fragments of putative unspliced *fkh2<sup>+</sup>* cDNA. B) Lanes 1 and 5, *Bst*XI digested product of putative unspliced *fkh2<sup>+</sup>* cDNA. Lanes 2,3 and 5, *Bst*XI digested product of putative spliced *fkh2<sup>+</sup>* cDNA. The appropriate sizes of expected fragments are indicated.

This confirmed that the slower-migrating PCR product was the intron-containing genomic *fkh2*<sup>+</sup> gene. We further confirmed this by a second digestion reaction using *Bst*XI, which generated 1300 bp and 3500 bp fragments for the spliced *fkh2*<sup>+</sup> cDNA, and 1600 bp and 3500 bp fragments for the genomic *fkh2*<sup>+</sup> (Fig. 4.14.B). Positive clones for the *fkh2*<sup>+</sup> cDNA were sent for sequencing using M13 forward and reverse sequencing primers, and an additional primer (GO602) to sequence the central region (Section 2.1.2).

DNA sequencing identified two clones with the least mutations: clone A with mutations at positions 1485 bp (A to G; silent mutation) and 1579 bp (T to C; tryptophan to cysteine) (GB387); and clone B with mutations at positions 651 bp (A to G; silent mutation) and 953 bp (T to C; silent mutation) (GB403) (Fig. 4.15.A). In addition, sequencing of *fkh2*<sup>+</sup> cDNA clones confirmed the proposed intron site as described in *S. pombe* genome database (Wood *et al.*, 2002). To rectify the mutations, we designed a strategy to digest full-length *fkh2*<sup>+</sup> cDNA into two halves and fuse the two error-free portions together.

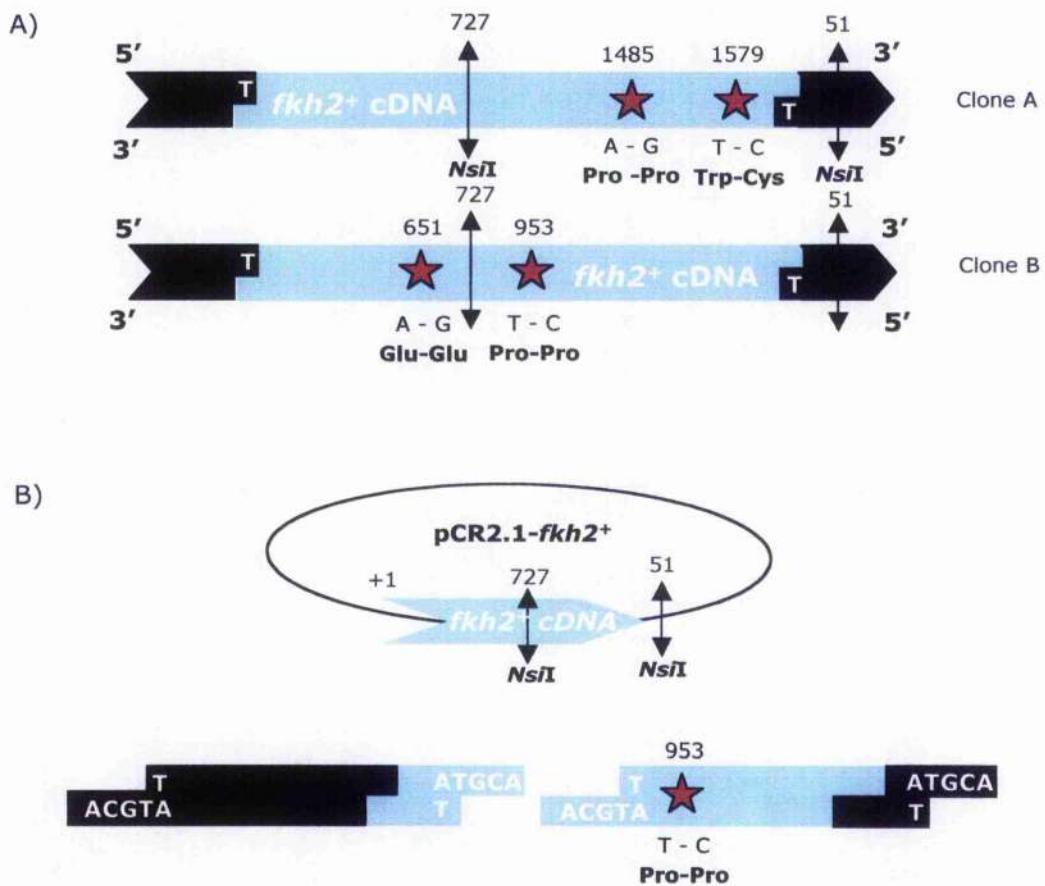
Purified clones were digested with *Nsi*I, which cleaves at position 727 bp in the *fkh2*<sup>+</sup> cDNA and position 51 bp in the vector (Fig. 4.15.B). Digested products of 5830 bp and 1240 bp were excised, purified and ligated (Fig. 4.16). Positive clones on LB-X-gal-Amp were selected and confirmed by digestions with *Bam*HI/*Xba*I, *Bst*I and *Nsi*I, respectively (Fig. 4.17.A-B). Sequencing confirmed that the new *fkh2*<sup>+</sup> clone contains just one silent mutation.

We have noted previously that full-length *fkh2*<sup>+</sup> does not rescue the mutants of mitotic catastrophic suppressor *mcs3*, while the C-terminus truncated *fkh2*<sup>+</sup>, designated *fkh2(t)*<sup>+</sup>, allowed entry into mitosis (Buck *et al.*, 2004). Therefore, we generated *fkh2(t)*<sup>+</sup> cDNA by PCR, amplified from *fkh2*<sup>+</sup> cDNA template corresponding to position 1-393 bp. Potential colonies were confirmed by nucleotide sequence analysis.

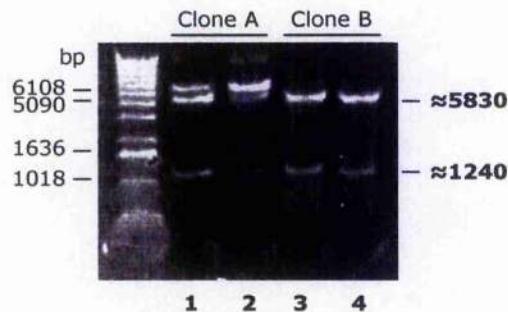
The *mbx1*<sup>+</sup> cDNA was generated by a colleague, Klki Papadopoulou, using a similar method.

#### **4.3.3.1 His-fkh2<sup>+</sup> and His-fkh2(t)<sup>+</sup> expression in bacteria**

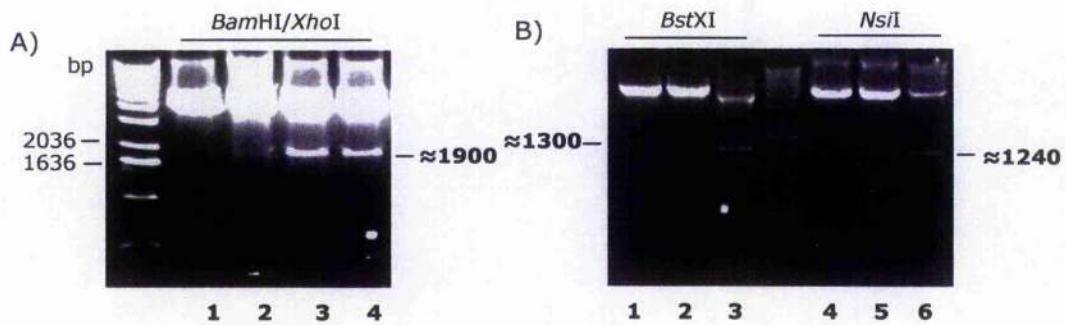
pCR2.1-*fkh2*<sup>+</sup> and pCR2.1-*fkh2(t)*<sup>+</sup> were digested and cloned into the pET-28c(+) expression vector (Sections 2.2.8.10.1 and 2.2.8.4). Plasmid DNAs were purified and sequenced to confirm that the cloned gene was in-frame to the N-terminal His-tag of the pET vector. Positive clones were transformed into the *E. coli* expression strain BL21 (DE3) CodonPlus-RIL (Section 2.2.8.5).



**Figure 4.15.** Schematic representation of “cut and paste” strategy to create *fkh2*<sup>+</sup> cDNA. A) Mutation sites identified in Clones A and B. B) Clones A and B were digested with *Nsi*I restriction enzyme and ligated to create single silent mutation of *fkh2*<sup>+</sup> cDNA.



**Figure 4.16.** *NsiI* digested fragments of *fkh2<sup>+</sup>* cDNA. Upper bands from lanes 1 and 2 (Clone A) and lower bands from lanes 3 and 4 (Clone B) were selected for "cut and paste" ligation. The appropriate sizes of expected digested fragments are indicated.



