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Control of M-G1 phase-specific expression in fission yeast

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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.

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Abstract

The mitotic cell cycle underlies propagation of eukaryotic cells, continually duplicating and dividing. The past few years have seen major advances in understanding of the regulatory mechanisms that impose on the cell cycle to tightly co-ordinate progression through its individual phases, safeguarding the timing and integrity of its hallmark events, DNA synthesis and mitosis. Transcription is prominent among these processes, manifesting its importance for cell cycle controls by the large number of eukaryotic genes that are expressed at specific cell cycle times. Certain genes are cell cycle regulated in a number of organisms, suggesting that their phase-specific transcription is important for all eukaryotic cells.

The budding and fission yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, have been used extensively as model organisms for the study of the eukaryotic cell cycle and cell cycle-regulated transcription, because the cell cycle machinery is conserved among eukaryotes and they are experimentally tractable. Recent microarray analyses have shown that cell cycle-specific expression is a frequent theme in the two yeasts, identifying consecutive, inter-dependent, waves of transcriptional activity, coinciding with the four main cell cycle transitions; G1-S, S, G2-M and M-G1 phases. Each phase-specific transcriptional wave corresponds to at least one group of co-regulated genes, sharing common *cis*- and *trans*- acting elements.

The work presented in this thesis delves into the regulatory network that drives phase-specific gene expression during late mitosis-early G1 phase in fission yeast. During this late cell cycle stage, fission yeast and, indeed, every eukaryotic cell, undergo major changes; each completes mitosis and cytokinesis, partitioning its duplicated genetic and cytoplasmic material into two progeny cells, which then themselves prepare for a new round of mitotic cell division. Consistent with their periodic pattern of expression, most of the genes transcribed during the M-G1 interval in *S. pombe* encode proteins that execute important functions during late mitosis and cytokinesis.

A DNA sequence promoter motif, the PCB (Pombe cell cycle box), has been identified in fission yeast that confers M-G1 specific transcription, and is bound by the PBF (PCB binding factor) transcription factor complex. PCB promoter motifs are present in several M-G1 transcribed genes, including *cdc15⁺*, *spo12⁺*, *sid2⁺*, *fin1⁺*, *slp1⁺*, *ace2⁺*, *mid1⁺/dmf1⁺* and *plo1⁺*, the latter encoding a Polo-like kinase that also regulates M-G1 gene expression and influences the PCB-dependent binding properties of PBF. Three transcription factors, Sep1p and Fkh2p, both forkhead-like transcription factors, and Mbx1p, a MADS-box protein, have been implicated in M-G1 specific gene expression and are thought to be components of PBF. Consistent with Fkh2p and Sep1p regulating

M-G1 specific transcription, forkhead-related sequences are present in the genes' promoters. Notably, *fkh2⁺* contains both PCB and forkhead promoter sequences and is transcribed during the M-G1 interval, implying that Fkh2p and Plo1p regulate gene transcription during late mitosis and ensuing passage through cytokinesis via feedback loops.

This study provides further evidence about transcriptional regulation late in the fission yeast cell cycle, revealing that the PCB sequence is crucial for M-G1 specific transcription, with forkhead-associated DNA motifs playing a parallel but smaller regulatory role. Consistent with this hypothesis, work here and elsewhere shows that both Fkh2p and Sep1p control phase-specific expression of their co-regulated genes through the PCB and forkhead sequences. Notably, data in this thesis reveal that these two forkhead transcription factors associate with each other *in vitro* and *in vivo* and bind *in vivo* to the PCB promoter regions of M-G1 transcribed genes, including *cdc15⁺* and *plo1⁺*, in a cell cycle specific manner, consistent with Fkh2p repressing and Sep1p activating transcription. Furthermore, Fkh2p contacts its own promoter, suggesting that it regulates its own expression via a negative feedback mechanism.

The Plo1p kinase is shown here to bind *in vivo* to Mbx1p throughout the cell cycle and in a manner that requires both its kinase and polo-box domains. In agreement with this observation, Plo1p can phosphorylate *in vitro* Mbx1p, itself known to become phosphorylated during late mitosis. This is the first time that a Polo-like kinase has been shown to bind and phosphorylate a MADS-box protein in any organism. Moreover, in concert with Plo1p binding and phosphorylating Mbx1p, ChIP assays here reveal that this kinase interacts *in vivo* with the PCB promoter DNA of M-G1 expressed genes, including *cdc15⁺* and *fkh2⁺*, in a cell cycle-dependent manner with a timing that coincides with low levels of expression, but follows promoter binding by Fkh2p. Given that Plo1p has previously been shown to positively influence M-G1 dependent transcription, its cell cycle pattern of promoter contact suggests that this Polo-like kinase functions at the genes' promoters, most-likely via binding and phosphorylation of Mbx1p, to re-stimulate transcription, following repression by Fkh2p. In parallel, these findings suggest that Plo1p regulates its own expression via a positive feedback loop.

Overall, the work presented in this thesis unravels crucial regulatory aspects of the transcriptional network that drives M-G1 specific transcription in *S. pombe*: it suggests an important role for the PCB promoter motif in transcriptional regulation; it proposes that Fkh2p acts as a repressor while Sep1p as an activator of late mitotic transcription; it reveals and proposes novel functions for Plo1p, a conserved Polo-like kinase family member, involving its association with Mbx1p, a MADS box protein, and its cell cycle specific recruitment to PCB promoters of M-G1 transcribed genes.

As transcriptional systems, encompassing homologues of most of the components of this *S. pombe* M-G1 specific transcriptional network operate both in *S. cerevisiae* and humans, this demonstrates their importance for mitotic cell cycle progression. Thus this work potentially offers new insights into M-G1 specific gene expression in all eukaryotes.

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Supporting publications

Papadopoulou, K., Ng, S.S., Ohkura, H., Geymonat, M., Sedgwick, S.G. and McNerny, C.J. (2008). Regulation of gene expression during M-G1-phase in fission yeast through Plo1p and forkhead transcription factors. *Journal of Cell Science* **121**, 38-47.

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Abbreviations

Amp ^R	ampicillin resistant
APC/C	anaphase promoting complex/cyclosome
ARK	Aurora related kinase
ARS	autonomous replication sequence
ATP	adenosine triphosphate
ATM	ataxia telangiectasia mutated
ATR	ATM-related
bp	base pair
CAK	Cdk-activating kinase
CAR	cytokinetic actomyosin ring
Cdc	cell division cycle
Cdk	cyclin dependent kinase
CTD	C-terminal domain
cDNA	complementary deoxyribonucleic acid
ChIP	Chromatin Immunoprecipitation
CKI	cyclin dependent kinase inhibitors
DDK	Dbf4p-dependent kinase
DNA	deoxyribonucleic acid
DPE	downstream promoter element
DSC1	DNA synthesis control 1
DTT	dithiothreitol
E2F	E2A binding factor
ECB	Early cell cycle box
ECL	Enhanced Chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EMM	Edinburgh minimal media
ETS	E twenty-six
g	gravity (relative centrifugal force; rcf)
GAP	GTPase activating protein
GEF	guanine-nucleotide-exchange factor

gr	grammes
GST	glutathione S-transferase
GTF	general transcription factor
h	hour
HA	hemagglutinin
Hepes buffer	N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) buffer
HIS	histidine
HNF	hepatocyte nuclear factor
HRP	horseradish peroxidase
IgG	immunoglobulin
Inr	initiator
IPTG	isopropyl β -thiogalactopyranoside
Kan ^R	kanamycin resistant
kb	kilo basepair
kDa	kilo Dalton
kV	kilo-Volt
l	litre
LB	Luria Broth
MADS-box	Mcm1p, Agamous, Deficiens SRF-box
MALDI-TOF	Matrix Assisted Laser Desorption-Ionization Time-of-Flight
MBF	<i>Mlul</i> cell cycle box binding factor complex
MCB	<i>Mlul</i> cell cycle box
MCM	minichromosome maintenance
MCP	morphogenesis checkpoint pathway
mcs	mitotic catastrophe suppressor
MEF2	myocyte-specific enhancer factor 2
MEN	mitotic-exit network
mg	milligram
min	minute
ml	millilitre
MNE	MOPS/NaOAc/EDTA
MOPS buffer	3-(N-Morpholino)-propanesulfonic acid buffer
mRNA	messenger RNA
MTOC	microtubule organising centre

N ₆	random hexanucleotide
NETO	new end take off
NIMA	never in mitosis A
nmt	no message in thiamine
NP-40	Nonidet P-40
Nrk	NIMA-related kinase
OD	optical density
ONPG	o-Nitrophenyl β-D-galactopyranoside
ORC	origin recognition complex
ORF	open reading frame
PBD	polo-box domain
PBF	PCB-binding factor
PBS	phosphate-buffered saline
PCB	Pombe cell cycle box
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PEG	polyethylene glycol
Plk	Polo-like kinase
PMSF	phenylmethanesulfonylfluoride
PNK	polynucleotide kinase
Pol I	RNA polymerase I
Pol II	RNA polymerase II
Pol III	RNA polymerase III
Post-RC	Post- replication complex
Pre-IC	pre-initiation complex
pre-RC	pre-replication complex
psi	pounds per square inch
Rb	Retinoblastoma
RFC	replication factor C
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
RPA	replication protein A
rpm	revolution per minute

rRNA	ribosomal RNA
RT-PCR	reverse transcriptase-polymerase chain reaction
SBF	SCB binding factor
SCB	Swi4/6 cell cycle box
S-CDK	Cdk bound to S phase cyclins
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoresis
sec	second
SFF	Swi five factor
SIN	septation initiation network
SPB	spindle pole body
SRF	serum response factor
SWI/SNF	mating-type switching/sucrose non-fermenting
TAF	TBP-associated factor
TBE	Tris/Borate/EDTA buffer
TBP	TATA-binding protein
TE	Tris/EDTA
TEMED	<i>N,N,N',N'</i> -tetramethylethylenediamine
THESIT	polyethylene glycol dodecyl ether
Tris	2-Amino-2-(hydroxymethyl)-1,3-propanediol
Triton X-100	Polyethylene glycol <i>tert</i> -octyl-phenyl ether
ts	temperature sensitive
U	units
UAS	upstream activating sequence
μCi	microcuries
μg	microgram
μl	microlitre
μM	micromolar
URS	upstream repressing sequence
w/v	weight for volume
YE	yeast extract
YPD	yeast extract with peptone and dextrose

Chapter 1

Introduction

1.1 Introduction

All eukaryotic cells, regardless of origin, ranging from unicellular yeasts to somatic human cells, are defined by their ability to grow and divide, culminating in their proliferation. The mitotic cell cycle, a highly coordinated sequence of events, is the process that the eukaryotic cell undergoes to accurately divide into two progeny, each with the same chromosomal and cytoplasmic content as the parent. DNA synthesis and mitosis are the hallmark events of the eukaryotic cell cycle. The former replicates the parental chromosomes and the latter partitions each copy of the duplicated hereditary material together with cytoplasm constituents and organelles, via the process of cytokinesis, into the two cell progeny.

A number of distinct regulatory networks operate within the cell to temporally separate DNA synthesis from mitosis and guarantee that chromosome segregation always follows chromosome duplication. Based on the temporal separation of the processes that copy and partition the duplicated genome during the lifespan of a eukaryotic cell, the mitotic cell cycle is conventionally perceived as a series of events that take place within four successive phases: G1, S, G2 and M phase, respectively. The S phase corresponds to the time when the chromosomes are replicated, while the M phase is the time when the two copies of each chromosome are allocated into the nuclei of the two progeny, which emerge as soon as cytokinesis is complete. G1 and G2 are the 'gap' phases that intervene between S and M phases. G1 commences after each M phase and precedes the onset of the next S phase, while the G2 phase follows completion of DNA synthesis (end of S phase) and paves the way to mitosis. The G1 and G2 phases provide the cell with sufficient time to grow and at the same time monitor its readiness for another round of DNA replication and division, respectively, thus maintaining its genetic identity and integrity in the ensuing generations of offspring.

Apart from asexual propagation through the process of mitotic cell division, a eukaryotic cell, depending on the organism and internal or external stimuli, can normally undergo distinct developmental fates. In multicellular eukaryotes, a somatic cell can stop dividing, exit the mitotic cell cycle and undergo differentiation into distinct cellular types, giving rise to different tissues and organs of the body. Alternatively, a eukaryotic cell can undergo meiosis, a modified type of cell division, generating haploid gametes, either eggs or sperm. During sexual reproduction, fusion of such germ cells from two different individuals yields a diploid zygote, which can then develop into a new individual. Meiosis, through the process of homologous recombination, greatly contributes to the generation of genetic variation within species, the driving force of evolution. The time when a eukaryotic cell decides whether to enter a mitotic cell cycle or pursue another developmental route, marks an irreversible point early in the cell cycle, during the G1 phase. This irreversible decision

point is termed 'START' in the budding and fission yeasts, or the 'Restriction point' in mammals. Once a cell progresses past the START/Restriction point, it becomes committed to the mitotic cell cycle and its successful completion.

Various interlocking mechanisms operate within the cell during the course of the mitotic cell cycle that co-ordinate DNA synthesis with mitosis and ensure that these events occur with high fidelity, in the correct order, and only after completion of the previous phase. Tight control of the mitotic cell cycle is of the utmost importance for proper growth and development for all living organisms. The significance of cell cycle control is exemplified most dramatically by the detrimental effects, for the survival of the cell, of errors in the execution of the regulatory processes that drive progression through the cell cycle. In multicellular organisms, such as humans, inactivation or abnormal behaviour of the regulatory networks for normal growth and division deregulates the mitotic cell cycle and results in genomic instability and the incidence of diseases, most prominently cancer.

The significance of the mitotic cell division cycle for the normal development and survival of eukaryotic organisms has prompted a vast amount of research over the past few decades to study the biological processes that constitute the mitotic cell cycle, and delineate the regulatory mechanisms that drive progression through S phase and mitosis. Genetic and biochemical studies in diverse species, including among others the budding and fission yeasts, sea urchin, *Drosophila*, *Xenopus* and, ultimately, humans, have shown most strikingly that the mitotic cell cycle operates in a similar way in all eukaryotes. Regulation of the cell cycle is exerted by mechanisms and networks that control the onset, execution and completion of DNA replication and mitosis. The activities of these modulatory processes converge on the master regulators of the cell cycle, the cyclin-dependent kinases (Cdks), which control S phase and mitosis by phosphorylating appropriate substrates and are themselves subject to tight regulation. Control of the activities of the cyclin-dependent kinases, and eventually the mitotic cell cycle, is brought about by the interplay between distinct molecular mechanisms, such as specific proteolytic degradation, changes in intracellular localisation and reversible phosphorylation (Nurse, 2000; Nasmyth, 2001; Nurse, 2002; Lodish *et al.*, 2003).

Cell cycle specific transcription, resulting in phase specific gene expression, also plays a major part in the regulation of the cell cycle. Global microarray analysis in the fission and budding yeasts, both of which are extensively used as models for the study of the eukaryotic cell cycle, has revealed a large number of genes that are transcribed in a cell cycle-specific manner. The mammalian homologues of a few of these genes are also transcribed and expressed at specific cell cycle times. For many of these genes, their ensuing phase-specific expression is essential for the

timing and completion of the key cell cycle events of DNA synthesis and mitosis, exemplifying the significance of transcription for normal cell cycle progression (McInerney, 2004; Ng *et al.*, 2006).

The regulatory mechanisms that govern cell cycle specific transcription during late mitosis-early G1 phase in the fission yeast are the research topic of this study. This introductory chapter describes the eukaryotic cell division cycle and its regulation, preceded by an outline of the life cycles, genetics and advantages of the fission yeast *Schizosaccharomyces pombe* and the budding yeast *Saccharomyces cerevisiae* as experimental systems for the analysis of cell cycle controls. Then, the focus turns to what is known about cell cycle specific transcription in the budding and fission yeasts, overviewing both the various groups of genes that are expressed in a cell cycle specific manner and the molecular components responsible for these patterns of periodic transcription. Finally, with relevance to this thesis, aspects of transcriptional control of eukaryotic gene expression are also discussed.

1.2 The budding and fission yeasts: powerful models for biological studies

The basic molecular processes that enable a eukaryotic cell to traverse through the mitotic cell cycle are conserved in organisms as diverse as fungi and mammals, exemplified by the ubiquity of the Cdks and many other components of the cell cycle machinery (Sections 1.3, 1.4, 1.5 and 1.6). The universality of the mitotic cell cycle has encouraged the use of different organisms, each with its own experimental benefits, as model systems for the elucidation of the regulatory mechanisms underlying normal cell cycle progression. Prominent among them are the budding yeast *S. cerevisiae* and the fission yeast *S. pombe*, largely due to their amenability to powerful genetics.

The budding and fission yeasts exist in nature as stable haploids, which make recessive mutations relatively straightforward to recognise. This aspect of yeast physiology is particularly useful for the isolation of conditional lethal cell cycle mutants that under restrictive conditions either accelerate or block progress past a specific cell cycle stage and define essential genes necessary for progression through cell cycle events, such as DNA synthesis and mitosis. Gene cloning in these organisms is greatly facilitated by complementation analysis. In this approach, mutants are transformed with plasmids carrying an appropriate gene library and screened for rescue of the mutant phenotype. *S. pombe* and *S. cerevisiae* are suitable for extragenic suppression analysis, unravelling the regulatory networks that underlie complex biological processes, such as the cell cycle. Extragenic suppressors define loss- or gain- of function mutants of a novel gene that rescue the mutant phenotype of the initial gene of interest. Extragenic suppression identifies genes whose encoded products interact directly or indirectly as part of a protein complex or are components of a

pathway, with the suppressor acting upstream of the original mutant in a positive or negative manner. Moreover, homologous recombination occurs in *S. cerevisiae* and in *S. pombe*, which has facilitated the development of gene disruption methods that generate null alleles of genes in their genomic loci. Such genetic techniques, combined with overexpression analyses and a broad spectrum of other molecular biology and biochemical applications, are powerful tools for the elucidation of gene function. The tractable genetics of the two yeasts combined with their ability to grow easily and promptly (completing a cell cycle within only a few hours) under laboratory conditions constitute them ideal experimental systems, not only for cell cycle studies but also for other biological processes (Hartwell *et al.*, 1974; Hartwell, 1978; Nurse *et al.*, 1976; Forsburg and Nurse, 1991; Hartwell, 1991; MacNeill and Nurse, 1997; Bähler *et al.*, 1998a; Nurse *et al.*, 1998; Forsburg, 1999; Nurse, 2002; Lodish *et al.*, 2003).

S. cerevisiae, the first eukaryote to enter the post-genomics era and have its genome sequenced, has extensively been used as model to address a wide range of biological phenomena, including, apart from the mitotic cell cycle, protein trafficking and transcriptional regulation. Relative to budding yeast, *S. pombe* has a shorter history as an experimental system. Nonetheless, fission yeast has become popular in the scientific community over recent years, culminating in the sequencing of its genome (Wood *et al.*, 2002). In parallel, the realisation that its genetic content is the smallest among eukaryotes and that it possesses features more characteristic of complex eukaryotes in various processes, including intron splicing, transcription initiation and protein modification, combined with its typical mitochondrial genome, has further boosted its use as a model organism (Russell and Nurse, 1986b; MacNeill and Nurse, 1997; Forsburg, 1999; Yanagida, 2002).

The budding and fission yeasts are free-living unicellular ascomycete fungi that, as their names denote, propagate by budding and medial fission, respectively. In terms of phylogenesis, *S. cerevisiae* and *S. pombe* appear to have diverged from each other very early during evolution and they are as distant from each other as either one is from higher eukaryotes (Forsburg, 1999; Sipiczki, 2000; Yanagida, 2002). This implies that cell cycle components and regulatory networks found in both yeasts are probably conserved between most eukaryotes, illustrating the importance of studying cell cycle control in both organisms (Russell and Nurse, 1986b; Forsburg, 1999; Yanagida, 2002).

The remote evolutionary relationship between *S. cerevisiae* and *S. pombe* accounts possibly for their distinct life styles and differences in the organisation of the genome, regulation of gene expression and in certain aspects of an otherwise conserved cell cycle (Forsburg and Nurse, 1991). Both yeasts contain a similar size genome (12-14 Mb), but *S. cerevisiae* partitions it into 16 relatively small chromosomes, while *S. pombe* contains only three larger chromosomes (Goffeau *et al.*, 1996; Wood *et al.*, 2002). This perhaps explains why the budding yeast possesses compact centromeres

and DNA replication origins with well defined sequences, as opposed to the large, disperse and less-defined sequences of these chromosome structures in fission yeast (Forsburg, 1999; Yanagida, 2002).

1.2.1 *S. pombe* and *S. cerevisiae* life cycles

S. pombe is characterised by a life cycle that comprises both haploid and transient diploid nuclear phases (Figure 1.1). Haploid cells are of two opposite mating types, termed *P* (h^+) and *M* (h^-). *S. pombe* haploids of both mating types occur naturally in homothallic strains, in which switch of mating type takes place. Diploid cells (zygotes) are formed transiently during conjugation and nuclear fusion of two haploids of opposite mating types. Zygotes undergo meiosis immediately and sporulate to yield four-spored ascospores. Each ascospore is enclosed in an ascus wall with all four surrounded by a cell wall, originating from the two initial haploids. Rupture of the asci releases four haploid spores. Under favourable growth conditions, such as ample nutrients, haploid cells propagate asexually by mitotic cell division. During the mitotic cell cycle, the cells grow by elongation at their tips and then divide by septation and medial fission. Under poor growth conditions, such as limiting nutrients, and in the presence of only one mating type, cells withdraw from the cell cycle and enter a stationary phase. Alternatively, if both mating types are present nutrient deprivation triggers formation of zygotes, followed by meiosis and sporulation, enabling the survival of the organism in a dormant state, due to the endurance of haploid spores under stress conditions, such as heat. As mentioned, the diploid phase in fission yeast is transient. However, if diploid zygotes are transferred to nutrient-rich media before meiosis, they can remain in a diploid state and undergo mitotic cell division. Haploid and diploid cells that undergo meiosis and sporulation are morphologically distinct, the former forming “rod-shape” un-ruptured azygotic asci and the latter “banana-shape” zygotic asci (Egel, 1989; Munz *et al.*, 1989; Forsburg and Nurse, 1991; Hayles and Nurse, 1992; Egel, 2003).

Unlike *S. pombe*, the life cycle of *S. cerevisiae* possesses both stable haploid and diploid cells (Figure 1.2). The two mating types in budding yeast are termed α and *a*. In the presence of only one mating type in the cell population, haploid cells undergo mitotic cell division. Early in the cell cycle, a bud forms in the dividing haploid which grows in size as the cell cycle progresses. Late during mitosis, the bud pinches off giving rise to a daughter cell, with the same genetic content but a smaller size than the mother cell (Forsburg and Nurse, 1991; Lew *et al.*, 1997). When both mating types are present in a cell population, haploids of opposite mating type fuse to form diploid cells (a/α) which, as long as there are sufficient nutrients, continue to grow and mitotically divide.

However, once starved, diploids undergo meiosis and sporulation to yield four haploid ascospores, enclosed by a cell wall of the original diploid cell.

In both budding and fission yeasts, conjugation leading to the formation of a stable or unstable diploid, respectively, is mediated by the mating pheromones, diffusible extracellular peptides, secreted by haploids. Each cell produces a mating type-specific pheromone, targeting the cell surface of a haploid cell of the opposite mating type. As a result, cells of both mating types arrest early in the G1 phase, prior to the irreversible decision point of the mitotic cell cycle, when a cell still has the choice to follow an alternative developmental fate (Section 1.1). Then, conjugation and nuclear fusion follows, leading to the formation of diploid zygote which, depending on the yeast species and availability of nutrients, undergoes either meiotic or mitotic cell division (Zaborowska *et al.*, 1975; Pringle and Hartwell, 1981; Miyata *et al.*, 1997).

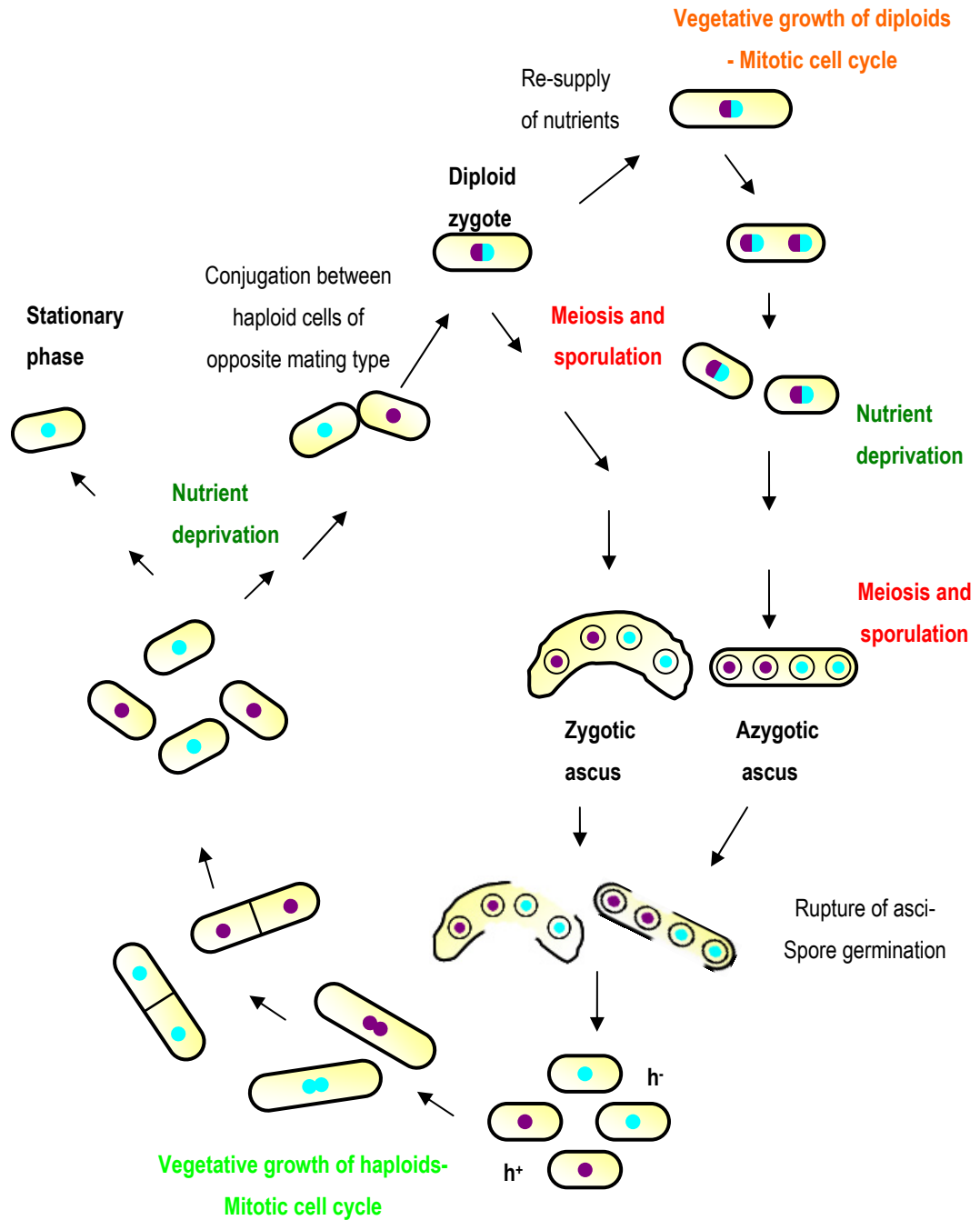


Figure 1.1: The life cycle of *Schizosaccharomyces pombe*. Fission yeast cells grow stably as haploids, multiplying by mitotic cell division. Upon nutrient deprivation cells exit from the mitotic cell cycle and enter into stationary phase. Nonetheless, if cells of both mating types (h⁺ and h⁻) are present, they conjugate to form a diploid zygote, which undergoes meiosis, yielding a zygotic ascus with four haploid ascospores. Upon re-supply of nutrients the haploid spores re-enter the mitotic cell cycle. Under certain conditions, including immediate re-supply of nutrients the diploid zygote can grow vegetatively by mitotic cell division until nutrients become limiting, where the diploid cells undergo meiosis and generate an azygotic ascus, morphologically distinct from the zygotic ascus.

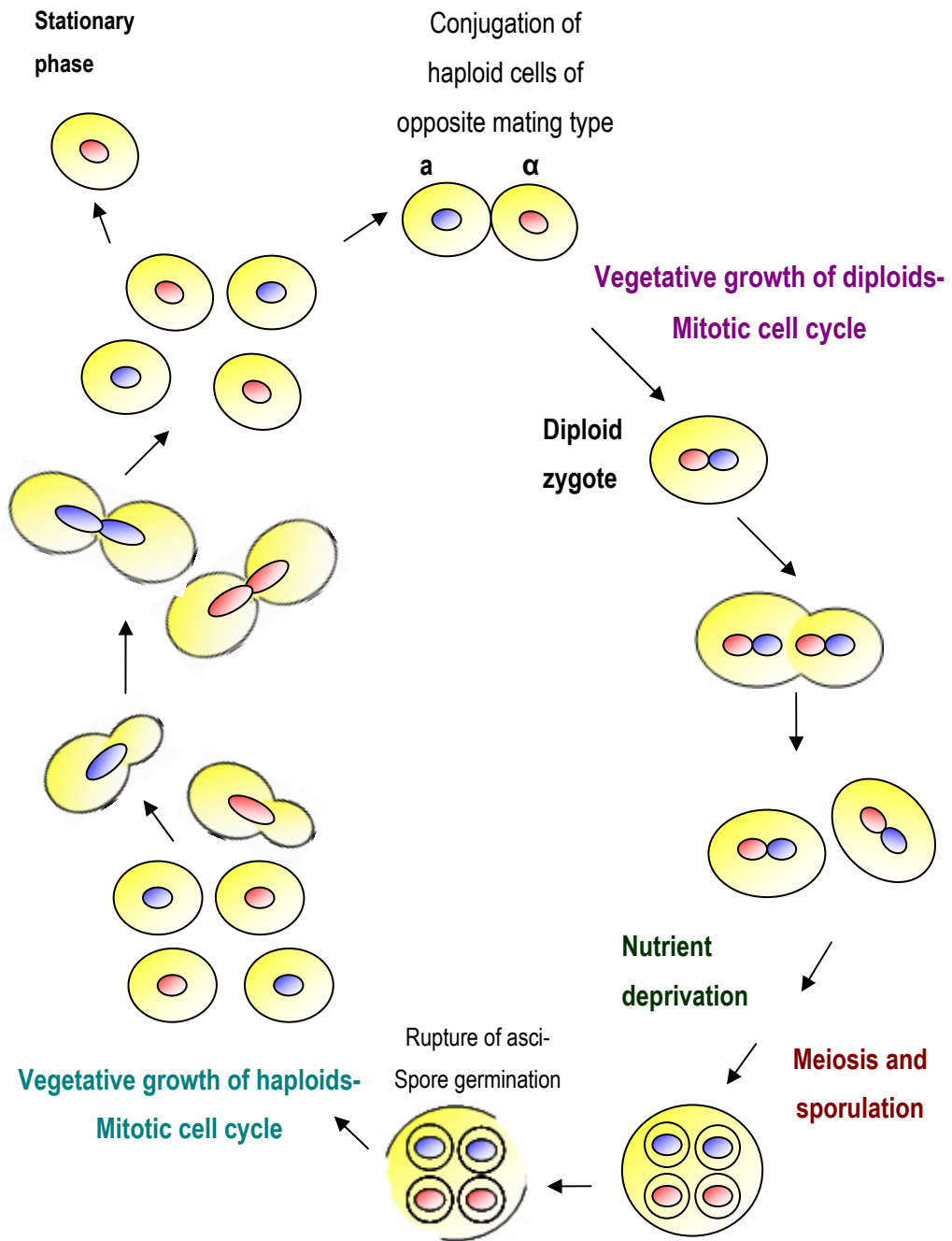


Figure 1.2: The life cycle of *Saccharomyces cerevisiae*. Budding yeast cells grow stably both as haploids and diploids. Upon nutrient deprivation, cells exit from the mitotic cell division cycle and enter into stationary phase. Nonetheless, if cells of opposite mating type (a and α) are present they conjugate to form a diploid zygote. In the presence of nutrients, diploid zygotes grow vegetatively by mitotic cycle division but when starved undergo meiosis and sporulation forming four haploid cells, which re-enter the mitotic cell cycle once nutrients are once more available.

1.2.2 Yeasts as models for the study of the eukaryotic cell cycle: a historical overview

A breakthrough in cell cycle research came from a combination of biochemical studies in *Xenopus* and marine invertebrates and genetic studies in the budding and fission yeasts. In the latter case, genetic dissection of the cell cycle machinery was greatly aided by the isolation of *S. pombe* and *S. cerevisiae* conditional lethal, temperature sensitive, mutants that are defective in normal cell cycle progression. Two distinct classes of cell cycle mutants were isolated; the cell division cycle (*cdc*) mutants, identified initially in budding and then in fission yeast, and the *wee* mutants in fission yeast (Hartwell, 1971; Nurse, 1975; Nurse *et al.*, 1976; Nurse and Thuriaux, 1980).

The temperature sensitive *cdc* mutants blocked mitotic cell division at a specific cell cycle stage, irrespective of their stage in the cell cycle at the time of the shift from the permissive to the restrictive temperature. *S. pombe* and *S. cerevisiae* *cdc* mutants were easily recognised morphologically because they blocked cell cycle progression but, critically, not cellular growth. Thus, once arrested, they continued to grow without dividing, resulting in cells of either extreme length or with uniform buds, respectively. The behaviour of *cdc* mutants suggested that they define mutant versions of genes that are necessary for progression through specific cell cycle events, such as DNA replication and mitosis (Hartwell, 1974; Nurse, 1975; Nurse *et al.*, 1976; Hartwell, 1978; Pringle, 1978).

Unlike fission yeast *cdc* mutants, *wee* mutants are small, because once shifted to the restrictive temperature they go through the cell cycle faster than wild-type cells, such that at the time of division they are half their size at the permissive temperature. Thus, the behaviour of *wee* mutants suggested that they define genes whose encoded products are rate limiting for the initiation of mitosis and depending on the nature of the mutations, whether loss- or gain- of function, they either inhibit or induce progress through the cell cycle (Fantes and Nurse, 1977; Fantes and Nurse, 1978; Nurse and Thuriaux, 1980).

Complementation analysis of the *cdc* and *wee* mutants has allowed the isolation of many of the genes necessary for cell cycle progression, including among others *cdc2⁺*, *cdc25⁺*, *wee1⁺* and *cdc13⁺* in fission yeast, and *CDC28* in budding yeast (Beach *et al.*, 1982; Russell and Nurse, 1986a; Russell and Nurse, 1987; Hagan *et al.*, 1988). Early genetic work in *S. cerevisiae* with various temperature sensitive *cdc28* mutants that arrest at G1 at the restrictive temperature has shown that passage through START and entry into S phase requires the function of the *CDC28* gene and is coupled to cell growth, such that only cells with a critical cell mass can initiate DNA synthesis and go through mitotic cell division. Another important observation from these studies was the dependency of late cell cycle events on the completion of earlier events of the cell division cycle (Hartwell *et al.*,

1974). Subsequently, a novel *cdc28* mutant was isolated that arrested at a time after START, suggesting that the function of this gene is required twice during the cell cycle (Piggott *et al.*, 1982).

In *S. pombe*, elaborate genetic analysis of the *cdc2* and *wee1* recessive mutants and the *wee2* dominant mutant, shown to map to the *cdc2* locus, revealed that *cdc2*⁺, like *CDC28* in budding yeast, is required for the onset of both S phase and mitosis, while *wee1*⁺ regulates the timing of mitosis and acts as an inhibitor of mitotic onset by acting upstream and negatively regulating *cdc2*⁺ (Nurse and Thuriaux, 1980; Nurse and Bissett, 1981). In parallel, analysis of these mutants, as well as others, unravelled that, similar to the situation in budding yeast, progression past START and entry into mitosis rely both on the attainment of a critical cell size (Fantes and Nurse, 1977; Fantes and Nurse, 1978). Later genetic studies with *cdc25* mutants, including suppression analysis with *wee1* mutants, showed that *cdc25*⁺ acts as a dosage-dependent inducer of mitosis that positively regulates *cdc2*⁺ and counteracts the inhibitory function of *wee1*⁺ (Fantes, 1979; Russell and Nurse, 1986a; Russell and Nurse, 1986b; Russell and Nurse, 1987). Finally, genetic work with *cdc13* mutants showed that *cdc13*⁺ is required for mitosis, while sophisticated suppression analyses, combined with overexpression studies, suggested that the encoded proteins of *cdc13*⁺ and *cdc2*⁺ physically interact with each other as part of a control mechanism that initiates mitosis (Nurse *et al.*, 1976; Nasmyth and Nurse 1981; Fantes, 1982; Booher and Beach, 1987).

The similarities in behaviour of *cdc2*⁺ and *CDC28* in the regulation of the mitotic cell cycle suggested that these genes perform similar functions in the two yeasts, portraying an evolutionary conserved component of the eukaryotic cell cycle machinery. Confirming this hypothesis, complementation studies revealed that *CDC28* could rescue the lethality of the *cdc2-33* mutant at the restrictive temperature and, *vice versa*, a DNA fragment corresponding to *cdc2*⁺ cDNA could rescue the defective phenotype of the *CDC28-4* mutant, showing that *cdc2*⁺ and *CDC28* are functional homologues (Beach *et al.*, 1982; Booher and Beach, 1986). Moreover, a clone from a human cDNA library, corresponding to the human *cdc2*⁺ homologue, was isolated that could also complement the *cdc2-33* mutant (Lee and Nurse, 1987). Peptide sequence analysis revealed that the polypeptides encoded by *cdc2*⁺ and *CDC28* are 63% identical, while the proteins encoded by *CDC28* and the human *cdc2*⁺ homologue share 58% sequence identity (Lörincz and Reed, 1984). Subsequently, *cdc2*⁺ homologues have been discovered in a variety of organisms and shown to encode further cyclin-dependent kinases (Nigg, 1995; Morgan, 1997). Apart from *cdc2*⁺, *cdc13*⁺, *wee1*⁺ and *cdc25*⁺ have also been shown to encode conserved cell cycle components, a mitotic cyclin, a kinase and a phosphatase, respectively, which modulate in concert the activity of *cdc2*⁺ (Section 1.3). These findings illustrate the value of *S. pombe* and *S. cerevisiae* as experimental systems for the study of the mitotic cell cycle.

1.3 The eukaryotic cell division cycle

Cell cycle studies with *cdc* mutants in both budding and fission yeasts have greatly contributed to the establishment of a basic scheme for the organisation and control of the mitotic cell cycle in eukaryotes. At the heart of cell cycle architecture lies the temporal separation of DNA synthesis from mitosis by the G1 and G2 gap phases (Nasmyth, 2001). Interphase defines the time that intervenes between the end of one mitosis and the onset of another, and encompasses the G1, S and G2 phases. During interphase the cell doubles both its DNA and mass content in preparation for the ensuing mitosis. DNA replication is restricted to S phase, while cellular growth, corresponding to RNA and protein synthesis, is more or less a continuous process during interphase.

The G1 phase encompasses the START/ Restriction point, during which the cell monitors whether the conditions are satisfactory to enter S phase and initiate a round of cell division. A number of different parameters, including internal stimuli, for instance growth rate and cell size, as well as external cues such as nutrients and mitogens, determine the cell's decision to commit to a cell division cycle. Upon favourable conditions for cell division, the cell progresses past the START/Restriction point and enters S phase. However, if the cell status is limiting for advancement into S phase and a consequent mitotic cell cycle, the cell withdraws from the cell cycle and arrests indefinitely at the so-called G0 phase in mammalian cells or at the stationary phase in yeasts, until conditions are once more favourable to re-enter the mitotic cell cycle (Lewin, 2000).

Once a cell progresses past the START/Restriction point it irreversibly commits to a round of mitotic division. Upon entry into S phase, DNA replication commences simultaneously at multiple replication origins. After initiation of DNA synthesis, the genome becomes refractory to re-replication until the G1 phase of the next cell cycle, when it is primed once again for replication. This process ensures that the genome replicates only once in the mitotic cell cycle. Following completion of S phase, the cell enters G2 phase, where it continues to increase its mass content until sufficient for mitosis (Moser and Russell, 2000). A crucial control point is exerted near the end of G2 where the cell monitors its readiness for division in terms of cell size and faithful replication of the genome. In the latter case, a number of checkpoints are superimposed on the pivotal components of the mitotic cell cycle machinery, the Cdks, to restrain the onset of mitosis in the presence of unreplicated or damaged DNA, preserving genome integrity. These checkpoints provide a means of linking initiation of cell cycle events to the achievement of previous ones (Russell, 1998).

After completion of DNA replication and attainment of the critical cell mass, the cell embarks on mitosis. During mitosis the cell reorganises its cytoskeleton dramatically to form the mitotic spindle, a microtubule-based structure designed to capture, align and separate the replicated

chromosomes, each composed of two sister chromatids held along their lengths by multiple protein cross-links, the cohesins. Based on these events, leading to the partition of the duplicated genome into the daughter nuclei, mitosis is sub-divided into five stages, prophase, prometaphase, metaphase, anaphase and telophase, respectively (Figure 1.3) (Hagstrom and Meyer, 2003).

During prophase, the chromosomes begin to condense into highly compact structures, a process mediated by multi-protein complexes called condensins. In higher eukaryotes, unlike the budding and fission yeasts, the nuclear envelope breaks down during prometaphase (MacNeill and Nurse, 1997). Around this time a complex protein structure assembles at the centromere of each sister chromatid and mediates its tethering to the mitotic spindle. The two kinetochores of each chromosome attach to microtubules emanating from opposite poles of the spindle.

By metaphase chromosomes are condensed maximally and are strong enough to endure the tension, generated as a result of the forces that pull the sister chromatids toward opposite spindle poles counteracting the force from chromosome cohesion. This tension facilitates alignment of the chromosomes at the central region of the spindle, along the metaphase plate. A checkpoint operates within the cell that monitors chromosome alignment during metaphase and delays advancement into anaphase until all the chromosomes are attached to the spindle and under tension.

Once all the chromosomes are properly aligned at the metaphase plate, anaphase commences. During anaphase, activation of the anaphase promoting complex/cyclosome (APC/C) triggers proteolytic degradation of securin, a protein that binds and sequesters separin. Separin is a protease that specifically cleaves cohesins. As a result, sister chromatids separate from each other and, as the spindle elongates, they move towards the opposite poles of the cell. During telophase, the newly separated chromosomes decondense, the mitotic spindle dissolves and the interphase cytoskeleton is re-established. In higher eukaryotes the nuclear envelope also reforms during this time. After mitosis, cytokinesis follows to physically separate the cytoplasm, giving rise to two daughter cells (Figure 1.3) (Hagstrom and Meyer, 2003).

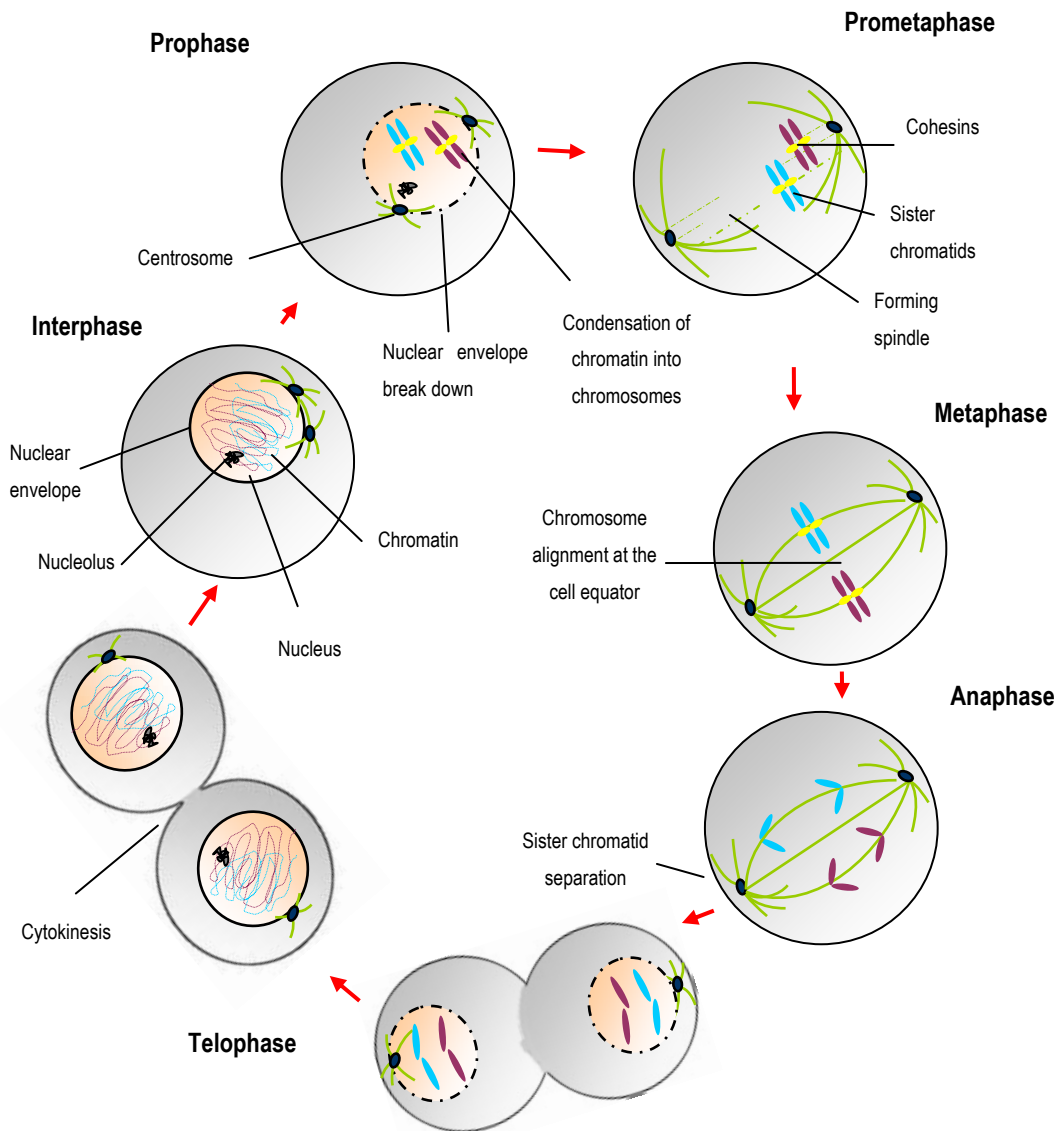


Figure 1.3: Schematic of mitosis in animal cells. Following duplication of the cell's DNA content during interphase, mitosis ensues to separate the two copies of the genetic material into the two progeny. During prophase, chromatin condenses into chromosomes, each comprising two sister chromatids held together by cohesins. In prometaphase, the nuclear envelope breaks down. Meanwhile, the mitotic spindle begins to form and the sister chromatids are tethered to spindle microtubules emanating from opposite spindle poles. By metaphase chromosomes are condensed maximally and align along the equatorial plane. Then, anaphase takes place, where cohesins are cleaved to liberate the two sister chromatids from each other and allow them to move towards the opposite poles of the cell. In telophase, the chromosomes decondense, the mitotic spindle dissolves and the nuclear envelope reforms. Then, cytokinesis follows to physically separate the two daughter cells.

Variations in the fundamental format of the mitotic cell cycle are encountered in eukaryotes to accommodate their specific needs and include differences in the time a cell spends in either G1 or G2 phase (Forsburg and Nurse, 1991). Higher eukaryotes regulate their cell cycle primarily at the Restriction point and spend a major part of the cell division cycle in G1 phase (Lewin, 2000). Similarly, *S. cerevisiae* also controls its cell division cycle mainly at START (Hartwell, 1974; Forsburg and Nurse, 1991; Lew *et al.*, 1997). A round of mitotic cell division in budding yeast generates a daughter cell that is smaller in size than the mother cell (Figure 1.4A). A lengthy G1 phase provides the daughter cell with sufficient time to grow and acquire the critical size before START and advancement on another cell cycle (Rupeš, 2002). Unlike other eukaryotes, however, *S. cerevisiae* has an unusual cell cycle with less-defined S, G2 and M phases, mainly due to its budding mode of division (Figure 1.4A). In budding yeast, bud formation initiates as soon as the cell progresses past START. Then, DNA replication, duplication of the spindle pole body, the equivalent microtubule organising centre (MTOC) structure of the animal centrosome, as well as the ensuing rearrangement of the interphase microtubule array into a mitotic spindle, follow closely at an early time during the cell cycle (Forsburg and Nurse, 1991; Lew *et al.*, 1997).

In *S. pombe*, similar to higher eukaryotes, the cell cycle is well-defined with discrete G1, S, G2 and M phases. However, unlike other eukaryotes, fission yeast spends most of its cell cycle time, ~ 70%, in G2 and regulates its cell cycle predominantly at the G2-M control point. Attainment of the crucial size, as well as successful completion of S phase and repair of any DNA damage, is a prerequisite for progression past the G2-M transition and entry into mitosis, which takes place within an intact nuclear envelope. Typical of eukaryotes, during mitosis in *S. pombe* the chromosomes condense and the interphase cytoplasmic array of microtubules disassembles to form the mitotic spindle (Forsburg and Nurse, 1991; MacNeill and Nurse, 1997; McCully and Robinow, 1971; Robinow, 1977; Hagan and Hyams, 1988).

Under conditions of normal growth and ample nutrients, fission yeast cells spend only a very short time in the G1 and S phases, corresponding in each case to no more than 10% of the cell cycle. G1 phase commences immediately after separation of the daughter nuclei during mitosis and by the time of cytokinesis cells have already completed S phase and entered G2 phase, attributed to the attainment of the critical size much earlier than START, at a time during cell division (Figure 1.4B). As a result, fission yeast exists for the bulk of the cell cycle in the diploid state. This situation is advantageous for an organism that lives primarily as a haploid, since the possession of two copies of the genome offers fission yeast protection against DNA damage. The control point operating at START, although cryptic in rapidly growing fission yeast cells, becomes important under limiting growth conditions, such as nutrient deprivation, or in certain mutants, for example *wee1* cells. In

these cases, the G1 phase extends to allow small cells to increase their mass and acquire the critical cell size (Nurse, 1975; Nasmyth *et al.*, 1979; Nurse, 1990; Forsburg and Nurse, 1991; MacNeill and Nurse, 1997; Humphrey, 2000).

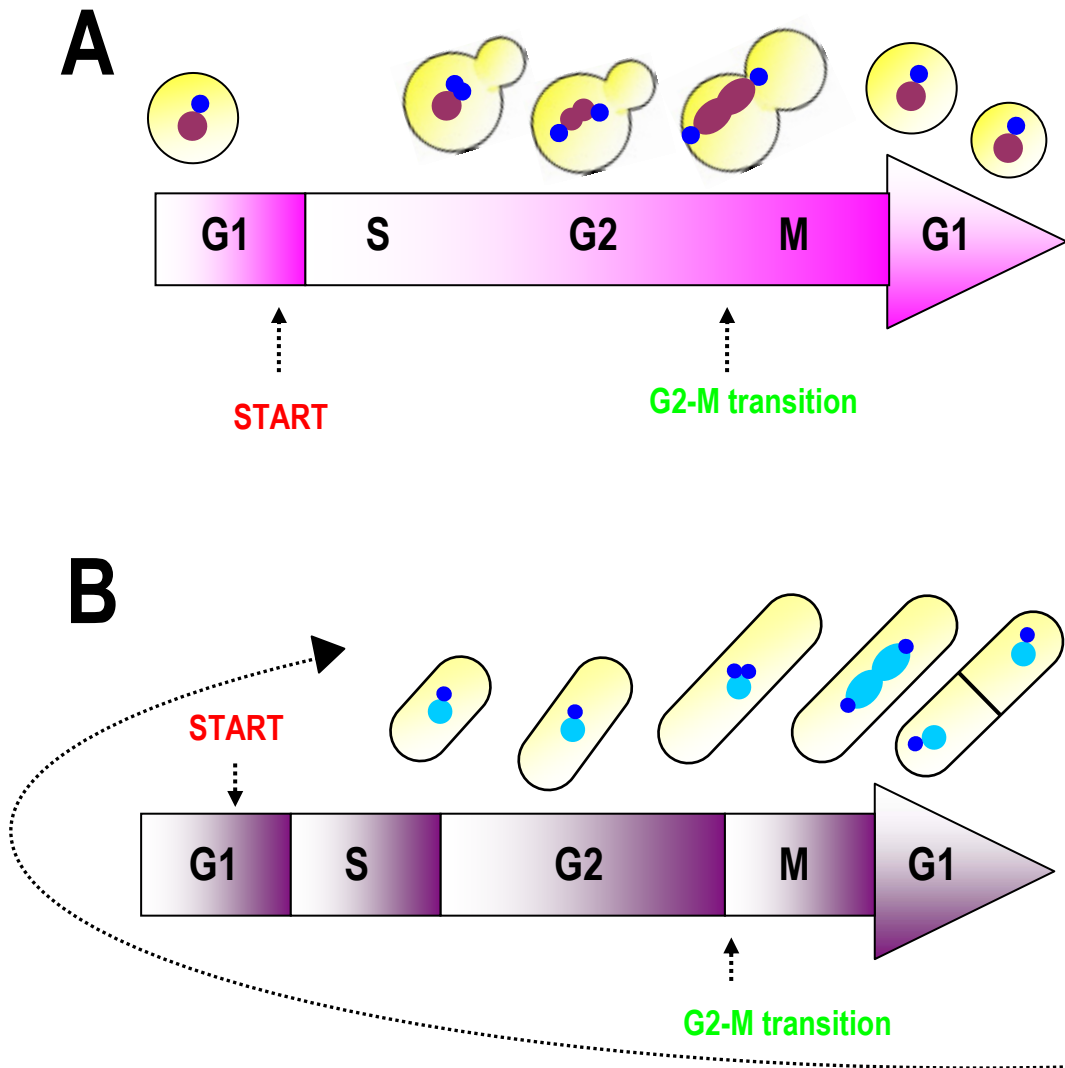


Figure 1.4: *S. cerevisiae* and *S. pombe* mitotic cell cycle. (A) Haploid *S. cerevisiae* cells at various stages of the mitotic cell cycle. SPB duplication and assembly of the mitotic spindle occur relatively early in the budding yeast cell cycle, resulting in less-defined individual S, G2 and M phases. Plum and blue circles represent the nucleus and spindle pole body, respectively. (B) Haploid *S. pombe* cells at various stages of the mitotic cell cycle. The fission yeast cell cycle is characterised by a lengthy G2 phase, occupying ~ 70% of the cell cycle, while G1 follows immediately after separation of the daughter nuclei, such that by the end of cytokinesis cells have already completed S phase and entered G2 phase. Arrows indicate the START and G2-M control cell cycle points. Aqua and blue circles represent the nucleus and spindle pole body, respectively.

1.3.1 Mechanisms of eukaryotic cell cycle control

The eukaryotic cell cycle is regulated by a highly complex but also fascinating network that at specific cell cycle times activates the Cdks, a special class of serine/threonine kinases, to trigger entry into S phase and mitosis (Nasmyth, 1996). The Cdks function as cell cycle regulators by phosphorylating their substrates in a cell cycle-dependent manner to elicit a variety of events including, among others, the initiation of DNA replication at replication origins, and at the same time the prevention of re-replication until the next S phase, as well as chromosome condensation, nuclear envelope breakdown and cytoskeletal reorganization during mitosis (Moser and Russell, 2000; Blow and Hodgson, 2002; Feng and Kipreos, 2003; Nielsen and Løbner-Olesen, 2008). The activity of Cdks varies greatly during the cell cycle, indicative of a high level of regulation. Cdk activity is controlled by various mechanisms, including reversible modification of the phosphorylation state of critical residues and cell cycle specific binding of regulatory subunits, either stimulatory, such as the cyclins, or inhibitory, the so-called *cyclin dependent kinase inhibitors* (CKIs) (Morgan, 1995b; Morgan, 1997; Nigg, 2001; Dorée and Hunt, 2002).

In *S. pombe* and *S. cerevisiae*, a unique Cdk, Cdc2p and Cdc28p respectively, drives entry into either S phase or mitosis. Unlike these yeasts, mammalian cells possess several stage specific Cdks, primarily Cdk1p, Cdk2p, Cdk4p and Cdk6p (Nigg, 1995; Morgan, 1997). Cdk1p is the mitosis specific Cdk, while Cdk2p is necessary for initiation and progress through S phase. Cdk4p and Cdk6p act early in G1 phase upon mitogen stimulation to promote a round of cell division by phosphorylating and inactivating Rb family members that repress expression of genes required for S-phase or by binding and sequestering CKIs (Sherr, 1996; Sherr and Roberts, 2004).

The Cdks are inactive as monomers. Activation of these kinases requires association with a cyclin to form a heterodimeric complex. Phosphorylation of Cdks reflects another crucial parameter controlling Cdk activation and involves changes in the phosphorylation status of either two (Tyr-15/Thr-167 in the fission yeast) or three (Tyr-15/Thr-14/Thr-161 in humans) amino acid residues, depending on the organism. Phosphorylation of Tyr-15 and Thr-14 near the N-terminus of the Cdk subunit is inhibitory and maintains the kinase in an inactive state, following its association with a cyclin subunit. Contrary to Tyr-15 and Thr-14, phosphorylation of Thr-161/Thr-167 is stimulatory and is necessary for complete activation of the kinase (Morgan, 1997).

Typically, eukaryotic cells contain various phase specific cyclins of distinct types. In contrast to the Cdks, the cyclins oscillate in protein levels during the cell cycle, hence the name. The periodic pattern of cyclin protein levels is predominantly the result of cell cycle dependent proteolysis, as well as of cell cycle specific expression. Cyclins are rate-limiting for cell cycle progression and together

with other regulatory mechanisms provide an elaborate means of regulating the timing of activation and ensuing inactivation of the associated Cdk (Morgan, 1997; Koepp *et al.*, 1999; Yamano *et al.*, 2000; Sullivan and Morgan, 2007).

Mammalian cells possess four distinct types of cyclins, A, B, D and E respectively. D- and E-type cyclins, the former associating with either Cdk4p or Cdk6p, and the latter with Cdk2p, are G1 specific cyclins and are required for entry and progression of G1 phase, as well as for initiation of DNA synthesis, in response to mitogenic stimulation. A-type cyclins associate with Cdk2p to promote progression through S and G2 phase, while B-type cyclins bind to Cdk1p and drive mitosis (Sherr, 1996; Sherr and Roberts, 2004).

In budding yeast, three G1 cyclins, Cln1p, Cln2p and Cln3p bind to Cdc28p in reverse order to accommodate progress past START and S phase entry by promoting among others early cell cycle events, such as bud formation, spindle pole body duplication and DNA replication (Nasmyth, 1996; Lew *et al.*, 1997; Rupeš, 2002). The G1 cyclins drive advancement past START and commencement of S phase by operating alongside the SBF and MBF transcription factor complexes, which regulate expression of many genes required for the G1-S phase transition, including among others *CLN1* and *CLN2* themselves (Nasmyth, 1996; Lew *et al.*, 1997; Rupeš, 2002; Bähler, 2005). SBF and MBF are in turn positively regulated by Cln3p associated with Cdc28p in an indirect manner, via inhibition of Whi5p, a negative regulator of G1-S specific transcription (Costanzo *et al.*, 2004; DeBruin *et al.*, 2004; Bähler, 2005). Following passage of START, Clb5p and Clb6p cyclins prompt DNA replication and advancement through S phase, while Clb1p, Clb2p, Clb3p and Clb4p are required for G2 and mitosis, Clb3-4p promoting mitotic spindle formation and Clb1-2p driving bud growth and nuclear division (Huberman, 1996; Nasmyth, 1996).

Following the G1 phase, Cdc28p-Cln1-2p complexes positively regulate the orderly and stable accumulation of the Clb1-6p cyclins and their ensuing association with Cdc28p by phosphorylating and targeting for proteolytic degradation Sic1p, a CKI that specifically inhibits Cdc28p-Clbp complexes. Clb1-2p cyclins bound to Cdc28p negatively regulate SBF and MBF transcription factors to turn off expression of Cln1-2p, terminate G1-S events and eventually progress cells through G2 phase and advance them into mitosis (Amon *et al.*, 1994; Morgan, 1995b; Futcher, 2002; Bähler, 2005). Moreover, Clb2p and Clb5p promote further the stable accumulation of the Clb1-2p mitotic cyclins via a sophisticated feedback loop, which activates transcription of *CLB1* and *CLB2* genes by the Mcm1p-Fkh2p-Ndd1p transcription factor complex, discussed in more detail in Sections 1.4, 1.5 and 3.1 (Koranda *et al.*, 2000; Darieva *et al.*, 2003; Reynolds *et al.*, 2003; Pic-Taylor *et al.*, 2004; Darieva *et al.*, 2006). Activated Cdc28p-Clb2p complexes drive entry and progression through mitosis. At the end of mitosis, the Clb2p mitotic cyclin is targeted for ubiquitin-

dependent proteolytic degradation, enabling the disruption and subsequent inactivation of the Cdc28p-Clb2p complex, a prerequisite for mitotic exit. Around the same time, the CKI inhibitor Sic1p re-accumulates and promotes further inactivation of Cdc28p-Clb2p (Futcher, 2002).

Fission yeast possesses four cyclins, namely Cdc13p, Cig1p, Cig2p and Puc1p, which associate with Cdc2p, a 34 kDa Cdk that is present at constant levels throughout the cell cycle. Cdc13p, Cig1p, Cig2p are B-type cyclins, while Puc1p is most similar to *S. cerevisiae* Cln1-3p cyclins (Forsburg and Nurse, 1991; Moser and Russell, 2000). All but Cdc13p are dispensable for the survival of the cell. Cdc13p associates with Cdc2p to drive mitosis and at the same time inhibit re-initiation of S phase (Hayles *et al.*, 1994). Consistent with its role as a mitotic cyclin, Cdc13p protein levels fluctuate in abundance during the cell cycle, increasing in mid-G2, peaking during mitosis and gradually decreasing as cells exit mitosis and enter G1 phase (Creanor and Mitchison, 1996). Similar to other B-type cyclins, Cdc13p contains near its N-terminus a nine amino acid sequence (RHALDDVSN), the so-called destruction box, which mediates ubiquitin-dependent proteolysis of Cdc13p near the end of mitosis to inactivate Cdc2p and allow the cell to exit mitosis (Yamano *et al.*, 1996).

Cig1p and Cig2p resemble *S. cerevisiae* cyclins Clb3p and Clb4p, and act early in the cell cycle, forming complexes with Cdc2p (Fisher and Nurse, 1995; Fisher and Nurse, 1996). In the absence of both Cig1p and Cig2p, the mitotic cyclin Cdc13p compensates for their roles in the G1-S transition. Cig2p is the most prominent S phase cyclin and promotes progression past START and entry into S phase, consistent with its levels and the activity of the associated Cdc2p kinase peaking during G1-S phase (Labib *et al.*, 1995; Martin-Castellanos *et al.*, 1996). A complex regulatory network underlies the periodic pattern of Cig2p protein levels. Cig2p contains a destruction box that mediates its ubiquitin-dependent proteolysis late in the cell cycle, during anaphase. Proteolytic degradation of Cig2p takes place also during the G2 phase via a distinct proteolytic pathway (Yamano *et al.*, 2000). Moreover, *cig2⁺* mRNA levels oscillate during the cell cycle, peaking during the G1-S transition, subject to transcriptional control by the *MluI* cell-cycle-box binding factor/DNA synthesis control 1 (MBF/ DSC1) complex, which regulates the expression of many genes required for S phase (Lowndes *et al.*, 1992; Kelly *et al.*, 1993; McInerny *et al.*, 1995; Whitehall *et al.*, 1999; Ayte *et al.*, 2001; White *et al.*, 2001). Apart from stage-specific proteolysis and expression, Cig2p is subject to regulation by Rum1p, an *S. pombe* CKI, which inhibits the activities of Cdc2p-Cdc13p and Cdc2p-Cig2p complexes during G1 phase (Correa-Bordes and Nurse, 1997; Benito *et al.*, 1998).

Puc1p is another *S. pombe* cyclin, acting during the G1 phase. Normally, the role of Puc1p is cryptic but it becomes apparent in the absence of both Cig1p and Cig2p, since only *cig1Δcig2Δpuc1Δ* but not *cig1Δcig2Δ* cells display an extended G1 phase and an increase in cell

size, combined with an accumulation of Rum1p. Moreover, loss of *rum1*⁺ drastically shortens the time that *cig1Δcig2Δpuc1Δ* cells spend in G1, while Cdc2p associated with either Puc1p or Cig1p is refractory to Rum1p and at the same time phosphorylates this CKI late in G1 phase (Benito *et al.*, 1998; Martin-Castellanos *et al.*, 2000). Therefore, Puc1p and Cig1p act during G1 phase to couple S phase entry to the attainment of the critical cell size by promoting the G1-S transition indirectly through inhibition of Rum1p (Moser and Russell, 2000; Gould, 2003).

1.3.2 Cell cycle control in fission yeast

1.3.2.1 G1-S transition

In fission yeast advancement through START and S phase entry underlies the co-ordinated activities of the MBF/DSC1 transcription factor complex and Cdc2p. As the cells exit mitosis and enter G1 phase, Cdc2p mitotic activity is eliminated owing to Cdc13p proteolysis and CKI inhibition. Cdc13p proteolysis is carried out by the 26S proteasome, a large complex that degrades ubiquitinated proteins. Three components, E1, E2 and E3, mediate the addition of ubiquitin to a target protein. Ubiquitin is a protein that once added to its substrate, targets it for proteolytic degradation (Hochstrasser, 1996; Hershko, 1997). E1 is a ubiquitin-activating enzyme, E2 is a ubiquitin-conjugating enzyme and E3 is a ubiquitin ligase. E3 acts as a specificity factor that determines both the type of substrate and the timing of degradation. The APC/C, a multi-subunit complex, is the E3 that targets for degradation, among others, B-type cyclins Cdc13p, Cig1p and Cig2p (Glotzer *et al.*, 1991; King *et al.*, 1996; Sudakin *et al.*, 1995; Blanco *et al.*, 2000; Yamano *et al.*, 2000). The APC/C is activated during mitosis at the metaphase-anaphase transition and persists until the end of G1 (Blanco *et al.*, 2000). Distinct proteins, members of the WD40 repeat family, associate with the APC/C at discrete cell cycle times and confer to it substrate specificity and stage-specific activation. *S. pombe* possesses various APC/C activators, including Slp1p and Ste9p. Slp1p fluctuates in abundance during the cell cycle, peaking around mid-mitosis, as a result of cell cycle specific transcription and proteolytic degradation (Yamada *et al.*, 2000; Buck *et al.*, 2004). Consistent with its periodicity, Slp1p interacts with the APC/C during mitosis to target for degradation Cut2p, the fission yeast securin, to promote sister chromatid separation during anaphase (Yanagida, 2000). Slp1p also mediates degradation of Cdc13p to bring about mitotic exit. Similar to Slp1p, Ste9p targets Cdc13p for APC/C-dependent proteolysis. Unlike Slp1p, however, Ste9p acts predominantly during the G1 phase to promote degradation of Cdc13p, remaining from the previous mitosis, as well as of Cig1p.

During S and G2 phase, Ste9p is phosphorylated in a Cdc2p-dependent manner, allowing its dissociation from the APC/C and ensuing degradation (Blanco *et al.*, 2000).

Apart from the activities of the APC/C, Rum1p also restrains mitotic Cdc2p activity during G1 phase by binding directly and inhibiting Cdc13p. Moreover, Rum1p binds to Cdc13p and targets it for ubiquitin-dependent proteolysis, thus preventing cells from undergoing mitosis before the initiation of S phase. Rum1p, the single CKI in *S. pombe*, oscillates in abundance during the cell cycle, starting to accumulate at anaphase, peaking in G1 phase and disappearing upon S phase entry, owing to regulation at the transcriptional and post-translational level (Correa-Bordes *et al.*, 1997; Benito *et al.*, 1998).

Many genes are periodically expressed during the G1-S interval, following transcriptional activation by the MBF/ DSC1 complex. These genes encode proteins required for S phase onset and progression, including among others the S phase cyclin Cig2p, the large subunit of ribonucleotide reductase Cdc22p, as well as Cdc18p and Cdt1p, both required for initiation of DNA replication. The MBF/DSC1 complex contains the encoded products of the *cdc10⁺*, *res1⁺*, *res2⁺* and *rep2⁺* genes, and binds to the *Mlul* cell cycle box (MCB) motif (5'-ACGCGT-3'), found in the genes' promoters (Lowndes *et al.*, 1992; Miyamoto *et al.*, 1994; Sugiyama *et al.*, 1994; Nakashima *et al.*, 1995; Whitehall *et al.*, 1999; Ayte *et al.*, 2001; White *et al.*, 2001; McInerney, 2004).

Although Cig2p starts to accumulate during G1 phase, the activity of Cdc2p-Cig2p complexes is inhibited during this time by Rum1p. The temporary inhibition of Cdc2p-Cig2p by Rum1p allows the cells to remain in the pre-START stage of the cell cycle until they acquire the critical size (Correa-Bordes and Nurse, 1995; Martin-Castellanos *et al.*, 1996; Benito *et al.*, 1998). In parallel, under conditions that promote mating, Rum1p inhibition of Cdc2p activity enables cells to arrest in G1 prior to START and conjugate (Moreno *et al.*, 1994).

As cells progress through G1 phase and reach the onset of S phase, Cig1p and Puc1p cyclins accumulate and associate with Cdc2p. As mentioned earlier, Cdc2p-Cig1p and Cdc2p-Puc1p complexes are resistant to Rum1p and can phosphorylate and target it for degradation. Ubiquitin dependent proteolysis of Rum1p is mediated by SCF, a distinct E3 ubiquitin ligase, following activation by Pop1p and Pop2p (Gould, 2003). Degradation of Rum1p is followed by a rise in Cdc2p-Cig2p activity, leading to the onset of S phase (Martin-Castellanos *et al.*, 1996; Monderset *et al.*, 1996; Benito *et al.*, 1998).

1.3.2.2 Onset of S phase

S phase is characterised by DNA synthesis to replicate the genome in preparation for the ensuing mitosis. Various regulatory mechanisms operate within the eukaryotic cell to ensure that the genome is replicated fully, accurately and only once per cell cycle, during S phase, maintaining genetic stability. DNA replication commences at multiple origins of replication, dispersed within intergenic chromosomal regions, forming replication forks that move along the DNA in both directions until they come across another replication fork, proceeding in the opposite direction.

The initiation of DNA replication, also called 'firing' at replication origins, involves cell cycle dependent and highly co-ordinated assembly and modification of multi-protein complexes at these genomic sites. These ordered series of events, collectively known as 'licensing', culminate in the formation of a pre-replicative complex (pre-RC) that mediates DNA replication during S phase and at the same time is unable to re-initiate replication ('re-fire') until the onset of the next S phase. This licensing system enables the cell to remember whether or not it has replicated its genome within each round of cell division (Wuarin and Nurse, 1996; Diffley, 2001; Lei and Tye, 2001; Nguyen *et al.*, 2001; Blow and Hodgson, 2002).

Many hundreds of replication origins exist in the genome of eukaryotes, such as *S. pombe* and humans. Surprisingly, however, only a subset of replication origins fires within each S phase. These replication origins tend not to initiate DNA synthesis simultaneously, but rather some of the origins fire earlier and others later during S phase. Indeed, passive replication is inevitable in eukaryotic DNA replication, since replication forks from early firing origins move along the DNA and replicate DNA regions that contain origins that have not yet fired, preventing them from firing until the onset of the next S phase. Therefore, in most eukaryotes, the mechanism that underlies selection of origins to initiate DNA replication and their time of firing appears to be more stochastic than deterministic in nature (Legouras *et al.*, 2006; Nielsen and Løbner-Olesen, 2008).

1.3.2.2.1 Replication origins

Depending on the organism, replication origins vary in their organisation, size and specificity. *S. cerevisiae* possesses relatively small replication origins, about 100 bp in length, consisting of a conserved 11 bp AT rich sequence, referred to as an autonomous replicating sequences (ARS) because it confers autonomous replication in plasmids (Marahrens and Stillman, 1992; Rao *et al.*, 1994; Forsburg, 1999). Unlike budding yeast, *S. pombe* contains large replication origins (between 500-1500 bp) that are localised in intergenic regions and close to promoter sequences, are rich in AT

content and comprise several short, highly asymmetric, AT stretches, which lack conservation and appear to act synergistically in terms of origin activity (Clyne and Kelly, 1995; Dubey *et al.*, 1996; Kim and Huberman, 1998; Okuno *et al.*, 1999). Similar to fission yeast, metazoans are also characterised by large replication origins, enriched for asymmetric AT stretches that lack definition of a consensus and are often located upstream of genes (Forsburg, 1999; Masukata *et al.*, 2003; Legouras *et al.*, 2006).

1.3.2.2.2 Pre-RC assembly and regulation of licensing at origins

Despite the differences in organisation of eukaryotic replication origins, the components of the regulatory network that underlies the formation of an active pre-RC, able to initiate DNA replication during S phase, appear to be conserved among eukaryotes. The first step in pre-RC assembly involves association of the origin recognition complex (ORC) with replication origins. Similar to other eukaryotes, the ORC in fission yeast is a six-subunit complex, comprising Orc1p-Orc6p proteins (Muzi-Falconi and Kelly, 1995; Moon *et al.*, 1999; Chuang and Kelly, 1999; Kelly and Brown, 2000). In *S. pombe*, the ORC binds to replication origins via the Orc4p subunit and remains bound to them throughout the cell cycle. Orc4p, like other Orcps, possesses an AAA⁺ ATP-binding domain, while its N-terminus consists of several AT-hook motifs that bind to the AT-rich tracts of replication origins (Chuang and Kelly, 1999; Lygerou and Nurse, 1999; Kong and DePamphilis, 2002).

The ORC serves as a scaffold for the highly co-ordinated recruitment from late mitosis and through G1 phase of another hexameric complex, the mini-chromosome maintenance complex (MCM), containing the Mcm2p-Mcm7p proteins, and believed to act as the replicative helicase (Miyake *et al.*, 1993; Forsburg and Nurse, 1994; Lee and Hurwitz, 2000; Labib and Diffley, 2001; Diffley and Labib, 2002). The genes encoding MCM components were initially identified in budding yeast in a screen for mutants unable to maintain plasmids (Tye and Sawyer, 2000). All MCM proteins share conserved ATPase motifs although, as shown in *S. pombe*, humans and mouse, it is the dimeric form of the Mcm4p-Mcm6p-Mcm7p complex that possesses substantial ATPase, DNA binding and DNA helicase activities (Ishimi, 1997; Lee and Hurwitz, 2000). Interactions of MCM components with other crucial replication factors and/or their modification are the trigger for increased helicase activity of the MCM complex. Overall, the MCM complex is thought to be the replicative helicase that binds and unwinds the DNA at origins during replication initiation and perhaps ahead of replication forks during the elongation phase of replication (Labib *et al.*, 2000; Lee and Hurwitz, 2000; Labib and Diffley, 2001; Nielsen and Løbner-Olesen, 2008).

Loading of MCM onto replication origins is restricted to a time early in the cell cycle and is mediated by two crucial replication initiation factors, Cdc18p (the equivalent of *S. cerevisiae* Cdc6p) and Cdt1p. Cdc18p is recruited to replication origins via direct interaction with the ORC (Chuang *et al.*, 2002). Cdt1p directly associates and co-operates with Cdc18p to load the MCM complex onto replication origins, completing pre-RC assembly (Figure 1.5). Both Cdc18p and Cdt1p are under tight cell cycle control, licensing replication origins for initiation of DNA synthesis only once during the S phase of each cell cycle (Lygerou and Nurse, 2000; Nishitani *et al.*, 2000; Nishitani and Lygerou, 2004).

Similar to MCM proteins and Orc1p, Orc4p and Orc5p, Cdc18p is yet another member of the AAA⁺ family of ATPases, while the exact biochemical role of Cdt1p is unclear (Kelly *et al.*, 1993; Nielsen and Løbner-Olesen, 2008). Both Cdc18p and Cdt1p are present in the cell's nucleus for only a short window of time, accumulating from late M phase and G1 and diminishing after the onset of S phase, as a result of cell cycle specific transcription and proteolysis. Both proteins are periodically transcribed, peaking during the G1-S transition, under the control of the MBF/DSC1 complex (Nishitani and Nurse, 1997; Baum *et al.*, 1998; Nishitani *et al.*, 2000). Moreover, following initiation of DNA replication, both Cdc18p and Cdt1p are targeted for proteolytic degradation. Cdt1p proteolysis in S phase involves a distinct E3 ubiquitin ligase, the Cul4p–Ddb1p complex, and requires Cdt2p, a WD40 repeat containing factor that confers substrate specificity. The Ddb1p/Cdt2p-dependent E3 pathway also mediates Cdt1p proteolysis in response to DNA damage (Ralph *et al.*, 2006). Unlike Cdt1p, Cdc18p is targeted for SCF-mediated proteolysis upon phosphorylation by Cdc2p-cyclin complexes, their activity rising as the cell enters S phase and progresses through the rest of the cell cycle (Jallepalli *et al.*, 1997; Drury *et al.*, 2000; Jallepalli *et al.*, 1998).

Apart from targeting Cdc18p for degradation, Cdc2p activity is believed to further contribute to prevention of DNA re-replication. Indeed, the Cdc2p-Cdc13p complex associates with the ORC during G2 and M phase and this interaction is mutually exclusive to that of the MCM complex (Wuarin *et al.*, 2002). Moreover, Orc2p becomes phosphorylated during S phase, G2 and mitosis, is dephosphorylated during G1 and has been shown to interact with Cdc2p (Leatherwood *et al.*, 1996; Vas *et al.*, 2001). These findings, in conjunction with the fact that *cdc13Δ* cells undergo multiple rounds of S phase without mitosis, show that mitotic Cdc2p activity is necessary both for promoting mitosis and preventing re-replication during the cell cycle (Hayles *et al.*, 1994; Nishitani and Lygerou, 2004).

1.3.2.2.3 Initiation of DNA replication

MCM recruitment heralds the formation of the pre-RC complex during late mitosis-G1 phase, conferring competence to replication origins ('licensing') to fire upon S phase onset. However, the actual firing during S phase awaits the loading of other replication factors at the pre-assembled pre-RCs, maturing into a pre-initiation complex (pre-IC). The pre-IC prompts local unwinding of the DNA, recruitment of DNA polymerases α , δ and ϵ (Pol α , Pol δ and Pol ϵ) and the establishment of replication forks at origins, leading to DNA synthesis (Figure 1.5). In eukaryotes, the forces that activate the pre-RCs and convert them into pre-ICs, driving S phase onset, are the activities of two kinases; Cdk bound to S phase cyclins (S-CDK) and the Dbf4p-dependent kinase (DDK), a member of the Cdc7 kinase family, initially identified in *S. cerevisiae* (Kelly and Brown, 2000; Lei and Tye, 2001; Bell and Dutta, 2002; Diffley and Labib, 2002; Kearsley and Cotterill, 2003; Nishitani and Lygerou, 2004; Legouras *et al.*, 2006; Nielsen and Løbner-Olesen, 2008).

Cdc2p and Hsk1p are the fission yeast S-CDK and DDK respectively, the former in complex with Cig2p and the latter with Dfp1p, accumulating during the G1-S transition, coincidentally with initiation of DNA replication, as a result of transcriptional and post-translational regulation (Brown and Kelly, 1998; Brown and Kelly, 1999). The activities of Cdc2p-Cig2p and Hsk1p-Dfp1p are required in S phase for a key event in the formation of the pre-IC; the loading to the pre-RC of Sna41p (the homologue of *S. cerevisiae* Cdc45p), via its association with the MCM complex. The interaction of Sna41p with MCM components is thought to promote unwinding of the DNA at origins and facilitate the recruitment of Pol α , with which Sna41p has been shown to interact (Uchiyama *et al.*, 2001).

Several other factors associate with replication origins to promote DNA replication initiation, including among others Drc1p (the homologue of *S. cerevisiae* Sld2p), Cdc23p (the homologue of *S. cerevisiae* Mcm10p), Sld3p, and Cut5p (the homologue of *S. cerevisiae* Dpb11p) (Nishitani and Lygerou, 2004; Legouras *et al.*, 2006; Nielsen and Løbner-Olesen, 2008). Sld3p and Cdc23p are required for Sna41p origin binding, while Cut5p and Drc1p associate with each other in a Cdc2p-dependent manner (Nakajima and Masukata, 2002; Gregan *et al.*, 2003). Indeed, mutation of Cdk phosphorylation sites in Drc1p abolishes binding to Cut5p and prevents DNA replication (Noguchi *et al.*, 2002). Notably, Dpb11p, the *S. cerevisiae* homologue of Cut5p, is required to recruit Pol ϵ to replication origins, an important step in the establishment of replication forks (Masumoto *et al.*, 2000). Overall, loading of Sna41p onto a pre-assembled pre-RC is essential for its activation to form, upon association of additional replication initiation factors, a pre-IC complex that facilitates unwinding of the DNA at origins. After origin unwinding, the replication protein A (RPA) binds to the single

stranded DNA and recruitment of Pol α , primase and Pol ϵ follows to initiate DNA synthesis (Takisawa *et al.*, 2000). Once short RNA-DNA primers are synthesised at the replication fork by the concerted actions of Pol α and primase the highly processive elongation phase of DNA replication ensues by recruitment of other components of the replication machinery, including among others Pol δ , Pol ϵ , proliferating cell nuclear antigen (PCNA) and replication factor C (RFC). PCNA acts as a 'sliding clamp' that binds to Pol δ /Pol ϵ and prevents it from falling off the DNA, whereas RFC acts as the 'clamp loader' that loads PCNA on the DNA (Diffley and Labib, 2002).

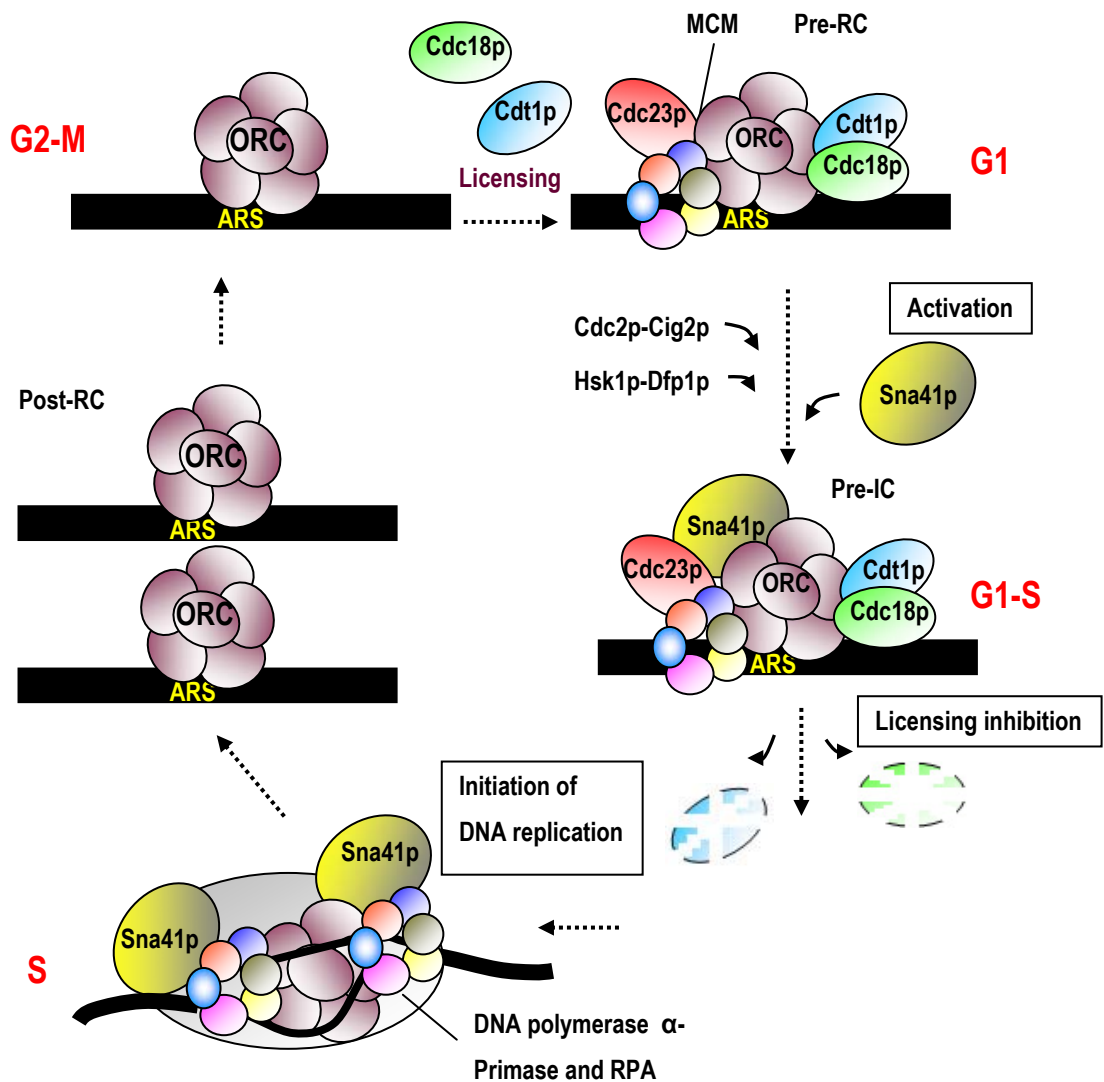


Figure 1.5: Pre-replicative complex (Pre-RC) assembly, licensing and initiation of DNA replication in *S. pombe*. Origin replication complex (ORC) binds to replication origins (ARS) throughout the cell cycle. During late M-early G1 phase, Cdc18p and Cdt1p associate via the ORC with origins to which Cdc23p is also bound. Then, the MCM complex is recruited at origins in a Cdc18p- and Cdt1p- dependent manner to form the pre-RC, thereby licensing the DNA for replication. The associated kinase activities of Cdc2p-Cig2p and Hsk1p-Dfp1p mediate the formation of the Pre-IC complex by promoting recruitment of Sna41p and ensuing unwinding of origin DNA. Meanwhile both Cdc18p and Cdt1p are targeted for proteolytic degradation, rendering replication origins refractory to re-initiation of DNA replication. Following recruitment of various replication factors, including DNA polymerase α , primase and RPA semi-conservative replication takes place.

1.3.2.3 Onset of mitosis (G2-M transition)

After DNA replication is complete, fission yeast cells embark on a lengthy G2 period and then proceed to mitosis to partition the duplicated genome, generating two progeny. A high content of mitotic Cdc2p kinase activity drives progress through mitosis and activation of Cdc2p-Cdc13p complexes at the G2-M transition is the rate-limiting step for mitotic onset (Nurse, 1990; MacNeill and Nurse, 1997; Moser and Russell, 2000). Cdc13p protein levels oscillate during the cell cycle, rising appreciably during mid-G2, peaking ahead of mitosis and declining gradually through G1 phase, as a result of proteolytic degradation (Creanor and Mitchison, 1996; Blanco *et al.*, 2000). Although the early presence of Cdc13p prompts the formation of Cdc2p-Cdc13p complexes from S phase onwards throughout G2, they remain in an inactive state due to inhibitory phosphorylation at Tyr-15 of Cdc2p by two conserved kinases, Wee1p and Mik1p, with homologues in many eukaryotes (Russell and Nurse, 1987; Gould and Nurse, 1989; Igarashi *et al.*, 1991; Lundgren *et al.*, 1991; Morgan, 1995b; Morgan, 1997; Mueller *et al.*, 1995b; MacNeill and Nurse, 1997; Nurse, 2002; Gould, 2003). In multicellular organisms, phosphorylation of the adjacent Thr-14 of the mitotic Cdk is also inhibitory, and in humans is carried out by Myt1p, a membrane-associated kinase, phosphorylating both Tyr-15 and Thr-14 of Cdk1p (Booher *et al.*, 1997).

The Cdc25p phosphatase activates Cdc2p-Cdc13p at the G2-M transition by removing the inhibitory phosphate group at Tyr-15, thus counteracting the activities of Wee1p and Mik1p (Russell and Nurse, 1986a; Millar *et al.*, 1991). Similar to Cdc2p and Wee1p, the role of the Cdc25p phosphatase as the activator of Cdc2p is ubiquitous and Cdc25p homologues exist in many eukaryotes, ranging from budding yeast to humans and capable to dephosphorylate both Tyr-15 and Thr-14 (Russell *et al.*, 1989; Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Kumagai and Dunphy, 1991; Strausfeld *et al.*, 1991; Alphey *et al.*, 1992; Millar and Russell, 1992; Lee *et al.*, 1992; Mata *et al.*, 2000; Lemaire *et al.*, 2004). Fission yeast contains another phosphatase, Pyp3p, which, although non-essential, when overexpressed rescues the mutant phenotype of *cdc25-22* cells and can also dephosphorylate Tyr-15 of Cdc2p *in vitro*, suggesting that it contributes to Cdc2p activation at mitotic onset (Millar *et al.*, 1992; Mondesert *et al.*, 1994; MacNeill and Nurse, 1997).

Apart from Cdc13p binding and relief of the inhibitory phosphate at Tyr-15, full activity of Cdc2p at the G2-M boundary requires phosphorylation at Thr-167, thought to promote conformational changes in the kinase subunit, stabilising the interaction with the cyclin subunit and enhancing substrate binding (Gould *et al.*, 1991; Gould, 2003). Phosphorylation-dependent activation of Cdks is a conserved phenomenon among eukaryotes, although the exact position of the

conserved threonine residue within the T-loop of the kinase varies slightly between individual Cdks (Morgan, 1995b; Morgan, 1997).

Two distinct types of Cdk-activating kinases (CAKs) are involved in phosphorylation of the conserved threonine residue in different eukaryotes. Higher eukaryotes possess only one type of CAK, itself a Cdk that consists of kinase Cdk7p bound to cyclin H and performs two distinct functions; it is a CAK that phosphorylates and activates Cdks and concomitantly is a component of the TFIID basal transcription factor that phosphorylates the C-terminal domain (CTD) of the largest subunit of RNA Polymerase II (Pol II), thus linking Cdk activity with the basal transcriptional apparatus (Nigg, 1996; Harper and Elledge, 1998; Liu and Kipreos, 2000; Fisher, 2005). In *S. cerevisiae*, Kin28p, the Cdk7p ortholog, is a Cdk that binds to Ccl1p, a cyclin H-related partner, but it only functions as a TFIID component, capable of phosphorylating the CTD of Pol II, while another enzyme, Cak1p, a single-subunit kinase with no cyclin partner, is the actual CAK that phosphorylates Cdc28p, as well as Kin28p (Feaver *et al.*, 1994; Cismowski *et al.*, 1995; Valay *et al.*, 1995; Kaldis *et al.*, 1996; Espinoza *et al.*, 1998).

S. pombe contains both types of CAKs with overlapping but also distinct functions; a Cdk containing Cdk7p (Mcs6p) and cyclin H orthologs (Mcs2p), and Csk1p, a Cak1p homologue capable of phosphorylating both Cdc2p and Mcs6p. Both Csk1p and Mcs6p-Mcs2p have redundant CAK roles in activation of Cdc2p, while the latter is also involved in phosphorylation of the CTD of Pol II (Buck *et al.*, 1995; Hermand *et al.*, 1998; Lee *et al.*, 1999; Saiz and Fisher, 2002; Lee *et al.*, 2005a). Although essential for complete kinase activity, CAK-dependent activation of Cdks does not appear to be a rate-limiting step for cell cycle progression. This perhaps explains why CAK activity itself is not subject to cell cycle control (Nigg, 1996; Harper and Elledge, 1998; Fisher, 2005). Indeed, in higher eukaryotes, such as *Xenopus* and humans, Cdk7p-cyclin H complexes are constitutively active and present at constant levels throughout the cell cycle (Brown *et al.*, 1994; Tassan *et al.*, 1994).

Unlike CAK-dependent phosphorylation on Thr-167, the phosphorylation status of Tyr-15 is rate-limiting for cell cycle progression in *S. pombe*. Indeed, it is the balance between the antagonistic activities of the inhibitory kinases (Wee1p and Mik1p) and the activating phosphatases (predominantly Cdc25p) that determines the phosphorylation state of Tyr-15 and consequently the activity of Cdc2p-Cdc13p complexes and entry into mitosis. As mentioned earlier, elaborate genetic analysis of *cdc25* and *wee1* mutants provided the first clues for the roles of Cdc25p and Wee1p as dose-dependent positive and negative regulators of mitosis, functioning through Cdc2p (Russell and Nurse, 1986a; Russell and Nurse, 1987; Section 1.2.2).

Distinct mechanisms tightly control the activities of Cdc25p, Wee1p and Mik1p during the cell cycle: at the G2-M transition these activate Cdc25p and simultaneously inactivate Wee1p and Mik1p to shift the balance towards activation of Cdc2p-Cdc13p complexes, and advance the cell into mitosis (Gould and Nurse, 1989; MacNeill and Nurse, 1997; Gould, 2003). Wee1p inactivation at the onset of mitosis is the result of post-translational regulation, since the mRNA and protein levels of *wee1+* remain relatively constant throughout the cell cycle (Aligue *et al.*, 1997). Phosphorylation is the principal mechanism that modulates Wee1p function and two Ser/Thr kinases, Cdr1p/Nim1p and Cdr2p, have been shown to promote mitosis by negatively regulating Wee1p activity. Cdr1p/Nim1p acts as a mitotic inducer that directly phosphorylates and inactivates Wee1p on mitotic entry (Coleman *et al.*, 1993; Parker *et al.*, 1993; Wu and Russell, 1993). Cdr2p, a kinase closely related to Nim1p/Cdr1p, is also thought to act as a mitotic inducer by negatively regulating Wee1p, although it is not clear whether it acts directly on Wee1p. It appears that the role of Cdr2p in mitosis becomes important under limiting nutrient conditions, while experimental evidence reveals additional significant roles for this kinase in cytokinesis and cell separation, since *cdr2+* overexpression is lethal and produces elongated, branched cells that possess multiple septa (Breeding *et al.*, 1998; Kanoh and Russell, 1998; Morrell *et al.*, 2004).

Mik1p is another kinase that functions as a mitotic inhibitor by phosphorylating Tyr-15 of Cdc2p. Unlike Wee1p, Mik1p oscillates in abundance during the cell cycle and peaks during S phase similar to the *mik1+* mRNA levels, the latter due to transcriptional regulation by the MBF/DSC1 complex (Christensen *et al.*, 2000; Ng *et al.*, 2001). The accumulation of a mitotic inhibitor, such as Mik1p, in S phase is believed to be part of an intrinsic cell cycle control mechanism that inhibits the occurrence of mitosis during DNA replication. Once S phase is complete, Mik1p is most probably degraded and Wee1p assumes control, maintaining Cdc2p-Cdc13p complexes in an inactive state during G2. Therefore, under normal conditions, Mik1p acts to ensure that the cell will not attempt to divide while it is still replicating the genome (Baber-Furnari *et al.*, 2000; Christensen *et al.*, 2000). Mik1p is also thought to be a target of checkpoint mechanisms that act through distinct signal transduction cascades to maintain the inhibitory phosphate at Tyr-15 of Cdc2p-Cdc13p and delay the onset of mitosis in the presence of either damaged or unreplicated DNA (Rhind and Russell, 1998; Rhind and Russell, 2001). The signal transduction cascades for both the DNA damage and the DNA replication checkpoints comprise a common group of proteins, encoded by the so-called "checkpoint *rad* genes", which sense and relay the damaged and unreplicated DNA signals to specific downstream effectors. Two protein kinases, Chk1p and Cds1p are the main effectors of the DNA-damage and DNA replication checkpoints, respectively (Rhind and Russell, 2000). Although the exact mechanisms are unclear, Chk1p and Cds1p up-regulate Mik1p activity and protein levels in

response to DNA damage or perturbed DNA replication, thereby keeping Cdc2p-Cdc13p activity low and preventing entry into mitosis until repair of the DNA damage or completion of DNA replication (Baber-Furnari *et al.*, 2000; Christensen *et al.*, 2000; Rhind and Russell, 2001).

Cdc25p is the major phosphatase that reverses Wee1p/Mik1p-dependent phosphorylation of Tyr-15 of Cdc2p upon activation at the G2-M boundary. The increase in Cdc25p activity at the onset of mitosis is the result of the co-ordinated action of transcriptional, translational and post-translational regulatory mechanisms. The mRNA and protein levels of *cdc25⁺* fluctuate during the cell cycle and peak once at the G2-M boundary (Moreno *et al.*, 1990). Cdc25p is also regulated at the level of translation initiation and this control is exerted via the translation initiation factor 4A (eIF4A). The translation initiation machinery is itself modulated in response to different nutritional and environmental conditions, and thus the susceptibility of *cdc25⁺* to translational regulation provides a link between the mitotic apparatus and growth rate signals (Daga and Jimenez, 1999). Moreover, upon mitotic entry Cdc25p is hyperphosphorylated and this leads to an increase in phosphatase activity and protein stability (Wolfe and Gould, 2004b).

Apart from activating phosphorylation of Cdc25p at mitotic onset, this mitotic inducer is also subject to checkpoint-induced inhibitory phosphorylation in response to DNA damage or incomplete DNA replication that is mediated by the Chk1p and Cds1p kinases, respectively (Rhind and Russell, 2000). Both Chk1p and Cds1p can phosphorylate Cdc25p directly and this phosphorylation inhibits its associated phosphatase activity (Furnari *et al.*, 1999). Chk1p-dependent phosphorylation of Cdc25p also promotes its association with Rad24, a 14-3-3 protein, which is thought to act as a nuclear export signal that leads to the export of Cdc25p from the nucleus, thereby sequestering this mitotic inducer away from the nuclear fraction of Cdc2p-Cdc13p complexes (Lopez-Girona *et al.*, 1999). Inhibition of Cdc25p and concomitant up-regulation of Mik1p by the DNA damage and DNA replication checkpoints ensures that Cdc2p-Cdc13p complexes are kept inactive, and so preventing entry into mitosis until DNA damage is repaired or replication is completed.

Studies in various eukaryotes, including humans, have shown that activating phosphorylation of Cdc25p-related phosphatases upon mitotic entry is mediated at least in part by a trigger level of activated mitotic Cdk complexes (Hoffmann *et al.*, 1993). At the same time, this 'primer' level of mitotic Cdk activity is also thought to phosphorylate (either directly or indirectly) and inactivate Wee1p homologues (Honda *et al.*, 1995; Mueller *et al.*, 1995a). This generates a positive feedback loop that promotes further mitotic Cdk activation and enables a rapid and irreversible advancement into mitosis (Lew and Kornbluth, 1996; Morgan, 1997; Ohi and Gould, 1999; Gould, 2003). Consistent with this, work in *S. pombe* shows that the increase in Cdc25p phosphorylation and activity observed at mitotic onset is dependent on Cdc2p activity and that the inhibitory

phosphorylation and inactivation of Wee1p at mitosis involves not only Cdr1p/Nim1p but another kinase, possibly Cdc2p (Kovelman and Russell, 1996; Tang *et al.*, 1993). Moreover, other studies show that Clp1p/Flp1p, a Cdc14p-like phosphatase that contributes to inhibition of Cdc2p activity during cytokinesis, when overexpressed blocks entry into mitosis in a Wee1p dependent manner (Trautmann *et al.*, 2001). Therefore, similar to other eukaryotes, a positive feedback loop also seems to exist in fission yeast that prompts the abrupt activation of Cdc2p-Cdc13p complexes at the G2-M transition and entry into mitosis.

In various eukaryotes, members of the conserved Ser/Thr Polo-like kinase (Plk) family (Sections 1.3.2.4 and 5.1.1), have been shown to enhance activation of Cdk1p-cyclin B complexes at mitotic onset by impinging on the positive feedback loop to promote Cdc25p and/or restrain Wee1p activity. For instance, in budding yeast the respective Plk (Cdc5p) is a potential negative regulator of Swe1p (the Wee1p homologue) and has been shown to interact with it directly (Bartholomew *et al.*, 2001). In *Xenopus*, the Polo-like kinase Plx1p binds, phosphorylates and activates a Cdc25p homologue (Cdc25Cp), while in non-immortalised human cells the introduction of antibodies against Plk1p (a Plk member) blocks entry into mitosis (Kumagai and Dunphy, 1996; Lane and Nigg, 1996). As shown from work in *Xenopus*, the Plks function in the positive feedback loop most likely to amplify rapidly the initial trigger level of mitotic Cdk activity, thereby affecting the rate of commitment to mitosis rather than mitotic commitment *per se* (Qian *et al.*, 1998a; Karaïskou *et al.*, 1999).

Plo1p, the *S. pombe* Plk with many essential functions in mitosis and cytokinesis (Sections 1.3.2.4.2, 1.3.2.5; Chapters 5 and 6), has also been proposed to impinge on the feedback loop that amplifies Cdc2p-Cdc13p activity. Plo1p activity itself oscillates periodically during the cell cycle and peaks during mitosis. The rise in Plo1p activity is the result of post-translational modifications, since Plo1p levels remain relatively constant throughout the cell cycle. Nonetheless, the *plo1⁺* mRNA levels oscillate periodically during the cell cycle, peaking during the M-G1 interval, as a result of transcriptional regulation by the concerted actions of the PBF complex, thought to comprise the Mbx1p, Fkh2p and Sep1p transcription factors, and Plo1p itself (Anderson *et al.*, 2002; Buck *et al.*, 2004; Bulmer *et al.*, 2004; Rustici *et al.*, 2004; Papadopoulou *et al.*, 2008; Chapters 5 and 6). Mitotic activation of Plo1p involves phosphorylation and is dependent on prior activation of Cdc2p-Cdc13p and Cdc25p, suggesting that Plo1p acts downstream of Cdc2p-Cdc13p in the feedback loop. However, Cdc2p-Cdc13p does not appear to activate Plo1p by direct phosphorylation, although a Cdc2p phosphorylation site (S/TP consensus) is present in Plo1p (Mulvihill *et al.*, 1999; Tanaka *et al.*, 2001).

The activities of Cdc2p and Cdc25p are also necessary for the recruitment of Plo1p to the spindle pole body (SPB). The SPB is a specialised microtubule organising centre (MTOC) structure

that associates with the nuclear envelope throughout the cell cycle and by late G2 duplicates to form two similar mitotic SPBs that separate and insert into the nuclear envelope to nucleate the formation of the microtubules of the mitotic spindle within the nucleus (Ding *et al.*, 1997; Hagan, 2003). Plo1p localises to the SPB from early mitosis until anaphase, where it physically interacts with the SPB component Cut12p (Tanaka *et al.*, 2001; Maclver *et al.*, 2003). Although both Plo1p and Cut12p associate with the SPB independently of each other, Cut12p function is required for maximal Plo1p activation and function in the positive feedback loop during mitotic commitment. Indeed, a dominant negative *cut12⁺* allele (*cut12.s11/stf1.1*), shown to relieve the requirement of Cdc25p for Cdc2p-Cdc13p activation and suppress the lethality of the *cdc25-22* mutant, causes Plo1p to associate with the interphase SPB, increases interphase Plo1p activity and prompts Plo1p activation in the absence of Cdc2p-Cdc13p activity. Plo1p activity itself is necessary for the role of Cut12p in mitotic commitment, since the *cut12.s11/stf1.1* mutant is unable to rescue the *cdc25-22* mutant when Plo1p function is compromised. Notably, expression of a constitutively active form of Plo1p significantly suppresses the defect in Cdc25p function of *cdc25.22* cells, whereas compromising Plo1p function greatly delays entry into mitosis. Therefore, it appears that the *cut12.s11/stf1.1* mutant bypasses the requirement of Cdc25p for Cdc2p-Cdc13p activation and promotes commitment to mitosis as a result of increased Plo1p activity that prompts inappropriate Cdc2p-Cdc13p activation via the positive feedback loop (Maclver *et al.*, 2003).

Fin1p is yet another fission yeast kinase that regulates various mitotic events and also plays a role in feedback loop-mediated amplification of Cdc2p-Cdc13p activity at mitotic entry (Grallert and Hagan, 2002). Fin1p is a member of the NIMA-related kinases (Nrks) that together with the Plks and the Aurora related kinases (Arks), another mitotic kinase family, cooperate with the Cdks to modulate mitotic progression (Nigg, 2001; Hagan, 2003; Section 1.3.2.4). Fin1p protein levels oscillate periodically and peak during mitosis, as a result of cell cycle specific transcription under control of the PBF transcription factor complex, a central topic of this thesis, and proteolysis (Krien *et al.*, 1998; Anderson *et al.*, 2002; Krien *et al.*, 2002; Buck *et al.*, 2004; Sections 1.3.2.4, 1.5.2.4 and 3.1). Fin1p kinase activity also fluctuates in abundance during the cell cycle and is maximal around the metaphase-anaphase transition. Similar to Plo1p, Fin1p also localises to the SPB, present from late G2 until early G1 phase. Fin1p is thought to promote Plo1p recruitment to the SPB, since its overexpression causes Plo1p to prematurely associate with the SPB in interphase, similar to the situation in the *cut12.s11/stf1.1* mutant. Consistent with this, Fin1p overexpression suppresses the *cdc25-22* mutant, while compromising Fin1p function abolishes the ability of the *cut12.s11/stf1.1* mutant to prematurely recruit Plo1p to the interphase SPB. Since Cdc2p-Cdc13p is also present in the SPB from late G2 to anaphase, it appears that Fin1p prompts the association of Plo1p with the

SPB, thus bringing Plo1p into a context whereby, through the positive feedback loop, it can enhance Cdc2p-Cdc13p activity (Alfa *et al.*, 1990; Decottingnies *et al.*, 2001; Grallert and Hagan, 2002; Hagan, 2003).

1.3.2.4 M phase

A high content of Cdc2p-Cdc13p activity irreversibly traverses *S. pombe* cells past the G2-M transition and into M phase, whereby they undergo mitosis and cytokinesis. Mitosis is characterised by a series of highly complex and dynamic events, including changes in chromosome morphology and behaviour and a reorganisation of the microtubule cytoskeleton.

Following DNA replication, chromosome cohesion holds the sister chromatids together from S phase onwards to the onset of anaphase, where APC/C dependent-proteolysis of the Cut2p securin, releases and activates its bound Cut1p separase, allowing it to cleave cohesins and trigger sister chromatid separation and spindle elongation. Chromosome cohesion is important for the proper attachment of the sister chromatids, via their kinetochores, to the spindle microtubules. Chromosome condensation, initiating during prophase, is also essential for the capture of sister chromatids by the spindle microtubules and their subsequent efficient separation without entanglement (Takahashi and Yanagida, 2003).

In tandem with the transitions in sister chromatid cohesion, condensation and separation, microtubule dynamics also dramatically change during mitosis. During interphase, a cytoplasmic array of microtubules spans the long axis of the cell, playing important roles in the control of growth polarity and the position of the nucleus. Upon mitotic entry, the interphase microtubule array disappears and a spindle forms, consisting of spindle microtubules. Spindle formation is preceded by SPB duplication and separation, giving rise to the two SPBs that nucleate distinct types of spindle microtubules; pole-to-pole ones expanding between the two SPBs, discontinuous ones that emanate from the two opposing SPBs and interdigitate with each other at the spindle midzone, and kinetochore microtubules that attach to the kinetochores of the chromosomes.

Following attachment of sister chromatids to kinetochore microtubules from opposite poles, their proper alignment at the metaphase plate and separation at anaphase onset, the kinetochore microtubules shorten, while discontinuous microtubules from opposing poles elongate and slide anti-parallel to each other, thus pushing the separated chromatids to opposite ends of the cell. The rearrangements of the microtubule cytoskeleton to form and elongate the mitotic spindle are tightly co-ordinated with the transitions in sister chromatid cohesion, condensation and separation during mitosis, thereby accommodating the faithful segregation of the duplicated genome between the

daughter nuclei (Su and Yanagida, 1997; Hagan, 2003). Two regulatory mechanisms underlie coordinated chromosome segregation and drive orderly progression through mitosis; protein phosphorylation and proteolysis. Both processes are closely linked and modulated by each other; phosphorylation controls the proteolytic machinery and *vice versa* proteolysis provides a means to regulate the availability and activity of protein kinases (Nigg, 2001).

The APC/C ubiquitinates and targets for degradation by the 26S proteasome Cut2p to promote sister chromatid separation and drive cells through anaphase, and Cdc13p to prompt inactivation of the mitotic form of Cdc2p to allow cells to exit mitosis and initiate cytokinesis. The spindle assembly checkpoint operates at the metaphase-anaphase transition to ensure that anaphase ensues only after all the sister chromatids are stably attached via their kinetochores to the mitotic spindle. The sensing components of the checkpoint are thought to assemble at the kinetochores to block the APC/C from promoting Cut2p degradation upon lack of tension, as a result of monopolar attachments (Takahashi and Yanagida, 2003).

1.3.2.4.1 Mitotic functions of Cdc2p-Cdc13p

Cdc2p-Cdc13p in *S. pombe* and the homologous Cdk1p-cyclin B complex(s) in other eukaryotes are the most prominent mitotic kinases. In higher eukaryotes, including mammals, Cdk1p-cyclin B complexes are thought to mediate several crucial mitotic events including, among others, nuclear envelope breakdown, centrosome separation, spindle formation and chromosome condensation. Consistent with this, Cdk1p-cyclin B complexes phosphorylate a number of related physiological substrates, such as nuclear lamins, microtubule-binding proteins and condensins (Nigg, 2001). Moreover, in human cells Cdk1p-cyclin B complexes have been shown to phosphorylate and activate the APC/C proteolytic apparatus. It is believed that this Cdk1p-dependent phosphorylation of the APC/C generates a negative feedback loop, through which Cdk1p-cyclin B promotes cyclin B degradation at the metaphase-anaphase transition, and therefore contributes to its own inactivation at the end of mitosis (Golan *et al.*, 2002).

In fission yeast, a few physiological mitotic substrates of Cdc2p-Cdc13p have also been identified and include Dis1p, a microtubule and SPB-associated protein that is necessary for sister chromatid separation, Cut3p, a subunit of the condensin complex that promotes chromosome condensation during mitosis, and Dis2p, a phosphatase homologous to the mammalian type-1 protein phosphatase (PP1) that is thought to promote mitotic exit and entry into G1 phase. Cdc2p-Cdc13p is thought to phosphorylate Dis1p to promote its re-localisation from the microtubules to the SPB, where it can interact with other proteins and mediate sister chromatid separation (Nabeshima

et al., 1995; Su and Yanagida, 1997). Phosphorylation of Cut3p by Cdc2p-Cdc13p also affects its localisation properties and facilitates its mitotic-specific accumulation in the nucleus to promote chromosome condensation (Sutani *et al.*, 1999). In the case of Dis2p, Cdc2p-Cdc13p-dependent phosphorylation inhibits its function, thereby allowing mitosis to proceed (Yamano *et al.*, 1994; Su and Yanagida, 1997). Apart from Cdc2p-Cdc13p, the Plx1p, Fin1p and Ark1p Ser/Thr kinases, members of the Polo, NIMA and Aurora family respectively, also have essential roles in mitotic progression, and in many cases their functions are conserved between eukaryotes (Nigg, 2001).

1.3.2.4.2 Mitotic functions of Plks: regulatory roles of Plx1p and its human homolog, Plk1p, in cell division

Plks are a conserved kinase family with homologues in many eukaryotes, ranging from the yeasts to humans. *Drosophila* and the budding and fission yeasts contain a single Plk, Polo, Cdc5p and Plx1p, respectively, while mammals, such as humans, possess four Plks, namely Plk1p, Plk2p, Plk3p and Plk4p. Plks were originally identified in *Drosophila*, where weak hypomorphic alleles of Polo were isolated that displayed several mitotic and meiotic abnormalities, including defects in spindle pole behaviour and bipolar spindle formation, whereby mitotic chromosomes cluster around a monopolar spindle in a ring arrangement, chromosome non-disjunction and, as shown later on, cytokinesis failure (Sunkel and Glover, 1988; Llamazares *et al.*, 1991; Carmena *et al.*, 1998). In mammals, Plk1p, the closest fly and yeast ortholog, is the most extensively studied Plk with prominent mitotic functions. Plk2p operates primarily during the G1 and S phases, where it is thought to modulate, among others, aspects of centriole duplication. Both Plk2p and Plk3p are encoded by immediate early genes, although Plk3p protein levels appear to remain constant throughout the cell cycle. Plk2p has been proposed to have a role in preventing mitotic catastrophe upon spindle damage, while Plk3p is shown to be activated in response to DNA damage (Bahassi *et al.*, 2002; Eckerdt *et al.*, 2005; van de Weerd and Medema, 2006).

Plks are crucial regulators of several mitotic events and in many cases their functions are well-preserved between metazoans and the yeasts (Glover *et al.*, 1998; Nigg, 1998; Lee *et al.*, 2005b). For example, Plks from various species, including fission yeast, have been shown to participate by various ways in feedback-loop amplification of mitotic Cdk activity at mitotic onset, thus influencing the timing of mitotic commitment (Section 1.3.2.3). In *Xenopus* and budding yeast the respective Plk (Plx1p and Cdc5p) phosphorylates cohesins and mediates sister chromatid separation, either by promoting the dissociation of cohesins from chromatin during

prophase/prometaphase or prompting their separin-dependent proteolysis at the metaphase-anaphase transition, respectively (Alexandru *et al.*, 2001; Sumara *et al.*, 2002).

Plks are also thought to modulate the function of the APC/C; activated mammalian Plk phosphorylates subunits of the APC/C, contributing, at least in part, to its activation and ensuing ubiquitination of cyclin B. Plx1p is also required for APC/C activation, while Cdc5p has been implicated in APC/C-dependent proteolysis of Clb2p (Descombes and Nigg, 1998; Kotani *et al.*, 1998; Shirayama *et al.*, 1998). Consistent with these observations, Plo1p has been shown to physically associate with the Cut23p/Apc8p APC/C subunit, suggesting a conserved role for Plks in regulation of APC/C activation (May *et al.*, 2002).

Plks play a key role in mitotic spindle formation in many eukaryotes. In *Drosophila*, *polo* mutations result in the formation of monopolar spindles due to a failure in centrosome separation in mitotic somatic cells (Llamazares *et al.*, 1991). Similarly, in *Xenopus* and human cells microinjection of appropriate anti-Plk antibodies generates monopolar spindles, while in the latter centrosomes are also unusually small suggesting a role for the mammalian Plk in centrosome maturation (Lane and Nigg, 1996; Qian *et al.*, 1998a). Consistent with a central role in mitotic spindle formation, Plks of either yeast or metazoan origin localise to the SPBs/centrosomes early during mitosis. For example, *Drosophila* Polo associates with the centrosomes upon entry into mitosis, while it translocates to the kinetochores in prophase and the mid-part of the central spindle during late anaphase (Glover *et al.*, 1998; Glover, 2005).

In addition to a highly conserved N-terminal catalytic domain, Plks also share significant similarity in their non-catalytic C-terminal domain, especially in two conserved regions, the polo-boxes, shown to act as a single domain that mediates interactions with target proteins and required for localisation of Plk function into specific subcellular structures (Dai and Cogswell, 2003; Lee *et al.*, 2005b). Indeed, mutations in a single polo-box of mammalian Plk1p or budding yeast Cdc5p disrupt their localisation at distinct sites of the mitotic apparatus during mitosis, while mutations and/or truncations within the polo-boxes of Plo1p abolish its localisation to the SPBs (Lee *et al.*, 1998; Song *et al.*, 2000; Seong *et al.*, 2002; Reynolds and Ohkura, 2003).

A series of studies with human Plk1p have shown that the polo-box domain functions as a phosphopeptide recognising module that preferentially binds and phosphorylates proteins previously phosphorylated by Pro-directed kinases, predominantly the Cdks (Elia *et al.*, 2003a; Elia *et al.*, 2003b). Nonetheless, recent work reveals that Plk1p can also phosphorylate itself and create a docking site for its own polo-box domain. A number of discrete partners have been identified that are 'primed' with phosphorylation by Cdk1p during early mitosis or Plk1p in anaphase to promote their interaction with the polo-box domain (Neef *et al.*, 2007; Rape, 2007; Lee *et al.*, 2008a; Lee *et al.*,

2008b). Although most Plk1p binding proteins are also Plk1p phosphorylation substrates, they also function as part of a scaffold that recruits the kinase to discrete sub-cellular structures at specific cell cycle times, thereby increasing local Plk1p concentrations (Petronczki *et al.*, 2008). Indeed, most Plk1p docking partners are localised within structures, such as the centrosomes, the kinetochores and the central spindle region, hence recruiting the kinase to the mitotic apparatus and allowing it to co-ordinate its function with earlier phosphorylation cell cycle events, mediated primarily by Cdk1p, the master regulator of mitosis (Casenghi *et al.*, 2003; Lowery *et al.*, 2005; Elowe *et al.*, 2007; Takaki *et al.*, 2008).

Human Plk1p localises to discrete structures of the mitotic apparatus in a cell cycle specific manner; during G2, Plk1p is present both at the cytoplasm and the nucleus, while from early mitosis is found at the centrosomes and the kinetochores where it persists until late anaphase. A sub-population of Plk1p also translocates to the central spindle region (spindle midzone) from anaphase to telophase, after which it is found in the midbody (Golsteyn *et al.*, 1995; Taniguchi *et al.*, 2002; van de Weerd and Medema, 2006; Lee *et al.*, 2008a; Takaki *et al.*, 2008). Consistent with a dynamic pattern of localisation during mitosis, Plk1p associates with and phosphorylates target molecules in a discrete spatial and temporal order to promote a multitude of mitotic events, including mitotic entry, resumption from a G2 arrest following activation of the DNA damage checkpoint, centrosome maturation, bipolar spindle assembly, APC/C activation, cohesin dissociation, chromosome alignment and cytokinesis (van Vugt and Medema, 2005; van de Weerd and Medema, 2006; Petronczki *et al.*, 2007; Takaki *et al.*, 2008).

Similar to its yeast and metazoan counterparts, human Plk1p has been proposed to impose on the feedback loop that rapidly amplifies an initial trigger level of mitotic Cdk1p activity during mitotic entry. In fact, research work shows that Plk1p promotes further activation of Cdk1p-cyclin B complexes at mitotic entry by more than one means; it phosphorylates Cdc25C to promote its translocation to the nucleus and ensuing activation, Wee1p to prompt its SCF-mediated proteolytic degradation and cyclin B to assist, at least in part, its nuclear localisation and/or activation of the associated Cdk1p kinase (Toyoshima-Morimoto *et al.*, 2001; Toyoshima-Morimoto *et al.*, 2002; Jackman *et al.*, 2003; Watanabe *et al.*, 2004; van Vugt and Medema, 2005; van de Weerd and Medema, 2006).

Plk1p ensures timely advancement past the G2-M transition but is not a prerequisite for mitotic entry *per se*. Indeed, compromising Plk1p activity, either by depletion of Plk1p levels with RNAi or by chemical inhibition with a small compound, delays but eventually permits entry into mitosis (Sumara *et al.*, 2004; Lénárt *et al.*, 2007). Nonetheless, Plk1p is necessary for resuming mitotic entry during recovery from a G2 cell cycle arrest upon DNA damage and ensuing activation of

the DNA damage checkpoint. Indeed, Plk1p negatively regulates several downstream mediators/ effectors of ATM- and ATR-dependent checkpoint pathways in order to re-activate Cdk1p-cyclin B complexes and restart the cell cycle upon recovery from the G2 DNA damage-induced arrest. For example, it phosphorylates Wee1p to promote its SCF-mediated degradation; it interacts with the p53 tumour suppressor to inhibit its transactivation activity; it phosphorylates Claspin, a mediator protein that promotes the activation of the ATR effector kinase Chk1p, targeting it for SCF-dependent proteolysis (Eckerdt *et al.*, 2005; van Vugt and Medema, 2005; Xie *et al.*, 2005; van de Weerd and Medema, 2006). Consistent with a positive role for Plk1p in recovery from DNA damage checkpoint-induced arrest, DNA damage checkpoints inhibit Plk1p activity. In fact, in response to DNA damage in G2 phase, the Cdc14B phosphatase (a paralog of *S. cerevisiae* Cdc14p) relocates from the nucleolus to the nucleoplasm, whereby it dephosphorylates and activates Cdh1p, enabling it to target Plk1p for APC/C-dependent proteolysis (Bassermann *et al.*, 2008).

Several Plk1p interacting proteins and/or substrates have been identified over recent years that localise at distinct sites of the mitotic apparatus, providing insight about Plk1p function in centrosome maturation and separation, spindle assembly, establishment of stable microtubule-kinetochore attachments and initiation of cleavage furrow ingression during cytokinesis (van de Weerd and Medema, 2006; Petronczki *et al.*, 2008; Takaki *et al.*, 2008). At the G2-M transition, Plk1p interacts with and phosphorylates Nlpp (ninein-like protein), a centrosomal protein, leading to its displacement from the centrosome. Nlpp binds to and recruits γ -tubulin complexes in the interphasic centrosome, whereas its Plk1p-dependent dissociation from this site during mitotic onset is thought to promote the recruitment of mitosis-specific γ -tubulin complex binding proteins, contributing to centrosome maturation and enhanced microtubule nucleation capacity during mitotic spindle formation (Casenghi *et al.*, 2003). Recently, Plk1p has been shown to phosphorylate another centrosomal protein, Kizuna, eliciting a function that enhances the mechanical stability of mitotic centrosomes (Petronczki *et al.*, 2008). Moreover, in prophase Plk1p phosphorylates and targets for SCF-mediated degradation Emi1p (early mitotic inhibitor 1), an inhibitor of the APC/C that is localised at the centrosomes, hence contributing to APC/C activation (Hansen *et al.*, 2004).

In kinetochores, Plk1p interacts with a variety of proteins. Initially, Plk1p is recruited to interphase centromeres during G2 phase, via its association with PBIP1p (polo-box interacting protein 1), a centromeric protein. Priming phosphorylation of PBIP1p is mediated by Plk1p itself, creating its own docking site and promoting its stable accumulation at centromeres and/or kinetochores. During prometaphase, a high level of Plk1p activity drives PBIP1p hyperphosphorylation. In turn, PBIP1p dissociates from kinetochores and targeted for SCF-dependent degradation, allowing Plk1p to bind to and phosphorylate other kinetochore components,

including among others BubR1p (Lee *et al.*, 2008a; Lee *et al.*, 2008b). Cdk1p pre-phosphorylates BubR1p and creates a docking site for the Plk1p polo-box domain. Maximum phosphorylation of BubR1p by Plk1p occurs during prometaphase and is thought to convey a lack of tension signal at kinetochores that promotes the establishment of stable kinetochore-microtubule attachments during bipolar attachment at metaphase (Elowe *et al.*, 2007).

At the metaphase-anaphase transition Plk1p translocates from the kinetochores to the spindle midzone, whereby it regulates the onset of cytokinesis. PRC1p (protein regulating cytokinesis 1), a microtubule associated protein, is a major delivery factor for Plk1p recruitment to the spindle midzone in anaphase. In fact, Plk1p phosphorylates and primes itself the interaction of its polo-box domain with PRC1p during anaphase. During prophase and prometaphase, Cdk1p also phosphorylates PRC1p and impedes its interaction with Plk1p, hence ensuring that Plk1p is not recruited prematurely to the central spindle region. APC/C-dependent proteolysis of cyclin B at the onset of anaphase promotes Cdk1p inactivation and allows Plk1p to interact with PRC1p and accumulate at the spindle midzone. Additional microtubule-associated proteins, such as Mklp1p and Mklp2p, interact with Plk1p and contribute to its localisation at the central spindle region ((Neef *et al.*, 2007; Petronczki *et al.*, 2007; Rape, 2007).

Localisation of Plk1p at the spindle midzone is crucial for cytokinesis, consistent with a role for this kinase in formation of the contractile actomyosin ring and ensuing ingression of the cleavage furrow. Specifically, Plk1p has been shown to promote the interaction between HsCyc-4p (a Rho GTPase activating protein) and Ect2p (a Rho guanine nucleotide exchange factor) to recruit the latter to the spindle midzone. Once in the central spindle, Ect2p activates the GTPase RhoA, which in turn drives actin polymerization and myosin activation; both functions are necessary for the formation of the contractile ring at the equatorial cortex and the initiation of cytokinesis (Petronczki *et al.*, 2007; Petronczki *et al.*, 2008; Takaki *et al.*, 2008).

The functions and localisation pattern of Plk1p during mitosis correlates well with its pattern of expression and activation during the cell cycle. Plk1p protein levels accumulate in G2 and M phase, as a result of both cell cycle regulated transcription and proteolysis (Eckerdt *et al.*, 2005; Winkles and Alberts, 2005). Indeed, the forkhead transcription factor FOXM1 drives G2-M specific transcription of *Plk1* and many other important genes for mitotic progression, whereas Cdh1p mediates APC/C-dependent degradation of Plk1p during late anaphase, an event that promotes mitotic exit (Lindon and Pines, 2004; Laoukili *et al.*, 2005). Interestingly, FOXM1 is not only a regulator of G2-M specific expression of *Plk1* but also a substrate for phosphorylation by Plk1p. Indeed, Plk1p associates via its polo-box domain with the C-terminal region of FOXM1 and phosphorylates this forkhead transcription factor to enhance its transcriptional activity, leading to

increased expression at mitotic onset of target genes, including *Plk1* itself. Cdk1p promotes the interaction between Plk1p and FOXM1 by phosphorylating the latter during late S-G2 phase and creating a docking site for the polo-box domain. Since Plk1p is both a target and a regulator of FOXM1 transcriptional activity, this suggests that Plk1p phosphorylation of FOXM1 is part of a positive feedback loop that operates at the G2-M transition and promotes further transcription of *Plk1*, as well as of many other genes necessary for mitotic progression (Fu *et al.*, 2008).

Plk1p activity is also regulated during the mitotic cell cycle, starting to increase at the G2-M interval and peaking during mitosis. Consistent with this, activation of Plk1p occurs at the G2-M transition and requires phosphorylation within its T-loop at Thr210. Indeed, in the absence of Thr210 phosphorylation, the polo-box domain interacts with the kinase domain of Plk1p, thereby inhibiting its associated kinase activity. Stimulatory phosphorylation at Thr210 relieves the inhibitory effect of the polo-box domain and activates Plk1p, which in turn can associate via the polo-box domain with its substrates, an event that further enhances its activity (García-Álvarez *et al.*, 2007). Aurora-A, an Ark family member, phosphorylates Plk1p at Thr210 in a manner that requires Bora, an Aurora-A specific co-factor that accumulates during G2 phase and associates with both Aurora-A and Plk1p. Bora binds to Plk1p in a phosphorylation-independent manner and promotes a conformational change that disrupts the inhibitory interaction between the polo-box domain and the kinase domain, hence allowing Aurora-A to gain access and phosphorylate Thr210 within the T-loop (Seki *et al.*, 2008a; Macůrek *et al.*, 2008; Eckerdt and Maller, 2008). Intriguingly, Plk1p has been shown to phosphorylate Bora during mitosis, promoting its association with β -TrCP (β -transducin repeat-containing protein), an F-box protein, and ensuing SCF-dependent degradation. Plk1p-driven proteolysis of Bora is thought to release Aurora-A, allowing it to interact with microtubule-associated co-factors and promoting its localisation to the mitotic spindle to perform its functions in bipolar spindle assembly (Chan *et al.*, 2008; Seki *et al.*, 2008b; Eckerdt and Maller, 2008). Finally, recent work reveals a prominent role for Protein Phosphatase 1 (PP1) in regulation of Plk1p kinase activity. Specifically, MYPT1 (Myosin phosphatase-targeting subunit 1), a PP1-specific regulatory subunit, interacts with the polo-box domain of Plk1p and promotes PP1-dependent dephosphorylation at Thr210, thereby restraining Plk1p activity. Given that PP1-MYPT1 complexes co-localise with Plk1p at centrosomes, kinetochores and the central spindle, it is possible that they also antagonise Plk1p activity indirectly by dephosphorylating Plk1p substrates at these sites (Archambault *et al.*, 2008; Yamashiro *et al.*, 2008).

Similar to Plk1p, Plo1p has been shown to regulate both mitotic spindle formation and cytokinesis in fission yeast. Disruption of Plo1p function, encoded by an essential gene, results in cells with overcondensed chromosomes associated with monopolar spindles, or in multinucleate

cells without septa that are unable to either form the actomyosin-based medial ring, a construct that forms early during mitosis and determines the site of septum formation (Section 1.3.2.5), or deposit septal material (Ohkura *et al.*, 1995). Meanwhile, temperature sensitive *plo1* mutants have also been identified that display a variety of defects in septum placement (Bähler *et al.*, 1998b). Over-expression of *plo1*⁺ also leads to the formation of monopolar spindles, suggesting a dependency of spindle formation on the proper levels or timing of Plo1p activation. Notably, *plo1*⁺ over-expression induces septum formation even in interphase cells (Ohkura *et al.*, 1995).

Consistent with a regulatory role for Plo1p in mitotic spindle, actomyosin ring and septum formation, this kinase localises to the SPBs from early mitosis until anaphase and can also be detected at the mitotic spindle and the medial ring (Bähler *et al.*, 1998b; Mulvihill *et al.*, 1999). Moreover, activation of Plo1p during mitosis is accompanied by two peaks of activity, correlating well with the timing of actomyosin medial ring assembly and septation, respectively (Glover *et al.*, 1998; Tanaka *et al.*, 2001; Lee *et al.*, 2005b).

Plo1p has been shown to interact with Mid1p/Dmf1p (a metazoan anillin-related protein), a protein that is required for the positioning and placement of the actomyosin medial ring (Bähler *et al.*, 1998b). Mid1p/Dmf1p is predominantly present in the nucleus during interphase, but early in mitosis it localises into the cell cortex to form a medial ring that precedes formation of the actomyosin-based ring (Chang *et al.*, 1996; Sohrmann *et al.*, 1996). Interestingly, Plo1p has been shown to be required for Mid1p/Dmf1p nuclear exit, as Mid1p/Dmf1p is unable to leave the nucleus in *plo1* mutant cells and *plo1*⁺ overexpression causes its premature exit from the nucleus. Taking into account that re-localisation of Mid1p/Dmf1p into the medial ring correlates with its phosphorylation, it is likely that Plo1p is the kinase that phosphorylates this actomyosin ring component (Bähler *et al.*, 1998b).

Consistent with the role of Plo1p in control of actomyosin ring formation and septation, and central to the topic of this thesis, the Plo1p kinase is also involved in transcriptional regulation by the PBF transcription factor complex of various genes that encode proteins with important mitotic and cytokinetic functions including Plo1p and Mid1p/Dmf1p themselves (Anderson *et al.*, 2002; Papadopoulou *et al.*, 2008; Chapters 5 and 6).

1.3.2.4.3 Mitotic functions of Fin1p

Fin1p is a member of the NIMA-related kinases (Nrks) that, apart from *S. pombe*, are also found in various eukaryotes, including humans, *S. cerevisiae* and *Aspergillus nidulans*. The *A. nidulans* Nrk, NIMA (never in mitosis A), is the founding member of this kinase family and was identified in a screen for conditional lethal mutants that are unable to enter mitosis (Morris, 1975). Despite the

arrest of *nimA* mutants before mitotic commitment, NIMA mitotic activation relies on prior mitotic Cdk activation. The activity of NIMA kinase fluctuates periodically during the cell cycle, peaking during mitosis ahead of maximal mitotic Cdk activity (Ye *et al.*, 1995). Its protein levels also accumulate in a cell cycle specific manner, attributed to proteolytic degradation at mitotic exit and cell-cycle specific transcription and/or translation (Pu and Osmani, 1995).

Unlike its *A. nidulans* counterpart, fission yeast *fin1+* is non-essential for viability. Fin1p shares similar functions with NIMA in regulation of mitotic progression, including roles in the timing of mitotic entry (Section 1.3.2.4), chromosome condensation, spindle organisation and cytokinesis, but the underlying molecular mechanisms seem to differ in the two fungi. Similar to NIMA and Fin1p, the mammalian Nrks also appear to contribute to regulation of distinct mitotic events, including spindle formation and late mitosis/cytokinesis (O'Regan *et al.*, 2007).

Apart from the role of Fin1p in feedback loop amplification of Cdc2p-Cdc13p activity at mitotic onset and its requirement for recruitment of Plo1p to the SPBs (Section 1.3.2.4), regulatory functions have emerged for this kinase in spindle formation and spindle pole maturation. Indeed, *fin1* mutants display monopolar spindles that are nucleated from a single SPB, while *fin1+* overexpression also promotes the formation of monopolar and/or metaphase spindles and causes premature chromosome condensation during interphase (Krien *et al.*, 1998; Grallert and Hagan, 2002). Moreover, Fin1p is also thought to function during cytokinesis, possibly through modulating the activity of the septation initiation network (SIN), a signalling cascade that brings about septation and cytokinesis (Section 1.3.2.5) (Grallert *et al.*, 2004). Finally, *fin1+* cell cycle gene expression is also under PBF control (Anderson *et al.*, 2002).

1.3.2.4.4 Mitotic functions of Ark1p

Arks regulate various processes during mitosis, including chromosome condensation and segregation, spindle formation, as well as cytokinesis. Typically, two Arks are present in metazoans, each associated with distinct functions. Mammalian cells also contain a third Ark, aurora-C, but its precise function is yet unclear (Bischoff and Plowman, 1999; Giet and Prigent, 1999; Nigg, 2001; Goto *et al.*, 2002).

aurora-A is a centrosome- and microtubule- associated protein that is thought to promote normal spindle assembly by sustaining centrosome separation. The role of aurora-A in spindle formation is exemplified by the phenotype of *aurora-A Drosophila* mutants, characterised either by monopolar spindles associated with one or two paired centrosomes or by bipolar spindles with two paired centrosomes per pole. aurora-B associates with chromatin in early mitosis and with the

spindle midzone late during mitosis, and is required for both proper chromosome condensation and cytokinesis. Indeed, *Drosophila* cells, when depleted of aurora-B display improperly condensed chromosomes and are unable to undergo cytokinesis. The defects in chromosome condensation result from inefficient phosphorylation of Ser-10 of histone H3 and the inability to localise condensin to chromosomes; both of these functions are thought to be mediated by aurora-B (Glover *et al.*, 1995; Giet and Glover, 2001).

S. cerevisiae and *S. pombe* each contain a single Ark, Ipl1p and Ark1p, both of which display a combination of aurora-A and aurora-B related functions (Hsu *et al.*, 2000; Petersen *et al.*, 2001). In fission yeast, deletion of *ark1⁺* results in viable cells that exhibit defects in chromosome condensation during prophase, in chromosome resolution during anaphase onset, in kinetochore function, and in spindle formation. Moreover, similar to metazoan aurora-B and Ipl1p, Ark1p can also phosphorylate Ser-10 of histone H3, a function thought to be important for proper chromosome condensation. Although Ark1p is present at constant levels throughout the cell cycle, it displays stage-specific localisation into the centromeres and the central mitotic spindle region during mitosis that resembles the localisation pattern of metazoan aurora-B (Petersen *et al.*, 2001). Finally, unlike Plo1p and Fin1p, Ark1p does not appear to associate with the SPB during mitosis (Petersen *et al.*, 2001).

1.3.2.5 Mitotic exit and cytokinesis

After chromosome segregation a co-ordinated series of events take place within the cell to promote exit from mitosis and initiate cytokinesis, thereby physically dividing the two daughter cells. The presence of a rigid cell wall in yeasts, such as *S. pombe* and *S. cerevisiae*, necessitates not only membrane separation but also cell wall separation of the two progeny; both processes in these organisms define cytokinesis.

The orderly execution and co-ordination of mitosis with cytokinesis is vital for the survival of the cell and the maintenance of its genomic identity, since premature cytokinesis before completion of sister chromatid separation would lead to aneuploidy and polyploidy (Guertin *et al.*, 2002). Control mechanisms exist in the cell that ensure that mitosis and cytokinesis are co-ordinated temporally and spatially. Indeed, early genetic work has shown that mitosis and cytokinesis are tightly coupled and established a dependency for cytokinesis on passage through mitosis (Nurse, 1975; Nurse *et al.*, 1976). In recent years many of the genes that drive actomyosin ring and septum formation in *S. pombe* have been isolated and characterised, making significant progress towards understanding

the regulatory network that underlies cytokinesis and couples it to nuclear division (Fankhauser and Simanis, 1994; Le Goff *et al.*, 1999; Balasubramanian and McCollum, 2003).

A prerequisite for mitotic exit in eukaryotic cells is the inactivation of mitotic Cdk activity. In *S. pombe* Cdc2p-Cdc13p complexes are inactivated after chromosome segregation by APC/C-mediated proteolysis of Cdc13p, through the function of the Slp1p APC/C activator, while during G1 phase another APC/C activator, Ste9p functions to maintain low mitotic Cdc2p activity (Bardin and Amon, 2001; Section 1.3.2.1). Moreover, the Clp1p/Flp1p phosphatase is thought to contribute to disruption of the Cdc2p-Cdc13p amplification loop and therefore promote inactivation of the associated Cdk in late mitosis, via inactivation of Cdc25p (Krapp *et al.*, 2004; Wolfe and Gould, 2004a; Wolfe and Gould, 2004b).

A signalling cascade, the septation-initiation network (SIN) operates late in the fission yeast cell cycle and regulates initiation of cytokinesis, coupling it to inactivation of Cdc2p-Cdc13p and therefore completion of chromosome segregation (Bardin and Amon, 2001; Balasubramanian and McCollum, 2003; Simanis, 2003; Krapp *et al.*, 2004). A similar signalling pathway functions in budding yeast, the mitotic-exit network (MEN), which is required for inactivation of mitotic Cdk activity and controls both mitotic exit and cytokinesis (Bardin and Amon, 2001; Simanis, 2003; Krapp *et al.*, 2004; Section 1.3.3).

In fission yeast, the microtubule cytoskeleton re-organises during mitosis to separate the two copies of the genome, whereas the actomyosin cytoskeleton re-shuffles to accommodate ensuing cell cleavage, yielding two individual cell entities. Concomitant with spindle formation in early mitosis a ring structure, composed of F-actin, myosin II and numerous other proteins, starts to assemble at the medial plane of the cell, at a site that predicts the future position of septum formation and cell division (Balasubramanian *et al.*, 2000; Guertin *et al.*, 2002; Balasubramanian and McCollum, 2003; Wolfe and Gould, 2005).

F-actin displays a dynamic localisation pattern during the mitotic cell cycle (Figure 1.6). In interphase and prior to a transition point in G2, termed new end take off (NETO), F-actin patches are found mainly at the 'old' end of the cell (the one inherited from the mother cell), thereby promoting monopolar growth. At NETO, F-actin is present at both cell ends, prompting bipolar growth and after mitotic entry it re-localises to the centre of the cell to form the medial ring structure, also termed the cytokinetic actomyosin ring (CAR) (Gachet *et al.*, 2003). Once the CAR has assembled, F-actin patches also become visible next to the CAR and are thought to contribute to the delivery of septal material. At the end of anaphase, after chromosome segregation and while the mitotic spindle is dissolving, the CAR constricts, providing the driving force that divides the cytoplasm (Wolfe and Gould, 2005).

The septation initiation network (SIN) is the signalling cascade that triggers constriction of the actomyosin ring and signals the commencement of cytokinesis, whereby new membrane and cell wall material is deposited inwards from the cell cortex and behind the constricting ring to accommodate cell division via the formation of a primary division septum. After formation of the primary septum, consisting of sugar polymers, secondary septa are formed on either of its sides, generating a three-layered septum structure. Degradation of the primary septum combined with rupture of the pre-existing cell wall then physically separates the two daughter cells (McCollum and Gould, 2001; Guertin *et al.*, 2002; Balasubramanian and McCollum, 2003; Simanis, 2003; Krapp *et al.*, 2004).

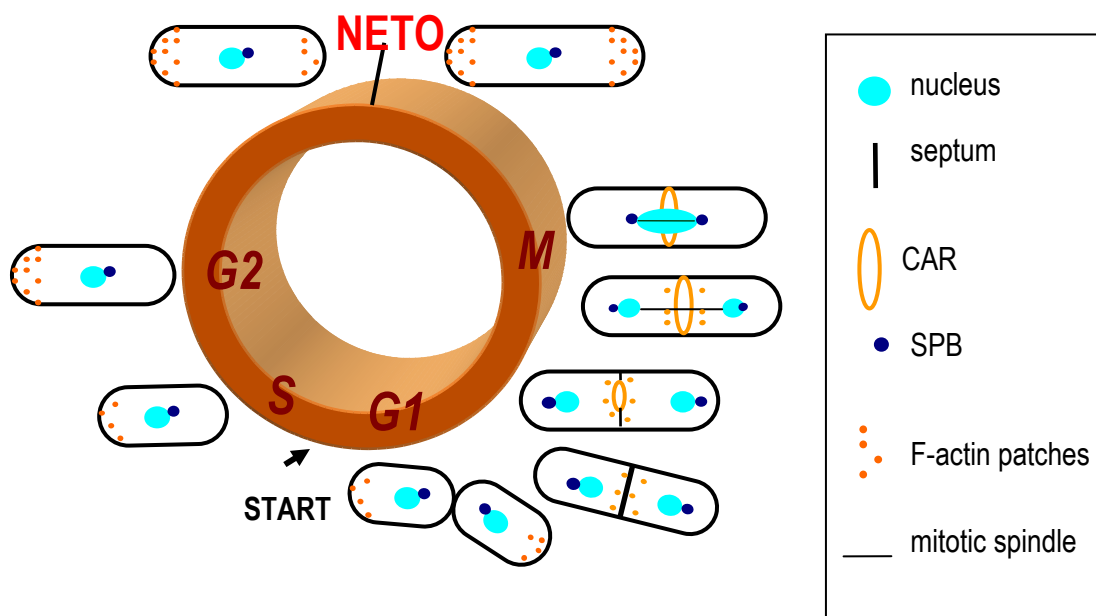


Figure 1.6: Rearrangements in actin distribution and structure during the cell cycle in *S. pombe*. During G1 and S phase and until the new end take off (NETO) transition point in G2, F-actin patches localise predominantly at one end of the cell (the one inherited from the mother cell). After NETO, F-actin patches are found at both cell ends and drive bipolar growth. In mitosis, F-actin is lost from both cell ends and forms the cytokinetic actomyosin ring (CAR) at the centre of the cell. After CAR assembly, F-actin patches appear next to the CAR and promote septal material deposition. At late mitosis and while the CAR constricts, the septum forms inwards from the cell cortex and behind the constricting CAR to enable cell division and generation of two independent cell entities.

1.3.2.5.1 Timing, positioning and assembly of the actomyosin ring

Formation of the actomyosin ring requires entry into mitosis since *cdc2* and *cdc25* mutant cells arrested at the G2-M transition continue to grow in the absence of nuclear division and fail to assemble an actomyosin ring (Snell and Nurse, 1994). This raises the possibility that activated

Cdc2p-Cdc13p complexes might promote actomyosin ring assembly by phosphorylating ring-nucleating proteins, but evidence so far is lacking (Balasubramanian *et al.*, 2000).

Eukaryotes employ discrete mechanisms to select the position of the actomyosin ring and, therefore, their future site of cell division. In *S. cerevisiae*, the site of division is determined early in the cell cycle during G1 and depends on the previous cell division site, forming either across or adjacent to it. Animal cells use the position of the mitotic spindle to define their divisional plane at anaphase onset. Unlike animal cells and budding yeast, the position of the interphase nucleus dictates the fission yeast cell division site, corresponding to the position of the actomyosin ring and the ensuing septum. The interphase array of microtubules is thought to mediate the maintenance of the interphase nucleus in the middle of the fission yeast cell (Chang and Nurse, 1996; Guertin *et al.*, 2002; Balasubramanian and McCollum, 2003).

Placement of the actomyosin ring in *S. pombe* requires the function of Plo1p to promote nuclear exit of Mid1p/Dmf1p and its ensuing localisation into the cell cortex overlying the nucleus, thus marking the future cell division site relative to the position of the nucleus. Mid1p/Dmf1p, although predominantly nuclear in interphase, is also seen during this time as a broad equatorial band overlying the nucleus. It is believed that Mid1p/Dmf1p shuttles between the nucleus and the cell cortex in interphase cells, thereby continuously marking the division plane. Following entry into mitosis, Mid1p/Dmf1p strongly localises into the medial cell cortex in a Plo1p-dependent manner to mediate the recruitment of actomyosin ring components (Figure 1.7A and B) (Bähler *et al.*, 1998b; Guertin *et al.*, 2002; Balasubramanian and McCollum, 2003; Wolfe and Gould, 2005; Section 1.3.2.4.2).

Various proteins accumulate at the medial plane of the cell from as early as mid-G2 to metaphase to drive the assembly of the actomyosin ring, which becomes fully organised after anaphase onset. Important actomyosin ring components include among others Myo2p (the type II myosin heavy chain that physically interacts with Mid1p/Dmf1p), Cdc4p and Rlc1p (the two myosin-associated light chain subunits), Cdc15p (a member of the PCH protein family), Cdc12p (a formin homologue) and Rng2p (an IQGAP-related protein) (Figure 1.7B and C) (Balasubramanian *et al.*, 2000; Guertin *et al.*, 2002; Balasubramanian *et al.*, 2004; Wolfe and Gould, 2005).

In a manner that requires Mid1p/Dmf1p function, these proteins are thought to form a precursor, spot-like, structure at the cell cortex that supports the assembly of the ensuing mature actomyosin ring, comprising F-actin and numerous other proteins. Cdc15p and Cdc12p interact with each other and promote the recruitment of actin-nucleating components, including the profilin Cdc3p and the Arp2/3p complex, thereby inducing actin polymerisation. Later, tropomyosin Cdc8p and the cross-linking protein α -actinin Ain2p are thought to bind spontaneously to polymerizing actin and

promote the transition of the equatorial actin band into a compact ring structure, which after the accumulation of additional components, such as Myp2p (type II myosin heavy chain), septins (filament-forming proteins) and F-actin patches matures into the actomyosin ring during late anaphase (anaphase B). Once the mitotic spindle disassembles, the actomyosin ring contracts, mediated by the motor activity of type II myosin that causes sliding of the actin filaments (Figure 1.7D and E). The SIN regulates actomyosin ring constriction and concomitant septum formation, bringing about cytokinesis (Wu *et al.*, 2003; Balasubramanian *et al.*, 2004; Wolfe and Gould, 2005).

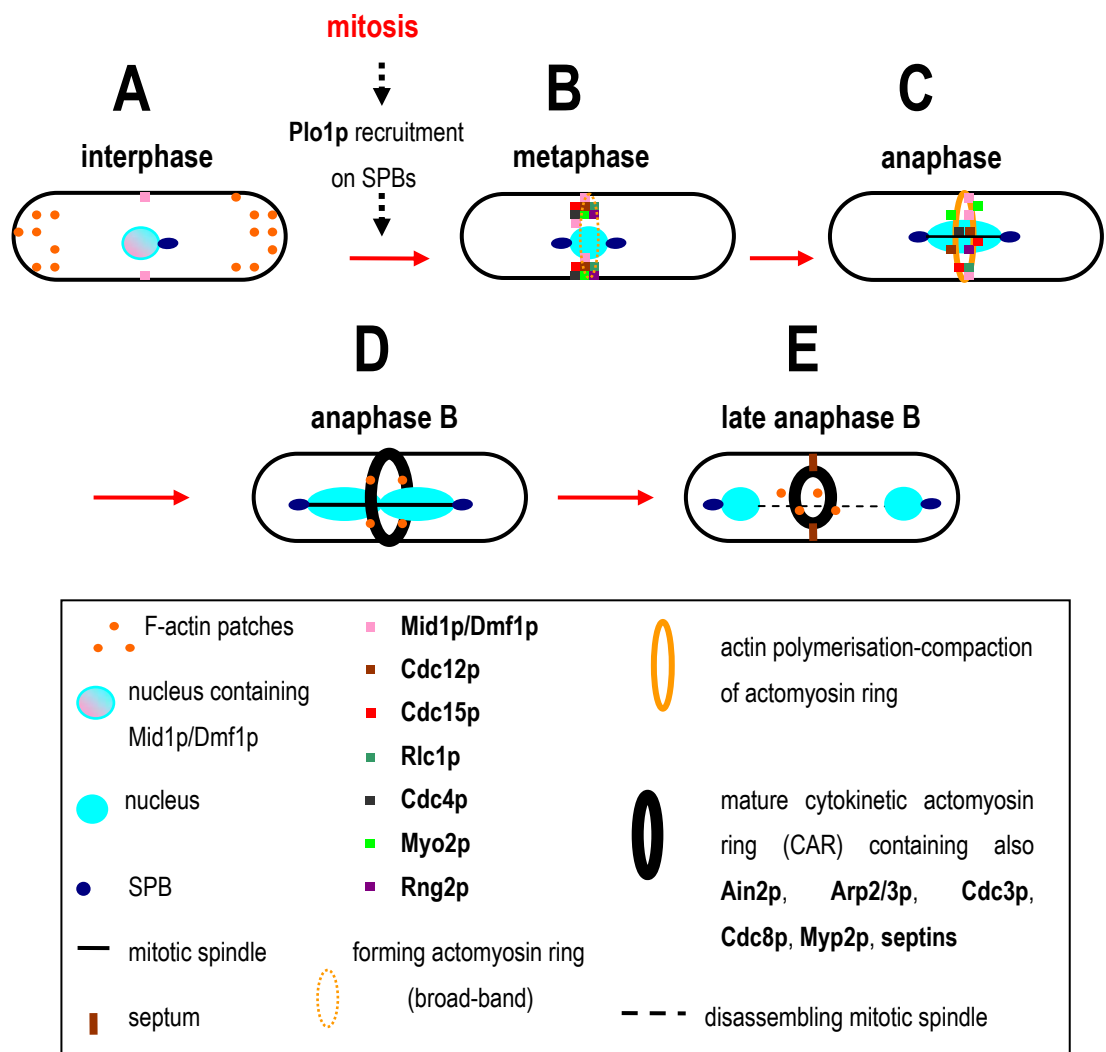


Figure 1.7: Actomyosin ring assembly and constriction to bring about septation and cytokinesis in *S. pombe*. (A) In interphase Mid1p/Dmf1p localises predominantly in the nucleus but also shuttles between the nucleus and the cell cortex to mark the division plane. (B) After mitotic entry, Mid1p/Dmf1p localises into the medial cell cortex in a Plo1p-dependent manner and promotes recruitment of actomyosin ring components. (C-E) Initially, Myo2p, Cdc4p, Rlc1p, Cdc12p, Cdc15p and Rng2p accumulate into a broad medial ring structure and promote the recruitment of Cdc3p and the Arp2/3p complex, which in turn induce actin polymerisation. Localisation of Cdc8p and Ain2p to the medial actomyosin ring enables its transformation into a compact ring structure. Finally, assembly of various proteins, including Myp2p, septins and F-actin patches leads to the formation of a mature actomyosin ring in late anaphase (anaphase B). After SIN activation, the actomyosin ring constricts to bring about septation and cytokinesis.

1.3.2.5.2 Regulation of cytokinesis-Septation Initiation Network (SIN)

Constriction of the actomyosin ring and septum formation is a highly co-ordinated process that necessitates the inactivation of Cdc2p-Cdc13p complexes, since the presence of a high content of mitotic Cdk activity inhibits cytokinesis. Coupling of Cdc2p-Cdc13p inactivation to cytokinesis to ensure that cell division occurs after chromosome segregation is mediated by the SIN signalling pathway (Figure 1.8) (Balasubramanian *et al.*, 2004; Krapp *et al.*, 2004).

The SIN is a GTPase-driven signalling cascade that comprises Spg1p, a small GTPase, Cdc7p, Sid1p and Sid2p protein kinases and Cdc14p and Mob1p, the associated subunits of Sid1p and Sid2p. The GTPase activating protein (GAP) that converts Spg1p from its GTP- (active) to its GDP- (inactive) bound form is a bi-partite complex, Cdc16p-Byr4p. Cdc16p shares similarities with other GAP proteins, while Byr4p is a scaffold protein that binds to both Spg1p and Cdc16p and is required for the GAP-associated activity of Cdc16p (Simanis, 2003). SIN signalling is mediated through the SPB(s), since all the members of the pathway localise at this sub-cellular structure, either throughout or at specific times during the cell cycle. Accumulation of SIN components to the SPB(s) requires Sid4p and Cdc11p, two proteins that are present at the SPB(s) at all times during the cell cycle and act as a scaffold to orderly anchor SIN proteins (Krapp *et al.*, 2004).

The role of the SIN pathway in the regulation of cytokinesis is exemplified by the phenotypes resulting from loss-of-function mutations and/or overexpression of any of the SIN components. *spg1*, *cdc7*, *sid1*, *sid2*, *cdc11*, *cdc14*, *mob1*, and *sid4* mutants are elongated multinucleate cells that undergo nuclear division in the absence of cytokinesis (SIN phenotype). Moreover, the SIN mutants also fail to contract the actomyosin ring. Unlike the SIN mutants, *cdc16* and *byr4* mutants, or cells overexpressing either *spg1*⁺ or *cdc7*⁺, undergo multiple rounds of septum formation with no cell cleavage (Nurse *et al.*, 1976; Minet *et al.*, 1979; Fankhauser and Simanis, 1994; Song *et al.*, 1996; Balasubramanian *et al.*, 1998; Simanis, 2003).

Despite the presence of a Spg1p-specific GAP, no Spg1p-specific guanine-nucleotide-exchange factor (GEF) exists in *S. pombe*. Nonetheless, in the absence of Cdc16p, Byr4p has been shown to inhibit GTP hydrolysis and release and thereby 'lock' Spg1p in its active form. This suggests that modulating binding of Cdc16p and Byr4p with Spg1p could somehow compensate for the lack of an Spg1p-specific GEF, but experimental evidence in support of such a regulatory model of Spg1p signalling is lacking (Simanis, 2003; Krapp *et al.*, 2004). The Plp1p kinase, shown to associate with Sid4p, is thought to modulate Spg1p signalling through Cdc16p-Byr4p, and promote formation of its active state (Balasubramanian and McCollum, 2003; Krapp *et al.*, 2004). This is a possibility taking into account that Cdc5p, the homologous Plk in budding yeast, regulates in a

phosphorylation-dependent manner the activity of the respective GAP of the MEN cascade, Bub2p-Bfa1p (Hu *et al.*, 2001). Consistent with this, Plo1p is both sufficient and necessary for septum formation and positively regulates SIN activity; compromising Plo1p function abolishes both actomyosin and septum formation, while *plo1⁺* overexpression induces septation even during interphase but only in cells with a functional SIN pathway (Ohkura *et al.*, 1995; Tanaka *et al.*, 2001; Sections 1.3.2.4.2 and 5.1.2).

Localisation of SIN components to SPB(s) during the cell cycle is central to the regulatory network that underlies SIN signalling and initiation of cytokinesis (Figure 1.8). In interphase, Spg1p localises at the SPB in its inactive, GDP-bound state, since Cdc16p-Byr4p is also present at the interphase SPB. Once cells enter mitosis, Byr4p remains but Cdc16p is lost from both SPBs. Meanwhile Spg1p is activated at both SPBs, present in its GTP-bound state. This transition promotes the recruitment to the SPBs of Cdc7p, preferentially binding to Spg1p-GTP. During anaphase B and while inactivation of Cdc2p-Cdc13p complexes takes place, Cdc16p re-appears in only one of the two SPBs to re-form the GAP and turn Spg1p back to its inactive state. As a result, Cdc7p dissociates from this SPB and SIN signalling is turned off. Meanwhile, the presence of both Spg1p-GTP and Cdc7p in the other SPB promotes the recruitment of Sid1p-Cdc14p kinase, which in turn activates the Sid2p-Mob1p kinase, localised at the SPB during mitosis, causing it to translocate to the actomyosin ring, where it is thought to prompt ring constriction and septation (Figure 1.8). Activated Cdc2p-Cdc13p complexes prevent localisation of Sid1p-Cdc14p kinase at the SPB, consistent with mitotic Cdk activity inhibiting cytokinesis and the dependency of the latter on prior Cdc2p-Cdc13p inactivation (Simanis, 2003).

Evidence accumulates for the existence of a cytokinesis checkpoint that monitors contractile ring structures and acts through the SIN pathway and the Clp1p/Flp1p phosphatase to ensure that the cell embarks on another cell cycle after completion of cytokinesis, thereby preventing a sudden increase in Cdc2p-associated activity as a result of delayed or perturbed cytokinesis. It is thought that when cytokinesis is delayed, an active SIN persists and allows Clp1p/Flp1p to remain in an active state at the actomyosin ring, preventing its return to the nucleolus, where it is found during interphase. In turn, Clp1p/Flp1p functions in a Wee1p-and/or Cdc25p-dependent manner to inhibit Cdc2p-associated kinase activity, thus enabling the SIN pathway to complete cytokinesis (Balasubramanian and McCollum, 2003; Krapp *et al.*, 2004; Wolfe and Gould, 2005).

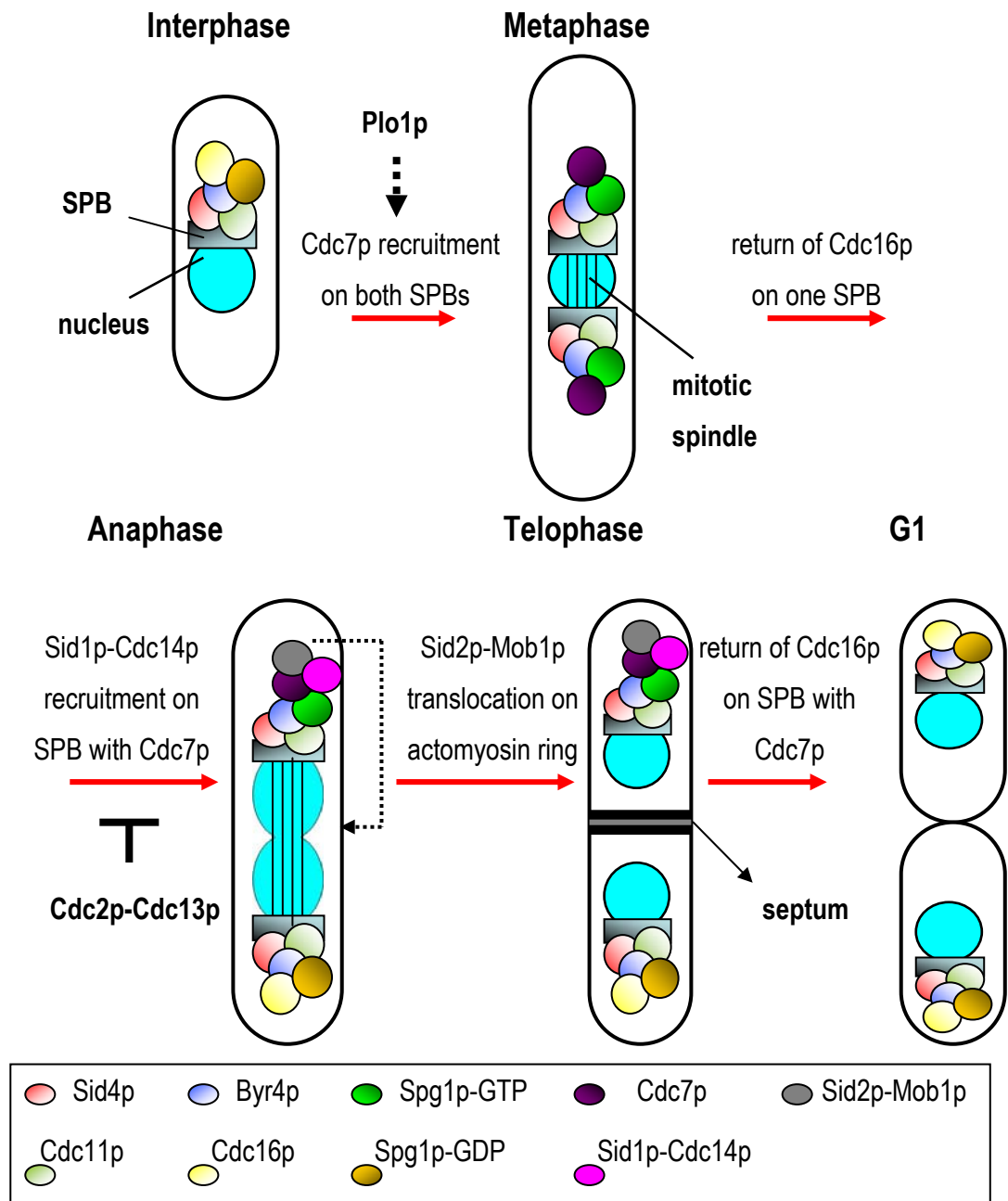


Figure 1.8: Activation of the septation initiation network (SIN) signalling cascade in *S. pombe*. Sid4p and Cdc11p localise to the SPB(s) throughout the cell cycle and function as a scaffold for the anchoring of SIN components. Spg1p, the GTPase, is also constantly present at the SPB(s), but is kept inactive during interphase through the action of the Cdc16p-Byr4p GAP. Plo1p localises at the SPBs upon mitotic entry and promotes formation of the active form of Spg1p (GTP-bound), possibly acting through Cdc16p-Byr4p. During metaphase Cdc16p leaves both SPBs enabling formation of the active state of Spg1p and ensuing recruitment of Cdc7p. At anaphase B, Cdc16p returns on one SPB, leading to Spg1p inactivation and loss of Cdc7p from this SPB. Meanwhile, the other SPB retaining Cdc7p promotes recruitment of Sid1p-Cdc14p concomitant with inactivation of Cdc2p-Cdc13p, which when active inhibits localisation of Sid1p-Cdc14p at the SPB. Next, Sid1p-Cdc14p mediates activation of Sid2p-Mob1p, constantly present at the SPB(s). Finally, activated Sid2p-Mob1p translocates to the actomyosin medial ring and promotes ring constriction and septation.

1.3.3 Cell cycle control in budding yeast: an overview

Whereas *S. pombe* divides by fission through the formation of a septum late in the cell cycle, *S. cerevisiae* divides by budding, mediated by a bud that appears soon after the cell progresses past START, almost coincidentally with other important cell cycle events, including initiation of DNA replication and SPB duplication. Bud formation requires Cdc28p-Clnp associated-activity, and is mediated by actin cortical patches and a septin-based ring structure, the latter initially marking the bud site and later on, as the bud emerges and grows, the bud neck between the mother and the daughter cell (Barral *et al.*, 2000; Cid *et al.*, 2002).

During G1 phase in budding yeast, the SPB embedded in the nuclear envelope, moves and orients itself towards the future bud site, either preceding or in parallel with bud emergence. Following SPB duplication, SPB separation occurs almost concomitant with S phase and requires the activity of Cdc28p-Clbp complexes. Nonetheless, the two SPBs remain next to each other until S phase completion. After this time and while the bud has already grown to a third of its size, the SPBs migrate away from each other towards the opposite sides of the nucleus and, via the activity of Cdc28p-Clbp complexes, a short pre-mitotic spindle forms between them, located in the mother cell. Prior to M phase the nucleus and the short spindle move into the mother-bud neck and orient themselves along the mother-bud axis. Once oriented, the nucleus awaits in this configuration with the short spindle until anaphase onset, where the spindle elongates through the bud neck and towards the daughter cell (Lim *et al.*, 1996; Lew *et al.*, 1997; Cid *et al.*, 2002).

In *S. cerevisiae*, the budding mode of division necessitates that events, such as SPB duplication and separation and ensuing mitotic spindle formation, take place early in the cell cycle to ensure timely migration of the nucleus into the bud neck, therefore precluding a clear distinction of S, G2 and M phases. Although the differences in the dividing pattern and life style between *S. pombe* and *S. cerevisiae* dictate some variations in the underlying regulatory network that drives progress through the mitotic cell cycle, the majority of its molecular components and their associated modes of function are well-conserved between the two species (Lew *et al.*, 1997).

Similar to *S. pombe*, *S. cerevisiae* possesses a single Cdk, Cdc28p, which binds to different cyclin subunits to control mitotic progression. Three G1 cyclins (Cln1-3p) associate with Cdc28p to promote advancement past START and prepare the cell for the ensuing S phase, promoting initiation of DNA replication, spindle pole body duplication and bud formation. START is a major cell cycle control point in budding yeast and its progression is determined by the availability of nutrients and the presence of mating factors (Madden and Snyder, 1998). Once the cells pass START, Cdc28p associates with six B-type cyclins (Clb1-6p) to drive the rest of the cell cycle. Advancement through

S phase requires Clb5p and Clb6p, while Clb1-4p are required for G2 and mitosis; Clb3-4p promote mitotic spindle formation, and Clb1-2p drive bud growth and nuclear division (Huberman, 1996; Nasmyth, 1996; Lew *et al.*, 1997; Section 1.3.1).

S. cerevisiae cyclin levels oscillate periodically during the cell cycle as a result of cell cycle specific transcription and/or proteolytic degradation. Cell cycle specific expression of *CLN1* and *CLN2*, peaking during the G1-S interval, is mediated by the SCB-binding factor (SBF) transcription factor complex, comprising Swi4p and Swi6p, and the SWI4/6 cell cycle box (SCB) promoter element (Ogas *et al.*, 1991; Koch and Nasmyth, 1994; Iyer *et al.*, 2001). *CLB5* and *CLB6* are also expressed coincidentally with *CLN1* and *CLN2* due to transcriptional activation by the bi-partite MBF complex that is related to SBF, containing Swi6p and Mbp1p, and binding to the MluI cell cycle box (MCB) promoter element (Koch *et al.*, 1993; Breeden, 2003).

Cln3p positively regulates G1-S specific transcription of *CLN1*, *CLN2*, *CLB5* and *CLB6* through the function of its associated Cdc28p kinase activity and is itself periodically transcribed, its mRNA levels peaking during the M-G1 boundary, subject to transcriptional regulation by the early cell cycle box (ECB) and the Mcm1p transcription factor (McInerney *et al.*, 1997; MacKay *et al.*, 2001; Wijnen *et al.*, 2002; Bähler, 2005).

Although present during the M-G1 interval, Clb5p and Clb6p are kept inactive during this time via their association with Sic1p, which also accumulates during the M-G1 interval due to transcriptional activation by the Swi5p transcription factor (Toyn *et al.*, 1997; Wittenberg and Reed, 2005). At this early time in the cell cycle, Sic1p inhibits both S phase (Clb5p and Clb6p) and mitotic cyclins (Clb1-4p) that persist from the previous cell cycle, having escaped APC/C-driven proteolysis, mediated either by Cdc20p (Clb3p and Clb5p), which also promotes advancement into anaphase and sister-chromatid separation by inducing the ubiquitination of the Pds1p securin, or Cdh1p/Hct1p (Clb3p and Clb2p) specificity factors (Zachariae, 1999). As a result, Clb-associated Cdc28p activity is kept minimal, allowing the loading of MCM and Cdc6p onto replication origins, thus promoting pre-RC assembly and licensing the DNA for replication during the ensuing S phase (Schwob *et al.*, 1994; Donovan *et al.*, 1997; Kramer *et al.*, 1998; Section 1.3.2.2.2). Sic1p persists until the end of G1 phase, where it is targeted for degradation, promoting an increase in Cdc28p-Clb5-6p activity and triggering S phase entry (Section 1.3.1).

Clb3p and Clb4p also accumulate periodically during the cell cycle, their transcripts peaking during S phase and G2, coincidentally with their functions in SPB separation (Lew *et al.*, 1997). Expression of Clb1p and Clb2p cyclins follows that of Clb3p and Clb4p, beginning to rise in late S phase, peaking during mitosis and disappearing during anaphase, as a result of transcriptional activation during the G2-M interval, mediated by the Mcm1p-Fkh2p-Ndd1p complex and the kinase

activities of Cdc28p-Clb2p and Cdc5p, as well as APC/C-driven proteolysis (Althoefer *et al.*, 1995; Maher *et al.*, 1995; Zachariae, 1999; Zachariae and Nasmyth, 1999; Koranda *et al.*, 2000; Kumar *et al.*, 2000; Pic *et al.*, 2000; Zhu *et al.*, 2000; Hollenhorst *et al.*, 2001; Darieva *et al.*, 2003; Reynolds *et al.*, 2003; Pic-Taylor *et al.*, 2004; Darieva *et al.*, 2006; Section 3.1.2).

Similar to other eukaryotes, mitotic Cdk activity drives entry into mitosis in *S. cerevisiae*, mediated by Cdc28p and its associated B-type cyclins, Clb3-4p, promoting spindle formation and Clb1-2p, the only indispensable pair of *S. cerevisiae* cyclins, driving nuclear division (Surana *et al.*, 1991; Lew *et al.*, 1997). The Swe1p kinase, a Wee1p homologue, is responsible for inhibitory phosphorylation of mitotic Cdc28p-Clbp complexes at Tyr-19 (the equivalent to Cdc2p Tyr-15), whereas Mih1p, a Cdc25p-related phosphatase, removes the inhibitory phosphate at this position (Russell *et al.*, 1989; Booher *et al.*, 1993). Swe1p-dependent phosphorylation of Cdc28p links entry into mitosis with correct bud formation and growth, via the so-called morphogenesis checkpoint pathway (MCP), the latter monitoring proper assembly of septin- and/or actin-based ring structures in the bud neck (Madden and Snyder, 1998; McMillan *et al.*, 1999; Cid *et al.*, 2002; Sakchaisri *et al.*, 2004).

In the course of a normal cell cycle, Swe1p accumulates periodically during late G1 and S phase, transcribed maximally during late G1, and then becomes hyperphosphorylated, leading to its SCF-dependent proteolytic degradation at the G2-M transition, hence allowing entry into mitosis (Kaiser *et al.*, 1998; Shulewitz *et al.*, 1999). However, defects in actin or septin filament assembly at the bud neck cause hypophosphorylation and stabilisation of Swe1p, which can then phosphorylate and inhibit Cdc28p-Clbp complexes, leading to a G2 delay. Therefore, tight regulation of Swe1 activity is an important determinant of the timing of mitotic onset (McMillan *et al.*, 1999; Sakchaisri *et al.*, 2004).

Hsl1p, an *S. pombe* Nim1p/Cdr1p homologue, in concert with Hsl7p, negatively regulates Swe1p, promoting its hyperphosphorylation and degradation (McMillan *et al.*, 1999; Shulewitz *et al.*, 1999). During unperturbed bud formation, septin-dependent localisation of Hsl1p to the bud neck drives together with Hsl7p, thought to act as an adapter, recruitment of Swe1p. Hsl7p then mediates hyperphosphorylation of Swe1p, either directly and/or more likely indirectly, perhaps by promoting the association with the bud neck of other kinases capable of phosphorylating Swe1p, including the *S. cerevisiae* Plk, Cdc5p. Consistent with this observation, Cdc5p interacts via its polo-box domain with septins at the bud neck, is necessary for Swe1p phosphorylation *in vivo*, and can phosphorylate Swe1p both *in vivo* and *in vitro*. Swe1p hyperphosphorylation leads to its ubiquitination and degradation, which in turn enables activation of Cdc28p-Clb complexes and entry into mitosis (Song *et al.*, 2000; Bartholomew *et al.*, 2001; Sakchaisri *et al.*, 2004).

Similar to Plo1p in fission yeast, Cdc5p has multiple roles in mitotic progression in budding yeast. Among others, Cdc5p phosphorylates the cohesin subunit Scc1p, promoting cleavage by the separase Esp1p; and is also a component and regulates at distinct levels the MEN pathway, which functions predominantly to promote inactivation of mitotic Cdc28p-Clbp complexes, thereby enabling exit from mitosis (Alexandru *et al.*, 2001; Bardin and Amon, 2001; Hu *et al.*, 2001; Lee *et al.*, 2001; Pereira and Schiebel, 2001; Cid *et al.*, 2002; Section 1.3.3.1). Moreover, Cdc5p is both a regulator and a substrate of the APC/C, since it is necessary for APC/C-dependent proteolysis of Clb2p during anaphase, and is itself degraded in an APC/C-dependent manner during late anaphase.

Unlike Plo1p, whose protein levels remain constant throughout the cell cycle, Cdc5p accumulates periodically, with levels peaking during G2-M phase and disappearing at late anaphase (Shirayama *et al.*, 1998). The oscillating cell cycle pattern of Cdc5p is the result of both cell cycle specific transcription and proteolysis. The Mcm1p-Fkh2p-Ndd1p complex regulates G2-M-specific transcription of *CDC5* and many other 'CLB2' cluster genes, mediated by the association of Mcm1p and Fkh2p transcription factors with their cognate promoter elements (Althoefer *et al.*, 1995; Maher *et al.*, 1995; Koranda *et al.*, 2000; Kumar *et al.*, 2000; Pic *et al.*, 2000; Zhu *et al.*, 2000; Hollenhorst *et al.*, 2001; Darieva *et al.*, 2003). Notably, Mcm1p-Fkh2p-Ndd1p-driven transcriptional activation requires the concerted functions of Cdc28p-Clbp complexes, as well as of Cdc5p itself, suggesting that both Clb2p and Cdc5p control their own expression via autoregulatory feedback mechanisms (Pic-Taylor *et al.*, 2004; Reynolds *et al.*, 2003; Darieva *et al.*, 2006; Section 1.5.1.4). Cell cycle specific APC/C-dependent proteolysis of Cdc5p requires two destruction boxes (absent from Plo1p) present in the N-terminus of the kinase, and is mediated by Hct1p/Cdh1p (Nigg, 1998; Shirayama *et al.*, 1998).

1.3.3.1 Mitotic exit network (MEN)

Similar to other eukaryotes, mitotic exit in *S. cerevisiae* requires inactivation of mitotic Cdc28p-Clbp complexes (Bardin and Amon, 2001; Pintard and Peter, 2001). The MEN, a signalling cascade analogous to SIN, is necessary both for exit from mitosis, promoting mitotic Cdk inactivation, and also regulates initiation of cytokinesis. Indeed, most MEN mutants arrest in late anaphase with separated chromosomes, elongated spindles and a high content of mitotic Cdc28p-Clbp activity, whereas a few other MEN mutants manage to exit mitosis but are unable to undergo cytokinesis, emphasising the importance of the MEN for both processes (McCollum and Gould, 2001; Simanis, 2003).

Inactivation of mitotic Cdk complexes in most eukaryotes initiates at the metaphase-anaphase transition, but in budding yeast a fraction of activated Cdc28p-Clbp complexes persists beyond this point until completion of spindle elongation. It is believed that a spindle orientation checkpoint operates in budding yeast that maintains this pool of mitotic Cdc28p activity until the spindle has traversed through the bud neck and is properly placed, with one pole in the mother and the other in the daughter cell (McCollum and Gould, 2001; Simanis, 2003).