**Figure 4.17.** Verification of *fkh2<sup>+</sup>* cDNA created by "cut and paste" ligation. A) Lanes 1,2,3 and 4, *BamHI/XhoI* digested fragments of *fkh2<sup>+</sup>* cDNA. B) Lanes 1,2 and 3, *BstXI* digested fragments of *fkh2<sup>+</sup>* cDNA. Lanes 4,5 and 6, *NsiI* digested fragments of *fkh2<sup>+</sup>* cDNA. The appropriate sizes of expected fragments are indicated.

An aliquot from overnight culture was subcloned into fresh 50 ml NZY medium. Cells were grown in orbital incubator at 37°C until the optical density approached density of approximately 0.4-0.5 (Section 2.2.4).

Expression of pET-28c(+)*-fkh2*<sup>+</sup> was then induced with the addition of 0.5 mM IPTG, and samples removed at the point of induction (0 h) and hourly intervals thereafter for 4 h at 30°C. However, to induce the expression of pET-28c(+)*-fkh2(t)*<sup>+</sup>, once the cells reached the optimal density they were heat-shocked at 42°C for 20 min and incubated at 15°C for not more than 15 h with 0.1 mM IPTG. Both samples were pelleted, resuspended in Laemmli buffer and analysed by SDS-PAGE.

Figure 4.18.A-B show induced recombinant His-Fkh2p and His-Fkh2(t)p, corresponding to the predicted molecular masses: approximately 74 kD for His-Fkh2p and 16 kD for His-Fkh2(t)p.

#### **4.3.3.2 GST-*sep1*<sup>+</sup> and GST-*mbx1*<sup>+</sup> expression in bacteria**

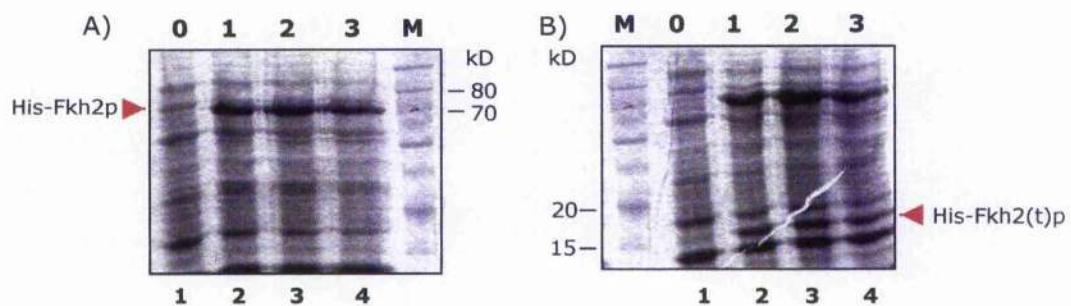
To generate recombinant GST-Sep1p and GST-Mbx1p, pCR2.1-*sep1*<sup>+</sup> and pCR2.1-*mbx1*<sup>+</sup> cDNAs and pGEX-KG expression vector were digested, ligated and transformed into *E. coli* DH5α cells. Potential GST-*sep1*<sup>+</sup> and GST-*mbx1*<sup>+</sup> plasmids were sequenced and positive clones were transformed into the BL21 (DE3) CodonPlus-RIL cells.

Both GST-*sep1*<sup>+</sup> and GST-*mbx1*<sup>+</sup> were induced using the same method as His-tagged *fkh2*<sup>+</sup> and their levels examined. Figure 4.19.A-B show successful expressions corresponding to their predicted masses: approximately 98 kD, GST-Sep1p; 77 kD, GST-Mbx1p.

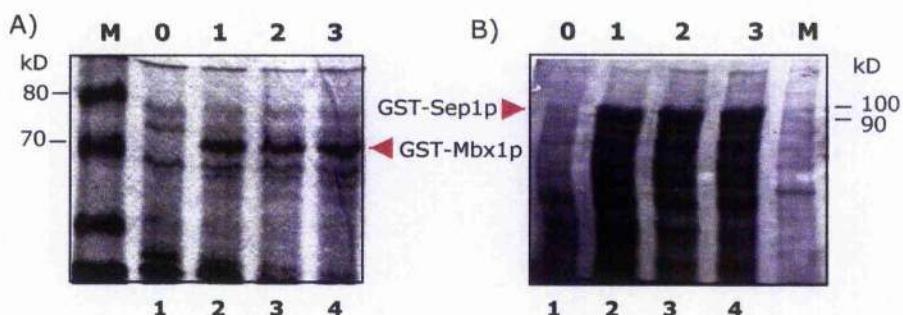
#### **4.3.3.3 Purification of His-Fkh2p, His-Fkh2(t)p, GST-Sep1p and GST-Mbx1p**

To study protein function, recombinant proteins must be soluble. Therefore, cell pellets from His-Fkh2p, His-Fkh2(t)p, GST-Sep1p and GST-Mbx1p cultures were resuspended in lysis buffer, either Hepes buffer for His-tagged or PBS buffer for GST-tagged purifications, and disrupted using the French Pressure apparatus (Sections 2.2.10.5, 2.2.10.5.1 and 2.2.10.5.2). An aliquot of cell sample was taken while the remaining whole cell extract (E) was clarified by high-speed centrifugation. Subsequently, two aliquots of sample, one containing clarified supernatant (S) and the other the cell pellet (P), were removed. Solubility was determined by visual comparison of protein levels present in the supernatant and pellet, following Coomassie Blue staining.

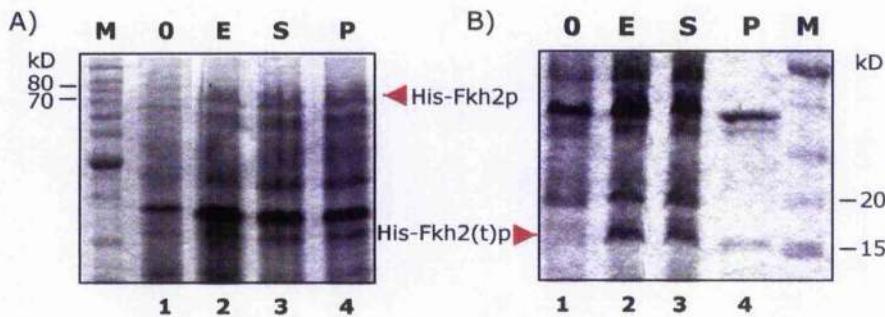
Figure 4.20.A-B show that His-Fkh2p and His-Fkh2(t)p were both detected in soluble fractions following induction. Similarly, Figure 4.21.A-B revealed that both GST-Sep1p and



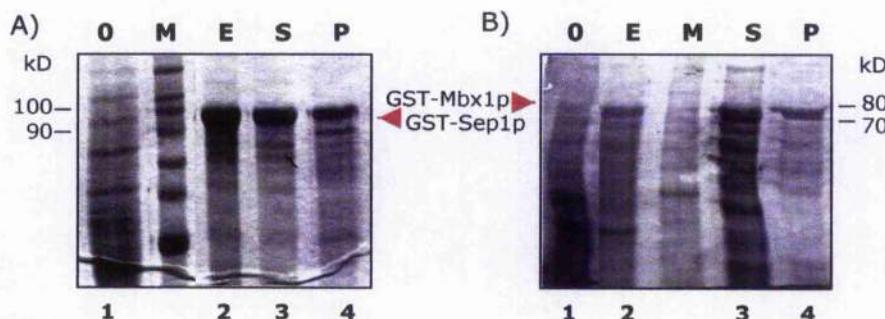
**Figure 4.18.** *His-fkh2<sup>+</sup>* and *His-fkh2(t)<sup>+</sup>* overexpression. *His-fkh2<sup>+</sup>* and *His-fkh2(t)<sup>+</sup>* were induced with IPTG and samples collected at the point of induction (0) and at hourly intervals thereafter for 3 hours. A) Lanes 1,2,3 and 4, expressions of His-Fkh2p at hourly intervals upon induction. B) Lanes 1,2,3 and 4, expressions of His-Fkh2(t)p at hourly hours upon induction. The appropriate sizes of overexpression products are indicated in red corresponding to the molecular weight marker (M).



**Figure 4.19.** *GST-sep1<sup>+</sup>* and *GST-mbx1<sup>+</sup>* overexpression. *GST-sep1<sup>+</sup>* and *GST-mbx1<sup>+</sup>* were induced with IPTG and samples collected at the point of induction (0) and at hourly intervals thereafter for 3 hours. A) Lanes 1,2,3 and 4, expressions of GST-Mbx1p at hourly intervals upon induction. B) Lanes 1,2,3 and 4, expressions of GST-Sep1p at hourly hours upon induction. The appropriate sizes of overexpression products are indicated in red corresponding to the molecular weight marker (M).