The APC/C together with Cdc20p mediates destruction of Clbps at the metaphase-anaphase transition, while after anaphase and during G1 phase Clbps are degraded by the APC/C in a Cdh1p/Hct1p-dependent manner. Sic1p also acts in late mitosis and G1 phase to inhibit Cdc28p-Clbp complexes, promoting their inactivation (Morgan, 1999). Nonetheless, Cdc28p-associated activity itself negatively regulates Cdh1p/Hct1p and Sic1p, thereby blocking mitotic Cdc28p inactivation; Cdc28p-Clb2p phosphorylates Cdh1p/Hct1p and inhibits its activity, Cdc28p-Clnp complexes phosphorylate and target for degradation Sic1p, while Swi5p, activating transcription of Sic1p during late M-G1 phase, is kept in the cytoplasm during G2 and mitosis in a Cdc28p-dependent manner (Moll *et al.*, 1991; Verma *et al.*, 1997; Jaspersen *et al.*, 1999).

The Cdc14p phosphatase (a Clp1p/Flp1p homologue) is a vital factor for inactivation of mitotic Cdc28p-Clbp complexes in late mitosis, destruction of mitotic Clbps, and ensuing exit from mitosis, since it reverses inhibitory Cdc28p-dependent phosphorylation events. Cdc14p dephosphorylates Sic1p to promote its stabilisation, Swi5p to allow its accumulation into the nucleus, and Cdh1p/Hct1p to enable its interaction with the APC/C proteolytic machinery (Jaspersen *et al.*, 1999; Morgan, 1999; Bardin and Amon, 2001).

Localisation is a major determinant of the availability of Cdc14p to its substrates. During G1, S, G2 and early mitosis, Cdc14p is sequestered away into the nucleolus, bound to Net1p/Cfi1p. In early anaphase, the combined actions of various proteins, including Cdc5p, transiently release Cdc14p into the nucleus and cytoplasm. Next, the MEN (of which Cdc14p is considered to be a component) functions at the end of anaphase and telophase to maintain Cdc14p out of the nucleolus and allow it to interact with the SPB (Yoshida *et al.*, 2002; Yoshida and Toh-e, 2002; Stegmeier *et al.*, 2002).

Similar to the SIN, the MEN is a GTPase-driven signalling cascade that comprises the Tem1p GTPase, the Bfa1p-Bub2p GAP, the Lte1p GEF, the Cdc5p, Cdc15p, Dbf2p and Dbf20p kinases, Mob1p (a Dbf2p-associated factor) and Nud1p an SPB component (Bardin and Amon, 2001; Pereira and Schiebel, 2001; Yoshida and Toh-e, 2002). The MEN components are asymmetrically distributed between the two SPBs, suggesting that asymmetry is a conserved feature between the SIN and the MEN. In budding yeast the asymmetric SPB localisation of MEN members

is thought to accommodate, via the action of the spindle orientation checkpoint, the dependency of activation of this signalling cascade on prior successful extension of the anaphase spindle through the bud neck (McCollum and Gould, 2001; Pereira and Schiebel, 2001; Simanis, 2003).

Lte1p is spatially restricted into the bud cortex until late anaphase, after which is released into the cytoplasm, while Tem1p, Cdc15p and Bub2p-Bfa1p localise during mitosis at the cytoplasmic phase of the SPB that eventually enters the bud. Nud1p is thought to act as a scaffold protein for the association of MEN components with the SPB. Tem1p is kept inactive (GDP-bound form), via the action of the GAP, until the SPB migrates into the bud during late anaphase. There, the GTPase comes into contact with Lte1p that converts it into the GTP-bound form, leading to MEN activation (Figure 1.9A). Cdc5p also impinges at various levels on the MEN pathway to regulate its activity. For example, Cdc5p is believed to phosphorylate Bfa1p, although it is still unclear whether this phosphorylation event promotes or inhibits its associated GAP activity, and is also thought to modulate activation of the Dbf2p kinase (Figure 1.9B) (Hu *et al.*, 2001; Lee *et al.*, 2001; Pereira and Schiebel, 2001).

Once active, Tem1p is thought to transduce a signal to Cdc15p, which then phosphorylates and activates the Dbf2p-kinase in a Mob1p-dependent manner. Although the exact mechanism is not yet clear, Dbf2p-Mob1p-associated activity promotes the dissociation of Cdc14p from Net1p/Cfi1p, and thus the release of the phosphatase out of the nucleolus (Figure 1.9) (Bardin and Amon, 2001; Pereira and Schiebel, 2001; Pintard and Peter, 2001; Stegmeier *et al.*, 2002). Once released, Cdc14p functions to promote both mitotic exit and cytokinesis. Indeed, Cdc14p has been shown to dephosphorylate Cdc15p, an event thought to allow the latter to perform a function required for cytokinesis (Figure 1.9B) (Menssen *et al.*, 2001).

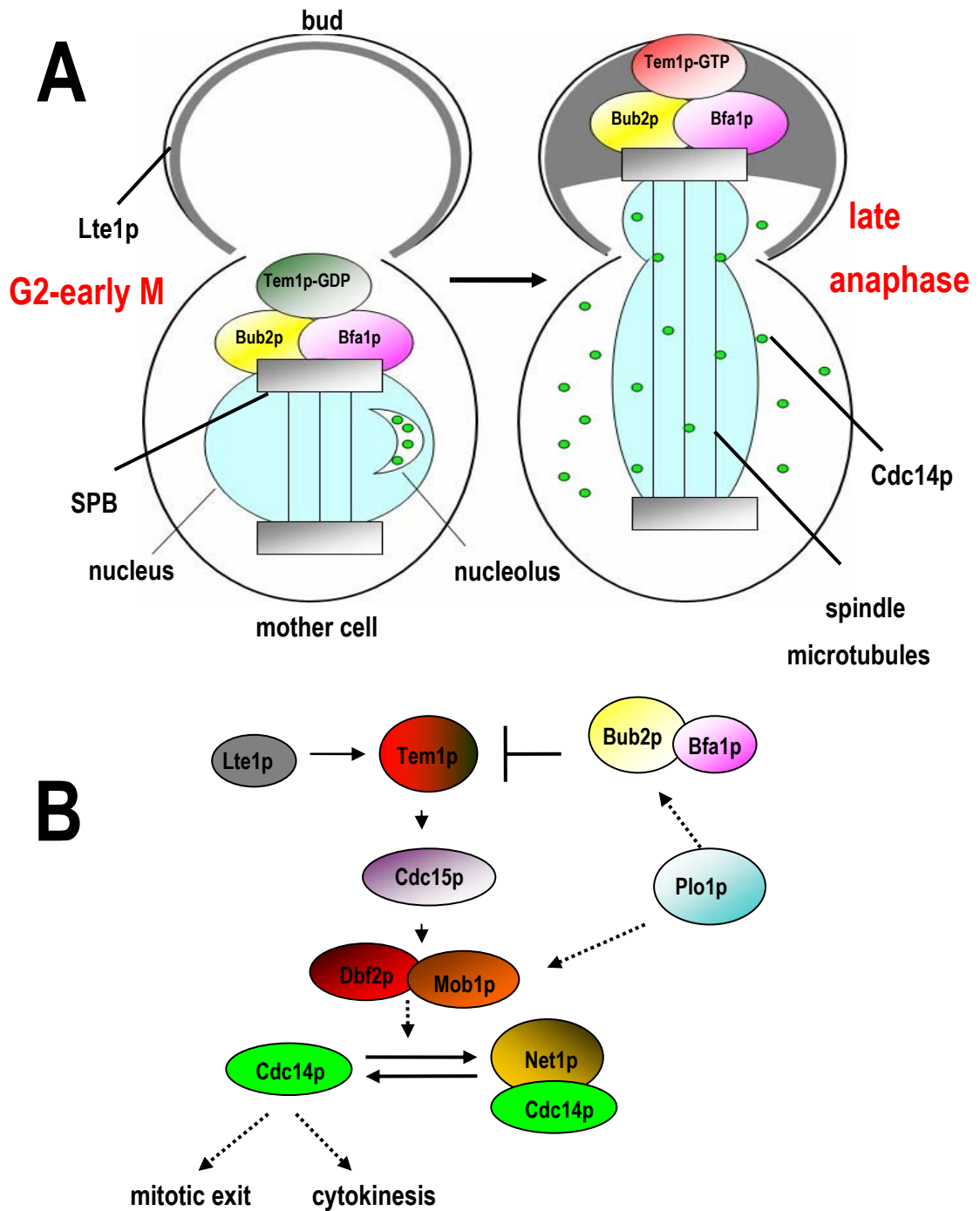


Figure 1.9: The mitotic exit network (MEN) pathway in *S. cerevisiae*. (A) Asymmetric distribution of the Tem1p GTPase, the Bub2p-Bfa1p GAP and the Lte1p GEF between the mother cell and the bud to ensure that the MEN signalling cascade is activated during late anaphase after the spindle has successfully extended through the bud neck and the SPB of the future daughter cell has entered the bud. (B) Active Tem1p (GTP-bound form) transduces a signal to Cdc15p, which then activates the Dbf2p-kinase. Dbf2p-associated kinase activity mediates the release of Cdc14p from the nucleolus by promoting its dissociation from Net1p/Cfi1p. After its release, Cdc14p functions to promote both mitotic exit and cytokinesis.

1.4 Eukaryotic transcription

Three distinct DNA-dependent RNA polymerases mediate transcription in eukaryotic cells: RNA polymerase I (Pol I) synthesises rRNA; RNA polymerase III (Pol III) makes a variety of small RNAs, including tRNAs, 5S rRNA, U6 snRNA and transcribes various gene families of repetitive short interspersed elements (SINEs), present in mammals; RNA polymerase II (Pol II) is responsible for transcription of all the protein coding genes, thereby synthesising a vast number of gene-specific mRNAs. The eukaryotic RNA polymerases are multisubunit protein complexes, comprising both polymerase-specific subunits, as well as polypeptides found in all three enzymes, providing a means of co-regulation of their activities. A common feature of Pols I, II and III, is their requirement for accessory factors, acting at the promoter region and directing RNA polymerase recruitment, as well as accurate initiation of transcription, at the start site, typically labelled '+1'. Each RNA polymerase has its own specific set of accessory and general transcription factors (GTFs), constituting, in conjunction with the corresponding polymerase, the basal transcription apparatus (Lewin, 2000; Paule and White, 2000).

1.4.1 Transcription by RNA polymerase II (Pol II)

The basal Pol II transcription machinery has been conserved between eukaryotes as distant as yeasts and humans, depicted by the resemblances between these species in Pol II composition and structure itself, as well as GTF mode of function in transcription initiation. Moreover, many features of the core promoter, encompassing the transcription start site and the region where the basal Pol II machinery is recruited to initiate transcription, are also shared among eukaryotic genes, both in terms of sequence content and/or position (Hampsey, 1998; White, 2001).

A typical core promoter element is the TATA box [TATA (a/t) AA (g/a) consensus], which is found in a significant proportion of eukaryotic genes. In many species, including humans and *S. pombe*, the TATA element is almost invariably present at a distance 25-30 bp upstream from the transcription start site, while in *S. cerevisiae* its position is more flexible, ranging between 40-120 bp upstream from the start site (Hampsey, 1998; Smale and Kadonaga, 2003). The initiator (Inr) is another core promoter element that surrounds the transcription start site; it is rich in pyrimidine content and can also support transcription initiation in a TATA-independent manner. Indeed, core promoters exist that contain either both a TATA box and an Inr or only a TATA-box or an Inr. Furthermore, in the case of metazoans, ranging from *Drosophila* to humans, promoters devoid of a TATA box contain an Inr, and in many cases another core promoter motif, the downstream promoter

element (DPE) that acts in concert with Inr and is typically found 28-32 bp downstream from the transcription start site (Hampsey, 1998; White, 2001; Kadonaga, 2002; Smale and Kadonaga, 2003). All three types of core promoter elements mediate, although via slightly distinct means, recruitment of TFIID, a multicomponent GTF that comprises the TATA box binding protein (TBP) and several TBP-associated factors (TAFs), a key initial step in the assembly and positioning of the basal Pol II apparatus at the start site (White, 2001; Smale and Kadonaga, 2003; Levine and Tjian, 2003; Section 1.4.1.1).

Similar to *S. cerevisiae* and humans, *S. pombe* Pol II comprises twelve subunits, Rpb1-12p (Ishihama *et al.*, 1998, Lee and Young, 2000). Four subunits form the catalytic core of the enzyme (Rpb1p, Rpb2p, Rpb3 and Rpb11p) and are structurally related to the β , β' , α and α' subunits of the *Escherichia coli* RNA polymerase that is responsible for synthesis of all types of RNAs. Five are also present in Pols I and III (Rpb5p, Rpb6p, Rpb8p, Rpb10p and Rpb12p), while the other three are Pol II-specific (Ishihama *et al.*, 1998; Kimura *et al.*, 2003).

An intriguing highly conserved structural feature of the largest Pol II subunit, Rpb1p, is its C-terminal domain (CTD), which contains tandem repeats of the YSPTSPS heptapeptide sequence. The number of the repeats varies between species, with *S. cerevisiae*, *S. pombe* and human CTD comprising twenty seven, twenty nine and fifty two such heptapeptide repeats, respectively (Hampsey, 1998; Lee and Young, 2000; Kimura *et al.*, 2003). The Rpb1p CTD is subject to phosphorylation, mediated in *S. pombe* at least in part by Mcs6p-Mcs2p, itself a Cdk that also phosphorylates Cdc2p (Section 1.3.2.3). Notably, the unphosphorylated-CTD form of Rpb1p is found only in transcription initiation-related Pol II complexes, whereas its hyperphosphorylated form is present in transcription elongation-related Pol II complexes, suggesting that CTD-dependent phosphorylation promotes the transition from the initiation to the elongation step of transcription (Hampsey, 1998).

1.4.1.1 Initiation of transcription-preinitiation complex (PIC) assembly

As revealed by several studies, initiation of basal transcription *in vitro* requires the ordered assembly at the promoter of the preinitiation complex (PIC), consisting of the TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH general transcription factors, as well as Pol II (Figure 1. 10). In the case of TATA-containing Pol II promoters, TFIID initially binds to the TATA element via sequence-specific interactions of TBP with the TATA motif. X-ray crystallographic analysis has shown that TBP resembles a saddle and binds to the promoter by sitting atop and sharply bending the DNA, making sequence specific interactions with the minor groove of the TATA element (Buratowski, 1994; White,

2001; Lee and Young, 2002). In the case of TATA-less promoters, TFIID binds to DPE and Inr elements, through the functions of its associated TAFs (Levine and Tjian, 2003).

TFIIA binds to TBP and is thought to assist the interaction of TFIID with the promoter, while its role becomes more apparent under conditions of transcriptional activation. Following association of TFIID with the TATA element, TFIIB is recruited and the DNA-TFIID-TFIIB complex serves as a platform that promotes the binding of a pre-assembled complex of Pol II (in its unphosphorylated CTD form) and TFIIF, both of which interact with TFIIB. Taking into account that TFIIB also interacts with TBP, this GTF acts as a bridge between Pol II and the TATA box and is thought to assist in proper positioning of Pol II relative to the transcription start site. TFIIF tightly binds to Pol II and stabilises the preinitiation complex. Finally, TFIIIE and TFIIH enter the complex forming the preinitiation complex (Figure 1. 10). TFIIIE association precedes and probably facilitates recruitment of TFIIH, a multisubunit complex that exhibits DNA-dependent ATPase and ATP-dependent DNA helicase activities, the latter participating in the unwinding of the DNA around the start site during initiation of transcription. Notably, TFIIH possesses Ser/Thr kinase activity that is responsible for the phosphorylation of the Pol II CTD. Indeed, as shown in both humans and the budding yeast, Cdk7p-cyclin H and Kin28p are themselves components of TFIIH (Buratowski, 1994; Nikolov and Burley, 1997; Hampsey, 1998; Tirode *et al.*, 1999; White, 2001; Lee and Young, 2002; Section 1.3.2.3).

Once the preinitiation complex is assembled, TFIIH-mediated phosphorylation of the CTD releases TFIIIE, which is thought to inhibit the TFIIH-associated helicase activity, allowing unwinding of promoter DNA and ensuing initiation of mRNA synthesis by Pol II. CTD phosphorylation also promotes the release of Pol II from TFIID and other GTFs, enabling it to move away from the start site, associate with appropriate elongation factors and catalyse the elongation step of transcription. In parallel, GTFs, including TFIIB, TFIIIE and TFIIH, also dissociate from the complex (White, 2001; Lee and Young, 2002).

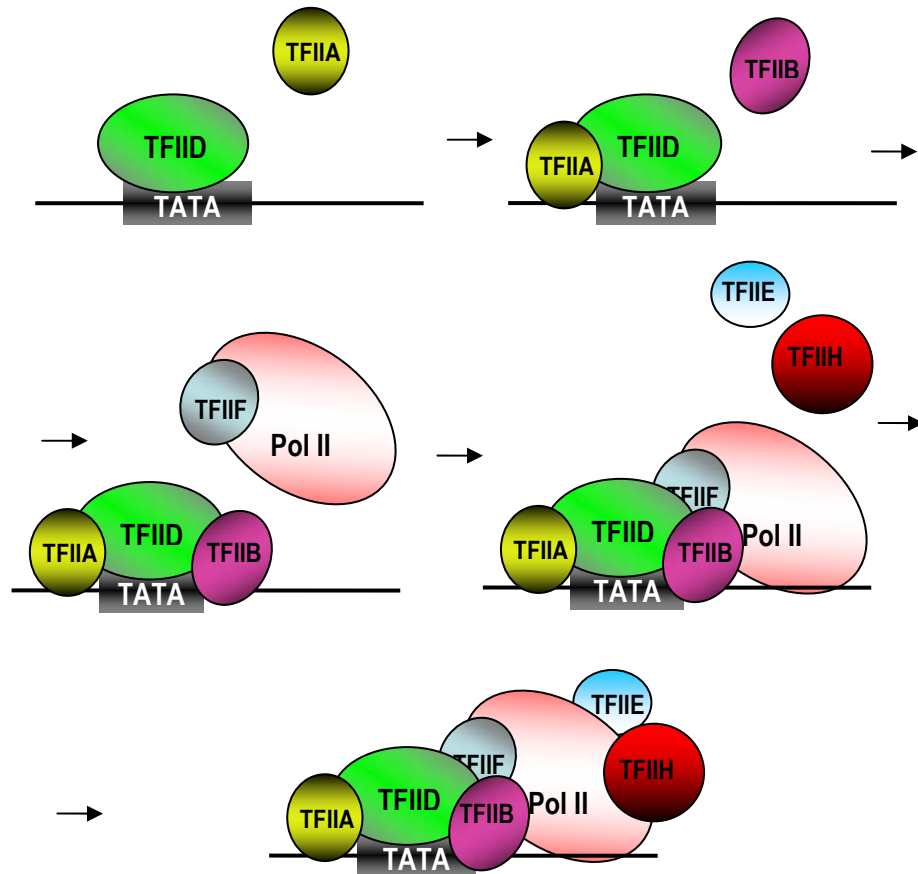


Figure 1.10: Stepwise assembly of the eukaryotic basal Pol II machinery on TATA-containing promoters. Ordered recruitment of TFIIA, TFIIB, TFIIID, TFIIF, TFIIE, TFIIH and Pol II to a TATA-containing promoter to form the preinitiation complex, required for transcription initiation.

1.4.1.2 Regulation of transcription initiation

Transcription initiation is a major control point for regulation of the activity of Pol II. Initiation of mRNA synthesis involves apart from GTFs, various gene-specific transcription factors, either transcriptional activators or repressors, which exert their function by associating with their cognate DNA motifs. The binding sites for activators are termed upstream activating sequences (UAS) or enhancers, while repressors associate with upstream repressing sequences (URS) and silencers.

Typically, the term UAS refers to DNA motifs located a few hundred base pairs upstream from the start site and containing up to three closely associated binding sites for one or two transcription factors. Generally, enhancers are regarded as relatively more complex DNA elements, which in higher eukaryotes encompass several binding sites for at least three transcription factors, are located many kilobases away from the start site regardless of orientation and are involved in cell-type and/or tissue specific gene expression. Similar to enhancers, silencers also function in a

distance or orientation independent manner. In metazoans, the transcriptional control modules of genes are characterised by an intricate organisation of several distinct enhancer and silencer elements, as well as proximal promoter elements, located within a few hundred base pairs from the start site, thus providing a means of combinatorial control by multiple transcription factors, necessary to bring about the elaborate cell-type and tissue specific gene expression patterns in these organisms (Figure 1.11B). Unlike metazoans, the transcriptional control units of unicellular eukaryotes, such as *S. cerevisiae*, are relatively simple, encompassing a UAS and a silencer element, both of which are found within a few hundred base pairs from the core promoter (Figure 1.11A) (Levine and Tjian, 2003; Lodish *et al.*, 2003). Transcription factors associate with their cognate DNA elements and regulate transcription initiation by means of either direct or indirect interactions with components of the basal transcription apparatus, such as the GTFs (Lee and Young, 2002).

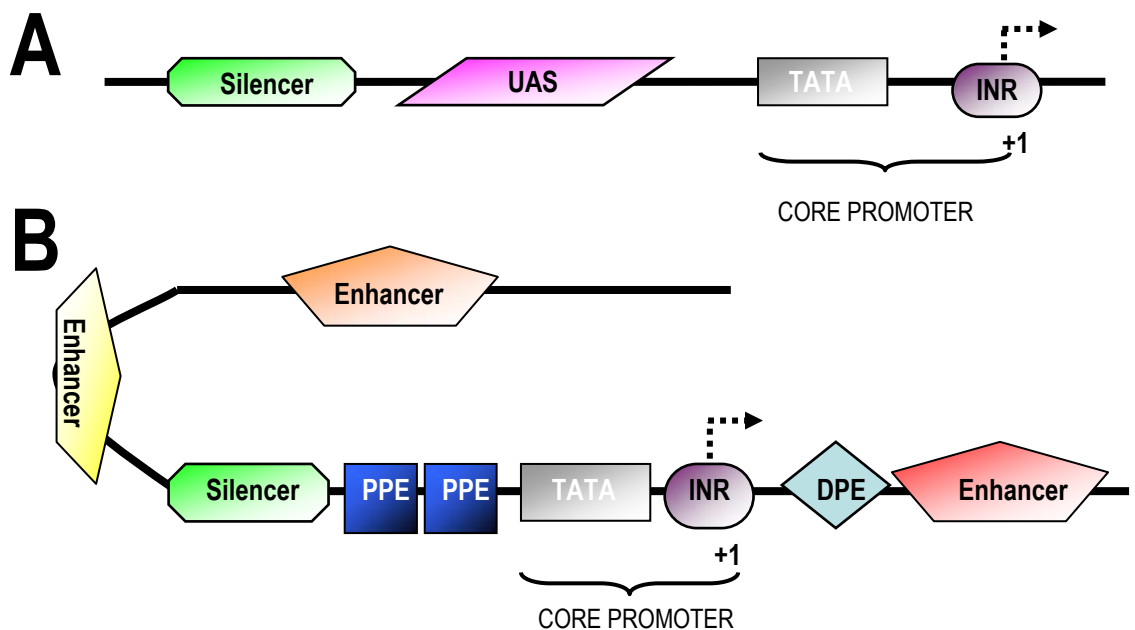


Figure 1.11: Arrangement of *cis*-acting elements in *S. cerevisiae* and metazoan Pol II promoters. (A) A typical Pol II promoter unit in budding yeast contains core promoter elements, such as a TATA box and an initiator element (Inr), as well as an upstream activating sequences (UAS) and a silencer at a distance of no more that a few hundred base pairs from the start site. (B) Metazoan transcriptional regulatory modules are complex and contain apart from the core promoter, itself encompassing a TATA box, an initiator element (Inr) and/or a downstream promoter element (DPE), promoter proximal elements (PPE) within 100-200 bp from the start site and multiple enhancer and silencer elements at distances of thousand of kilobases away from the start site and in either orientation (adapted from Levine and Tjian, 2003).

1.4.1.2.1 *Srb/Mediator*

A subset of GTFs, including TFIIIE, TFIIH, TFIIIF and TFIIIB, either together or individually, have been detected in DNA-free complexes, termed holoenzymes, containing Pol II (Conaway and Conaway, 1993; White, 2001). As initially shown in budding yeast, another multisubunit complex, the Srb/Mediator, has also been identified as a holoenzyme component that interacts with the Pol II CTD (Kim *et al.*, 1994; Koleske and Young, 1994). Metazoans appear to possess more than one Srb/Mediator-like complex. Srb/Mediator constitutes a distinct class of transcriptional regulatory co-factor that functions as an interface between gene-specific transcription factors and the basal transcriptional apparatus, thereby relaying modulatory information between them. Indeed, many transcriptional activators exert their stimulatory role on transcription by directly associating with the Srb/Mediator, which, although itself unable to specifically contact promoter DNA, physically interacts with the Pol II machinery. Apart from its role as a bridge between transcriptional activators and the basal transcription apparatus, Srb/Mediator has also been shown to stimulate the CTD-specific kinase activity of TFIIH (Lee and Young, 2000; Levine and Tjian, 2003; Malik and Roeder, 2000; Myers and Kornberg, 2000; Woychik and Hampsey, 2002).

1.4.1.2.2 *Specific transcription factors: activators and repressors*

Activators are a prerequisite for eukaryotic transcription within cells, since the basal Pol II apparatus is inefficient in accessing DNA packaged into chromatin. Indeed, as shown in budding yeast, TBP associates with core promoters *in vivo* in an activator-dependent manner. Contrary to the basal transcription apparatus, many activators are only modestly inhibited by chromatin and can therefore bind to their cognate DNA and function via distinct mechanisms to promote Pol II recruitment and transcription initiation (Struhl, 1999; White, 2001; Lee and Young, 2002). Unlike transcriptional activators, repressors are not a necessity for maintaining a low level of transcriptional activity in general, but they are important for transcriptional regulation of various eukaryotic genes (Struhl, 1999).

A shared structural feature between transcriptional activators and repressors is their modular nature; they consist of functionally autonomous domains, typically a DNA-binding domain and an activation or repressor domain as appropriate, the former stimulating and the latter inhibiting transcription. The structure of the DNA binding domain confers DNA sequence-binding specificity to a transcription factor, allowing it to associate with only a distinct type of UAS or enhancer in the case of an activator, or a URS or silencer in the case of a repressor (Lodish *et al.*, 2003). These *cis*-acting elements are normally part of the transcriptional control module of more than one gene, thereby

providing a means to the respective transcription factor to co-ordinately regulate transcription and ensuing expression of a group of genes.

There are various types of DNA-binding domains, each containing a characteristic structural motif that mediates binding of the transcription factor to its cognate DNA sequence. In fact, transcription factors are classified according to the type of the structural motif of their DNA-binding domain. Common types of DNA-binding domains found in eukaryotic transcription factors include the homeodomain, consisting of a region with similarity to the helix-turn-helix motif of prokaryotic transcription factors, the related POU domain, found in metazoan transcription factors, the bZIP domain, containing the leucine zipper motif, and the bHLH domain, comprising the helix-loop-helix motif. Moreover, the DNA-binding domains of a plethora of transcription factors contain distinct classes of the zinc finger structural motif, which is also found in non-DNA binding proteins (White, 2001; Lodish *et al.*, 2003). Finally, with relevance to the topic of this thesis, other types of DNA-binding domains include those found in the MADS-box and forkhead/HNF-3 families of transcription factors.

1.4.1.2.3 MADS-box transcription factors

MADS-box transcription factors are a large protein family with members present in a diverse range of eukaryotes (Shore and Sharrocks, 1995). The signature feature of these transcription factors is the MADS-box motif, a 56 amino acid region that is found within the DNA-binding domain and supports both DNA-binding specificity and dimer formation. MADS-box proteins are named after five family members; Mcm1p and Arg80p in budding yeast, Agamous and Deficiens in plants and SRF (serum response factor) in humans (Dubois *et al.*, 1987; Norman *et al.*, 1988; Passmore *et al.*, 1989; Sommer *et al.*, 1990; Yanofsky *et al.*, 1990). Thus far, several MADS-box transcription factors have been identified, while as revealed by sequence database searches in plants, many more putative MADS-box genes await their discovery.

A diverse array of biological roles have been assigned to MADS-box transcription factors; SRF is required for the expression of immediate-early genes, such as *c-fos*, following mitogenic stimulation in proliferative cells, as well as of muscle-specific genes in post-mitotic muscle cells; Arg80p regulates the expression of genes involved in arginine metabolism; Deficiens controls the expression of floral organ identity genes. Meanwhile, Mcm1p modulates the expression of genes involved in diverse biological processes, including among others arginine metabolism, cell-type determination, mating and cell cycle regulation (Norman *et al.*, 1988; Sommer *et al.*, 1990; Treisman

and Ammerer, 1992; Shore and Sharrocks, 1995; Althoefer *et al.*, 1995; McInerney *et al.*, 1997; Kumar *et al.*, 2000; Messenguy and Dubois, 2003).

MADS-box transcription factors are classified into two types. Type I members, such as Mcm1p and Arg80p, share greatest similarity with SRF, while type II members are more similar to mammalian myoocyte-specific enhancer factor 2 (MEF2) proteins, which regulate expression of muscle-specific genes. Typically, both SRF-like and MEF2-like transcription factors exist in each eukaryotic species. Sequence alignments have shown that the 56 amino acid MADS-box motif is highly conserved, its N-terminal and C-terminal halves required for DNA binding and dimerisation, respectively. Variations between family members exist in the adjacent, ~30 amino acid, C-terminal region of the MADS-box motif, thought to confer dimerisation specificity and mediate interactions with other proteins (Figure 1.12). In most cases, the MADS-box is present at the N-terminus but in certain proteins, such as SRF, is found near the middle, or in others even towards the C-terminus (Shore and Sharrocks, 1995; Messenguy and Dubois, 2003).

MADS-box family members share similarities in their cognate DNA-binding sites, characterised by a 10 bp AT-rich core sequence, although individual proteins display distinct sequence preferences; SRF-like proteins bind to CC(A/T)₆GG, whereas MEF2-like proteins associate with CTA(A/T)₄TAG (Pollock and Treisman, 1991). Consistent with this, X-ray crystallography studies with MADS-box proteins have shown that the DNA-binding domains of SRF and Mcm1p contact DNA in a similar manner, inducing a comparable degree of DNA bending, while MEF2-like proteins bend the DNA to a lesser extent. Nonetheless, both SRF- and MEF2- like proteins bind to DNA as dimers, their DNA-binding domains adopting an overall similar three-layered dimer structure that fits well with the dyad symmetry of their cognate DNA sequences (Pellegrini *et al.*, 1995; Tan and Richmond, 1998; Huang *et al.*, 2000).

A common characteristic of MADS-box transcription factors is their combinatorial nature; these proteins, apart from homo- and hetero- dimerisation with other MADS-box members, can interact with other transcription factors or co-factors to modulate either positively or negatively transcription of genes with diverse functions. Therefore, the associated DNA-binding partner(s) of MADS-box transcription factors confer regulatory specificity to the resulting modulatory transcription factor complex. For example, in α -haploid cells, Mcm1p, when combined with the a2 protein, represses expression of α -specific genes, whereas together with the a1 protein stimulates expression of α -specific genes (Shore and Sharrocks, 1995; Messenguy and Dubois, 2003). Meanwhile, with relevance to the topic of this thesis, Mcm1p acts in concert with Fkh2p, a forkhead/HNF-3 family member, and the Ndd1p co-activator to mediate G2-M specific expression of the 'CLB2' gene cluster. Mcm1p and Fkh2p bind co-operatively to the promoters of 'CLB2' cluster

genes, with the latter requiring prior binding of the former (Koranda *et al.*, 2000; Kumar *et al.*, 2000; Pic *et al.*, 2000; Zhu *et al.*, 2000; Hollenhorst *et al.*, 2001; Boros *et al.*, 2003; Darieva *et al.*, 2003).

Thus far, three genes encoding MADS-box proteins have been identified in *S. pombe*; *map1⁺* is involved in expression of cell-type specific genes in response to mating-pheromone signalling but has no apparent roles in mitotic cell cycle control (Yabana and Yamamoto, 1996); *pvg4⁺* has been suggested to regulate genes involved in the biosynthesis of the pyruvylated Gal β 1,3-epitope in N-linked glycans (Andreishcheva *et al.*, 2004); *mbx1⁺*, a major topic of this thesis (Chapters 4, 5 and 6), encodes a protein mostly related to Map1p, Mcm1p and Arg80p and is involved together with Fkh2p and Sep1p, two forkhead/HNF-3 transcription factors, in M-G1 specific expression of many genes that control various aspects of mitosis, including sister chromatid separation, septation and cytokinesis (Buck *et al.*, 2004; Bulmer *et al.*, 2004; Rustici *et al.*, 2004; Alonso-Nuñez *et al.*, 2005; Petit *et al.*, 2005; Papadopoulou *et al.*, 2008).

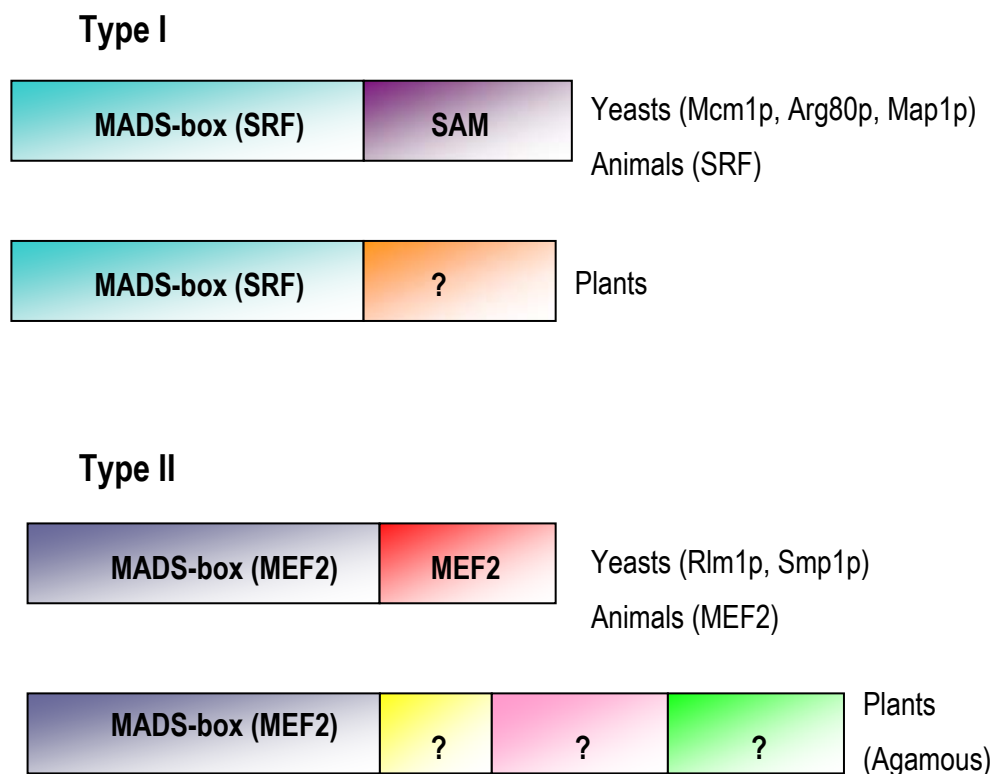


Figure 1.12: Schematic representation of type I and type II classes of MADS (Mcm1p, Agamous or Arg80p, Deficiens and SRF)-box domains and the distinct types of adjacent C-terminal regions. MADS-box domains are classified into type I (SRF-like) and type II (MEF2-like). Both types of MADS-box domains are found in eukaryotes, including fungi, animals and plants. MADS-box family members differ in the region found at the C-terminus of the MADS-box domain; SAM (SRF, Arg80p and Mcm1p) and MEF2 (myocyte-specific enhancer factor 2) represent such C-terminal extensions, the former present in Mcm1p and Arg80p (budding yeast), Map1p (fission yeast) and SRF (animal) and the latter in Rlm1p and Smp1p (budding yeast) and MEF2 (animal). “?” denotes C-terminal domains of plant family members not well characterised (adapted from Messenguy and Dubois, 2003).

1.4.1.2.4 Forkhead/HNF-3 transcription factors

The forkhead/HNF-3 family of transcription factors derives its name from its founding members; the encoded protein of the *Drosophila* forkhead gene, shown to be necessary for the formation of terminal structures in the embryo, and a small group of hepatocyte enriched transcription factors in rodents, the so-called hepatocyte nuclear factor 3 (HNF-3) family (Lai *et al.*, 1990; Weigel and Jäckle, 1990). The nuclear localisation of the forkhead protein in *Drosophila* provided the first clues about the role of forkhead/HNF-3 (termed also forkhead for convenience) proteins as transcriptional regulators. The forkhead gene was shown to be necessary for proper formation of the tissues and organs where it was expressed, since its mutation led to homeotic transformation of gut structures into head-derived components (Kaufmann and Knochel, 1996).

Many diverse eukaryotic species, ranging from the budding and fission yeasts to humans, have been shown to possess several forkhead proteins, with roles in development, differentiation and cell cycle control (Yang *et al.*, 1997; Kaestner *et al.*, 2000; Hollenhorst *et al.*, 2000; Buck *et al.*, 2004; Bulmer *et al.*, 2004; Costa, 2005; Papadopoulou *et al.*, 2008). Forkhead proteins share a 110 amino acid monomeric DNA-binding domain. As revealed by X-ray crystallography studies with an HNF-3 protein, when bound to DNA, the DNA-binding domain adopts a butterfly-like structure, related to the helix-turn-helix motif and consisting of three α helices, an anti-parallel three stranded β -sheet and two intervening loop, 'wing'-like, regions. Due to the presence of the two 'wings' in the DNA-binding domain, forkhead proteins are also termed 'winged helix' (Clark *et al.*, 1993; Kaufmann and Knochel, 1996).

Consistent with the conservation within their DNA-binding domain, forkhead transcription factors recognise a common 7 bp core sequence (RTAAAYA, where R and Y correspond to G/C and C/T). Sequences flanking this core promoter element vary between individual family members and are thought to contribute to binding specificity (Kaufmann *et al.*, 1995; Kaufmann and Knochel, 1996). A subset of forkhead transcription factors contain a phosphopeptide recognition module, the so-called forkhead-associated domain (FHA), mediating binding to proteins phosphorylated at threonine residues. Apart from forkhead/HNF-3 family members, the FHA domain is also present in many other prokaryotic and eukaryotic proteins, involved in several processes, including signal transduction and transcription (Durocher and Jackson, 2002).

As already discussed in the previous section, two forkhead transcription factors in *S. cerevisiae*, Fkh1p and Fkh2p, are together with Mcm1p part of the regulatory network that drives G2-M specific transcription of the 'CLB2' gene cluster. A similar, yet distinct, circuit operates in *S. pombe*, also involving two forkhead transcription factors and a MADS-box protein.

1.4.1.2.5 Mechanisms of transcriptional activation and repression

Activators employ distinct mechanisms to stimulate transcription. In many cases, activators promote recruitment of the basal Pol II apparatus to the promoter region by association via their activation domains either with GTFs, or transcriptional co-activators, such as Srb/Mediator. Alternatively, activators stimulate transcription by mediating changes in chromatin structure, since they are capable of recruiting chromatin remodelling complexes (White, 2001; Lee and Young, 2002). For example, in budding yeast the Swi5p transcription factor positively regulates transcription of the *HO* gene by mediating recruitment to the promoter of the Swi/Snf chromatin modification complex, which in turn facilitates the recruitment of the SAGA complex. The associated histone acetylase activity of SAGA then enables binding of the SBF transcriptional activator, which recruits the basal Pol II apparatus at the *HO* promoter (Cosma *et al.*, 1999). In a few instances, activators stimulate transcription by affecting transcriptional post-initiation steps, such as the elongation rate of Pol II (Blau *et al.*, 1996).

Various repressors have been identified in organisms ranging from the budding yeast to humans, demonstrating the effectiveness of repression as a regulatory mechanism of eukaryotic transcription. Repressor molecules can affect transcription either generally or in a gene-specific manner. Many general repressors negatively regulate transcription by associating with components of the basal transcriptional apparatus. Examples include budding yeast Mot1p and human Dr1p-Drap1p complex, both of which bind to TBP, the former causing it to dissociate from the TATA box, while the latter blocking its interaction with TFIIA and TFIIB. Other repressors function by mediating changes in chromatin structure, including the recruitment of histone deacetylases, which themselves operate as gene-specific or general repressors via their distinct effects on chromatin but are unable to directly interact with DNA. Finally, many gene-specific repressors exert their negative role on transcription by interacting and sequestering an activator or, alternatively, by competing with an activator for overlapping DNA binding sites (Inostroza *et al.*, 1992; Hampsey, 1998; Lee and Young, 2002).

1.5 Cell cycle-regulated transcription in the budding and fission yeasts

Classical single-gene studies and consequent genome-wide microarray analyses in the budding and fission yeasts, combined with the availability of genomic sequence data, have provided a description of the global mRNA program of these model organisms. These genome-wide transcriptional analyses have unravelled the existence of consecutive waves of transcriptional activity during the

mitotic cell cycle, each corresponding to a cluster of co-regulated genes that are periodically transcribed at a specific cell cycle time under control of the same *trans*-acting transcription factors bound to their cognate, *cis*-acting, promoter elements.

Cell cycle-specific transcription is common among genes whose products are required only once at a specific cell cycle point, for instance the proteins necessary for DNA replication, as well as genes encoding proteins functioning as molecular switches within the cell, such as transcription factors. This mode of cell cycle-regulated transcription and ensuing expression might be crucial for normal advancement through the cell cycle, as in the case of cyclins, whose phase-specific expression drives orderly mitotic progression and aberrant accumulation is detrimental for cell viability. Alternatively, cell cycle specific transcription might offer a means of maximising cell economy, ensuring that a gene is transcribed coincidentally with the time of function of its encoded protein within the cell (Futcher, 2000; Breeden, 2003; McInerney, 2004).

In budding yeast, microarray data analysis has demonstrated that the transcripts of up to 800 genes, corresponding to more than 10% of the total number of genes in the genome, fluctuate in abundance in a cell cycle-dependent manner. Based on their cell cycle-dependent transcriptional patterns, these genes are clustered into distinct groups containing co-regulated genes, including the M-G1-, G1- and S- specific gene clusters, as well as the 'CLB2' cluster, with genes maximally transcribed during the G2-M phase (Figure 1.13A) (Cho *et al.*, 1998; Spellman *et al.*, 1998; Wittenberg and Reed, 2005).

Relative to budding yeast, in fission yeast a smaller number of genes are periodically transcribed during the mitotic cell cycle. Depending on individual microarray studies, the mRNA levels of 400-750 genes have been shown to oscillate in abundance during the cell cycle, of which 140 show high-amplitude changes and are classified into four major transcriptional waves, namely the G1-S, S-, G2- and M-G1- specific gene clusters (Figure 1.13B) (McInerney, 2004; Rustici *et al.*, 2004; Oliva *et al.*, 2005; Peng *et al.*, 2005).

Cell cycle specific transcription appears to be a conserved theme among eukaryotes, since large-scale transcriptional analysis in human cells reveals ~800 genes with fluctuations in their transcript levels (Cho *et al.*, 2001). Comparisons between the global transcription programs of the budding and fission yeasts show that they share a common group of ~40 periodically transcribed genes, which also seems to be conserved in human cells, suggesting that for these genes regulation of their expression is of great importance (McInerney, 2004; Wittenberg and Reed, 2005).

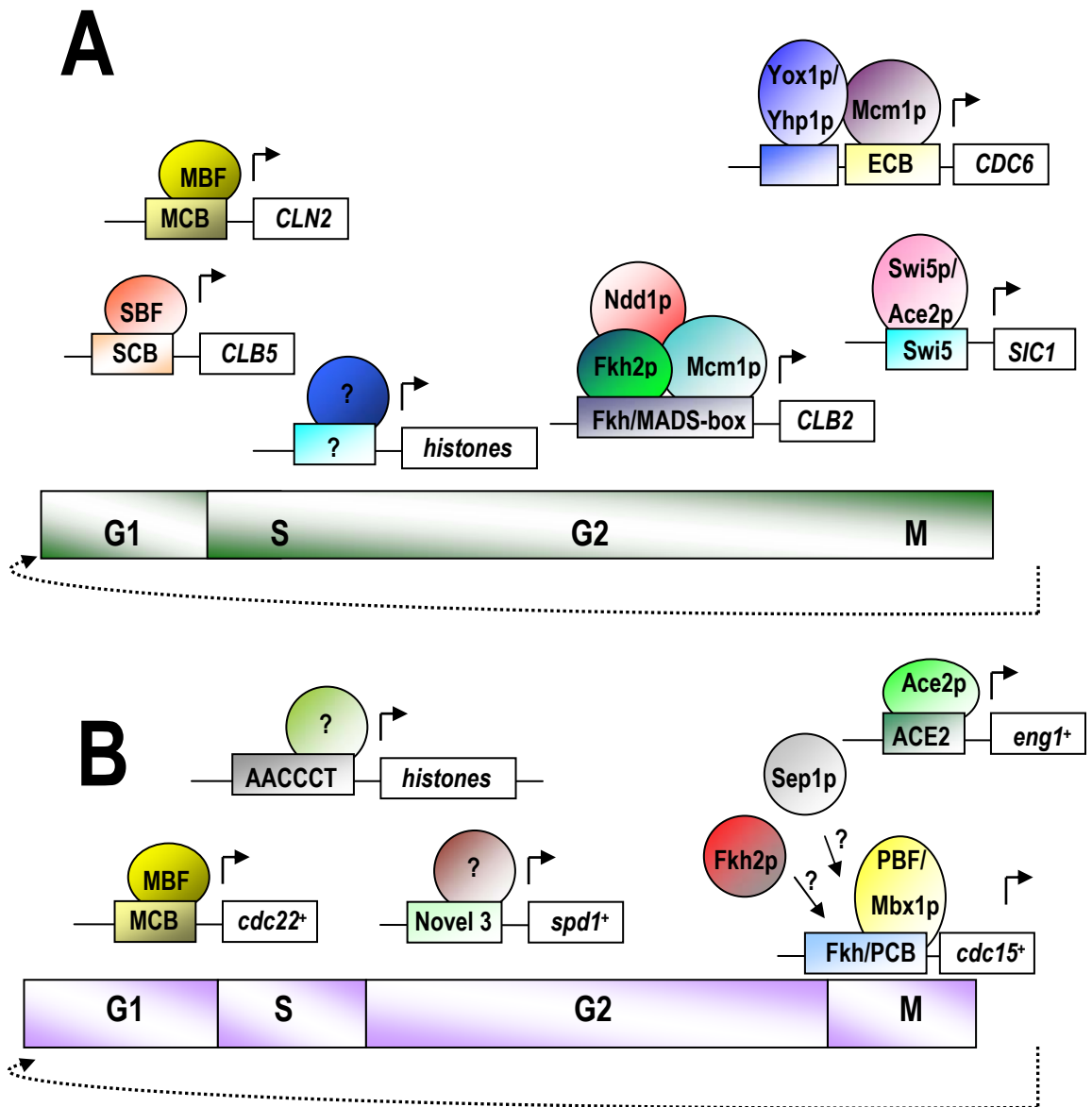


Figure 1.13: Phase-specific gene clusters and their regulatory transcriptional networks in budding and fission yeasts. (A) Six groups of co-transcribed genes exist in *S. cerevisiae*. Two gene groups are expressed during G1 phase under MCB-MBF and SCB-SBF control, respectively. Histone genes are expressed in S phase, while the ‘CLB2’ gene cluster is transcribed during the G2-M interval and is regulated by forkhead/HNF-3 and MADS-box transcription factors and the Ndd1p co-activator protein. Finally, the ‘SIC1’ gene cluster is expressed during the M-G1 interval, subject to Swi5p/Ace2p regulation, while several other genes, such as *CDC6*, are expressed during the M-G1 interval due to transcriptional regulation by the Mcm1p MADS-box transcription factor and the Yox1p and Yhp1p repressors. (B) Five phase-specific groups of co-regulated genes have been identified in *S. pombe*. A gene cluster is specifically transcribed in G1-S phase under MBF/DSC1 control. Histone genes are expressed in S phase, while a diverse group of genes is expressed during G2. Two gene clusters are periodically transcribed during M-G1 phase, the one under control of forkhead/HNF-3 and MADS-box transcription factors, related to and/or part of the PBF complex, while the other is regulated by the Ace2p transcription factor (see Section 1.5 for details of transcriptional mechanisms).

1.5.1 *S. cerevisiae* M-G1, G1, S and G2-M gene clusters and their regulation

1.5.1.1 M-G1 specific gene clusters

Numerous genes are specifically expressed during the M-G1 interval in *S. cerevisiae* (Cho *et al.*, 1998; Spellman *et al.*, 1998). A subset of these genes, including *SWI4* and *CLN3*, as well as *CDC6*, *CDC46* and *CDC47*, encoding proteins with functions in DNA replication, are periodically transcribed during the M-G1 interval due to transcriptional regulation by Mcm1p and the Yox1p and Yhp1p repressors. The early cell cycle box (ECB), a 16 bp palindrome, is present in the promoter regions of these genes and provides a binding site for Mcm1p (McInerney *et al.*, 1997). Yox1p and Yhp1p also bind to their cognate sequence, found nearby the ECB, and can also associate with Mcm1p (Pramila *et al.*, 2002). Mcm1p associates with the ECB throughout the cell cycle, while Yox1p and Yhp1p bind to their cognate sequence and repress transcription at all times but for the M-G1 interval, when they are absent from the cell, thereby allowing transcription to ensue only during this time (Mai *et al.*, 2002; Wittenberg and Reed, 2005).

Another subset of M-G1 expressed genes is the 'SIC1' cluster, named after one of its members, the Cdk inhibitor gene *SIC1* (Spellman *et al.*, 1998). Phase-specific transcription of the 'SIC1' cluster genes is mediated by the Swi5p and Ace2p transcription factors, both of which bind to the same set of DNA-binding sequences (the Swi5p binding site) at the genes' promoters and they can either activate or repress transcription, determined by their associated co-factors (McBride *et al.*, 1999; Wittenberg and Reed, 2005).

1.5.1.2 G1 phase-specific gene cluster

The heterodimeric MBF and SBF transcription factors, sharing a common subunit (Swi6p) and containing different DNA-binding subunits (Mbp1p and Swi4p), regulate late G1 transcription of genes involved in initiation of DNA replication and other events that promote the G1-S transition. MBF and SBF associate with related DNA promoter sequences, termed Mlul cell cycle box (MCB) and Swi4p cell cycle box (SCB), consistent with a partial overlap between these transcription factors in terms of function, DNA-binding specificity and SCB and MCB presence within promoters (Breedon and Nasmyth, 1987; Andrews and Herskowitz, 1989; Koch *et al.*, 1993; Breedon, 1996; Wittenberg and Reed, 2005). Nonetheless, despite these similarities, MBF and SBF appear to regulate functionally discrete groups of genes; MBF-regulated genes, including *RNR1*, *POL2*, *CLB5* and *CLB6*, are implicated in DNA replication and repair, while SBF-regulated genes, including *PCL1*,

PCL2, *GIN4*, *CLN1* and *CLN2*, are important, amongst others, for cell morphogenesis and SPB duplication (Iyer *et al.*, 2001; Simon *et al.*, 2001). SBF- and MBF- dependent transcription is positively regulated by Cdc28p-Cln1-3p complexes. Notably, Cdc28p-Cln3p activates late G1-S specific transcription by phosphorylating and inhibiting Whi5p, a transcriptional repressor that binds to promoters through SBF. During late G1 phase, the associated kinase activity of Cdc28p is thought to activate transcription by promoting Whi5p dissociation from SBF and its exit from the nucleus (Costanzo *et al.*, 2004; DeBruin *et al.*, 2004; Bähler, 2005).

1.5.1.3 S phase-specific gene clusters

Nine genes encode histones in *S. cerevisiae* and are specifically transcribed during S phase. Cell cycle specific transcription of the histone genes involves the Hir1p and Hir2p repressors, which bind to the genes' promoters and are thought to function via the recruitment of chromatin remodelling complexes. Interestingly, SBF and possibly MBF also bind to histone promoters, which contain SBF-related binding sites, but it is so far unclear whether and how these transcription factors are implicated in transcriptional regulation of the histone genes.

Another group of genes, the so-called 'MET' cluster, is also specifically transcribed during S phase. The mechanisms underlying transcriptional control of these genes, most of which are involved in methionine biosynthesis, are not known (Osley *et al.*, 1986; Sherwood *et al.*, 1993; Cho *et al.*, 1998; Spellman *et al.*, 1998; Iyer *et al.*, 2001; Simon *et al.*, 2001; Kato *et al.*, 2004).

1.5.1.4 G2-M specific gene cluster

Several genes, members of the 'CLB2' cluster, are periodically transcribed from late S phase until nuclear division, their transcript levels peaking during the G2-M interval. The *CLB1* and *CLB2* mitotic cyclin genes are constituents of this cluster, hence the name. Other 'CLB2' cluster members include the genes encoding the Cdc5p kinase, the Cdc20p APC/C-dependent specificity factor and the Ace2p and Swi5p transcription factors (Surana *et al.*, 1991; Cho *et al.*, 1998; Spellman *et al.*, 1998).

Early work in transcriptional control of *SWI5* and *CLB2* provided insight into the underlying network that mediates G2-M specific transcription, encompassing a 55-bp UAS and two *trans*-acting factors, Mcm1p and Swi five factor (SFF), the latter an unknown at the time protein. Mcm1p associated as a dimer with its DNA binding site in the UAS on its own, while binding of SFF to its cognate sequence required the formation of a ternary complex with Mcm1p (Lydall *et al.*, 1991; Althoefer *et al.*, 1995; Maher *et al.*, 1995). Later studies have shown that the SFF-binding site

(RTAAAYA), located in close proximity to the Mcm1p-binding site, is identical to the forkhead DNA motif and SFF corresponds to Fkh2p, which is recruited to 'CLB2' cluster promoters in an Mcm1p-dependent manner. Fkh1p also associates with the forkhead DNA motif within 'CLB2' promoter regions and is involved in control of G2-M transcription, displaying both redundant but also distinct functions relative to Fkh2p.

Fkh1p and Fkh2p are highly related, sharing an overall 47% identity and 82% similarity, while their DNA-binding domains are 72% identical and they both contain an FHA domain. Consistent with the similarities between Fkh1p and Fkh2p and partially overlapping functions, deletion of both *FKH1* and *FKH2*, but not either one alone, causes cell morphology and separation defects, delayed mitotic entry and disrupts periodic, G2-M specific transcription of 'CLB2' genes, resulting in basal transcript levels throughout the cell cycle (Hollenhorst *et al.*, 2000; Kumar *et al.*, 2000; Pic *et al.*, 2000; Zhu *et al.*, 2000; Hollenhorst *et al.*, 2001). Differences between Fkh1p and Fkh2p also exist, since Fkh2p- but not Fkh1p- promoter binding requires Mcm1p. The distinct promoter-binding dependencies of the two forkhead transcription factors result from two Fkh2p features; a motif that promotes co-operative binding with Mcm1p and an autoinhibitory domain that prevents DNA binding in the absence of Mcm1p (Hollenhorst *et al.*, 2001; Boros *et al.*, 2003).

Fkh2p and Mcm1p associate with the promoters of G2-M transcribed genes constantly during the cell cycle, necessitating the presence of additional regulatory elements to drive periodic transcriptional activity. Ndd1p, a co-activator molecule that, when overexpressed, up-regulates transcription of 'CLB2' genes, transiently associates with the promoters of these genes during the G2-M interval, leading to transcriptional activation. Ndd1p is itself periodically expressed during S and G2 phases and is recruited to the promoters in a Fkh2p-dependent manner, via its interaction with the FHA domain (Loy *et al.*, 1999; Koranda *et al.*, 2000; Darieva *et al.*, 2003; Reynolds *et al.*, 2003). It is thought that Fkh2p plays both positive and negative roles in transcription of 'CLB2' genes and that Ndd1p recruitment relieves Fkh2p-mediated transcriptional repression. Indeed, deletion of *FKH2* suppresses the lethality caused by deletion of *NDD1*, an essential gene. Apparently, Fkh1p exerts its modulatory role in transcription of the 'CLB2' cluster via an Ndd1p-independent mechanism, since mutants lacking both *FKH1* and *NDD1* are inviable (Koranda *et al.*, 2000).

Early studies have suggested a requirement for Cdc28p-Clbp-related activity for G2-M specific transcription (Amon *et al.*, 1993). Consistent with this, recent work has shown that Cdc28p-Clb2p phosphorylates Ndd1p and promotes its association with the FHA domain of Fkh2p (Darieva *et al.*, 2003; Reynolds *et al.*, 2003). Taking into account that *CLB2* is regulated by the Mcm1p-Fkh2p-Ndd1p complex, it appears that Cdc28p-Clb2p-dependent phosphorylation of Ndd1p is part of a positive feedback loop that operates to further enhance *CLB2* transcription.

Fkh2p is also periodically phosphorylated during the cell cycle with a timing that immediately precedes the onset of expression of 'CLB2' genes (Pic *et al.*, 2000). Cdc28p-Clb5p complexes are predominantly responsible for phosphorylation of Fkh2p, which contains several potential Cdk target sites (S/TP) preferentially located within its extended C-terminal region. Cdc28p-Clb5p-dependent phosphorylation of Fkh2p is thought to enhance intermolecular interactions with Ndd1p, contributing to its recruitment to Mcm1p-Fkh2p complexes and ensuing transcriptional activation (Pic-Taylor *et al.*, 2004).

Regulation of G2-M specific transcription involves yet another kinase, Cdc5p, which is recruited to 'CLB2' cluster promoters in a cell cycle specific manner, coincidentally with transcriptional activation of these genes. Fkh2p interacts with Cdc5p and mediates its recruitment to 'CLB2' promoter regions. Cdc5p then specifically phosphorylates Ndd1p at Ser 85 within a Cdc5p target site that matches the mammalian Plk1p consensus phosphorylation sequence (D/E-X-S/T-φ-X-D/E, where φ is a hydrophobic and X any amino acid). Cdc5p-dependent phosphorylation of Ndd1p is thought to promote cell cycle-specific recruitment of Ndd1p to the promoters of 'CLB2' cluster genes (Darieva *et al.*, 2006). Similar to *CLB2*, *CDC5* is under transcriptional control of the Mcm1p-Fkh2p-Ndd1p complex and at the same time regulates its activity, suggesting that it operates via a positive feedback loop to promote G2-M specific gene expression.

1.5.2 *S. pombe* G1-S, S, G2 and M-G1 gene clusters and their regulation

1.5.2.1 G1-S specific gene cluster

In fission yeast, various genes promoting S phase entry and progression, including those encoding the S phase cyclin Cig2p, the replication factors Cdc18p and Cdt1p and the large subunit of the ribonucleotide reductase Cdc22p, are specifically expressed during the G1-S interval. The MBF/DSC1 transcription factor complex (Section 1.3.2.1), related to MBF in budding yeast, regulates G1-S specific transcription of these genes by binding to promoter sequences identical to the *S. cerevisiae* MCB motif (Lowndes *et al.*, 1992; Kelly *et al.*, 1993; Hofmann and Beach, 1994; Ayte *et al.*, 2001; Maqbool *et al.*, 2003).

At least three proteins are components of the MBF/DSC1 complex: Cdc10p, Res1p and Res2p. Association of the MBF/DSC1 complex with the promoters of its target genes is mediated via the N-terminal DNA-binding domains of Res1p and Res2p, which interact through their C-termini with Cdc10p, a regulatory subunit. Rep2p, a co-regulatory protein that specifically interacts with Res2p, is also considered to be part of the MBF/DSC1 complex (Ayte *et al.*, 1995; Whitehall *et al.*, 1999; White

et al., 2001). An MBF/DSC1-related complex also operates during the fission yeast meiotic cell cycle to regulate transcription of genes involved in pre-meiotic DNA replication (Cunliffe *et al.*, 2004).

The MBF/DSC1 complex exerts both positive and negative regulatory roles in G1-S specific transcription (McInerny, 2004). Early work has implicated Cdc2p-cyclin G1 complexes in regulation of MBF/DSC1-specific transcriptional activity, although recent work suggests that rather another kinase, the Pas1p-Pef1p complex, mediates MBF/DSC1-dependent transcriptional activation during the G1-S interval (Reymond *et al.*, 1993; Whitehall *et al.*, 1999; Bähler, 2005). Moreover, Cig2p has been shown to bind to Res2p and then phosphorylate Res1p, via its associated Cdc2p kinase activity, leading to inhibition of G1-S specific transcription. Since Cig2p itself is periodically transcribed in the G1-S phase under MBF/DSC1 control, it appears that a negative feedback loop operates to down-regulate MBF/DSC1-dependent transcriptional activity, following Cig2p transcription and the ensuing rise of Cdc2p-Cig2p kinase activity (Ayte *et al.*, 2001).

1.5.2.2 *S phase-specific gene cluster*

Similar to *S. cerevisiae*, histone genes are periodically expressed during S phase in *S. pombe*. Thus far, there is limited knowledge about the promoter elements and transcription factors that mediate transcription of these genes. A 17 bp DNA promoter element, consisting of AACCT repeats is present in histone promoters that might be involved in transcriptional regulation, but its associated transcription factor complex is yet unknown (McInerny, 2004).

1.5.2.3 *G2 phase-specific gene cluster*

During G2 phase a heterogeneous population of genes are expressed, with most of them only weakly regulated, displaying low fluctuations in their mRNA levels. *spd1⁺*, a negative regulator of DNA replication, is one of most highly regulated genes of this cluster, its transcript levels peaking in early G2. In terms of transcriptional regulation, a single DNA motif, termed Novel 3, has been shown to be present within the promoters of a subset of these genes, while no transcription factor has yet been identified. It is assumed that many of these genes are preferentially regulated at the level of mRNA stability (Rustici *et al.*, 2004).

1.5.2.4 M-G1 specific gene clusters

Several *S. pombe* genes are specifically expressed late in the cell cycle, their mRNA levels peaking during late mitosis-early G1 phase (Anderson *et al.*, 2002; Rustici *et al.*, 2004; Oliva *et al.*, 2005; Peng *et al.*, 2005). Consistent with their periodic transcriptional pattern, many of these genes encode proteins with functions in late mitotic stages, including among others *plo1⁺*, *fin1⁺*, *mid1⁺*, *sid2⁺*, *slp1⁺*, *cdc15⁺* and *fkh2⁺*, their functions discussed in earlier sections, as well as *ace2⁺*, encoding a transcription factor that controls expression of another subset of genes during M-G1 phase (Fankhauser *et al.*, 1995; Balasubramanian *et al.*, 1998; Bähler *et al.*, 1998; Kim *et al.*, 1998; Krien *et al.*, 1998; Tanaka *et al.*, 2001; Grallert and Hagan, 2002; Buck *et al.*, 2004; Bulmer *et al.*, 2004; Alonso-Nunez *et al.*, 2005; Ng *et al.*, 2006).

A number of studies have begun to unravel the regulatory network underlying periodic transcription of this M-G1 gene cluster. Earlier work has revealed a role for Plo1p in the regulation of these M-G1 transcribed genes, including *plo1⁺* itself, whilst in parallel identified a transcription factor complex that binds *in vitro* to a 6 bp sequence, related to the forkhead sequence and found within the respective promoters. This promoter element and its associated transcription factor complex have been termed Pombe cell cycle box (PCB) and PCB-binding factor, respectively (Anderson *et al.*, 2002; Buck *et al.*, 2004; Section 3.1.1). Later work has revealed that an Mcm1p-related transcription factor, Mbx1p, is a component of PBF that is important for the association of this complex, at least *in vitro*, with the PCB element. Meanwhile, the forkhead transcription factors Sep1p and Fkh2p, the former already known to be necessary for cell cycle-specific expression of *cdc15⁺*, have been shown to be necessary for M-G1-dependent transcription, consistent with the presence of forkhead DNA motifs within the genes' promoters (Zilahi *et al.*, 2000; Buck *et al.*, 2004; Bulmer *et al.*, 2004; Rustici *et al.*, 2004).

Both Fkh2p and Mbx1p are phosphoproteins that are periodically phosphorylated during late M phase (Buck *et al.*, 2004; Bulmer *et al.*, 2004). Since Plo1p is implicated in transcriptional regulation of M-G1 transcribed genes it is tempting to speculate that this kinase exerts its regulatory role via direct phosphorylation of Fkh2 and/or Mbx1p. Indeed, this study reveals that Plo1p binds *in vitro* and *in vivo* to Mbx1p. Consistent with this, Plo1p directly phosphorylates this MADS-box transcription factor *in vitro*. In parallel, as also shown here and elsewhere, Sep1p and Fkh2p appear to regulate cell-cycle specific transcription of this gene cluster in a positive and negative manner, respectively (Rustici *et al.*, 2004; Papadopoulou *et al.*, 2008; Chapters 5 and 6).

Another group of at least seven genes are expressed during the M-G1 interval under control of the encoded product of *ace2⁺*, itself a member of the Sep1p/Fkh2p-regulated gene cluster and a

homologue of *S. cerevisiae* ACE2. These genes, such as *eng1⁺*, are required for cell separation and contain in their promoters a DNA motif similar to the *S. cerevisiae* Ace2p/Swi5p binding sequence (Martín-Cuadrado *et al.*, 2003; Buck *et al.*, 2004; Rustici *et al.*, 2004; Alonso-Nuñez *et al.*, 2005).

1.6 Conserved aspects of the cell cycle transcriptional circuitry

A combination of single-gene and genome-wide studies in model organisms, such as the budding and fission yeasts, and in metazoans, including humans, reveals that parallels exist in the underlying regulatory networks that drive cell cycle specific transcription in these distantly related organisms. Examples of common features of cell cycle-regulated transcription include, among others, functional similarities of the MBF/SBF and MBF/DSC1 complexes in *S. cerevisiae* and *S. pombe* with the mammalian E2F-DP bipartite transcription factor complex that regulates G1-S-dependent transcription of genes that mediate progress past the Restriction point and entry into S phase and encode cell cycle regulators, such as cyclins A and E and Cdk1p, as well as proteins required for DNA replication, including thymidine kinase and DNA polymerase α . Indeed, although the E2F family members lack amino acid sequence similarity with their related SBF/MBF and MBF/DSC1 components, they adopt an analogous winged helix-related fold and associate with DNA promoter sequences that closely resemble the MCB/SCB motifs (Dyson, 1998; Zheng *et al.*, 1999; Ren *et al.*, 2002; Breeden, 2003; Bähler, 2005). Moreover, certain aspects of cyclin A-mediated regulation of E2F-specific gene expression resemble the Cig2p-dependent feedback loop that is thought to inhibit MBF/DSC1 activity, since cyclin A is itself an E2F-DP target gene (Ayte *et al.*, 2001; Stevens and LaThangue, 2003).

Apart from similarities between SBF/MBF, MBF/DSC1 and E2F-DP-dependent regulatory mechanisms, *S. cerevisiae*, *S. pombe* and mammalian cells also appear to share common features in transcriptional control by MADS-box and forkhead-related transcription factor complexes. For example, the budding yeast Mcm1p-Fkh2p complex is related to the mammalian SRF-Elk-1 complex, the latter operating during cell cycle entry in response to mitogenic signalling. Elk-1 is the founding member of the ternary complex factor (TCF) proteins, which constitute a subfamily of the ETS-domain transcription factor family. Mcm1p and SRF share significant sequence and structural similarities, while both forkhead and E-twenty-six (ETS)-domain transcription factors contain winged helix-turn-helix DNA binding domains (Treisman, 1994; Sharrocks, 2002; Boros *et al.*, 2003). Notably, mammalian forkhead transcription factors have been shown to participate in transcriptional regulation of the genes encoding mitotic regulators, such as Plks (Plk1p) and cyclins (cyclin B1), which contain forkhead consensus binding sites in their promoters (Alvarez *et al.*, 2001; Breeden,

2003; Martin and Strebhardt, 2006; Fu *et al.*, 2008). Since MADS-box and forkhead-related transcription factor complexes also regulate homologous cyclin B1 and/or Plk1p genes in the budding (*CLB2* and *CDC5*) and fission (*plo1⁺*) yeasts (Sections 1.5.1.4 and 1.5.2.4), this implies that crucial components of the underlying networks that regulate these mitotic regulators have been preserved during evolution.

1.7 Aims of work

The intention of this thesis has been to elucidate the mechanisms by which Sep1p, Fkh2p and Mbx1p transcription factors, thought to constitute PBF components, and Plo1p bring about M-G1 phase-specific transcription in fission yeast. Initially, experiments were performed to establish the importance of the PCB promoter sequence, implicated in regulation of PBF-dependent genes, for M-G1 specific transcription *in vivo*. Moreover, experiments were carried out to evaluate the role of the TGTTTAY forkhead-related promoter motif in transcriptional regulation of these genes. For this, mutations in these two motifs, either individually or in combination, were tested by Northern blot for their ability to support M-G1 specific transcription. In parallel, experiments were performed to examine whether Sep1p and Fkh2p control M-G1 dependent gene expression through the PCB and/or TGTTTAY promoter motifs.

The relationship between Sep1p, Fkh2p and Mbx1p has also been examined here by GST pull-down and co-immunoprecipitation assays, to determine whether these three transcription factors regulate M-G1 phase-specific expression by directly interacting with each other. Another aim of this work has been to investigate by the yeast two-hybrid system and/or co-immunoprecipitation assays whether Plo1p influences M-G1 specific expression by associating with PBF components, either Mbx1p, Fkh2p or Sep1p, and, if so, determine which Plo1p domains, the kinase, the polo-box or both, mediate such interaction(s). Finally, chromatin immunoprecipitation (ChIP) assays were performed as part of this thesis to examine whether and if so how Fkh2p, Sep1p, Mbx1p and Plo1p interact *in vivo* with the promoters of their co-regulated genes, including *fkh2⁺* and *plo1⁺* themselves, which have been proposed to regulate their own expression via feedback mechanisms.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals used were of analytical grade and obtained commercially. Distilled water was of Millipore-Q quality. All antibiotics (ampicillin, kanamycin, chloramphenicol and G418), protease inhibitors [chymostatin, phenylmethanesulfonylfluoride (PMSF), pepstatin, antipain, leupeptin and aprotinin] and detergents (NP-40, SDS, and sodium deoxycholate) were obtained from Sigma. Protease Inhibitor Cocktail Tablets (Complete, Mini) were purchased from Roche. p-Nitrophenylphosphate (pNPP) and β -Glycerophosphate were obtained from Sigma, while Triton X-100 was from Fisons.

The chemicals used for *S. pombe* and bacterial maintenance were obtained from BD Biosciences while NZ amine was obtained from Sigma. Specialised chemicals for growth of *Saccharomyces cerevisiae* including synthetic drop-out (SD) media (lacking tryptophan, leucine, histidine and uracil) were purchased from BIO 101/QBIOgene and YPD was obtained from USB. Low-melting point agarose was purchased from SeaPlaque and o-Nitrophenyl β -D-galactopyranoside (ONPG) was obtained from Sigma.

Sephadex G-50, [α -P32] dCTP (3000 Ci/mmol) and dIdC (1 mg/ml) were obtained from GE Healthcare. N6, Klenow enzyme and glass beads (425-600 μ m) were obtained from Sigma.

The DNA Molecular Weight Marker X (0.07-12.2 kb) was obtained from Roche, 1 kb DNA ladder was from Promega and N3232S DNA ladder was from NEB. The protein Full-Range Rainbow™ Molecular Weight Markers (10-250 kD) was obtained from GE Healthcare, while the BenchMark™ Protein Ladder (10-220 kD) was obtained from Invitrogen. The Bradford reagent was purchased from Bio-Rad.

Electrophoretic chemicals such as acrylamide/bis-acrylamide 40% solution [(37.5:1 mix ratio of acrylamide/bis-acrylamide (w/w))] and TEMED were obtained from Sigma.

All enzymes used including T4 DNA ligase, calf intestinal alkaline phosphatase (CIAP), *Taq* DNA polymerase, Pfu DNA polymerase, Vent_R DNA polymerase and T4 polynucleotide kinase, as well as dNTPs used for PCR amplification were obtained from Promega. Expand High Fidelity PCR system was obtained from Roche. The QIAprep® Spin Miniprep Kit, the QIAquick Gel Extraction Kit and the QIAquick PCR Purification Kit were supplied by QIAGEN. The Superscript® II Reverse transcriptase kit was purchased from NEB. The TA Cloning® Kit was obtained from Invitrogen, while X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) was obtained from Sigma.

Protein staining dyes, Ponceau S solution, Brilliant Blue R-250 and Coomassie Blue G-250 colloidal stain, were all obtained from Sigma.

All antibodies used in the present study are listed in Table 2.1. Affinity purified IgGs from mouse, rabbit and sheep used as negative controls in chromatin immunoprecipitation assays were supplied by Santa Cruz Biotechnology.

The protein expression inducer IPTG was obtained from Melford Lab. Ltd and thiol-reducing agent DTT was supplied by ALEXIS. 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 and Chelating Sepharose™ Fast Flow slurry were obtained by GE Healthcare, while Protein A-Sepharose 4B Fast Flow and Protein G-Sepharose 4B Fast Flow were purchased from Sigma. The Econo-Pac Chromatography Columns were obtained from Bio-Rad and the Disposable Polypropylene Columns were purchased from PIERCE.

Table 2.1. List of antibodies used in the current study. WB, IP and ChIP indicates antibody used in the current study for Western blot, Immunoprecipitation and Chromatin Immunoprecipitation assay, respectively.

Antibody Name	Isotype	Application	Supplier
HA-probe (F-7)	Mouse monoclonal IgG _{2a}	WB (primary), IP,ChIP	Santa Cruz Biotechnology
c-Myc (9E10)	mouse monoclonal IgG ₁	WB (primary), IP,ChIP	Santa Cruz Biotechnology
Anti-Glutathione-S- Transferase (GST)	rabbit polyclonal	WB (primary)	Sigma
Anti-HIS	mouse monoclonal IgG ₂	WB (primary)	GE Healthcare
Histone H3	rabbit polyclonal	ChIP	abcam
Anti-Plo1p (HO)	rabbit polyclonal	IP, ChIP	(Ohkura <i>et al.</i> , 1995)
Anti-Plo1p (IH)	sheep	ChIP	Lab stock
Anti-Mouse Immunoglobulins/ HRP (HorseRadish Peroxidase conjugated)	rabbit polyclonal	WB (secondary)	DAKO
Anti-Rabbit Immunoglobulins/ HRP (HorseRadish Peroxidase conjugated)	swine polyclonal	WB (secondary)	DAKO
Anti-sheep Immunoglobulins/ HRP (HorseRadish Peroxidase conjugated)	rabbit polyclonal	WB (secondary)	DAKO
Anti-Fkh2p	rabbit polyclonal	WB	Laboratory stock
Anti-Mbx1p	rabbit polyclonal	WB	Laboratory stock
Anti-Sep1p	rabbit polyclonal	WB	Laboratory stock
Anti-GST-Mbx1p	sheep	WB	Laboratory stock

2.1.2 DNA

All oligonucleotides used were custom synthesized by DNA Technology A/S. DNA sequencing was performed by MWG-AG Biotech. Oligonucleotides used in this thesis are listed in Appendix I. The annotations GO refer to Glasgow laboratory oligonucleotide collection number.

2.1.3 Bacteria strains and plasmid vectors

All bacterial strains and vectors (maintained in bacteria) used in this thesis are listed in Appendix II. The annotation GB refers to the Glasgow laboratory bacteria collection number. One Shot™ ultracompetent InVαF *Escherichia coli* cells were purchased from Invitrogen, *E. coli* BL21 CodonPlus (DE3)–RIL cells (GB 445), utilized for high level protein expression, were initially obtained from Stratagene and *E. coli* DH5α cells (GB 4), used for the propagation and storage of plasmid DNA, 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 was 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 pCR®2.1 for one step cloning of a polymerase chain reaction product with polydeoxyadenosine 3' overhangs, were obtained from Invitrogen.

2.1.4 S. pombe strains and plasmid vectors

All fission yeast strains used in this thesis are described in Appendix II. The annotation GG refers to the Glasgow laboratory fission yeast collection number. Fission yeast plasmid vectors maintained in bacteria are also listed in Appendix II.

2.1.5 Budding yeast strains and plasmid vectors

All *S. cerevisiae* strains used in this thesis are described in Appendix II. The annotation GGBY refers to the Glasgow laboratory budding yeast collection number. *S. cerevisiae* plasmid vectors maintained in bacteria are also described in Appendix II.

2.1.6 Bacterial media

E. coli strains were grown in Luria Broth (LB; 10 g Bacto tryptone, 5 g Bacto yeast extract and 10 g NaCl per litre, pH 7.5) or NZY⁺ broth (5 g NaCl, 2 g MgSO₄·7H₂O, 5 g yeast extract, 10 g NZ amine per litre, pH 7.5). LB agar plates were made by adding 7.5 g Bacto agar per 500 ml LB. Media were autoclaved before use and supplemented with ampicillin (100 µg/ml), kanamycin (70 µg/ml) or chloramphenicol (34 µg/ml), as required (Sambrook and Russell, 2001).

2.1.7 Fission yeast media

S. pombe strains were grown in either liquid or on solid YE (Appendix III), as described by Moreno *et al.* (1991). Strains carrying plasmids or used for physiological experiments were propagated in EMM supplemented with the appropriate concentration of amino acids and/or uracil (Appendix III). *S. pombe* stocks were prepared in YE supplemented with 20% glycerol and stored at -70°C.

2.1.8 Budding yeast media

Routinely *S. cerevisiae* strains were grown in YPD (2% Bacto tryptone, 1% yeast extract, 2% glucose) liquid or solid (as for liquid YPD plus 2% agar). Strains containing plasmids were propagated in synthetic drop-out (SD) media supplemented with the appropriate amino acids and/or uracil whenever necessary (Appendix III). For SD agar plates 2% agar was added (Sambrook and Russell, 2001).

2.1.9 Equipment

Electro-transformation of bacterial or fission yeast cells was achieved in a Bio-Rad *E. coli* pulser using 0.2 cm electroporation cuvettes. Bacterial cultures were grown in an orbital incubator (Sanyo-Gallenkamp) and yeast cultures were grown in a shaking water-bath (Grant – OLS200). The number of growing *S. pombe* cells in liquid culture was measured in a Z2 Coulter Counter. Bacteria and yeast cells were routinely collected in 50 ml centrifuge tubes in a chilled benchtop Eppendorf Centrifuge 5810R. Bacterial cells were lysed using a French Pressure cell at either 750 or 950 psi depending on the culture volume, while breakage of fission yeast cells was performed in a HYBAID Ribolyser. Disrupted yeast and bacterial cells were pelleted in a benchtop Eppendorf Centrifuge 5810R and the supernatants clarified using a temperature adjustable Helena Biosciences Centrifuge

5415R benchtop microcentrifuge. Concentration of protein samples was accomplished with a Microcon YM-50 centrifugal concentrator from Millipore. Bacterial or budding yeast cell mass, as well as protein, DNA and RNA concentration was measured in a spectrophotometer, set to the appropriate wavelength.

A Diagenode Bioruptor Sonicator was used for the production of high quality sheared chromatin from formaldehyde fixed fission yeast cells. Bacterial and fission yeast protein extracts were routinely processed in a cold room via shaking in a Labinco test tube rotator.

Electrophoretic assays were performed in a Bio-Rad Mini-Protean gel kit system, while PAGE gels were dried in a Bio-Rad Gel Dryer Model 583. PCR reactions were carried out in a Primus Thermal Cycler with 0.5 ml thin walled PCR tubes (Anachem). A Techne Hybridizer HB-1D was used for hybridisation of DNA probes on RNA blots. Autoradiography film (Fujifilm) was processed in an X-Omat 100 from Kodak.

The *S. pombe* DIC microscopic images were captured with a Sony DS-75 digital camera attached to Zeiss AxioStar R-grade microscope. Tetrad dissection in *S. pombe* was carried out with the Singer Micromanipulator System Series 200.

2.1.10 Computational databases

Fission yeast gene 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/clustalw/index.html>, [/emboss/align/index.html](http://www.ebi.ac.uk/emboss/align/index.html) and [/InterproScan/](http://www.ebi.ac.uk/InterproScan/), and <http://www.ncbi.nlm.nih.gov/BLAST/>.

2.2 Methods

2.2.1 Manipulation of bacterial cells

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

2.2.2 Preparation of competent bacterial cells

The appropriate bacterial *E. coli* strain (DH5 α or BL21 CodonPlus (DE3)–RIL) was streaked onto an LB plate and grown overnight at 37°C. A single colony was used to inoculate a 5 ml LB culture with shaking for 16 hours, which was then sub-cultured into 100 ml of LB.

In the case of DH5 α (GB 4) strain, once the cells reached an OD₆₀₀ of 0.5, they were put on ice for 30 min and then harvested by centrifugation at 2500 g for 10 min. The supernatant was removed and cells were then resuspended in 10 ml of ice-cold 10% glycerol. The process was repeated once more with 20 ml ice-cold 10% glycerol. Finally, the cells were centrifuged, resuspended in 1-2 ml of ice-cold 10% glycerol and stored into 50 μ l aliquots at -70°C .

BL21 CodonPlus (DE3)-RIL cells (GB 455) were grown at 37°C with shaking until they reached an OD₅₅₀ of 0.48, chilled on ice for 5 min and harvested by centrifugation at 3000 rpm for 10 min at 4°C . The supernatant was discarded and pelleted cells were resuspended in 40 ml Buffer 1 (100 mM RbCl, 10 mM CaCl₂, 50 mM MnCl₂, 15% (v/v) glycerol in 30 mM KC₂H₃O₂, pH 5.8). Cells were harvested with centrifugation as before and resuspended in 4 ml Buffer 2 (10 mM RbCl, 75 mM CaCl₂, 15% (v/v) glycerol in 10 mM 3-[N-morpholino] propanesulfonic acid (MOPS), pH 6.5). Finally, cells were stored into 50 μ l aliquots at -70°C .

2.2.3 Electro-transformation of bacteria

Competent *E. coli* DH5 α cells prepared as described above, were thawed on ice for 10-15 min and 1-2 μ l of the appropriate plasmid DNA (1-10 ng) was added. The cell-DNA suspension was transferred to a pre-chilled 0.2 cm electroporation cuvette and pulsed at 2.4 kV. 1 ml of SOC media (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose) was added to the cells, which were then transferred to a microcentrifuge tube and incubated at 37°C with shaking for 45 min. Cells were then plated on an LB-agar plate containing the appropriate antibiotic to allow for selection overnight at 37°C .

2.2.4 Heat-shock transformation of bacteria

BL21 CodonPlus (DE3)-RIL and OneShot™ InV α F cells were preferably used for the expression of recombinant proteins and transformed as follows: 1-10 ng of plasmid DNA was added to 50 μ l of competent bacteria, which was then chilled for 15 min before heat shocking at 42°C for 90 sec. Cells were chilled on ice for 2 min, supplemented with 450 μ l of SOC and incubated at 37°C with shaking for 45 min. Finally, cells were plated on an LB-agar plate containing the appropriate antibiotic and incubated overnight at 37°C .

2.2.5 Preparation of *S. pombe* competent cells

500 ml of EMM plus appropriate supplements was inoculated with a 10 ml overnight culture. The culture was grown at 25°C until it reached a density of 1×10^7 cells/ml ($A_{595} \sim 0.5$). The cells were harvested in 50 ml tubes by centrifugation at 3000 rpm for 5 min. The supernatant was removed and pelleted cells were resuspended in a total of 50 ml of ice-cold dH₂O. The cells were centrifuged and resuspended in 50 ml ice-cold 1 M sorbitol. Harvesting by centrifugation followed once more and cells were resuspended in 20 ml ice-cold 1 M sorbitol. Finally, cells were pelleted by centrifugation, resuspended in 1-2 ml ice-cold 1 M sorbitol and stored into 50 μ l aliquots at -70°C.

2.2.6 Electro-transformation of competent *S. pombe* cells

Competent *S. pombe* cells were thawed at room temperature and added to chilled microcentrifuge tubes containing 1-2 μ l of plasmid DNA (1-10 ng). The cell-DNA suspension was incubated on ice for 5 min, transferred into a pre-chilled 0.2 cm electroporation cuvette and pulsed at 1.5 kV. 0.9 ml of 1 M sorbitol was immediately added to the cuvette and the cell suspension was returned to the microcentrifuge tube and placed on ice. Cells were then plated onto EMM supplemented with appropriate amino acids and/or uracil to allow for selection by incubation for 3-4 days at 25°C-30°C.

2.2.7 Preparation of competent *S. cerevisiae* cells

The appropriate budding yeast strain was streaked onto an YPD agar plate or appropriate selective SD for 2-3 days at 30°C. A single colony was used to inoculate 5 ml of YPD or selective SD overnight at 30°C. 0.5 ml of this culture was then added to 4.5 ml of fresh media and the OD₆₆₀ nm was measured. Then, a suitable amount of cells was added into 60 ml of fresh media to give an OD₆₆₀ of 0.2 (2×10^6 cells/ml). This culture was grown for 3-4 hours with shaking at 30°C until it reached an OD₆₆₀ of 0.7 (1×10^7 cells/ml). The cells were centrifuged for 5 min at 3000 g, the supernatant was discarded and the pellet was washed in sterile 10 ml dH₂O. This step was repeated once more but instead the pelleted cells were resuspended in 1 ml dH₂O. Cells were transferred to a 1.5 ml microcentrifuge tube, centrifuged 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). Finally, cells were pelleted by centrifugation, resuspended in LiAc/TE at a density of 2×10^9 cells/ml and kept on ice.

2.2.8 Lithium acetate transformation of competent *S. cerevisiae* cells

50 µl of competent *S. cerevisiae* cells were mixed with 5 µl of 10 mg/ml salmon sperm DNA that had been denatured at 95°C for 5 min and 1-3 µl of the appropriate plasmid DNA. 300 µl sterile 40% PEG/LiAc/TE (prepared from 50% PEG, 10x LiAc and 10x TE) was added to the cell suspension, which was then incubated at 30°C for 30 min with gentle agitation. 40 µl DMSO (Dimethyl sulfoxide) was added to the mix prior to heat-shock for 15 min at 42°C. Finally, the cells were centrifuged for 5 sec at high speed and resuspended in 1 ml TE. This step was repeated once again and cells were plated out onto selective SD agar plates at 30°C for 3-4 days.

2.2.9 DNA Manipulation

2.2.9.1 Purification of plasmid DNA from bacterial cultures

The QIAprep[®] Spin Miniprep Kit (QIAGEN) was routinely used to prepare plasmid DNA from bacterial cultures. The kit was used according to the manufacturer's instructions.

2.2.9.2 Preparation of *S. pombe* genomic DNA

10 ml of cell culture was grown for 2-3 days until saturated at 25°C-30°C as appropriate. Cells were harvested by centrifugation at 3,000 rpm for 5 min, resuspended in 0.5 ml dH₂O and transferred to a 1.5 ml screw cap microcentrifuge tube. Cells were centrifuged briefly, the supernatant was discarded and 0.2 ml solution A (1% Triton, 10% SDS, 1 M NaOH, 1 M Tris-HCl pH 7.5, 0.5 M EDTA), 0.2 ml phenol: chloroform (1:1), as well as 0.3 g acid washed glass beads, was added to the pellet. Breakage of cells was performed in a HYBAID Ribolyser for 3 x 40 sec at speed setting 4. 400 µl of TE (10 mM Tris-HCl pH 8, 1 mM EDTA) was added to the mix prior to centrifugation for 5 min at 13,000 rpm. The aqueous layer only, containing both DNA and RNA, was transferred to a fresh 1.5 ml microcentrifuge tube. 1 ml of 100% ethanol was added to the tube, which was then inverted repeatedly to gently mix its content. The tube was centrifuged at 13,000 rpm for 5 min and the supernatant discarded. The centrifugation step was repeated once more to remove residual ethanol. The pellet was resuspended in 400 µl of TE and 3 µl Ribonuclease A (50 µg/ml) prior to incubation at 37°C for 5 min to digest RNA. 1 ml 100% ethanol and 8 µl 5M ammonium acetate was added to the tube, which was then inverted repeatedly and stored at -70°C for a few hours. Finally, the tube was

centrifuged at 13,000 rpm for 10 min and the supernatant discarded. The resulting DNA pellet was air-dried shortly, resuspended in 100 µl TE and stored at -20°C.

2.2.9.3 Quantification of nucleic acids by spectrophotometry

Nucleic acid samples were diluted 100 fold in dH₂O and the concentration estimated by measuring the OD at 260 nm with a spectrophotometer. The following formula was used to calculate nucleic acid concentrations:

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

2.2.9.4 Polymerase chain reaction

All oligonucleotides used for PCR amplifications are listed in Appendix I. PCR reactions were routinely carried out in a Biotech Primus Thermal Cycler with one of the following polymerases: *Taq* DNA polymerase, Vent_R DNA polymerase, Expand High Fidelity PCR System. The general program for PCR amplification was as follows:

Initial Denaturation	94°C	2 min	1 cycle
Denaturation	94°C	1 min	} (variable between 23-30 cycles)
Annealing*1	54°C	1 min	
Extension*2	68°C or 72°C*3	1 min	
Final Extension	68°C or 72°C	5 min	1 cycle

*1 Annealing temperatures varied depending on oligonucleotides used (T_m was calculated using the equation: T_m = 4 x (G + C) + 2 x (A + T)). *2 extension time 1 min per kb of product. *3 68°C was used for Vent_R DNA polymerase and 72°C for *Taq* DNA polymerase and Expand High Fidelity enzyme system.

2.2.9.4.1 PCR using *Taq* DNA polymerase

PCR reactions were carried out using 0.2 ml thin walled PCR tubes. In general, 50 µl or 100 µl PCR reactions were prepared. A typical 50 µl reaction mixture contained 250 µM of each dNTP, 50 pmole

of each primer, 50 ng of plasmid-DNA template or 1 µg of genomic-DNA template, 1x Thesit buffer (prepared from a 10x stock: 300 mM Tris-HCl pH 8.5, 20 mM MgCl₂, 50 mM β-mercaptoethanol, 1% THESIT) and 1.25 U *Taq* DNA polymerase (Promega). Alternatively, Thesit buffer was replaced with 1x *GoTaq* Flexi Buffer (Promega, 5x stock) and 500 µM MgCl₂ (25 mM stock).

2.2.9.4.2 PCR using *Vent_R* DNA polymerase

The PCR reaction using *Vent_R* DNA polymerase (NEB) was carried out as described for *Taq* DNA polymerase but 1x Thesit buffer was replaced with 1x Thermopol buffer (prepared from a 10x stock: 100 mM KCl, 100 mM (NH₄)₂SO₄, 200 mM Tris-HCl pH 8.8, 80 mM MgSO₄, 1% Triton X-100) instead. The reaction mixture also contained 2 mM MgSO₄ (100mM stock).

2.2.9.4.3 PCR using Expand High Fidelity PCR system

The PCR reaction was carried out as described for *Taq* DNA polymerase but Thesit buffer was replaced with 10x Expand High Fidelity buffer with 15 mM MgCl₂ used at a 1 x final concentration. 2.6 U of Expand High Fidelity enzyme was used per 50 µl reaction.

2.2.9.5 Agarose gel electrophoresis

The appropriate amount of agarose was dissolved in 100 ml 0.5 x TBE (45 mM Tris-borate pH 8.3, 1 mM EDTA), based on the percentage of agarose gel required (1-2%). 5 µl of a 10mg/ml ethidium bromide stock solution (Sigma) was added. DNA samples were diluted 5 fold by the addition of 6 x blue/orange loading dye (Promega) and loaded on the agarose gel. Gels were run at 100 V in 0.5 x TBE for 40 min to 1 h. Following electrophoresis, DNA was visualised on a UV transilluminator and photographed using E.A.S.Y imaging software.

2.2.9.6 Restriction digestion and generation of blunt-ended DNA fragments

Plasmid DNA and PCR products were digested, prior to ligation, as follows:

DNA (≈200 ng)	1-5 µl
Appropriate buffer (10x)	2 µl
Appropriate enzyme (10 U)	1 µl
dH ₂ O	to 20 µl

Reactions were incubated for 1 h at 37°C or 25°C depending on the restriction enzyme. In certain cases, blunt-ended DNA fragments were generated from 3' or 5' overhangs, following restriction digestion with appropriate enzyme. In the former case, the 3' overhang was removed using the 3' to 5' exonuclease activity of T4 DNA polymerase. In the latter case the 5' overhang was repaired using the polymerase activity of the Klenow enzyme. Specifically, following restriction digestion, 0.5 µl of 500 mM dNTPs were added into the reaction along with 1 µl Klenow enzyme (1 unit of Klenow enzyme/µg of DNA) and incubated at 30°C for 15 min. The reaction was then heat-inactivated at 75°C for 10 min.

2.2.9.7 Dephosphorylation of digested plasmid

Digested vector was dephosphorylated by the addition of 1 U of calf intestinal alkaline phosphatase and incubated at 37°C for a further 30 min. This was routinely carried out to prevent self-ligation of digested vector.

2.2.9.8 Annealing of single stranded oligonucleotides

Complementary custom made single stranded DNA oligonucleotides (DNA Technology A/S) were annealed to produce double stranded DNA prior to ligation into the appropriate plasmid DNA (Section 2.2.9.11). Both complementary oligonucleotides were resuspended at the same molar concentration, using Annealing Buffer (10mM Tris-HCl pH 7.5-8.0, 50mM NaCl, 1mM EDTA). Equal volumes of both complementary oligonucleotides were mixed (at equimolar concentration) in a 1.5ml microcentrifuge tube, which was then placed in a heat block at 90-95°C for 10 min. The tube was removed from the apparatus and allowed to cool to room temperature for 1-2 hours. Finally, the resulting double stranded DNA was purified using the QIAquick PCR Purification Kit (Section 2.2.9.10) to remove any unwanted salt and EDTA that might interfere with subsequent enzymatic reactions, and stored at -20°C until ready to use.

2.2.9.9 Phosphorylation of DNA with 5' protruding ends

T4 Polynucleotide kinase (PNK) catalyses the transfer of the γ phosphate of ATP to the 5' terminus of single and double stranded DNA or RNA molecules. In this study, T4 Polynucleotide kinase (Promega) was used to phosphorylate the 5' ends of DNA fragments generated from annealing custom made single stranded DNA oligonucleotides (Section 2.2.9.8) lacking a 5' phosphate prior to

ligation into the appropriate plasmid DNA. The following reaction was assembled in a microcentrifuge tube:

DNA 5' ends	1-50 pmole*
10x Kinase buffer	4 µl
10 mM ATP	4 µl
T4 Polynucleotide kinase	10 U
dH ₂ O	to 40 µl

The reaction mixture was incubated at 37°C for 30 min and then 2 µl 0.5 M EDTA was added to stop the reaction. Following PNK treatment, the DNA was purified by gel extraction using the QIAquick Gel Extraction Kit as described below (Section 2.2.9.10).

*The amount of DNA 5' ends in a double-stranded DNA molecule was calculated using the following formula:

Amount of 5' ends (in pmoles) = $[X/(Y \times 660)] \times 10^{12} \times 2$, where X is mass in gr and Y is the length of DNA.

2.2.9.10 DNA fragment purification

DNA fragments were purified from various enzymatic reactions, such as restriction digestion, dephosphorylation, 5' end phosphorylation and PCR, by extraction from 1-2% agarose gels using the QIAquick Gel Extraction Kit (QIAGEN). Following electrophoresis, DNA fragments were excised from the gel and purified according to the manufacturer's instructions. Alternatively, DNA fragments generated from PCR and other enzymatic reactions were purified using the QIAquick PCR Purification Kit (QIAGEN).

2.2.9.11 Ligations

The DNA fragment of interest was ligated into the appropriate digested and dephosphorylated plasmid DNA at various molar ratios (5:1, 3:1, 1:1, 1:3, and 1:5) in the following ligation reaction:

DNA insert	1-5 µl
Linearised plasmid DNA	2-3 µl

10x T4 DNA ligase buffer	1 μ l
T4 DNA ligase (3 U/ μ l)	0.5 μ l
dH ₂ O	to 10 μ l

Ligation reactions were incubated overnight at 4°C and then transformed into the appropriate competent *E. coli* cells (One Shot™ ultracompetent InVaF, DH5 α or BL21 CodonPlus (DE3)–RIL) as described above (Sections 2.2.3 and 2.2.4).

2.2.9.12 Cloning of PCR products into the pCR® 2.1 vector

The TA Cloning® Kit (Invitrogen) was routinely used for efficient cloning of PCR products into the pCR®2.1 vector according to the manufacturer's instructions.

2.2.9.13 RT-PCR and *S. pombe* cDNA synthesis

Gene-specific cDNA was synthesised from *S. pombe* mRNA purified from total RNA extracts (Section 2.2.10.1) using the SuperScript™ Reverse Transcriptase (Invitrogen) as follows: A 20 μ l reaction mixture was assembled in a nuclease-free microcentrifuge tube containing 2 pmole gene-specific primer (GSP), 1-500 ng mRNA, 1 μ l dNTP mix (10 mM of each) and 12 μ l dH₂O. The mixture was heated to 65°C for 5 min and chilled on ice. Following a brief centrifugation of the tube, 4 μ l 5 x First-Strand buffer, 2 μ l 0.1 M DTT and 1 μ l RNaseOUT™ (optional) were added to the mixture. The contents of the tube were mixed gently and incubated at 42°C for 2 min. 1 μ l of Superscript™ II Reverse Transcriptase (200 units) was added and the tube contents were mixed by gentle pipetting. The mixture was incubated at 42°C for 50 min and then the reaction was inactivated by heating at 70°C for 15 min. 10% of the resulting first-strand cDNA was then used as a template for PCR amplification of the appropriate gene in the following reaction:

10x High Fidelity enzyme (Buffer #2)	5 μ l
50 mM dNTP Mix (12.5 mM each)	1 μ l
Forward primer (100 pmol/ μ l)	0.5 μ l
Reverse primer (100 pmol/ μ l)	0.5 μ l
High Fidelity enzyme (2.6 U/ μ l)	0.75 μ l
cDNA from first-strand reaction	2 μ l
PCR water	to 50 μ l

In each case, a PCR amplification programme of 34 cycles was employed.

2.2.9.14 Preparation of radio-labelled DNA probe

Radio-labelled DNA probes for Northern blot analysis were prepared by random hexanucleotide (N₆) primed labelling of PCR amplified DNA fragments according to the method of Feinberg and Vogelstein (1983). Briefly, a reaction mix was assembled in a screw-cap microcentrifuge tube containing 11 µl denatured DNA probe (~25 ng of DNA-heated at 94°C for 5 min and chilled on ice for 5 min), 2 µl N₆ (random hexanucleotide mixture at 100pm/µl), 2 µl Klenow Buffer (5u/ µl-prepared from 10x stock: 100 mM MgCl₂, 500 mM Tris-HCl pH 7.5), 2 µl -dCTP mix [250 µM of each dNTP (dTTP, dGTP and dATP)], 2 µl [α -P³²] dCTP (3000 Ci/mmol) and 1 µl Klenow enzyme (Promega). The mixture was incubated at 37°C for 1-4 h. Then, 100 µl of nuclease-free H₂O was added to the tube and the probe was purified by passing through a Sephadex G-50 size-exclusion column. Briefly, Sephadex G-50 was prepared by adding two volumes of TE and autoclaving. Then, the plunger of a 1 ml syringe was removed and siliconised glass wool was used to plug the end of the syringe. A microcentrifuge tube was placed on the end of the syringe, which was then filled with Sephadex G-50, placed into a 50 ml centrifuge tube and centrifuged for 2-5 min at 2,000 rpm. TE was removed and the process repeated until 0.8 ml of Sephadex G-50 remained in the syringe. A fresh tube was placed on the end of the syringe and the radio-labelled probe was added to the Sephadex G-50 column. The column was centrifuged at 2,000 rpm for 2 min and the purified probe was collected in the microcentrifuge tube. Separation of labelled DNA fragment from unincorporated radio-nucleotide was confirmed by monitoring the DNA probe with a Geiger counter.

2.2.10 RNA Manipulation

2.2.10.1 Preparation of total RNA

S. pombe total RNA was prepared according to the method of Kaufer *et al.* (1985). A 20-200 ml mid-exponentially growing culture was harvested by centrifugation at 3000 rpm for 5 min in 50 ml centrifuge tubes. The cell pellet was resuspended in 1 ml STE (0.32 M sucrose, 20 mM Tris-HCl pH 7.5 and 10 mM EDTA pH 8.0) and transferred to a 1.5 ml microcentrifuge tube, which was then spun shortly. The supernatant was removed and pelleted cells were stored at -70°C or processed immediately for RNA extraction.

To extract the RNA, cells were resuspended in 200 µl STE and 600 µl NTES (100 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.5 and 1% w/v SDS), transferred to a screw-top tube containing 500 µl of hot phenol and acid-washed glass beads (pre-heated at 65°C for 10-15 min) and then lysed in a HYBAID Ribolyser for 3 x 40 sec at speed setting 4. The tube containing the cell lysate was centrifuged for 5 min at maximum speed and the aqueous and protein interface were transferred into a fresh tube containing 500 µl hot phenol. The tube was agitated in the Ribolyser for 1x40 sec at setting 4 and the aqueous phase was transferred into a fresh tube with 400 µl of phenol (room temperature). The tube was agitated in the Ribolyser as before, centrifuged for 5 min and the aqueous phase was transferred to a tube containing phenol/chloroform (1:1). Agitation and centrifugation followed and the aqueous phase was placed in a tube with 300 µl of chloroform. Once agitated in the Ribolyser, the tube was centrifuged at maximum speed and the aqueous phase was transferred to a fresh tube with 1/10 volume of 3M sodium acetate and 3 volumes of 100% ethanol added to precipitate the RNA at -70°C overnight.

The RNA was centrifuged at maximum speed for 10 min and the supernatant discarded. The RNA pellet was washed in 1 ml 70% ethanol, air-dried for 10 min and dissolved in 50-100 µl RNase-free H₂O prior to storage at -70°C.

2.2.10.2 mRNA purification

Prior to cDNA synthesis mRNA was purified from total *S. pombe* RNA using the polyA Spin™ mRNA isolation kit (New England Biolabs) according to the manufacturer's manual.

2.2.10.3 Formaldehyde gel electrophoresis of RNA

1% agarose formaldehyde gels were used to separate RNA samples. 1 g agarose was dissolved in 63 ml dH₂O and 20 ml 5x MNE (120 mM MOPS, 25 mM NaOAc, 5 mM EDTA pH 7.0) by boiling. Once cooled to 60°C, 17 ml formaldehyde (38% aqueous solution) was added to the melted agarose mixture, which was then poured onto a horizontal casting mould.

10 µg of RNA were mixed with 20 µl RNA buffer (600 µl formaldehyde, 200 µl formamide, 240 µl 5x MNE buffer and 160 µl dH₂O) and 1 µl of 0.5mg/ml ethidium bromide prior to heating at 65°C for 5 min and loading onto the gel. Gels were run in 1x MNE buffer at 50 V for 3-4 h, visualized on a UV transilluminator and photographed using E.A.S.Y imaging software.

2.2.10.4 Northern blot

2.2.10.4.1 RNA transfer by capillary action

Following RNA gel electrophoresis, the RNA gel was soaked in 50 mM NaOH for 15 min to break down large transcripts and then in 0.1 M Na₂HPO₄/NaH₂PO₄ (pH 6.5) for 15 min, to remove residual formaldehyde. The RNA was then transferred from the gel onto the Genescreen nylon membrane using the capillary method (Sambrook *et al.*, 2001). Briefly, a few sheets of Whatman paper (four short ones and two long ones) and a piece of Genescreen membrane, cut to a size slightly larger than the gel, were soaked in 0.1 M Na₂HPO₄/NaH₂PO₄. Two long Whatman sheets were placed on a glass plate to form a wick over a reservoir of 0.1 M Na₂HPO₄/NaH₂PO₄, and another two short Whatman pieces were placed on top of that. The RNA gel was then placed upside down on the Whatman papers with gel spacers placed along the edges of the gel. Genescreen membrane was placed on top of the gel, while another two Whatman sheets were placed on top of the membrane. Finally, a stack of dry towels was put on top of the Whatman papers and a heavy weight on top of that. Transfer took place for 16-24 h. The membrane was then irradiated at 1200 MJ to UV crosslink the RNA to it, as well as baked at 80°C for 3 h.

2.2.10.4.2 RNA hybridisation with radio-labelled DNA probe

Prior to hybridisation, the RNA membrane was incubated in 18 ml pre-hybridisation buffer [10 ml formamide, 4 ml P Buffer [1% (w/v) BSA (Bovine serum albumin), 1% (w/v) pyrrolidine, 1% (w/v) ficoll, 250 mM Tris-HCl pH 7.5, 0.25% (w/v) sodium pyrophosphate, 5% (w/v) SDS], 4 ml 50% w/v Dextran sulphate, 1.16 g NaCl, 200 µl 10 mg/ml denatured salmon sperm DNA) at 42°C for 3 h in a Techne oven. The appropriate denatured radio-labelled DNA probe was then added and allowed to hybridise overnight.

The next day, the membrane was washed twice for 5 min at 42°C in 2x SSPE (20x stock: 3 M NaCl, 20 mM NaHPO₄, and 200 mM EDTA pH 7.4). The membrane was then washed in 2x SSPE + 0.5% SDS at 65°C for 15 min. Finally, the membrane was rinsed in 0.1x SSPE and wrapped in Saran wrap before overnight exposure to an autoradiography film at -70°C.

Following processing of the autoradiography film, the membrane was stripped of the DNA probe by incubation in strip buffer (5 mM Tris-HCl pH 8, 0.2 mM EDTA, 0.05% (w/v) sodium pyrophosphate, 0.002% (w/v) pyrrolidine, 0.002% (w/v) BSA, 0.002% (w/v) ficoll) at 70°C for 1 h. Stripping of the membrane allowed for re-probing with another DNA fragment of interest.

2.2.10.4.3 Quantification of Northern blots

Whenever necessary, quantification of radioactivity signals following Northern blot analysis was performed with the Image J programme, where each sample signal was normalised against the control signal from the corresponding rRNA band. In each case, triplicate measurements were made for each sample.

2.2.11 Protein Manipulation in *E. coli*

2.2.11.1 Growth of bacterial cells for protein induction

A single colony of BL21 CodonPlus (DE3)-RIL cells transformed with the appropriate expression vector was used to inoculate 10 ml of NZY⁺ media supplemented with the necessary antibiotic. Cells were grown for 16 h at 37°C with shaking. The next day, the pre-culture was used to inoculate an appropriate volume of NZY⁺ with antibiotic (1 ml pre-culture per 50 ml of NZY⁺ plus antibiotic). Based on the amount of protein required, 50-400 ml cell cultures were set up.

The cell culture was propagated at 37°C with shaking until OD₆₀₀ reached 0.5. To induce expression of the protein of interest, a final concentration of 0.2-0.5 mM IPTG (1M IPTG stock) was added to the culture which was incubated at 30°C for 3 h or at 15°C for 16 h with shaking. The cell culture was then collected in 50 ml centrifuge tubes and harvested by centrifugation at 3,000 rpm for 5 min. The supernatant was discarded and the pelleted cells were resuspended in the appropriate lysis buffer.

1 ml cell samples were collected from the cell culture immediately before induction and every hour following induction to confirm protein over-expression (for 16 h induction samples were taken before induction and at 16 h). In each case, the 1 ml sample was centrifuged at high speed, the supernatant was discarded and the cell pellet was resuspended in Laemmli sample buffer [2% (w/v) SDS, 10% (w/v) sucrose, 62.5 mM Tris-HCl pH 6.8, 0.02% (w/v) Pyronin Y dye] using 10 µl per 0.1 OD₆₀₀ units.

2.2.11.2 Preparation of bacterial protein extracts

GST- and HIS- tagged proteins were expressed from appropriate vectors as described (Section 2.2.11.1).

The pellet from a 50 ml bacterial culture expressing a GST-tagged protein of interest was resuspended in 3 ml of 1 x PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH

7.3) or in 20 ml 1x PBS for a 400 ml cell culture. For HIS-tagged proteins, pelleted cells were resuspended in 3 or 20 ml of Buffer A (20 mM KH_2PO_4 , 5 mM imidazole, 100 mM NaCl pH 7.5), respectively. The cells were then disrupted under high pressure using a French Pressure cell at 750 or 950 psi for 3 or 20 ml cell suspensions, respectively. 3 passes were made and protease inhibitors were added (2 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ pepstatin A, 0.5 $\mu\text{g}/\text{ml}$ leupeptin, 0.5 $\mu\text{g}/\text{ml}$ antipain, 1 $\mu\text{g}/\text{ml}$ chymostatin and 1 mM PMSF final concentration). The cell lysate was then centrifuged at 13,000 rpm for 15 min at 4°C and the clarified supernatant was collected.

Prior to supernatant clarification, a 200 μl sample from the cell lysate was collected. 100 μl of cell lysate were resuspended in 100 μl 2x Laemmli sample buffer, while the remaining 100 μl of cell lysate were centrifuged at 13,000 rpm for 2 min at 4°C. The supernatant was transferred to a fresh tube and resuspended in 100 μl 2x Laemmli sample buffer, while the pellet was resuspended in 100 μl 2x Laemmli sample buffer and 100 μl of the appropriate lysis buffer. The lysate, supernatant and pellet samples were subjected to SDS-PAGE electrophoresis to assess the solubility of the tagged protein of interest.

2.2.11.3 Column purification of GST-tagged proteins

The column for purification of a GST-tagged protein, expressed from 200 ml of bacterial culture, was set as follows: 200 μl of Glutathione Sepharose 4B (75% slurry in ethanol) were transferred into a 15 ml centrifuge tube and the medium was sedimented by centrifugation at 500 x g for 5 min at 4°C. The supernatant was discarded and 1.5 ml 1 x PBS was added to Glutathione Sepharose 4B. The tube was agitated gently by repeated inversion, centrifuged for 5 min and the supernatant was discarded. This process was repeated three times ensuring adequate washing of Glutathione Sepharose 4B with PBS to remove the ethanol. 150 μl 1 x PBS was then added to the tube, resulting in 300 μl 50% Glutathione Sepharose 4B slurry equilibrated with PBS. The clarified supernatant was then added and the Glutathione Sepharose 4B-protein suspension was incubated for 1h at 4°C with end-over-end rotation to facilitate binding of the GST-moiety of the protein of interest to the Glutathione Sepharose 4B.

Following incubation, the mixture was transferred into an Econo-Pac column (Bio-Rad), which was tapped to allow the medium to settle. Then, the end cap was removed to allow the supernatant containing all unbound material to flow through. The gel was washed with 10 bed volumes of 1 x PBS (1 bed volume equals 0.5 x volume of 50% slurry) and the column was drained. The process was repeated twice more for a total of three washes. Then, the column was tapped and 400 μl of glutathione elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8) were added

and incubated for 10 min to elute the GST-fusion protein. Several 400 µl elution fractions were collected and stored at -70°C .

A 10 µl aliquot of each elution fraction was retained for SDS-PAGE analysis (Section 2.2.11.6) along with lysate, supernatant and pellet samples (Section 2.2.11.2). Elution fractions where GST-fusion protein of interest was detected with SDS-PAGE were pooled and dialysed in 1 x PBS (Section 2.2.11.7).

2.2.11.4 Column purification of HIS-tagged proteins

The column for purification of a HIS-tagged protein, expressed from 200 ml of bacterial culture, was prepared as follows: 200 µl of Chelating Fast Flow slurry was loaded into an Econo-Pac column (~13 ml volume capacity). Once drained, the column was washed with 5-10 column volumes (CV) of dH_2O to remove ethanol. The column was then loaded with 0.5 column volumes of 0.1 M ZnCl_2 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 column was washed with 5-10 CV of dH_2O and then with 5 CV of binding Buffer A.

Clarified supernatant containing the HIS-fusion protein of interest was loaded on to the column and incubated at 4°C with end-over-end rotation for 1 h. The column was then drained to allow flow through of unbound material, washed with 5-10 column volumes of Buffer A and incubated for 10 min in elution Buffer B (20 mM KH_2PO_4 , 0.5 M imidazole, 100 mM NaCl, pH 7.5). 2-5 elution Buffer B fractions (400 µl each) were collected and stored at -70°C . A 10 µl aliquot of each elution fraction was retained for SDS-PAGE analysis (Section 2.2.11.6) along with lysate, supernatant and pellet samples (Section 2.2.11.2). Elution fractions where HIS-fusion protein was detected with SDS-PAGE were pooled and dialysed in 1 x PBS (Section 2.2.11.7).

2.2.11.5 Determination of protein concentration

The Bradford method (1976) was routinely employed. A standard curve was prepared based on known concentrations of BSA. The OD of the sample [1:1000 dilution of protein sample in Bradford reagent (Bio-Rad) prepared according to manufacturer's manual] was then measured at 595 nm and the protein concentration was extrapolated from the standard curve.

2.2.11.6 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The Laemmli method (1970) for protein resolution under denaturing conditions was adopted. Gel Recipes for Bio-Rad 0.75 mm thick gels are described in Table 2.2. 60 μ l 10% APS (Ammonium Persulfate) and 22 μ l TEMED were added per gel to facilitate polymerisation.

Table 2.2. Gel Recipe for Bio-Rad 0.75 mm thick gels. All recipes are in ml.

	Stacking	6%	7.8%	10%	12%	15%
dH ₂ O	3.2	6	5.5	5.0	4.5	4.0
4x Resolving Gel Buffer *	-	2.5	2.5	2.5	2.5	2.5
4x Stacking Gel Buffer +	1.3	-	-	-	-	-
40% acrylamide	0.5	1.5	2.0	2.5	3.0	3.5

***4x Resolving buffer:** 1.5M Tris-HCl pH 8.8, 0.4% SDS.

+**4x Stacking buffer:** 0.5M Tris-HCl pH 6.8, 0.4% SDS.

10 μ l protein samples for SDS-PAGE electrophoresis were resuspended in 10 μ l 2x Laemmli buffer and 1 M DTT was added to a final concentration of 150 mM before boiling for 5 min. 10 -20 μ l of sample was loaded onto the gel alongside 10 μ l of Full-Range Rainbow marker or BenchMark marker. Gels were run in 1x Running buffer [10x Running buffer: 250 mM Tris-HCl, 1.92 M glycine, 1% SDS (w/v), pH adjusted to 8.3] using the Bio-Rad Mini-Protean gel kit system at 120 V for 1 h 30 min. Gels were then either stained in Coomassie stain (0.1% w/v Brilliant Blue R250, 10% v/v acetic acid and 50% v/v methanol) for 20-45 min, followed by de-staining in 10% (v/v) acetic acid, 10% (v/v) methanol overnight, or subjected to Western blotting (Section 2.2.11.11).

2.2.11.7 Dialysis of protein samples

Dialysis tubing was boiled in 10 mM sodium bicarbonate pH 8, 1 mM EDTA for 10 min, rinsed in sterile dH₂O and stored in 100% (v/v) ethanol. Prior to use the tubing was thoroughly rinsed in dH₂O. Protein dialysis took place at 4°C overnight with frequent changes of dialysis buffer (1x PBS).

2.2.11.8 Concentration of protein samples

Following dialysis, protein samples were concentrated by centrifugation in a Centricon Plus-20 centrifugal concentrator filter (Amicon), with cut off 30,000 Da, according to the manufacturer's instructions.

2.2.11.9 GST pull-down

Following dialysis and concentration of protein samples, interaction between two proteins of interest, expressed the one as a GST N-terminal fusion and the other as a HIS N-terminal fusion protein, was assayed by a GST pull-down. 500 μ l of Glutathione Sepharose 4B slurry was added to a microcentrifuge tube. Glutathione Sepharose 4B beads were washed with 1ml 1x PBS, centrifuged and supernatant discarded. Three more washes of the beads were performed prior to addition of 375 μ l 1x PBS. 100 μ l of resulting 50% Glutathione Sepharose 4B were transferred into a fresh tube and 3-4 μ g of dialysed GST-fusion protein sample were added.

The beads were incubated with the GST-fusion protein for 1 h at 4°C by end-over-end rotation and an equal amount of dialysed HIS-fusion protein sample (1:1 relative to the GST-fusion protein) was added and the suspension was incubated for another hour at 4°C. The mix was then centrifuged for 1 min at 500 x g and the supernatant was removed and kept aside on ice. 1 ml PBS supplemented with 0.1% NP-40 was added to the Glutathione Sepharose 4B beads and the mix was agitated gently before centrifuging for 1 min at 500 x g. The supernatant was removed and kept on ice, while the beads were washed twice more with 1 ml PBS plus 0.1% NP-40. Once the supernatant was removed, the Glutathione Sepharose 4B beads were resuspended in 150 μ l 2x Laemmli sample buffer and 1 M DTT was added to a final concentration of 150 mM before boiling for 5 min. The suspension was centrifuged for 10 min at 13,000 rpm and the supernatant was removed into a fresh tube to subject its protein content to TCA precipitation (Section 2.2.11.10). All samples including washes were also TCA precipitated.

2.2.11.10 TCA (tri-chloroacetic acid) precipitation

10 μ l TCA (100% w/v) was added to each protein sample (Section 2.2.11.9), which was then incubated overnight at 4°C. The next day the sample was centrifuged for 15 min at 13,000 rpm. The supernatant was removed and the pellet was washed in 500 μ l acetone. The sample was centrifuged, the supernatant removed and the pellet was finally resuspended in 20 μ l Laemmli

sample buffer and 3 μ l 1 M DTT. 10 μ l of the sample was boiled for 5 min and loaded onto an SDS-PAGE gel. Western blot analysis then followed (2.2.11.11).

2.2.11.11 Immunoblotting using Enhanced Chemiluminescence (ECL)

The Western blotting method was adapted from Towbin *et al.* (1979). Following SDS-PAGE electrophoresis, proteins were transferred from the gel to a nitrocellulose membrane using the Bio-Rad gel kit system at 50 V for 2 h in 1x Transfer buffer (10x Transfer buffer: 250 mM Tris-HCl pH 7.6, 1.92 M glycine) supplemented with 20% v/v methanol. The nitrocellulose membrane was then stained for 5 min with the non-fixative Ponceau S stain solution [0.1% w/v in 5% acetic acid (Sigma)] to confirm complete protein transfer prior to extensive rinsing with dH₂O to remove Ponceau S.

The nitrocellulose membrane was submerged into blocking buffer (25 mM Tris-HCl pH 7.6, 15 mM NaCl, 0.5% v/v Tween-20, 5% non-fat milk powder) and incubated with rotation for 1 h to block non-specific binding sites. The membrane was washed briefly in 1x wash buffer (32.5 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.2% v/v Tween-20 pH to 7.4) and then incubated in appropriate primary antibody (1:1000 to 1:5000 dilution in wash buffer) for 3 h or overnight at 4°C.

The membrane was washed 3 times at 10 min intervals in wash buffer, then incubated in appropriate secondary antibody (1:1000 dilution) for 1 h and washed 3 times in wash buffer (10 min each). The detection process was carried out as described in the GE Healthcare protocol for ECL detection. In the dark room, autoradiography film was placed onto the membrane and exposed for various times depending on signal strength.

2.2.12 Protein Manipulation in S. pombe

2.2.12.1 Preparation of protein extracts for immunoprecipitation and Western blotting

0.5-1 l of *S. pombe* cells grown to mid-exponential phase in YE media, were collected in 50 ml centrifuge tubes by centrifugation at 2000 rpm for 5 min at 4°C. The supernatant was discarded and pelleted cells were washed once with sterile dH₂O. Once the supernatant was discarded, the cells were resuspended in ice-cold HB extraction buffer (25 mM Tris-HCl pH 7.5, 60 mM β -Glycerophosphate, 15 mM p-Nitrophenylphosphate (pNPP), 15 mM EGTA, 15 mM MgCl₂, 5 mM DTT, 0.5 mM Na₃VO₄, 0.1 mM NaF, 0.1% NP-40, 1 mM PMSF, 1 μ g/ml leupeptin, 10 μ g/ml aprotinin) at 3 x 10⁹ cells/ml and transferred to screw-top microcentrifuge tubes. In each case, the

cell suspension was mixed with an equal volume of acid washed glass beads and the tube was chilled on ice for 2-3 min prior to cell lysis using a HYBAID Ribolyser (4 x 20 sec at setting 4). During cell lysis, the cells were chilled on ice for 2-3 min between each disruption step. Following cell breakage, cell lysates were centrifuged at 3000 rpm for 5 min and the supernatants were transferred into fresh tubes, which were then spun at 13000 rpm for 30 min. Following centrifugation, all clarified material, referred to as crude lysate, was transferred into a 15 ml tube on ice and its protein concentration was measured, using the Bradford method (2.2.11.5). The protein concentration of the crude lysate was adjusted to 5 µg/µl with HB extraction buffer before storing at -70°C. Typically, 2 mg of crude lysate was used per immunoprecipitation (IP) reaction of the protein of interest (Section 2.2.12.2).

2.2.12.2 Immunoprecipitation

400 µl of crude lysate (Section 2.2.12.1) was transferred into a 1.5 ml tube and pre-cleared with 40 µl 50% (v/v) protein A- or protein G-Sepharose beads (beads were washed repeatedly and resuspended in an equal volume of HB extraction buffer), depending on the antibody used for immunoprecipitation, via shaking in a Labincro rotator for 1 h at 4°C. Following incubation, the tube was centrifuged at 500 x g for 1 min at 4°C and the supernatant was transferred into a fresh tube. 10% of the supernatant was removed and stored at -70°C as the control non-immunoprecipitated protein sample (input).

To the rest of the pre-cleared lysate, the appropriate antibody [2-3 µg for HA-probe (F-7) and c-Myc (9E10) antibody, 3-4 µl for Plo1p (HO) antibody (Table 2.1)] was added and the suspension was incubated for 3 h at 4°C to bind the antibody to protein of interest. 40 µl 50% (v/v) protein A- or protein G-Sepharose beads were then added and the suspension was incubated for a further 1-2 h at 4°C with rocking. The bead mixture was then centrifuged for 1 min at 500 x g and the supernatant was removed. 0.5 ml HB extraction buffer was added to the bead mixture, which was then agitated gently and centrifuged for 1 min at 500 x g. The supernatant was removed and kept on ice, while the beads were washed twice more with 0.5 ml HB extraction buffer. Once the supernatant was removed, the beads were resuspended in 40 µl 2x Laemmli sample buffer and 1 M DTT was added to a final concentration of 150 mM before boiling for 5 min. The suspension was centrifuged for 10 min at 13,000 rpm and the supernatant (containing the protein of interest and other associated protein (s)) was removed into a fresh tube and stored at -70°C. SDS-PAGE electrophoresis and Western blotting with appropriate antibodies followed to detect the immunoprecipitated protein (IP) as well as an associating, co-immunoprecipitating protein (Co-IP) of interest.

2.2.12.3 Chromatin Immuno-Precipitation assay (ChIP)

Chromatin Immuno-Precipitation (ChIP) was employed to study transcription factor occupancy of transcribed promoters by using formaldehyde to rapidly fix multimeric complexes within living cells, followed by chromatin extraction and immunoprecipitation (Ekwall and Partridge, 1999; Gomez-Roman *et al.*, 2003; Takahashi *et al.*, 2000).

2.2.12.3.1 Fixation of *S. pombe* cells

0.5 l of culture was grown to 5×10^6 cells/ml in YE or EMM (plus appropriate amino acids and/or uracil), as appropriate, at 25°C with shaking. The next day, formaldehyde (FA) from a 37% stock was added to the culture to a final concentration of 1% and cells were incubated for 30 min at 25°C with shaking. Fixation was stopped by adding 2.5 M glycine to the culture to 0.125 M final concentration and a further 5 min incubation with shaking at room temperature. Cells were then collected in 10 x 50 ml centrifuge tubes and harvested by centrifugation at 3000 rpm for 5 min at 4°C. In each case, the supernatant was discarded in a fume hood and pelleted cells were resuspended gently in 20 ml 1 x ice-cold TBS (per l: 137 mM NaCl, 2.683 mM KCl, 24.8 mM Tris base pH to 7.4 with HCl) prior to centrifugation at 3000 rpm for 5 min at 4°C. Once the supernatant was removed, the cell pellet was resuspended in 1 ml 1 x ice-cold TBS and transferred into a 2 ml screw-cap tube. Each tube was spun for 30 sec at 13000 rpm, the supernatant was discarded and cells were stored at -70°C.

2.2.12.3.2 Preparation of *S. pombe* chromatin extracts

The fixed cell pellets from Section 2.2.12.3.1 were each resuspended in 400 µl ice-cold lysis buffer [50 mM Hepes-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) sodium deoxycholate] supplemented with protease inhibitors (Complete, Mini Protease Inhibitor Cocktail Tablets) according to manufacturer's instructions. An equal volume of acid washed glass beads was added to each cell suspension and the tubes were chilled on ice for 2-3 min prior to cell lysis using a HYBAID Ribolyser (3 x 40 sec, 1 pulse at power speed 4.5, 2nd and 3rd pulse at power speed 5). During cell lysis, the cells were chilled on ice for 2-3 min between each disruption step. Following cell breakage, the base of each screw-cap tube was punctured with a 19-gauge needle and placed within a 15 ml centrifuge tube, which was then centrifuged at 2000 rpm for 2-3 min at 4°C to separate the glass beads from the cell lysate. The cell lysates were then transferred into fresh 15 ml tubes (2 ml maximum mixture volume in lysis buffer) before sonication in a Diagenode Bioruptor™ Sonicator,

following the manufacturer's manual [Bioruptor™ on position "high": 30 sec "ON", 30 sec "OFF", total time 10 min (10 cycles= 5 min cumulative sonication time)]. Routinely, a high proportion of 300-1000 bp fragments of chromatin were obtained (Figure 2.1).

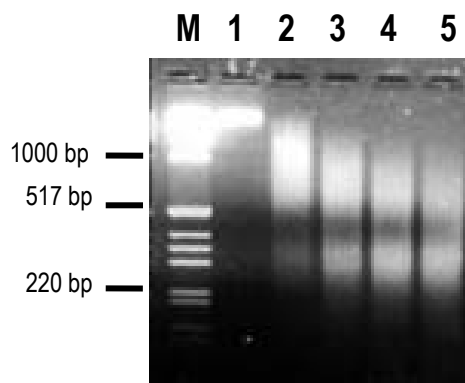


Figure 2.1: Sonication of *S. pombe* lysates from cells fixed with 1% FA. Following cell breakage, lysates were subjected to time courses of sonication in a Diagenode Bioruptor™ Sonicator (Bioruptor™ on position "high": 30 sec "ON", 30 sec "OFF") to establish the conditions for preparation of 300-1000 bp chromatin fragments. Lysates were sonicated for total time 3 min (Lane 2), 5 min (Lane 3), 8 min (Lane 4) and 10 min (Lane 5) and sonicated samples were run on a 2% agarose gel. *S. pombe* genomic DNA was run in lane 1. Lane M indicates DNA Molecular Weight Marker X.

Sonicated material was transferred to microcentrifuge tubes, which were then spun at 13000 rpm for 5 min at 4°C to remove cell debris. The supernatants were transferred to fresh tubes and centrifuged at 13000 rpm for a further 20-30 min at 4°C. Following centrifugation, all clarified material, referred to as crude lysate, was transferred into a 15 ml tube on ice and its protein concentration was measured, using the Bradford method (2.2.11.5). The protein concentration of the crude lysate was adjusted to 10 µg/µl with ice-cold lysis buffer plus protease inhibitors. 4 mg of crude lysate was used per immunoprecipitation (IP) reaction of the chromatin protein of interest (Section 2.2.12.3.3).

2.2.12.3.3 Immunoprecipitation of chromatin extracts

400 µl of crude lysate (Section 2.2.12.3.2) was transferred into a 1.5 ml tube and pre-cleared with 80 µl 50% (v/v) protein A- or protein G-Sepharose beads (beads were washed repeatedly and resuspended in an equal volume of lysis buffer), depending on the antibody used for immunoprecipitation, via shaking in a Labinco rotator for 1 h at 4°C. Following incubation, the tube was centrifuged at 500 x g for 1 min at 4°C and the supernatant was transferred into a fresh tube. 10% of the supernatant was removed and stored at -20°C as the control non-immunoprecipitated chromatin sample.

To the rest of the sample, the appropriate antibody [4-5 µg for HA-probe (F-7) and c-Myc (9E10) antibody, 2 µg for histone H3 antibody, 6-8 µl for Plo1p (HO) or Plo1p (IH) antibody] was added and the suspension was incubated overnight at 4°C with rocking to bind the antibody to the protein of interest. The next day, 80 µl 50% (v/v) protein A- or protein G-Sepharose beads were added to the crude lysate and the suspension was incubated for a further 1-2 h at 4°C with rocking.

Following incubation, the suspension was transferred to a disposable Polypropylene Column (PIERCE). Once the column was drained to remove all unbound material, the beads within the column were washed successively with 2 x 10 ml of RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40), 2 x 10 ml LiCl buffer (10 mM Tris-HCl pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA pH 8.0) and 2 x 10 ml 1x TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). Following washes, the column was tapped and 400µl of 1x TE/1% SDS buffer were added and incubated for 15 min at room temperature to elute the antibody-protein-DNA complexes of interest. Once the end cap of the column was removed, the resulting immunoprecipitated (IP) chromatin was collected into a microcentrifuge tube. 400 µl of 1x TE/1% SDS buffer were also added into the control non-immunoprecipitated (non-IP) chromatin sample.

2.2.12.3.4 Cross-linking reversal and purification of DNA

To reverse the cross-linking of protein to the DNA, the IP and non-IP chromatin samples from Section 2.2.12.3.3 were incubated overnight in a heat-block at 42°C in the presence of 5 µl proteinase K (10 µg/µl stock solution). The next day, both samples were incubated for a further 6 h in a 65°C heat-block and in either case the DNA was then purified using the QIAquick PCR Purification Kit according to the QIAGEN protocol. Finally, the resulting IP and non-IP DNA samples (each eluted in 40 µl 1x TE) were incubated with 1 µl RNase A (10 µg/µl stock solution) for 30 min at 37°C prior to storage at -20°C. PCR amplification of DNA was performed using *Taq* polymerase (Sections 2.2.9.4 and 2.2.9.4.1).

2.2.13 Random Spore analysis in *S. pombe*

Strains of opposite mating type were streaked onto YE plates and grown for 2-3 days at 25°C or 30°C, as necessary. A scoop of cells of the one mating type was then transferred on an ME plate next to a scoop of cells of the opposite mating type (Appendix III). 100 µl of sterile dH₂O was added

on top and the two strains were mixed together with a wooden stick and incubated at 25°C for 2-3 days.

Once mating was confirmed with the presence of ascospores, observed with a light microscope, a scoop of mated cells was mixed with 100 µl sterile dH₂O and 0.5% β-glucuronidase and incubated overnight at 36°C. The next day, the suspension was plated onto solid YE and incubated at 25°C for 4-5 days until colonies were seen. 100 colonies were picked and transferred to another YE plate and incubated at 25°C. The plate was then replica plated onto different selective EMM plates to determine genotypes. Cells of the desired genotype were streaked onto a YE plate to obtain single colonies and the genotype was checked once more.

2.2.14 Tetrad analysis in *S. pombe*

S. pombe strains were mated as described for random spore analysis (Section 2.2.13). A scoop of mated cells was then spread onto a YE plate. Ascospores were picked using a Singer micromanipulator. The cells were incubated at 37°C for 2-5 h until asci walls were broken down. Each ascus was then dissected forming four isolated spores. The spores were incubated until distinct colonies formed. The plate with the colonies was then replica plated onto appropriate selective media to determine the genotype of each progeny.

2.2.15 Light and Microscopic Observations of *S. pombe* Cells

Appropriate *S. pombe* cultures were grown to a density of 5x10⁶ 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 a Sony DS-75 digital camera attached to Zeiss Axiostar R-grade microscope integrated with 40x and 100x DIC filter set.

2.2.16 Yeast Two-Hybrid System

The yeast two-hybrid system in *S. cerevisiae* was employed to analyse potential interactions between *S. pombe* proteins. The budding yeast strain and plasmid vectors used for the two-hybrid screen are listed in Appendix II.

2.2.16.1 X-gal overlay assay

S. cerevisiae cells carrying both bait and prey plasmids were grown on an SD agar plate plus appropriate amino acids and/or uracil for 2-3 days at 30°C. A solution of 9.3 ml 0.5 M potassium phosphate buffer (1x stock: 39 ml 1 M KH₂PO₄, 61 ml 1 M K₂HPO₄ plus 100 ml dH₂O), 0.6 ml dimethyl formamide (DMF) and 0.1 ml 10% SDS was mixed with 80 mg low-melting agarose, which was then melted in a microwave for 40 sec. The agarose solution was allowed to cool down to a temperature of 60°C and 30 µl X-gal (40 mg/ml stock solution in DMF) and 5-6 µl β-mercaptoethanol were added. A Pasteur pipette was then used to cover the cells on the surface of the SD agar plate with the warm solution. The agarose was allowed to set and the plate was placed within a 30°C incubator. The plate was checked for blue colour development every hour during a 6 h period, after which it was left to incubate overnight.

2.2.16.2 Quantitative β-galactosidase assay

To quantify protein interactions identified with the X-gal overlay assay the β-galactosidase assay was carried out as follows: 5 ml of *S. cerevisiae* cells carrying the appropriate bait and prey plasmids were grown in SD media plus any required amino acids and/or uracil to an OD₆₀₀ of 0.2-0.5. 1 ml aliquots of each cell culture were transferred to individual 1.5 ml microcentrifuge tubes, which were then spun for 30 sec at maximum speed. Each cell pellet was resuspended in 500 µl buffer Z (1x stock: 60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, pH adjusted to 7.0 and autoclaved), β-mercaptoethanol to a final concentration of 50 mM and 10 µl chloroform. The tubes were then agitated vigorously by vortex for 20 sec and 100 µl ONPG (4 mg/ml stock in buffer Z) was added to each before incubating at 30°C for 30 min. The reaction was stopped by adding 250 µl 1 M Na₂CO₃. The tubes were cooled on ice for 2-3 min, centrifuged at high speed briefly to remove cell debris and the OD₄₂₀ of the respective supernatants was measured.

Results were expressed as OD₄₂₀ per minute for 1 ml of culture at OD₆₀₀ in Miller Units. 1 Miller Unit is defined as the amount of β-galactosidase activity which hydrolyses 1 µmole of ONPG to o-nitrophenol and D-galactose per minute (Miller, 1992).

$$\beta\text{-galactosidase activity in Miller Units} = 1000 \times \text{OD}_{420} / (t \times V \times \text{OD}_{600})$$

In each case, the assay was performed in triplicate for two individual transformants and statistical analysis was carried out using SPSS version 14.

Chapter 3

*Analysis of the role of cis- and trans- acting
elements in regulation of M-G1 specific
transcription in fission yeast*

3.1 Introduction

Eukaryotic cells undergo a sequence of precise events, known as the mitotic cell cycle, to ensure their proliferation by division into two identical daughter cells. Various processes control advancement through the mitotic cell cycle; one of these is transcription, the regulation of gene expression (Futcher, 2000).

A paradigm of eukaryotic transcription-dependent cell cycle events is the G1-S transition in the budding yeast *S. cerevisiae* (Breedon, 2003). Here, the timing of G1-S progression depends on the prior transcription at peak levels of the *CLN3* and *SWI4* genes during late M-early G1 phase, since Cln3p and Swi4p are rate limiting for the induction of a second wave of transcripts, whose products facilitate progression into S phase.

Cell cycle-specific transcription is a frequent theme in eukaryotic organisms, ranging from budding and fission yeasts to metazoans (Sections 1.5 and 1.6). In the fission yeast *S. pombe*, traditional methods and microarray analysis revealed 400-700 genes that are expressed at specific cell cycle phases (McInerney, 2004; Rustici *et al.*, 2004; Bähler, 2005; Oliva *et al.*, 2005; Peng *et al.*, 2005). Approximately 140 of these genes display significant fluctuations in their mRNA levels during the mitotic cell cycle and are classified into four groups, based on their cell cycle stages of peak transcription (Rustici *et al.*, 2004; Section 1.5.2). The first group of genes is maximally transcribed at the G1-S boundary, the second during S phase, the third during G2 phase and the fourth during the M-G1 interval. In some cases, the molecular mechanisms, including promoter sequences and transcription factors, regulating transcription of each group have been identified (Section 1.5.2).

3.1.1 M-G1 specific transcription in *S. pombe*

Several genes are members of the gene cluster transcribed maximally during the M-G1 transition in fission yeast (Anderson *et al.*, 2002; Buck *et al.*, 2004; Bulmer *et al.*, 2004; Rustici *et al.*, 2004). This gene family includes among others, *plo1⁺*, *cdc15⁺*, *spo12⁺*, *ppb1⁺*, *slp1⁺*, *ace2⁺*, *sid2⁺* and *mid1⁺/dmf1⁺*, and all are involved in a variety of late mitotic events, including spindle formation, the onset of anaphase, positioning, formation and constriction of the actin-based medial ring, septation and cell separation (Fankhauser *et al.*, 1995; Simanis, 1995; Bähler *et al.*, 1998b; Mulvihill *et al.*, 1999; Samuel *et al.*, 2000; Utzig *et al.*, 2000; Tanaka *et al.*, 2001; Alonso-Núñez *et al.*, 2005; Section 1.5.2.4).

M-G1 specific transcription was first established for *cdc15⁺*, with this gene displaying maximum mRNA levels during metaphase (Fankhauser *et al.*, 1995). Several approaches were

adopted to reveal the *cis*- and *trans*- acting elements underlying cell cycle control of *cdc15⁺* expression (Utzig *et al.*, 2000; Anderson *et al.*, 2002). Such studies have shown that a ~100 bp *cdc15⁺* promoter fragment encompassing a 6 bp sequence, termed the Pombe cell cycle box (PCB), is sufficient to impart periodic M-G1 specific transcription to the heterologous *lacZ* gene, expressed ectopically from a reporter plasmid, within *S. pombe* cells. In parallel, gel retardation assays with *S. pombe* protein extracts and the same promoter fragment as a radio-labelled probe revealed a PCB specific binding factor complex, named the PCB-binding factor (PBF).

As discussed earlier, DNA sequences similar to the PCB are present in the promoter regions of *plo1⁺*, *spo12⁺*, *ppb1⁺*, *slp1⁺*, *fin1⁺*, *ace2⁺*, *sid2⁺* and *mid1⁺/dmf1⁺* genes, which all display the same cyclic pattern of M-G1 specific transcription as *cdc15⁺* (Anderson *et al.*, 2002, Buck *et al.*, 2004). Competitive gel retardation assays have identified the cognate PCB sequence for a few of these genes, resulting in the prediction of the GNAACR consensus for the PCB motif (Anderson *et al.*, 2002); this sequence has some similarities with the forkhead transcription factor binding motif (RTAAAYA) (Hromas and Costa, 1995; Kaufmann *et al.*, 1995). Later, computer based database analyses revealed that M-G1 transcribed genes contain another promoter element resembling a forkhead binding motif present in either orientation (Rustici *et al.*, 2004; Peng *et al.*, 2005). Consistent with these observations, two forkhead-like transcription factors are implicated in regulation of M-G1 specific transcription in *S. pombe*, suggesting that the forkhead promoter motif might be involved in M-G1 specific transcription, exerting its role either together or in parallel with the PCB promoter sequence (Buck *et al.*, 2004; Bulmer *et al.*, 2004).

3.1.2 Regulation of M-G1 specific transcription

The discovery of the PBF protein complex prompted the identification of its component(s) and other regulatory factors. So far the products of four genes, *flh2⁺*, *sep1⁺*, *mbx1⁺* and *plo1⁺* have been implicated in the regulation of M-G1 specific transcription in fission yeast (Zilahi *et al.*, 2000; Anderson *et al.*, 2002; Buck *et al.*, 2004; Bulmer *et al.*, 2004).

plo1⁺ is itself periodically transcribed during M-G1, suggesting that Plo1p regulates not only its own transcription but all the other M-G1 transcribed genes in the group by an autoregulatory loop (Anderson *et al.*, 2002). Since *plo1⁺* encodes a Ser/Thr kinase (Ohkura *et al.*, 1995), it is possible that Plo1p regulates transcription by interacting with and phosphorylating PBF component(s) and/or other regulatory factors important for M-G1 gene expression. Indeed, it has been shown recently that Plo1p interacts *in vitro* and *in vivo*, and directly phosphorylates Mbx1p (Papadopoulou *et al.*, 2008). The role of Plo1p in M-G1 transcription will be examined later in this thesis (Chapters 5 and 6).

fkh2⁺ and *sep1⁺* encode members of the conserved family of forkhead/HNF-3 transcription factors (Section 1.4.1.2.4), possessing a 110 amino acid monomeric DNA binding domain, the winged helix motif, present in multi-cellular eukaryotes, including *Drosophila*, *Xenopus*, rat and human (Weigel *et al.*, 1989; Dirksen and Jamrich, 1992; Clark *et al.*, 1993; Lai *et al.*, 1993; Kaufmann and Knochel, 1996). Forkhead transcription factors have been assigned various functions in early developmental events, cell differentiation and cell cycle progression (Hromas and Costa, 1995; Kaufmann and Knochel, 1996; Yang *et al.*, 1997; Costa, 2005).

mbx1⁺ encodes a homologue of the MADS-box family of transcription factors (Section 1.4.1.2.3), found in a variety of eukaryotes ranging from budding yeast to humans, each containing a conserved 56 amino acid motif (MADS-box) within the DNA-binding domain (Jarvis *et al.*, 1989; Treisman and Ammerer, 1992; Shore and Sharrocks, 1995). MADS-box proteins regulate transcription of genes mediating distinct biological processes; for example muscle cell cycle progression and cell differentiation in mammals (Han and Prywes, 1995; Shore and Sharrocks, 1995), floral organ development in plants (Davies and Schwarz-Sommer, 1994; Ma, 1994), and arginine metabolism, cell type determination, mating and cell cycle progression in *S. cerevisiae* (Dolan and Fields, 1991; Lydall *et al.*, 1991; Messenguy and Dubois, 1993; Althoefer *et al.*, 1995).

Most MADS-box transcription factors bind their cognate DNA sequence [CC(AT)₆GG] as homodimers and mediate the formation of multi-component regulatory complexes by recruiting other transcription factors, which in turn activate or repress transcription of several target genes. Examples of such transcription factors, assembled into complexes with MADS-box proteins on the promoter region of target genes, are several myogenic basic-helix-loop-helix (bHLH) proteins in mammalian cells (Buckingham, 1994; Molkenin and Olson, 1996) and the forkhead transcription factor Fkh2p in budding yeast (Althoefer *et al.*, 1995; Kumar *et al.*, 2000; Pic *et al.*, 2000).

Mcm1p is the *S. cerevisiae* MADS-box transcription factor recruiting Fkh2p on the promoters of several 'CLB2' cluster genes (Maher *et al.*, 1995; Hollenhorst *et al.*, 2000; Jorgensen and Tyers, 2000; Kumar *et al.*, 2000; Pic *et al.*, 2000; Zhu *et al.*, 2000; Hollenhorst *et al.*, 2001). These genes are periodically transcribed during late G2-early M phase as a result of transcriptional regulation by the Fkh2p-Mcm1p complex, bound to the genes' promoters throughout the cell cycle, along with Fkh1p, and the co-activator Ndd1p protein. Fkh1p binds to the promoters of 'CLB2' cluster genes in a Mcm1p-independent manner (Kumar *et al.*, 2000; Hollenhorst *et al.*, 2001). Ndd1p is recruited to the promoters of 'CLB2' cluster genes exclusively during the G2-M interval to activate their transcription. Phosphorylation of Ndd1p by Cdc28p-Clb2p complexes, as well as Cdc5p, is a prerequisite for its recruitment and subsequent association with the FHA domain of Fkh2p (Koranda *et al.*, 2000; Darieva *et al.*, 2003; Reynolds *et al.*, 2003; Darieva *et al.*, 2006), binding serine or

threonine phosphorylated residues (Durocher and Jackson, 2002). Cell cycle specific recruitment of Cdc5p onto the promoters of 'CLB2' cluster genes requires Fkh2p, which is itself phosphorylated at its C-terminus in a Cdc28p-Clbp dependent manner prior to its interaction with phosphorylated Ndd1p (Darieva *et al.*, 2003; Pic-Taylor *et al.*, 2004; Darieva *et al.*, 2006).

The products of the *S. pombe fkh2⁺* and *sep1⁺* genes, Fkh2p and Sep1p, are homologues of *S. cerevisiae* Fkh1p and Fkh2p, whereas Mbx1p, encoded by the *S. pombe mbx1⁺* gene, is similar to Mcm1p (Buck *et al.*, 2004). These observations suggest that M-G1 specific transcription in fission yeast might engage common control mechanisms to those seen in budding yeast, although the absence of an Ndd1p homologue or obvious Mcm1p-binding sequences within the promoters of M-G1 periodically expressed genes, argues that distinct regulatory networks may operate in this organism.

Several lines of evidence support a role for *fkh2⁺*, *sep1⁺* and *mbx1⁺* in transcription at the M-G1 transition. Gel retardation assays with protein extracts from *mbx1 Δ* cells and the *cdc15⁺* promoter fragment revealed that deletion of *mbx1⁺* abolishes binding of the PBF complex to the PCB sequence, suggesting that Mbx1p is a core PBF component. Nonetheless, as shown by Northern blot analysis, various genes, including *cdc15⁺*, *spo12⁺*, *fin1⁺*, *slp1⁺* and *ace2⁺*, are still periodically transcribed during the M-G1 phase in the absence of *mbx1⁺*, implying that other transcription factors must be responsible for regulating the oscillating transcriptional pattern of these genes. In support of this conjecture, deletion of either *fkh2⁺* or *sep1⁺* eliminates periodic transcription of all M-G1 genes, with their mRNA levels present at constant levels throughout the cell cycle, suggesting that Sep1p and Fkh2p transcription factors are required for M-G1 specific transcription (Buck *et al.*, 2004). Notably, *fkh2⁺* is also periodically transcribed during the cell cycle, its mRNA levels peaking during late mitosis-G1 phase, and its promoter contains PCB and forkhead sequences, implying that *fkh2⁺* regulates its own expression and that of other M-G1 transcribed genes by feedback mechanisms (Buck *et al.*, 2004; Bulmer *et al.*, 2004; Rustici *et al.*, 2004).

3.2 Role of the PCB sequence in M–G1 specific transcription in *S. pombe*

Previous work has shown that a *cdc15⁺* promoter fragment (-157 to -53 relative to the ATG) confers M-G1 specific transcription to *lacZ*, when cloned upstream of the reporter gene in the UAS reporter plasmid pSP Δ 178 (Utzig *et al.*, 2000; Anderson *et al.*, 2002). The ability of the *cdc15⁺* promoter fragment to confer M-G1 specific transcription to a heterologous gene allows two conclusions: firstly,

it shows that this promoter region contains the necessary *cis*-acting element(s) mediating M-G1 transcription of *cdc15⁺*; and, secondly, it suggests that the periodic mRNA pattern of *cdc15⁺* is a result of regulation at the level of transcription and not mRNA stability.

Gel retardation assays revealed a 6 bp sequence, GCAACG, termed the PCB, within the same *cdc15⁺* promoter fragment that binds *in vitro* to a protein complex, termed PBF. All the genes displaying the same oscillating cell cycle-specific mRNA pattern as *cdc15⁺*, including *spo12⁺*, *sid2⁺*, *fin1⁺*, *slp1⁺*, *ace2⁺* and *plo1⁺*, possess sequences similar to GCAACG in their promoters. This has allowed the prediction of a consensus sequence for the PCB, GNAACR (Anderson *et al.*, 2002).

Following these findings, one of the first aims of this study was to confirm whether the PCB controls M-G1 specific transcription in *S. pombe*. To accomplish this, mutated versions of the PCB sequence (GCAACG substituted for AAGGTT or TTGGTT), within the *cdc15⁺* promoter fragment, were assayed by Northern blot for their effect on cell cycle transcription of *lacZ*, expressed ectopically from the pSPΔ178 reporter construct.

Two pSPΔ178 reporter constructs were generated, pSPΔ178.15UAS.MUT and pSPΔ178.15UAS.MUT2, the former carrying the AAGGTT-, while the latter the TTGGTT- containing *cdc15⁺* promoter fragment. The two plasmids were transformed separately into *cdc25-22* fission yeast cells, which were then synchronised by transient temperature arrest and allowed to progress through the mitotic cell cycle. Samples were then collected at successive time points and analysed by Northern blot to monitor levels of *lacZ* mRNA.

3.2.1 Construction of pSPΔ178.15UAS.MUT and pSPΔ178.15UAS.MUT2 reporter plasmids

3.2.1.1 The pSPΔ178 reporter plasmid

The pSPΔ178 reporter plasmid is a high copy vector derived from the budding yeast shuttle vector pLG669-Z (Guarente and Ptashne, 1981; Guarente and Mason, 1983; Figure 3.1). pSPΔ178 carries the *E. coli* selectable marker Amp^R, the *S. cerevisiae* URA3 marker (selectable also in *S. pombe* since it complements the fission yeast *ura4* allele), as well as appropriate *E. coli* and *S. pombe* origins of replication, *ori* and ARS6, respectively. It also possesses the *lacZ* gene fused at its 5' end to a fragment from the promoter of the *S. cerevisiae* cytochrome c (*CYC1*) gene, functioning in fission yeast as a minimal promoter, which by itself is unable to activate *lacZ* transcription (Guarente and Mason, 1983; Lowndes *et al.*, 1992). A *Xho*I restriction site, present immediately upstream of the *CYC1* minimal promoter, facilitates insertion of the *S. pombe* promoter sequence of interest, such as

a potential upstream activation sequence (UAS), which can then be assessed by Northern blot analysis for its effect on *lacZ* transcription.

The pSP Δ 178 reporter construct has been successfully employed to elucidate the role of the MCB promoter motif in regulation of G1-S cell cycle specific transcription in *S. pombe* (Lowndes *et al.*, 1992; Maqbool *et al.*, 2003) and to show that a PCB-containing *cdc15*⁺ promoter fragment imparts M-G1 cell cycle specific transcription to the *lacZ* gene (Anderson *et al.*, 2002). In the present study, this vector was used to determine whether the PCB sequence is a requirement for M-G1 cell cycle specific transcription in *S. pombe*, by studying the effect of mutated versions of the *cdc15*⁺ PCB sequence on *lacZ* cell cycle transcription.

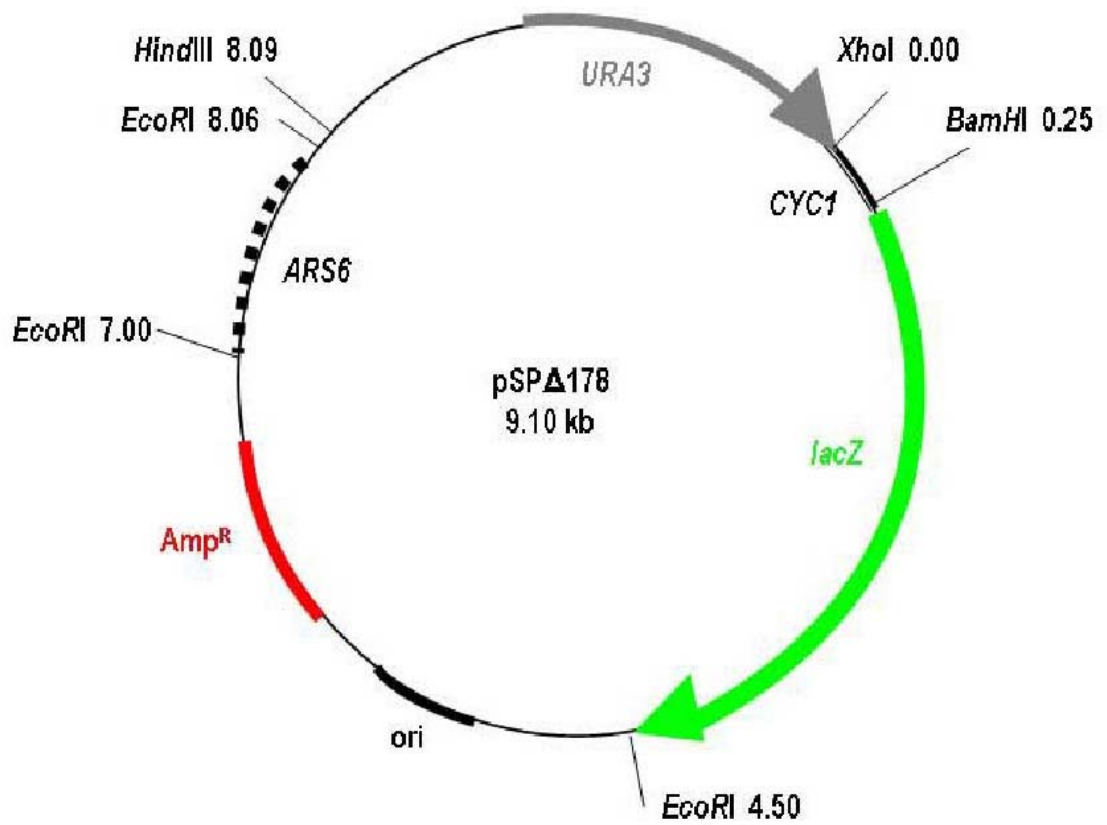


Figure 3.1: The fission yeast UAS reporter plasmid pSPΔ178. pSPΔ178 contains the minimal *CYC1* promoter from budding yeast upstream of the *lacZ* gene which, by itself, does not activate *lacZ* transcription (Guarente and Mason 1983; Lowndes *et al.*, 1992). A potential UAS is inserted into the *XhoI* restriction site upstream of the *CYC1* promoter and UAS activity is assayed by *lacZ* transcription.

3.2.1.2 *pSPΔ178.15UAS.MUT* and *pSPΔ178.15UAS.MUT2* constructs

Classical molecular procedures were adopted to construct the *pSPΔ178.15UAS.MUT* and *pSPΔ178.15UAS.MUT2* plasmids, each carrying an appropriate *cdc15⁺* promoter fragment with a mutated version of the PCB sequence.

The first step in the process involved creation of two ~100 bp DNA fragments, corresponding to the *cdc15⁺* promoter fragment that drives M-G1 specific *lacZ* transcription (Anderson *et al.*, 2002), but carrying either the mutated AAGGTT or the TTGGTT sequence instead of GCAACG, the wild-type PCB promoter motif. To generate the AAGGTT- and TTGGTT- containing *cdc15⁺* promoter fragments, termed for convenience “PCB mut1” and “PCB mut2”, two complementary single stranded DNA oligonucleotides were custom-made and annealed to produce in each case a double stranded DNA fragment (Section 2.2.9.8) with appropriate protruding 5' ends to facilitate cloning into the *Xho*I site of the *pSPΔ178* plasmid (GB 10). Oligonucleotides GO 590 and GO 591 yielded PCB mut1 DNA, whereas oligonucleotides GO 588 and GO 589 produced the PCB mut2 DNA fragment (Appendix I). The 5' ends of the PCB mut1 and PCB mut2 DNA fragments were then phosphorylated by T4 polynucleotide kinase before ligation with the respective ends of *Xho*I-digested and dephosphorylated *pSPΔ178* vector (Sections 2.2.9.6-2.2.9.11). In each case, transformation into DH5α cells (GB 4) (Section 2.2.3) was followed by overnight growth at 37°C on solid media supplemented with ampicillin, with successful transformants picked, grown overnight in liquid media and subjected to plasmid purification to screen for successful cloning.

Successful cloning of the PCB mut1 or PCB mut2 DNA fragment into the *Xho*I site of *pSPΔ178*, was verified by PCR analysis (Section 2.2.9.4). Primers GO 47 (forward) and GO 46 (reverse) (Appendix I), the former corresponding to a sequence ~50 bp upstream of the *Xho*I site and the latter ~20 bp downstream, were used to screen for the presence of insert, either the PCB mut1 or the PCB mut2 DNA fragment. Next, primers GO 400 (forward) and GO 46, the first corresponding to a sequence within the *cdc15⁺* promoter fragment (-132 to -114 relative to the ATG), were used to test for cloning of DNA fragments into the correct orientation. As a positive control, the *pSPΔ178.15UAS* reporter plasmid (GB 194), carrying the wild-type PCB-containing *cdc15⁺* promoter fragment (Anderson *et al.*, 2002), was used as a template for PCR amplification with primer pair GO 46 and GO 47, or GO 46 and GO 400, respectively.

PCR analysis identified one isolate of *pSPΔ178.15UAS.MUT* (GB 464) and one isolate of *pSPΔ178.15UAS.MUT2* (GB 469). As shown in Figures 3.2A and 3.2B, PCR amplification with primers GO 46 and GO 47 with *pSPΔ178.15UAS.MUT* (Lane A-3) or *pSPΔ178.15UAS.MUT2* (Lane B-2), yielded a ~180 bp fragment, consistent with the presence of a ~100 bp insert. Similarly, PCR

amplification with primers GO 46 and GO 400 generated a ~100 bp DNA, verifying that cloning of the PCB mut1 (Lane A-4) or PCB mut2 (Lane B-4) fragment occurred in the correct orientation. Finally, both constructs were confirmed by DNA sequencing (MWG Biotech).

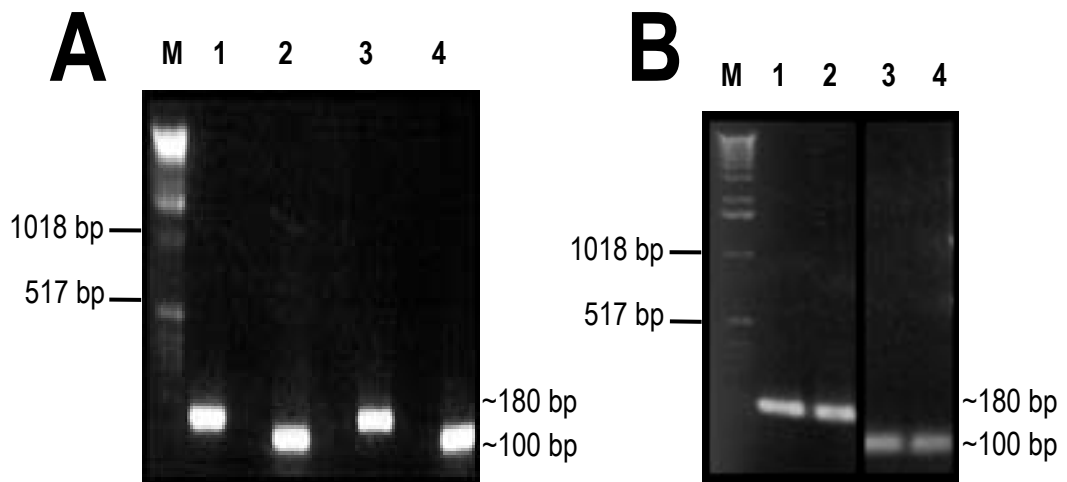


Figure 3.2: Verification of pSP Δ 178.15UAS.MUT and pSP Δ 178.15UAS.MUT2 reporter constructs. PCR analysis was performed to verify cloning of (A) PCB mut1 DNA fragment and (B) PCB mut2 DNA fragment into the *Xho*I site of pSP Δ 178, yielding plasmid pSP Δ 178.15UAS.MUT (GB 464) and pSP Δ 178.15UAS.MUT2 (GB 469), respectively. In each case, primer pair GO 46 and GO 47, flanking the *Xho*I site, was used to PCR amplify a ~180 bp fragment (Lanes A-3 and B-2) to check for cloning into pSP Δ 178, while primer pair GO 46 and GO 400 was used to PCR amplify a ~100 bp fragment (Lanes A-4 and B-4) to check for cloning into the correct orientation. As a positive control, pSP Δ 178.15UAS plasmid (GB 194) was used as a template for PCR amplification with primer pair GO 46 and GO 47 (Lanes A-1 and B-1) or primer pair GO 46 and GO 400 (Lanes A-2 and B-3). Lane M (A-B) indicates DNA Molecular Weight Marker X.

3.2.2 Requirement of the PCB sequence for M-G1 specific transcription in fission yeast

To elucidate the role of the PCB promoter motif in regulation of *cdc15⁺* transcription, two *cdc15⁺* promoter fragments, termed PCB mut1 and PCB mut2 DNA, each containing a mutated version of the PCB sequence, AAGGTT or TTGGTT, were separately introduced upstream of *lacZ* into the pSPΔ178 vector (Section 3.2.1.2). Northern blot analysis was then performed to test the ability of each of these fragments to drive M-G1 specific transcription of the *lacZ* reporter gene within synchronously dividing *S. pombe* cells. As a positive control, the wild-type *cdc15⁺* PCB sequence, GCAACG, was also monitored for its effect on M-G1 specific transcription of *lacZ*.

Creation of the pSPΔ178.15UAS.MUT and pSPΔ178.15UAS.MUT2 reporter plasmids, carrying the PCB mut1 and PCB mut2 DNA fragments, respectively, was described in Section 3.2.1.2. The pSPΔ178.15UAS reporter construct encompassing the *cdc15⁺* promoter fragment with the PCB sequence has been described (Anderson *et al.*, 2002).

Each of the three reporter constructs, pSPΔ178.15UAS, pSPΔ178.15UAS.MUT or pSPΔ178.15UAS.MUT2, was separately transformed into *cdc25-22 ura4-D18 leu1-32* (GG 308) *S. pombe* cells (Section 2.2.6), carrying the *cdc25-22* temperature sensitive allele (Fantes, 1979), as well as a complete deletion of the *ura4* gene (Grimm and Kohli, 1988; Grimm *et al.*, 1988). The *cdc25-22* mutation causes cells to arrest at the G2 cell cycle phase upon transfer from the permissive temperature of 25°C to the restrictive temperature of 36°C; on return to 25°C the cells begin dividing again in a highly synchronous manner, hence facilitating the synchronisation of a cell population by a simple transient temperature arrest. The *ura4-D18* deletion allows the maintenance in *S. pombe* cells of plasmids, such as pSPΔ178 (Section 3.2.1.1), possessing the *S. cerevisiae* *URA3* gene that complements *S. pombe ura4* mutations, upon growth in medium lacking uracil.

Following transformation, a single colony of *cdc25-22 ura4-D18 leu1-32* cells carrying either one of the three pSPΔ178 reporter constructs, was added separately to 5 ml of leucine-supplemented EMM and grown overnight at 25°C. The next day, each 5 ml culture was transferred into 500 ml of the same selective media and cells were grown at 25°C to mid-log phase before transfer to 36°C for 3.5 hours to arrest at G2 phase. After the transient temperature arrest, each cell culture was returned and incubated at the permissive temperature (25°C), allowing cells to progress through mitosis and subsequent phases of the cell cycle in synchrony. In each case, cell samples were collected every 15 min for a four hour period. Total RNA was prepared from each sample and resolved by formaldehyde gel electrophoresis (Section 2.2.10.1 and 2.2.10.3). Northern blot analysis (Section 2.2.10.4) followed using appropriate DNA probes to assay the cell cycle mRNA levels of *lacZ*, as well as endogenous *cdc15⁺*, the latter used as a positive control, to confirm synchrony of the

cells. As a loading control the rRNA content of each RNA sample was detected by ethidium bromide staining. In some experiments, *adh1⁺*, a gene with an invariant mRNA profile during the mitotic cell cycle, was also used as a probe for Northern blotting (Buck *et al.*, 2004). As another measure of cell synchrony, the septation index of each sample was counted microscopically and plotted as a percentage of cell septa against time.

The results obtained from these experiments are shown in Figures 3.3, 3.4 and 3.5, displaying the cell cycle transcript profile of *lacZ* under control of the PCB-, AAGGTT- and TTGGTT-containing *cdc15⁺* promoter fragment, respectively.

As expected, the fragment with the wild-type *cdc15⁺* PCB sequence conferred M-G1 specific transcription to *lacZ*, since the cell cycle mRNA profile of the reporter gene was similar to that of the endogenous *cdc15⁺* gene (Figure 3.3; Buck *et al.*, 2004). This result confirmed earlier work showing the M-G1 specific mRNA profile of *lacZ*, mediated by the PCB-containing *cdc15⁺* promoter fragment, in synchronously dividing wild-type cells prepared by centrifugal elutriation (Anderson *et al.*, 2002).

In contrast, changing the PCB sequence from GCAACG to AAGGTT within the *cdc15⁺* promoter fragment abolished transcription of *lacZ* throughout the cell cycle but not of the endogenous wild-type *cdc15⁺* gene (Figure 3.4), confirming a requirement of the PCB sequence for PBF-dependent transcription of *cdc15⁺* in *S. pombe* (Buck *et al.*, 2004). Nonetheless, the second type of *cdc15⁺* PCB mutation, changing GCAACG to TTGGTT, drastically reduced the amplitude of *lacZ* mRNA levels at all cell cycle times observed, but did not affect cell cycle-dependent transcription *per se*. Indeed, as shown in Figure 3.5, the *cdc15⁺* promoter fragment containing the TTGGTT sequence instead of the PCB was still able to support cell cycle specific transcription of the reporter gene, since the *lacZ* mRNA levels fluctuated in abundance in a periodic manner and peaked at times corresponding to the M-G1 interval, despite their radical decline in terms of relative amount throughout the cell cycle. Taken together, these findings reveal that the PCB sequence positively regulates transcription of *cdc15⁺* in a cell cycle independent manner.

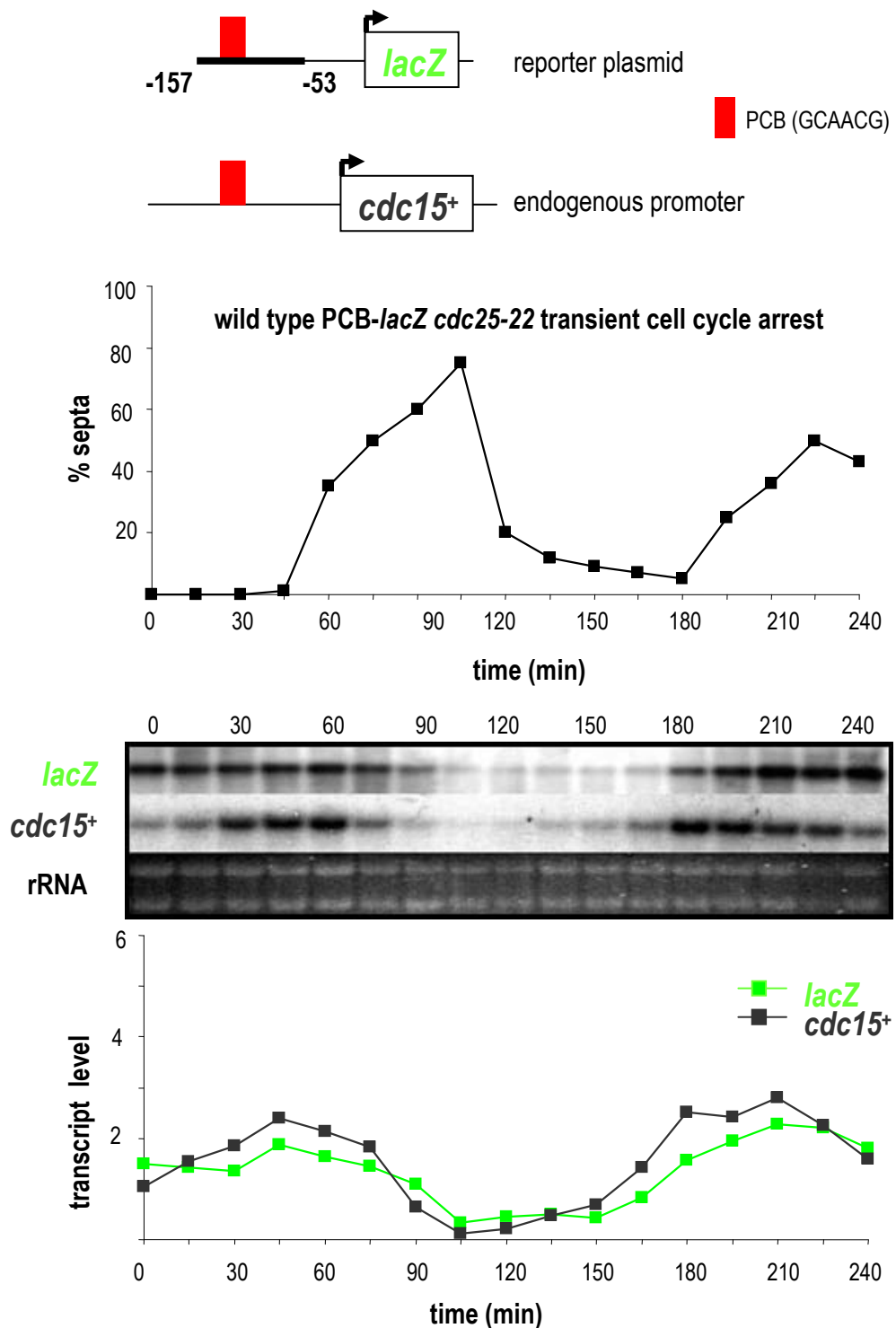


Figure 3.3: A *cdc15+* promoter fragment containing the PCB sequence (GCAACG) confers M-G1 specific transcription on the *lacZ* reporter. *cdc25-22* cells (GG 308) transformed with the pSPΔ178.15UAS plasmid (GB 194) were synchronised by transient temperature arrest. Samples were collected every 15 min upon release to the permissive temperature and total RNA was prepared for Northern blot analysis. The transient cell cycle arrest with *cdc25-22* cells transformed with pSPΔ178.15UAS plasmid was The blot was hybridised consecutively with *cdc15+* and *lacZ* probes. Ethidium staining of rRNA was used as a loading control. Quantification of mRNA levels against rRNA is shown. Septation indices were counted microscopically and are plotted to indicate the synchrony of the culture. Northern blot analysis was carried out twice with samples prepared from two individual cultures of synchronous cells. The results obtained from Northern blot analysis of samples from one of the two cultures are shown here and are representative of the results from both experiments.

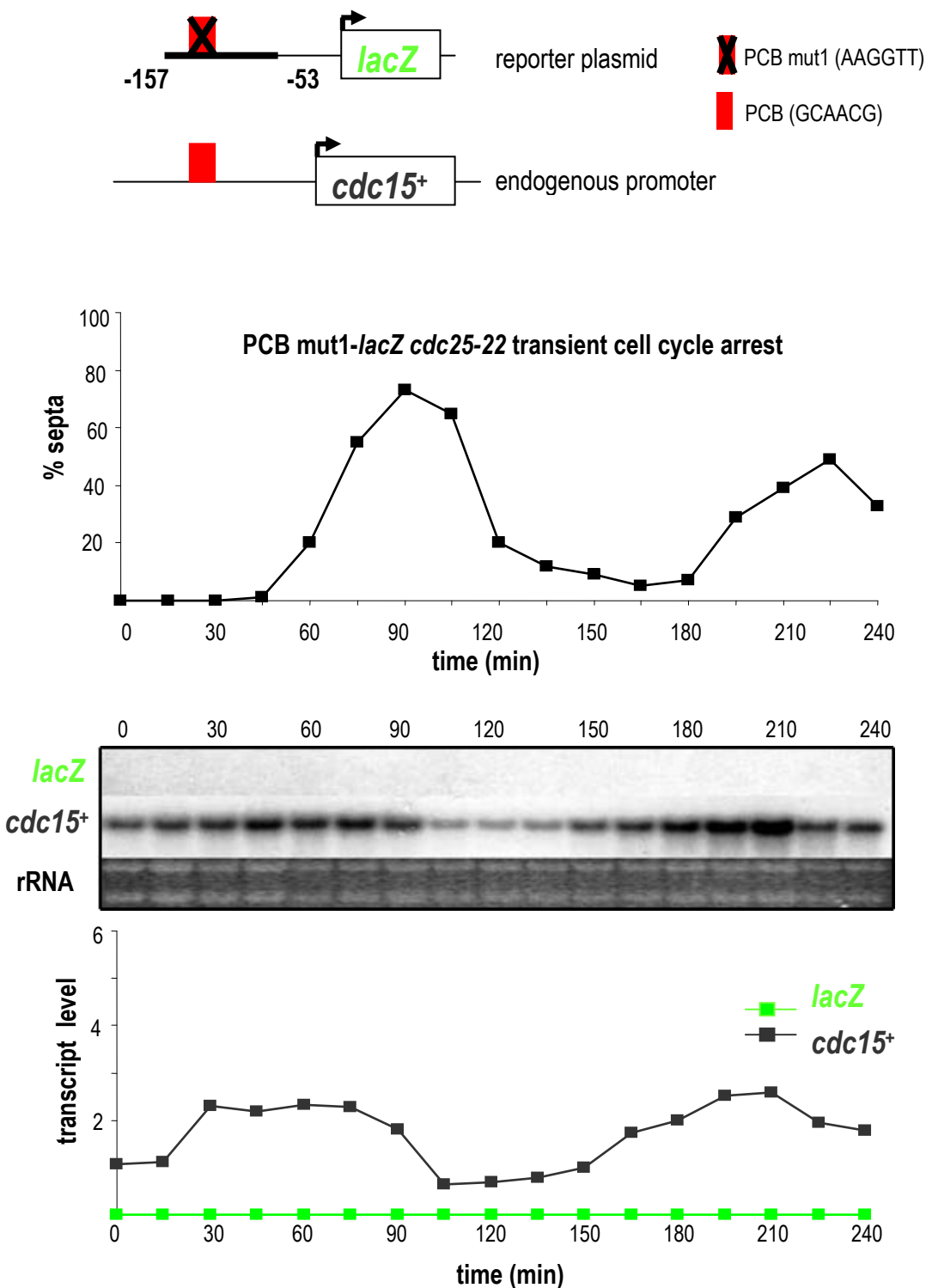


Figure 3.4: A *cdc15+* promoter fragment containing AAGGTT instead of the PCB sequence (GCAACG) abolishes transcription of the *lacZ* reporter gene. *cdc25-22* cells (GG 308) transformed with the pSPΔ178.15UAS.MUT plasmid (GB 344) were synchronised by transient temperature arrest. Samples were collected every 15 min upon release to the permissive temperature and total RNA was prepared for Northern blot analysis. The blot was hybridised consecutively with *cdc15+* and *lacZ* probes. Ethidium staining of rRNA was used as a loading control. Quantification of mRNA levels against rRNA is shown. Septation indices were counted microscopically and are plotted to indicate the synchrony of the culture. Northern blot analysis was carried out twice with samples prepared from two individual cultures of synchronous cells. The results obtained from Northern blot analysis of samples from one of the two cultures are shown here and are representative of the results from both experiments.