**Figure 4.20.** Solubility of His-Fkh2p and His-Fkh2(t)p. Overexpressing His-Fkh2p and His-Fkh2(t)p cells were lysed and samples collected from cell extract (E) prior to lysis, supernatant fraction (S) and pellet fraction (P), upon lysis. Sample collected prior to IPTG induction is denoted with (0). A) Lanes 1,2,3 and 4, expression of His-Fkh2p in 0, E, S and P fractions. B) Lanes 1,2,3 and 4, expression of His-Fkh2(t)p 0, E, S and P fractions. The appropriate fragment sizes of products are indicated in red corresponding to the molecular weight marker (M).



**Figure 4.21.** Solubility of GST-Sep1p and GST-Mbx1p. Overexpressing GST-Sep1p and GST-Mbx1p cells were lysed and samples collected from cell extract (E) prior to lysis supernatant fraction (S) and pellet fraction (P), upon lysis. Sample collected prior to IPTG induction is denoted with (0). A) Lanes 1,2,3 and 4, expression of GST-Sep1p in 0, E, S and P fractions. B) Lanes 1,2,3 and 4, expression of GST-Mbx1p in 0, E, S and P fractions. The appropriate fragment sizes of products are indicated in red corresponding to the molecular weight marker (M).

GST-Mbx1p were detected in high concentration in the supernatants, showing that both proteins were also soluble.

#### 4.3.4 Cloning of His-plo1<sup>+</sup> and GST-plo1<sup>+</sup>

We have proposed that Plo1p controls PBF-PCB gene expression during M/G1 in *S. pombe*. One way for this to occur is through Plo1p directly phosphorylating components of PBF. To test this possibility we performed *in vitro* kinase assays with Plo1p and Mbx1p, Fkh2p and Sep1p. This experiment required pure and active Plo1p, and so a eukaryotic expression host, *S. cerevisiae*, was exploited to ensure correctly folded protein, containing any necessary post-translational modifications (Sudbery, 1996).

To express *plo1*<sup>+</sup>, we constructed pMG1-*plo1*<sup>+</sup> and pMH919-*plo1*<sup>+</sup> expression vectors, containing *GST* and *His* N-terminal tags using the pMG1 (GB404) and pMH919 (GB405) vectors, respectively, a gift of Dr. Sedgwick (NIMR, London). *plo1*<sup>+</sup> was PCR amplified from pHN180-*plo1*<sup>+</sup> (GB188; obtained from Prof. Hagan) as template to yield a product containing *Bam*H I and *Xba*I sites (GO605/606). The product obtained was approximately 2000 bp in length, as predicted from *S. pombe* genome database (Fig. 4.22) (Wood *et al*, 2002).

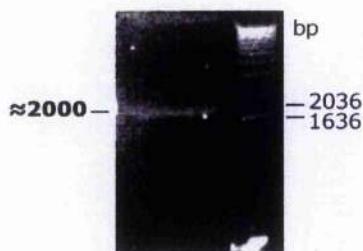
The PCR products were digested, purified, ligated into pMG1 and pMH919 vectors and transformed into DH5 $\alpha$  cells. Potential *plo1*<sup>+</sup>-containing clones were digested with *Bam*H I and *Xba*I for verification. Figure 4.23.A-B show digested product of approximately 2000 bp. Positive pMG1-*plo1*<sup>+</sup> (GB428) and pMH919-*plo1*<sup>+</sup> (GB430) clones were further confirmed by *Pvu*II digestion and a 4000 bp fragment was obtained (Fig. 4.24.A-B). Following this, further manipulations were completed in the laboratory of Dr. Sedgwick to introduce the two *plo1*<sup>+</sup> expression constructs into separate *S. cerevisiae* strains (*GST-plo1*<sup>+</sup>: GGBY143 and *His-plo1*<sup>+</sup>: GGBY144).

##### 4.3.4.1 His-plo1<sup>+</sup> expression in *S. cerevisiae*

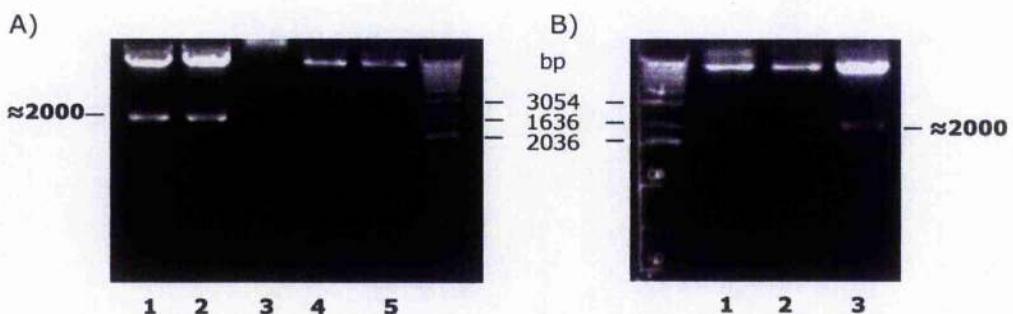
*His-plo1*<sup>+</sup> and *GST-plo1*<sup>+</sup> were expressed in *S. cerevisiae* according to the methods suggested by Dr. Sedgwick (Section 2.2.5). These vectors contained promoters regulated by glucose, so that when glucose is removed from the growth medium transcription is induced.

An aliquot from an overnight culture was added to fresh medium lacking glucose, but instead containing sucrose. When the cells reached an appropriate density, the culture was diluted to induce *plo1*<sup>+</sup> expression. Samples were harvested after 9 h of induction, cells broken using acid glass beads, and protein extracts prepared (Section 2.2.10.4).

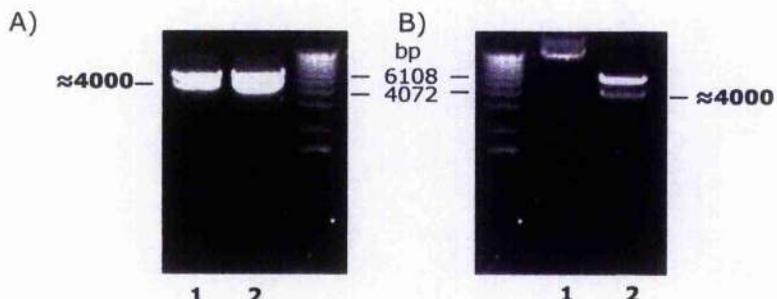
GST-Plo1p was purified using glutathione-Sepharose beads. The beads were washed using GST washing buffer and eluted with 20 mM reduced glutathione (Section 2.2.10.4.1).



**Figure 4.22.** PCR fragment amplified from pHN180-*plo1*<sup>+</sup>. The appropriate size of expected fragment are indicated.



**Figure 4.23.** Verification of PCR-based cloning to generate pMG1-*plo1*<sup>+</sup> and pMH919-*plo1*<sup>+</sup>. A) Lanes 1,2,3,4 and 5, *Bam*HI/*Xba*I digested fragments of pMGI-*plo1*<sup>+</sup>. B) Lanes 1,2 and 3, *Bam*HI/*Xba*I digested fragments of pMH919-*plo1*<sup>+</sup>. The appropriate sizes of expected fragments are indicated.



**Figure 4.24.** Verification of PCR-based cloning to generate pMG1-*plo1*<sup>+</sup> and pMH919-*plo1*<sup>+</sup>. A) Lanes 1 and 2, *Pvu*II digested fragments of pMGI-*plo1*<sup>+</sup>. B) Lanes 1 and 2, *Pvu*II digested fragments of pMH919-*plo1*<sup>+</sup>. The appropriate sizes of expected fragments are indicated.

His-Plo1p was instead purified using Zn-bound-Sepharose beads, with the beads washed using His washing buffer, and eluted with 250 mM imidazole (Section 2.2.10.4.2).

Figure 4.25.A lanes 5-6 shows purified His-Plo1p and GST-Plo1p, separated by SDS-PAGE and stained with Coomassie Blue. To test for purity of these proteins, equal amounts were analysed by Western blot analysis using anti-His and anti-GST antibodies. Figure 4.25.B lanes 3 and 6 show the autoradiogram of fusion Plo1p corresponding to the sizes as predicted from *S. pombe* genome database: GST-Plo1p at approximately 103 kD, and His-Plo1p at approximately 78 kD.

#### 4.3.5 *Plo1p* *in vitro* kinase assay

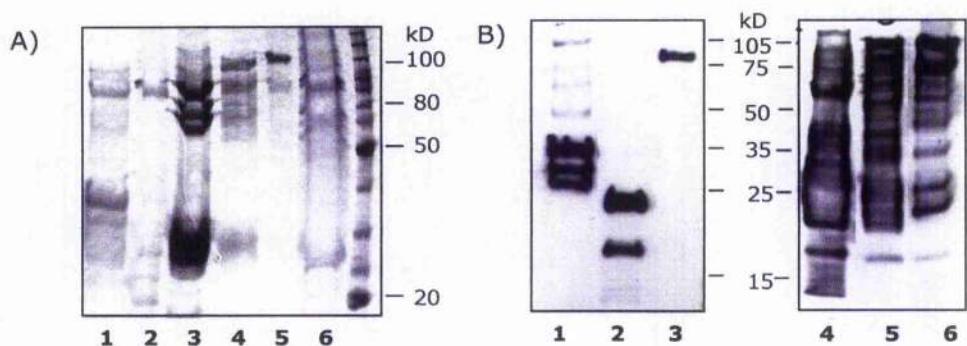
To ensure that purified Plo1p preparations were biologically active, assays were performed with MBP (myelin basic protein) as a control substrate. Phosphorylated MBP was detected with both GST-Plo1p and His-Plo1p kinase, confirming that both tagged forms of Plo1p were active (Dr Sedgwick, data not shown).

Next, the ability of GST-Plo1p and His-Plo1p to phosphorylate His-Fkh2p, GST-Mbx1p, GST-Sep1p and His-Fkh2(t)p was tested. The quantity of 1:5 (Plo1p to substrate) was added to kinase buffer containing [<sup>32</sup>P]-ATP and the reaction mixes were incubated (Section 2.2.11.2). Once the incubation was completed, the reaction was stopped by the addition of Laemmli buffer, and analysed by SDS-PAGE. The gel was stained with colloidal Coomassie Blue and autoradiographed.

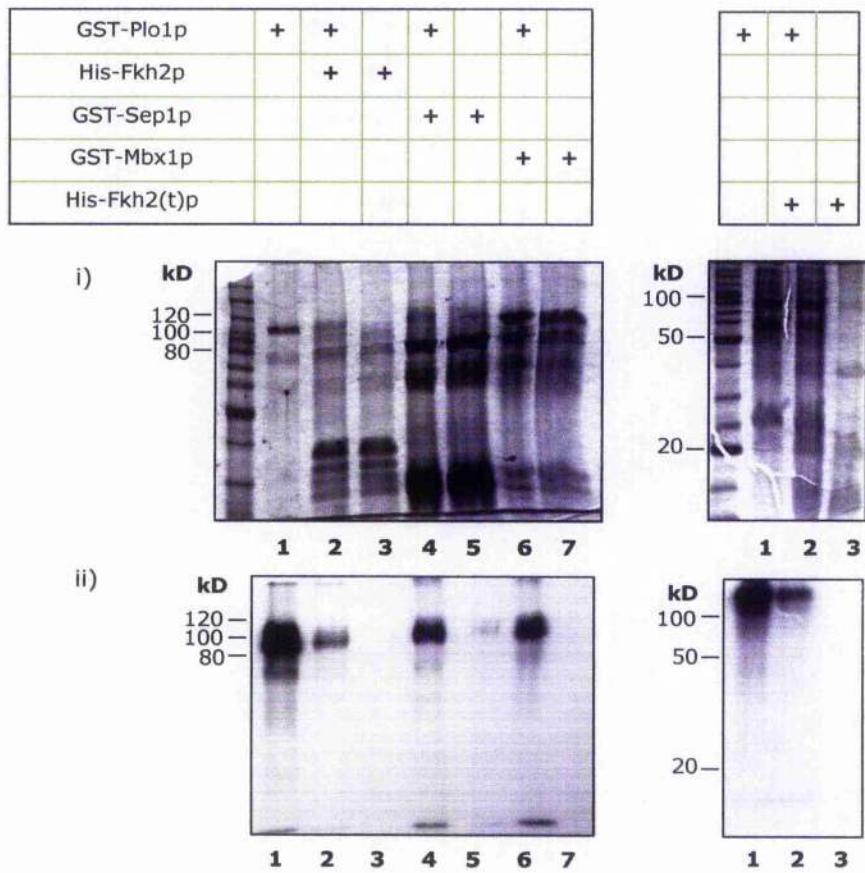
Figure 4.26.A.i shows kinase activity of GST-Plo1p towards the recombinant substrates His-Fkh2p, GST-Mbx1p, GST-Sep1p and His-Fkh2(t)p. No substrate-specific phosphorylation was detected, possibly because GST-Plo1p and some of the substrates had the same mobility on the SDS gel, so potentially masking substrate phosphorylation. However, the fact that a phosphorylated protein of the correct size was detected in the lane where only Plo1p was added confirmed that the addition of GST-tag protein does not alter its kinase activity (Figure 4.26.A.ii lane 1).

To discriminate against the potential size masking, the experiment was repeated with His-Plo1p, as this form of Plo1p has a different predicted molecular weight. Under these conditions, we observed specific phosphorylation of GST-Mbx1p (Fig. 4.27.A.ii lane 6), but not His-Fkh2p, GST-Sep1p and His-Fkh2(t)p.

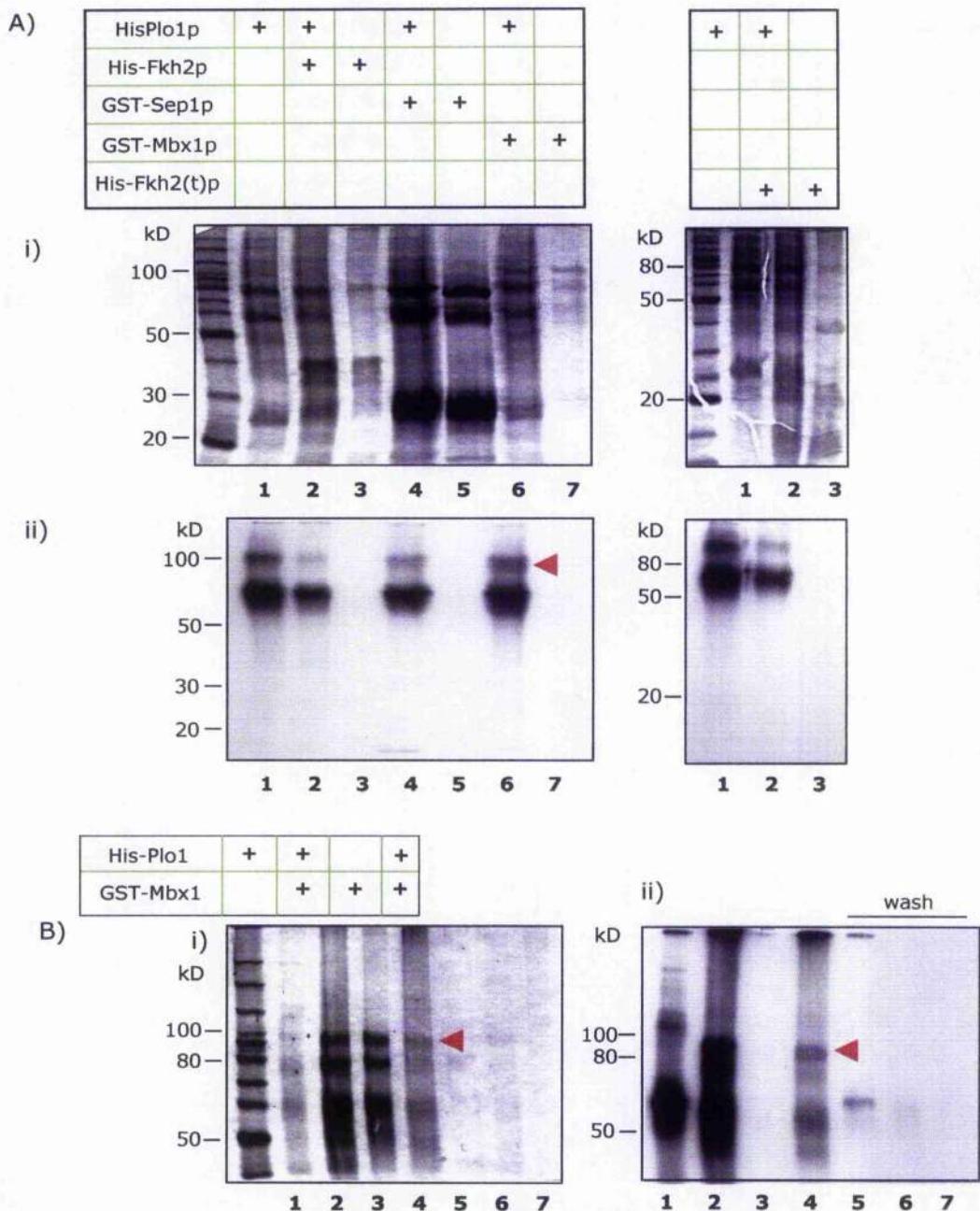
To confirm this result, an alternative purification protocol was adapted, to address whether Plo1p phosphorylation was specific to Mbx1p. In this method, *GST-mbx1<sup>+</sup>* was overexpressed and purified using glutathione-Sephadex beads, however, the immobilised protein was immediately subjected to a kinase assay without eluting with reduced glutathione. This method allows the recovery of GST-Mbx1p-beads after kinase



**Figure 4.25.** SDS-PAGE analysis of recombinant proteins. A) Coomassie Blue staining of purified His-Fkh2p, His-Fkh2(t)p, GST-Sep1p, GST-Mbx1p, GST-Plo1p and His-Plo1p, corresponding to lanes 1-6, respectively. B) Western blotting of purified His-Fkh2p, His-Fkh(t)p, Plo1p-His, GST-Sep1p, GST-Mbx1p and GST-Plo1p, corresponding to lanes 1-6 respectively. Sizes of molecular weight marker are indicated in kD.