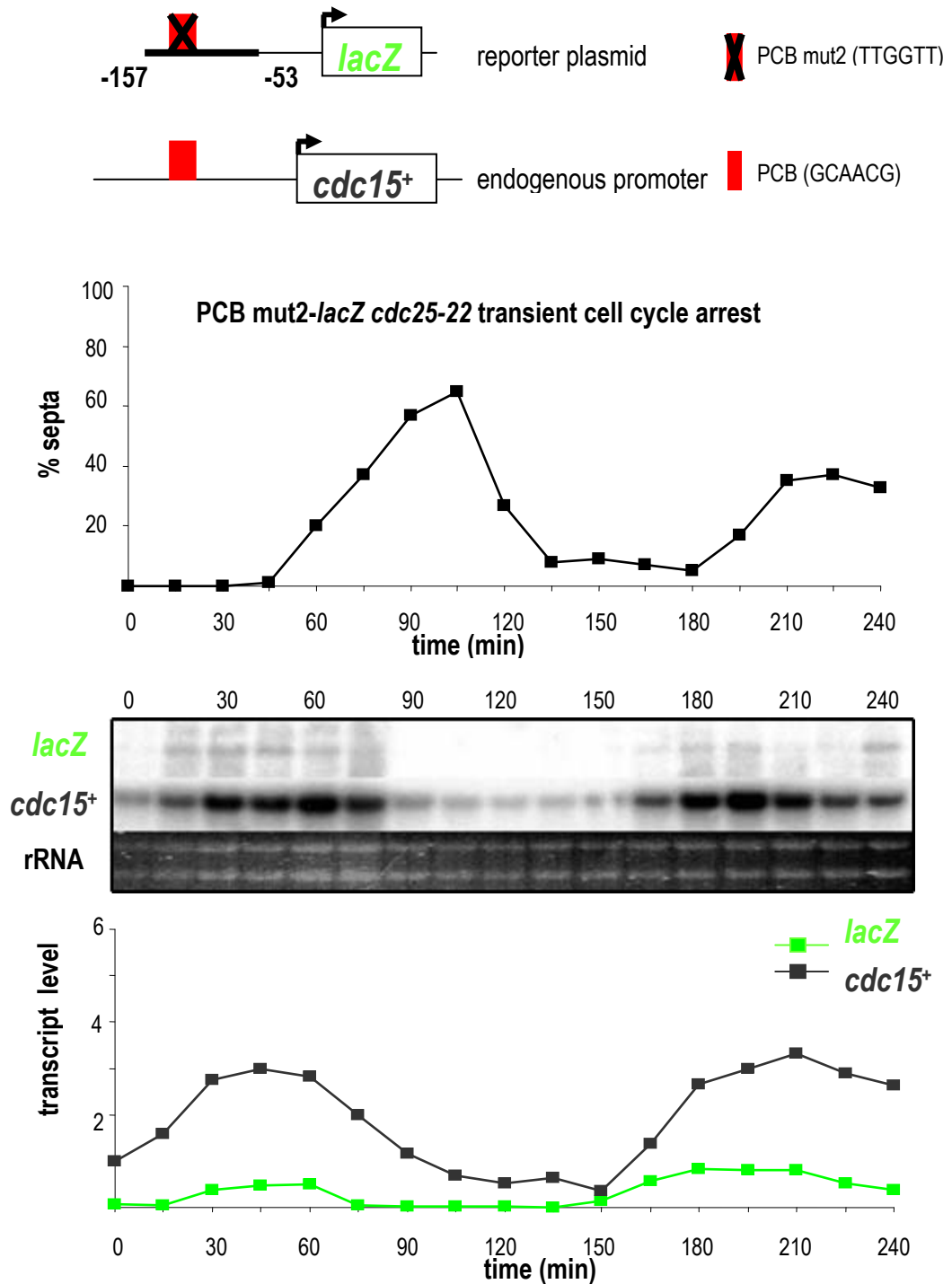


Figure 3.5: A *cdc15⁺* promoter fragment containing TTGGTT instead of the PCB sequence (GCAACG) drastically affects the amplitude but not the cell cycle specific pattern of transcription of the *lacZ* reporter gene. *cdc25-22* cells (GG 308) transformed with the pSPΔ178.15UAS.MUT2 plasmid (GB 469) were synchronised by transient temperature arrest. Samples were collected every 15 min upon release to the permissive temperature and total RNA was prepared for Northern blot analysis. The blot was hybridised consecutively with *cdc15⁺* and *lacZ* probes. Ethidium staining of rRNA was used as a loading control. Quantification of mRNA levels against rRNA is shown. Septation indices were counted microscopically and are plotted to indicate the synchrony of the culture. Northern blot analysis was carried out in two separate occasions with total RNA samples prepared from two individual cultures of synchronous *cdc25-22* cells transformed with the pSPΔ178.15UAS plasmid. Northern blot analysis was carried out twice with samples prepared from two individual cultures of synchronous cells. The results obtained from Northern blot analysis of samples from one of the two cultures are shown here and are representative of the results from both experiments.

3.3 Role of the Fkh2p and Sep1p transcription factors in regulation of M-G1 specific transcription in *S. pombe*

Various studies have revealed that the encoded products of *fkh2⁺*, *sep1⁺* and *mbx1⁺* are involved in regulation of M-G1 transcription in fission yeast (Buck *et al.*, 2004; Bulmer *et al.*, 2004; Rustici *et al.*, 2004).

mbx1⁺ was identified by database searches based on its similarity to genes encoding MADS-box proteins (Treisman and Ammerer, 1992; Buck *et al.*, 2004). It is a non-essential gene, since the *mbx1Δ* mutant is viable and grows both on solid and in liquid media, comparable to wild-type, although it is defective in cytokinesis (Buck *et al.*, 2004). Moreover, *mbx1Δ* cells display aberrant mating as they are able to cross with either h⁻ or h⁺ cells (Appendix V), indicating a role for *mbx1⁺* in sexual differentiation.

Fission yeast *fkh2⁺* was initially identified in a genetic screen when a truncated version of the gene acted as a multi-copy suppressor of the G2-M cell cycle arrest phenotype of the *mcs3-12 cdc25-22 wee1-50* triple mutant (Molz *et al.*, 1989; Samuel *et al.*, 2000). Although *fkh2⁺* is non-essential, *fkh2Δ* cells grow more slowly and are longer relative to wild-type, while a small proportion exhibits unconstricted, multiple or misplaced septa, suggesting a role for Fkh2p in the control of mitotic events and cytokinesis (Buck *et al.*, 2004; Bulmer *et al.*, 2004). Moreover, *fkh2Δ* cells exhibit conjugation and sporulation defects and are partially sterile (Szilagyi *et al.*, 2005), consistent with the recent finding that Fkh2p in a Cdc2p-dependent manner, regulates transcription of *ste11⁺*, a transcription factor modulating the expression of genes necessary for sexual differentiation (Shimada *et al.*, 2008). Perhaps also of note is the observation that the slow growth and septation phenotype of *fkh2Δ* cells is only seen upon growth on rich (YE) but not minimal (EMM) solid medium (Appendix VI).

The *sep1⁺* gene is also not essential for viability, but *sep1Δ* cells are defective in cell separation, forming branched hyphae, showing regulatory functions in late mitotic events, such as septation and cytokinesis (Sipiczki *et al.*, 1993; Gould and Simanis, 1997; Ribàr *et al.*, 1997; Ribàr *et al.*, 1999; Zilahi *et al.*, 2000). Consistent with the phenotype of *sep1* mutants, *sep1⁺* genetically interacts with a number of genes, including *wee1⁺*, *cdc2⁺* and *cdc25⁺*, all known regulators of entry into mitosis, as well as *cdc4⁺*, which is necessary for the assembly of the actin-based medial ring (Sipiczki *et al.*, 1993; Grallert *et al.*, 1998). Finally, other studies have shown that *sep1⁺* is required for periodic transcription of *cdc15⁺* and that it encodes a protein required for the positioning and assembly of the actin-based medial ring during late mitosis (Simanis, 1995; Zilahi *et al.*, 2000).

As discussed in Section 3.1.2, Fkh2p and Sep1p show peptide similarity to the budding yeast Fkh1p and Fkh2p proteins, which control periodic G2-M specific transcription of the 'CLB2' gene cluster. Fkh2p, similar to its *S. cerevisiae* counterparts, possesses an N-terminal forkhead-binding domain (FHD) and a forkhead associated domain (FHA), whereas Sep1p only possesses the FHD domain (Buck *et al.*, 2004).

Previous work revealed that in the absence of Fkh2p or Sep1p, but not Mbx1p, periodic M-G1 specific transcription of genes, such as *cdc15⁺*, is lost in fission yeast (Buck *et al.*, 2004; Bulmer *et al.*, 2004). These findings show that *fkh2⁺* and *sep1⁺* are required for periodic transcription during the M-G1 interval in the fission yeast. In parallel, previous work and experiments presented in Figure 3.3 demonstrate that a ~ 100 bp region from the *cdc15⁺* promoter supports M-G1 specific transcription of the *lacZ* reporter and contains among others the PCB sequence (Anderson *et al.*, 2002; Buck *et al.*, 2004). Taken together, these findings suggest that Fkh2p and Sep1p regulate periodic M-G1 transcription of *cdc15⁺*, either directly or indirectly, through *cis*-acting element(s), including the PCB sequence, present within the *cdc15⁺* promoter fragment.

To determine whether Fkh2p and Sep1p exert their function on transcriptional regulation of *cdc15⁺* through *cis*-acting element(s) within the 100 bp PCB-containing *cdc15⁺* promoter fragment Northern blot analysis was performed to examine the effect of deleting either *fkh2⁺* or *sep1⁺* on cell cycle transcription of both endogenous *cdc15⁺* and also *lacZ*, the latter expressed ectopically under control of the PCB-containing promoter fragment (Section 3.2).

pSPΔ178.15UAS was transformed into *fkh2Δ cdc25-22 ura4-D18 leu1-32* (GG 756) or *sep1Δ cdc25-22 ura4-D18 leu1-32* (GG 776) cells. For both transformed strains, synchronous cultures were created by transient temperature arrest (Section 3.2.2) and cell samples collected every 15 min. Total RNA was then prepared and resolved by formaldehyde gel electrophoresis (Section 2.2.10.1 and 2.2.10.3). Northern blot analysis was then completed, to monitor the cell cycle mRNA levels of *lacZ* and *cdc15⁺*. Septation indices were counted only for *fkh2Δ cdc25-22* cells containing pSPΔ178.15UAS, as the defective septation and cell separation phenotype of *sep1Δ* cells precluded such analysis in this strain. As shown in Figure 3.6, *fkh2Δ cdc25-22* cells displayed a slightly delayed septation, which persisted thereafter, when compared to *cdc25-22* cells (Figure 3.3). This reflects the septation defect seen in this strain.

The results from these experiments are shown in Figures 3.6 and 3.7. In the absence of either *fkh2⁺* or *sep1⁺*, the mRNA levels of endogenous *cdc15⁺* no longer fluctuate in abundance during the cell cycle in an M-G1 specific manner. Deletion of either *fkh2⁺* or *sep1⁺* also abolishes periodic M-G1 specific transcription of *lacZ*, expressed under control of the *cdc15⁺* promoter fragment containing the wild-type PCB sequence GCAACG (Buck *et al.*, 2004). Taken together,

these results suggest that the encoded products of *fkh2⁺* and *sep1⁺* control M-G1 specific transcription of *cdc15⁺* through sequences present within the *cdc15⁺* promoter fragment that contains the PCB element. Interestingly however, experiments shown in Figures 3.4 and 3.5 have revealed that the PCB sequence mediates transcription of *cdc15⁺* throughout the cell cycle. Hence, it is likely that promoter elements other than the PCB exist within the *cdc15⁺* promoter fragment that are responsible for Fkh2p- and/or Sep1p- mediated transcription at the M-G1 interval, exerting their role either together or in parallel with the PCB.

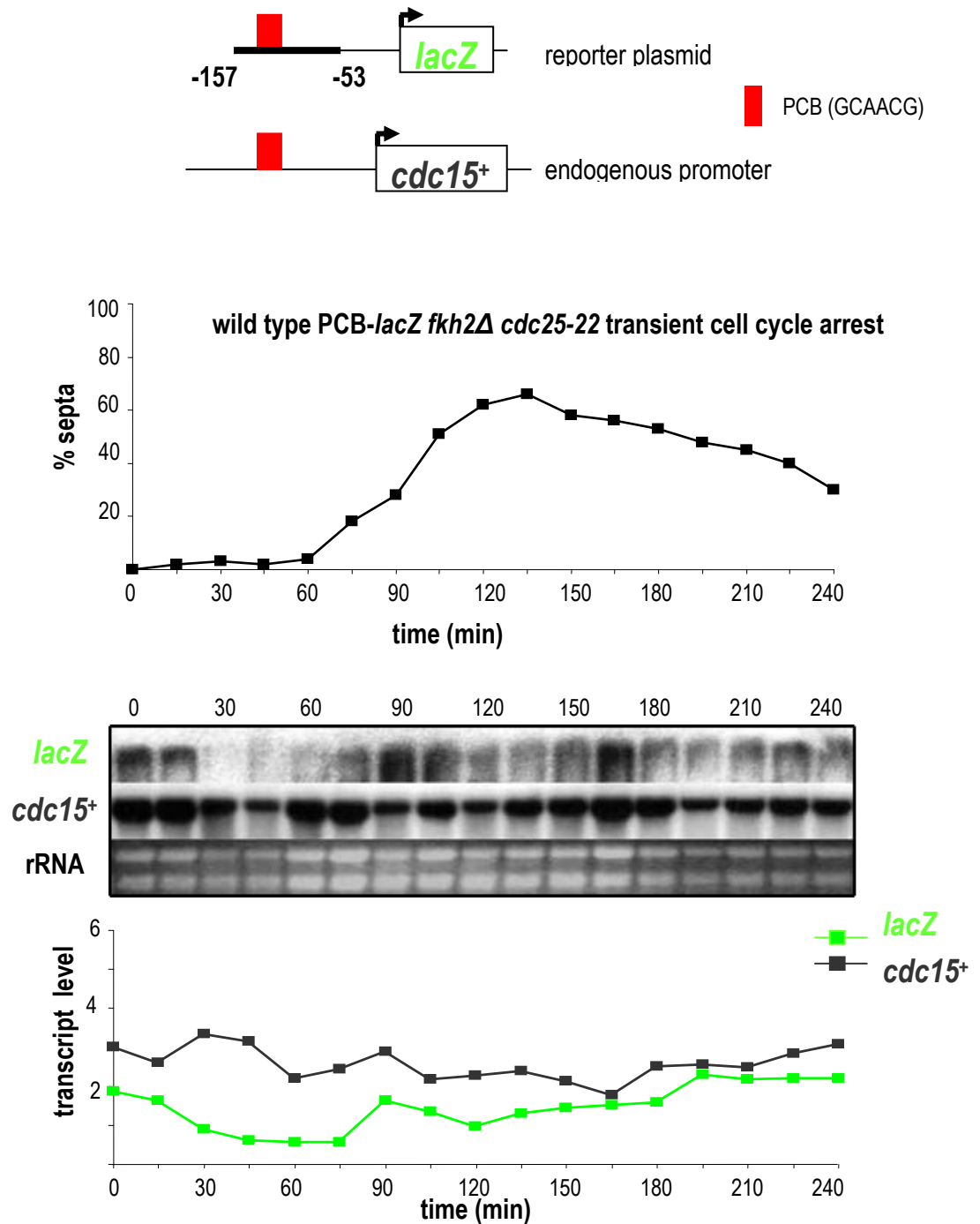
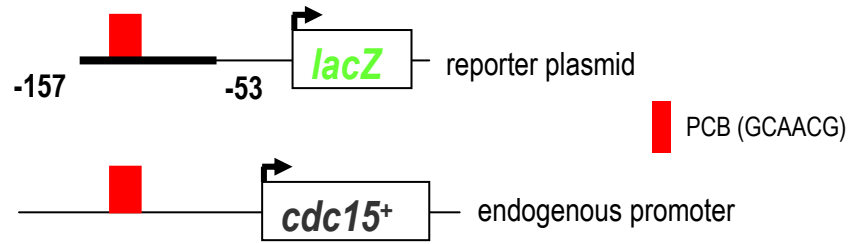


Figure 3.6: Loss of periodic transcription of *lacZ* in *fkh2*Δ cells. *fkh2*Δ *cdc25-22* cells (GG 756) transformed with pSPΔ178.15UAS plasmid (GB 194) were synchronised by transient temperature arrest. Samples were collected every 15 min upon release to the permissive temperature and total RNA was prepared for Northern blot analysis. The blot was hybridised consecutively with *cdc15⁺* and *lacZ* probes. Ethidium staining of rRNA was used as a loading control. Quantification of mRNA levels against its rRNA is shown. Septation indices were counted microscopically and are plotted to indicate the synchrony of the culture. Northern blot analysis was carried out twice with samples prepared from two individual cultures of synchronous cells. The results obtained from Northern blot analysis of samples from one of the two cultures are shown here and are representative of the results from both experiments.



wild type PCB-*lacZ* *sep1Δ cdc25-22* transient arrest cell cycle

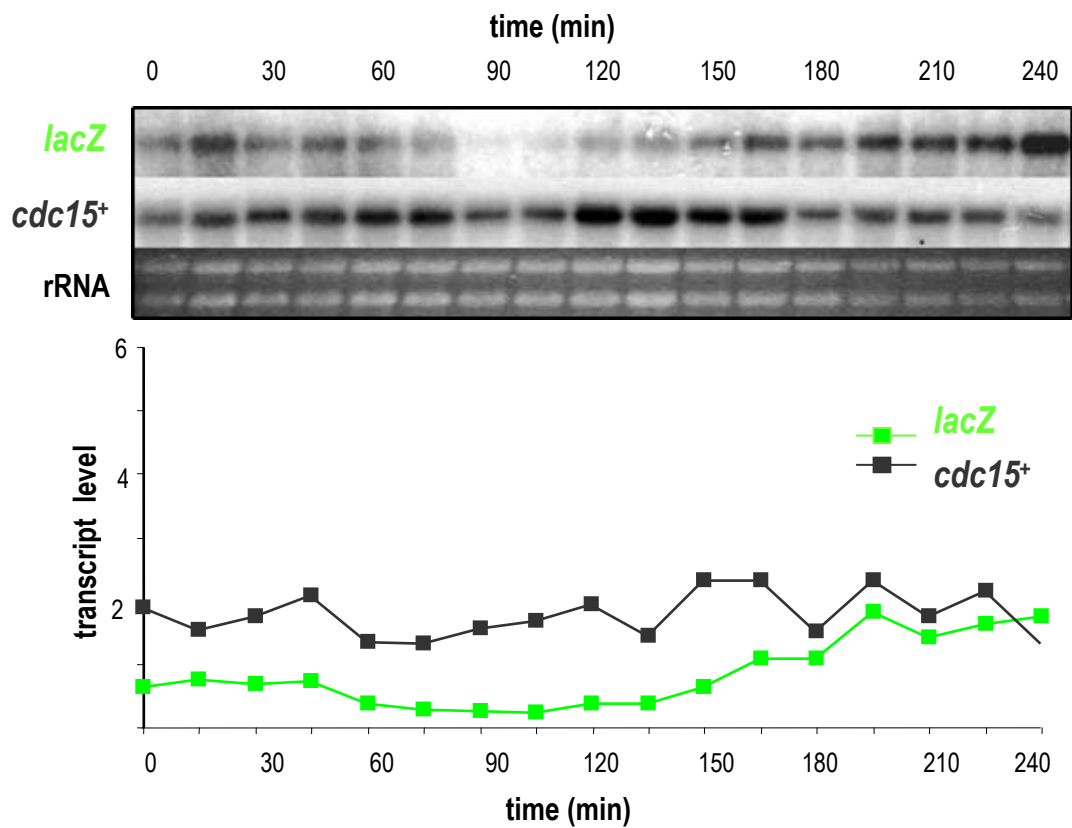


Figure 3.7: Loss of periodic transcription of *lacZ* in *sep1Δ* cells. *sep1Δ cdc25-22* cells (GG 776) transformed with pSPΔ178.15UAS plasmid (GB 194) were synchronised by transient temperature arrest. Samples were collected every 15 min upon release to the permissive temperature and total RNA was prepared for Northern blot analysis. The blot was hybridised consecutively with *cdc15+* and *lacZ* probes. Ethidium staining of rRNA was used as a loading control. Quantification of mRNA levels against rRNA is shown. Septation indices were counted microscopically and are plotted to indicate the synchrony of the culture. Northern blot analysis was carried out twice with samples prepared from two individual cultures of synchronous cells. The results obtained from Northern blot analysis of samples from one of the two cultures are shown here and are representative of the results from both experiments.

3.4 Role of the TGTTTAC forkhead-related motif in M-G1 specific transcription in *S. pombe*

As described in Section 3.1.1, the promoter regions of *S. pombe* M-G1 transcribed genes, regulated by Fkh2p and Sep1p, are enriched for another promoter sequence that is similar to the conserved forkhead motif and found in either orientation (Rustici *et al.*, 2004). Genes from a variety of organisms, ranging from the budding yeast to humans, contain in their promoter conserved forkhead sequences, (RTAAAYA), bound by forkhead transcription factors (Pierrou *et al.*, 1994; Hromas and Costa, 1995; Kaufmann *et al.*, 1995; Kaufmann and Knöchel, 1996; Pic *et al.*, 2000; Zhu *et al.*, 2000; Hollenhorst *et al.*, 2001) (Section 1.4.1.2.4).

In *S. pombe*, database analyses show that the forkhead motif is present in the promoters of M-G1 transcribed genes in both orientations, although TRTTTAY is statistically more significant than RTAAAYA (Rustici *et al.*, 2004, Bähler, 2005). Experimental evidence for a role of this forkhead motif in transcriptional regulation of M-G1 transcribed genes in fission yeast comes from work showing that Sep1p-mediated transcription of *ace2⁺* is dependent on the TGTTTAY sequence, present within the *ace2⁺* promoter region in multiple copies and in both orientations (Alonso-Nuñez *et al.*, 2005). Northern blot analysis showed that the *ace2⁺* transcript is absent from *ace2Δ* cells transformed with a plasmid carrying the *ace2⁺* ORF under control of a promoter fragment lacking these forkhead sequences.

Interestingly, a sequence search within the *cdc15⁺* promoter fragment that confers M-G1 specific transcription to *lacZ* (Anderson *et al.*, 2002), shows that it contains not only the PCB element but also two forkhead sequences, one TGTTTAC (-87 to -81 relative to *cdc15⁺* ATG) and one GTAAACA (-98 to -92). This observation implies that these forkhead motifs might have a role in Fkh2p-/Sep1p-mediated cell cycle specific transcription of *cdc15⁺*, as well as of other M-G1 transcribed genes. Figure 3.8 displays a feature map of the PCB and the forkhead motifs found within the promoters of fission yeast M-G1 transcribed genes, created by using the Regulatory Sequence Analysis Tools (**RSAT**) (<http://rsat.ulb.ac.be/rsat/>) (Van Helden, 2003). A detailed list of the positions of the PCB and forkhead sequences within the promoters of these genes is shown in Appendix VII.

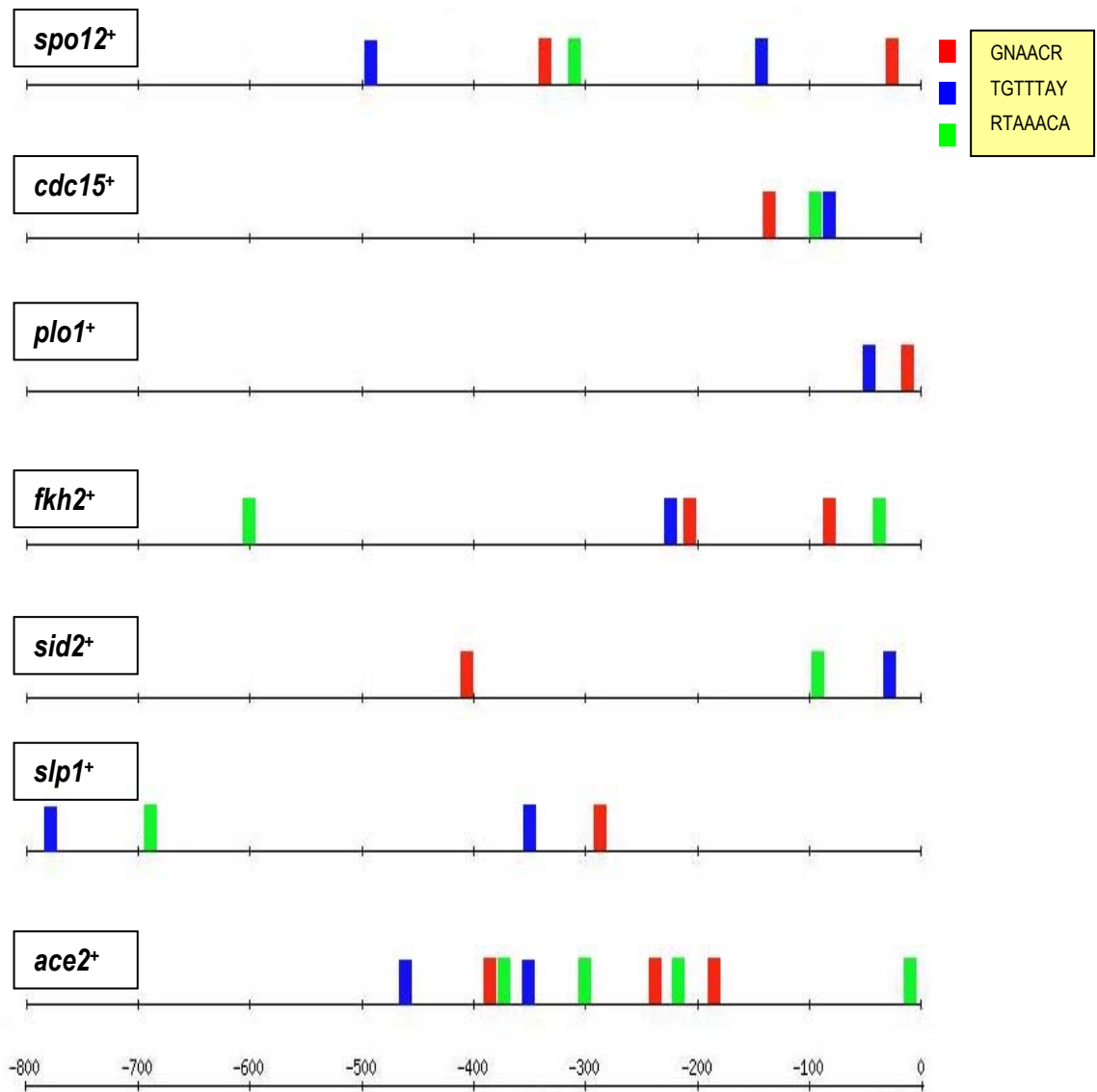


Figure 3.8: Feature map of the positions of the PCB and forkhead motifs within the promoters of *S. pombe* M-G1 transcribed genes. The RSAT web resource was used to retrieve *spo12+*, *cdc15+*, *plo1+*, *fkh2+*, *slp1+*, *ace2+* and *sid2+* promoter sequences (spanning an upstream 800 bp region), which were then searched with the DNA-pattern matching tool for the positions of the PCB (GNAACR) and the forkhead motifs, the latter in both orientations, TGTTTAY and RTAAACA, respectively. Red, blue and green boxes correspond to GNAACR, TGTTTAY and RTAAACA sequence motifs. Scale bar denotes distance every 100 bp for a 800 bp upstream promoter sequence. R stands for G or A, Y for C or T and N for G, C, T or A.

Based on these findings, Northern blot analysis was employed to examine whether the TGTTTAC forkhead-related sequence is involved in cell cycle specific transcription of *cdc15⁺*. To accomplish this, a mutated version of the forkhead sequence (TGTTTAC substituted for GTGGGGT) within the *cdc15⁺* promoter fragment (Section 3.2), was monitored, either alone or in combination with a mutated version of the PCB sequence (GCAACG substituted for TTGGTT), for its effect on cell cycle transcription of *lacZ*.

Two pSPΔ178 plasmids were created, one containing a *cdc15⁺* promoter fragment with the mutated forkhead sequence and the wild-type PCB (pSPΔ178.15UAS.MUT3), while the other a fragment with the mutated forkhead sequence and the mutated PCB (pSPΔ178.15UAS.MUT4). The plasmids were transformed separately into *cdc25-22* cells, which were then synchronised by transient temperature arrest and allowed to progress through the mitotic cell cycle. Samples were then collected and analysed by Northern blot to monitor the cell cycle transcriptional profile of *lacZ* and that of endogenous *cdc15⁺*.

3.4.1 Construction of pSPΔ178.15UAS.MUT3 and pSPΔ178.15UAS.MUT4 reporter plasmids

Standard molecular procedures were adopted to make the pSPΔ178.15UAS.MUT3 and pSPΔ178.15UAS.MUT4 plasmids (Section 3.2.1.2). Two ~100 bp *cdc15⁺* promoter fragments were generated, forkhead mut3 and PCB/forkhead mut4, the first containing the mutated forkhead sequence alone and the other the mutated forkhead together with the mutated PCB.

To create forkhead mut3 and PCB/forkhead mut4 fragments two single stranded (ssDNA) oligonucleotides were annealed in each case, GO 701 with GO 702 and GO 703 with GO 704, respectively (Section 2.2.9.8) (Appendix I), and cloned into the pSPΔ178 vector (GB 10). PCR analysis was performed on transformants to identify inserts in the correct orientation (Section 3.2.1.2). This analysis identified one isolate of pSPΔ178.15UAS.MUT3 (GB 475) and one isolate of pSPΔ178.15UAS.MUT4 (GB 471), as shown in Figure 3.9. Finally, both constructs were confirmed by sequencing (MWG Biotech).

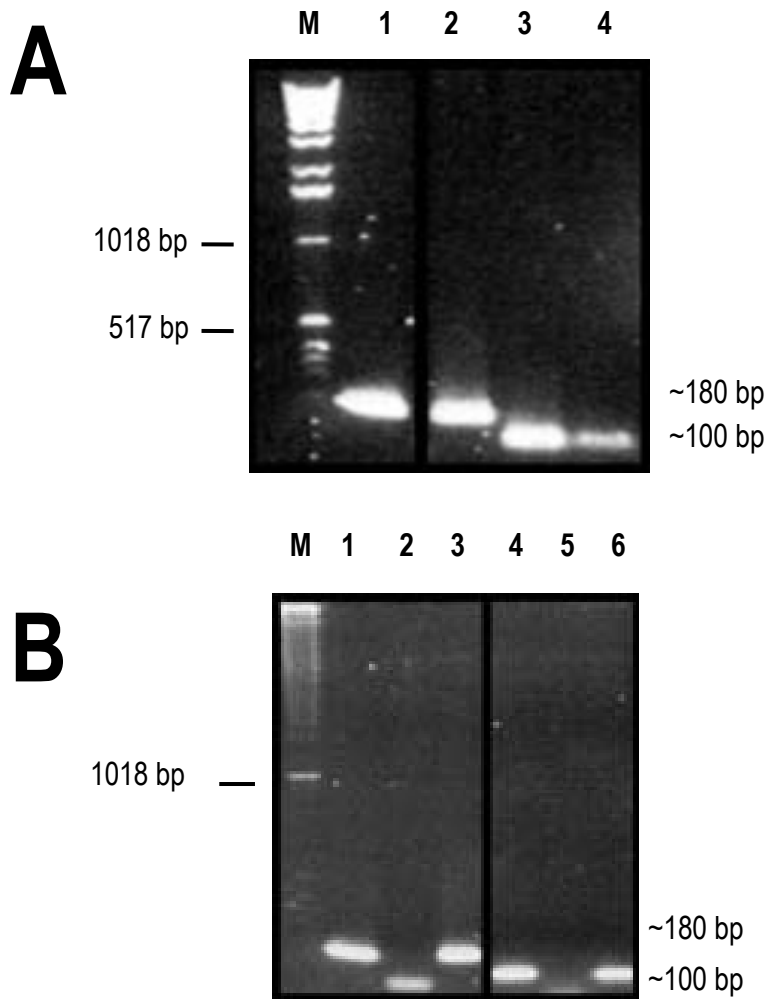


Figure 3.9: Verification of pSPΔ178.15UAS.MUT3 and pSPΔ178.15UAS.MUT4 reporter constructs. (A-B) PCR analysis was performed to verify cloning of forkhead mut3 and PCB/forkhead mut4 DNA fragments into the *Xho*I site of pSPΔ178, yielding plasmid pSPΔ178.15UAS.MUT3 (GB 475) and pSPΔ178.15UAS.MUT4 (GB 471). Primers GO 46 and GO 47, flanking the *Xho*I site, were used to PCR amplify a ~180 bp fragment (Lanes A-2 and B-3 corresponding to forkhead mut3 and PCB/forkhead mut4 DNA fragment, respectively) to check for cloning into pSPΔ178, while primer pair GO 46 and GO 398 was used to PCR amplify a ~100 bp fragment (Lanes A-4 and B-6 corresponding to forkhead mut3 and PCB/forkhead mut4 DNA fragment, respectively) to check for cloning into the correct orientation. As a positive control, pSPΔ178.15UAS plasmid (GB 194) was used as a template for PCR amplification with primer pair GO 46 and GO 47 (Lanes A-1 and B-1) or primer pair GO 46 and GO 398 (Lane A-3 and B-4). (B) Lanes B-2 and B-5 show PCR amplification using as a template empty pSPΔ178 plasmid (GB 10) as a negative control with primer pair GO 46 and GO 47 or GO 46 and GO 398, respectively. (A-B) Lane M indicates DNA Molecular Weight Marker X.

3.4.2 Requirement of TGTTTAC sequence for M-G1 specific transcription of *cdc15⁺*

To determine whether the TGTTTAC sequence motif regulates M-G1 specific transcription of *cdc15⁺*, either in the presence or in the absence of the PCB motif, Northern blot analysis was performed to examine the effect of either mutating only this forkhead-related sequence or both the forkhead and the PCB sequence on cell cycle transcription of *lacZ*, expressed ectopically from pSPΔ178.15UAS.MUT3 or pSPΔ178.15UAS.MUT4, respectively (Section 3.4.1). Initially, each reporter plasmid was transformed into *cdc25-22 ura4-D18 leu1-32* cells. For each transformed strain, a synchronous culture was created by transient temperature arrest (Section 3.2.2), and Northern blot analysis completed on cell samples collected every 15 min, to detect cell cycle mRNA levels of *lacZ* and endogenous *cdc15⁺* (Sections 2.2.10.1 and 2.2.10.3).

Figures 3.10 and 3.11 display the results from these analyses, showing the cell cycle mRNA levels of *lacZ* and *cdc15⁺* in *cdc25-22* cells containing the pSPΔ178.15UAS.MUT3 or pSPΔ178.15UAS.MUT4 plasmid, respectively. In the absence of the wild-type forkhead sequence, but in the presence of the PCB sequence, the *lacZ* mRNA levels were still found to oscillate in abundance in a cell cycle manner, their amplitude relatively similar to that of endogenous *cdc15⁺*. Nonetheless, mutating the TGTTTAC motif compromised M-G1 specific transcription of the reporter gene, since the mRNA levels of *lacZ* peaked 15-30 minutes later than the mRNA levels of endogenous *cdc15⁺* (Figure 3.10). It is important to note that the loss of the TGTTTAC sequence only delayed but did not abolish periodic transcription of *lacZ* relative to *cdc15⁺*, implying that other promoter elements might also contribute to the control of M-G1, phase-specific, transcription. Interestingly, the *cdc15⁺* promoter fragment contains another forkhead motif in the reverse orientation, GTAAACA (Figure 3.8). Hence, it is possible that there is redundancy between the two forkhead motifs in their role in cell cycle specific transcription of *cdc15⁺*, with one substituting for the loss of the other. Overall, the TGTTTAY forkhead-related sequence appears to have a role in maintaining the temporal, M-G1 specific, transcriptional pattern of *cdc15⁺* and, presumably, of other co-regulated genes. Contrary to the temporal effect on the cell cycle mRNA pattern of *lacZ* as a result of changing the TGTTTAC sequence, mutating both the TGTTTAC and the PCB sequences dramatically reduced *lacZ* mRNA levels throughout the cell cycle but did not compromise M-G1 transcription (Figure 3.11), generating a *lacZ* mRNA profile similar to that seen with the mutated PCB alone (Figure 3.5). Taken together, these findings suggest that the TGTTTAY motif and the PCB sequence possibly function in a parallel, but not additive, manner, with the former regulating the timing of phase-specific *cdc15⁺* transcription and the latter the amplitude of *cdc15⁺* transcription but in a cell cycle non-specific manner.

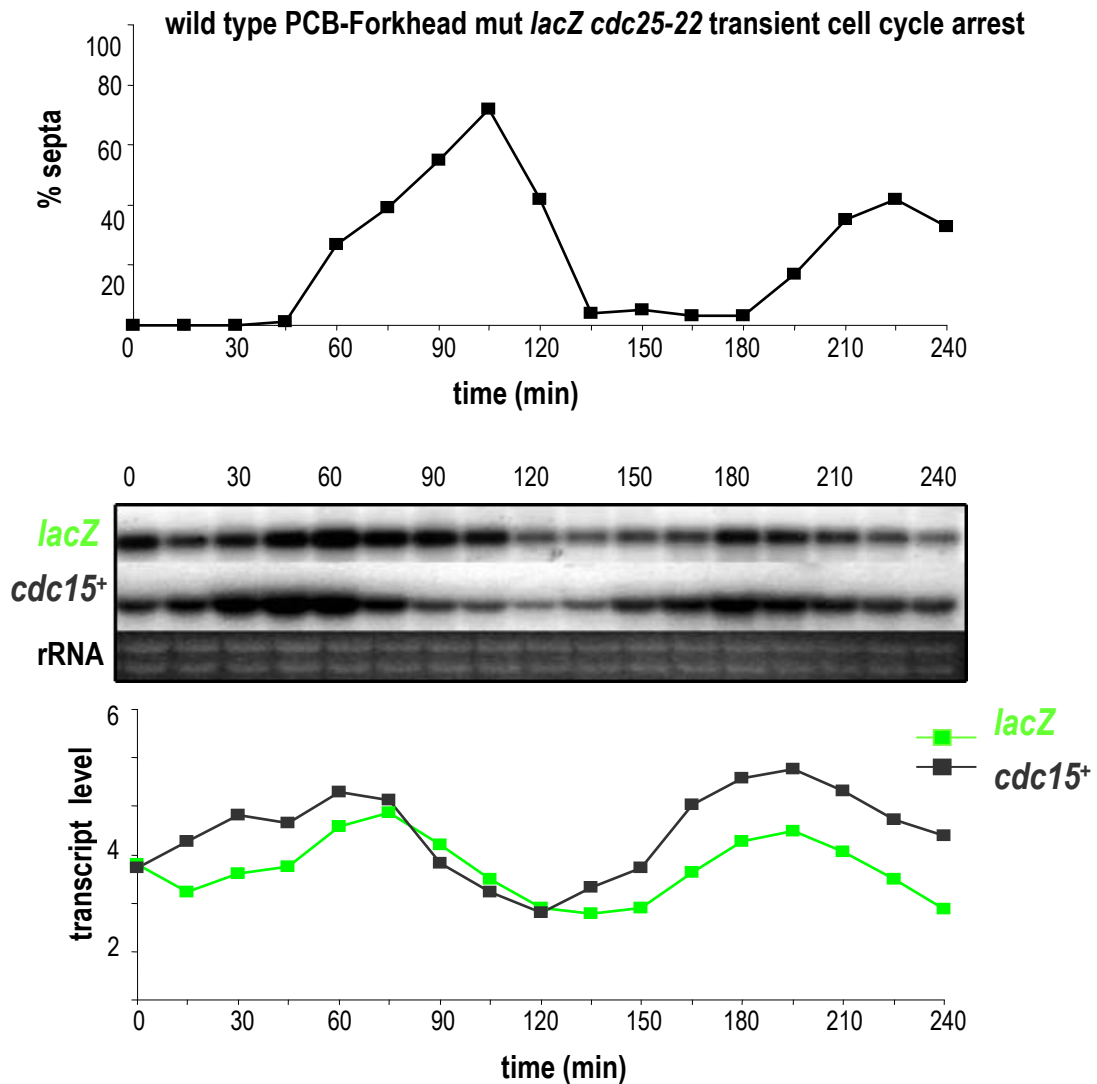
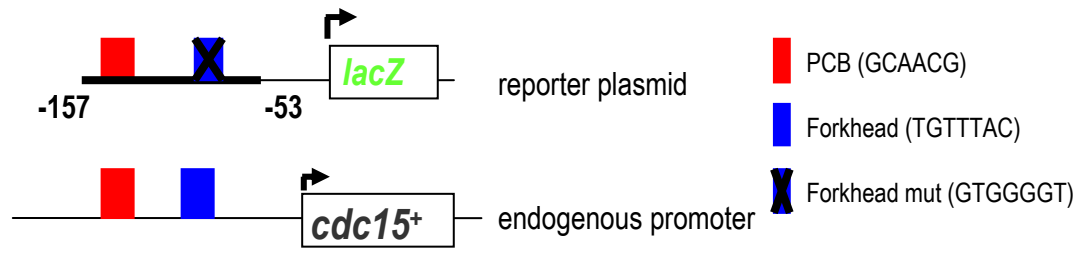


Figure 3.10: Effect of mutating the TGTTTAC forkhead sequence on M-G1 specific transcription in fission yeast. *cdc25-22* cells (GG 308) transformed with pSPΔ178.15UAS.MUT3 plasmid (GB 475) were synchronised by transient temperature arrest. Samples were collected every 15 min upon release to the permissive temperature and total RNA was prepared for Northern blot analysis. The blot was hybridised consecutively with *cdc15+* and *lacZ* probes. Ethidium staining of rRNA was used as a loading control. Quantification of mRNA levels against rRNA is shown. Septation indices were counted microscopically and are plotted to indicate the synchrony of the culture. Northern blot analysis was carried out twice with samples prepared from two individual cultures of synchronous cells. The results obtained from Northern blot analysis of samples from one of the two cultures are shown here and are representative of the results from both experiments.

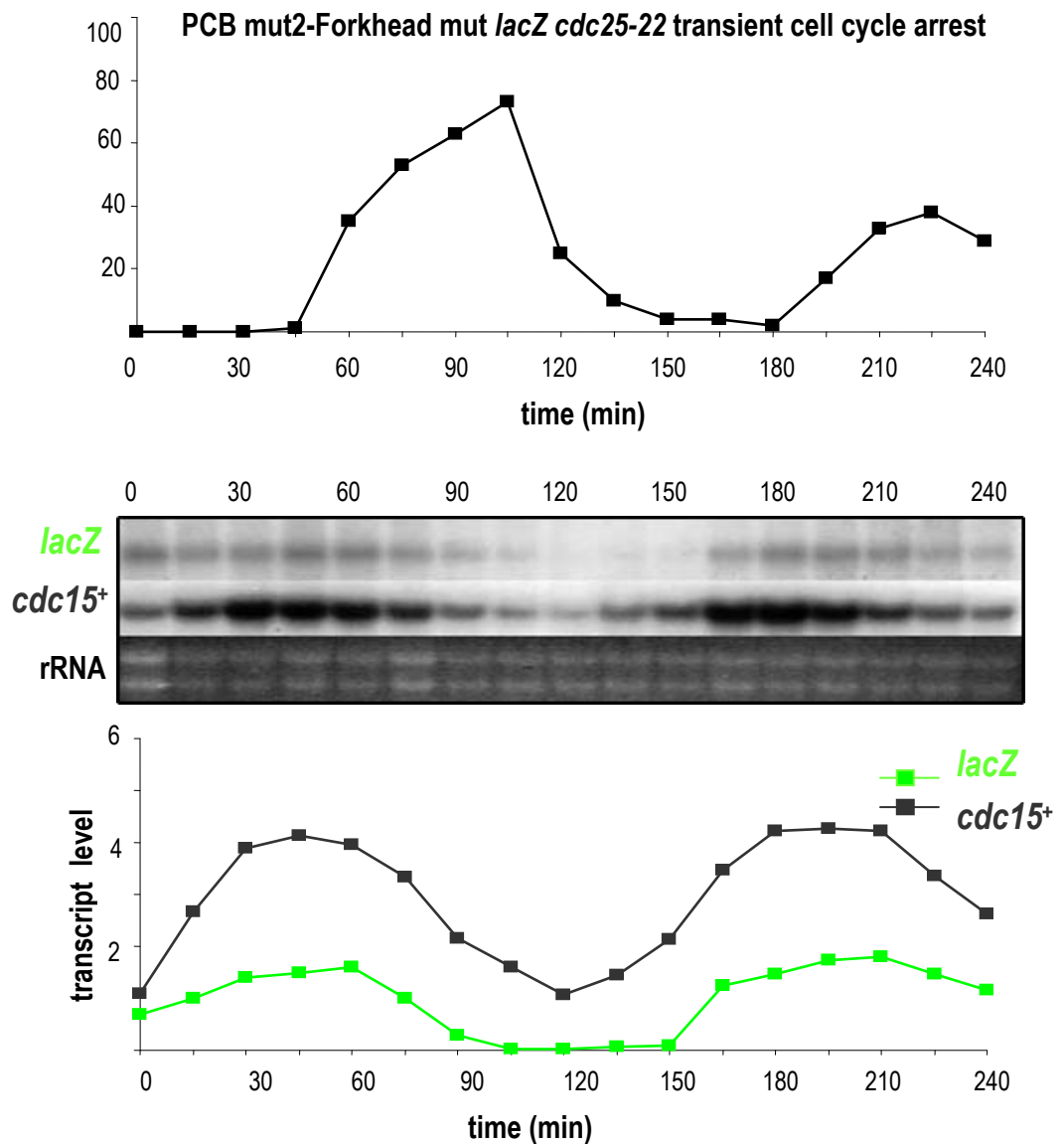
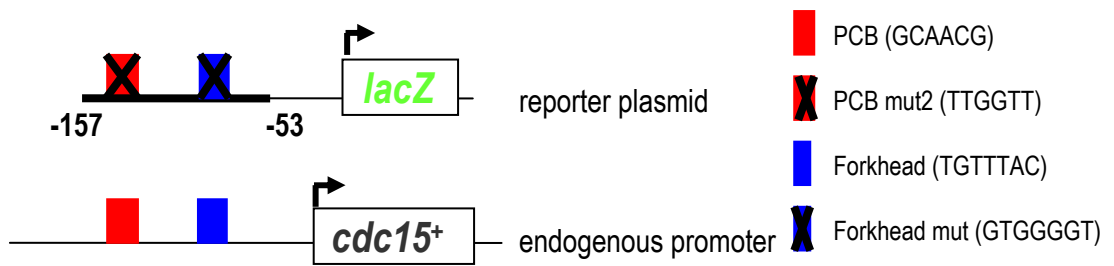


Figure 3.11: Effect of mutating both the PCB and the TGTTTAC sequences on M-G1 specific transcription in fission yeast. *cdc25-22* cells (GG 308) transformed with pSPΔ178.15UAS.MUT4 plasmid (GB 471) were synchronised by transient temperature arrest. Samples were collected every 15 min upon release to the permissive temperature and total RNA was prepared for Northern blot analysis. The blot was hybridised consecutively with *cdc15+* and *lacZ* probes. Ethidium staining of rRNA was used as a loading control. Quantification of mRNA levels against rRNA is shown. Septation indices were counted microscopically and are plotted to indicate the synchrony of the culture. Northern blot analysis was carried out twice with samples prepared from two individual cultures of synchronous cells. The results obtained from Northern blot analysis of samples from one of the two cultures are shown here and are representative of the results from both experiments.

3.4.3 Comparative analysis of *lacZ* mRNA levels upon mutation of the PCB, the TGTTTAC or both the PCB and the TGTTTAC sequence

Experiments presented in Sections 3.2.2 and 3.4.2 analysed the effect of mutations in the PCB and TGTTTAC sequence on cell cycle transcription of *cdc15⁺* in *S. pombe*. To allow a direct comparison between these mutations on the levels of *cdc15⁺* transcription, separate cultures of asynchronous *cdc25-22* cells containing either pSPΔ178.15UAS, pSPΔ178.15UAS.MUT, pSPΔ178.15UAS.MUT2, pSPΔ178.15UAS.MUT3 or pSPΔ178.15UAS.MUT4 were prepared and grown to mid-log phase. Once cells were harvested, total RNA extracts were prepared and resolved on the same formaldehyde gel prior to Northern blot analysis with DNA probes to detect *lacZ* and *cdc15⁺* mRNA levels. The results obtained from this experiment are shown in Figure 3.12.

In confirmation of previous results changing the PCB sequence within the *cdc15⁺* promoter fragment to either AAGGTT or TTGGTT resulted in a complete absence or a dramatic reduction of *lacZ* mRNA levels respectively, relative to that under control of the wild-type PCB sequence. Similarly, changing the TGTTTAC forkhead sequence to GTGGGGT reduced but did not abolish *lacZ* mRNA levels, while upon mutation of both promoter motifs (GCAACG to TTGGTT and TGTTTAC to GTGGGGT) *lacZ* mRNA levels were dramatically reduced, similar to when changing the PCB from GCAACG to TTGGTT. These findings are consistent with the results of the cell cycle experiments, showing that mutations in the *cdc15⁺* PCB sequence more drastically affect the amplitude of transcription relative to mutations in the TGTTTAC forkhead-related sequence. Moreover, changing both the PCB and the forkhead motif almost completely compromises *lacZ* mRNA levels, as is also the case for *lacZ* transcript levels upon changes in the PCB sequence alone (GCAACG replaced by TTGGTT), suggesting that the effects of these two promoter elements on transcription are not additive.

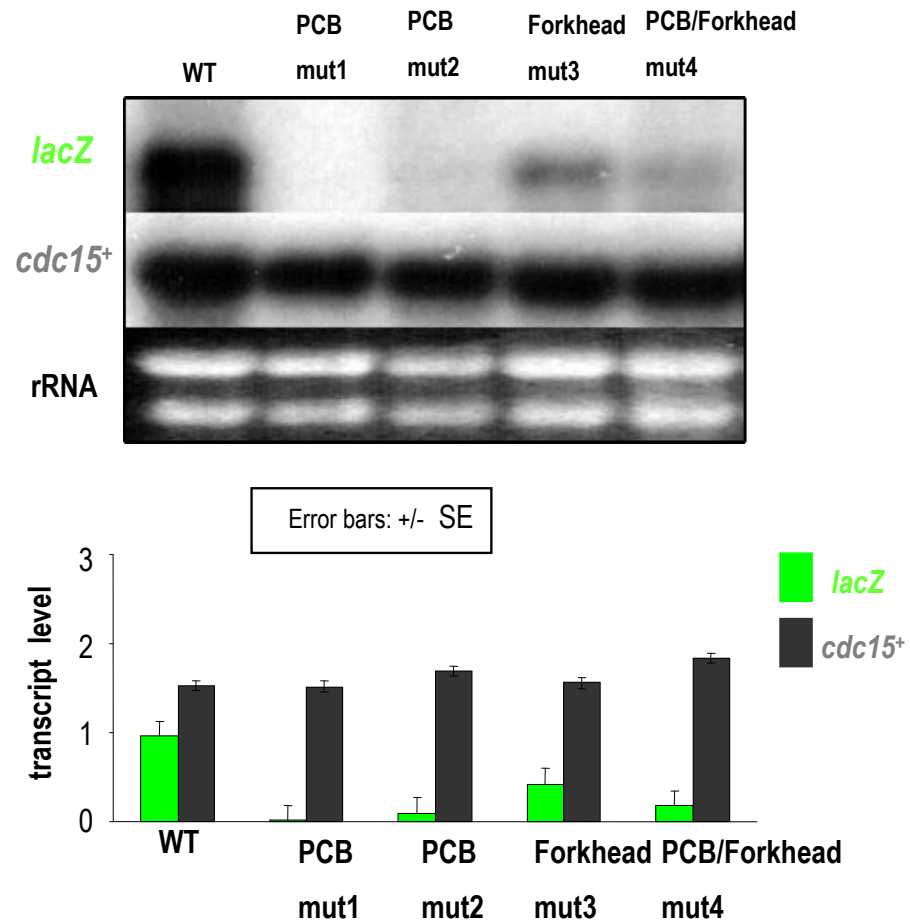


Figure 3.12: Northern blot analysis comparing the effects on *lacZ* transcription of changes in either the PCB sequence, the TGTTTAC forkhead sequence or both the PCB and the TGTTTAC sequence. Individual populations of asynchronous *cdc25-22* cells (GG 308) transformed with plasmid pSPΔ178.15UAS (wt), pSPΔ178.15UAS.MUT (PCB mut1), pSPΔ178.15UAS.MUT2 (PCB mut2), pSPΔ178.15UAS.MUT3 (forkhead mut3) or pSPΔ178.15UAS.MUT4 (PCB/forkhead mut4) were grown to mid-log phase, harvested and total RNA was prepared for Northern blot analysis. The blot was hybridised consecutively with *cdc15+* and *lacZ* probes. Ethidium staining of rRNA was used as a loading control. Quantification of mRNA levels against rRNA is shown. Northern blot analysis of total RNA samples was carried out in triplicate. The results obtained from one of these Northern blot analyses are shown here and are representative of the results obtained in all three instances.

3.5 Summary

Previous studies have shown that a ~100bp DNA fragment from the promoter of *cdc15⁺* is sufficient to confer M-G1 specific transcription to *lacZ*, strongly suggesting that this promoter region contains the *cis*-acting element(s) mediating the cell cycle-dependent pattern of *cdc15⁺* expression. The PCB sequence within the *cdc15⁺* promoter fragment is a *cis*-acting element previously implicated in regulation of *cdc15⁺* and many other M-G1 transcribed genes (Anderson *et al.*, 2002). Here, experiments were performed to assess the requirement of the PCB motif for cell cycle specific transcription of *cdc15⁺*. For this, changed versions of the *cdc15⁺* PCB sequence (GCAACG substituted for AAGGTT or TTGGTT) were assayed by Northern blot analysis for their effect on *lacZ* cell cycle transcription. Both PCB sequence substitutions, when compared to the wild-type sequence, severely affected the amplitude of *lacZ* transcription throughout the cell cycle with AAGGTT having a stronger effect than TTGGTT and completely abolishing transcription of the reporter gene (Figures 3.3, 3.4 and 3.5).

The stronger effect of the AAGGTT mutation on transcription of *lacZ* relative to TTGGTT suggests that the first two bases of the PCB element are important for binding by the transcription factor(s), associating directly or indirectly with the PCB sequence. In support of this, gel retardation assays have shown that the first two bases of the PCB sequence of *spo12⁺*, another M-G1 transcribed gene, are crucial for the interaction with the PBF complex (Anderson *et al.*, 2002). Thus far, the Mbx1p transcription factor has been shown to be necessary for the PCB-mediated interaction of the PBF complex with the promoters of M-G1 transcribed genes, including *cdc15⁺* and *spo12⁺*. Interestingly, Mbx1p is dispensable for M-G1 specific transcription of *cdc15⁺* and other co-regulated genes, although it appears to have a role in regulating the amplitude of transcription of these genes. Taking into account that the PCB sequence itself is necessary for transcription of *cdc15⁺* regardless of the time in the cell cycle (Figures 3.4 and 3.5), it is possible that Mbx1p exerts its effect on transcription of *cdc15⁺* either directly or indirectly through this promoter element. Overall, these results suggest that the PCB sequence regulates transcription of *cdc15⁺* in a cell cycle independent manner and indicate that this promoter motif might also modulate the amplitude of transcription of many other similarly co-regulated genes, including *plo1⁺*, *spo12⁺*, *ppb1⁺*, *slp1⁺*, *ace2⁺*, *sid2⁺* and *mid1⁺/dmf1⁺* (Buck *et al.*, 2004).

Various studies have shown that Fkh2p and Sep1p are required for periodic, M-G1-dependent, transcription in fission yeast. Taking into account that the *cdc15⁺* promoter fragment contains all necessary *cis*-acting elements for efficient periodic gene expression (Anderson *et al.*, 2002), experiments were performed here to examine whether these transcription factors function

through such sequences (Buck *et al.*, 2004; Section 3.2.2). As shown in Figures 3.6 and 3.7, in the absence of either Fkh2p or Sep1p, M-G1 specific transcription is lost both for endogenous *cdc15⁺* and *lacZ*, expressed ectopically under control of the *cdc15⁺* promoter fragment (Buck *et al.*, 2004). This suggests that Fkh2p and Sep1p regulate M-G1 specific transcription through sequences found in the *cdc15⁺* promoter fragment, including the PCB motif. Nonetheless, the PCB element mediates transcription of *cdc15⁺* throughout the cell cycle, suggesting that other sequences within the *cdc15⁺* promoter fragment might have a role in Fkh2p/Sep1p-mediated transcription during the M-G1 interval.

Apart from the PCB, the promoters of M-G1 transcribed genes also contain the forkhead motif (RTAAAYA), which displays sequence similarities with the PCB. Notably, the forkhead motif is present in the promoters of these fission yeast genes in both orientations. Since forkhead motifs are also found within the *cdc15⁺* promoter fragment that drives cell cycle-dependent transcription of *lacZ* (Figure 3.8) and two forkhead transcription factors, Fkh2p and Sep1p, regulate M-G1 transcription, it is tempting to speculate that these sequences also contribute to periodic expression of these genes (Zilahi *et al.*, 2000; Buck *et al.*, 2004; Bulmer *et al.*, 2004; Rustici *et al.*, 2004). Indeed, forkhead sequences appear to be required for expression of *ace2⁺*, an M-G1 transcribed gene (Alonso-Nuñez *et al.*, 2005). To determine whether forkhead elements are important for M-G1-dependent transcription, the TGTTTAC sequence within the *cdc15⁺* promoter fragment was mutated either alone or in combination with PCB and the effects of these sequence changes were assayed on cell cycle transcription of *lacZ*. Upon TGTTTAC sequence substitution (changed to GTGGGGT), *lacZ* phase-specific transcription remained periodic but was delayed 15-30 minutes relative to wild-type (Figure 3.10). On the contrary, changing both the forkhead and PCB sequences, substituted for GTGGGGT and TTGGTT respectively, significantly reduced the amplitude of *lacZ* mRNA levels at all cell cycle times but did not compromise M-G1 specific transcription *per se*, similar to when mutating the PCB alone (compare Figures 3.11 with 3.3 and 3.5). Following individual examination of the effects on cell cycle *lacZ* transcription of substitutions in the *cdc15⁺* PCB, TGTTTAC or both these sequences, comparative Northern blot analysis was performed with asynchronous cell samples to directly contrast the effects of these mutations on *lacZ* mRNA levels.

As shown in Figure 3.12 and consistent with the results from the cell cycle experiments, distinct substitutions in the *cdc15⁺* PCB sequence, when compared to wild-type, either dramatically reduce or even abolish *lacZ* mRNA levels. Moreover, changing the *cdc15⁺* TGTTTAC significantly reduces but does not eliminate *lacZ* transcript levels, whereas when TGTTTAC and PCB mutations are combined, *lacZ* mRNA levels are compromised to an extent identical to that observed upon respective changes in the PCB sequence alone.

Overall, the results of this chapter suggest that the PCB sequence is required for transcription of *cdc15⁺* throughout the cell cycle and reveal a role for the TGTTTAC forkhead-related motif in regulation of the M-G1 specific transcriptional pattern of *cdc15⁺*. Furthermore, these findings imply that the two *cis*-acting elements function in transcriptional control of *cdc15⁺* and, presumably, of other co-regulated genes in a parallel but not additive manner, with each sequence mediating distinct functions. Finally, taking into account that the promoters of many of these genes, including *cdc15⁺*, possess the forkhead motif in either orientation, it is possible that forkhead sequences, in either orientation, have redundant roles in M-G1 specific transcription of these genes.

In the next results chapter, analysis of PCB-PBF-dependent transcriptional controls in fission yeast is continued by exploring the interactions between Fkh2p, Sep1p and Mbx1p to understand how this may contribute to their mechanism of action.

Chapter 4

*In vivo and in vitro analysis of interactions
between PBF components Fkh2p, Sep1p and
Mbx1p*

4.1 Introduction

In fission yeast two forkhead transcription factors, Fkh2p and Sep1p, regulate periodic transcription of several genes, such as *cdc15⁺*, *plo1⁺* and *ace2⁺*, through the PCB and forkhead promoter DNA sequences (Buck *et al.*, 2004; Bulmer *et al.*, 2004; Alonso-Nuñez *et al.*, 2005; Chapter 3). A MADS-box protein Mbx1p is also implicated in M-G1 specific transcription, as it is necessary for PCB-mediated binding of the PBF complex to the genes' promoters *in vitro*, and *mbx1⁺* shows genetic interactions with *fkh2⁺* and *sep1⁺* (Anderson *et al.*, 2002; Buck *et al.*, 2004). Both Mbx1p and Fkh2p appear to negatively influence *ace2⁺* expression, since deleting either cognate gene leads to an increase in Ace2p protein levels. In contrast, Sep1p has been shown to positively regulate *ace2⁺* expression (Alonso-Nuñez *et al.*, 2005; Petit *et al.*, 2005). Consistent with these findings, genome-wide microarray analysis in *S. pombe* has uncovered a positive role for Sep1p and a negative role for Fkh2p in transcriptional regulation of many genes expressed coincidentally with *ace2⁺* (Rustici *et al.*, 2004).

mbx1Δ, *fkh2Δ* and *sep1Δ* cells all display cytokinetic defects. Furthermore, analyses of all the respective double and triple mutants have shown that the cytokinetic defects are aggravated relative to the single mutants (Buck *et al.*, 2004). Additionally, deleting *mbx1Δ* rescues the slow growth phenotype of *fkh2Δ*, since *mbx1Δ fkh2Δ* grow at a similar rate to wild type cells. Moreover, strong overexpression of *fkh2⁺* is lethal in wild-type cells but not in *sep1Δ* cells, indicating that Sep1p is required for Fkh2p function, and this might be explained by Sep1p and Fkh2p interacting with each other as part of their role in regulation of periodic gene expression (Buck *et al.*, 2004). Combined, these genetic observations imply that Mbx1p, Fkh2p and Sep1p share both common and distinct functions. To explore further these findings using biochemical methods, co-immunoprecipitation and GST pull-down assays were performed to examine whether Mbx1p, Fkh2p and Sep1p interact with each other *in vivo* and *in vitro*.

4.2 Co-immunoprecipitation analysis of interactions between Sep1p, Mbx1p and Fkh2p

Co-immunoprecipitation analysis was employed to determine whether Fkh2p, Sep1p and Mbx1p associate with each other *in vivo*. For this, soluble protein extracts were prepared from appropriate *S. pombe* strains, either *sep1-3HA fkh2-13myc*, *sep1-3HA mbx1-13myc* or *fkh2-3HA mbx1-13myc*, which express one protein as an HA fusion and the other as a myc fusion, tagged in each case at the

C- terminus in the endogenous locus and so expressed from the native promoter at normal levels (Section 4.2.1). Immunoprecipitation and Western blotting with HA and myc antibodies then followed (Sections 4.2.2, 4.2.3 and 4.2.4).

It is important to mention that an attempt was made to produce antibodies against native Fkh2p, Sep1p and Mbx1p in rabbits (Table 2.1), using as antigens synthetic, gene-specific peptides (Eurogentec). In parallel, efforts also focused on raising an antibody against Mbx1p in sheep (Table 2.1) (NHSScotland), using as antigen bacterially expressed and purified GST-Mbx1p, prepared as described in Sections 4.3.4 and 4.3.6. Following immunisation, first, second (data not shown) and third bleed (Appendix VIII) sheep polyclonal GST-Mbx1p antisera, first, second and third bleed rabbit polyclonal Fkh2p, Sep1p and Mbx1p antisera (data not shown) and the respective affinity purified antibodies (third bleed) (Appendix VIII) were tested for their specificity by Western blotting against *S. pombe* protein extracts and/or bacterially expressed and purified GST-/HIS-fusions of Mbx1p, Fkh2p and Sep1p, the latter prepared as described in Sections 4.3.2-4.3.7. Unfortunately, as shown in Appendix VIII, with the exception of the Sep1p affinity purified antibody that detected a specific band of the expected size corresponding to Sep1p only in the bacterially purified GST-/HIS-Sep1p fraction, all antisera and the respective affinity purified antibodies failed to detect a specific band of the expected size corresponding to Mbx1p, Fkh2p or Sep1p in *S. pombe* protein extracts. Therefore, these antisera were inappropriate for use in co-immunoprecipitation assays with extracts from *S. pombe* cells, and were not used.

4.2.1 Construction of double tag strains *sep1-3HA fkh2-13myc*, *sep1-3HA mbx1-13myc* and *fkh2-3HA mbx1-13myc*

A classical genetic cross between two haploid *S. pombe* strains of opposite mating type, each containing the appropriate HA- or myc- tagged version of *sep1*⁺, *fkh2*⁺ or *mbx1*⁺, was the first step in the construction of the three double tagged strains, *sep1-3HA fkh2-13myc*, *sep1-3HA mbx1-13myc* and *fkh2-3HA mbx1-13myc*.

The *fkh2-13myc:kanR* (GG 507), *mbx1-13myc:kanR* (GG 504) and *fkh2-3HA:kanR* (GG 558) strains have been described previously (Buck *et al.*, 2004). The *sep1-3HA:kanR* (GG 313), *mbx1-13myc:kanR* pIRT2u:*mbx1*⁺ (GG 527) and *fkh2-3HA:kanR* pUR19:*fkh2*⁺ (GG 539) strains derive from the Glasgow laboratory *S. pombe* collection (Appendix II).

All *sep1*⁺, *fkh2*⁺ or *mbx1*⁺ specific HA and myc tagged strains used for mating (Figure 4.1, 4.2 and 4.3) were generated by the PCR-based gene targeting method of Bähler *et al.* (1998a) and/or Krawchuk and Wahls (1999), which enables the C-terminal tagging of genes in their

chromosomal locus by homologous recombination. Briefly, appropriate primers are used to PCR amplify a DNA fragment with 60-80 bp gene-specific C-terminus sequences, flanking not only the 3HA or 13myc tagging module but also the heterologous selectable marker *KanMX6*, which allows for selection of G418-resistance upon transformation into *S. pombe* cells. G418-resistant transformants are then screened by PCR for integration by homologous recombination of the gene-specific 3HA or 13myc *KanMX6* construct into the correct chromosomal location, replacing the respective genomic fragment between the gene-specific sequences. As a result, the encoded protein of the tagged gene is expressed as a C-terminus 3HA or 13myc fusion under control of the endogenous promoter, a situation closely resembling expression of the untagged wild-type gene.

4.2.1.1 The *sep1-3HA fkh2-13 myc double tagged strain*

To generate the *sep1-3HA:kanR fkh2-13myc:kanR* double tagged strain, freshly grown h⁺ *sep1-3HA:kanR* (GG 313) and h⁻ *fkh2-13myc:kanR* (GG 507) cells were mixed and allowed to mate for 2-3 days at 25°C on solid ME medium (Section 2.2.13), since nutrient starvation is a prerequisite for conjugation and sporulation in *S. pombe*. Following mating, meiotic progeny were analysed by tetrad dissection (Section 2.2.14). Several four spore-containing zygotic asci were picked with a Singer micromanipulator, orderly placed on solid YE, and incubated at 36°C for 2-4 hours to induce break down of the asci walls. Once cell walls were broken, the four spores were separated with the micromanipulator, placed in a row, and left to grow at 30°C. Upon colony formation, cells were replica plated onto solid YE supplemented with G418 to select for the *Kan^R* marker.

As revealed by G418 selection (Figure 4.1A), all possible types of tetrad were produced, including the parental ditype (*Kan^R* marker segregating at a 1:1:1:1 ratio, no recombination; row A1-4), the tetratype (*Kan^R* marker segregating at a 3:1 ratio, two recombinants/ascus; row B1-4) and the non-parental ditype (*Kan^R* marker segregating at a 2:2 ratio, all recombinants; row C1-4) whose two G418-resistant progeny (C2 and C4) have the desired *sep1-3HA:kanR fkh2-13myc:kanR* genotype. One of the G418-resistant isolates (GG 1030) was then selected and backcrossed to wild-type (GG 217) for confirmation. After tetrad dissection and G418 selection, various tetrads were identified in which the *Kan^R* marker segregated at a 3:1 ratio (row A and B; Figure 4.1B), possible only if the G418-resistant parent in the backcross possesses two *Kan^R* markers.

Following backcross verification, *sep1-3HA fkh2-13myc* (GG 1030) soluble protein extracts were prepared and analysed by Western blot together with protein extracts from *sep1-3HA* (GG 313) and *fkh2-13myc* cells (GG 507) used as negative controls. As expected, both Sep1p-3HA and Fkh2p-13myc were detected in protein extracts from *sep1-3HA fkh2-13myc* cells with HA and myc

antibodies and resolved at sizes that are consistent with their predicted molecular masses (~72 kDa for Fkh2p and 73 kDa for Sep1p) and the presence of the 3HA and 13myc tag, respectively. On the contrary, Sep1p-3HA was absent from *fkh2-13myc* cells and Fkh2p-13myc from *sep1-3HA* cells (Figure 4.1C). It is important to mention that the 13myc tag resolves at a molecular mass of ~35-40 kDa, instead of the predicted molecular mass of ~20 kDa (data not shown), consistent with previous observations (Jansen *et al.*, 2005).

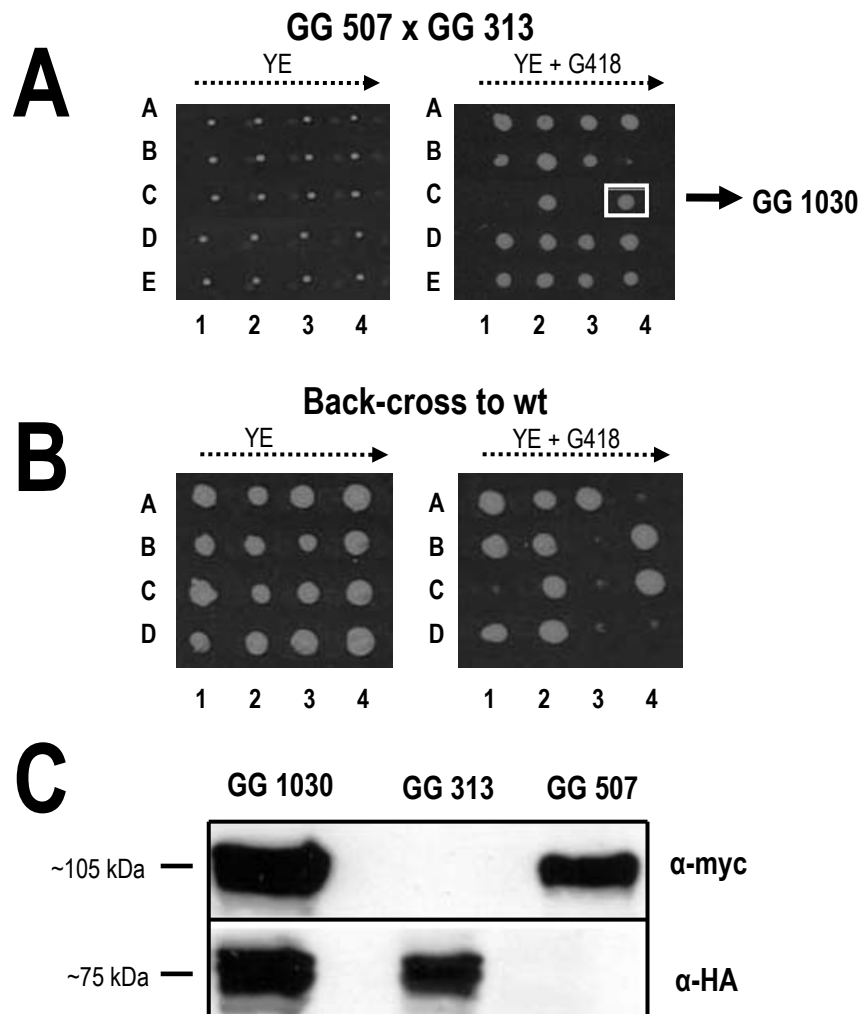


Figure 4.1: Generation of a *sep1-3HA fkh2-13myc* double tagged strain. (A) Tetrad analysis following mating of *sep1-3HA:kanR* (GG 313) and *fkh2-13myc:kanR* cells (GG 507) to select for *sep1-3HA:kanR fkh2-13myc:kanR* cells. After mating, ascospores were picked on solid YE medium and once asci walls were broken each ascus was dissected into four individual spores. Upon colony formation, cells were replica plated onto solid YE supplemented with G418 to select for the *Kan^R* marker segregating at a 2:2 ratio. (B) *sep1-3HA:kanR fkh2-13myc:kanR* cells (GG 1030) were backcrossed to wild-type cells (GG 217) for confirmation. (C) Western blot analysis of soluble protein extracts from *sep1-3HA fkh2-13myc* cells. *sep1-3HA fkh2-13myc* extracts were analysed by Western blot with antibodies against HA and myc to detect Sep1p-3HA and Fkh2p-13myc. *sep1-3HA* (GG 313) and *fkh2-13myc* (GG 507) extracts were used as negative controls. Protein molecular masses are indicated in kDa.

4.2.1.2 The *sep1-3HA mbx1-13myc* double tagged strain

The *sep1-3HA:kanR mbx1-13myc:kanR* strain was constructed by the same genetic approach as described in Section 4.2.1.1. Briefly, h⁺ *sep1-3HA:kanR* (GG 313) cells were mated with h⁻ *mbx1-13myc:kanR* (GG 504) cells and meiotic progeny were analysed by tetrad dissection. Replica plating onto G418 supplemented solid YE medium allowed selection of the *Kan^R* marker. Figure 4.2A shows all types of dissected tetrads, including the non-parental ditype (*Kan^R* marker segregating at a 2:2 ratio; row A1-4) containing two G418-resistant progeny (A2 and A4) of the desired *sep1-3HA:kanR mbx1-13myc:kanR* genotype. One of the G418-resistant isolates (GG 1032) was backcrossed to wild-type for confirmation (Figure 4.2B; row B, 3:1 *Kan^R* segregation ratio), before Western blot analysis of *sep1-3HA mbx1-13myc* protein extracts to detect Sep1p-3HA and Mbx1p-13myc using HA and myc antibodies, respectively (Figure 4.2C). As shown, Mbx1p-13myc resolved at a molecular mass of ~100 kDa. Taking into account that the 13myc tag resolves with a molecular mass of ~35-40 kDa, this suggests that Mbx1p resolves at a size slightly larger than the predicted value of ~51 kDa, consistent with results presented in Section 4.3.4.

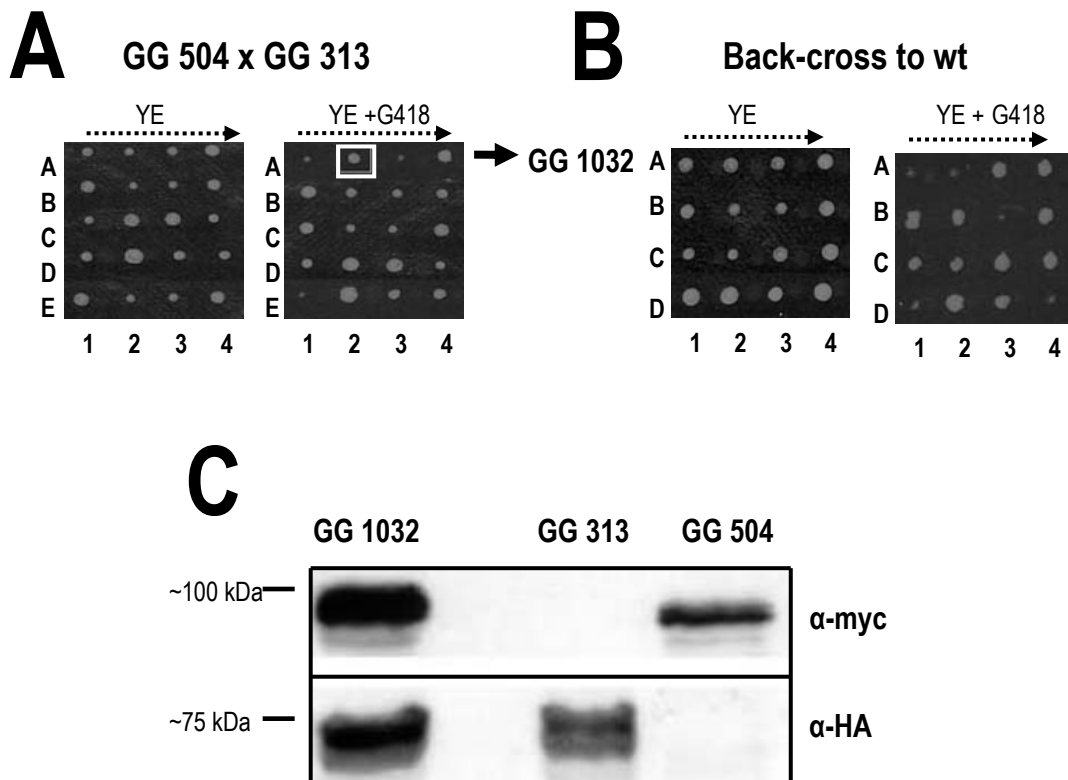


Figure 4.2: Generation of a *sep1-3HA mbx1-13myc* double tagged strain. (A) Tetrad analysis following mating of *sep1-3HA* (GG 313) and *mbx1-13myc* cells (GG 504) to select for *sep1-3HA mbx1-13myc* cells. Following mating, ascospores were picked on solid YE medium and once asci walls were broken each ascus was dissected into four individual spores. Upon colony formation the cells were replica plated onto solid YE supplemented with G418 to select for the *Kan^R* marker segregating at a 2:2 ratio. (B) *sep1-3HA mbx1-13myc* cells (GG 1032) were backcrossed to wild-

type cells (GG 218) for confirmation. (C) Western blot analysis of soluble protein extracts from *sep1-3HA mbx1-13myc* cells. *sep1-3HA mbx1-13myc* extracts were analysed by Western blot with antibodies against HA and myc to detect Sep1p-3HA and Mbx1p-13myc. *sep1-3HA* (GG 313) and *mbx1-13myc* (GG 504) extracts were used as negative controls. Protein molecular masses are indicated in kDa.

4.2.1.3 The *fkh2-3HA mbx1-13myc* double tagged strain

To create the *fkh2-3HA:kanR mbx1-13myc:kanR* strain, mating was performed between h^+ *fkh2-3HA:kanR* pUR19:*fkh2*⁺ (GG 539) and h^- *mbx1-13myc:kanR* pIRT2u:*mbx1*⁺ cells (GG 527). For this cross both the *fkh2-3HA* and the *mbx1-13myc* cells carried plasmids with untagged versions of *fkh2*⁺ and *mbx1*⁺, respectively, to improve mating efficiency. Upon mating, tetrad analysis of meiotic progeny and G418-resistance selection followed. Various tetrad types were identified, including the non-parental ditype (Figure 4.3A, row D) with two G418-resistant spores of the desired *fkh2-3HA:kanR mbx1-13myc:kanR* genotype (D3 and D4). One of the G418-resistant spores (GG 1035) was then backcrossed to wild-type for confirmation (Figure 4.3B; row B, 3:1 *Kan*^R segregation ratio), before Western blot analysis of *fkh2-3HA mbx1-13myc* protein extracts to detect Fkh2p-3HA and Mbx1p-13myc using HA and myc antibodies, respectively (Figure 4.3C).

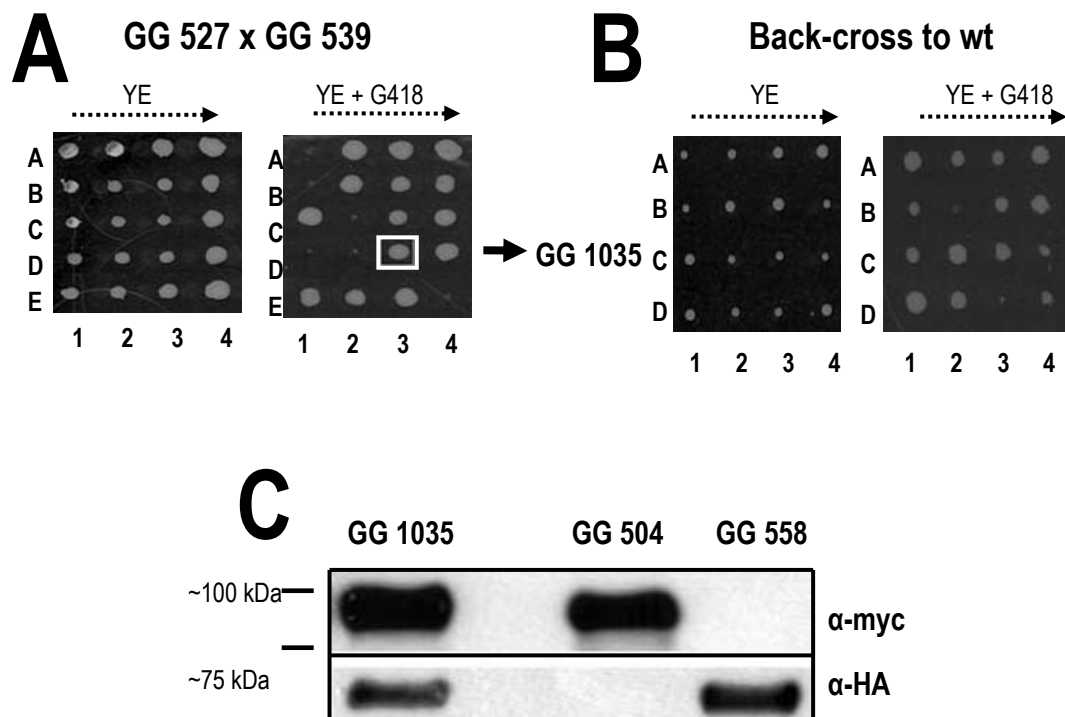


Figure 4.3: Generation of a *fkh2-3HA mbx1-13myc* double tagged strain. (A) Tetrad analysis following mating of h^+ *fkh2-3HA:kanR* pUR19:*fkh2*⁺ (GG 539) and h^- *mbx1-13myc:kanR* pIRT2u:*mbx1*⁺ cells (GG 527) to select for *fkh2-3HA mbx1-13myc* cells. Following mating, ascospores were picked on solid YE medium and once asci walls were broken each ascus was dissected into four individual spores. Upon colony formation, the cells were replica plated onto solid YE medium supplemented with G418 to select for the *Kan*^R marker segregating at a 2:2 ratio. (B) *fkh2-3HA mbx1-13myc* cells (GG 1035) were backcrossed to wild-type cells (GG 218) for confirmation. (C) Western blot analysis of soluble protein extracts from GG 1035, GG 504, and GG 558 cells. The top panel shows α -myc bands at approximately 100 kDa, and the bottom panel shows α -HA bands at approximately 75 kDa.

protein extracts from *fkh2-3HA mbx1-13myc* cells. *fkh2-3HA mbx1-13myc* extracts were analysed by Western blot with antibodies against HA and myc to detect Fkh2p-3HA and Mbx1p-13myc. *fkh2-3HA* (GG 558) and *mbx1-13myc* (GG 504) extracts were used as negative controls. Protein molecular masses are indicated in kDa.

4.2.2 Co-immunoprecipitation analysis of sep1-3HA fkh2-13myc protein extracts reveals an *in vivo* interaction between Sep1p and Fkh2p

To determine whether Sep1p and Fkh2p interact with each other *in vivo*, soluble protein extracts (Section 2.2.12.1) were prepared from *sep1-3HA fkh2-13myc S. pombe* cells (Section 4.2.1.1) and analysed by co-immunoprecipitation assay (Section 2.2.12.2).

Sep1p-3HA was immunoprecipitated from *sep1-3HA fkh2-13myc* protein extracts with an HA antibody followed by SDS-PAGE electrophoresis and Western blotting with HA and myc antibodies to detect Sep1p-3HA and co-immunoprecipitated Fkh2p-13myc. As a negative control, *sep1-3HA* protein extracts were also prepared and subjected to immunoprecipitation with an HA antibody before Western blotting with HA and myc antibodies.

Two milligrammes of protein extract were incubated with an appropriate volume of protein A-Sepharose beads for an hour at 4°C prior to centrifugation to remove all proteins that might bind non-specifically to the beads. The pre-cleared *sep1-3HA fkh2-13myc* and *sep1-3HA* protein extracts were then incubated with 2-3 µg of HA antibody (Table 2.1) for 3 h at 4°C before addition of protein A-Sepharose beads and further incubation for an hour. Pre-cleared *sep1-3HA fkh2-13myc* extract was also incubated for an hour with beads alone (no HA antibody). In each case, beads were washed thoroughly (Section 2.2.12.2) before re-suspension and boiling in 2x Laemmli buffer supplemented with DTT to release immunoprecipitated protein material. SDS-PAGE electrophoresis and Western blotting with HA and myc antibodies was then employed to detect Sep1p-3HA and any co-immunoprecipitated Fkh2p-13myc.

As shown in Figure 4.4, Fkh2p-13myc specifically co-immunoprecipitated with Sep1p-3HA in *sep1-3HA fkh2-13myc* protein extracts, a finding indicating an *in vivo* interaction between Fkh2p and Sep1p (Papadopoulou *et al.*, 2008). The weak nature of the interaction between Sep1p and Fkh2p, exemplified by the low amount of Fkh2p-13myc co-immunoprecipitating with Sep1p-3HA, may be explained by these two forkhead-like transcription factors binding to each other transiently during the course of the mitotic cell cycle (see Section 7.1).

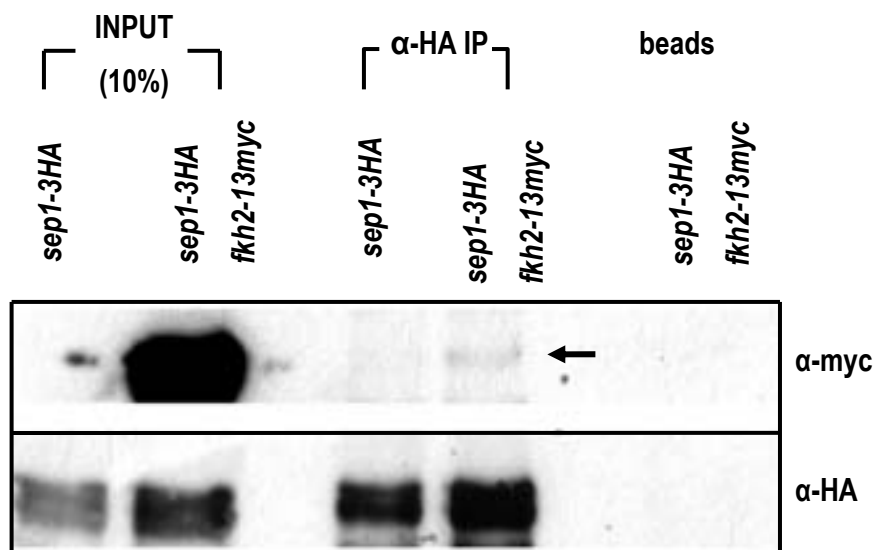


Figure 4.4: Fkh2p co-immunoprecipitates with Sep1p from *sep1-3HA fkh2-13myc* *S. pombe* protein extracts. Sep1p-3HA was immunoprecipitated with antibody against HA (α -HA) from soluble protein extracts of *sep1-3HA fkh2-13myc* fission yeast cells, expressing Sep1p-3HA and Fkh2p-13myc, in each case under control of the endogenous promoter. The soluble extract (input) and the immunoprecipitated sample (IP) were analysed by Western blotting with antibodies against HA and myc (the former to detect Sep1p-3HA, while the latter Fkh2p-13myc). As negative controls, immunoprecipitated sample from *sep1-3HA* fission yeast cells and precipitated sample without HA antibody (beads only) from *sep1-3HA fkh2-13myc* cells were also included. In each case, the input was used in a 1:10 ratio relative to the IP sample. The arrow indicates co-immunoprecipitated Fkh2p-13myc using a α -myc antibody. Immunoprecipitation assays of *sep1-3HA fkh2-13myc* and *sep1-3HA* soluble protein extracts with the HA antibody were carried out twice. The results of Western blot analysis of immunoprecipitated material with HA and myc antibody in one of the two assays are shown here and are representative of the results of Western blot analysis of the other assay.

4.2.3 Co-immunoprecipitation analysis of sep1-3HA mbx1-13myc protein extracts reveals no *in vivo* interaction between Mbx1p and Sep1p

Following the discovery that Fkh2p interacts with Sep1p *in vivo*, co-immunoprecipitation assays (Section 2.2.12.2) were employed to examine whether either of these two forkhead proteins also interacts with Mbx1p. Immunoprecipitations were carried out as described in Section 4.2.2.

In the case of Sep1p and Mbx1p, an HA antibody was used to immunoprecipitate Sep1p-3HA from *sep1-3HA mbx1-13myc* (Section 4.2.1.2) and *sep1-3HA* protein extracts, the latter as a negative control. Western blot analysis was then performed to detect Sep1p-3HA and co-immunoprecipitated Mbx1p-13myc, using HA and myc antibodies, respectively. As shown by immunoblotting, no Mbx1p-13myc was co-immunoprecipitated with Sep1p-3HA in *sep1-3HA mbx1-13myc* protein extracts (Figure 4.5A).

To exclude the possibility that the failure to identify an interaction between Mbx1p and Sep1p is an indirect result of inefficient co-immunoprecipitation of Mbx1p-13myc itself, another co-immunoprecipitation assay was performed to examine whether Sep1p-3HA is detected in co-immunoprecipitates with Mbx1p-13myc. Briefly, Mbx1p-13myc was immunoprecipitated from *sep1-3HA mbx1-13myc* protein extracts with a myc antibody before Western blotting with an HA antibody to detect any co-immunoprecipitated Sep1p-3HA. As a negative control, Mbx1p-13myc was also immunoprecipitated from *mbx1-13myc* soluble protein extracts prior to Western blot analysis. Immunoprecipitation was carried out as before (Section 4.2.2), except for the use of protein G instead of protein A-Sepharose beads. Immunoblotting using an HA antibody detected no Sep1p-3HA co-immunoprecipitating with Mbx1p-13myc (Figure 4.5B), consistent with the inability to detect Mbx1p-13myc in co-immunoprecipitates with Sep1p-3HA (Figure 4.5A). Taken together, these findings suggest that Mbx1p and Sep1p do not bind to each other *in vivo*.

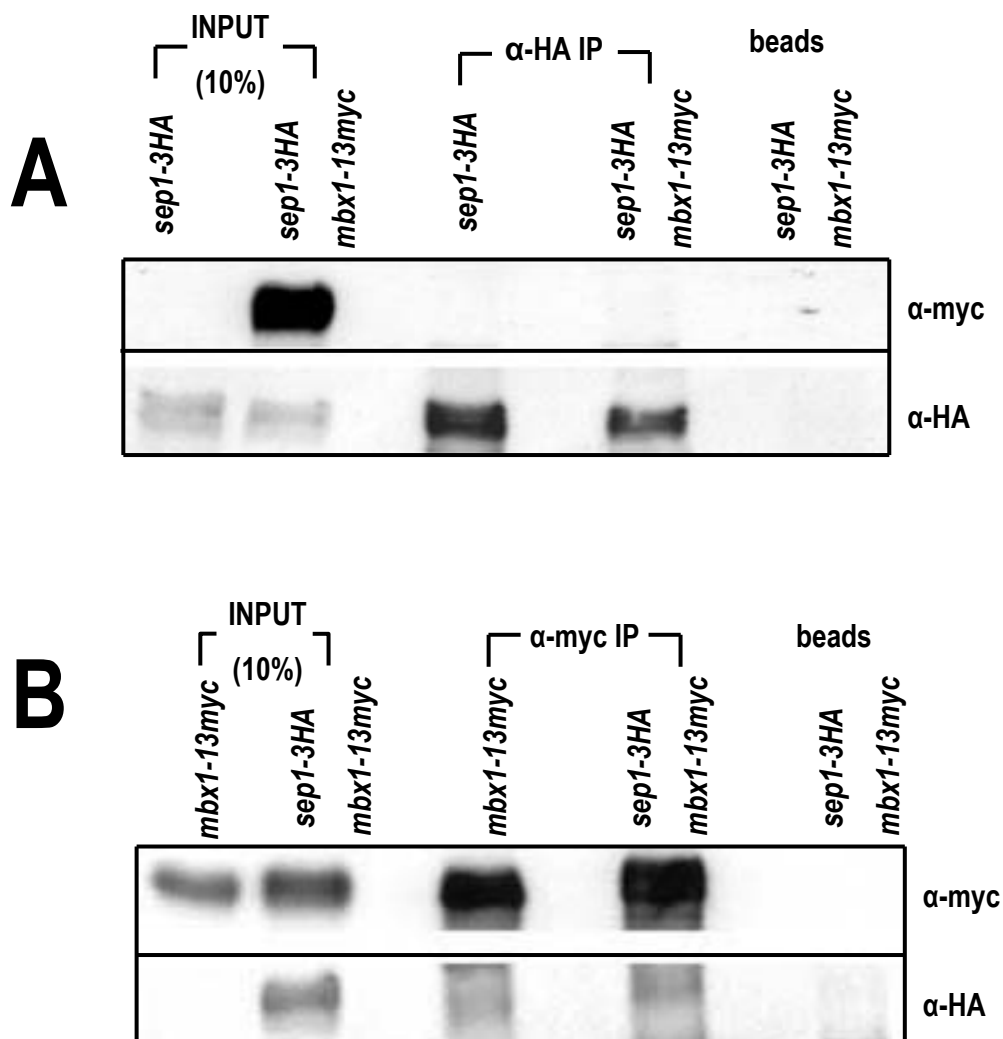


Figure 4.5: Mbx1p does not co-immunoprecipitate with Sep1p and vice versa in *sep1-3HA mbx1-13myc S. pombe* protein extracts. (A) Sep1p-3HA was immunoprecipitated with antibody against HA (α -HA) from *S. pombe sep1-3HA mbx1-13myc* protein extracts. The soluble extract (input) and the immunoprecipitated sample (IP) were analysed by Western blotting with antibodies against HA and myc (the former to detect Sep1p-3HA, while the latter Mbx1p-13myc). As negative controls, immunoprecipitated sample from *sep1-3HA* fission yeast cells and precipitated sample without HA antibody (beads only) from *sep1-3HA mbx1-13myc* cells were also included. (B) Mbx1p-13myc was immunoprecipitated with antibody against myc (α -myc) from soluble protein extracts of *sep1-3HA mbx1-13myc* fission yeast cells. The soluble extract (input) and the immunoprecipitated sample (IP) were analysed by Western blotting with antibodies against myc and HA (the former to detect Mbx1p-13myc, while the latter Sep1p-3HA). As negative controls, immunoprecipitated sample from *mbx1-13myc* fission yeast cells and precipitated sample without myc antibody (beads only) from *sep1-3HA mbx1-13myc* cells were also included. (A-B) In each case, the input was used in a 1:10 ratio relative to the IP sample. (A-B) Immunoprecipitation assays of *sep1-3HA mbx1-13myc* and *sep1-3HA* soluble protein extracts with either the HA or myc antibody were carried out twice. In each case, the results of Western blot analysis of immunoprecipitated material with HA and myc antibody in one of the two assays are shown here and are representative of the results of Western blot analysis of the other assay.

4.2.4 Co-immunoprecipitation analysis of *fkh2-3HA mbx1-13myc* protein extracts reveals no *in vivo* interaction between *Mbx1p* and *Fkh2p*

To investigate a potential interaction between *Mbx1p* and *Fkh2p*, soluble protein extracts were prepared from *S. pombe fkh2-3HA mbx1-13myc* cells (Section 4.2.1.3) and analysed by co-immunoprecipitation assays.

As for *Mbx1p* and *Sep1p* (Section 4.2.3), immunoprecipitations were carried out with either an HA or a myc antibody to immunoprecipitate *Fkh2p-3HA* or *Mbx1p-13myc*, respectively. Western blotting was then performed to detect *Fkh2p-3HA* and any co-immunoprecipitated *Mbx1p-13myc*, or *Mbx1p-13myc* and any co-immunoprecipitated *Fkh2p-3HA*. As negative controls, *fkh2-3HA* and *mbx1-13myc* protein extracts were prepared and subjected to immunoprecipitation analysis with HA and myc antibodies.

As shown in Figure 4.6A, co-immunoprecipitation analysis of *fkh2-3HA mbx1-13myc* protein extracts revealed that no *Mbx1p-13myc* was co-immunoprecipitated with *Fkh2p-3HA*. The reverse was also true, since no *Fkh2p-3HA* was detected in co-immunoprecipitates with *Mbx1p-13myc* (Figure 4.6B). These results show that at least under the current experimental conditions *Mbx1p* and *Fkh2p* do not interact with each other *in vivo*.

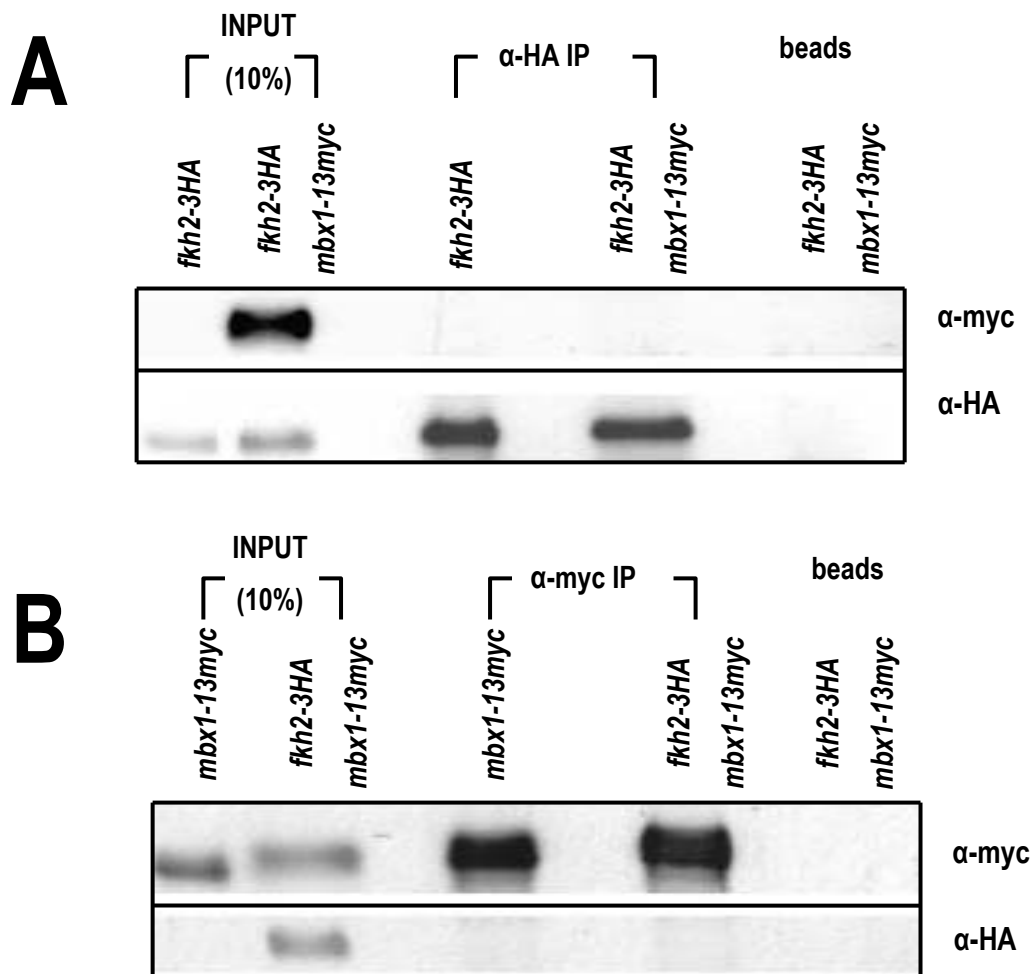


Figure 4.6: Mbx1p does not co-immunoprecipitate with Fkh2p and vice versa in *fkh2-3HA mbx1-13myc S. pombe* protein extracts. (A) Fkh2p-3HA was immunoprecipitated with antibody against HA (α -HA) from soluble protein extracts of *fkh2-3HA mbx1-13myc* fission yeast cells, expressing Fkh2p-3HA and Mbx1p-13myc, in each case under control of the endogenous promoter. The soluble extract (input) and the immunoprecipitated sample (IP) were analysed by Western blotting with antibodies against HA and myc (the former to detect Fkh2p-3HA, while the latter Mbx1p-13myc). As negative controls, immunoprecipitated sample from *fkh2-3HA* fission yeast cells and precipitated sample without HA antibody (beads only) from *fkh2-3HA mbx1-13myc* cells were also included. In each case, the input was used in a 1:10 ratio relative to the IP sample. (B) Mbx1p-13myc was immunoprecipitated with antibody against myc (α -myc) from soluble protein extracts of *fkh2-3HA mbx1-13myc* fission yeast cells. The soluble extract (input) and the immunoprecipitated sample (IP) were analysed by Western blotting with antibodies against myc and HA (the former to detect Mbx1p-13myc, while the latter Fkh2p-3HA). As negative controls, immunoprecipitated sample from *mbx1-13myc* fission yeast cells and precipitated sample without myc antibody (beads only) from *fkh2-3HA mbx1-13myc* cells were also included. In each case, the input was used in a 1:10 ratio relative to the IP sample. (A-B) Immunoprecipitation assays of *fkh2-3HA mbx1-13myc* and *fkh2-3HA* soluble protein extracts with either the HA or myc antibody were carried out twice. In each case, the results of Western blot analysis of immunoprecipitated material with HA and myc antibody in one of the two assays are shown here and are representative of the results of Western blot analysis of the other assay.

4.3 GST pull down analysis of interactions between Sep1p, Mbx1p and Fkh2p

The GST (Glutathione S-Transferase) pull-down assay is an *in vitro* method used routinely to determine whether two proteins interact directly with each other. A bacterially expressed and purified GST-tagged protein, immobilised on a glutathione affinity gel, is tested for its ability to bind and 'pull down' another protein, which can also be bacterially expressed and purified as a fusion with a different tag, such as HIS. The GST-tagged protein serves as a 'bait' to capture a putative binding partner, the so-called 'prey' protein.

As already described, a series of co-immunoprecipitation experiments have shown that Fkh2p and Sep1p bind to each other *in vivo*, whereas under the same experimental conditions no interaction was observed between Mbx1p and either Fkh2p or Sep1p. To analyse further these findings, the GST pull-down method was employed to examine *in vitro* whether Fkh2p and Sep1p interact with each other directly, as well as whether under these conditions an interaction between Mbx1p and either Fkh2p or Sep1p could be detected. The first step in the process involved cloning of *fkh2⁺*, *sep1⁺* and *mbx1⁺* into an appropriate expression vector with a fusion tag to allow their bacterial overexpression and purification.

4.3.1 Cloning of *sep1⁺*, *mbx1⁺* and *fkh2⁺*

The cloning of *fkh2⁺*, *sep1⁺* and *mbx1⁺* was accomplished using standard DNA manipulation methods (Section 2.2.9). Genomic ORFs of *fkh2⁺*, *sep1⁺* and *mbx1⁺* were obtained from the *S. pombe* database (Wood *et al.*, 2002), showing that while introns are absent from *sep1⁺*, *fkh2⁺* contains one intron and *mbx1⁺* three introns. Therefore, in the case of *fkh2⁺* and *mbx1⁺*, cDNA was generated and used as a template for PCR amplification to remove the introns to permit expression of these genes in bacteria, which cannot process intervening sequences.

Full-length *sep1⁺* DNA was amplified by PCR from *S. pombe* genomic DNA using primers GO 570 and GO 571 to introduce *Sma*I and *Xho*I restriction sites at the 5' and 3' ends, respectively. PCR amplification was carried out with Vent_R DNA polymerase, which has extensive 3' to 5' exonuclease proofreading activity, to minimise errors in base incorporation. As shown in Figure 4.7A, analysis of the PCR product on a 1% agarose gel revealed a ~2 kb DNA fragment, corresponding to the predicted size of *sep1⁺* (ORF: 1992 bp).

The PCR product was next cloned into the linearised pCR[®]2.1 vector (~3.9 kb), carrying single 3' deoxythymidine (T) residues. Since Vent_R polymerase does not leave single 3' deoxyadenosine (A) overhangs, the PCR product was incubated at 72°C for 10 min with *Taq* polymerase, which adds single deoxyadenosine (A) to the 3' ends of PCR products in a template independent manner, to facilitate efficient ligation to pCR[®]2.1 vector, followed by transformation into InVαF' cells (Section 2.2.4). To allow for selection of successful transformants, cells were grown overnight at 37°C on solid LB medium supplemented with ampicillin, as well as X-gal. Individual white colonies were picked and cell cultures were set and grown overnight.

Following plasmid purification (Section 2.2.9.1), successful cloning of the *sep1*⁺ PCR product into the pCR[®]2.1 vector was confirmed by a restriction digest with *Eco*RI (Figure 4.7B) and a double digest with *Eco*RI and *Bam*HI (Figure 4.7C). As expected, digestion with *Eco*RI alone yielded a ~2 kb DNA fragment corresponding to full-length *sep1*⁺, since no *Eco*RI site is present within *sep1*⁺, but pCR[®]2.1 contains two *Eco*RI sites flanking the *sep1*⁺ DNA fragment (Figure 4.7B). Digestion with *Eco*RI and *Bam*HI generated two DNA fragments around 1.8 kb and 0.2 kb, consistent with the presence within *sep1*⁺ of a *Bam*HI site at position +1784 (Figure 4.7C). Various isolates of pCR[®]2.1 plasmid constructs carrying *sep1*⁺ were DNA sequenced (MWG Biotech) to search for mutations. One isolate free of mutations was identified (GB 444) and used for further experiments.

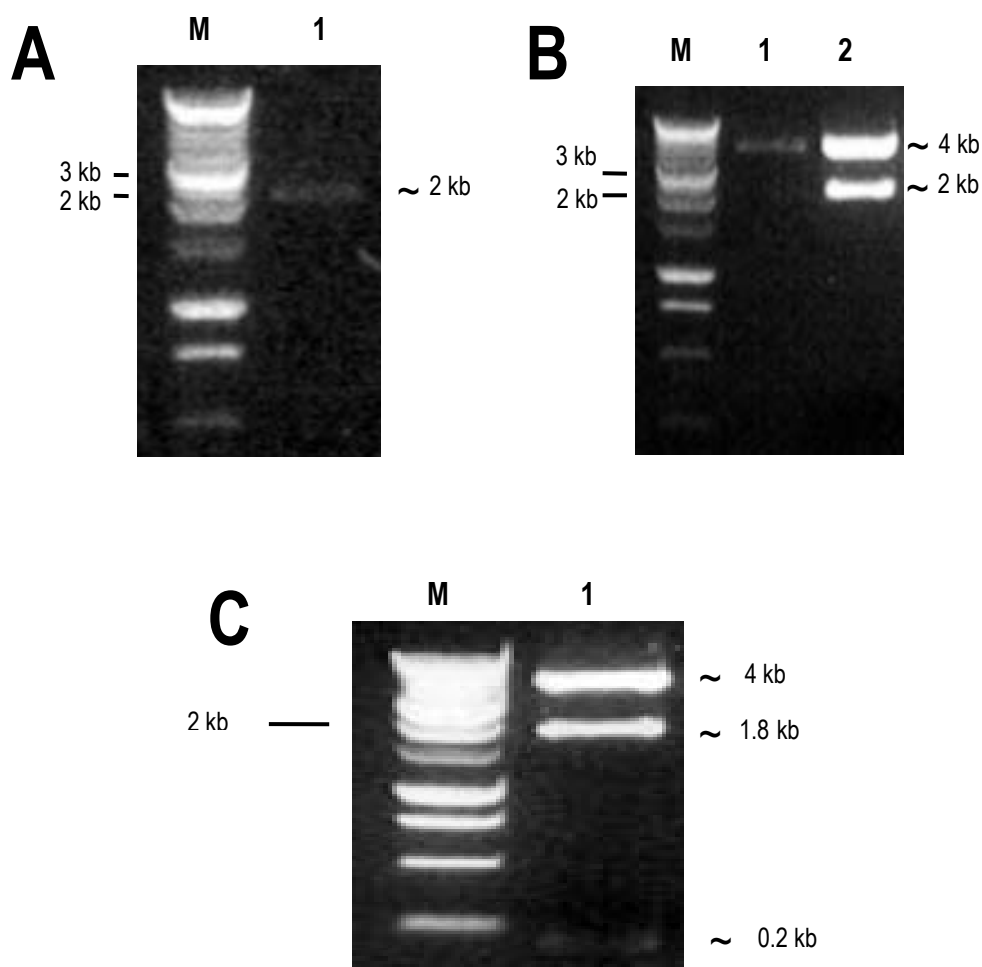


Figure 4.7: Cloning of the *S. pombe sep1⁺* gene. (A) Lane 1, PCR amplified full-length *sep1⁺* DNA using as a template *S. pombe* genomic DNA. Lane M, 1 kb DNA ladder (Promega). (B) Lane 1, *EcoRI* restriction digest of empty pCR[®]2.1 vector. Lane 2, *EcoRI* restriction digest of pCR[®]2.1 vector containing full-length *sep1⁺*. Lane M, 1 kb DNA ladder (Promega). (C) Lane 1, *BamHI/EcoRI* restriction digest of pCR[®]2.1 vector containing full-length *sep1⁺*. *BamHI* cuts within the *sep1⁺* DNA sequence, generating two DNA fragments around 1.8 kb and 0.2 kb, respectively. Lane M, 1 kb DNA ladder (Promega). (A-C) Sizes of DNA fragments are indicated.

The presence of introns within *mbx1⁺* necessitated the use of *mbx1⁺* cDNA as a template for PCR amplification. In order to generate *mbx1⁺* cDNA, mRNA was purified from total *S. pombe* RNA (Section 2.2.10.1-2.2.10.2) and used as a template for gene-specific cDNA synthesis with an *mbx1⁺* anti-sense primer (GO 573) (Section 2.2.9.13). The *mbx1⁺*-specific cDNA was used as a template for PCR amplification with primers GO 572 and GO 573 to introduce *SmaI* and *XhoI* restriction sites at the 5' and 3' ends, respectively. As shown in Figure 4.8A, analysis of the PCR products on a 1%

agarose gel identified two DNA fragments, ~1.4 kb and ~1.6 kb, respectively. As expected, the ~1.4 kb DNA fragment corresponded to the predicted size of *mbx1*⁺ cDNA (1437 bp). In contrast, the ~1.6 kb fragment corresponded to the predicted size of *mbx1*⁺ genomic DNA (1664 bp), suggesting that this DNA fragment was PCR amplified from contaminating genomic DNA, present in the cDNA sample. Consequently, the ~1.4 kb DNA fragment was excised from the gel and purified (Figure 4.8B), before cloning into the pCR[®]2.1 vector and transformation into InVαF' cells. Once cells were grown overnight on solid LB-ampicillin supplemented with X-gal, white colonies were selected and cell cultures were set and grown overnight.

Following plasmid purification, successful cloning of the *mbx1*⁺ PCR product into pCR[®]2.1 was confirmed by a restriction digest with *Sma*I and *Xho*I (Figure 4.9A), as well as with *Eco*RI and *Bam*HI (Figure 4.9B). Restriction digest with *Sma*I and *Xho*I yielded an ~1.4 kb DNA fragment corresponding to full-length *mbx1*⁺ cDNA, since neither *Sma*I nor *Xho*I cut within *mbx1*⁺. Digestion with *Eco*RI and *Bam*HI generated three DNA fragments, ~0.9 kb, ~0.5 kb and ~0.04kb, since *Bam*HI cuts within *mbx1*⁺ at position +1187, as well as within pCR[®]2.1. Different isolates of pCR[®]2.1 plasmid constructs carrying *mbx1*⁺ were DNA sequenced (MWG Biotech). One isolate was identified (GB 445) carrying only a silent mutation at position 203 (TCT mutated to TCC; both encode serine) and used for further experiments. Importantly, DNA sequencing revealed that the third intron was shorter than predicted in the *S. pombe* genome database (Wood *et al.*, 2002). Sequencing the cDNA showed that the third intron was present from position +501 to +572 instead of +501 to +635 (Figure 4.10), with this part of the *mbx1*⁺ DNA sequence from +573 to +635 comprising part of the final exon. This corrected genomic sequence has now been added to the *S. pombe* genome database (<http://www.genedb.org/genedb/pombe/coordChanges.jsp>).

Cloning of *fkh2*⁺ was performed by a colleague, Dr Szu Shien Ng, using a similar approach to that used for *mbx1*⁺, since *fkh2*⁺ also contains an intron. Briefly, *fkh2*⁺-specific cDNA was generated and used as a template for PCR amplification with primers GO 568 and GO 569 to introduce *Bam*HI and *Xho*I restriction sites at the 5' and 3' ends, respectively. The resulting PCR product was cloned into pCR[®]2.1 vector and transformed into InVαF' cells. Successful cloning was confirmed by DNA sequencing (MWG Biotech), identifying one isolate (GB 403), carrying a silent mutation at position +903, which was used for further experiments.

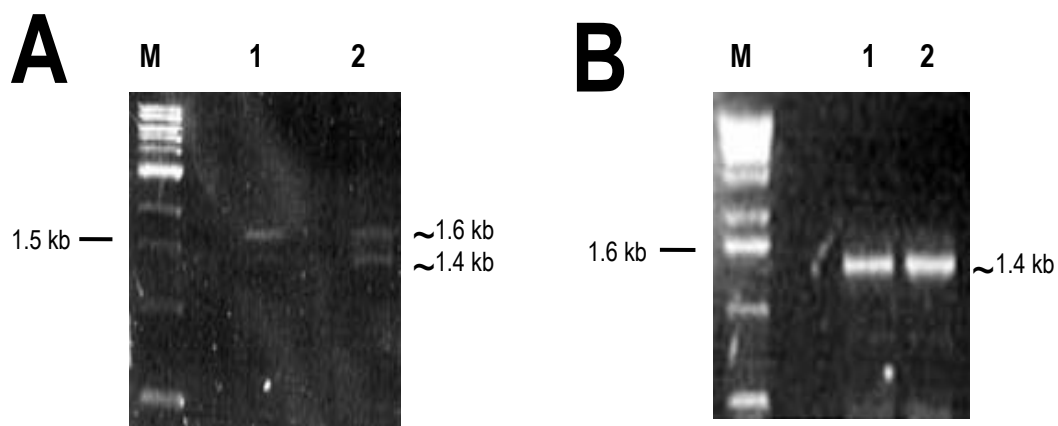


Figure 4.8: *S. pombe mbx1+* cDNA. (A) Lanes 1 and 2, PCR amplified DNA products using as a template *mbx1+* cDNA. Lane M, 1 kb DNA Ladder (NEB). (B) Lanes 1 and 2, purified DNA products of *mbx1+* cDNA. Lane M, DNA Molecular Weight marker X (Roche). (A-B) Sizes of DNA fragments are indicated.

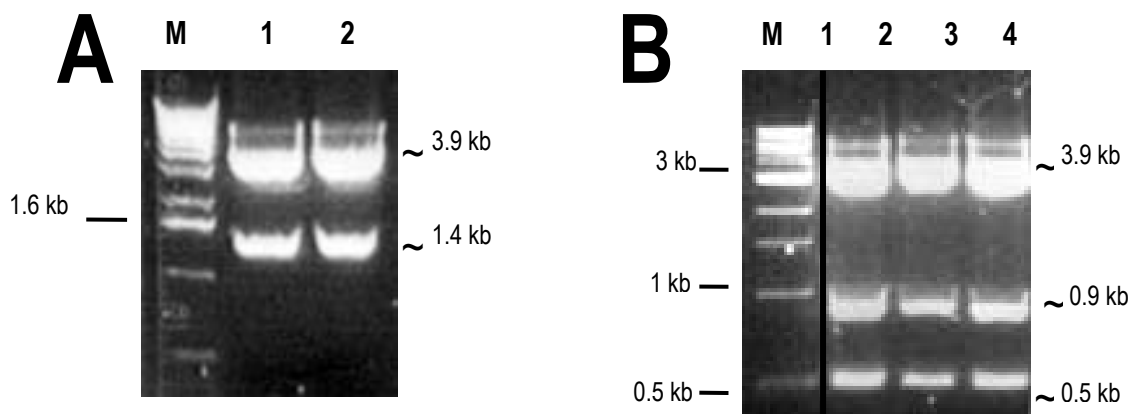


Figure 4.9: Cloning of *S. pombe mbx1+* cDNA. (A) Lanes 1 and 2, *SmaI/XhoI* restriction digest of pCR[®]2.1 vector containing full-length *mbx1+*. Lane M, DNA Molecular Weight marker X (Roche). (B) Lanes 1, 2 and 3, *BamHI/EcoRI* restriction digest of pCR[®]2.1 vector containing full-length *mbx1+*. *BamHI* cuts within the *mbx1+* DNA sequence, generating two DNA fragments around 0.9 kb and 0.5 kb, respectively. Lane M, 1 kb DNA ladder (NEB). (A-B) Sizes of DNA fragments are indicated.

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ATGGATATTA  ATCCTCCTCC  TAGTACTGCT  CCTTCATCTC  CTCGCCGTTT  T[GTATGTGCT
ACATAGTGGT  TTGGTTCCAT  TCATACACCC  CTTTTCTCCT  CGCTAACGTA  CCATTTAG]*1  AT
ACAGCGTATT  TCCGATGCAA  AAAATAAGGC  TCTTACTTTT  AATCGTCGAC  GACTTGGCTT
GATAAAAAAA  GCACATGAAC  TCc#CCGTTTT  GTGTGATGCT  AAAGTGGTGG  TCATGATTTT
TGATTCTAAA  AATGCTTGTC  ACGTT[GTATG  TTTCATATTT  CCCGTTTTTC  TGATCCTTTT
CTGTCAATATC  CTTTCAATGT  AAAGTGAATA  CATTATAGGG  AAGGTGATGA  AAGTCAACAA
CCAGAAAACG  TGGCAATGTT  TTTACAAGTT  TTTAATATTA  ACATACACCT  CTTTAG]*2  TATT
CTTCTGAGGA  ACCGGAAGAG  CAAAGAGATG  CCTTGCTGCA  AAAATTCCTG  AACCAAGGACT
TTGTCACGGT  TGACCCTCTG  [GTAAGTCACT  TATCAGCAAT  CATTATGCTT  GTCCTGTATT
CTGTTTACTA  ACATAATCCC  TTGGGGAGAA  AG]*3  AGAAACAT  AAATCCTAAT  ATACCATCTG
ATGAATCCCT  TCATAATTGG  AGACCTAAAG  ATAAGCGGAT  TGCTTCTGTT  ACCACATACA
GTGCTCAACC  TTCTAATAAT  TGCTCTTCTG  CTACCGACAG  TGAAAATGAC  TTTCAATCTT
TCACGATCAA  ATCCTCTACC  ACGTACCATA  CTACTCCAAC  TACTGCAAGC  GAGAACAAGA
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CAACAGTCAA  ACATTCTTTT  GTCTCTCCAG  TTTCTGGAGA  TFACTCTGAC  TCACCTTTAG
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TTCAACATAA  TGATGTTCCG  CAAACCGATA  ATTTTATCCC  TTTCTTACT  CCTAAGCGTC
AAGCATATGG  ACAATCATCT  TCCCGAGCTG  ATCGTAGCTC  TGTTCTGTCG  AGCCAATCTT
TTAAAAATCG  TCGTAATGGA  AAACCGCGAA  TATCCAGGCT  GCATACTTCT  CATGCTTCTA
TTGACGGGCT  AACTGATTTT  ATTCAAAGTC  CTTCTTCTGG  TTATTTGGAT  CCCAGCTCAA
CACCTATAAC  ACCTTTGGAC  TCTGCTATAA  ATCAAATTAC  GCCTCCTTTT  CTTCCCGATA
ATTTGGGTCA  AGAAAACCGT  GGAGAATTAT  ATTCTCACGA  TAATCCTACT  TCTATGGTAT
ATGAACATCC  AAAATTTGAT  GAATGCCCCA  ACGGATTTAT  TGATACTCAT  GAGTTGAACA
TACTAAGTCG  CTCATTTACA  GCATCTCCTA  ACCAAATCTT  TCGCGAATCG  AATATGGTTA
ATCAAGACTC  TTTTACCGAC  AATCCAGTTG  ATGCAACTTG  GGATGCTTTA  ATTGGTACAA
CGCAAATTGA  TTTAGATTTA  GATTATGAGA  GGTCAATCAAT  ACCCTCATCC  ACTATTCCTG
CTGATCAACT  GAAAGACGGC  GTCCCCACAA  ATTCGGTATA  TCGAAATAAC  ATGGTTGATC
ATAATTTATA  TCCGTCTTTA  AATATTGAAC  GAAATGCCCC  TTAA

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Figure 4.10: Sequence of *mbx1⁺* cDNA and inferred genomic structure. DNA sequencing of *mbx1⁺* cDNA cloned into the pCR[®]2.1 vector. Introns are in red. *1 indicates the position of the 1st intron, *2 the position of the 2nd intron and *3 the position of the 3rd intron. # in yellow denotes T changed to C; silent mutation. This corrected sequence has been added to the Sanger pombe genomic data base (<http://www.genedb.org/genedb/pombe/coordChanges.jsp>).

4.3.2 Generation of GST-tagged versions of *sep1*⁺ and *mbx1*⁺

sep1⁺ and *mbx1*⁺ were cloned into pGEX-KG (GB 159), a bacterial expression vector that upon addition of IPTG allows over-expression of the cloned gene as an N-terminal GST-fusion protein (Appendix IV). pCR[®]2.1 containing *mbx1*⁺ (GB 445) or *sep1*⁺ (GB 444) was digested with *Sma*I and *Xho*I, the respective *mbx1*⁺ or *sep1*⁺ DNA fragment was gel extracted, purified and cloned into linearised pGEX-KG vector digested with *Sma*I and *Xho*I (Section 2.2.9.6). pGEX-KG containing either *sep1*⁺ or *mbx1*⁺ was then transformed into the *E. coli* BL21 CodonPlus bacterial strain (Section 2.2.4) to allow protein overexpression and purification. Successful cloning was confirmed by a *Sma*I/*Xho*I restriction digest of pGEX-KG containing either *sep1*⁺ (GB 441) or *mbx1*⁺ (GB 448), revealing an ~2 kb or an ~1.4 kb DNA fragment, respectively (Figure 4.11A and 4.11B). The integrity of the pGEX-KG-*sep1*⁺ construct was also confirmed by DNA sequencing (MWG Biotech).

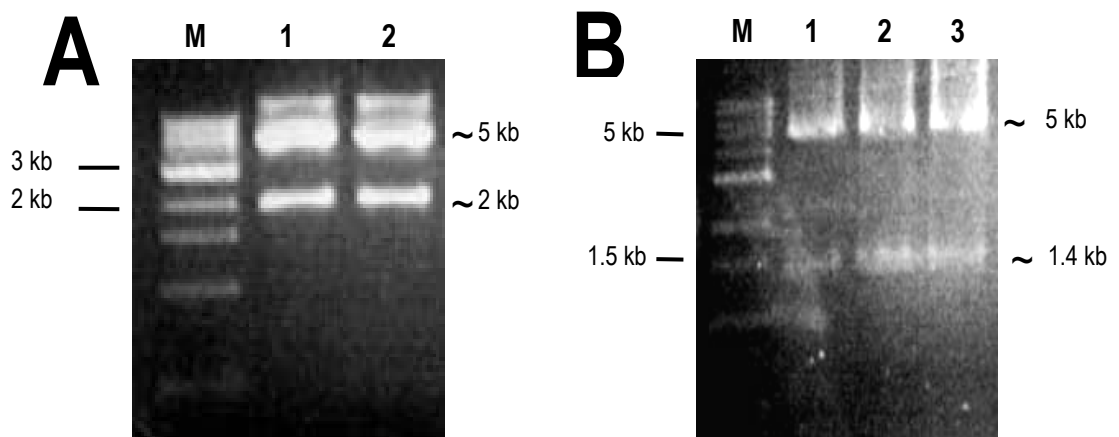


Figure 4.11: Cloning of the *sep1*⁺ and *mbx1*⁺ genes into the pGEX-KG expression vector. (A) Lanes 1 and 2, *Sma*I/*Xho*I restriction digest of the pGEX-KG expression vector containing full-length *sep1*⁺. Lane M, 1 kb DNA Ladder (NEB). (B) Lanes 1, 2 and 3, *Sma*I/*Xho*I restriction digest of the pGEX-KG expression vector containing full-length *mbx1*⁺. Lane M, 1 kb DNA Ladder (NEB). (A-B) Sizes of DNA fragments are indicated.

4.3.3 Generation of HIS-tagged versions of *sep1*⁺ and *fkh2*⁺

pET-28-c (+) and pET-28-a (+) are bacterial expression vectors that carry a N-terminal 6xHIS tag, which, in the presence of IPTG, allows over-expression of the cloned gene as an N-terminal HIS fusion protein (Appendix IV). *fkh2*⁺ and *sep1*⁺ were cloned into appropriate restriction sites of pET-28-c (+) and pET-28-a (+) vector respectively, by Dr Szu Shien Ng, using standard molecular cloning procedures (Section 2.2.9). Briefly, pCR[®]2.1 vector containing *fkh2*⁺ (GB 403) or *sep1*⁺ (GB 444)

was digested with appropriate restriction enzymes, the respective *fkh2*⁺ or *sep1*⁺ DNA fragment was gel extracted, purified and cloned into linearised pET-28-c (+) and pET-28-a (+) vector digested with the same enzymes. pET-28-a (+) containing *sep1*⁺ (GB 425) or pET-28-c (+) containing *fkh2*⁺ (GB 417) was then transformed into the *E. coli* BL21 CodonPlus bacterial strain (Section 2.2.4). In each case, successful cloning was confirmed by DNA sequencing (MWG Biotech).

4.3.4 Bacterial overexpression of GST-fusion proteins

Following successful cloning of *mbx1*⁺ and *sep1*⁺ into pGEX-KG, the resulting plasmids were transformed into the BL21 CodonPlus bacterial strain (GB 448 and GB 441). BL21 (DE3) CodonPlus-RIL cells are ideal for the overexpression of proteins from species other than *E. coli*, since they contain extra copies of several tRNA genes that are 'rare' to *E. coli* but more frequent in other species, thus dramatically improving overexpression of heterologous proteins.

Small-scale protein inductions (50 ml) were carried out to examine the overexpression efficiency and solubility of GST-Mbx1p and GST-Sep1p. BL21 CodonPlus cells transformed with empty pGEX-KG vector were also induced for protein overexpression, since GST was required as a control for the subsequent GST pull-down assay.

Optimal conditions of protein overexpression in the BL21 CodonPlus strain were selected to allow the production of GST-Mbx1p, GST-Sep1p and GST. Specifically, a single colony of BL21 CodonPlus cells transformed with empty pGEX-KG (GB 159) or pGEX-KG carrying either *mbx1*⁺ (GB 448) or *sep1*⁺ (GB 441) was picked from a colony grown overnight on appropriate solid medium and inoculated into 10 ml of NZY⁺ broth supplemented with ampicillin. Following growth for 16 h at 37°C with shaking, 1 ml of culture was added to 50 ml of fresh media. The culture was grown further at 37°C with shaking until the OD_{600nm} reached 0.5 and IPTG was added at a final concentration of 0.5 mM to induce over-expression of GST, GST-Mbx1p or GST-Sep1p for 3-4 h at 30°C. In each case, 1 ml samples were collected from the culture immediately before addition of IPTG (0 h), as well as at hourly intervals after IPTG induction (1 h, 2 h, 3 h and 4 h). Following protein overexpression, cells were harvested by centrifugation, resuspended in PBS and lysed by French pressure disruption. 1 ml of lysate (L) was collected and kept on ice, while 1 ml of lysate was centrifuged to separate the supernatant (S) and pellet fraction (P). From each sample, 10 µl were taken and resuspended in an equal volume of 2x Laemmli sample buffer, denatured by boiling for 5 min in the presence of 150 mM DTT and analysed by SDS-PAGE electrophoresis and Coomassie blue staining to examine the overexpression and solubility of GST, GST-Mbx1p and GST-Sep1p (Figures 4.12, 4.13 and 4.14).

The solubility of each protein was determined by comparing the fraction of protein present in the supernatant (soluble fraction) with that in the pellet (insoluble fraction).

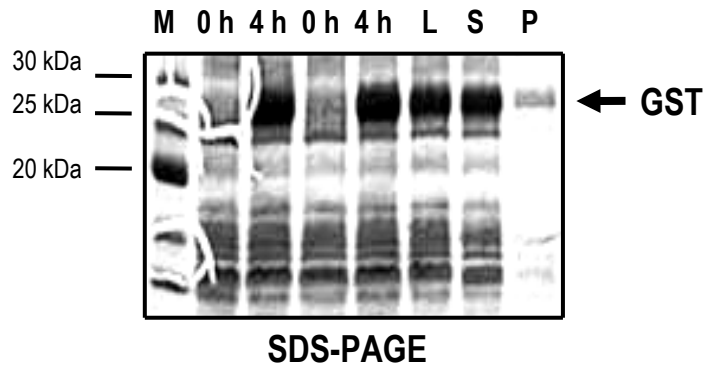


Figure 4.12: GST overexpression and solubility. GST overexpression was induced in BL21 CodonPlus (DE3)-RIL cells with 0.5 mM IPTG at 30°C for 4 h. Samples were collected at the point of induction (0 h) and at 4 h following induction. Cells were harvested by centrifugation, resuspended in PBS and lysed by French pressure disruption. The lysate (L) was centrifuged to separate the supernatant (S) and pellet fraction (P). 1 ml samples were collected at all stages. All samples were resuspended in Laemmli sample buffer, denatured by boiling for 5 min in the presence of DTT and analysed by SDS-PAGE and Coomassie blue staining. Molecular weight marker is shown (M), with sizes indicated in kDa. The arrow indicates GST.

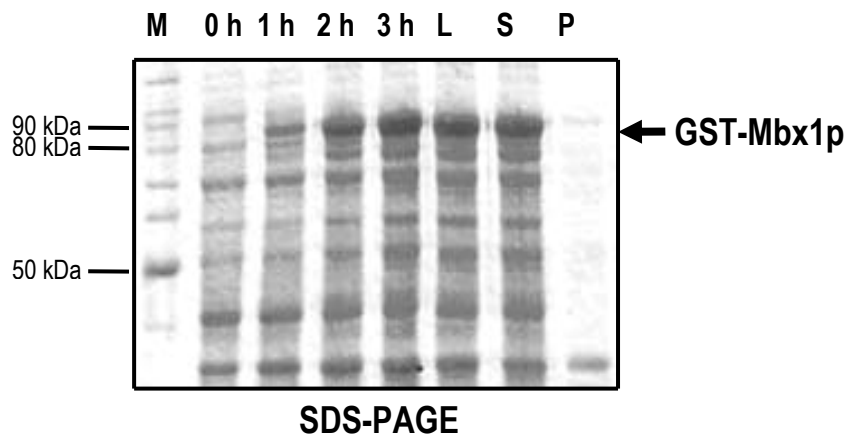


Figure 4.13: GST-Mbx1p overexpression and solubility. GST-Mbx1p overexpression was induced in BL21 CodonPlus (DE3)-RIL cells with 0.5 mM IPTG at 30°C for 3 h. Samples were collected at the point of induction (0 h) and at hourly intervals thereafter (1 h, 2 h and 3 h). Following 3 h induction, cells were harvested by centrifugation, resuspended in PBS and lysed by French pressure disruption. The lysate (L) was then centrifuged to separate the soluble and insoluble protein material into the supernatant (S) and pellet fraction (P), respectively. 1 ml samples were collected at all stages. All samples were resuspended in Laemmli sample buffer, denatured by boiling for 5 min in the presence of DTT and analysed by SDS-PAGE and Coomassie blue staining. Molecular weight marker is shown (M), with sizes indicated in kDa. The arrow indicates full-length GST-Mbx1p.

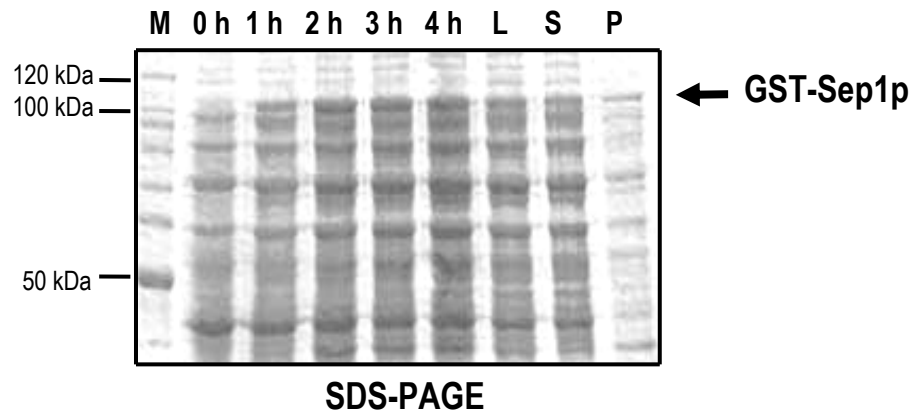


Figure 4.14: GST-Sep1p overexpression and solubility. GST-Sep1p overexpression was induced in BL21 CodonPlus (DE3)-RIL cells with 0.5 mM IPTG at 30°C for 4 h. Samples were collected at the point of induction (0 h) and every hour thereafter. Following 4 h induction, cells were harvested by centrifugation, resuspended in PBS and lysed by French pressure disruption. The lysate (L) was then centrifuged to separate the supernatant (S) and pellet fraction (P). 1 ml samples were collected at all stages. All samples were resuspended in Laemmli sample buffer, denatured by boiling for 5 min in the presence of DTT and analysed by SDS-PAGE and Coomassie blue staining. Molecular weight marker is shown (M), with sizes indicated in kDa. The arrow indicates full-length GST-Sep1p.

As shown in Figures 4.12, 4.13 and 4.14, overexpression of GST, GST-Mbx1p and GST-Sep1p was successful. As expected, GST resolved at ~26 kDa and was present almost exclusively in the supernatant (Figure 4.12). GST-Mbx1p resolved at a size slightly higher than the predicted value of ~77 kDa, while GST-Sep1p resolved at a size close to the predicted value of ~100 kDa. Approximately 100% of GST-Mbx1p was recovered in the soluble fraction (Figure 4.13), whereas in the case of GST-Sep1p a significant proportion of the fusion protein (>70%) was also detected in the supernatant (Figure 4.14).

4.3.5 Bacterial overexpression of HIS-fusion proteins

Overexpression and solubility of 6HIS-Fkh2p and 6HIS-Sep1p was examined by a procedure similar to that followed for overexpression of GST-Mbx1p and GST-Sep1p, but with modifications. In particular, a single colony of BL21 CodonPlus cells transformed with either pET-28-a (+) vector containing *sep1*⁺ (GB 425) or pET-28-c (+) containing *fkh2*⁺ (GB 417) were grown overnight on solid medium before transfer to 10 ml of kanamycin supplemented NZY⁺ broth, which was then grown at 37°C for no more than 16 h. 1 ml of culture was removed, added to 50 ml of fresh NZY⁺ plus kanamycin and allowed to grow until an OD₆₀₀ of ~0.5 was reached. IPTG was added to a final concentration of 0.5 mM and the culture was grown at 30°C for 3 h to induce overexpression of 6HIS-Fkh2p or 6HIS-Sep1p. 1 ml samples were removed at the point of induction (0 h) and every

hour onwards for 3 h. Following overexpression, cells were collected by centrifugation, resuspended in Buffer A and lysed by French pressure. 1 ml of lysate (L) was collected and kept on ice, while 1 ml of lysate was centrifuged to separate the supernatant (S) and pellet fraction (P). From each sample, 10 μ l were taken and resuspended in an equal volume of 2x Laemmli sample buffer, denatured by boiling for 5 min in the presence of 150 mM DTT and analysed by SDS-PAGE electrophoresis and Coomassie blue staining to examine the overexpression and solubility of 6HIS-Fkh2p and 6HIS-Sep1p (Figures 4.15 and 4.16).

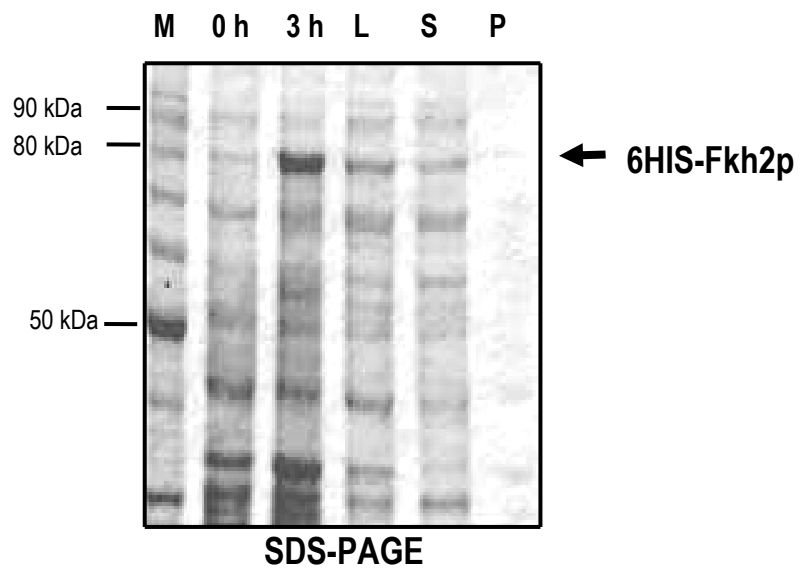


Figure 4.15: 6HIS-Fkh2p overexpression and solubility. 6HIS-Fkh2p overexpression was induced in BL21 CodonPlus (DE3)-RIL cells with 0.5 mM IPTG at 30°C for 3 h. Samples were collected at the point of induction (0 h) and 3 h after induction (3 h). Cells were harvested by centrifugation, resuspended in Buffer A and lysed by French pressure disruption. The lysate (L) was centrifuged to separate the supernatant (S) and pellet fraction (P). 1 ml samples were collected at all stages. All samples were resuspended in Laemmli sample buffer, denatured by boiling in the presence of DTT and analysed by SDS-PAGE and Coomassie blue staining. Molecular weight marker is shown (M), with sizes indicated in kDa. The arrow indicates full-length 6HIS-Fkh2p.

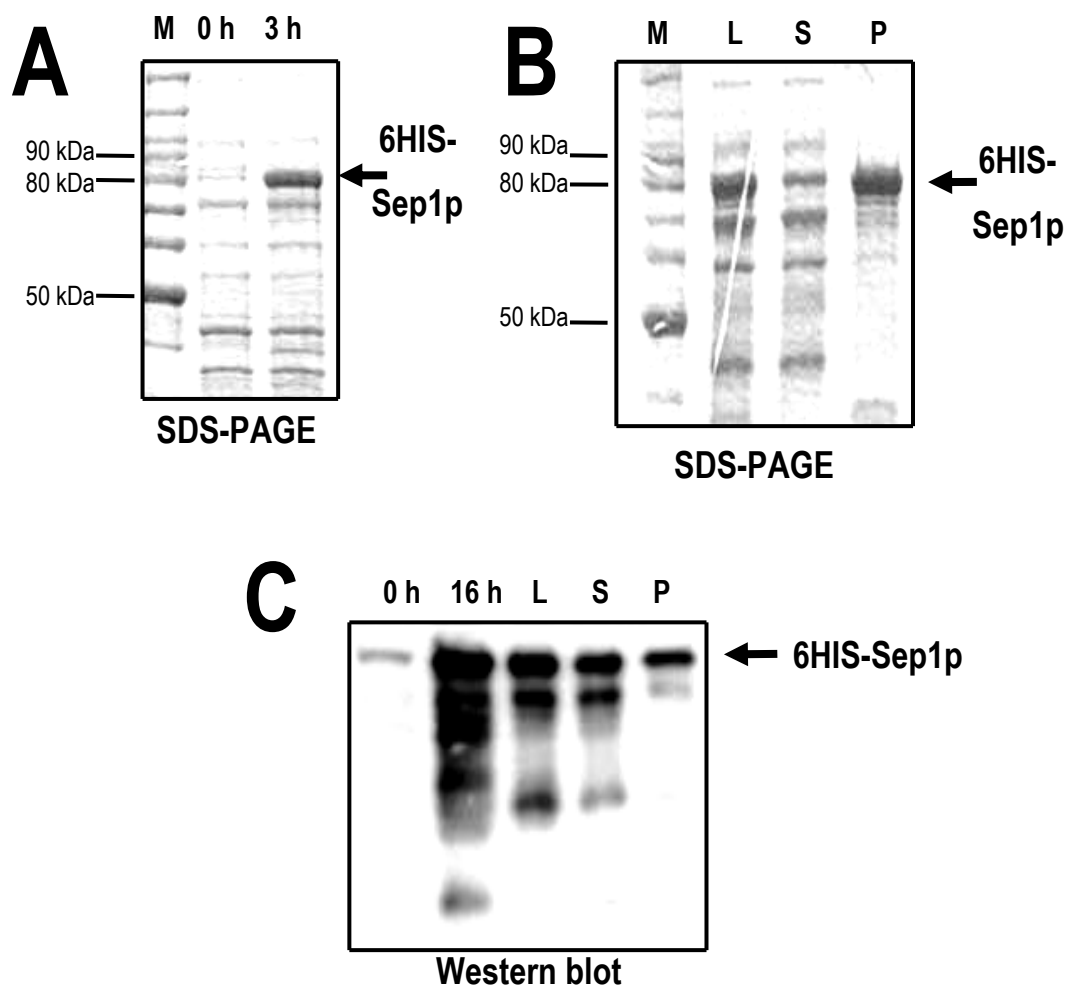


Figure 4.16: 6HIS-Sep1p overexpression and solubility. (A-B) 6HIS-Sep1p overexpression was induced in BL21 CodonPlus (DE3)-RIL cells with 0.5 mM IPTG at 30°C for 3 h. Samples were collected at the point of induction (0 h) and 3 h after induction (3 h). Cells were harvested by centrifugation, resuspended in Buffer A and lysed by French pressure. The lysate (L) was centrifuged to separate the supernatant (S) and pellet fraction (P). 1 ml samples were collected at all stages. All samples were resuspended in Laemmli sample buffer, denatured by boiling in the presence of DTT and analysed by SDS-PAGE and Coomassie blue staining. (C) 6HIS-Sep1p was overexpressed for 16 h at 15°C with 0.15 mM IPTG. Prior to IPTG induction, cells were heat-shocked for 20 min at 42°C. Samples were collected at the point of induction (0 h) and 16 h after induction (16 h). Following induction, cells were harvested by centrifugation, resuspended in Buffer A and lysed by French pressure. The lysate was centrifuged to separate the supernatant (S) and pellet fraction (P). 1 ml samples were collected at all stages. All samples were resuspended in Laemmli sample buffer, denatured by boiling in the presence of DTT and analysed by SDS-PAGE, followed by Western blotting. To detect 6HIS-Sep1p, the blot was probed with anti-HIS antibody. (A-B) Molecular weight marker is shown (M), with sizes indicated in kDa. (A-D) The arrow indicates full-length 6HIS-Sep1p.

As shown in Figures 4.15 and 4.16, overexpression of 6HIS-Fkh2p and 6HIS-Sep1p was successful with both HIS-tagged proteins resolving at sizes close to their predicted molecular masses, ~72 kDa for 6HIS-Fkh2p and ~73 for 6HIS-Sep1p. Almost 100% of 6HIS-Fkh2p was detected in the soluble fraction (Figure 4.15), whereas in contrast more than 70% of 6HIS-Sep1p was present in the pellet, with only 25-30% of protein recovered in the soluble fraction (Figure 4.16A and B). Therefore, in order to improve 6HIS-Sep1p solubility, alternative overexpression conditions were adopted, including heat-shock at 42°C for 20 min prior to IPTG induction. Heat shock has been shown to improve solubility of heterologous proteins expressed in bacteria due to the synthesis of bacterial heat shock proteins, known to facilitate proper folding of proteins and prevent their aggregation (Chen *et al.*, 2003; Oganessian *et al.*, 2007). After heat-shock of cells, 6HIS-Sep1p expression was induced with 0.15 mM IPTG for sixteen hours (16 h) at 15°C (Figure 4.16C), since high level 6HIS-Sep1p overexpression, observed upon induction with 0.5 mM IPTG at 30°C over a short three hour period (Figure 4.16A and B), might itself have contributed to protein insolubility. Following 16 h IPTG induction at 15°C, cells were collected, resuspended in Buffer A and lysed by French pressure disruption. The lysate was centrifuged to separate the supernatant (S) and pellet fraction (P). Samples collected at all stages during IPTG induction and lysis were analysed by SDS-PAGE electrophoresis and Western blot with an anti-HIS antibody (Table 2.1) to detect 6HIS-Sep1p (Figure 4.16C). As revealed by the Western blot, heat-shock combined with IPTG induction at a low temperature significantly improved 6HIS-Sep1p solubility, since more than 60% of the protein was detected under these conditions in the supernatant (soluble fraction).

4.3.6 Purification of GST-fusion proteins

Once conditions for protein induction of GST-tagged proteins were optimised (Section 4.3.4), large amounts of GST-Mbx1p, GST-Sep1p and GST were overexpressed from 200 ml (GST-Mbx1p and GST) or 500 ml (GST-Sep1p) cultures, and purified in preparation for the GST pull-down assay. Overexpression and column purification of all GST-tagged proteins using Glutathione Sepharose 4B slurry was performed as described in detail in Sections 2.2.11.2-2.2.11.3. For each protein several 0.4 ml (GST-Mbx1p and GST) or 1 ml (GST-Sep1p) elution fractions were collected. 10 µl of each elution fraction were removed and analysed by SDS-PAGE, followed by Coomassie blue staining (Figure 4.17). All elution fractions containing in each case the purified protein (GST, GST-Mbx1p or GST-Sep1p) were combined prior to overnight dialysis in 1x PBS (Section 2.2.11.7) and subsequent concentration (Section 2.2.11.8).

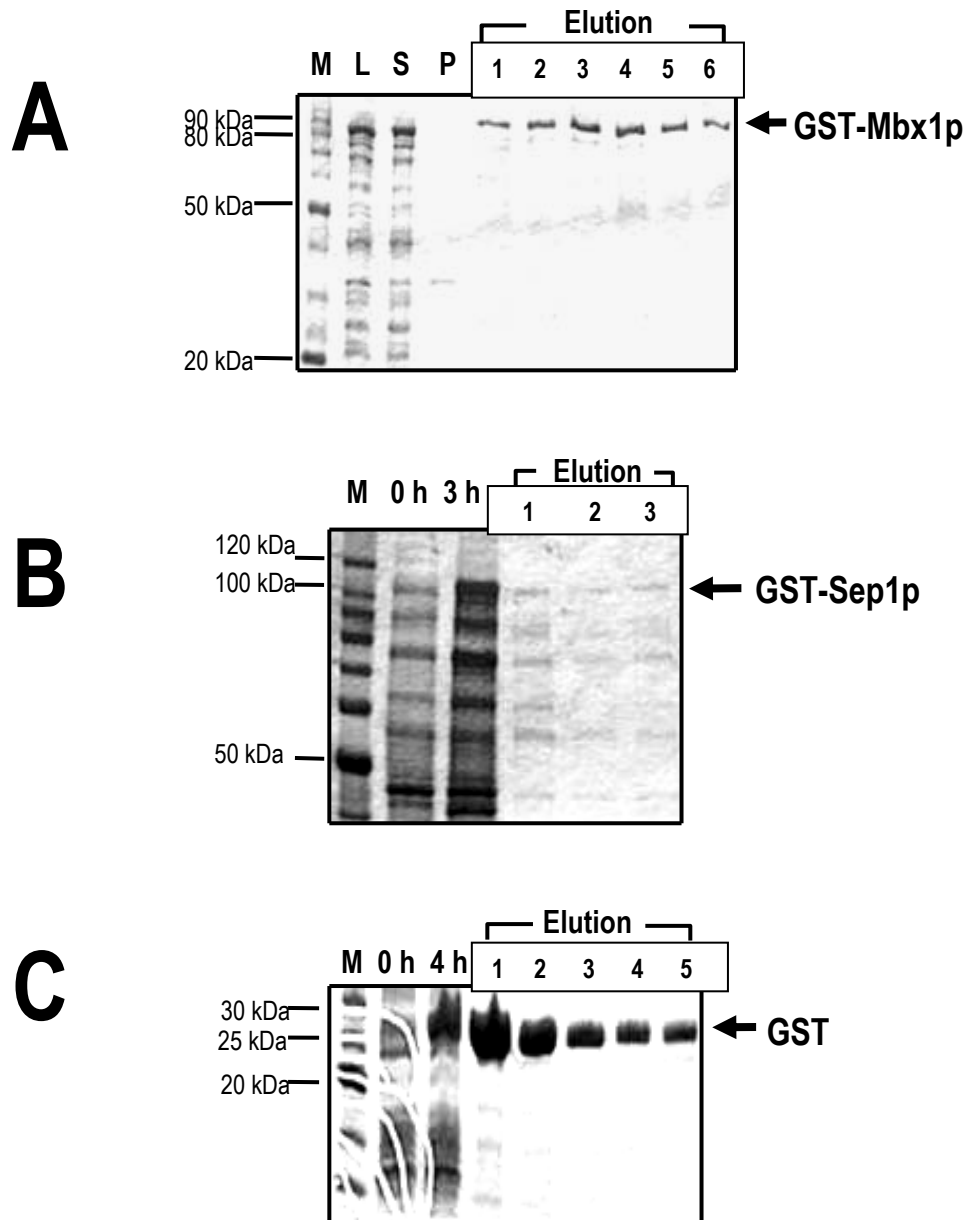


Figure 4.17: Purification of GST-fusion proteins. (A-C) GST-Mbx1p, GST-Sep1p or GST were separately overexpressed in BL21 CodonPlus (DE3)-RIL cells (200-500 ml) for 3-4 h at 30°C with 0.5 mM IPTG. Following induction, cells were harvested, resuspended in PBS and lysed by French pressure disruption. The lysate was centrifuged and the supernatant (soluble protein fraction) was collected and subjected to GST column purification. In each case, various elution fractions were collected and 10 μ l samples were taken (Elution lanes). All samples were diluted in an equal volume of Laemmli sample buffer, denatured by boiling in the presence of DTT and analysed by SDS-PAGE and Coomassie blue staining. (A) The lysate (L), supernatant (S) and pellet (P) samples are also shown. (B-C) Samples taken at the time of induction (0 h) and following induction (3 h) or (4 h) are also shown. Lane M, Molecular weight marker with sizes indicated in kDa. The arrow indicates GST-Mbx1p (A), GST-Sep1p (B) or GST (C).

4.3.7 Purification of HIS-fusion proteins

The most favourable conditions were used to overexpress large quantities of 6HIS-Fkh2p and 6HIS-Sep1p (Section 4.3.5). Following overexpression in 500 ml cultures, 6HIS-Fkh2p and 6HIS-Sep1p were purified for use in the GST pull-down. Overexpression and column purification of HIS-tagged proteins using Chelating Fast Flow slurry was performed as described in detail in Sections 2.2.11.2 and 2.2.11.4. For each protein several 1ml elution fractions were collected. 10 μ l of each elution fraction were removed and analysed by SDS-PAGE, followed by Coomassie blue staining. As shown in Figure 4.18, 6HIS-Sep1p and 6HIS-Fkh2p were successfully purified. As in the case of GST-tagged proteins, elution fractions containing either 6HIS-Fkh2p or 6HIS-Sep1p were combined prior to overnight dialysis in 1x PBS (Section 2.2.11.7) and subsequent concentration (Section 2.2.11.8).

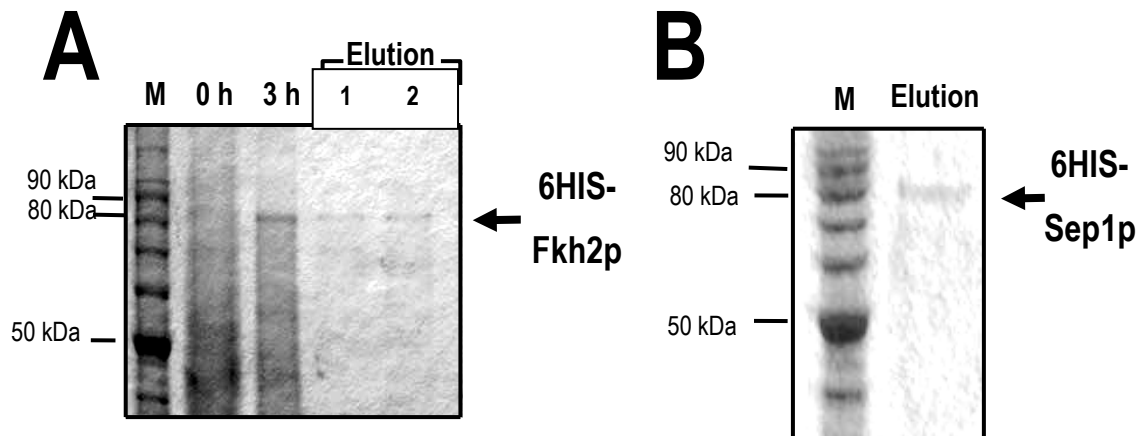


Figure 4.18: Purification of HIS-fusion proteins. (A-B) 6HIS-Fkh2p was overexpressed in BL21 CodonPlus (DE3)-RIL cells for 3 h at 30°C with 0.5 mM IPTG, while 6HIS-Sep1p was overexpressed for 16 h at 15°C with 0.15 mM IPTG. In the case of 6HIS-Sep1p, cells were also heat-shocked before IPTG induction. Samples were collected at the point of induction (0 h) and after induction, (3 h) or (16 h). Cells were then harvested, resuspended in Buffer A and lysed by French pressure disruption. The lysate was centrifuged and the supernatant was collected and subjected to HIS column purification. Elution fractions were collected and 10 μ l samples were taken (Elution lanes). Samples were diluted in an equal volume of Laemmli sample buffer, denatured by boiling in the presence of DTT and analysed by SDS-PAGE, followed by staining with Coomassie brilliant blue. (A) Samples taken at the time of induction (0 h) and following induction (3 h) are also shown. Lane M, Molecular weight marker with sizes indicated in kDa. The arrow indicates full-length 6HIS-Fkh2p (A) or 6HIS-Sep1p (B).

4.3.8 GST pull down unravels a direct *in vitro* interaction between Sep1p and Fkh2p

Following purification, dialysis and concentration of GST- and HIS- tagged proteins, GST pull-down assays were performed to examine whether GST-Sep1p interacts with 6HIS-Fkh2p, as well as whether GST-Mbx1p interacts with 6HIS-Fkh2p or 6HIS-Sep1p. In each case, the GST pull-down was carried out as described in Section 2.2.11.9. Briefly, 3-4 µg of GST-tagged protein (GST-Sep1p or GST-Mbx1p) or GST alone, used as a negative control, were incubated with Glutathione Sepharose beads for one hour to allow binding. In each case, 3-4 µg of the appropriate HIS-tagged protein (6HIS-Fkh2p or 6HIS-Sep1p) were added to the beads suspension and incubated for another hour to enable interaction between the two proteins. As another negative control 6HIS-Fkh2p or 6HIS-Sep1p were incubated for one hour with Glutathione Sepharose beads. After removal of the supernatant and sufficient washes the beads with all bound protein(s) were resuspended in 2x Laemmli buffer supplemented with 150 mM DTT and boiled to release the protein(s) from the beads. Following centrifugation, the supernatant containing all the protein was TCA precipitated. Finally, the pelleted protein material was resuspended in 10 µl Laemmli buffer with 150 mM DTT, boiled and analysed by SDS-PAGE electrophoresis, followed by Western blot using a HIS antibody to detect whether 6HIS-Fkh2p is pulled down by GST-Sep1p or GST-Mbx1p, as well as whether 6HIS-Sep1p is pulled down by GST-Mbx1p. In each case, all samples were also analysed by a Western blot using a GST antibody to confirm the presence of the respective GST protein, GST-Sep1p, GST-Mbx1p or GST itself, in the pull-down fraction (data not shown).

As shown in Figure 4.19A, 6His-Fkh2p was detected in the GST-Sep1p pull-down fraction, whereas no 6His-Fkh2p was found in the GST pull-down fraction or in the Glutathione Sepharose beads fraction, showing that the interaction between GST-Sep1p and 6His-Fkh2p is specific and not the indirect result of 6His-Fkh2p non-specific binding to either the GST moiety or Glutathione Sepharose beads. Thus, the pull-down experiment not only confirmed the physical interaction between Sep1p-3HA and Fkh2p-13myc, observed *in vivo* by co-immunoprecipitation, but also revealed that Sep1p and Fkh2p can bind to each other directly, at least *in vitro* (Papadopoulou *et al.*, 2008). In contrast, neither 6His-Fkh2p nor 6HIS-Sep1p was detected in the GST-Mbx1p pull-down fraction (Figure 4.19B and C). This result is in agreement with the findings of co-immunoprecipitation experiments (Sections 4.2.3 and 4.2.4), showing that at least under these conditions no physical interaction was observed between Mb1p-13myc and either Sep1p-3HA or Fkh2p-3HA. Taken together, the combination of co-immunoprecipitation and GST pull-down experiments have demonstrated a direct interaction between Sep1p and Fkh2p, whilst no interaction was observed between the MADS box-like protein Mb1p and either of the two forkhead transcription factors.

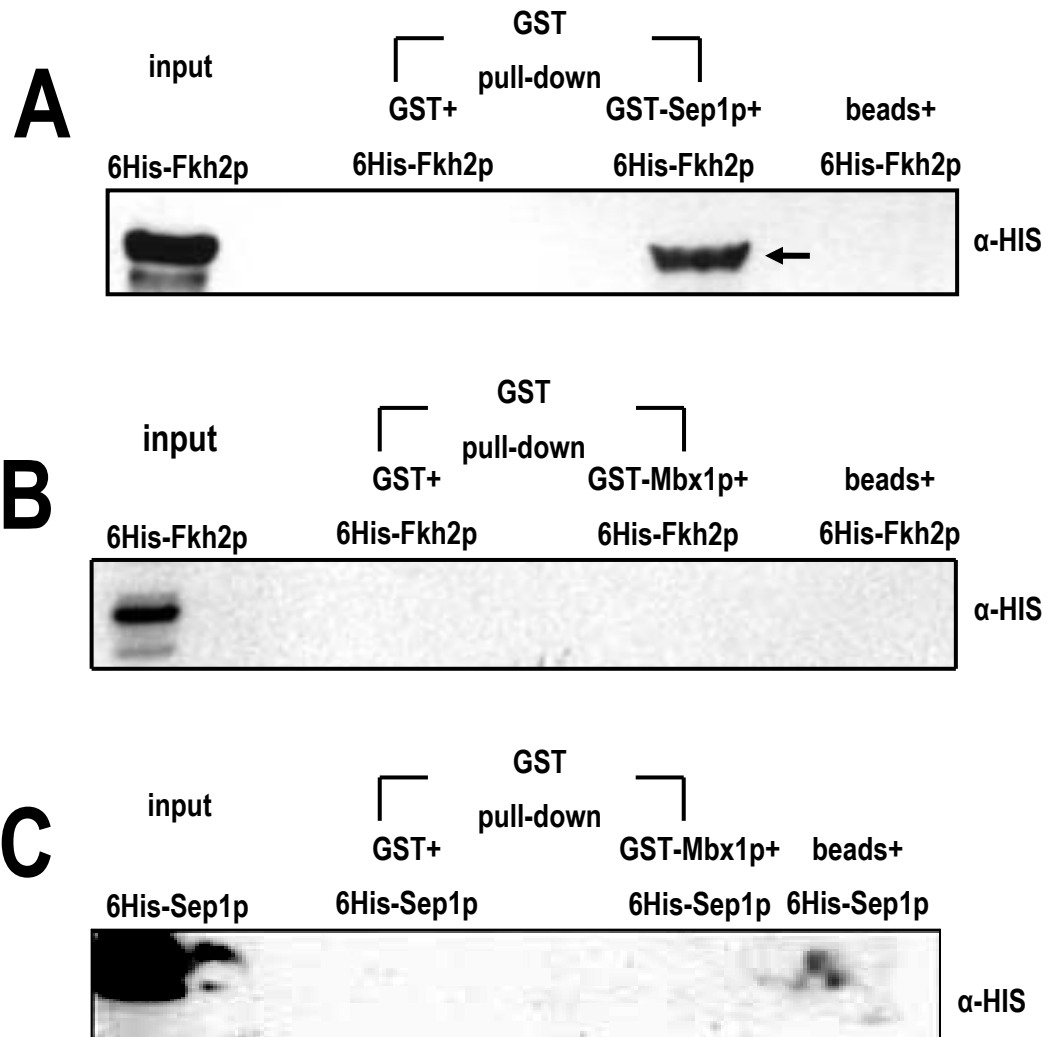


Figure 4.19: GST pull-down analysis reveals that Fkh2p and Sep1p directly interact with each other *in vitro*, while no interaction is detected between Mbx1p and either Fkh2p or Sep1p. (A) 6His-Fkh2p is pulled down by GST-Sep1p *in vitro*. Bacterially expressed and purified 6HIS-Fkh2p was mixed with purified GST-Sep1p bound to Glutathione Sepharose 4B beads. Following GST pull-down analysis, the GST-Sep1p pull down fraction was resuspended in Laemmli sample buffer supplemented with DTT and analysed by SDS-PAGE, followed by Western blotting. To detect 6HIS-Fkh2p the blot was probed with an antibody against HIS. As negative controls, 6His-Fkh2p was mixed with purified GST bound to beads, as well as with beads alone. Input indicates purified 6His-Fkh2p. The arrow indicates 6His-Fkh2p present in the GST-Sep1p pull down fraction. (B) GST pull-down analysis using purified 6His-Fkh2p and GST-Mbx1p. As in (A), appropriate negative controls were included and Western blot with an antibody against HIS was performed to examine whether 6HIS-Fkh2p is present in the GST-Mbx1p pull-down fraction. (C) GST pull-down analysis using purified 6His-Sep1p and GST-Mbx1p. As negative controls, 6His-Sep1p was mixed with purified GST bound to beads, as well as with beads alone. Input indicates purified 6His-Sep1p. To detect 6HIS-Sep1p the blot was probed with an antibody against HIS. (A-C) GST pull-down assays of 6HIS-Fkh2p with either GST-Sep1p or GST-Mbx1p and of 6His-Sep1p with GST-Mbx1p were carried out three times. In each case, the results of Western blot analysis of pulled-down material with the HIS antibody in one of these assays are shown here and are representative of the results of Western blot analysis of all three assays.

4.4 Summary

In fission yeast Fkh2p, Sep1p and Mbx1p are all involved in the co-ordinate expression of several genes during late mitosis and early G1 phase, are components and/or related to the PBF complex and, as suggested by genetic analyses, they exhibit both overlapping and separate functions (Buck *et al.*, 2004; Bulmer *et al.*, 2004; Bähler, 2005; Ng *et al.*, 2006). To examine further the relationship between these transcription factors, co-immunoprecipitation analysis was performed here to determine whether they associate with each other *in vivo*. Due to the unavailability of antibodies against native Fkh2p, Sep1p and Mbx1p (Section 4.2), immunoprecipitations were carried out with appropriate double-tagged *S. pombe* strains (*sep1-3HA fkh2-13myc*, *sep1-3HA mbx1-13myc* or *fkh2-3HA mbx1-13myc*), expressing one protein as an HA fusion and the other as a myc fusion, tagged in each case at the C-terminus (Figures 4.1, 4.2 and 4.3). Immunoprecipitation and ensuing Western blot analyses with HA and myc antibodies detected a specific *in vivo* interaction between Sep1p-3HA and Fkh2p-13myc, but not between Mbx1p-13myc and either Sep1p-3HA or Fkh2p-3HA (Figures 4.4, 4.5 and 4.6; Papadopoulou *et al.*, 2008).

To confirm the observed interaction between Fkh2p and Sep1p and determine whether these forkhead transcription factors can bind to each other directly, GST pull-down assays were performed with bacterially expressed and purified GST and HIS-fusions of Sep1p and Fkh2p respectively, tagged in each case at their N-terminus. Moreover, GST pull-down assays were carried out with an N-terminal GST-fusion of Mbx1p and HIS-fusions of Sep1p and Fkh2p to examine whether Mbx1p, although not shown to associate *in vivo* with Fkh2p or Sep1p, binds to either of these proteins under these conditions *in vitro*. For this, cDNA or genomic DNA preparations of *mbx1*⁺, *fkh2*⁺ and *sep1*⁺ were cloned into GST- and/or HIS- specific expression vectors as appropriate, followed by bacterial overexpression and purification of the respective fusion proteins (Sections 4.3.1-4.3.7). In agreement with the results of the co-immunoprecipitation assays, GST pull-down analyses showed that GST-Sep1p and 6HIS-Fkh2p bind to each other directly *in vitro*, while no direct interaction was detected, at least under these experimental conditions, between GST-Mbx1p and either 6HIS-Fkh2p or 6HIS-Sep1p (Figure 4.19; Papadopoulou *et al.*, 2008).

The detected *in vivo* and *in vitro* interaction between Fkh2p and Sep1p validates previous genetic observations that have established a requirement of Fkh2p for Sep1p function and suggested a potential interaction between the two forkhead proteins. Moreover, the inability to identify here an interaction between Mbx1p and Fkh2p or Sep1p, might imply that this MADS-box protein exerts its role on transcriptional regulation of M-G1 genes, not by direct association with the

forkhead proteins but indirectly as a PBF component, bound to the PCB sequence within the genes' promoters. Consistent with this, Mbx1p has been shown to be necessary for binding of PBF to the PCB sequence, at least in the case of *cdc15⁺*, and most probably binds close to Fkh2p and/or Sep1p on the *cdc15⁺* promoter (Anderson *et al.*, 2002; Buck *et al.*, 2004). Intriguingly, MADS-box motifs are absent from the promoters of M-G1 transcribed genes (Rustici *et al.*, 2004). Presumably, unlike the situation in *S. cerevisiae*, whereby MADS-box and forkhead sequences mediate G2-M specific expression of the 'CLB2' gene cluster, in fission yeast PCB sequences in concert with forkhead sequences accommodate M-G1 specific expression of the PBF-dependent gene cluster (Bähler, 2005).

Apart from Fkh2p, Sep1p and Mbx1p, the Plo1p kinase is also involved in control of PBF-mediated M-G1 specific transcription. The next chapter examines the relationship between Plo1p and these three transcription factors to determine whether this kinase exerts its role on M-G1-specific gene expression via its interaction with PBF components.

Chapter 5

*In vivo analysis of interactions between Plo1p
kinase and PBF components Fkh2p, Sep1p
and Mbx1p*

5.1 Introduction

Previous studies and research work presented in Chapter 3 have demonstrated that PBF components Mbx1p, Sep1p and Fkh2p regulate M-G1 specific transcription, through the PCB and forkhead promoter sequences (Anderson *et al.*, 2002; Buck *et al.*, 2004; Bulmer *et al.*, 2004; Rustici *et al.*, 2004; Alonso-Nuñez *et al.*, 2005; Petit *et al.*, 2005). *fkh2⁺* is itself periodically transcribed during the M-G1 phase, indicating that it modulates its own expression as part of an autoregulatory loop (Buck *et al.*, 2004, Ng *et al.*, 2006). Moreover, experiments presented in Chapter 4 have shown that Fkh2p associates with Sep1p *in vivo* and *in vitro*, providing further information about the mechanism that regulates cell cycle specific transcription at the M-G1 boundary. Consistent with the M-G1 pattern of late cell cycle expression, most M-G1 transcribed genes encode proteins that are necessary for mitosis and cytokinesis (Ng *et al.*, 2006). Prominent among them is *plp1⁺*, encoding a Ser/Thr Polo-like kinase (Sections 1.3.2.3 and 1.3.2.4.2).

5.1.1 Polo-like kinases

Polo-like kinases (Plks) are found in organisms as diverse as yeast and humans. Well-known members include several human Plks, *Drosophila* Polo, *Xenopus* Plx1p, *S. cerevisiae* Cdc5p and *S. pombe* Plo1p kinase. They all share a common structure with an N-terminal catalytic domain and a C-terminal regulatory domain containing characteristic motifs; the so-called polo-boxes. The Plks regulate a plethora of events during mitosis, including bipolar spindle assembly, chromosome segregation and in some organisms cytokinesis (Sections 1.3.2.3 and 1.3.2.4.2). In humans, Plks are also crucial for centrosome separation and maturation (Barr *et al.*, 2004). Moreover, Plks from various species have been shown to interact with and regulate the activity of the anaphase promoting complex/cyclosome, contributing to degradation of B-type cyclins and mitotic exit (Descombes and Nigg, 1998; Kotani *et al.*, 1998; Shirayama *et al.*, 1998; May *et al.*, 2002).

Plks are also key players in early mitotic events. They are thought to phosphorylate Cdc25p in a p34^{cdc2}/Cdk1p-dependent manner, thus forming part of an amplification loop that further activates the p34^{cdc2}/Cdk1p-cyclin B complex at the G2-M transition and promotes irreversible commitment to mitosis (Barr *et al.*, 2004; Section 1.3.2.3). Consistent with their roles in early and late mitosis, Plks localise to specific substructures of the mitotic apparatus at distinct times during mitosis (Golsteyn *et al.*, 1995; Lee *et al.*, 1995; Shirayama *et al.*, 1998; Bähler *et al.*, 1998b; Mulvihill *et al.*, 1999; Tanaka *et al.*, 2001). Similarly, as shown by studies in mammals and the budding and fission

yeasts, the pattern of Plk activity appears to correlate well with their functions in space and time in the various organisms (Glover *et al.*, 1998).

Research in *S. cerevisiae* and *S. pombe* has also uncovered a role for Plks in the regulation of cell-cycle specific expression of genes that are required for progression through mitosis and cytokinesis (Anderson *et al.*, 2000; Darieva *et al.*, 2006; Papadopoulou *et al.*, 2008). In *S. cerevisiae*, Cdc5p controls G2-M specific transcription of the 'CLB2' gene cluster by phosphorylating the co-activator protein Ndd1p, promoting its recruitment to the genes' promoters in a cell cycle-specific manner. Ndd1p is recruited to the promoters of the 'CLB2' cluster genes via interaction with the FHA domain of Fkh2p, which is found in the promoters as a complex with Mcm1p throughout the cell cycle. Apart from Cdc5p, the Cdc28p-Clb2p and Cdc28p-Clb5p complexes also promote the association between Ndd1p and Fkh2p, phosphorylating the former Ndd1p and the latter Fkh2p (Darieva *et al.*, 2003; Reynolds *et al.*, 2003; Pic-Taylor *et al.*, 2004; Darieva *et al.*, 2006). Markedly, since *CDC5* is a 'CLB2' cluster gene, Cdc5p seems to regulate its own expression as part of a positive feedback loop.

5.1.2 Plo1p: a regulator of mitotic events and M-G1 transcription

Analyses in *S. pombe* of *plo1* disruptants and *plo1* temperature sensitive mutants have demonstrated that Plo1p function is essential for viability and necessary for various mitotic events and cytokinesis, including bipolar spindle assembly, the arrangement of the acto-myosin medial ring and septum formation. As shown by overexpression studies, Plo1p is both necessary and sufficient to induce septum formation from any point in the cell cycle, without prior commitment to mitosis, suggesting that Plo1p acts as a septum promoting factor (Ohkura *et al.*, 1995; Bähler *et al.*, 1998b).

As well as these functions, Plo1p also regulates M-G1 specific transcription. This is shown by the fact that *plo1*⁺ overexpression leads to an increase in the mRNA levels of M-G1 transcribed genes, such as *cdc15*⁺ and *spo12*⁺, while loss of Plo1p function, in the temperature sensitive *plo1-ts35* kinase mutant, compromises M-G1 specific transcription of these same genes. Notably, loss of Plo1p function also abolishes PCB-mediated binding of the PBF complex. These findings suggest that Plo1p regulates M-G1 specific transcription by controlling the activity of PBF (Anderson *et al.*, 2002). Since Plo1p is a Polo-like kinase it could modulate PBF activity by binding and phosphorylating one or more of its components, Mbx1p, Fkh2p and Sep1p. Interestingly, both Mbx1p and Fkh2p have previously been shown to be phosphorylated specifically during mitosis (Buck *et al.*, 2004; Bulmer *et al.*, 2004).

5.2 Two-Hybrid analysis of interactions between Plo1p and PBF components Mbx1p, Sep1p and Fkh2p

For more than a decade the yeast two-hybrid system has been used extensively as an *in vivo* means to study whether two proteins of interest interact with each other, as well as to identify novel binding partners of a known protein (Fields, 2005). This yeast genetic system, initially developed by Fields and Song (1989), exploits the observation that transcription factors consist of functionally autonomous domains, such as the DNA-binding domain (DBD) that recognises and binds to its cognate promoter sequence, and an activation domain (AD) that activates transcription, via interactions with the RNA polymerase II machinery (Hope and Struhl, 1986). The independency of these domains is exemplified by the ability of the DBDs or ADs of different transcription factors to compensate functionally for one another. For example, the DBD of LexAp, an *E.coli* transcriptional repressor, successfully substitutes for the DBD of Gal4p, an *S. cerevisiae* transcriptional activator, since a chimera resulting from the fusion of the LexAp DBD with the Gal4p AD activates ectopic expression of β -galactosidase in budding yeast, provided that LexAp binding sites are present upstream of the *lacZ* reporter gene (Brent and Ptashne, 1985).

Despite the autonomy of the DBD and AD, transcriptional activation takes place only in the presence of both a DBD and AD. On its own a DNA-binding domain, although able to associate with its cognate DNA, is unable to activate transcription. Similarly, an AD domain without a DBD domain cannot target itself to the promoter region, resulting in no transcription. The inability of the AD to activate transcription in the absence of the DBD and *vice versa* allows screening for an interaction between two proteins by yeast two-hybrid, outlined in Figure 5.1B. In this system, the cDNA of gene X is cloned into the “bait” vector that expresses protein X as a fusion with a DBD (e.g. LexAp or Gal4p), while the cDNA of gene Y is cloned into the “prey” vector that expresses protein Y as a fusion with the AD of Gal4p. An *S. cerevisiae* strain containing a reporter gene (the *HIS3* or *ADE2* auxotrophic marker or *lacZ* colour marker) with multiple upstream DNA-binding sites for the respective DBD, either on a plasmid or integrated into the genome, is then transformed with both the “bait” and “prey” vectors. When proteins X and Y physically interact with each other, the DBD and the AD are brought close enough to reconstitute a functional transcriptional factor that activates transcription and ensuing expression of the reporter gene. Conversely, if proteins X and Y do not associate with each other, no transcriptional activation of the reporter takes place, since the DBD-X fusion protein, although bound to its promoter sequence, lacks a nearby AD, while the AD-Y fusion protein lacks a DBD and is unable to locate itself to the promoter of the reporter gene.