**Figure 4.26.** *In vitro* kinase assay with purified GST-Plo1p and recombinant components of PBF. A) Separate kinase reactions with GST-Plo1p and His-Fkh2p, GST-Sep1p and GST-Mbx1p were separated by SDS PAGE, stained with Coomassie Blue (i) and exposed for autoradiography (ii).



**Figure 4.27.** *In vitro* kinase assay with purified His-Plo1p and recombinant components of PBF. A) Separate kinase reactions with His-Plo1p and His-Fkh2p, GST-Sep1p and GST-Mbx1p were separated by SDS PAGE, stained with Coomassie Blue (i) and exposed for autoradiography (ii). B) Kinase reaction of Plo1p-His with GST-Mbx1p bound to glutathione beads after washes (lanes 5-7), separated by SDS PAGE, stained with Coomassie Blue (i) and exposed for autoradiography (ii). Arrows in red indicate Plo1p-His phosphorylating GST-Mbx1p.

reaction when the reaction mix were subjected to extensive washes with GST wash buffer supplemented with 1% NP-40, thus removing material non-specific to the beads. GST-Mbx1p was then recovered from the glutathione-beads by boiling in Laemmli buffer, and separated on a SDS gel. Interestingly, the autoradiogram revealed the presence of a 90 kD band, corresponding to the lane containing only GST-Mbx1p (Fig. 4.27.B.i lanes 3-4 and 4.29.B.ii lane 4).

Therefore, we conclude that Plo1p can phosphorylate Mbx1p *in vitro*.

#### 4.4 Discussion

The Plks were initially identified in *D. melanogaster* and subsequent work identified both sequence and functional homologues in other organisms, including *S. pombe* and *S. cerevisiae* (Sunkel and Glover, 1988; Llamazares *et al.*, 1991; Ohkura *et al.*, 1995).

To date, much work has shown that Plks control many functions during mitosis. Ohkura *et al* (1995) also identified an important role for Plo1p in *S. pombe* during cytokinesis. Following this, evidence in *S. cerevisiae* suggested additional roles for Cdc5p during mitotic exit (Shirayama *et al*, 1998). Both Plks have important roles in signalling pathways that control mitotic exit in *S. cerevisiae* and cytokinesis in *S. pombe*. These pathways have been designated MEN and SIN, respectively (McCollum and Gould, 2001; Bardin and Amon, 2001).

##### 4.4.1 Characterisation of PBF activity through Plo1p

Phosphorylation as a form of control in cell cycle events is widely used and well studied (Nigg, 2001). We established that a group of genes, including *cdc15<sup>+</sup>*, *fkh2<sup>+</sup>* and *plo1<sup>+</sup>* are transiently expressed during M/G1 (Anderson *et al*, 2002). We went on to show that Plo1p regulates M/G1-specific transcription through PBF, possibly through an autofeedback loop. The fact that Plo1p is a protein kinase suggests that it may regulate PBF through phosphorylation. In this chapter, the main aim was to characterise the effect of Plo1p on PBF, through both *in vitro* and *in vivo* experiments.

First we showed that *plo1-ts35* affected the periodic transcription of PCB genes at 25°C. We observed that the profiles of *cdc15<sup>+</sup>* and *spo12<sup>+</sup>* mRNA were delayed, becoming coincident with *cdc22<sup>+</sup>*, a known G1/S-specific gene. Furthermore, septation timing was similarly retarded suggesting roles for M/G1 genes during late mitotic events. These observations supported our proposal for Plo1p as a regulator of M/G1-specific genes, possibly through the activity of PBF transcription factor complex.

Through genetic analyses, we generated combinatorial double mutants between *fkh2Δ*, *sep1Δ*, *mbx1Δ* with *plo1-ts35* cells. Despite no synthetic lethality being observed in these double mutants, they exhibited multiple levels of growth and viability defects, relative to single mutant cells. Microscopic inspection revealed genetic interactions among PBF transcription factor proteins with *plo1<sup>+</sup>*, as demonstrated by exacerbated defects in septation, cytokinesis and cell separation. These additive phenotypes were especially strong in *sep1Δ plo1-ts35* cells. The synthetic phenotypes suggest genetic interactions between *plo1<sup>+</sup>* with *fkh2<sup>+</sup>*, *sep1<sup>+</sup>* and *mbx1<sup>+</sup>*.

*plo1<sup>+</sup>* overexpression causes multiple rounds of septation, even in interphase cells (Ohkura *et al.*, 1995). Given that Plo1p is required for the correct timing of PCB periodic transcription, we asked whether overexpression of *plo1<sup>+</sup>* would induce PCB genes and found that it caused higher mRNA levels of *cdc15<sup>+</sup>* and *spo12<sup>+</sup>*.

Septation is controlled through a signal transduction pathway, the SIN, which is activated by Plo1p (Tanaka *et al.*, 2001). We therefore tested whether Plo1p regulates PCB genes through the SIN by using *spg1.B8 cdc7.A20* cells. We concluded that PCB-specific gene transcription is independent of SIN signalling, although how PBF-PCB and SIN modulate septation independently is not clear.

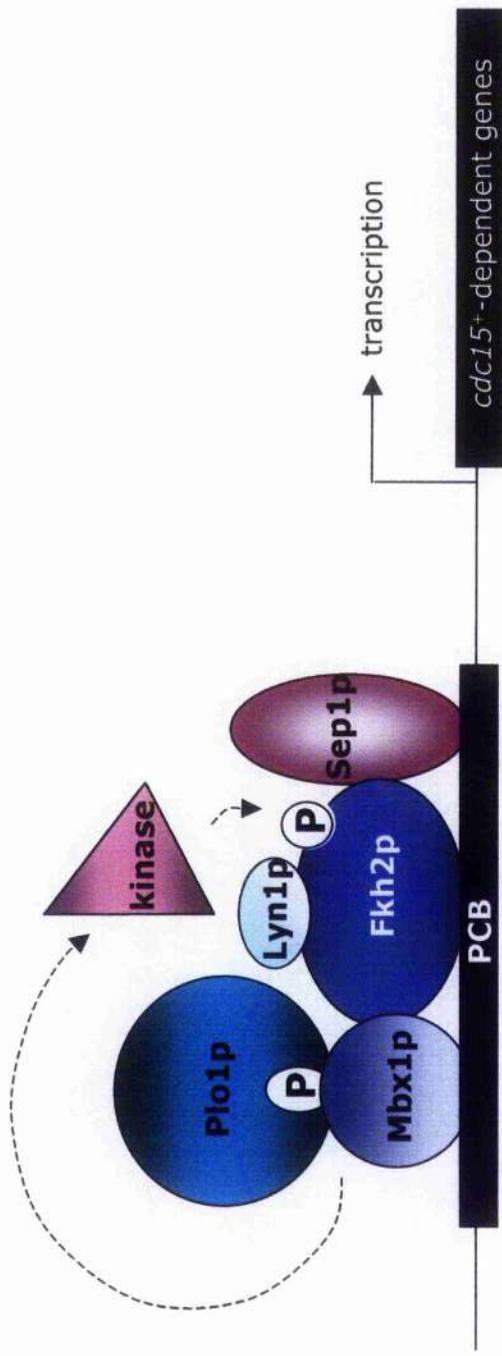
To define functional domains of Plo1p required for M/G1 transcription, we assayed PCB activity using *plo1* mutants. Overexpressions of the KD mutant *plo1K69R* abolished *cdc15<sup>+</sup>* and *spo12<sup>+</sup>* transcription, but this was not observed when the PBD mutant, *plo1DHK625*, was overexpressed. This is consistent with our proposal that Plo1p kinase may regulate M/G1-specific transcription through phosphorylation.

We next showed that Fkh2p and Mbx1p are transiently hyperphosphorylated during mitosis, coincident with septation. Both Fkh2p and Mbx1p migrated as multiple forms and were modified through mitosis. Following treatment of protein extracts with CIP, the multiple forms collapsed to a single band. These results indicate that Fkh2p and Mbx1p are phosphoproteins that are phosphorylated in a cell cycle-dependent manner.

This led us to test whether Fkh2p and Mbx1p were potential substrates of the Plo1p kinase. However, we failed to demonstrate effects on Fkh2p and Mbx1p phosphorylation status by overexpressing *plo1<sup>+</sup>* *in vivo*. In addition, the overexpression of a *plo1* KD mutant had no effect.

We then took a different approach to investigate the potential phosphorylation of Fkh2p, Sep1p and Mbx1p by Plo1p. We generated pure and active Plo1p to assay phosphorylation *in vitro*, and we added this to bacterial expressed Fkh2p, Sep1p and Mbx1p. Interestingly, we found that Plo1p specifically phosphorylated Mbx1p, but not the Sep1p and Mbx1p.

From these observations, we propose that Plo1p might regulate M/G1 transcription through phosphorylation of Mbx1p. The fact that Fkh2p is also a cell cycle-dependent phosphoprotein implies a different kinase complex is important in M/G1-specific cell cycle control. Given that Fkh2p abundance and timing of its activation is similar to Mbx1p, it is tempting to speculate that sequential changes in phosphorylation state of one protein before another is important in mediating PBF control. Alternatively, we are yet to rule out other levels of control through Fkh2p. Combined, we propose a model suggesting that Plo1p directly controls the expression of *cdc15<sup>+</sup>* group of genes through the phosphorylation activity of Mbx1p, a component of PBF transcription machinery (Fig. 4.28).



**Figure 4.28.** Model for Plo1p activation of M/G1 transcription in *S. pombe*. Plo1p targets Mbx1p for phosphorylation which then stimulate phosphorylation activity of Fkh2p by an activating kinase, thus initiating transcription of *cdc15<sup>+</sup>* and other genes at the M/G1 interval.

## **Chapter 5: Conclusions**

### **5.1 Introduction**

The current model for cell cycle transcription in yeasts is defined by consecutive waves of gene expression, corresponding to the four stages of the cell cycle (Breeden, 2000; McInerny, 2004). One focus in our laboratory is to study cell cycle regulated genes and their control mechanisms in *S. pombe*. We have identified a group of genes whose transcript levels peak during M/G1 interval (Anderson *et al*, 2002). Most of these encode proteins required for septation, cytokinesis and cell separation. In this thesis, we investigated the mechanisms that control M/G1-dependent genes in *S. pombe*.

One key question is how this subset of genes is periodically expressed through the cell cycle. This is important, as we believe that their periodic transcription is a limiting step for cell cycle progression, and their regulation is responsible for ensuring that cell cycle events take place at the correct time.

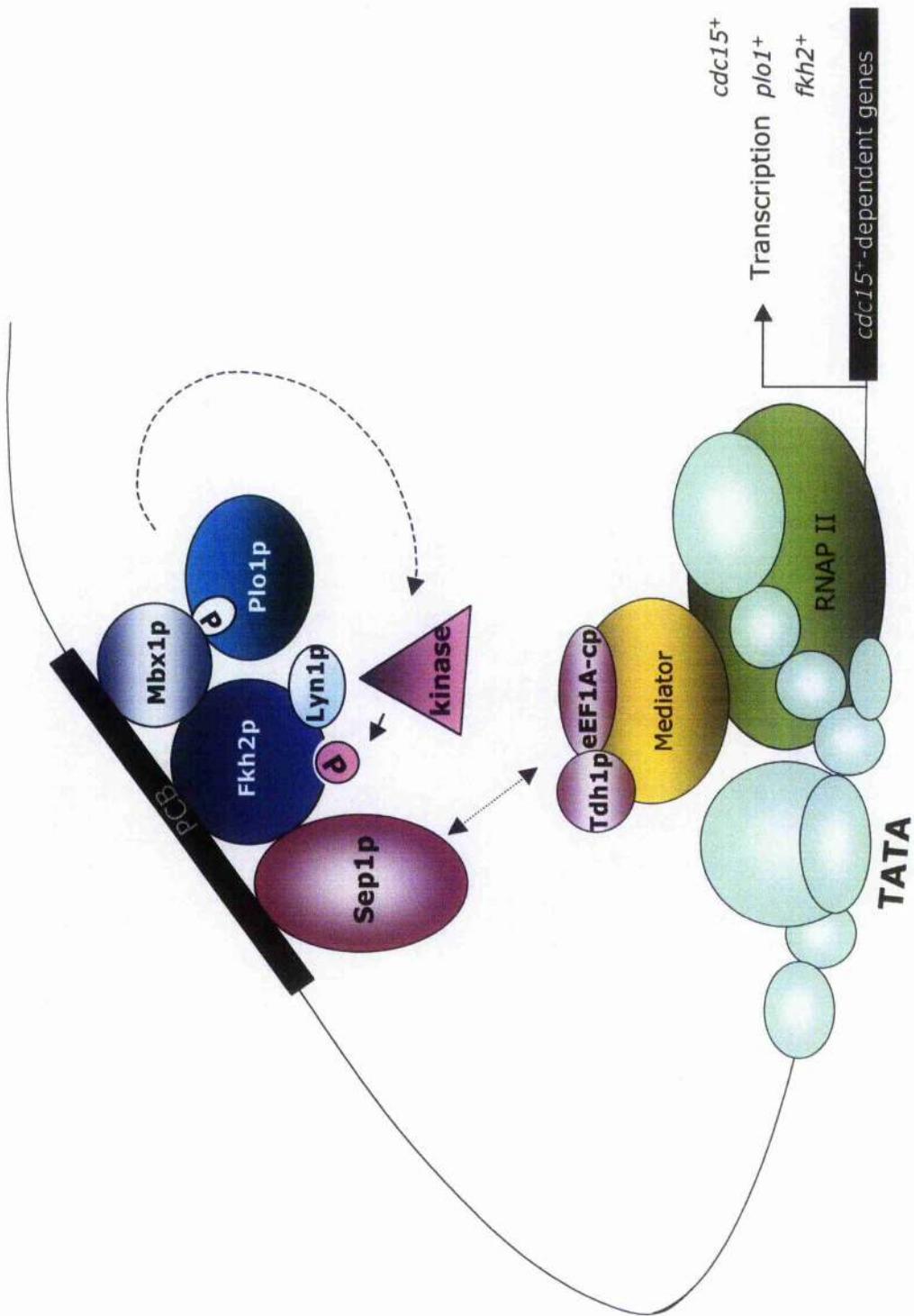
Periodic transcription is usually regulated by transcription factors that can have either activating or repressing roles. We have identified a transcription factor complex (PBF-PCB) required for the periodic transcription of M/G1 genes (Anderson *et al*, 2002). Subsequent work isolated at least three transcription factors, two FHD proteins (Fkh2p and Sep1p) and a MADS-box protein (Mbx1p), that are likely to be components of PBF (Buck *et al*, 2004). A similar transcription factor complex, SFF, operates in *S. cerevisiae* also contain two FHD and a MADS-box protein, suggesting conservation in control mechanisms (Kumar *et al*, 2000). This is confirmed by observations that a similar mechanism has been found in mammalian cells (Alvarez *et al*, 2001).

In this thesis we describe the identification of a protein that binds to Fkh2p, which we named Lyn1p. Although the *lyn1Δ* cells are viable, they are morphologically elongated, a characteristic of cell separation phenotype seen in *fkh2Δ* cells, suggesting the two have related function. Using a similar strategy, we isolated two proteins that bind to Sep1p, Tdh1p and eEF1A-cp. Several lines of evidence suggest a role for Tdh1 and eEF1A-cp as components of the Mediator complex, hypothesized as the bridging property between transcriptional activators and RNAP II machinery (Kimura *et al*, 2001; Mitsuzawa *et al*, 2004). This prompted us to suggest Sep1p as a component of PBF that binds to the RNAP II machinery.

Phosphorylation is a common form of control in transcription. We have demonstrated that both Fkh2p and Mbx1p are cell cycle-dependent phosphoproteins and *in vitro* kinase assays showed that Mbx1p is phosphorylated by Plo1p. In addition, we found that *plo1K69R*, mutated at its KD, prevents M/G1-specific expression when overexpressed. Combined, these

observations suggest that Plo1p, through its KD, is involved in regulating M/G1-specific expression in *S. pombe*, through controlling the phosphorylation status of Mbx1p.

Based on these observations, we propose a model for M/G1 transcription regulation in *S. pombe* (Fig. 5.1). It is likely that the PBF complex binds to PCB promoter throughout the cell cycle, with its activation regulated at the post-translational level (Anderson *et al.*, 2002). In response to an unknown upstream signal, Plo1p, whose expression is self-regulated through PBF-PCB complex, phosphorylates Mbx1p and this stimulates a modification either through Mbx1p itself or a separate kinase-Fkh2p phosphorylation reaction. Together, these result in a change of structure allowing Sep1p to recruit and interact with the Mediator complex. Finally, the protein-DNA complex aids the association of RNAP II machinery to its core promoter thus initiating transcription.



**Figure 5.1.** Model for PBF-PCB regulation of M/G1 transcription by Plo1p in *S. pombe*. Phosphorylation of Mbx1p by Plo1p stimulates the phosphorylation of Fkh2p by either Plo1p itself or a separate kinase. This change of modification causes a conformational change in the protein-DNA complex which enables Sep1p to bind to components of the Mediator complex and RNAP II machinery leading to transcription activation.

## **5.2 Future Work**

We have suggested a model for the PBF-PCB control of M/G1 transcription in *S. pombe*. Future experiments would test this model.