Research has revealed that Fkh2p and Sep1p regulate M-G1 specific transcription, while Mbx1p was shown to be a core component of the PBF complex that binds via the PCB sequence to the promoters of M-G1 transcribed genes (Buck *et al.*, 2004; Bulmer *et al.*, 2004; Chapter 3). As discussed above, the Ser/Thr kinase Plo1p controls M-G1 specific expression in fission yeast and is necessary for binding of the PBF complex to PCBs, at least *in vitro* (Anderson *et al.*, 2002). These findings, combined with the fact that *plo1*⁺ genetically interacts with *fkh2*⁺, *sep1*⁺ and *mbx1*⁺, suggest that Plo1p influences M-G1-specific expression by interacting with and phosphorylating one or more PBF components. Consistent with this, the yeast two-hybrid system was employed here to examine whether Plo1p physically associates with either Mbx1p, Fkh2p or Sep1p and if so, determine which Plo1p domain(s), the kinase and/or the polo-box, mediate the interaction. To do this, *fkh2*⁺, *mbx1*⁺ and *sep1*⁺ were cloned separately into the pACT2 prey vector (Appendix IV), allowing expression of each gene as a fusion protein with the Gal4p activation domain (Section 5.2.1). Cloning of *plo1*⁺, as well as of the *plo1.K69R*, *plo1.DHK625AAA* and *plo1.472-684 plo1* mutants, into the pBTM116 bait vector (Appendix IV), expressing a fusion protein with the LexAp DNA-binding domain, was described previously (May *et al.*, 2002; Reynolds and Ohkura, 2003).

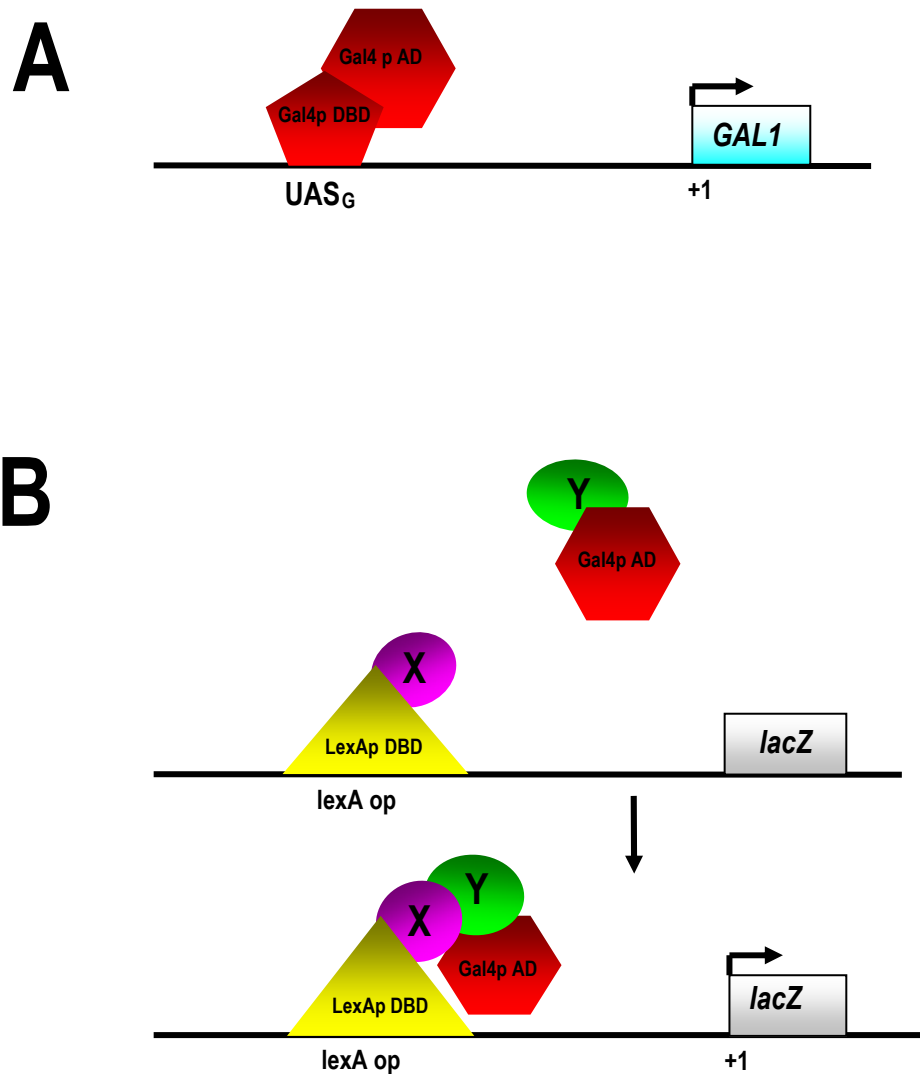


Figure 5.1: Model of yeast two hybrid-mediated transcriptional activation. (A) Gal4p-regulated expression of *GAL1* in *S. cerevisiae*. The budding yeast Gal4p transcription factor binds via its DNA binding domain (DBD) to the upstream activation sequence of the *GAL1* promoter (UAS_G) and activates transcription, mediated by its activation domain (AD). (B) The yeast two-hybrid system. In the yeast two hybrid assay, transcription of a reporter gene, e.g. *lacZ*, takes place in *S. cerevisiae* only if protein X, expressed as a fusion with a DNA-binding domain (here the LexAp DBD) from the “bait” vector, interacts with protein Y, expressed as a fusion with the Gal4p activation domain (Gal4p AD) from the “prey” vector. The association between X and Y proteins brings in close proximity the promoter bound LexAp DNA-binding domain with the Gal4p activation domain, enabling transcriptional activation. LexA op denotes the *lexA* operator recognised by the DBD of LexAp.

5.2.1 Cloning of *sep1*⁺, *fkh2*⁺ and *mbx1*⁺ into the pACT2 prey vector

fkh2⁺, *sep1*⁺ or *mbx1*⁺ full-length cDNA was introduced separately into pACT2 (GB 331), a shuttle *E. coli* and *S. cerevisiae* expression vector that facilitates the expression of the cloned gene as an N-terminal GAL4AD-fusion protein from the constitutive *ADH1* promoter (Appendix IV). Cloning of *sep1*⁺ genomic DNA and *fkh2*⁺ and *mbx1*⁺ cDNA into pCR[®]2.1 vector was described in Section 4.3.1. Standard DNA manipulation methods were employed to insert each gene into pACT2 in the correct orientation and reading frame relative to the upstream GAL4AD domain (Section 2.2.9).

pCR[®]2.1 containing *mbx1*⁺ or *sep1*⁺ was digested with *Sma*I and *Xho*I, whereas pCR[®]2.1 containing *fkh2*⁺ was digested with *Bam*HI and *Xho*I. Each DNA fragment was then gel extracted and purified before cloning into linearised pACT2 vector digested with either *Sma*I and *Xho*I (*mbx1*⁺ or *sep1*⁺) or *Bam*HI and *Xho*I (*fkh2*⁺). Transformation of pACT2 containing *sep1*⁺, *mbx1*⁺ or *fkh2*⁺ into *E. coli* DH5 α cells followed. Once cells were grown overnight on solid LB supplemented with ampicillin, transformants were selected and grown overnight in liquid selective media. Following plasmid purification, successful cloning of *sep1*⁺, *mbx1*⁺ or *fkh2*⁺ into pACT2 was confirmed by a *Sma*I/*Xho*I or a *Bam*HI/*Xho*I restriction digest as appropriate, revealing a ~2 kb, ~1.4 kb or ~2 kb DNA fragment, respectively (Figures 5.2, 5.3 and 5.4). One isolate of pACT2-*mbx1*⁺ (GB 447), pACT2-*sep1*⁺ (GB 442) and pACT2-*fkh2*⁺ (GB 438) was selected and used for subsequent transformation into the budding yeast strain CTY10-5d (Section 5.2.2). The integrity of the pACT2-*sep1*⁺ and pACT2-*fkh2*⁺ constructs was also confirmed by DNA sequencing (MWG Biotech).

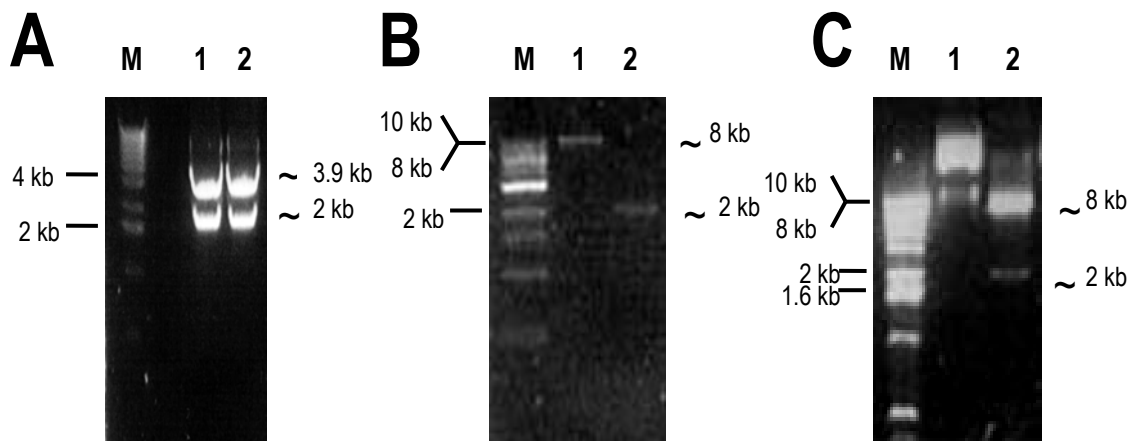


Figure 5.2: Cloning of full-length *sep1*⁺ into pACT2 two-hybrid prey vector. (A) Lanes 1 and 2, *Sma*I/*Xho*I restriction digest of pCR[®]2.1 vector containing full-length *sep1*⁺. Lane M, 1 kb DNA Ladder (NEB). (B) Lane 1, purified fragment of *Sma*I/*Xho*I digested pACT2 vector. Lane 2, purified gel excised DNA fragment of full-length *sep1*⁺, following *Sma*I/*Xho*I restriction digest of pCR[®]2.1 vector containing full-length *sep1*⁺, shown in (A). Lane M, 1 kb DNA Ladder (NEB). (C) Lane 1, undigested circular pACT2 vector. Lane 2, *Sma*I/*Xho*I restriction digest of pACT2 vector containing full-length *sep1*⁺. Lane M, DNA Molecular Weight marker X (Roche). (A-C) Sizes of DNA fragments are indicated.

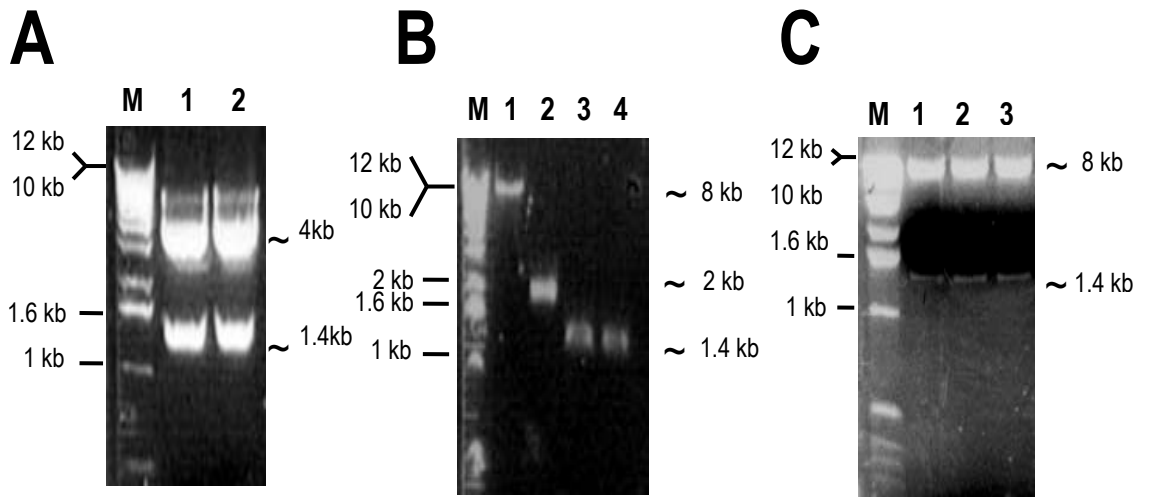


Figure 5.3: Cloning of full-length *mbx1*⁺ into pACT2 two-hybrid prey vector. (A) Lanes 1 and 2, *Sma*I/*Xho*I restriction digest of pCR^{2.1} vector containing full-length *mbx1*⁺. Lane M, DNA Molecular Weight marker X (Roche). (B) Lane 1, purified fragment of *Sma*I/*Xho*I digested pACT2 vector. Lane 2, purified gel excised DNA fragment of full-length *sep1*⁺, following *Sma*I/*Xho*I restriction digest of pCR^{2.1} vector containing full-length *sep1*⁺. Lanes 3 and 4, purified gel excised DNA fragment of full-length *mbx1*⁺, following *Sma*I/*Xho*I restriction digest of pCR^{2.1} vector containing full-length *mbx1*⁺, shown in (A). Lane M, DNA Molecular Weight marker X. (C) Lanes 1, 2 and 3, *Sma*I/*Xho*I restriction digest of pACT2 vector containing full-length *mbx1*⁺. Lane M, DNA Molecular Weight marker X. (A-C) Sizes of DNA fragments are indicated.

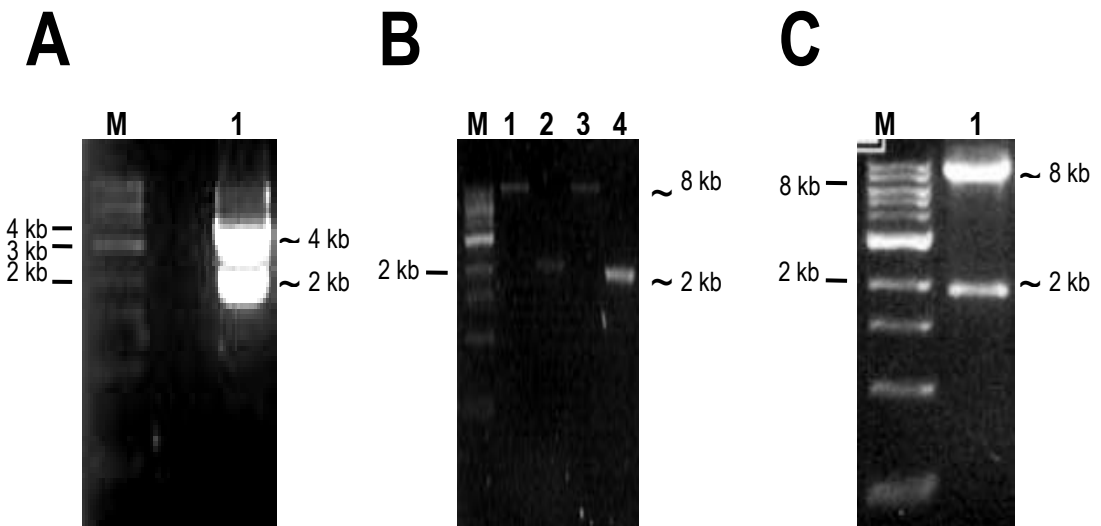


Figure 5.4: Cloning of full-length *fkh2*⁺ into pACT2 two-hybrid prey vector. (A) Lane 1, *Bam*HI/*Xho*I restriction digest of pCR^{2.1} vector containing full-length *fkh2*⁺. Lane M, 1 kb DNA Ladder (NEB). (B) Lane 1, purified fragment of *Sma*I/*Xho*I digested pACT2 vector. Lane 2, purified gel excised DNA fragment of full-length *sep1*⁺, following *Sma*I/*Xho*I restriction digest of pCR^{2.1} vector containing full-length *sep1*⁺. Lane 3, purified fragment of *Bam*HI/*Xho*I digested pACT2 vector. Lane 4, purified gel excised DNA fragment of full-length *fkh2*⁺, following *Bam*HI/*Xho*I restriction digest of pCR^{2.1} vector containing full-length *fkh2*⁺, shown in (A). Lane M, 1 kb DNA Ladder (NEB). (C) Lane 1, *Bam*HI/*Xho*I restriction digest of pACT2 vector containing full-length *fkh2*⁺. Lane M, 1 kb DNA Ladder (NEB). (A-C) Sizes of DNA fragments are indicated.

5.2.2 *Plo1p* interacts with *Mbx1p* but not *Fkh2p* or *Sep1p* in the Two-Hybrid assay

After cloning, each of the pACT2-*sep1*⁺, pACT2-*fkh2*⁺ and pACT2-*mbx1*⁺ constructs was co-transformed with pBTM116-*plo1*⁺ (GB 329) into competent *S. cerevisiae* CTY10-5d cells (GGBY 138), which carry the *lacZ* gene integrated into the genome together with multiple upstream binding sites for the LexAp DBD (Section 2.2.7 and 2.2.8). As negative controls to ensure that Sep1p, Fkh2p, Mbx1p, Plo1p or even the LexAp DBD and Gal4p AD can not alone stimulate *lacZ* transcription, pACT2-*sep1*⁺, pACT2-*fkh2*⁺ and pACT2-*mbx1*⁺ were co-transformed into budding yeast with empty pBTM116 vector (GB 332), pBTM116-*plo1*⁺ was co-transformed with empty pACT2 vector and empty pACT2 vector was co-transformed with empty pBTM116 vector. Moreover, as a positive control to confirm the efficiency of the yeast two-hybrid system, pBTM116-*plo1*⁺ was co-transformed into CTY10-5d cells with pGADGH-*sck1*⁺ (GB 520), which expresses a fusion of the Gal4p AD with Sck1p, a known Plo1p-interacting protein (Reynolds and Ohkura, 2003).

After transformation, cells were grown on solid SD medium lacking tryptophan and leucine for 3-4 days at 30°C. Successful transformants were selected in each case and screened for *lacZ* expression by a semi-quantitative X-gal overlay assay, which detects the activity of β-galactosidase, the encoded product of *lacZ* (Section 2.2.16.1). For this, all the transformants were grown on solid media for 2-3 days before covered with an X-gal solution and incubated at 30°C to allow for blue colour development. Cells were checked for blue colour formation at hourly intervals for a 6 hour period and then were left to incubate overnight. The X-gal overlay assay was performed three times for two transformants of each *S. cerevisiae* strain. The results obtained from the X-gal overlay assay in one of these occasions are displayed in Figure 5.5A. As shown, a strong blue colour, representing a high level of β-galactosidase activity, was observed only in cells expressing *plo1*⁺ and either *sck1*⁺ (positive control) or *mbx1*⁺. In the case of *sck1*⁺, blue colour was observed within 2 hours, while for *mbx1*⁺ after 5-6 hours. In contrast, no blue colour was seen in cells expressing *plo1*⁺ and either *sep1*⁺ or *fkh2*⁺, as well as in cells expressing *plo1*⁺, *mbx1*⁺, *fkh2*⁺ and *sep1*⁺ alone, even after overnight incubation at 30°C. These observations suggest a specific *in vivo* interaction between Plo1p and Mbx1p.

Following these results, a quantitative β-galactosidase assay was also performed to measure the interaction between Plo1p and Mbx1p relative to the positive and all negative controls (Section 2.2.16.2). Protein interactions were quantified in 1 ml samples from *S. cerevisiae* cells grown in liquid medium using the following formula:

$$\beta\text{-galactosidase activity (Miller Units)} = 1000 \times \text{OD}_{420} / (t \times V \times \text{OD}_{600})$$

The assay was performed in triplicate for two transformants of each *S. cerevisiae* strain. The results obtained from statistical analysis, shown in Figure 5.5B, confirm the findings of the X-gal overlay assay and, moreover, reveal that the interaction between Plo1p and Mbx1p is ~40-60% the strength of that between Plo1p and Sck1p.

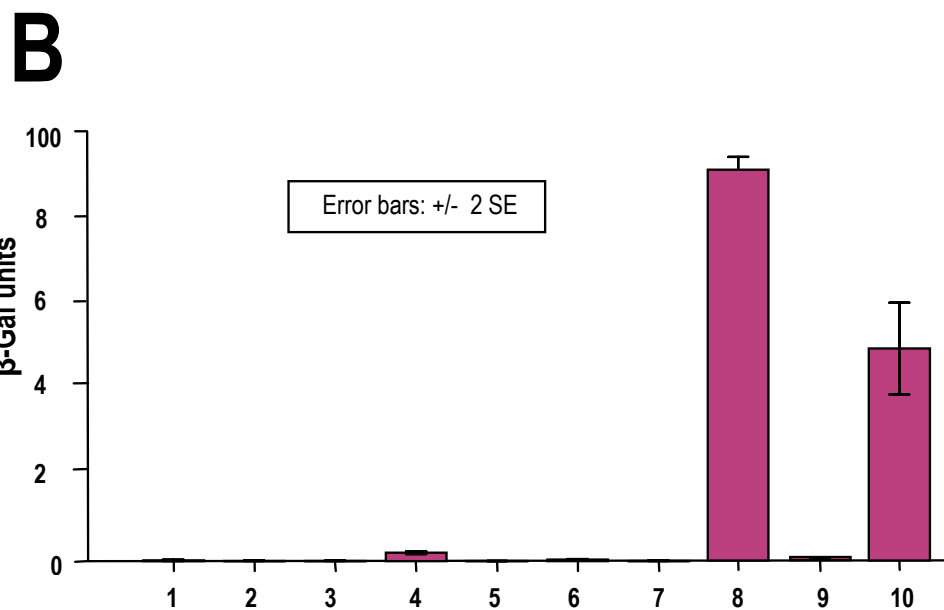
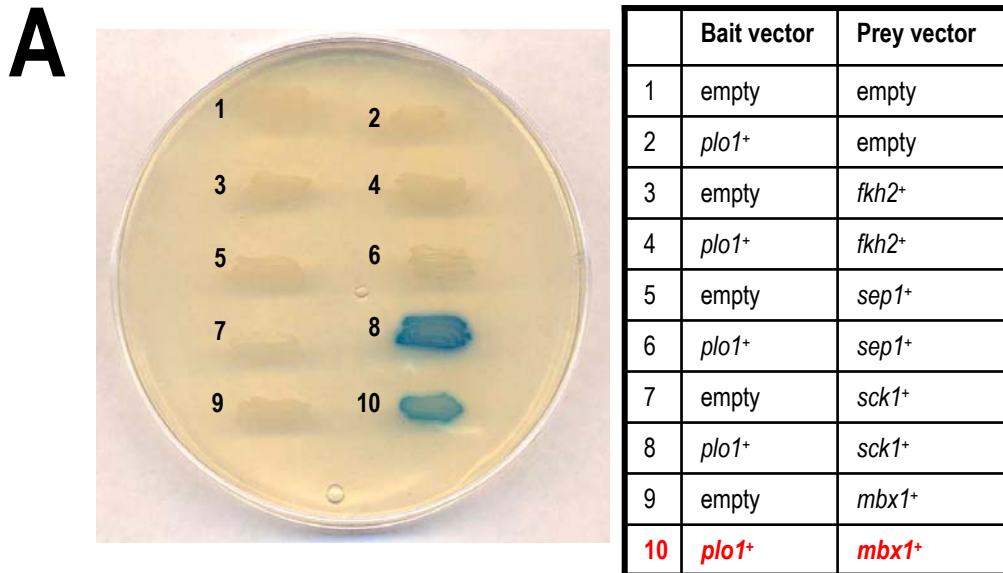


Figure 5.5: Yeast two hybrid analysis reveals a specific *in vivo* interaction between Plo1p and Mbx1p. (A) The budding yeast-two hybrid system was used to examine whether Plo1p interacts with Mbx1p, Fkh2p or Sep1p. *plo1*⁺ fused to the LexAp DNA binding domain of the pBTM116 bait vector (GB 329) was co-transformed with either *mbx1*⁺ (GB 447), *fkh2*⁺ (GB 438) or *sep1*⁺ (GB 442), fused to the Gal4p activation domain of prey vector pACT2, into budding yeast strain CTY105d (GGBY 138) and transcriptional activation was monitored by an X-gal overlay assay. As a positive control, prey vector with *sck1*⁺ (GB 520), encoding a known Plo1p-interacting protein, was co-transformed into budding yeast with bait vector containing *plo1*⁺. In each case as negative control, the prey vector with *mbx1*⁺, *fkh2*⁺, *sep1*⁺ or *sck1*⁺ was co-transformed into budding yeast with empty bait vector, while bait vector with *plo1*⁺ was co-transformed with empty prey vector. The table indicates combinations of bait and prey vectors co-transformed into budding yeast for two-hybrid analysis. The X-gal overlay assay was performed three times for two transformants of each *S. cerevisiae* strain. The

results obtained from the X-gal overlay assay in one of these occasions are displayed here and are representative of the results of all experiments. (B) Quantitative β -galactosidase assay to quantify protein interactions identified with the X-gal overlay assay. In each case colour reactions were quantified in cells grown in liquid medium for two individual transformants in triplicate. Numbers 1-10 correspond to the combinations of bait and prey vectors co-transformed into budding yeast, shown in Table of upper panel. Error bars indicate two standard errors (SE).

5.2.3 The Two-hybrid interaction between Plo1p and Mbx1p requires both the kinase and the polo-box domains

Section 5.2.2 revealed that the Plo1p kinase physically associates with Mbx1p, but not Fkh2p or Sep1p. As described in Section 1.3.2.4.2, Plo1p contains both N-terminal catalytic and C-terminal polo-box domains, the latter a signature polo-kinase region that is necessary and sufficient for various functions, such as subcellular localisation (Lee *et al.*, 1998; Jang *et al.*, 2002; Seong *et al.*, 2002; Reynolds and Ohkura, 2003). Notably, the polo-box domain of human Plk1p has been shown to bind to phospho-serine/-threonine peptides (Elia *et al.*, 2003a; Elia *et al.*, 2003b). In fission yeast, the polo-box domain is also necessary and sufficient for the interaction of Plo1p with several proteins and comprises three polo-box motifs that behave as a single entity (May *et al.*, 2002; Reynolds and Ohkura, 2003). Finally, recent data have demonstrated a dependency of the ability of Plo1p to stimulate M-G1 specific transcription on the kinase domain but not on the polo-box domain (Papadopoulou *et al.*, 2008).

Here, the yeast two-hybrid system was employed to determine whether the interaction of Plo1p with Mbx1p requires the kinase domain, the polo-box domain, or both. To accomplish this, each of the pBTM116-*plo1.K69R* (GB 333), pBTM116-*plo1.472-684* (GB 334) and pBTM116-*plo1.DHK625AAA* (GB 335) constructs was co-transformed with pACT2-*mbx1+* into *S. cerevisiae* CTY10-5d cells. The *plo1.K69R* mutant contains a point mutation in the kinase domain (lysine at position 69 changed to arginine), *plo1.DHK625AAA* contains a point mutation in polo-box 3, whereas pBTM116-*plo1.472-684* corresponds to a C-terminal truncated version of *plo1+* (amino acids 472-684), containing only the polo-box domain (Tanaka *et al.*, 2001; Reynolds and Ohkura, 2003). As negative controls, pBTM116-*plo1.K69R*, pBTM116-*plo1.472-684* and pBTM116-*plo1.DHK625AAA* were co-transformed into budding yeast with empty pACT2 vector.

Successful transformants, capable of growing in the absence of leucine and tryptophan, were selected in each case and screened for *lacZ* expression by the X-gal overlay assay, as well as by the quantitative β -galactosidase assay, together with two *S. cerevisiae* strains, the one containing pBTM116-*plo1+* and pACT2-*mbx1+* and the other pBTM116-*plo1+* and pACT2-*sck1+*, as positive controls (Section 5.2.2). As before, for the X-gal assay all *S. cerevisiae* strains were grown on solid media for 2-3 days before covered with an X-gal solution, followed by incubation at 30°C to allow for

blue colour development (Section 2.2.16.1). In the case of the quantitative assay, colour reactions were quantified in samples from cells grown in liquid medium (Section 2.2.16.2).

The results obtained from the X-gal overlay and quantitative β -galactosidase assays are displayed in Figure 5.6. As shown, mutations either in the Plo1p kinase domain (*plo1.K69R*) or the polo-box domain (*plo1.DHK625AAA*) abolish the interaction with Mbx1p. Consistent with this, in the absence of the kinase domain the polo-box domain alone (*plo1.472-684*) is unable to associate with Mbx1p. Thus, these findings suggest that the interaction between Plo1p and Mbx1p requires both the kinase and the polo-box domain of Plo1p (Papadopoulou *et al.*, 2008).

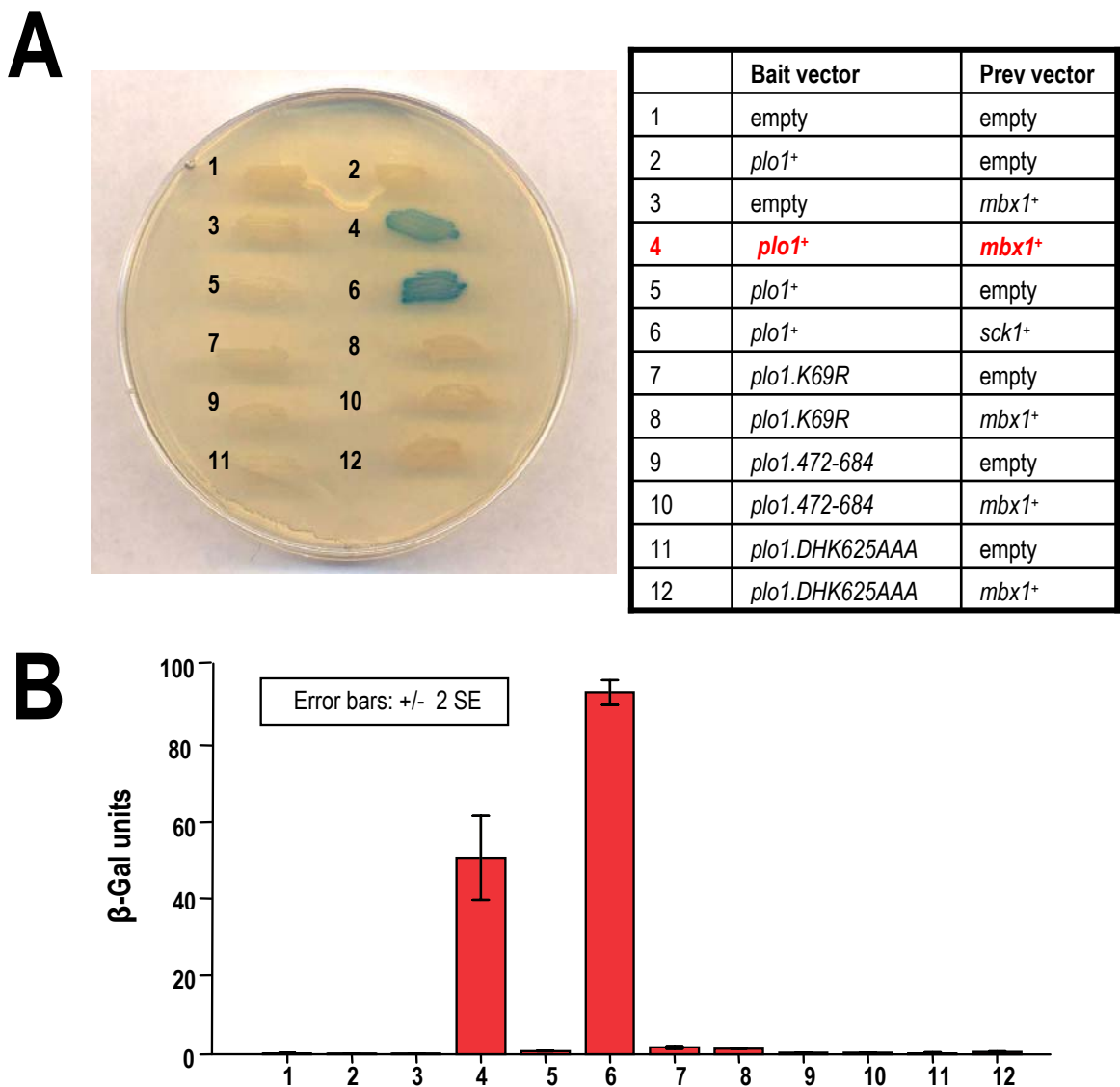


Figure 5.6: Two-hybrid interaction between Plo1p and Mbx1p requires both the kinase and the polo-box domains. (A) The two hybrid system was used to examine the requirement of the Plo1p kinase domain and the polo-box domain for the interaction of Plo1p with Mbx1p. Bait vector containing the *plo1.K69R* kinase mutant (GB 333), the *plo1.DHK625AAA* polo-box mutant (GB 335) or the *plo1.472-684* C-terminal truncation (GB 334) was co-transformed into CTY10-5d cells with prey vector containing *mbx1*⁺. As positive controls, bait vector with *plo1*⁺ was co-transformed into

budding yeast with either *mbx1⁺* or *sck1⁺*. In each case as negative control, bait vector with *plo1.K69R*, *plo1.472-684* or *plo1.DHK625AAA* was co-transformed into budding yeast with empty prey vector, while bait vector with *plo1⁺* was co-transformed with empty prey vector. The table indicates combinations of bait and prey vectors co-transformed into budding yeast cells. . The X-gal overlay assay was performed three times for two transformants of each *S. cerevisiae* strain. The results obtained from the X-gal overlay assay in one of these occasions are displayed here and are representative of the results of all experiments. (B) Quantitative β -galactosidase assay. In each case colour reactions were quantified in cells grown in liquid medium for two individual transformants in triplicate. Numbers 1-12 correspond to the combinations of bait and prey vectors co-transformed into budding yeast, shown in Table of upper panel. Error bars indicate two standard errors (SE).

5.3 Co-immunoprecipitation analysis of the interaction between Plo1p and Mbx1p

To confirm that the two-hybrid interaction between Plo1p and Mbx1p represents the situation in fission yeast, soluble protein extracts from *mbx1-13myc S. pombe* cells, expressing Mbx1p-13myc from its endogenous promoter and thus at normal wild-type levels (Section 4.2.1), were analysed by co-immunoprecipitation assay (Sections 2.2.12.1 and 2.2.12.2). Plo1p was immunoprecipitated from *mbx1-13myc* protein extracts with an antibody against the native protein (Ohkura *et al.*, 1995) before SDS-PAGE electrophoresis and Western blotting with Plo1p and myc antibodies to detect Plo1p and co-immunoprecipitated Mbx1p-13myc. As a negative control, Plo1p was also immunoprecipitated from *mbx1 Δ S. pombe* extracts before Western blotting with Plo1p and myc antibodies.

5.3.1 Mbx1p co-immunoprecipitates with Plo1p from *mbx1-13myc* fission yeast extracts

For immunoprecipitation, pre-cleared *mbx1-13myc* (GG 504) and *mbx1 Δ* (GG 513) protein extracts (2 mg of crude lysate/IP reaction) were incubated with 3-4 μ l of Plo1p (HO) antibody (Table 2.1) for 3 hours at 4°C before addition of protein A-Sepharose beads and further incubation for an hour. Pre-cleared *mbx1-13myc* extract was also incubated for one hour with beads without Plo1p (HO) antibody, as an additional control. After extensive washes, beads were re-suspended and boiled in 2x Laemmli buffer supplemented with DTT, followed by SDS-PAGE and Western blotting with Plo1p and myc antibodies to detect Plo1p and co-immunoprecipitated Mbx1p-13myc.

As shown in Figure 5.7, Mbx1p-13myc (~100 kDa) is present in co-immunoprecipitates with Plo1p (~78kDa) from *mbx1-13myc* but not *mbx1 Δ S. pombe* extracts. Moreover, neither Plo1p nor Mbx1p-13myc is detected in *mbx1-13myc* precipitates that were incubated with beads in the absence of Plo1p (HO) antibody. Thus, Mbx1p-13myc specifically co-immunoprecipitates with Plo1p in *mbx1-13myc* protein extracts. These results confirm the two-hybrid interaction between Plo1p and Mbx1p (Section 5.2.2) and show that these proteins physically associate with each other in *S. pombe* cells.

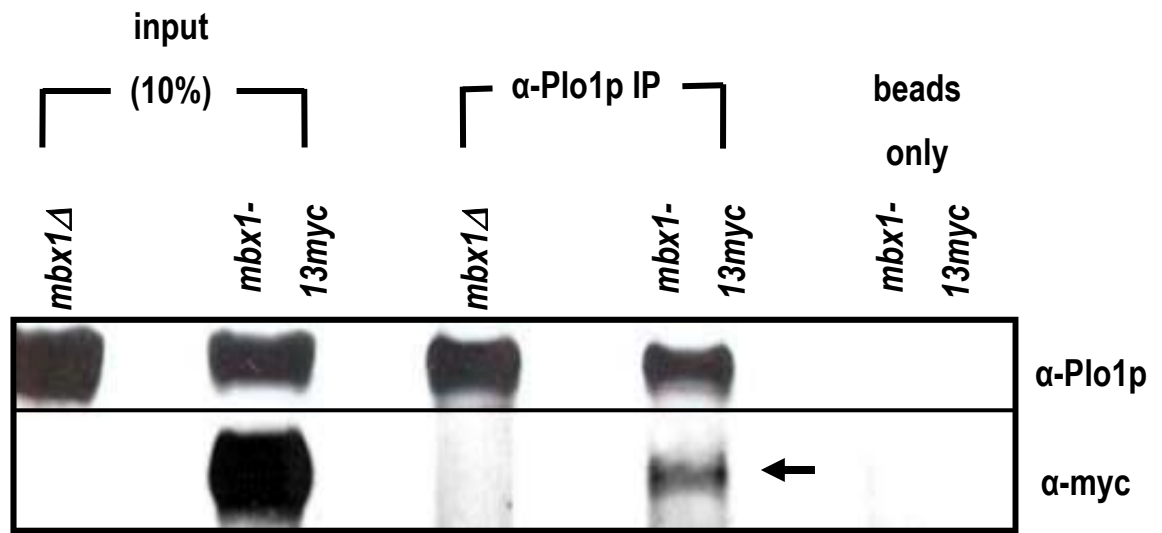


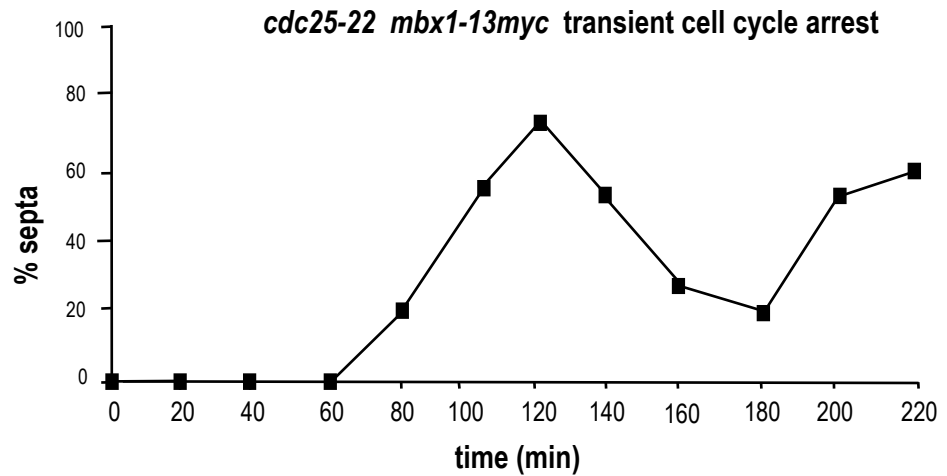
Figure 5.7: Mbx1p co-immunoprecipitates with Plo1p from fission yeast protein extracts. Plo1p was immunoprecipitated with antibody against the native protein (α -Plo1p) from soluble protein extracts of *mbx1-13myc* fission yeast cells (GG 504), expressing Mbx1p-13myc from its endogenous promoter. The soluble extract (input) and the immunoprecipitated sample (IP) were analysed by Western blotting with antibodies against Plo1p and myc (the latter to detect Mbx1p-13myc). As negative controls, immunoprecipitated sample from *mbx1 Δ* (GG 513) fission yeast cells and precipitated sample without antibody (beads only) from *mbx1-13myc* cells were also included. In each case, the input was used in a 1:10 ratio relative to the IP sample. The arrow indicates co-immunoprecipitated Mbx1p-13myc using an α -myc antibody. Immunoprecipitation assays of *mbx1-13myc* and *mbx1 Δ* soluble protein extracts with the Plo1p antibody were carried out three times. In each case, the results of Western blot analysis of immunoprecipitated material with Plo1p and myc antibody in one of the three assays are shown here and are representative of the results of Western blot analysis of all three assays.

5.3.2 Mbx1p co-immunoprecipitates with Plo1p throughout the mitotic cell cycle

Following the finding that Plo1p not only interacts with Mbx1p *in vivo* within *S. pombe* cells but also phosphorylates this MADS-box transcription factor *in vitro* (Papadopoulou *et al.*, 2008), a co-immunoprecipitation assay was performed to determine whether Plo1p regulates M-G1 specific transcription by associating with Mbx1p in a cell cycle dependent manner. For this, soluble protein extracts were prepared from synchronous *mbx1-13myc cdc25-22* fission yeast cells (GG 743) and subjected to co-immunoprecipitation analysis with the Plo1p (HO) and myc antibodies.

1.5 l of YE were inoculated with a 10 ml pre-culture of *mbx1-13myc cdc25-22* cells, and grown overnight at 25°C. The cell culture was grown to mid-log phase at 25°C and then transferred to 36°C for 3.5 hours, allowing the cells to arrest at the G2 phase. After this transient temperature arrest, the culture was returned and left to grow at the permissive temperature (25°C), allowing cells to progress through the cell cycle in synchrony. Samples were collected at 20 min intervals and soluble protein extracts were prepared and processed for immunoprecipitation. Moreover, as a measure of cell synchrony, the septation index of each sample was counted microscopically and plotted as a percentage of cell septa against time.

Plo1p was immunoprecipitated from each *mbx1-13myc cdc25-22* extract with the Plo1p (HO) antibody as before (Section 5.3.1). SDS-PAGE electrophoresis and Western blotting with Plo1p and myc antibodies followed to detect Plo1p and co-immunoprecipitated Mbx1p-13myc. As shown in Figure 5.8, Mbx1p-13myc is present in co-immunoprecipitates with Plo1p throughout the cell cycle at similar levels, suggesting that Mbx1p interacts with Plo1p at all times during the mitotic cell cycle.



Co-IP

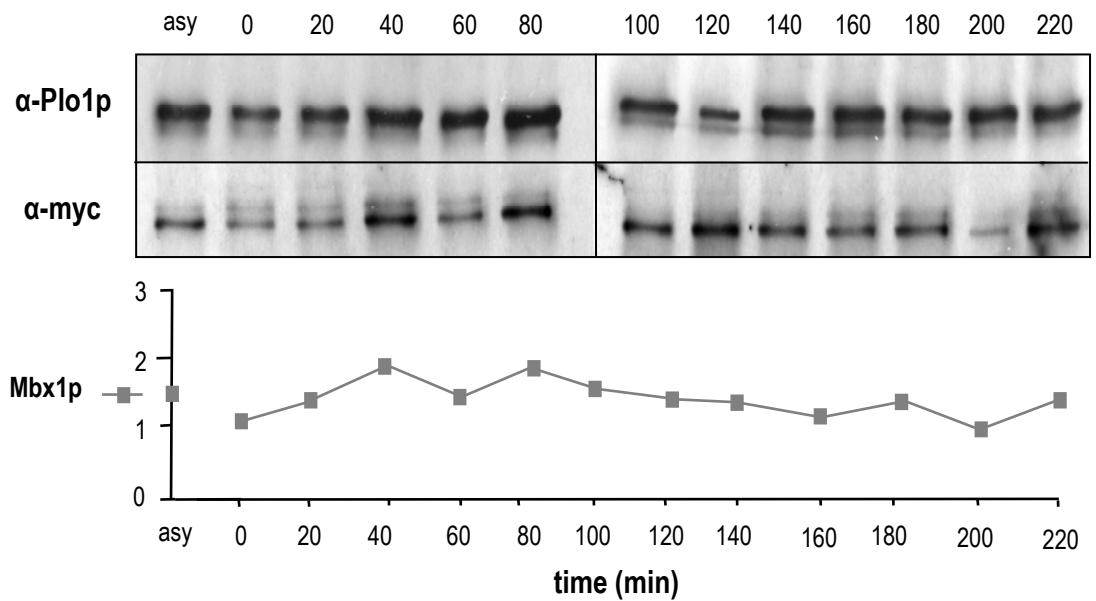


Figure 5.8: Mbx1p binds to Plo1p throughout the mitotic cell cycle. *cdc25-22 mbx1-13myc* cells (GG 743) were synchronised by transient temperature arrest and samples were taken every 20 min after returning the cells to the permissive temperature. Septation indices were counted microscopically and were plotted to indicate the synchrony of the culture. Soluble protein extracts were prepared from each sample and Plo1p was immunoprecipitated with antibody against the native protein. The immunoprecipitated samples were analysed by Western blotting with antibodies against Plo1p and myc (the latter to detect Mbx1p-13myc). asy, control sample from asynchronous cells prior to synchronisation. Quantification of the amount of co-immunoprecipitated Mbx1p-13myc against immunoprecipitated Plo1p is shown for each time point.

5.4 Summary

Earlier work unravelled a positive role for Plo1p in M-G1 specific transcription and showed that Plo1p function is required for PCB-dependent binding of the PBF complex (Anderson *et al.*, 2002). Plo1p is a Ser/Thr kinase raising the possibility that its effect on PBF-mediated transcription is exerted by association and ensuing phosphorylation of PBF components, Mbx1p, Fkh2p and Sep1p. Indeed, both Mbx1p and Fkh2p are periodically phosphorylated during the cell cycle with a timing that coincides with M-G1 specific gene expression (Buck *et al.*, 2004; Bulmer *et al.*, 2004). Furthermore, recent data show that *plo1*⁺ genetically interacts with *fkh2*⁺, *sep1*⁺ and *mbx1*⁺ and overexpression of a *plo1* kinase mutant (*plo1.K69R*) compromises transcription of genes, such as *cdc15*⁺ and *spo12*⁺ (Papadopoulou *et al.*, 2008).

In this study, the budding yeast two-hybrid system was employed to explore whether Plo1p influences M-G1 specific expression by associating with either Mbx1p, Fkh2p or Sep1p and, if so, which domain(s) of the kinase mediate such interaction(s). These analyses revealed a specific, direct, interaction only between Plo1p and Mbx1p and established a requirement for this association for both the kinase and the polo-box domains of Mbx1p (Figures 5.5 and 5.6). Subsequent co-immunoprecipitation experiments with appropriate fission yeast extracts confirmed the findings of the yeast two-hybrid assay and in parallel showed that Plo1p and Mbx1p can associate with each other *in vivo* at all times during the mitotic cell cycle (Figures 5.7 and 5.8; Papadopoulou *et al.*, 2008).

In agreement with the observed *in vivo* interaction between Plo1p and Mbx1p, kinase assays have shown that this Plk directly phosphorylates *in vitro* Mbx1p, but not Fkh2p or Sep1p. Notably, this is the first time a Polo-like kinase has been shown to bind and phosphorylate a MADS-box protein in any organism (Papadopoulou *et al.*, 2008).

The next chapter explores further how Plo1p, Mbx1p, Fkh2p and Sep1p control M-G1 phase-specific gene expression by analysing *in vivo* their interactions with the PCB promoter regions of their co-regulated genes, including *plo1*⁺ and *fkh2*⁺ themselves.

Chapter 6

*In vivo analysis of Fkh2p, Sep1p, and Plo1p
binding to the promoters of M-G1 transcribed
genes*

6.1 Introduction

In fission yeast a group of genes is periodically transcribed during late mitosis and early G1 phase. Since most of the genes encode proteins involved in late mitotic events and cytokinesis it is possible that the transcriptional control of their expression might provide a means of promoting progression through these later cell cycle stages.

Previous studies and experiments in Chapter 3 have shown that expression of M-G1 transcribed genes is regulated by the Sep1p, Fkh2p and Mbx1p transcription factors, the Plo1p kinase and two *cis*-acting DNA promoter motifs, the PCB and forkhead-like sequences (Utzig *et al.*, 2000; Anderson *et al.*, 2002; Buck *et al.*, 2004; Bulmer *et al.*, 2004; Rustici *et al.*, 2004; Alonso-Núñez *et al.*, 2005; Petit *et al.*, 2005). Sep1p, Fkh2p and Mbx1p are thought to be components of the PBF complex, which has been shown to associate *in vitro* with PCB sequences in the promoters of M-G1 transcribed genes. Mbx1p is necessary for the interaction *in vitro* of the PBF complex with the PCB sequence (Buck *et al.*, 2004). Interestingly, Plo1p is also required for PCB-mediated binding of PBF, suggesting that it exerts its function in M-G1 specific gene expression by modulating the properties of PBF, via interaction and phosphorylation of one or more PBF components (Anderson *et al.*, 2002). Indeed, experiments presented in this thesis show that Plo1p interacts *in vivo* with Mbx1p, with two-hybrid assays revealing that binding of Plo1p to Mbx1p relies on both the kinase and polo-box domains of Plo1p. Furthermore, Plo1p binds directly and phosphorylates Mbx1p *in vitro*, and Sep1p and Fkh2p associate with each other *in vivo*, possibly directly (Papadopoulou *et al.*, 2008).

plo1⁺ not only regulates periodic expression of M-G1 transcribed genes, but is itself transcribed during the M-G1 interval and contains PCB and forkhead sequences in its promoter (Anderson *et al.*, 2002). Similarly, *fkh2⁺* possesses PCB and forkhead promoter motifs and is transcribed during late M-early G1 phase (Buck *et al.*, 2004). These observations suggest that both Plo1p and Fkh2p regulate their own expression via feedback mechanisms. Indeed, Fkh2p varies in abundance during the cell cycle, both in terms of protein levels and phosphorylation status, with higher and lower protein levels coinciding with un-phosphorylated and phosphorylated forms of Fkh2p, respectively. These findings suggest that regulation of cell-cycle specific *fkh2⁺* expression might also be important for the functional properties of Fkh2p (Buck *et al.*, 2004; Bulmer *et al.*, 2004). In contrast to Fkh2p, however, Plo1p protein levels remain constant during the cell cycle, although its kinase activity varies (Tanaka *et al.*, 2001).

As another way to explore the regulatory mechanisms underlying periodic gene expression at the M-G1 interval in fission yeast, chromatin immunoprecipitation analysis (ChIP) was performed

to determine whether Fkh2p, Sep1p, Mbx1p and Plo1p interact with the promoter regions of M-G1 transcribed genes *in vivo*.

6.2 ChIP analysis of interactions of PBF components and Plo1p with the promoters of genes transcribed during M-G1 phase

Chromatin immunoprecipitation analysis (ChIP) is a powerful tool to study the *in vivo* interaction of proteins with chromosomal DNA. A great advantage of this method is that it allows the recovery of protein-DNA complexes in a near-native state via chemical cross-linking of living cells with formaldehyde. Moreover, this initial fixation step stabilises bulky chromatin complexes, and thus enables the detection not only of proteins that directly bind to their cognate DNA, but also of proteins that indirectly associate with chromosomal DNA, via protein-protein interactions (Ekwall and Partridge, 1999; Takahashi *et al.*, 2000).

A schematic outline of the ChIP method is shown in Figure 6.1. Once cells are fixed, cell extracts are prepared and sonicated to fragment the chromatin to appropriate, smaller sizes. The desired DNA-protein complexes are then recovered by immunoprecipitation with a suitable antibody. Finally, cross-linking is reversed to allow purification of precipitated DNA, which is then subjected to PCR amplification to screen for binding of the protein(s) of interest to specific target sequences.

The ChIP method was employed to detect Fkh2p, Sep1p and Mbx1p transcription factors and the Plo1p kinase at the promoters of genes transcribed during the M-G1 interval, including *cdc15⁺*, *plo1⁺* and *fkh2⁺*. Due to the unavailability of suitable antibodies against native Fkh2p, Sep1p and Mbx1p (Section 4.2; Appendix VIII), ChIP analysis was performed with cross-linked chromatin extracts from *S. pombe* cells that express each one of these transcription factors as an HA and/or myc fusion, tagged in each case at the C-terminus of the protein, and so expressed under control of the native promoter at normal levels. In the case of Plo1p, ChIP analysis was performed with extracts from either wild-type cells, cells expressing Plo1p as an HA fusion from the endogenous promoter at normal levels, or in cells mildly over-expressing Plo1p as an HA fusion from the *nmt41* promoter. For the immunoprecipitation step, HA and myc antibodies were used for HA- and myc-tagged versions of Fkh2p, Sep1p, Mbx1p and Plo1p, whereas for immunoprecipitation of native Plo1p from wild-type *S. pombe* cells two Plo1p antibodies (HO and IH) were utilised (Table 2.1). In each case, following immunoprecipitation and reversal of cross-linking, purified immunoprecipitated DNA was analysed by PCR with primers to amplify a 130-200 bp fragment from the promoter region of *cdc15⁺*, *plo1⁺* and *fkh2⁺* that contains the PCB sequence(s), as well as the TGTTTAC sequence

in the case of *plo1*⁺ (Figure 6.2). As a negative control, appropriate primers were also used to PCR amplify a DNA fragment from the coding region of the *act1*⁺ gene, encoding actin, which is not under PCB control and is not expressed at the M-G1 interval.

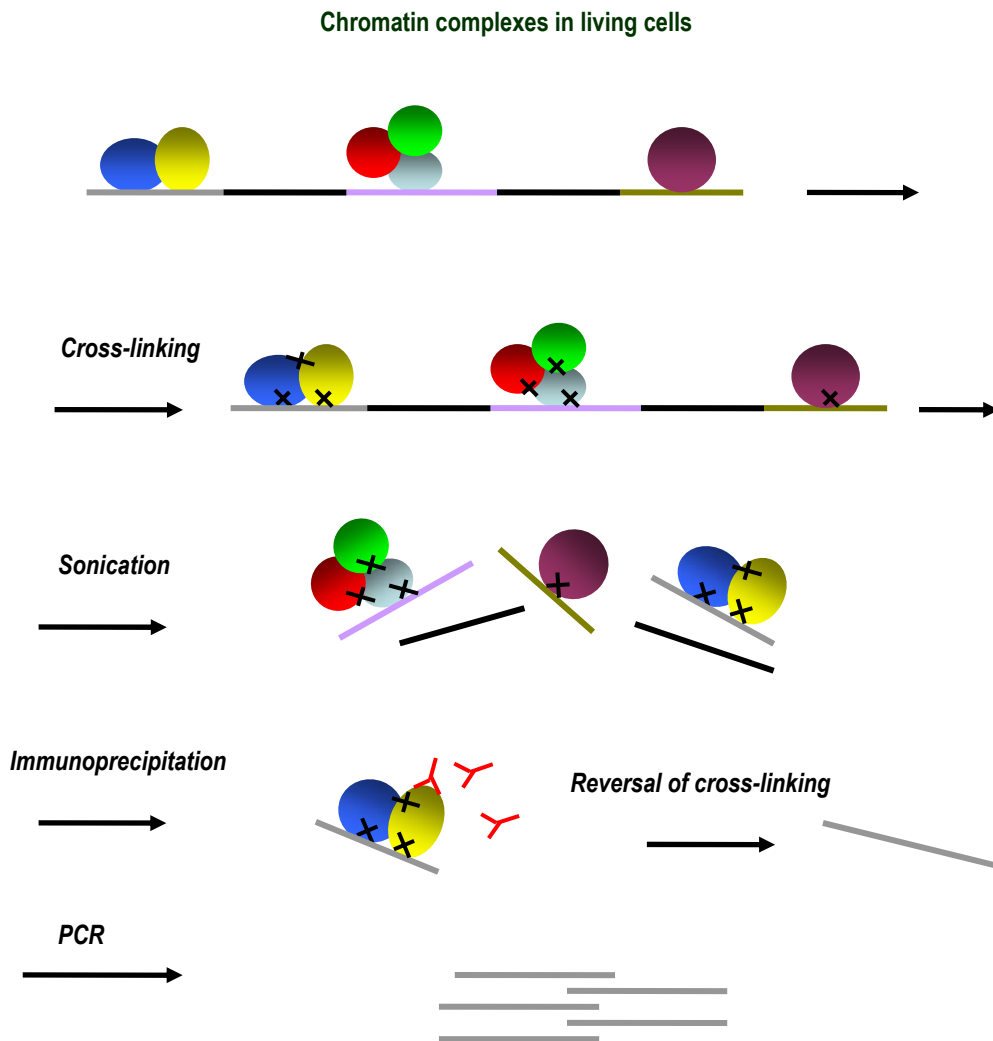


Figure 6.1: Schematic outline of the ChIP method. Living cells are fixed with formaldehyde followed by sonication of cell extracts to shear the chromatin to a small size. Specific DNA-protein complexes are then recovered by immunoprecipitation with an antibody against the protein of interest. The cross-linking is then reversed and the precipitated DNA purified before PCR amplification to analyse for association of the protein of interest with a specific DNA sequence. The letter x denotes chemical cross-linking of chromatin complexes. Precipitated DNA is shown as a grey line. **Black, lavender and dark yellow** lines denote non-precipitated DNAs. Antibodies are shown in **red**.

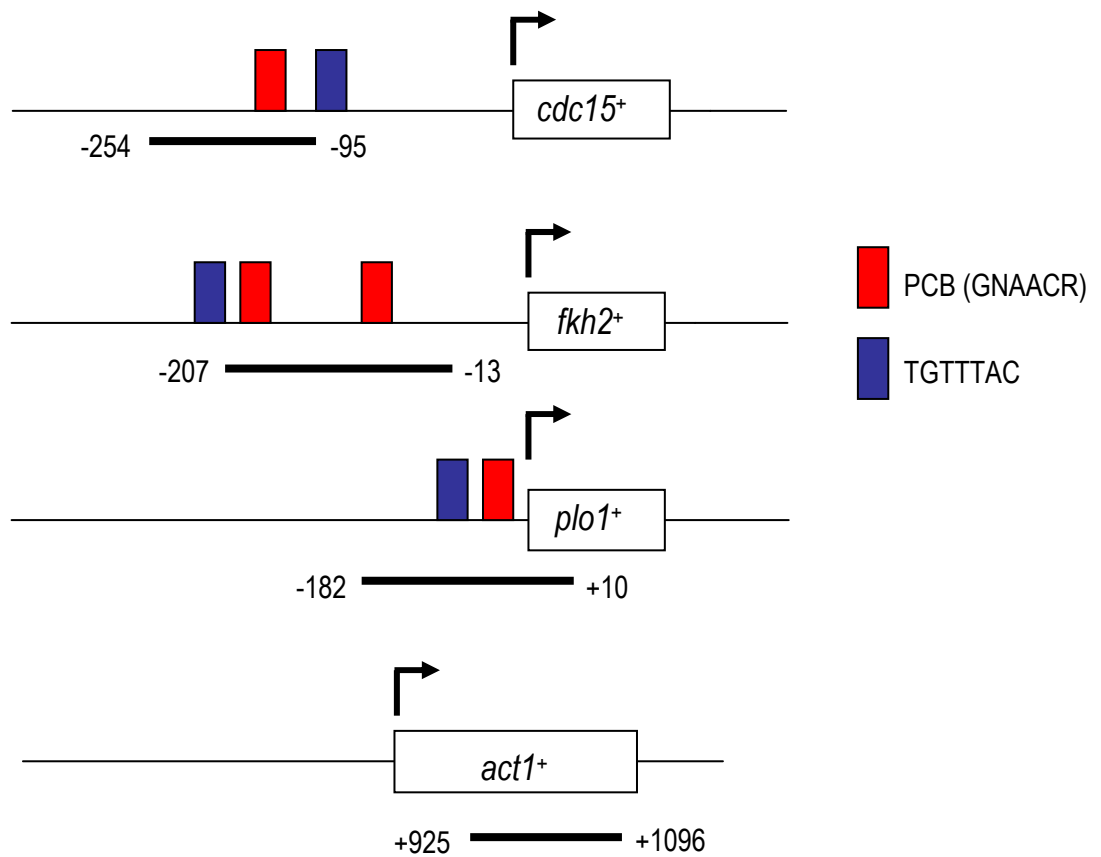


Figure 6.2: DNA fragments amplified by PCR for ChIP analysis. For *cdc15+*, *fkh2+*, or *plo1+*, appropriate primers were designed to PCR amplify in each case a 130-200 bp DNA promoter fragment containing the PCB or both the PCB and forkhead-related sequences. As a negative control primers were designed to amplify by PCR a DNA fragment from the coding region of the *act1+* gene. Positions of the PCB and TGTTTAC sequences within the promoters of *cdc15+*, *fkh2+* and *plo1+* are shown.

6.2.1 Fkh2p, Sep1p and Plo1p bind in vivo to the promoters of genes transcribed during M-G1 phase

ChIP assays were employed to determine whether Fkh2p, Sep1p, Mbx1p and Plo1p associate *in vivo* with the PCB promoter regions of *cdc15⁺*, *fkh2⁺* and *plo1⁺* (Section 2.2.12.3). ChIP analysis was performed with either *fkh2-13myc* (GG 507) or *fkh2-3HA* (GG 558) cells for Fkh2p, *sep1-13myc* (GG 767) cells for Sep1p, *mbx1-13myc* (GG 504) cells for Mbx1p and either wild-type (GG 217), *plo1-3HA* (GG 1110) or *nmt41:plo1-3HA* (GG 704) cells for Plo1p.

The first step in the ChIP process involved chemical cross-linking of cells. Routinely, a 500 ml culture of complete or selective media was inoculated with 5-10 ml of an overnight pre-culture of an appropriate *S. pombe* strain. This culture was then grown overnight to 5x10⁶ cells/ml before fixation with a final concentration of 1% formaldehyde for 30 min at 25°C with shaking. Following cross-linking, glycine addition and appropriate washes, fixed cells were harvested by centrifugation and processed for preparation of chromatin extracts. As shown in Figure 2.1, optimal cell lysis and sonication conditions were established to generate a high proportion of 300-1000 bp fragments of sheared chromatin (Sections 2.2.12.3.1 and 2.2.12.3.2).

After cell breakage and sonication, the resulting crude lysates were clarified by centrifugation prior to immunoprecipitation (Section 2.2.12.3.3). Briefly, 4 mg of each crude lysate was incubated with either protein A- or protein G- Sepharose beads as appropriate for an hour at 4°C to remove material that binds non-specifically to the beads. Following incubation, the pre-cleared lysate was separated from the beads by centrifugation. 10% of the pre-cleared lysate was removed and used as the control non-immunoprecipitated chromatin sample. An HA, myc or Plo1p (HO or IH) antibody was then added as appropriate to the remainder of the pre-cleared lysate and the suspension was incubated overnight at 4°C before addition of protein A- or protein G- Sepharose beads, and further incubation for 1-2 hours. As a positive control to confirm the efficiency of the ChIP assay, pre-cleared lysates were also incubated overnight with an antibody against histone 3 (H3) (Table 2.1). As negative controls, pre-cleared lysates were incubated overnight without antibody or with normal mouse, rabbit or sheep IgG, since the HA and myc antibodies were raised in mouse, while the Plo1p (HO and IH) antibodies in rabbit and sheep, respectively. Furthermore, pre-cleared lysate prepared for ChIP analysis from wild-type, un-tagged, *S. pombe* strains was also incubated with the HA or myc antibody as another negative control for antibody specificity.

Following incubation, each bead suspension was recovered on a polypropylene column and after successive washes the immunoprecipitated antibody-protein-DNA complexes were eluted. Appropriate treatment followed to reverse the cross-linking and purify the immunoprecipitated (IP)

DNA from all associated antibodies and proteins. The non-immunoprecipitated sample was also treated in a similar way to remove all associated proteins from the DNA, termed “whole cell extract” (WCE) (Sections 2.2.12.3.3 and 2.2.12.3.4). Finally, PCR analysis was performed with primer pairs GO 708/GO 709, GO 710/GO 711 and GO 712/GO 713 to amplify a 130-200 bp fragment from the *cdc15⁺*, *fkh2⁺* and *plo1⁺* promoters, respectively, while primer pair GO 714/GO 715 was used to amplify a fragment from the coding region of *act1⁺* (Sections 2.2.9.4 and 2.2.9.4.1). Routinely, PCR amplification of IP DNA (1 µl) was performed with *Taq* Polymerase under optimal cycling parameters as follows: 95°C for 2 min, 23-26 cycles of 95°C for 1 min, 54°C for 1 min and 72°C for 1 min, and 72°C for 5 min. Moreover, in each case PCR analysis was performed with five-fold serially diluted WCE DNA to ensure that PCR amplification was within a linear range. Following PCR analysis, amplified products were resolved on 2% agarose gels and visualised by ethidium bromide staining. The results obtained from ChIP analysis of the association of Fkh2p, Sep1p, Mbx1p and Plo1p with the *cdc15⁺*, *fkh2⁺* and *plo1⁺* promoter regions are shown in Figures 6.3-6.8.

As shown in Figures 6.3 and 6.4, ChIP assays with myc and HA antibodies and fixed cell extracts from *fkh2-13myc* and *fkh2-3HA* cells specifically detected Fkh2p-13myc and Fkh2p-3HA bound to the promoters of *cdc15⁺*, *plo1⁺* and *fkh2⁺*, but not to the coding region of *act1⁺*. As expected, ChIP analysis of *fkh2-13myc* and *fkh2-3HA* cells with the H3 antibody also detected histone 3 associated with the *cdc15⁺*, *fkh2⁺* and *plo1⁺* promoters and the *act1⁺* coding region, yielding a strong PCR signal, similar to that following PCR amplification of the WCE DNA, consistent with the fact that histones are abundant chromatin proteins. Furthermore, no PCR signal was retrieved in any of the negative controls, including precipitated samples from *fkh2-13myc* and *fkh2-3HA* cells in the absence of a myc or HA antibody and immunoprecipitated samples from wild-type, un-tagged, cells in the presence of a myc or HA antibody. Finally, the ChIP was shown to be quantitative, since a serial dilution of the WCE DNA resulted in a corresponding reduction of the observed PCR signal.

Similar to Fkh2p, as seen in Figure 6.5, ChIP analysis of *sep1-13myc* cells with the myc antibody to examine the interaction of Sep1p with the promoter regions of M-G1 transcribed genes, could detect specific binding of Sep1p-13myc to the promoters of *cdc15⁺* and *plo1⁺*. Unlike Fkh2p however, Sep1p-13myc could not be detected associated with the promoter of *fkh2⁺*. As before, appropriate positive and negative controls were completed.

To examine the association of Plo1p with the promoters of *cdc15⁺*, *fkh2⁺* and *plo1⁺*, ChIP analysis was performed with a Plo1p antibody and fixed extracts from wild-type cells, or with an HA antibody and extracts from cells expressing Plo1p-3HA from the endogenous promoter at normal levels or cells mildly over-expressing Plo1p-3HA from the medium-strength thiamine-repressible

nmt41 promoter integrated into the genome. As shown in Figure 6.6A, using one Plo1p (HO) antibody and extracts from wild-type cells, Plo1p could not be detected at the promoters of *cdc15⁺*, *fkh2⁺* and *plo1⁺*. Similar results were obtained when ChIP assays were performed with wild-type extracts and another Plo1p (IH) antibody (data not shown) or, as observed in Figure 6.6B, with an HA antibody and extracts from *plo1-3HA* cells, expressing Plo1p-3HA from the native promoter. However, mild over-expression of Plo1p-3HA under control of the *nmt41* promoter drastically improved the sensitivity of the ChIP assay, since under these conditions, and with the use of an HA antibody, Plo1p-3HA was detected at the promoter regions of *cdc15⁺* and *fkh2⁺* (Figure 6.7). As expected, PCR analysis of IP and WCE DNAs with primers specific for the *plo1⁺* promoter region did not yield a PCR signal, since in the *nmt41:plo1-3HA* strain the *plo1⁺* promoter is replaced by *nmt41*.

Finally, Figure 6.8 shows the results obtained from ChIP analysis of *mbx1-13myc* extracts with a myc antibody to study binding of Mbx1p to the *cdc15⁺*, *plo1⁺* and *fkh2⁺* promoters. As seen, specific binding of Mbx1p-13myc to the promoters of these genes could not be detected. Nonetheless, the inability of the ChIP assay to detect promoter bound Mbx1p is likely due to technical reasons, because previous studies have shown that Mbx1p is a core component of the PBF complex and binds *in vitro* to the PCB promoter region of *cdc15⁺* throughout the cell cycle (Anderson *et al.*, 2002; Buck *et al.*, 2004). Notably, Plo1p has also been shown to both bind to Mbx1p (Figure 5.7) and associate with the promoter regions of *cdc15⁺* and *fkh2⁺*, raising the possibility that it is through the interaction with Mbx1p that Plo1p is recruited to the promoters of these genes (Section 7.1). Thus, it is possible that the C-terminal tagging of Mbx1p has interfered with its binding to the promoters of these genes or, alternatively, the myc antibody might have been unable to recognise Mbx1p because the respective epitopes were masked, preventing detection by ChIP.

In summary, a series of ChIP experiments were performed with antibodies against HA- and/or myc- tagged versions of Fkh2p, Mbx1p and Sep1p, expressed under the control of their native promoter, or an HA-tagged version of Plo1p, mildly over-expressed from the *nmt41* promoter. Under these conditions, Fkh2p-13myc or Fkh2p-3HA were shown to associate with the promoters of *cdc15⁺*, *fkh2⁺* and *plo1⁺*, Plo1p-3HA was shown to interact with the promoters of *cdc15⁺* and *fkh2⁺*, while Sep1p-13myc was shown to bind to the promoters of *cdc15⁺* and *plo1⁺* but not *fkh2⁺* (Papadopoulou *et al.*, 2008).

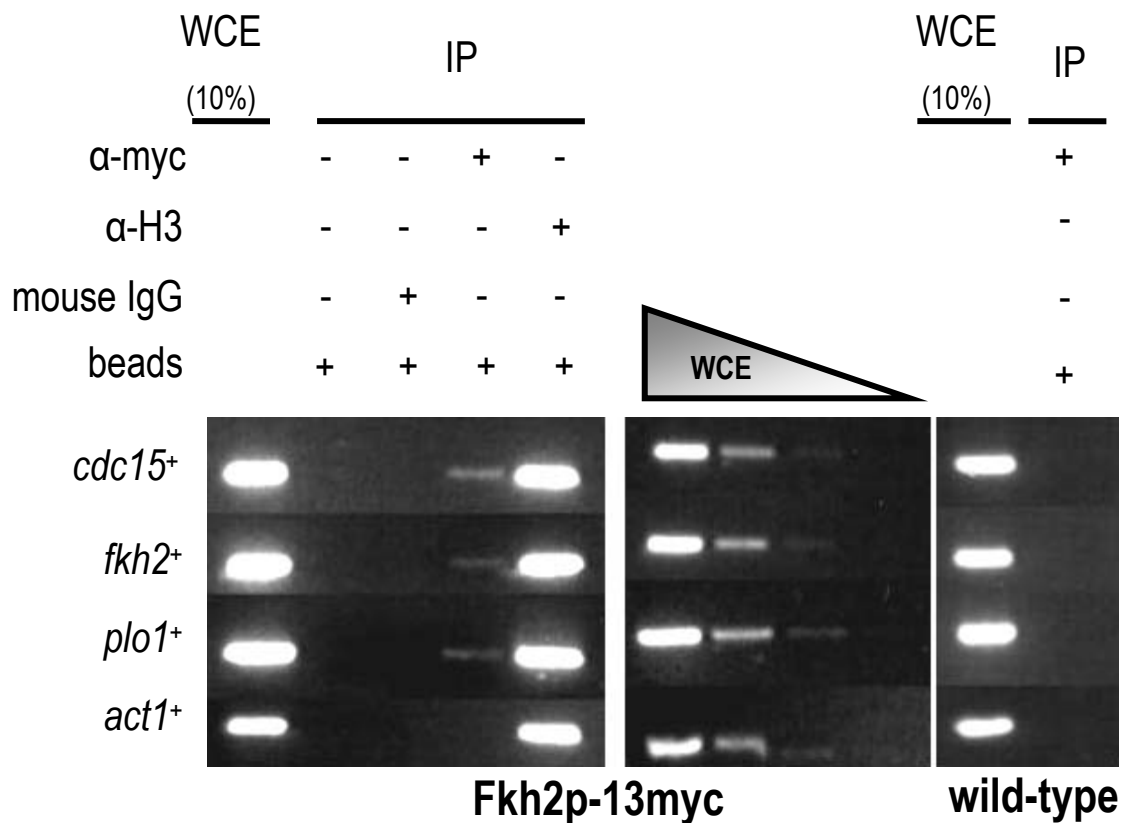


Figure 6.3: Fkh2p binds to the promoters of *cdc15*⁺, *fkh2*⁺ and *plo1*⁺ genes *in vivo*. ChIP analysis in *fkh2-13myc* cells (GG 507) to detect Fkh2p binding the promoters of *cdc15*⁺, *fkh2*⁺ and *plo1*⁺ using an anti-myc antibody. All primers used for PCR amplification following ChIP analysis are shown in Figure 6.2. WCE (whole cell extract) refers to the non-immunoprecipitated sample and IP to the immunoprecipitated samples. The WCE sample was used in a 1:10 ratio relative to the IP samples. As negative controls, precipitates without antibody and precipitates with normal mouse IgG were used. As another control for antibody specificity, ChIP analysis was performed in wild-type untagged cells using the anti-myc antibody. As a positive control, histone 3 antibody was utilised to detect histone 3 on the *cdc15*⁺, *fkh2*⁺ and *plo1*⁺ promoters, as well as on the *act1*⁺ coding region. PCR amplification was performed within a linear range, such that a serial dilution of the WCE DNA results in a reduction of the PCR signal. ChIP assays with cross-linked chromatin extracts from *fkh2-13myc* cells were carried out twice. The results of PCR analysis of immunoprecipitated DNA with the myc and histone 3 antibodies and of precipitated material from the two negative controls in one of the two assays are shown here and are representative of the results of PCR analysis of both assays.

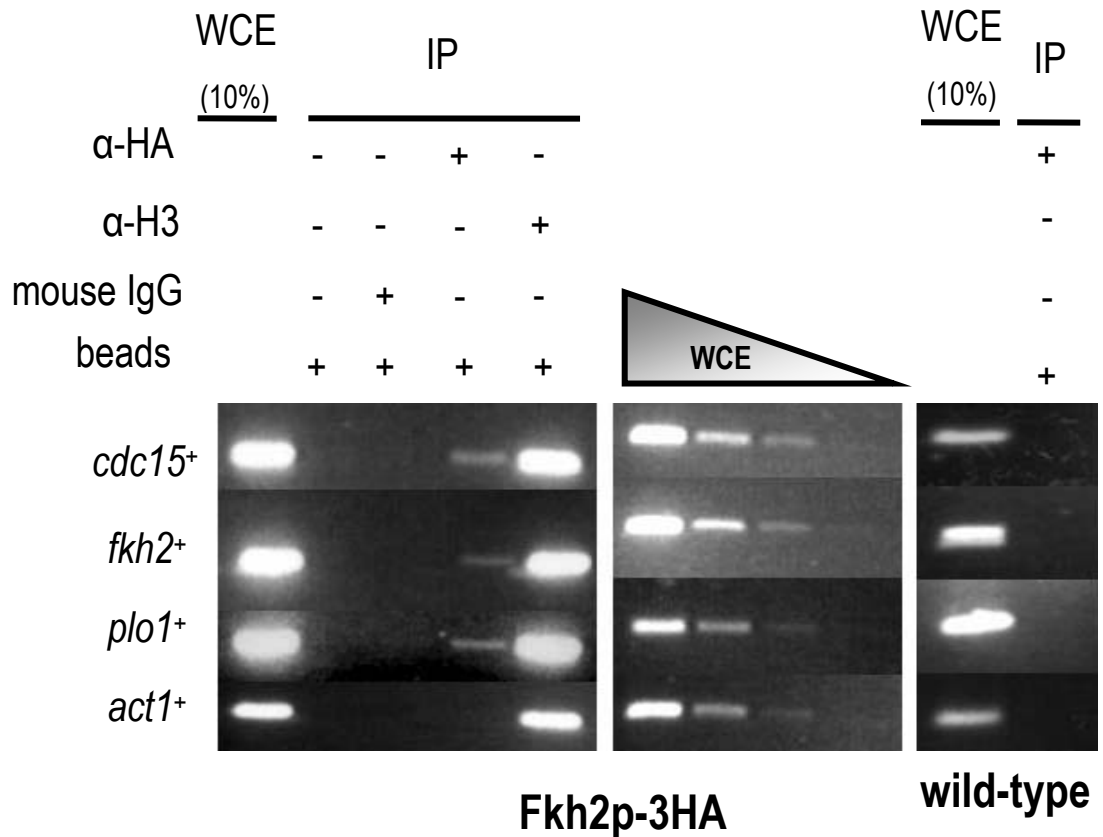


Figure 6.4: Fkh2p binds to the promoters of *cdc15⁺*, *fkh2⁺* and *plo1⁺* *in vivo*. ChIP analysis in *fkh2-3HA* cells (GG 558) to detect Fkh2p binding to the promoters of *cdc15⁺*, *fkh2⁺* and *plo1⁺* using an anti-HA antibody. All primers used for PCR amplification following ChIP analysis are shown in Figure 6.2. WCE (whole cell extract) refers to the non-immunoprecipitated sample and IP to the immunoprecipitated samples. The WCE sample was used in a 1:10 ratio relative to the IP samples. As negative controls, precipitates without antibody and precipitates with normal mouse IgG were used. As another control for antibody specificity, ChIP analysis was performed in wild-type untagged cells using the anti-HA antibody. As a positive control, histone 3 antibody was utilised to detect histone 3 on the *cdc15⁺*, *fkh2⁺* and *plo1⁺* promoters, as well as on the *act1⁺* coding region. PCR amplification was performed within a linear range, such that a serial dilution of the WCE DNA results in a reduction of the PCR signal. ChIP assays with cross-linked chromatin extracts from *fkh2-3HA* cells were carried out twice. The results of PCR analysis of immunoprecipitated DNA with the HA and histone 3 antibodies and of precipitated material from the two negative controls in one of the two assays are shown here and are representative of the results of PCR analysis of both assays.

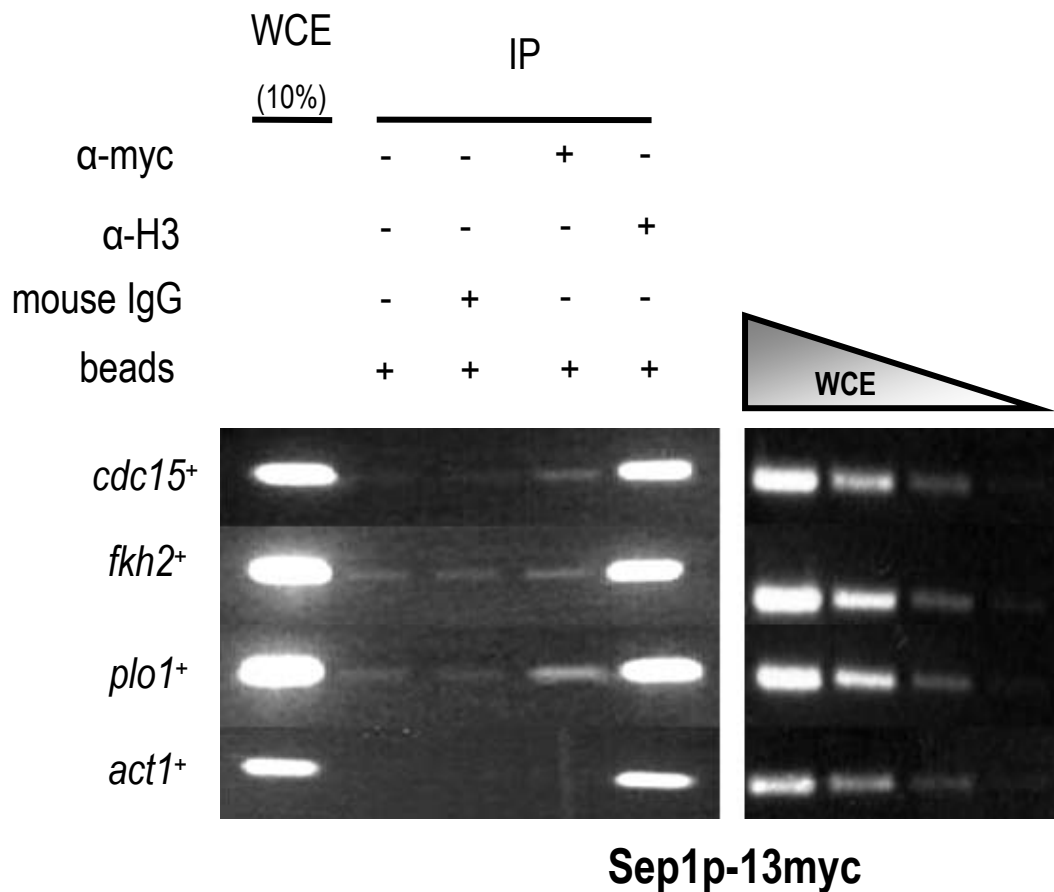
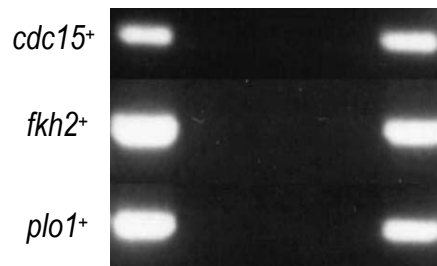


Figure 6.5: Sep1p binds to the promoters of *cdc15⁺* and *plo1⁺* *in vivo*. ChIP analysis in *sep1-13myc* cells (GG 767) to detect Sep1p binding to the promoters of *cdc15⁺*, *fkh2⁺* and *plo1⁺*, using an anti-myc antibody. As a negative control, primers were used to amplify a DNA fragment from the coding region of the *act1⁺* gene. All primers used for PCR amplification following ChIP analysis are shown in Figure 6.2. WCE (whole cell extract) refers to the non-immunoprecipitated sample and IP to the immunoprecipitated samples. The WCE sample was used in a 1:10 ratio relative to the IP samples. As negative controls, precipitates without antibody and precipitates with normal mouse IgG were used. As a positive control, histone 3 antibody was utilised to detect histone 3 on the *cdc15⁺*, *fkh2⁺* and *plo1⁺* promoters, as well as on the *act1⁺* coding region. PCR amplification was performed within a linear range, such that a serial dilution of the WCE DNA results in a reduction of the PCR signal. ChIP assays with cross-linked chromatin extracts from *sep1-13myc* cells were carried out twice. The results of PCR analysis of immunoprecipitated DNA with the myc and histone 3 antibodies and of precipitated material from the two negative controls in one of the two assays are shown here and are representative of the results of PCR analysis of both assays.

A

	WCE (10%)		IP	
α -Plo1p	-	-	+	-
α -H3	-	-	-	+
rabbit IgG	-	+	-	-
beads	+	+	+	+

**Plo1p****B**

	WCE (10%)		IP	
α -HA	-	-	+	-
α -H3	-	-	-	+
mouse IgG	-	+	-	-
beads	+	+	+	+

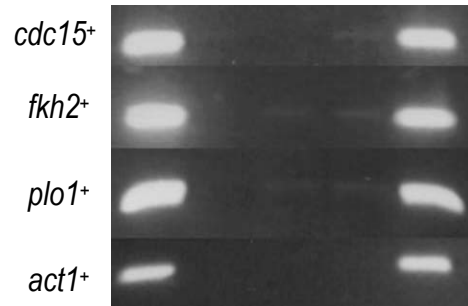
**Plo1p-3HA**

Figure 6.6: ChIP analysis in (A) wild-type cells (GG 217) and (B) *plo1-3HA* cells (GG 1110) to detect Plo1p on the promoters of *cdc15⁺*, *fkh2⁺* and *plo1⁺*. An anti-Plo1p (A) and an anti-HA antibody (B) were used to immunoprecipitate Plo1p and Plo1p-3HA, respectively. (A-B) For *cdc15⁺* and *fkh2⁺* and *plo1⁺* appropriate primers were used to PCR amplify a promoter fragment containing the PCB (*cdc15⁺* and *fkh2⁺*) or both the PCB and forkhead-related sequences (*plo1⁺*) (Figure 6.2). WCE refers to the non-immunoprecipitated sample and IP to the immunoprecipitates. The WCE sample was used in a 1:10 ratio relative to the IP samples. As negative controls, precipitates without antibody and precipitates with (A) normal rabbit IgG and (B) normal mouse IgG were used. Histone 3 antibody was used as a positive control. (B) As a negative control, primers were used to amplify a DNA fragment from the coding region of the *act1⁺* gene. (A-B) ChIP assays with cross-linked chromatin extracts from either wild-type or *plo1-3HA* cells were carried out twice. In each case, the results of PCR analysis in one of the two assays are shown here and are representative of the results of PCR analysis of both assays.

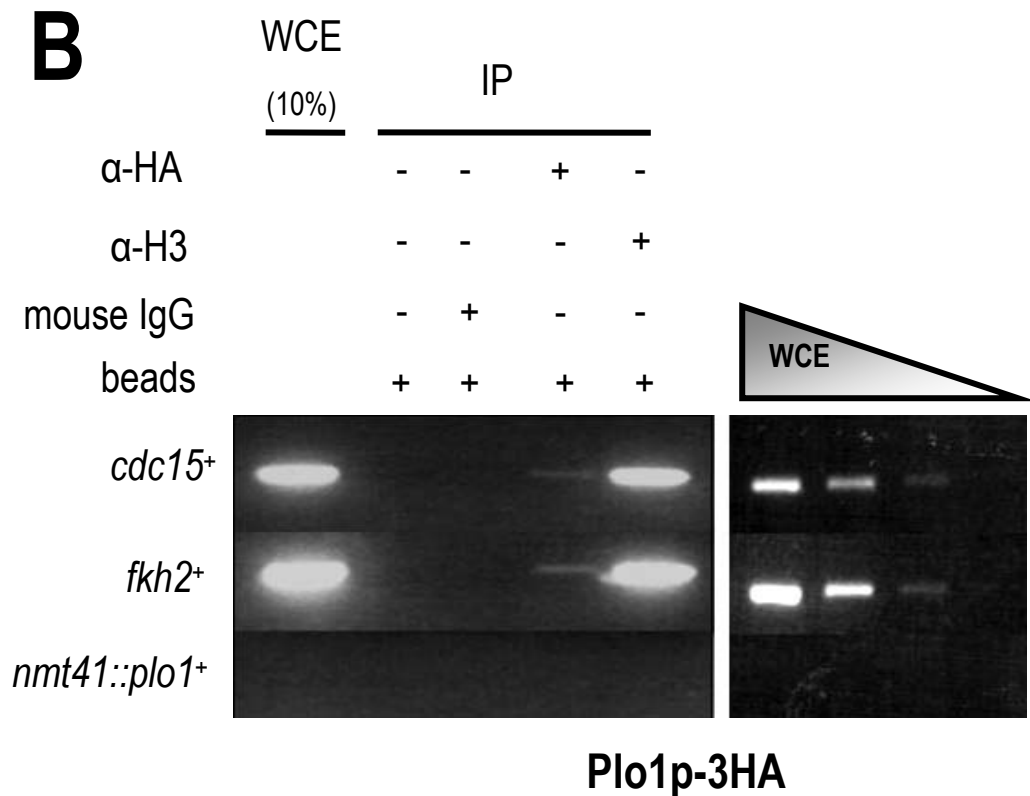
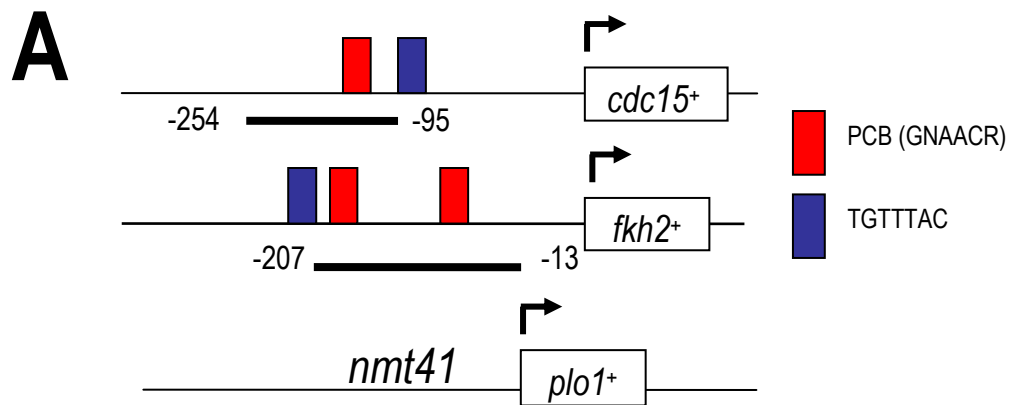


Figure 6.7: Plo1p binds to the promoters of *cdc15*⁺ and *fkh2*⁺ in vivo. ChIP analysis in *nmt41::plo1-3HA* cells (GG 704) to detect Plo1p binding to the promoters of *cdc15*⁺ and *fkh2*⁺ using an anti-HA antibody. (A) For *cdc15*⁺, *fkh2*⁺ and *plo1*⁺ appropriate primers were used to PCR amplify a promoter fragment containing the PCB. (B) WCE refers to the non-immunoprecipitated sample and IP to the immunoprecipitates. The WCE sample was used in a 1:10 ratio relative to the IP samples. As negative controls, precipitates without antibody and precipitates with normal mouse IgG were used. Histone 3 antibody was used as a positive control. PCR amplification was performed within a linear range, such that a serial dilution of the WCE DNA results in a reduction of the PCR signal. ChIP assays with cross-linked chromatin extracts from *nmt41::plo1-3HA* cells were carried out twice. The results of PCR analysis of immunoprecipitated DNA with the HA and histone 3 antibodies and of precipitated material from the two negative controls in one of the two assays are shown here and are representative of the results of PCR analysis of both assays.