### **5.2.1 *Lyn1p***

In our studies we have recovered interacting partners of Fkh2p and Sep1p. Lyn1p, the Fkh2p-associating protein, exhibited a cell separation function, so it is possible that Lyn1p is also a component of PBF, which is required for M/G1-specific transcription. A way to test this is to measure the mRNA levels of PCB-dependent genes in *lyn1Δ* cells. Using the same deletion cells, we would also determine whether Lyn1p is required for PBF binding *in vitro* by EMSA. If it is required for PBF binding, we will test whether it is a component of PBF by constructing a tagged version to permit super-shift experiments.

We would also test for genetic interactions between *lyn1<sup>+</sup>* and components of PBF. We would do this by combining *lyn1Δ* with individual *fkh2Δ*, *sep1Δ* and *mbx1Δ* mutants, to search for synthetic phenotypes. We would also examine genetic interactions by overexpression studies, by introducing an *nmt1-lyn1<sup>+</sup>* plasmid into *fkh2Δ*, *sep1Δ* and *mbx1Δ* cells, to search for high copy suppression and synthetic phenotypes.

### **5.2.2 *Sep1p*-interacting proteins**

We also propose to characterise the Sep1p-interacting proteins we have identified, Tdh1p and eEF1A-cp. We suggest that Sep1p potentially links PBF to the RNAP II machinery. Therefore, we would make *tdh1<sup>+</sup>* and *ef1a-c<sup>+</sup>* cDNA to enable the study of protein-protein interactions through 2-hybrid analysis. In addition, deletion fragments of Sep1p would allow the mapping of its binding sites, using the same assay.

### **5.2.3 *Plo1p* phosphorylation of *Mbx1p***

We showed that Plo1p can directly phosphorylate Mbx1p *in vitro*, and that the *plo1-ts35 mbx1Δ* double mutant demonstrated a synthetic separation phenotype. In addition, overexpression of the Plo1p KD mutant, *plo1K69R*, specifically abolished M/G1 gene expression. Considering these observations, we plan to assay if *plo1K69R* prevents the phosphorylation of Mbx1p, by *in vitro* kinase assays. We would do this by expressing and purifying Plo1K69Rp in *S. cerevisiae*, exactly we did with wild-type Plo1p, and test its ability to phosphorylate Mbx1p.

Next, we would plan to mutate potential phosphorylation sites in Mbx1p and test for phosphorylation activity, to discover which amino acid residues are phosphorylated by Plo1p. Again this will use the *in vitro* kinase assay, but instead with wild-type Plo1p and various purified mutated forms of Mbx1p.

Having mapped the phosphorylation sites of Mbx1p, this would allow us to test for any effects that mutating these sites might have on biological activity. For example, we can test the mutants for *in vitro* binding to the PCB promoter fragment, through EMSA. We would also mutate these residues in the *mbx1<sup>+</sup>* in its native locus to test for *in vivo* effects on M/G1 transcription, through Northern blot analysis.

#### **5.2.4 Plo1p phosphorylation of Fkh2p**

It is possible that Plo1p phosphorylates Fkh2p as part of PBF to control M/G1 transition, even though *in vitro* assays have so far failed to show this. Potentially, phosphorylation of Fkh2p requires the initial phosphorylation of Mbx1p. We suggest testing this possibility by a co-*in vitro* kinase assay containing active Plo1p with both Fkh2p and Mbx1p, purified using a bacterial coexpression system.

#### **5.2.5 Sep1p binding to RNAP II machinery in vivo**

To date, very little is known about the links between activator proteins, Mediator complexes and RNAP II machinery. Therefore, it would be interesting to establish if Sep1p interacts with components of the Mediator complex. We aim to test this hypothesis by gel filtration chromatography of protein extracts purified from *S. pombe*.

Using the *sep1-TAP* strain, we plan to isolate Sep1p containing complexes under low salt conditions, which preserve a higher order of protein-protein interactions, potentially allowing the maintenance of the PBF-RNAP II complex. We would follow the presence of Sep1p after gel filtration by Western blot analysis using antibody specific to the TAP-tag. Once the eluted fractions have been selected, they would be size separated by SDS-PAGE, and individual proteins identified by MALDI-TOF and confirmed by Silver staining.

#### **5.2.6 PBF binding to PCB promoter in vivo**

In this thesis we provided *in vitro* and *in vivo* evidence for at least three transcription factor proteins as components of the PBF complex, binding specifically to the PCB UAS. To complement these observations we plan to analyse PBF complexes, expressed at endogenous levels *in vivo*, by ChIP analysis.

In this method protein complexes are initially crosslinked with formaldehyde to their DNA sites *in vivo* and subsequently purified by immunoprecipitation using specific antibodies. We plan to use the HA/Myc tagged versions of Fkh2p, Sep1p and Mbx1p to allow us to immunoprecipitate the protein using anti-HA/Myc antibodies, after crosslinking to DNA. Once the crosslink is reversed we will assay the potential PCB promoter fragments by PCR, using oligonucleotide specific to the PCB fragment. Alternatively, we would identify the PCB fragment by DNA microarrays through fluorescently labeled DNA. These experiments would allow us to study the interaction of PBF to PCBs *in vivo*, for example through the cell cycle, which may give clues to how it functions.

#### **5.2.7 In vivo localisation of Fkh2p, Sep1p and Mbx1p**

We also plan to GFP tag Fkh2p, Sep1p and Mbx1p at their chromosomal locus to allow *in vivo* localisation studies. There is precedence in both yeast and mammalian systems for cell cycle transcription factors to move between the cytoplasm and the nucleus through the cell cycle; this shuttling is important for their function. The GFP-tagged strains of Fkh2p, Sep1p and Mbx1p will allow us to examine their distribution through the cell cycle, which also may be important for their activity.

To complement these GFP studies, we have already started making polyclonal antibodies against Fkh2p, Sep1p and Mbx1p, to allow indirect immunofluorescence studies.

Both of these localisation studies also offer the advantage of analysing the interactions among components of PBF and its upstream regulators, such as Plo1p. For example, we speculate that Plo1p may control the distribution of Fkh2p, Sep1p and Mbx1p, and so the *plo1-ts35* mutant might cause their delocalisation.

Combined, all these proposed experiments will contribute greatly to our understanding of PBF-PCB mediated transcription in *S. pombe*.

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## ***Appendix I***

### **YE media**

	g/litre
D-Glucose	30
Bacto-yeast extract	5
Adenosine	0.225
Uracil	0.225

To make solid media, add 8 g of Bacto agar in every 400 ml.

### **ME media**

	g/300 ml
Malt extract	9
Bacto agar	6

Prepare fresh plate for mating each time. Autoclaved bottle of ME can be stored on the bench for months.

## ***Appendix II***

### **EMM**

	g/litre
D-Glucose	20
Ammonium chloride	5
Magnesium chloride-6-hydrate	1
Sodium sulphate	0.1
Calcium chloride	0.015
Potassium hydrogen phthalate	3
di-sodium hydrogen orthophosphate anhydrous	1.8

### **Vitamins**

	ml/litre
Vitamins	1
Trace	0.1

	g/500 ml
Inositol	5
Nicotinic acid	5
Calcium pantothenate	0.5
Biotin	0.005

Aliquot into 5 ml in 15 ml centrifuge tubes and store at -20°C. Microwave to defrost.

### **Trace**

	g/200 ml
Boric acid	1
Manganese sulphate	1.04
Zinc sulphate	0.8
Ferric chloride	0.4
Molybdic acid	0.288
Copper sulphate	0.08
Citric acid	2
Potassium iodide	0.02

Aliquot into 5 ml in 15 ml centrifuge tubes and store in the fridge.

## ***Appendix III***

### **SDS-PAGE**

Mini Gel Recipe for Bio-Rad 1.0 or 1.5 mm thick gels: all recipes are in ml unless otherwise stated.

	Stacking	5%	6%	7.8%	10%	12%	15%
dH <sub>2</sub> O	3.2	6.4	5.9	5.4	5.0	4.5	4.0
4x Resolving Gel Buffer	-	2.5	2.5	2.5	2.5	2.5	2.5
4x Stacking Gel Buffer	1.3	-	-	-	-	-	-
40% Solution Acrylamide	0.5	1.7	1.5	2.0	2.5	3.0	3.5

Add 60 µl 10% APS and 22 µl TEMED per gel to set.

### **Resolving buffer (4x)**

1.5M Tris, 0.4% SDS

Dissolve in 50 ml, adjust pH with 5 M HCl to pH 8.8 and make up to 100 ml.

### **Stacking buffer (4x)**

500 mM Tris, 0.4% SDS

Dissolve in 85 ml, adjust pH with 5 M HCl to pH 6.8 and make up to 100 ml.

## **Appendix IV**

### **S. pombe strains**

GG214	<i>leu1-32</i> wild-type
GG217	wild-type (prototrophic for all nutritional marker)
GG481	<i>h<sup>-</sup> plo1-ts35 cdc2-33ura4-D18/leu1-32</i>
GG501	<i>h<sup>-</sup> pREP1:plo1<sup>+</sup> cdc7.A20sgp1.B8/leu1-32ura4-D18</i>
GG503	<i>h<sup>-</sup> mbx1::kan<sup>R</sup>/leu1-32ura4-D18ade6-M210his7-366</i>
GG507	<i>h<sup>-</sup> fkh2-13myc::kan<sup>R</sup>/leu1-32ura4-D18ade6-M210</i>
GG509	<i>h<sup>-</sup> mbx1-13myc::kanR/leu1-32ura4-D18ade6-M210his7-366</i>
GG515	<i>h<sup>-</sup> sep1::ura4<sup>+</sup>/ura4-D18</i>
GG524	<i>h<sup>-</sup> fkh2::kanR/ura4-D18/leu1-32</i>
GG543	<i>h<sup>+</sup>sep1::ura4<sup>+</sup>mbx1::kanR/leu1-32ura4-D18ade6-M210his7-366</i>
GG551	<i>h<sup>+</sup> fkh2::kanRmbx1::kanR/leu1-32ura4-D18ade6-M210his7-366</i>
GG558	<i>h<sup>+</sup> fkh2-3HA::kan<sup>R</sup>/leu1-32ura4-D18</i>
GG614	<i>h<sup>-</sup> pREP:plo1<sup>+</sup>/leu1-32</i>
GG650	<i>h<sup>+</sup> pREP1:plo1<sup>+</sup> fkh2-3HA::kan<sup>R</sup>cdc2-33ura4-D18/leu1-32</i>
GG651	<i>h? pREP1:plo1<sup>+</sup> mbx1-13myc::kan<sup>R</sup>cdc2-33/leu1-32ura4-D18his7-366</i>
GG668	<i>h<sup>-</sup> pREP1: plo1-DHK625AAA leu1-32</i>
GG670	<i>h<sup>-</sup> pREP1: plo1-K69R leu1-33</i>
GG695	<i>h<sup>-</sup> pREP3x plo1-ts35ura4-D18/leu1-32</i>
GG697	<i>h<sup>+</sup> pREP3X:mbx1<sup>+</sup> plo1-ts35ura4-D18/leu1-32</i>
GG699	<i>h<sup>+</sup> pREP3x:fkh2<sup>+</sup> plo1-ts35ura4-D18/leu1-32</i>
GG701	<i>h<sup>-</sup> pREP3x:sep1<sup>+</sup> plo1-ts35ura4-D18/leu1-32</i>
GG743	<i>h<sup>+</sup> mbx1-13myc::kan<sup>R</sup>cdc25-22ura4-D18/leu1-32</i>
GG745	<i>h<sup>+</sup> fkh2-3HA::kan<sup>R</sup>cdc25-22/leu1-32ura4-D18</i>
GG746	<i>h? sep1::ura4<sup>+</sup>mbx1::kan<sup>R</sup>fkh2::kan<sup>R</sup>/leu1-32ura4-D18ade6-M210his7-366</i>
GG749	<i>h<sup>-</sup> pREP1:plo1-K69R mbx1-13myc::kanR/leu1-32ura4-D18ade6-M210his7-366</i>
GG753	<i>h<sup>+</sup> pREP1:plo1-K69R fkh2-3HA::kanR/leu1-32ura4-D18</i>
GG757	<i>h<sup>-</sup> fkh1-13myc</i>
GG760	<i>h<sup>+</sup> mbx1::kan<sup>R</sup>plo1-ts35/leu1-32ura4-D18ade6-M210his7-366</i>
GG761	<i>h<sup>-</sup> sep1::ura4<sup>+</sup>plo1-ts35/leu1-32</i>
GG763	<i>h<sup>+</sup> fkh2::kan<sup>R</sup>plo1-ts35ura4-D18/leu1-32</i>
GG878	<i>h<sup>-</sup> sep1<sup>+</sup>-2PA-cTAP:kan<sup>R</sup></i>
GG886	<i>h<sup>-</sup> fkh2<sup>+</sup>-4PA-cTAP:kan<sup>R</sup></i>
GG908	<i>h<sup>+</sup>/h<sup>-</sup> /leu1-32 /leu1-32ura4-D18/ura4-D18ade6-210/ade6-216</i>
GG950	<i>h<sup>+</sup>/h<sup>-</sup>lyn1::URA4/lyn1<sup>+</sup>/leu1-32/leu1-32ura4-D18/ura4-D18ade6-210/216</i>

GG985	<i>h<sup>+</sup> lyn1::URA4leu1-32ura4-D18ade6-210</i>
GG989	<i>h?</i> <i>lyn1::Kan<sup>R</sup> leu1-32ura4-D18ade6-210</i>

### ***S. cerevisiae* strains**

GGBY143	<i>plo1<sup>+</sup>-GST</i>
GGBY144	<i>plo1<sup>+</sup>-His</i>

### **Bacteria strains**

GB4	DH5 $\alpha$
	F <sup>-</sup> <i>ompT</i> <i>hsdS(r</i> <sub>5</sub> <i>m</i> <sub>6</sub> <i>)dcm</i> <sup>+</sup> <i>Tet</i> <sup>r</sup> <i>ga</i> $\lambda$ (DE3) <i>endA</i> <i>Hte[argUileWleuWCam</i> <sup>r</sup> <i>]</i>
GB455	BL21 DE3 codon(+)-RIL <i>supE44ΔlacU169(φ80lacZΔM15)hsdR17recA1endA1 byrA96thi1relA1</i>

### **Plasmid vectors**

GB28	pREP3X
GB131	pFA6a-kanMX6
GB133	pFA6a-13Myc-kanMX6
GB148	KS-ura4
GB263	pREP3X: <i>fkh2</i> <sup>+</sup>
GB290	pREP3X: <i>sep1</i> <sup>+</sup>
GB291	pREP3X: <i>mbx1</i> <sup>+</sup>
GB293	pREP81X: <i>mbx1</i> <sup>+</sup>
GB295	pREP1: <i>plo1</i> <sup>+</sup>
GB297	pREP1: <i>plo1</i> -DHK625AAA
GB301	pREP81X: <i>sep1</i> <sup>+</sup>
GB338	pREP1: <i>plo1</i> K69R
GB372	2PA-C-TAP pFA6a-KanMX6
GB374	4PA-C-TAP pFA6a-KanMX6
GB387	pCR2.1: <i>fkh2</i> <sup>+</sup> -cDNA
GB403	pCR2.1: <i>fkh2</i> <sup>+</sup> -cDNA
GB404	pMG1-GST
GB405	pMH919-His
GB428	pMG1- <i>plo1</i> <sup>+</sup> -GST
GB430	pMH919- <i>plo1</i> <sup>+</sup> -His
GB444	pCR2.1: <i>sep1</i> <sup>+</sup> -cDNA

## Oligonucleotide

GO183	GCAAATGAACAAGTAACATCGGGGTTGAAAGTTATTAAAGCAACCAAGG TAAAAGATATGTGAAAATTAGGAATTCCGAATTGAGCTCGTTAAC
GO184	CAGCCGCGCAAGATGATACTTACTTGCCCTCCTACCAAGAGGAAAATGC CAAACCTCAACACTATTCCGGATCCCCGGGTTAATTAA
GO195	GCT TTCGTAAATTAAAAAAATTGCGCAC
GO196	GCTAGGATAACAGTTCTCACATCACATCCG
GO507	CCGGACGTCGACATGACTGTTCGCAGACTCGAAAGC
GO508	CCGGACGGATCCTTAAGCACTACTTTAACATTAGA
GO515	CCGCACCTCGAGATGAATTAAATTCTACTAACCCCT
GO516	CCGCACGTCGACTTAGAATAGTGTGAAGTTGACG
GO517	CCGCACCTCGAGATGGATATTAAATCCTCCTCTAGT
GO518	CCGCACCCCGGGTTAAGGGCATTGTTCAATATT
GO570	CCG CCCCCGGGATGAATTAAATTCTACTAACCCCT
GO571	CCCTCTCGAGCITAGAATAGTGTGAAGTTGACG
GO574	CGCCAGAGTATTGGAGACCCG
GO575	GGGGATCCGTCGACCTGCAGCGTACGAGGTATAAGAGGATGATGTCG
GO576	GTTTAAACGAGCTCGAATTCTCGATTGAGCACAACCTGGCTTGGTGG
GO577	GAGCTGCCCGACAGG
GO578	CGCTGAATATGAGTTGTTGTTTC
GO594	CCAATTACAAAAACCTCGTAAAGAAAAATCTCCCCAGCAGTACATTAGG TTCCCTCAATCTAATGTTAAAGTAGTGCTCGGATCCCCGGGTTAATTAA
GO595	GGAAAACTTCGCAAGTTAACGGAAACGAACAATTCCAAAATTCTAG CAATAATTGTGATGAAAGAGCATAATGTCGAATTGAGCTCGTTAAC
GO605	TAGTGGATCCATGGCGAGTGTGCAATT
GO606	GTCACTCGAGTTAACTCACTCCATTTCG