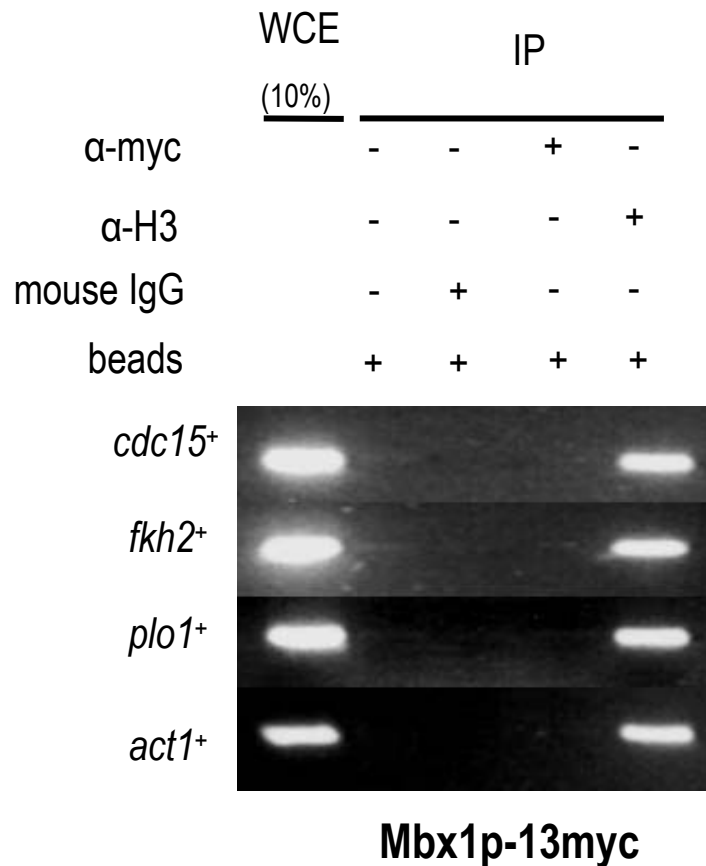


Figure 6.8: ChIP analysis in *mbx1-13myc* cells (GG 504) to detect MbX1p on the promoters of *cdc15⁺*, *fkh2⁺* and *plo1⁺*. An anti-myc antibody was used to immunoprecipitate MbX1p-13myc and appropriate primers were used to PCR amplify a fragment containing the PCB for the promoters of *cdc15⁺* and *fkh2⁺* or both the PCB and forkhead-related sequences for the promoter of *plo1⁺* (Figure 6.2). As a negative control, primers were used to amplify a DNA fragment from the coding region of the *act1⁺* gene. WCE refers to the non-immunoprecipitated sample and IP to the immunoprecipitates. The WCE sample was used in a 1:10 ratio relative to the IP samples. As negative controls, precipitates without antibody and precipitates with normal mouse IgG were used. Histone 3 antibody was used as a positive control. ChIP assays with cross-linked chromatin extracts from *mbx1-13myc* cells were carried out twice. The results of PCR analysis of immunoprecipitated DNA with the myc and histone 3 antibodies and of precipitated material from the two negative controls in one of the two assays are shown here and are representative of the results of PCR analysis of both assays.

6.2.2 Requirement of Plo1p, Mbx1p and Sep1p for Fkh2p promoter binding *in vivo*

In the previous section, Fkh2p was shown to bind *in vivo* to the promoter regions of *cdc15⁺*, *plo1⁺* and *fkh2⁺*. Previous work has shown that although deletion of *sep1⁺* is sufficient to rescue the lethal phenotype caused by strong *fkh2⁺* overexpression, deleting *fkh2⁺* does not rescue the lethality of cells strongly overexpressing *sep1⁺* (Buck *et al.*, 2004). These findings suggest that these two forkhead transcription factors have both overlapping and separate functions, and that Sep1p is important for Fkh2p function but itself can function without Fkh2p. Consistent with this proposal, experiments presented in this thesis have revealed that Fkh2p and Sep1p associate with each other both *in vivo* and *in vitro* (Figures 4.4 and 4.19). Furthermore, both Mbx1p and Plo1p are necessary for binding of the PBF complex to the PCB sequences *in vitro*, while Plo1p not only interacts with and phosphorylates Mbx1p but also associates *in vivo* with the promoters of M-G1 transcribed genes, including *cdc15⁺* and *fkh2⁺*. Taken together, these findings imply that Sep1p, Mbx1p and Plo1p, apart from their other functions in transcriptional regulation of M-G1 expressed genes, might also have a role in modulating the binding properties of Fkh2p to the promoters of these genes. Therefore, ChIP assays were performed to determine whether Fkh2p can still bind to the *cdc15⁺*, *plo1⁺* and *fkh2⁺* promoter regions in the absence of either Sep1p, Mbx1p or Plo1p.

To examine whether Plo1p is necessary for promoter binding of Fkh2p, ChIP assays were performed with fixed cell extracts from the temperature-sensitive *plo1-ts35 fkh2-3HA* (GG 555) mutant either at the permissive (25°C) or the restrictive temperature (36°C), the former used as a positive control. An 800 ml culture of *plo1-ts35 fkh2-3HA* cells was grown overnight to mid-log phase at 25°C before splitting into two 400 ml cultures. One culture was immediately fixed with a final concentration of 1% formaldehyde for 30 min at 25°C, while the other culture was transferred to 36°C for 3 hours to inactivate Plo1p prior to fixation with formaldehyde. Following cross-linking, both *plo1-ts35 fkh2-3HA* chromatin extracts were processed for ChIP analysis with the HA antibody. As another positive control, ChIP assays were also performed with the HA antibody and fixed extracts from *fkh2-3HA* cells, as described in Section 6.2.1. Additional negative and positive controls were included in the ChIP assay to confirm antibody specificity and the efficiency of the ChIP procedure. PCR analysis was then performed to check whether Fkh2p could be detected at the *cdc15⁺*, *plo1⁺* and *fkh2⁺* promoters in the absence of Plo1p function. PCR-amplified DNAs prepared from *fkh2-3HA* and *plo1-ts35 fkh2-3HA* cells grown at the permissive and restrictive temperatures were run on the same agarose gel to allow a direct comparison of their levels. Finally, PCR analysis was performed with serial dilutions of each WCE DNA to ensure that PCR amplification was within a linear range. Figure 6.9 displays the results from the ChIP analysis. As expected, ChIP assays with the HA

antibody could detect specific binding of Fkh2p-3HA to the *cdc15⁺*, *plo1⁺* and *fkh2⁺* promoters in *fkh2-3HA* cells. Similarly, Fkh2p-3HA was still detected at these promoters in *plo1-ts35 fkh2-3HA* cells at both the permissive and restrictive temperatures, suggesting that Fkh2p can associate with the promoters of M-G1 transcribed genes in the absence of Plo1p.

To investigate the requirement for Mbx1p and Sep1p for promoter binding of Fkh2p, cross-linked chromatin extracts were prepared from *mbx1Δ fkh2-13myc* (GG 1047) and *sep1Δ fkh2-3HA* (GG 1040) cells and processed for ChIP analysis with a myc and HA antibody, respectively. As before, appropriate negative and positive controls were included in the assay to confirm antibody specificity and the efficiency of the ChIP procedure. PCR analysis then followed to determine whether Fkh2p could be detected at the *cdc15⁺*, *plo1⁺* and *fkh2⁺* promoters in the absence of Mbx1p or Sep1p. PCR analysis was also performed with serial dilutions of each WCE DNA to ensure that PCR amplification was within a linear range. The results obtained from ChIP analysis are shown in Figures 6.10 and 6.11. These analyses revealed that Fkh2p-13myc and Fkh2p-3HA were still detected binding to the promoters of *cdc15⁺*, *fkh2⁺* and *plo1⁺*, showing that the absence of Mbx1p or Sep1p had no effect on the ability of Fkh2p to bind to these promoters (Papadopoulou *et al.*, 2008).

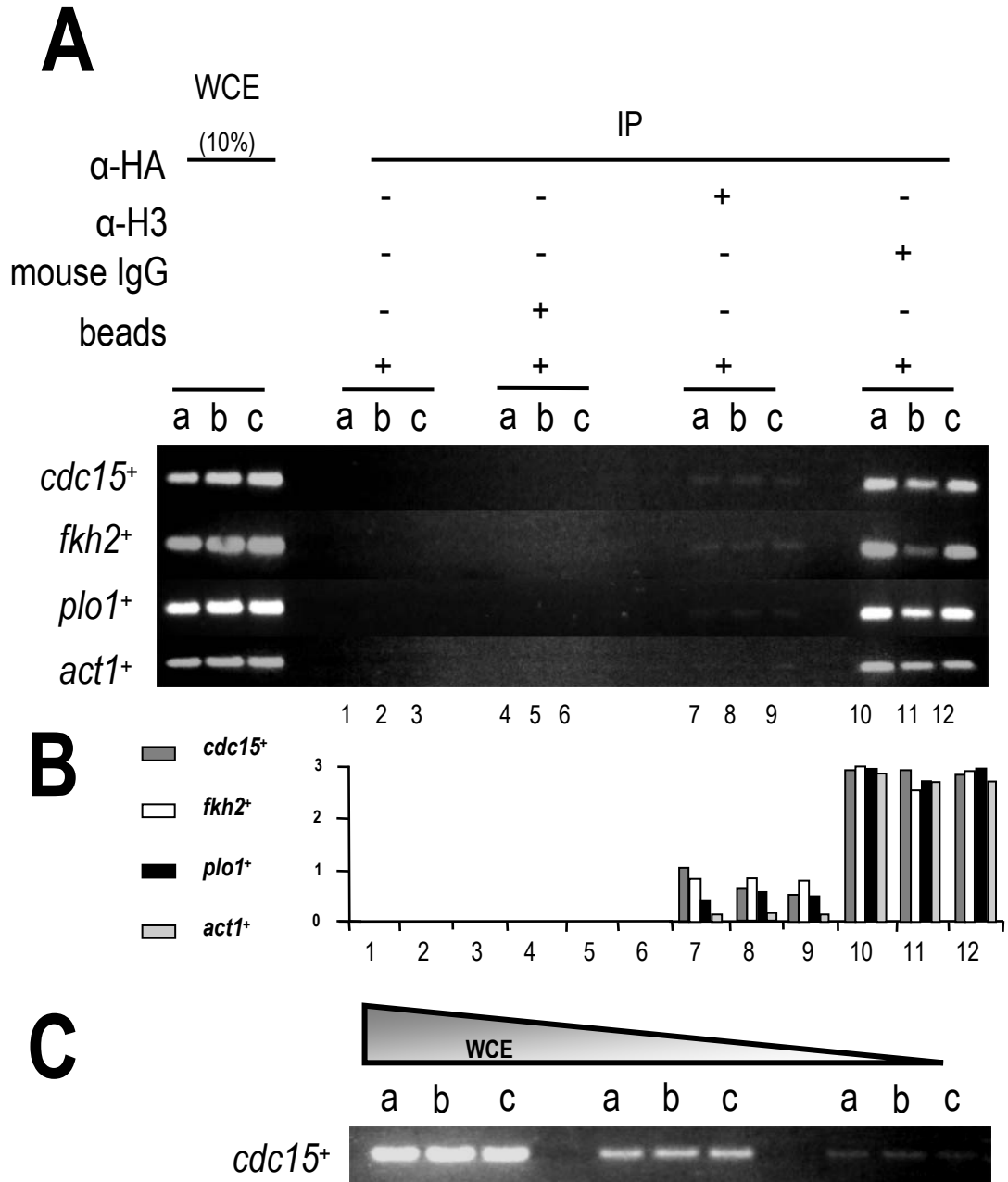


Figure 6.9: Requirement of Plo1p for Fkh2p promoter binding *in vivo*. ChIP analysis to examine whether Fkh2p binds the promoters of *cdc15⁺*, *fkh2⁺* and *plo1⁺* in the absence of Plo1p. Fkh2p-3HA was immunoprecipitated from (a) *fkh2-3HA* (GG 558) cells and *plo1-ts35 fkh2-3HA* cells (GG 555) at (b) permissive and (c) restrictive temperature using an anti-HA antibody. For (a), (b) and (c), Fkh2p binding to PCB and forkhead promoter regions of *cdc15⁺*, *fkh2⁺* and *plo1⁺* was detected by PCR. As a negative control, primers were used to amplify a DNA fragment from the coding region of the *act1⁺* gene. WCE refers to non-immunoprecipitated sample and IP to immunoprecipitates. Precipitates without antibody and precipitates with normal mouse IgG were used as negative controls, whereas histone 3 antibody was used as a positive control. (B) In all cases, the PCR signal from amplification of DNA immunoprecipitated with anti-HA (7-9) or histone 3 antibody (10-12) was quantified against the signal from PCR amplification of WCE DNA. (C) PCR amplification was performed within a linear range, such that a serial dilution of the WCE DNA results in a reduction of the PCR signal.

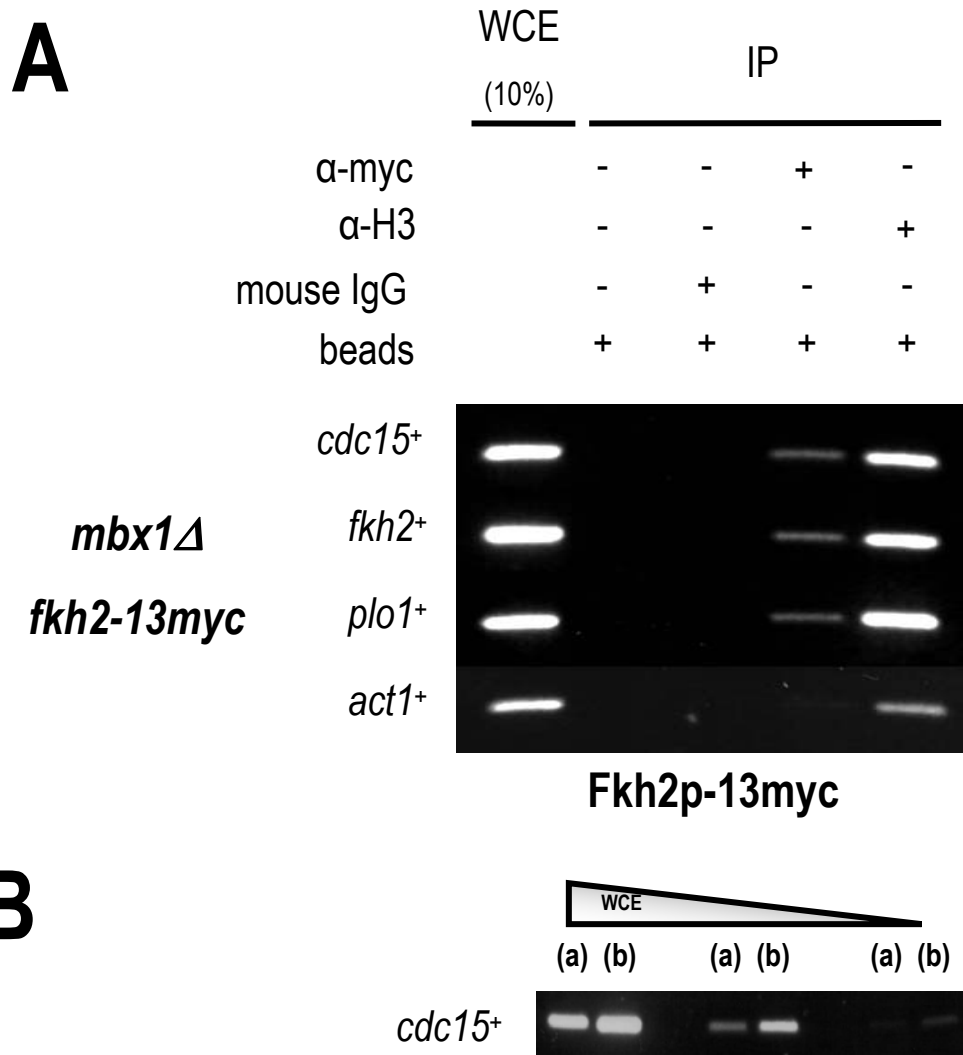


Figure 6.10: Requirement of Mbx1p for Fkh2p promoter binding *in vivo*. (A) ChIP analysis in *mbx1Δ fkh2-13myc* (GG 1047) cells to examine whether Fkh2p binds the promoters of *cdc15⁺*, *fkh2⁺* and *plo1⁺* in the absence of Mbx1p. Fkh2p-13myc was immunoprecipitated from cells using an anti-myc antibody and binding to PCB and forkhead promoter regions of *cdc15⁺*, *fkh2⁺* and *plo1⁺* was detected by PCR. All primers used for PCR amplification following ChIP analysis are shown in Figure 6.2. WCE refers to non-immunoprecipitated sample and IP to immunoprecipitates. As negative controls, precipitates without antibody and precipitates with normal mouse IgG were used, whereas histone 3 antibody was used as a positive control. (B) PCR amplification was performed within a linear range, such that a serial dilution of the WCE DNA results in a reduction of the PCR signal. (a) refers to WCE DNA prepared from *fkh2-13myc* (GG 507) cells and (b) to WCE DNA prepared from *mbx1Δ fkh2-13myc* cells. ChIP assays with cross-linked chromatin extracts from *mbx1Δ fkh2-13myc* cells were carried out twice. The results of PCR analysis of immunoprecipitated DNA with the myc and histone 3 antibodies and of precipitated material from the two negative controls in one of the two assays are shown here and are representative of the results of PCR analysis of both assays.

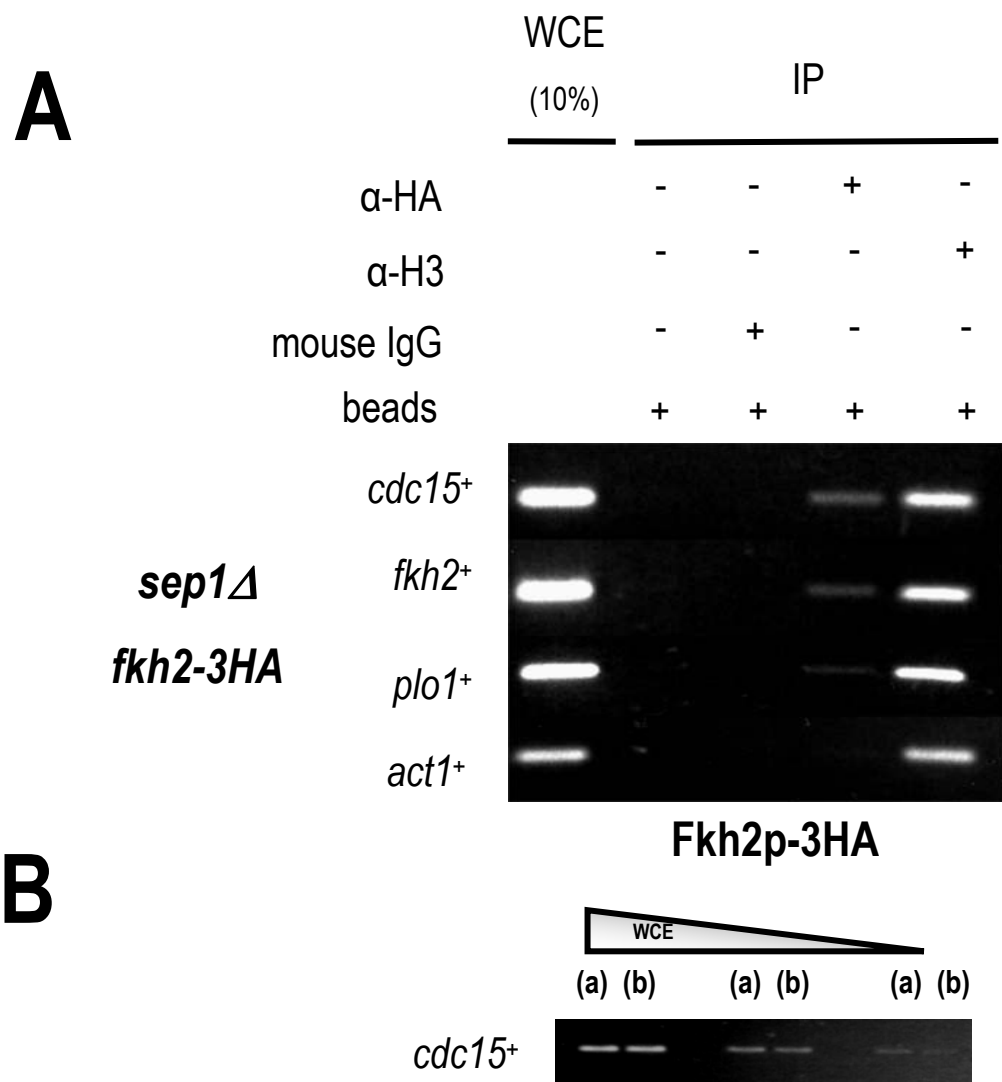


Figure 6.11: Requirement of Sep1p for Fkh2p promoter binding *in vivo*. ChIP analysis in *sep1 Δ fkh2-3HA* (GG 1040) cells to examine whether Fkh2p binds the promoters of *cdc15⁺*, *fkh2⁺* and *plo1⁺* in the absence of Sep1p. Fkh2p-3HA was immunoprecipitated from cells using an anti-HA antibody and binding to PCB and forkhead promoter regions of *cdc15⁺*, *fkh2⁺* and *plo1⁺* was detected by PCR. As a negative control, primers were used to amplify a DNA fragment from the coding region of the *act1⁺* gene. All primers used for PCR amplification following ChIP analysis are shown in Figure 6.2. WCE refers to non-immunoprecipitated sample and IP to immunoprecipitates. As negative controls, precipitates without antibody and precipitates with normal mouse IgG were used, whereas histone 3 antibody was used as a positive control. (B) PCR amplification was performed within a linear range, such that a serial dilution of the WCE DNA results in a reduction of the PCR signal. (a) refers to WCE DNA prepared from *fkh2-3HA* (GG 558) cells and (b) to WCE DNA prepared from *sep1 Δ fkh2-3HA* cells. ChIP assays with cross-linked chromatin extracts from *sep1 Δ fkh2-3HA* cells were carried out twice. The results of PCR analysis of immunoprecipitated DNA with the HA and histone 3 antibodies and of precipitated material from the two negative controls in one of the two assays are shown here and are representative of the results of PCR analysis of both assays.

6.2.3 Cell-cycle-specific binding of Fkh2p, Sep1p and Plo1p to the PCB promoter regions of M-G1 transcribed genes

A series of ChIP experiments presented in Section 6.2.1 revealed that Fkh2p, Sep1p and Plo1p associate *in vivo* with the promoters of M-G1 transcribed genes, including *cdc15⁺*, as well as *plo1⁺* and *fkh2⁺*. To explore further how Fkh2p, Sep1p and Plo1p might contribute to the control of M-G1 phase-specific gene expression, ChIP analysis was also employed to study their binding to PCB promoter regions throughout the cell cycle. To accomplish this, fixed cell extracts were prepared from synchronously dividing *cdc25-22 fkh2-3HA* (GG 745), *cdc25-22 nmt41:plo1-3HA* (GG 1097) and *cdc25-22 sep1-13myc* (GG 1118) cells and processed for ChIP analysis with HA and myc antibodies as appropriate. Furthermore, total RNA extracts were also prepared from these synchronous populations of cells and subjected to Northern blot analysis with appropriate DNA probes to determine how binding of Fkh2p, Sep1p and Plo1p to the promoters of *cdc15⁺*, *plo1⁺* and *fkh2⁺* during the cell cycle correlates with the cell cycle mRNA levels of the same genes, which are known to fluctuate during the cell cycle, with peak levels at M-G1 (Anderson *et al.*, 2002; Buck *et al.*, 2004).

For cell cycle ChIP and Northern blot analysis, 2 l of complete or selective media were inoculated with a 10 ml pre-culture of either *cdc25-22 fkh2-3HA*, *cdc25-22 nmt41:plo1-3HA* and *cdc25-22 sep1-13myc* cells and grown overnight at 25°C. Each cell culture was grown to mid-log phase at 25°C and then transferred to 36°C for 3.5 hours, allowing the cells to arrest at the G2 phase. After the transient temperature arrest, the cultures were returned and left to grow at the permissive temperature (25°C), allowing cells to progress through the cell cycle in synchrony. Samples were collected at 20 min intervals and immediately processed for ChIP and Northern blotting. To measure cell synchrony, a small sample was collected at each time point to count microscopically its septation index, which was then plotted as a percentage of cell septa against time.

For the ChIP assay, cross-linked chromatin was prepared from each sample, immunoprecipitated with an HA or myc antibody and the resulting IP DNA was analysed by PCR with primers specific for the *cdc15⁺*, *plo1⁺* and *fkh2⁺* promoter regions. All PCR-amplified DNAs were resolved on the same agarose gel to allow direct comparison of their levels. As a loading control, the respective WCE DNAs were also analysed by PCR and resolved by agarose electrophoresis. For Northern blotting, total RNAs were prepared from successive samples, resolved by formaldehyde gel electrophoresis and subjected to Northern blot analysis with DNA probes to assay the cell cycle mRNA levels of *cdc15⁺*, *plo1⁺* and *fkh2⁺*. In all cases, *cdc22⁺*, a gene periodically transcribed during

the G1-S interval independent of PBF-PCB controls, was also used as a probe for Northern blotting, to allow comparison between experiments. Finally, as a loading control the rRNA content of each RNA sample was detected by ethidium bromide staining. The results obtained from the three experiments are displayed in Figures 6.12, 6.13 and 6.14.

As seen in Figure 6.12, ChIP assays with the synchronous *cdc25-22 fkh2-3HA* cells revealed that Fkh2p-3HA contacted the promoters of *cdc15⁺*, *fkh2⁺* and *plo1⁺* in a cell cycle dependent manner. As shown by comparison of the ChIP and Northern blot data, detection of Fkh2p-3HA at the promoters of *cdc15⁺*, *fkh2⁺* and *plo1⁺* coincided with low mRNA levels of these genes during S phase and G2. Notably, maximum Fkh2p-3HA binding was observed when mRNA levels of *cdc15⁺*, *fkh2⁺* and *plo1⁺* were beginning to decrease, while no detectable binding of Fkh2p-3HA was seen when their mRNA levels reappeared. Residual Fkh2p binding was observed at zero times, corresponding to G2-arrested cells, when some gene expression occurred, but it is possible that this is an artefact of the experimental procedure. Overall, this cell cycle-specific pattern of promoter binding is consistent with a role for Fkh2p as a repressor of M-G1 specific transcription. In agreement with these findings, single gene and genome-wide microarray analyses have previously suggested that Fkh2p negatively regulates transcription of genes expressed during the M-G1 interval (Bulmer *et al.*, 2004; Rustici *et al.*, 2004; Petit *et al.*, 2005).

The results obtained from ChIP assays with the *cdc25-22 nmt41:plo1-3HA* cells are shown in Figure 6.13. Plo1p-3HA was detected at the promoters of *cdc15⁺* and *fkh2⁺* at specific cell cycle times, when the mRNA levels of the genes were low. However, the highest amounts of Plo1p-3HA were observed at the promoter regions of *cdc15⁺* and *fkh2⁺* after gene repression has occurred. Notably, when compared to cell cycle promoter binding of Fkh2p-3HA, the highest levels of Plo1p-3HA were found at the promoter regions later than those of Fkh2p-3HA, suggesting that Plo1p binding at the *cdc15⁺* and *fkh2⁺* promoters follows that of Fkh2p. Since previous work has shown that Plo1p positively modulates M-G1 specific transcription (Anderson *et al.*, 2002; Papadopoulou *et al.*, 2008), it is possible that the presence of Plo1p at the PCB promoter regions is part of a process that re-activates gene expression, following transcriptional repression by Fkh2p.

Figure 6.14 shows the cell cycle pattern of Sep1p binding to the promoters of *cdc15⁺* and *plo1⁺* in synchronously dividing *cdc25-22 sep1-13myc* cells. Sep1p-13myc bound to promoter DNA in a cell cycle dependent manner but, in contrast to Fkh2p-3HA, the highest levels of Sep1p-13myc were detected at the promoters of *cdc15⁺* and *plo1⁺* concurrent with maximum mRNA levels of these genes, while the lowest amounts of Sep1p-13myc binding coincided with the lowest *cdc15⁺* and *plo1⁺* mRNA levels. This behaviour is consistent with Sep1p being an activator of M-G1 specific transcription. This suggestion is in agreement with previous studies that have also shown that Sep1p

positively regulates transcription of M-G1 expressed genes (Bulmer *et al.*, 2004; Rustici *et al.*, 2004; Alonso-Nuñez *et al.*, 2005; Petit *et al.*, 2005).

It is important to note that *cdc15⁺* and *plo1⁺* transcription, as well as septation, was delayed by 40 minutes in the Sep1p-13myc experiment compared with the cell cycle experiments examining Fkh2p and Plo1p promoter binding (Figures 6.12 and 6.13). This observation implies that C-terminal tagging of Sep1p affects its function, resulting in both delayed transcription and septation (Papadopoulou *et al.*, 2008).

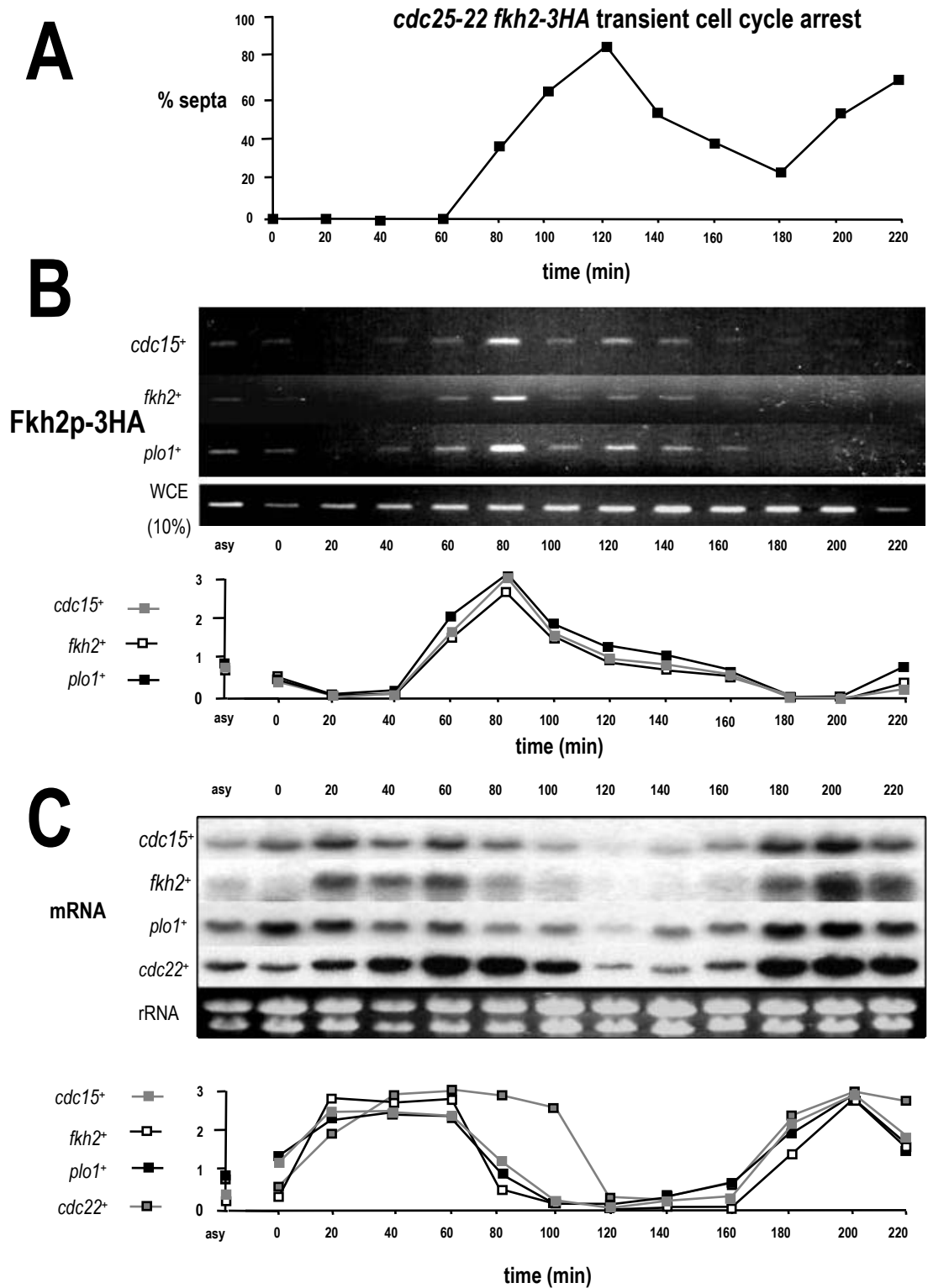
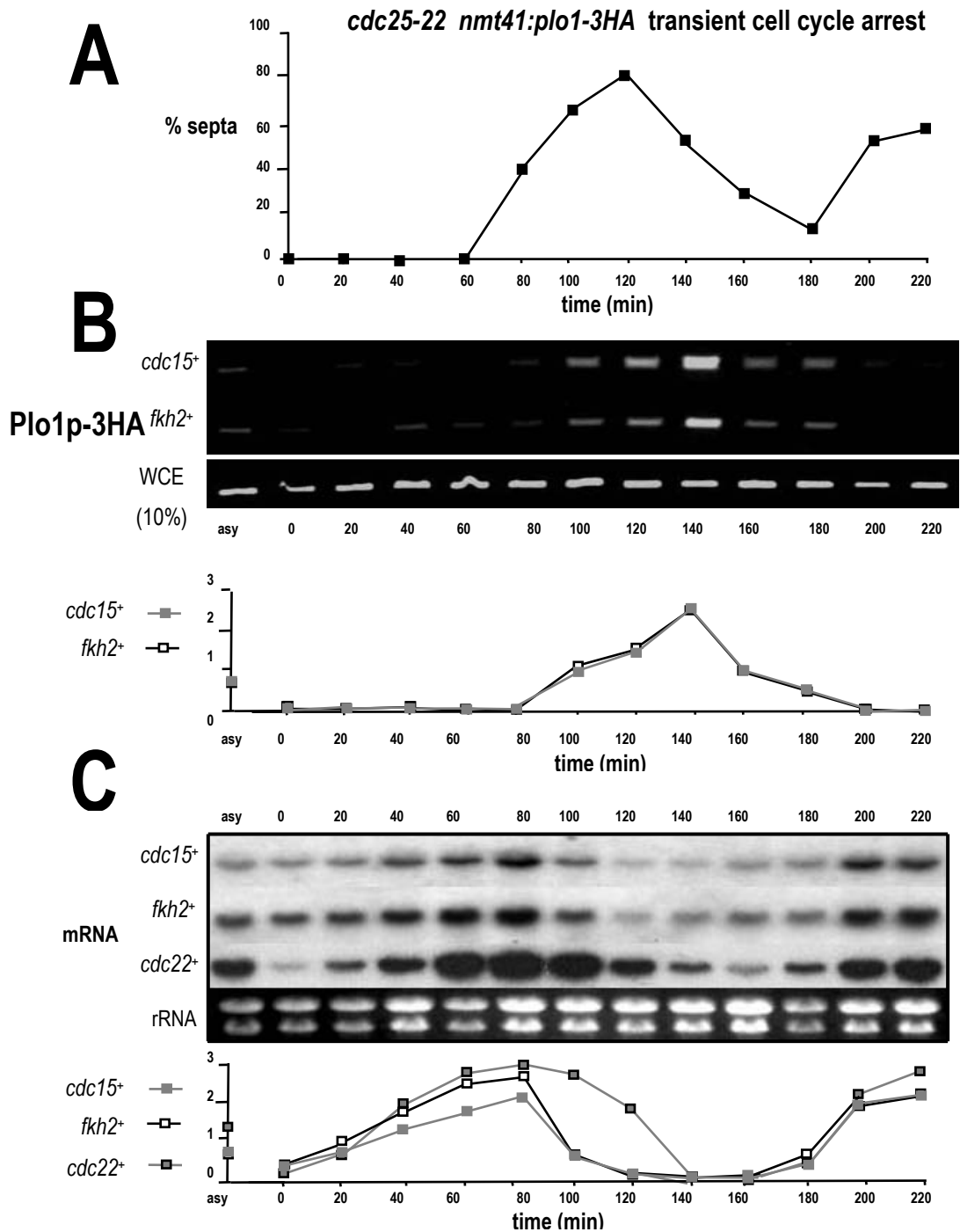


Figure 6.12: Cell cycle specific binding of Fkh2p to PCB promoter regions of *cdc15⁺*, *fkh2⁺* and *plo1⁺*. *cdc25-22 fkh2-3HA* (GG 745) cells were synchronised for cell division by transient temperature arrest. After returning the cells to the permissive temperature, samples were taken every 20 minutes and processed for ChIP and Northern blot analysis. (A) Septation indices were counted microscopically and plotted against time to measure cell synchrony. (B) For ChIP analysis, each cell sample was fixed with FA to cross-link DNA to protein and Fkh2p was immunoprecipitated from sheared cross-linked chromatin using an anti-HA antibody. Binding of Fkh2p-3HA to the *cdc15⁺*, *fkh2⁺* and *plo1⁺* promoters was detected by PCR. As a loading control, PCR was performed with 10% WCE DNA (WCE; non-

immunoprecipitated sample), and *plo1*⁺ oligonucleotides. Quantification of each immunoprecipitated sample against its non-immunoprecipitated control at the respective time point is shown. The ChIP assay was carried out twice with duplicate cell samples taken at successive time points from a culture of synchronous *cdc25-22 fkh2-3HA* cells. PCR analysis of DNA material (corresponding to either DNA immunoprecipitated with the HA antibody or WCE DNA) prepared from each set of successive samples was repeated twice and identical results were obtained. The results of PCR analysis of DNA prepared from one of the two sets of cross-linked cell samples, once processed for ChIP analysis, are shown here and are representative of the results of PCR analysis of both sets of samples. (C) For Northern blot analysis, total RNA was prepared from each cell sample. To detect mRNA, the blot was hybridised consecutively with *cdc15*⁺, *fkh2*⁺, *plo1*⁺ and *cdc22*⁺ probes, the latter a known G1-S phase expressed transcript independent of PBF-PCB controls. Quantification of each mRNA sample against its rRNA control at the respective time point is shown. For either ChIP or Northern blot analysis, asy indicates DNA or RNA samples respectively, prepared from asynchronous cells prior to temperature shift to the restrictive temperature.



time points from a culture of synchronous *cdc25-22 nmt41::plo1-3HA* cells. PCR analysis of DNA material (corresponding to either DNA immunoprecipitated with the HA antibody or WCE DNA) prepared from each set of successive samples was repeated twice and identical results were obtained. The results of PCR analysis of DNA prepared from one of the two sets of cross-linked cell samples, once processed for ChIP analysis, are shown here and are representative of the results of PCR analysis of both sets of samples. (C) For Northern blot analysis, total RNA was prepared from each cell sample. To detect mRNA, the blot was hybridised consecutively with *cdc15⁺*, *fkh2⁺* and *cdc22⁺* probes. Quantification of each mRNA sample against its rRNA control at the respective time point is shown. For either ChIP or Northern blot analysis, asy indicates DNA or RNA samples respectively, prepared from asynchronous cells prior to temperature shift to the restrictive temperature.

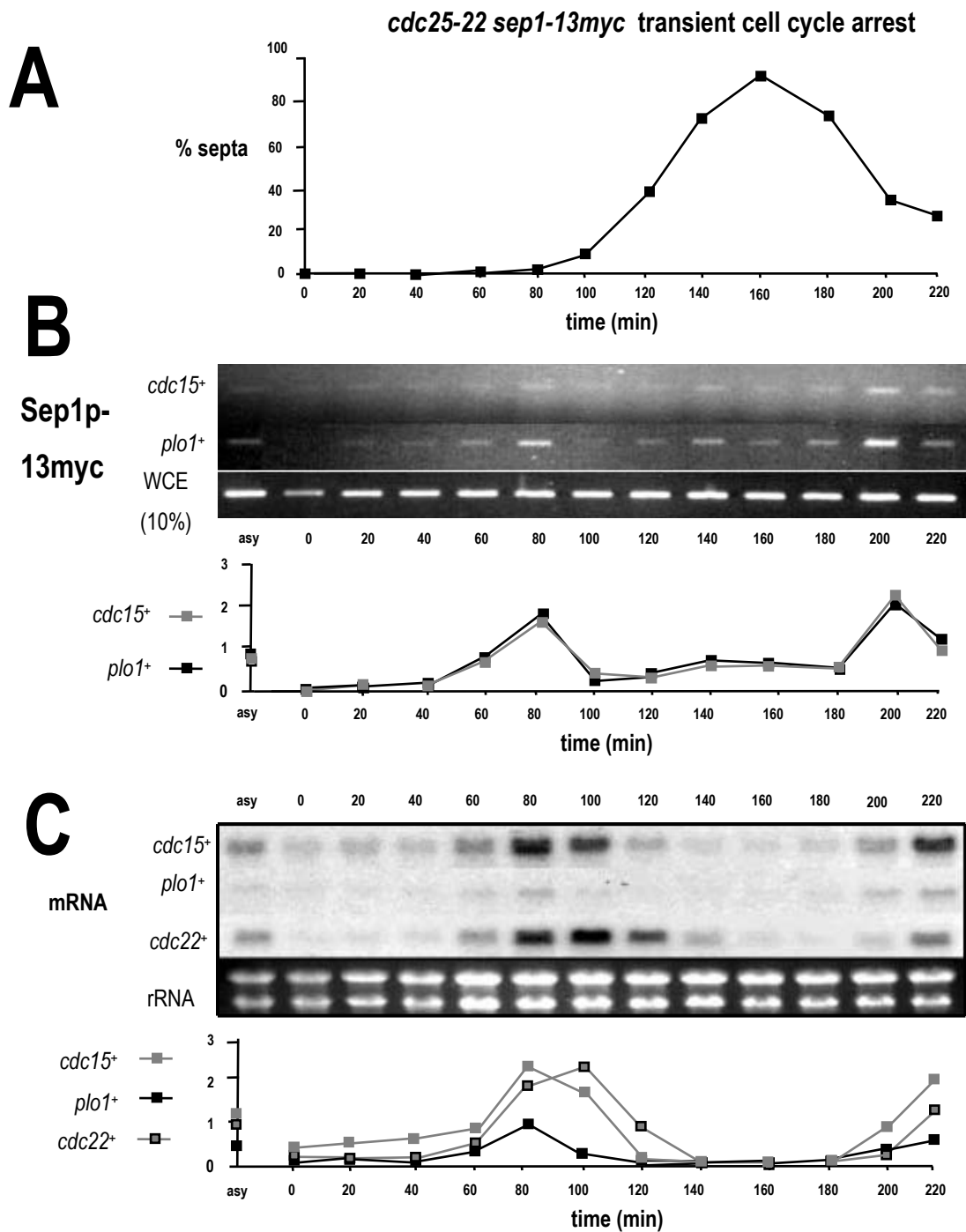


Figure 6.14: Cell cycle specific binding of Sep1p to PCB promoter regions of *cdc15+* and *plo1+*. *cdc25-22 sep1-13myc* (GG 1118) cells were synchronised by transient temperature arrest. After returning the cells to the permissive temperature, samples were taken every 20 minutes and processed for ChIP and Northern blot analysis. (A) Septation indices were counted microscopically and plotted against time to measure cell synchrony. (B) For ChIP analysis, each cell sample was fixed with FA and Sep1p was immunoprecipitated from sheared cross-linked chromatin using an anti-myc antibody. Binding of Sep1p-13myc to the *cdc15+* and *plo1+* promoters was detected by PCR. As a loading control, PCR was performed with 10% WCE DNA (WCE; non-immunoprecipitated sample), and *plo1+* oligonucleotides. Quantification of each immunoprecipitated sample against its non-immunoprecipitated control at the respective time point is shown. The ChIP assay was carried out twice with duplicate cell samples taken at successive time points from a

culture of synchronous *cdc25-22 sep1-13myc* cells. PCR analysis of DNA material (corresponding to either DNA immunoprecipitated with the HA antibody or WCE DNA) prepared from each set of successive samples was repeated twice and identical results were obtained. The results of PCR analysis of DNA prepared from one of the two sets of cross-linked cell samples, once processed for ChIP analysis, are shown here and are representative of the results of PCR analysis of both sets of samples. (C) For Northern blot analysis, total RNA was prepared from each cell sample. To detect mRNA, the blot was hybridised consecutively with *cdc15⁺*, *plo1⁺* and *cdc22⁺* probes. Quantification of each mRNA sample against its rRNA control at the respective time point is shown. For either ChIP or Northern blot analysis, *asy* indicates DNA or RNA samples respectively, prepared from asynchronous cells prior to temperature shift to the restrictive temperature.

6.3 Summary

Previous studies and work presented in this thesis have addressed, via distinct approaches, the regulatory network that drives phase-specific transcription at late mitotic stages in fission yeast. Here, ChIP assays were performed as another way to examine how components of this network, including the Fkh2p, Sep1p and Mbx1p transcription factors and the Plo1p kinase, regulate M-G1-dependent transcription in this model organism. Such analyses, using HA and myc antibodies against cross-linked chromatin extracts from strains expressing Sep1p, Fkh2p or Plo1p as HA and/or myc C-terminal fusions, revealed that Fkh2p, Sep1p and Plo1p associate *in vivo* with the promoters of genes transcribed during M-G1 phase. Specifically, either Fkh2p-3HA or Fkh2p-13myc was shown to bind to the promoters of *cdc15⁺*, *plo1⁺* and *fkh2⁺* itself, the latter showing that Fkh2p also regulates its own expression, consistent with previous observations (Buck *et al.*, 2004) (Figures 6.3 and 6.4). Moreover, Fkh2-3HA was detected at these promoters even in the absence of either Mbx1p or Sep1p or after compromising Plo1p function, in the *plo1-ts35* mutant at the restrictive temperature, suggesting that neither of these proteins is required for Fkh2p promoter binding (Figures 6.9, 6.10 and 6.11).

As shown in Figure 6.5, Sep1p-13myc bound at the promoters of *cdc15⁺* and *plo1⁺* but not *fkh2⁺*, the latter implying that Fkh2p and Sep1p might regulate common but also distinct M-G1 transcribed genes. Finally, Plo1p-3HA was detected at the promoters of *cdc15⁺* and *fkh2⁺*, whereas analysis of Plo1p-3HA binding to the *plo1⁺* promoter was precluded due to Plo1p-3HA being expressed under control of the *nmt41* and not the *plo1⁺* promoter (Figure 6.7).

cdc15⁺, *plo1⁺* and *fkh2⁺* contain PCB sequences in their promoters (Figure 3.8) and it was these PCB-containing regions that were amplified during the ChIP assay (Figure 6.2), in agreement with results in Sections 3.2 and 3.4, suggesting a cell cycle non-specific role for this motif in transcriptional regulation of these genes. However, these results are also consistent with other findings in Sections 3.3 and 3.4, revealing a regulatory role for the TGTTTAC forkhead-like sequence in M-G1 specific, Sep1p/Fkh2p-mediated, transcription of *cdc15⁺* and, presumably, of *plo1⁺* and *fkh2⁺*, since, as shown in Figure 6.2, the promoters of these genes contain this motif in close

proximity to the PCB. Overall, the presence, as shown here *in vivo* by ChIP analysis, of Fkh2p, Sep1p and Plo1p at the promoters of M-G1 transcribed genes is in concurrence with the previously ascribed roles for these proteins in regulation of the PBF-dependent gene cluster, as well as the proposition that Fkh2p and Plo1p exert their functions in M-G1 specific transcription through feedback mechanisms (Anderson *et al.*, 2002; Buck *et al.*, 2004; Bulmer *et al.*, 2004; Rustici *et al.*, 2004; Papadopoulou *et al.*, 2008).

Unlike Fkh2p, Sep1p and Plo1p, ChIP analysis, at least under the experimental conditions employed here, failed to detect Mbx1p bound at the *cdc15⁺*, *fkh2⁺* and *plo1⁺* promoters (Figure 6.8). Nonetheless, other findings support a role for Mbx1p in transcriptional regulation of these genes, including its requirement, at least *in vitro*, for binding of the PBF complex to the PCB sequence within the *cdc15⁺* promoter and its association with Plo1p, the latter implying that this transcription factor might be involved in Plo1p recruitment to the promoters of M-G1 transcribed genes, such as *cdc15⁺*, *fkh2⁺* and *plo1⁺* (Anderson *et al.*, 2002; Papadopoulou *et al.*, 2008; Section 7.1).

Previous work has suggested that Sep1p and Plo1p positively regulate M-G1 specific transcription, while Fkh2p appears to possess also negative modulatory roles (Anderson *et al.*, 2002; Buck *et al.*, 2002; Bulmer *et al.*, 2004; Rustici *et al.*, 2004; Alonso-Nuñez *et al.*, 2005; Petit *et al.*, 2005). In this study, Fkh2p, Sep1p and Plo1p promoter binding through the cell cycle was examined by ChIP to investigate how these proteins contribute to transcriptional control of their target genes, including *cdc15⁺*, *fkh2⁺* and *plo1⁺*. These studies showed that Fkh2p promoter binding coincides with declining transcript levels during G2 and S phase, suggesting that this forkhead transcription factor negatively influences transcription (Figure 6.12). Unlike Fkh2p, Sep1p binds to promoters when peak mRNA levels of its target genes are present, consistent with it behaving as a transcriptional activator instead (Figure 6.14). Finally, Plo1p contacts promoter DNA when low mRNAs levels of PCB-regulated genes are observed. Notably, however, Plo1p is found at the promoters later than Fkh2p, implying that Plo1p exerts its function after Fkh2p, which represses gene expression (compare Figures 6.12 and 6.13). This implies that Plo1p could participate, presumably in a manner that involves binding and phosphorylation of Mbx1p, in transcriptional reactivation of M-G1 expressed genes, following inhibition by Fkh2p (Papadopoulou *et al.*, 2008).

Chapter 7

Discussion

7.1 General discussion

Studies in *S. cerevisiae* and *S. pombe* have advanced current knowledge of the control mechanisms that drive mitotic cell cycle progression, unravelling the existence of exquisite regulatory components, predominantly the Cdks, as well as many other proteins, which are conserved among eukaryotes, a feature that exemplifies their significance for cell cycle controls (Sections 1.2 and 1.3). More recently, a combination of single-gene and global microarray analyses in these yeasts has provided insight into the ways by which discrete transcriptional networks accommodate phase-dependent expression of groups of co-regulated genes, encoding proteins that are required for functions concomitantly with their time of synthesis (Cho *et al.*, 1998; Spellman *et al.*, 1998; Rustici *et al.*, 2004; Oliva *et al.*, 2005; Peng *et al.*, 2005; Sections 1.5 and 1.6).

Four major consecutive waves of transcription characterise the mitotic cell cycle in fission yeast, coinciding with the G1, S, G2-M and M-G1 cell cycle stages (Section 1.5.2). This thesis has explored the regulatory network that underlies the expression of genes whose transcript levels peak during the M-G1 interval (Anderson *et al.*, 2002; Rustici *et al.*, 2004; Oliva *et al.*, 2005; Peng *et al.*, 2005). Consistent with their time of expression, many of these genes, including *sid2⁺*, *ace2⁺*, *slp1⁺*, *mid1⁺*, *spo12⁺*, *plo1⁺*, *fin1⁺*, *fkh2⁺* and *cdc15⁺*, encode proteins required for mitosis, cytokinesis and cell separation (McInerny *et al.*, 2004; Ng *et al.*, 2006; Chapter 1).

Earlier work has identified a transcription factor complex, PBF, which binds *in vitro* to the promoters of M-G1 transcribed genes in a manner that requires the 6 bp PCB sequence motif (GNAACR). In parallel, a PCB-containing *cdc15⁺* promoter fragment has been shown to confer M-G1-dependent transcription to *lacZ*, strongly suggesting that it encompasses the necessary element(s), presumably the PCB and other motif(s), for periodic expression of *cdc15⁺* and other co-regulated genes. Plo1p, a conserved kinase with a plethora of functions in mitosis and cytokinesis, has been shown to regulate M-G1-dependent transcription and is also required *in vitro* for PCB-dependent binding of PBF to the promoters of its targeted genes. Notably, *plo1⁺* is itself transcribed at the M-G1 transition and contains PCB sequences in its promoter suggesting that this kinase regulates periodic expression via feedback mechanisms (Anderson *et al.*, 2002).

Three transcription factors, two forkhead proteins, Fkh2p, and Sep1p, and a MADS-box protein, Mbx1p, have been implicated in M-G1 transcriptional control and are thought to be components of PBF. Similar to *plo1⁺*, *fkh2⁺* has also been proposed to exert its function via feedback mechanisms, since it is periodically expressed coincidentally with its target genes and contains PCB sequences in its promoter.

Mbx1p, although not required for periodic transcription *per se*, is necessary for binding of PBF to the PCB sequence. Unlike Mbx1p, Fkh2p and Sep1p are dispensable, at least *in vitro*, for PCB-dependent binding of the PBF complex but are absolutely required for M-G1 specific transcription. Moreover, given the involvement of Plo1p in M-G1-specific transcriptional control, both Fkh2p and Mbx1p have been shown to be periodically phosphorylated during late mitosis (Zilahi *et al.*, 2000; Buck *et al.*, 2004; Bulmer *et al.*, 2004).

Consistent with the requirement of forkhead transcription factors for M-G1-specific gene expression, database searches revealed that forkhead-related sequences are present at the promoters of M-G1 transcribed genes in both orientations and have been shown to be required for the expression of *ace2⁺*, one of these genes (Rustici *et al.*, 2004; Alonso-Nuñez *et al.*, 2005). Nonetheless, although Mbx1p is part of the regulatory network that mediates Sep1p/Fkh2p-dependent expression, the promoters of Sep1p/Fkh2p-regulated genes lack DNA-binding sequences for MADS-box transcription factors (Rustici *et al.*, 2004; Bähler, 2005). This situation is dissimilar to the modulatory network that drives G2-M specific expression in budding yeast, whereby the presence of both forkhead- and MADS-box- related sequences in the promoters of 'CLB2' genes accompanies their transcriptional regulation by Fkh2p/Sep1p (Fkh1p/Fkh2p) and Mbx1p (Mcm1p) homologues (Section 1.5.1.4). Therefore, it is assumed that fission yeast employs PCB sequences instead, which, either together or in parallel with forkhead motifs, mediate M-G1 specific transcription. In this study, various approaches have elucidated further how Fkh2p, Sep1p and Mbx1p transcription factors operate, in concert with the Plo1p kinase and through the PCB and forkhead-associated sequences, to drive M-G1 specific transcription of genes, including *cdc15⁺*, as well as *fkh2⁺* and *plo1⁺* themselves.

The PCB sequence has been shown here to be necessary for transcription of *cdc15⁺* in an M-G1 independent manner, since mutating the *cdc15⁺* PCB motif is sufficient to dramatically reduce or even abolish transcription of the *lacZ* reporter throughout the cell cycle (Buck *et al.*, 2004; Section 3.2). Presumably, the PCB has a similar role in transcriptional control of many other genes, co-regulated with *cdc15⁺* and possessing this DNA motif in their promoters (Figure 3.8). Unlike the PCB, the TGTTTAC forkhead-related motif appears to regulate the temporal, M-G1 specific, transcriptional pattern of *cdc15⁺*, since mutating the *cdc15⁺* TGTTTAC delays periodic transcription of *lacZ* mRNA levels, which no longer peak during late mitosis (Section 3.4). Interestingly, in the absence of the TGTTTAC sequence the cell cycle transcription of *lacZ* remains periodic, suggesting that other promoter elements can also support phase-specific transcription of *cdc15⁺*. Consistent with this, the *cdc15⁺* promoter fragment contains another forkhead motif in the reverse orientation, GTAAACA, also present in the promoters of many genes transcribed co-incidentally with *cdc15⁺* (Figure 3.8).

Hence, forkhead-related sequences could have redundant roles in M-G1 specific gene expression, although such a hypothesis necessitates further investigation. Finally, mutating both the PCB and the TGTTTAC sequence within the *cdc15⁺* promoter fragment compromises *lacZ* transcription in a cell cycle independent manner, similar to when mutating the PCB sequence alone, indicating that these promoter elements function in a parallel, but not additive, manner (Section 3.4).

Consistent with the involvement of PCB and forkhead sequences in transcriptional regulation of *cdc15⁺*, experiments presented in Section 3.3 demonstrate that in the absence of either Fkh2p or Sep1p, periodic transcription of *lacZ* under control of the PCB- and TGTTTAC- containing *cdc15⁺* promoter fragment is lost. Notably, ChIP assays in Section 6.2.1 reveal that these transcription factors bind *in vivo* to the promoter regions of M-G1 transcribed genes, including *cdc15⁺* and *plo1⁺*. Fkh2p also associates with the *fkh2⁺* promoter, thereby confirming the proposal that this factor regulates its own expression, while in parallel implying that it shares both common and distinct gene targets with Sep1p. Intriguingly, the two forkhead transcription factors are shown here to contact promoter DNA in a cell cycle specific manner. Indeed, Fkh2p is detected at the *cdc15⁺*, *fkh2⁺* and *plo1⁺* promoters during S and G2 phases when gene expression is minimal, whereas maximal Sep1p binding to the promoters of *cdc15⁺* and *plo1⁺* is observed coincidentally with their transcription at the M-G1 interval (Section 6.2.3). In concert with previous observations, these phase-specific patterns of promoter binding suggest that Fkh2p and Sep1p exert negative and positive roles in M-G1-specific gene expression, respectively (Buck *et al.*, 2004; Bulmer *et al.*, 2004; Rustici *et al.*, 2004).

Fkh2p and Sep1p are shown here to interact with each other *in vitro* and *in vivo* (Sections 4.2.2 and 4.3.8), in agreement with the observations of earlier overexpression studies, suggesting also a requirement of Sep1p for Fkh2p function (Buck *et al.*, 2004). Nonetheless, as shown in Section 6.2.2, Fkh2p is detected at the *cdc15⁺*, *fkh2⁺* and *plo1⁺* promoters even in the absence of Sep1p. Hence, at least the promoter binding properties of Fkh2p are not affected by Sep1p, although it is still possible that Sep1p influences the cell cycle pattern of Fkh2p-dependent promoter association and not binding *per se*. Furthermore, Mbx1p and Plo1p are also not required for the interaction of Fkh2p with the *cdc15⁺*, *fkh2⁺* and *plo1⁺* promoters (Section 6.2.2).

Protein phosphorylation, mediated by a suitable kinase, is commonly employed by the cell machinery to regulate transcription by affecting the properties of transcription factors, including DNA binding, protein stability, cellular localisation or interactions with co-regulators or components of the basal transcriptional apparatus (Whitmarsh and Davis, 2000). Interestingly, recent work showed that Plo1p phosphorylates Mbx1p *in vitro*, while overexpression of a *plo1* kinase mutant prevents expression of M-G1 transcribed genes, such as *cdc15⁺* and *spo12⁺*. This is the first time a Polo-like kinase has been shown to bind and phosphorylate a MADS-box protein in any organism. Moreover,

Plo1p is shown here to associate *in vivo* with Mbx1p in a manner that requires both its kinase and polo-box domain and irrespective of cell cycle stage (Papadopoulou *et al.*, 2008; Chapter 5). Taken together, these findings indicate that Plo1p regulates M-G1-specific gene expression in a manner that involves binding and phosphorylation of Mbx1p.

Consistent with the ability of Plo1p to bind and phosphorylate Mbx1p and regulate M-G1 specific transcription, this kinase is shown in Section 6.2.1 to interact *in vivo* with the promoters of *cdc15⁺* and *fkh2⁺*. Plo1p interacts with the promoters of these genes in a cell cycle-dependent manner with a timing that coincides with low levels of expression, but follows promoter binding by Fkh2p. This implies that Plo1p promoter contact causes a function later than that of Fkh2p, which is thought to negatively affect transcription of their co-regulated genes (Section 6.2.3).

Given that Plo1p can associate directly with Mbx1p and contact the promoters of their co-regulated genes, it is possible that this kinase is recruited to these promoters in an Mbx1p-dependent manner. Indeed, although ChIP assays described in Section 6.2.1 have been unable to detect Mbx1p at the promoters of M-G1 transcribed genes, recent studies reveal that Plo1p is unable to interact *in vivo* with the promoters of *cdc15⁺* and *fkh2⁺* in the absence of Mbx1p (Mead, E. and McInerney, C.J., unpublished data). Since previous experiments have also shown that Mbx1p is necessary for PCB-dependent binding of the PBF complex to the promoters of M-G1 transcribed genes, at least *in vitro*, these findings suggest that Mbx1p associates with the promoters of PBF-regulated genes and that technical reasons have prevented this to be shown here *in vivo* by ChIP. Furthermore, Mbx1p binds to Plo1p throughout the cell cycle, while Plo1p interacts with PCB promoter DNA in a cell-cycle specific manner, suggesting that a sub-population of Mbx1p is not in contact with gene promoters (Papadopoulou *et al.*, 2008).

Taken together, this study has provided further insight into the regulatory network that promotes M-G1 phase-specific transcription in fission yeast. Presumably, additional molecular mechanisms and components await discovery, but work so far shows that Fkh2p, Sep1p and Mbx1p transcription factors and the Plo1p kinase control periodic gene expression during late mitosis through the PCB and forkhead promoter sequences, the former modulating the amplitude of transcription throughout the cell cycle and the latter its periodicity during the M-G1 phase (Figure 7.1). Possibly, Mbx1p contacts the PCB promoter regions of M-G1 transcribed genes throughout the cell cycle, while Fkh2p and Sep1p interact with the forkhead-related promoter sequences of these genes in a cell cycle specific manner, consistent with Fkh2p repressing and Sep1p activating gene expression (Anderson *et al.*, 2002; Papadopoulou *et al.*, 2008). Regulation of transcription probably occurs through one forkhead transcription factor replacing the other in a stepwise manner, with transcriptional activation by Sep1p preceding repression by Fkh2p and *vice versa* (Figure 7.1). The

fact that Fkh2p and Sep1p interact, suggests that this process may occur through the two forkhead factors transiently binding to each other.

In concert with a role for Sep1p as a transcriptional activator, genetic analyses reveal that *sep1⁺* genetically interacts with *sep15⁺*, itself encoding a component of the Mediator complex (Grallert *et al.*, 1999; Lee *et al.*, 2005a). Thereby, Sep1p could stimulate gene expression via its association with the Mediator, promoting M-G1-specific promoter recruitment of the basal Pol II machinery and ensuing initiation of transcription. By contrast, Fkh2p could repress transcription, perhaps via the recruitment of factors that silence transcription, including histone deacetylases.

It is possible that Fkh2p and Sep1p antagonise each other for binding to the same promoter sequences, while the function of additional proteins, such as Plo1p, might help shift the balance in favour of either one forkhead transcription factor. Indeed, Plo1p contacts the promoters of M-G1 transcribed genes in an Mbx1p-dependent manner, with a timing that follows transcriptional repression by Fkh2p.

Presumably, given its positive roles in gene expression, Plo1p invokes a function that involves binding and phosphorylation of Mbx1p and resulting in transcriptional re-activation, mediated by Sep1p, during the ensuing late mitosis-early G1 phase. Interestingly, Plo1p and Fkh2p appear to be self-regulated, thereby controlling their own expression via positive and negative feedback loops, respectively (Figure 7.1) (Papadopoulou *et al.*, 2008). Furthermore, Fkh2p is itself phosphorylated almost coincidentally with Mbx1p, but not by Plo1p (Buck *et al.*, 2004; Bulmer *et al.*, 2004). Hence, it is tempting to speculate that a kinase other than Plo1p, mediating changes in the phosphorylation status of Fkh2p, might also be involved in transcriptional regulation during the M-G1 interval. One possible candidate is Cdc2p, though this seems unlikely as it is known that this Cdk must be inactivated to allow cytokinesis to occur in fission yeast (Krapp *et al.*, 2004); hence, another kinase must instead play a role.

Overall, this and earlier studies have explored the transcriptional network that drives M-G1 phase-specific gene expression in fission yeast, encompassing a Plk and members of the MADS-box and forkhead/HNF-3 transcription factor families. Related transcriptional systems operate both in budding yeast and humans and regulate important mitotic regulators, including Plo1p homologues, hence, exemplifying their importance for cell cycle controls (Kumar *et al.*, 2000; Pic *et al.*, 2000; Zhu *et al.*, 2000; Alvarez *et al.*, 2001; Darieva *et al.*, 2006; Fu *et al.*, 2008; Section 1.6).

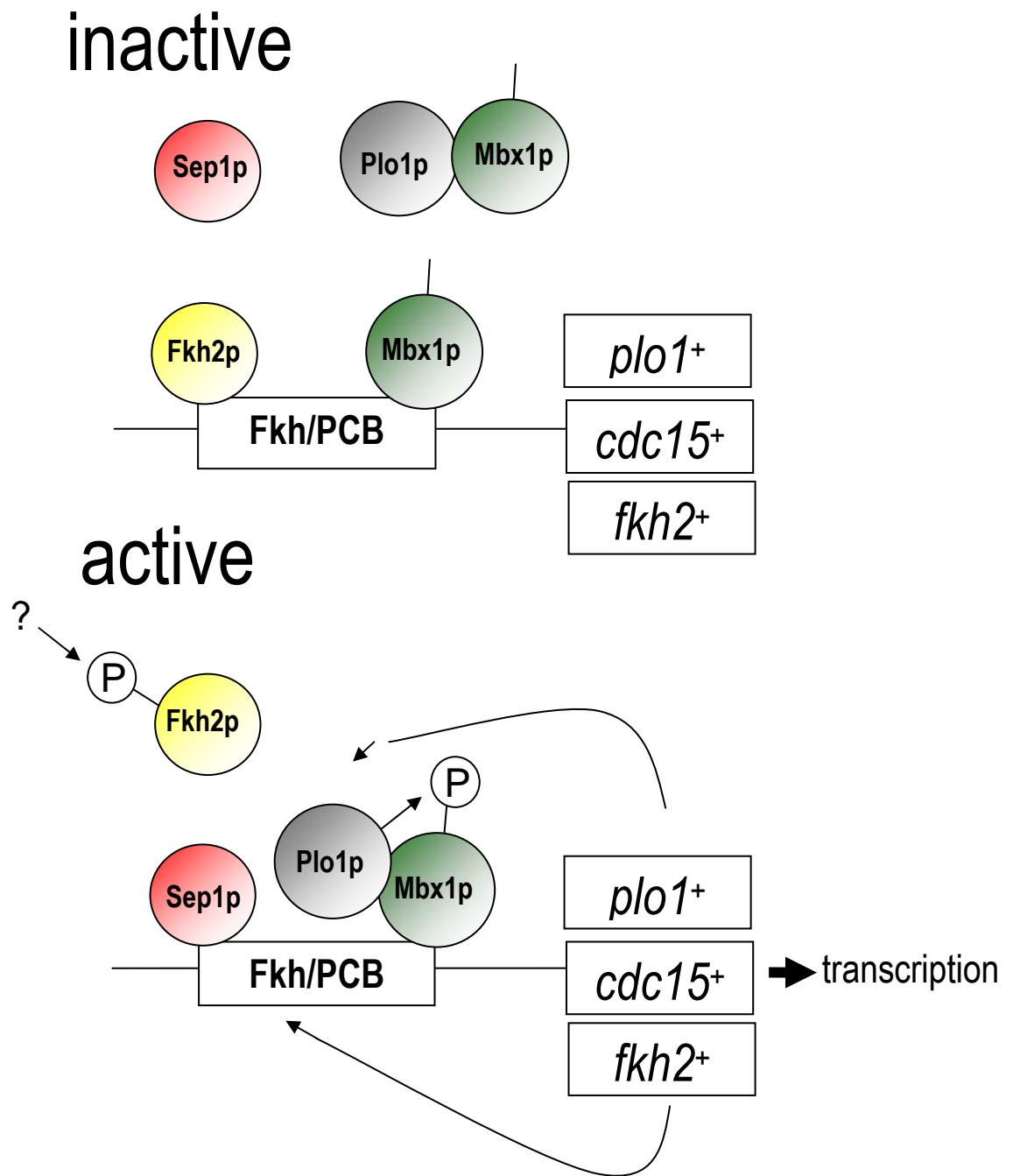


Figure 7.1: Model of Plo1p, Fkh2p and Sep1p regulation of M-G1 gene expression in fission yeast. Periodic expression of several genes, including *cdc15⁺*, during late M-early G1 phase in fission yeast is controlled by at least three transcription factors, Mbx1p, a MADS-box protein, and Fkh2p and Sep1p, both forkhead transcription factors. Fkh2p and Sep1p associate with the promoters of these genes, possibly through forkhead sequences (TGTTTAC and/or GTAAACA), in a cell cycle specific manner, consistent with Fkh2p repressing and Sep1p activating gene expression. Moreover, Mbx1p appears to associate constantly with the promoters of genes, such as *cdc15⁺*, in a PCB-dependent manner. Following transcriptional repression by Fkh2p during S and G2 phases, the Plo1p kinase is recruited at the PCB promoter regions and directly binds Mbx1p, thereby invoking a function that re-stimulates transcription. Plo1p itself contacts promoter DNA in a manner that requires Mbx1p, a sub-population of which is not in contact with the promoters of Fkh2p/Sep1p-regulated genes. Finally, *plo1⁺* and *fkh2⁺* appear to regulate their own expression, transcribed coincidentally with their co-regulated genes and interacting with their own promoters (see Section 7.1 for details) (adapted from Papadopoulou *et al.*, 2008).

7.2 Future Work

7.2.1 Elucidation of the role of the GTAAACA sequence in M-G1 specific transcription of *cdc15*⁺

In fission yeast, studies so far have implicated two promoter motifs, the PCB and forkhead-like sequences, in M-G1 specific transcription of genes under control of Mbx1p, Fkh2p and Sep1p PBF-related components and the Plo1p kinase (Anderson *et al.*, 2002; Buck *et al.*, 2004; Rustici *et al.*, 2004; Alonso-Nuñez *et al.*, 2005). Interestingly, computer-based searches reveal that the forkhead motif is present in both orientations, either TRTTTAY or RTAAAYA, with the former being statistically more significant than the latter. Nonetheless, recent data have shown that Fkh2p binds to RTAAAYA-like sequences (also called FLEX) in the promoter of *ste11*⁺, which encodes a transcription factor required for conjugation and meiosis during sexual development, to positively regulate its expression. The involvement of Fkh2p in regulation of *ste11*⁺ explains the partial sterile phenotype of *fkh2*Δ cells (Shimada *et al.*, 2008).

Here, the PCB promoter sequence was shown to be required for transcription of *cdc15*⁺ throughout the cell cycle, whereas the TGTTTAC forkhead-related motif appears to play a role in M-G1 specific transcription of *cdc15*⁺. Notably, the *cdc15*⁺ promoter also contains a GTAAACA forkhead-related sequence, suggesting that it could contribute to phase-specific transcription of *cdc15*⁺ and perhaps of other M-G1 transcribed genes. Hence, it would be interesting to evaluate whether, and if so how, mutating the GTAAACA sequence affects M-G1 specific transcription of *cdc15*⁺. Similar to experiments performed to assess the role of the *cdc15*⁺ PCB and TGTTTAC sequences, the GTAAACA of the *cdc15*⁺ promoter fragment driving transcription of *lacZ* could be mutated and then Northern blot analysis performed to monitor the cell cycle transcriptional profile of the reporter. Moreover, it would be interesting to test by similar Northern blot analyses-based approaches the contribution of PCB, as well as TGTTTAC and/or GTAAACA-like sequences in cell cycle transcription of M-G1 transcribed genes other than *cdc15*⁺.

7.2.2 Significance of *plo1*⁺ and *fkh2*⁺ M-G1 specific transcription in cell cycle controls

A recurrent theme emerging from earlier work and data presented in this thesis is that *plo1*⁺ and *fkh2*⁺ are components of the regulatory network that brings about M-G1 phase-specific gene expression and they are also themselves cell cycle regulated, transcribed coincidentally with the genes under their control (Anderson *et al.*, 2002; Buck *et al.*, 2004; Bulmer *et al.*, 2004; Rustici *et al.*,

2004). The *plo1⁺* and *fkh2⁺* promoters contain PCB and forkhead motifs (Figure 3.8) while, as shown in Sections 6.2.1 and 6.2.3, Fkh2p binds to its own promoter *in vivo* (Papadopoulou *et al.*, 2008). Although ChIP analysis of Plo1p interactions with its own promoter was not feasible (Section 6.2.1), other findings suggest that Plo1p also regulates its own transcription and that of other PBF-regulated genes via the PCB sequence (Anderson *et al.*, 2002). Moreover, strong *fkh2⁺* over-expression is not tolerated by fission yeast, suggesting that *fkh2⁺* expression is significant for cell cycle controls, necessitating the existence of an auto-regulatory feedback mechanism to modulate *fkh2⁺* expression in a subtle manner (Buck *et al.*, 2004). Interestingly, budding yeast and human homologues of *plo1⁺* and *fkh2⁺* are also cell cycle-regulated, suggesting that their phase-specific expression is important in eukaryotic cell cycle controls (Alvarez *et al.*, 2001; Darieva *et al.*, 2006). Hence, it will be interesting to assess the importance of PCB- or TGTTTAC- driven transcription of *fkh2⁺* and *plo1⁺* on their own expression, and that of their co-regulated genes.

Appropriate fission yeast strains could be constructed that contain mutations in the PCB or TGTTTAC motif(s) in the native chromosomal *plo1⁺* or *fkh2⁺* promoter regions. Northern blot analyses would then be performed with these strains to initially monitor how mutating either the PCB or TGTTTAC sequence(s) of endogenous *fkh2⁺* and *plo1⁺* influences the cell cycle transcriptional profile of *fkh2⁺* and *plo1⁺* themselves. Experiments presented in Chapter 3 have shown that the PCB and TGTTTAC sequence modulate transcription of *cdc15⁺*, either in terms of amplitude or M-G1 specific periodicity respectively, suggesting that manipulating the endogenous *fkh2⁺* or *plo1⁺* PCB or TGTTTAC promoter sequence(s) would also interfere, in each case, with transcription of the cognate gene. Finally, having established the importance of the PCB or TGTTTAC promoter sequence in cell cycle transcription of endogenous *fkh2⁺* and *plo1⁺*, it would be interesting to determine the effects of aberrant *plo1⁺* and/or *fkh2⁺* transcriptional patterns on cell cycle expression of all M-G1 transcribed genes by micro-array analysis and also examine other cellular phenotypes in these strains, particularly those related to cytokinesis and septation.

7.2.3 Identification of Plo1p phosphorylation site(s) in Mbx1p

In this thesis, Plo1p was shown to associate *in vivo* with Mbx1p in a manner that requires both an intact kinase and polo-box domain (Chapter 5). Consistent with this, Plo1p can directly phosphorylate Mbx1p *in vitro* (Papadopoulou *et al.*, 2008). To further explore Plo1p-driven phosphorylation of Mbx1p, it is essential to map the target phosphorylation site(s) of this Plk. The D/E-X-S/T-φ-X-D/E sequence has already been identified as the peptide phosphorylation consensus for human Plk1p and has been used successfully in budding yeast to determine the target

phosphorylation site of Cdc5p within the Ndd1p co-activator protein (Nakajima *et al.*, 2003; Darieva *et al.*, 2006; Section 1.5.1.4).

Based on the Plk1p phosphorylation consensus, examination of the Mbx1p peptide sequence shows that it contains two potential Plo1p target sites, one serine at position 46 and another at position 106 (Figure 7.2). To determine whether Plo1p phosphorylates serine-46 and/or serine-106, site-directed mutagenesis could be performed to mutate these residues to alanine, express and purify in bacteria the resulting mutant forms of Mbx1p and then test their susceptibility to phosphorylation by Plo1p, expressed and purified in budding yeast, in an *in vitro* kinase assay. It is anticipated that if serine-46 and/or serine-106 of Mbx1p are normally phosphorylated by Plo1p *in vivo*, Mbx1p mutants, containing either alanine-46 or alanine-106, when compared to wild-type Mbx1p, would be inefficiently phosphorylated *in vitro* by Plo1p.

Although serine-46 and serine-106 represent potential Plo1p phosphorylation sites, it is possible that Mbx1p is phosphorylated by this kinase at other sites instead or in parallel with these residues. To explore this possibility, MALDI-TOF analysis could be employed to map Plo1p-dependent phosphorylation sites in Mbx1p. Specifically, the phosphorylated products of an *in vitro* kinase assay with Plo1p and Mbx1p would be resolved by SDS-PAGE, followed by in-gel protein digestion with trypsin and analysis of the respective peptide fragments by MALDI-TOF mass fingerprinting and MALDI-TOF/TOF peptide sequencing. Finally, any identified Mbx1p phosphorylated residues, other than serine-46 and serine-106, would be once again mutated to alanine and the respective Mbx1p mutants tested by *in vitro* kinase assays for their Plo1p-specific phosphorylation efficiency.

```
MDINPPPSTA PSSPRRSIQR ISDAKNKALT FNRRRLGLIK KAHELSVLCD AKVVVMIFDS
KNACHVYSSE EPEEQRDALL QKFLNKDFVT VDPLRNINPN IPSDESLHNW RPKDKRIASV
TTYSAQPSNN CSSATDSEND FQSFTIKSST TYHTTPTTAS ENKKIESITI PDHASVYNDL
PLSPTVKHSF VSPVSGDYSD SPLEPSSSSS FSVPPESLNP TLSFQHNDVP QTDNFIPFLT
PKRQAYGQSS SRADRSSVRR SQSFKNRRNG KPRISRLHTS HASIDGLTDF IQSPSSGYLD
PSSTPITPLD SAINQITPPF LPDNLGQENR GELYSHDNPT SMVYEHPKFD ELPNGFIDTH
ELNILSRST ASPNQILRES NMVNQDSFTD NPVDATWDAL IGTTQIDL DL DYERSSIPSS
TIPADQLKDG VPTNSVYRNN MVDHNLPSL NIERNAP
```

Figure 7.2: Potential Plo1p phosphorylation sites within the Mbx1p amino acid sequence. Two potential Plo1p phosphorylation sites, which resemble the human Plk1p phosphorylation consensus (D/E-X-S/T-φ-X-D/E, where X and φ are any and hydrophobic amino acids), are present within the Mbx1p amino acid sequence (www.sangercenter.org.uk), highlighted in yellow and flanking the one serine at position 46 and the other serine at position 106. Both putative Plo1p target serine residues are highlighted in red.

7.2.4 Role of Mbx1p phosphorylation by Plo1p in regulation of M-G1 specific transcription

After mapping the Mbx1p serine and/or threonine residues phosphorylated by Plo1p *in vitro*, it would be important to assess their *in vivo* biological significance, predominantly how their phosphorylation affects regulatory aspects of M-G1 specific transcription, including binding of Mbx1p to Plo1p, association of Mbx1p, Plo1p, Sep1p and Fkh2p with the promoters of M-G1 transcribed genes and transcription of these genes *per se*. For this, appropriate fission yeast strains could be constructed that express Mbx1p mutant versions, where the relevant serine and/or threonine residues are replaced by either alanine or aspartic acid, thereby mimicking un-phosphorylatable and constitutively phosphorylated protein forms, respectively. Initially, the cellular phenotypes of these strains would be examined, in particular whether they display defects in cytokinesis, similar to *mbx1Δ* cells (Buck *et al.*, 2004; Section 3.3). Co-immunoprecipitation experiments could be performed with these strains to determine how distinct mutations in the Plo1p phosphorylation site(s) of Mbx1p affect the association with Plo1p. In parallel, yeast two-hybrid analysis could be completed with prey constructs expressing the mutant forms of Mbx1p and a previously described bait vector expressing Plo1p (Reynolds and Ohkura, 2003; Papadopoulou *et al.*, 2008) to test the requirement of individual Plo1p phosphorylation sites of Mbx1p and their status for interaction with this Plk.

Fission yeast strains expressing either un-phosphorylatable or constitutively phosphorylated forms of Mbx1p could also be used in ChIP assays to determine whether and/or how the phosphorylation status of Mbx1p and thus, Plo1p-driven phosphorylation of this PBF component, influences the association of Mbx1p, as well as that of Plo1p, Sep1p and Fkh2p with the promoters of M-G1 transcribed genes. In the case of Mbx1p, it would be interesting to elucidate whether, and if so how, changes in its phosphorylation status alter its binding properties to promoter DNA. ChIP experiments so far have failed to detect promoter binding of Mbx1p *in vivo* (Section 6.2.1; Papadopoulou *et al.*, 2008). Nonetheless, since Mbx1p is absolutely required *in vitro* for PBF binding to PCB promoters (Anderson *et al.*, 2002), it is reasonable to assume that Mbx1p also binds to the promoters of the respective genes *in vivo*, and thus that it is due to a technical failure that its promoter binding escapes detection with ChIP. Indeed, as mentioned earlier, recent ChIP experiments establish a dependency of Plo1p promoter association on the presence of Mbx1p (Mead, E. and McInerney, C.J., unpublished data). Hence, it would be interesting to study, either by ChIP and/or gel retardation assays, how mutating distinct Plo1p phosphorylation sites in Mbx1p affects promoter association of Mbx1p itself, as well as of Plo1p. In light of data presented here showing that Plo1p interacts with PCB promoter regions in a cell cycle specific manner (Section

6.2.3) it would also be appealing to investigate whether there are any changes in the cell cycle pattern of Plo1p promoter contact in these Mbx1p mutants.

Apart from Plo1p, Fkh2p and Sep1p also bind to promoters in a cell cycle-dependent manner that is suggestive of Fkh2p repressing and Sep1p activating transcription (Section 6.2.3; Papadopoulou *et al.*, 2008). Data presented in Section 6.2.2 show that Fkh2p can bind to promoter DNA in the absence of Mbx1p. Nonetheless, it is still possible that mutating the Plo1p-specific phosphorylation sites, although not affecting Fkh2p promoter binding, might have an impact on Fkh2p cell cycle promoter binding *in vivo*. Unlike Fkh2p, it is yet unclear whether Sep1p requires Mbx1p for binding to promoters. Therefore, it would be interesting to examine whether and how the presence of un-phosphorylatable or constantly phosphorylated versions of Mbx1p impacts on the binding of Fkh2p or Sep1p to the promoters of PBF co-regulated genes.

Previous work and data shown here have revealed that Plo1p function mediates M-G1 specific transcription (Anderson *et al.*, 2002; Papadopoulou *et al.*, 2008; Sections 6.2.1 and 6.2.3). Given that Plo1p binds and phosphorylates Mbx1p, it is possible that mutating the respective phosphorylation residues in Mbx1p influences the cell cycle expression pattern of target M-G1 transcribed genes. Therefore, Northern blot analysis could be performed with strains expressing the phosphorylation mutant versions of Mbx1p to assay their effect on cell cycle transcription of these genes.

7.2.5 *In vivo* analysis of Sep1p and Plo1p promoter association dependencies

Although both Sep1p and Plo1p were shown in this study to interact *in vivo* with the promoters of *cdc15⁺* and either *plo1⁺* or *fkh2⁺*, it is yet unclear whether they can associate with promoter DNA in the absence of one another, Fkh2p and, at least for Sep1p, Mbx1p as well. Such ChIP analyses of promoter binding requirements have so far been performed for Fkh2p, which, as shown in Section 6.2.2, can still bind to promoter DNA in the absence of Mbx1p, Sep1p or functional Plo1p. The presence of Plo1p at the promoters of M-G1 transcribed genes has recently been shown to depend on Mbx1p (Mead, E. and McInerney, C.J., unpublished data). It would be tempting to examine whether Fkh2p or Sep1p are also required for Plo1p promoter contact.

Sep1p and Fkh2p have been shown here to interact with each other (Sections 4.2.2 and 4.3.8), while both Plo1p and Mbx1p are required for PCB-dependent promoter binding of PBF. Taken together, it would be interesting to study whether the presence of Fkh2p, Plo1p or Mbx1p affects Sep1p binding to the promoters of M-G1 transcribed genes.

7.2.6 Genome-wide analysis of Plo1p, Fkh2p, Sep1p and Mbx1p promoter binding sites

In this study Fkh2p and Sep1p were shown to associate *in vivo* with the promoters of a limited number of genes, including *cdc15⁺*, *plo1⁺* and/or *fkh2⁺* (Chapter 6; Papadopoulou *et al.*, 2008). Nonetheless, global microarray analyses reveal that more than forty M-G1 transcribed genes are positively regulated by Sep1p, whilst these genes also appear to be negatively regulated by Fkh2p (Rustici *et al.*, 2004). Therefore, it would be interesting to assess whether Sep1p and Fkh2p bind to the promoters of all of these genes. Since, Plo1p and Mbx1p are also involved in regulation of M-G1 transcription, it would be tempting to monitor whether they also interact with the promoters of these Sep1p and Fkh2p gene targets. To identify which of the M-G1 transcribed genes are targeted by Sep1p, Fkh2p, Plo1p and Mbx1p, as well as impartially and globally screen the DNA-binding sites targeted by these proteins, ChIP-chip analysis could be employed. ChIP-chip is a novel high-throughput technology that combines chromatin immunoprecipitation with microarrays to unravel the genome-wide targets of transcription factors and/or other proteins (Wu *et al.*, 2006). Here, the exploitation of ChIP-chip technologies could allow the identification of all the Fkh2p, Sep1p, Plo1p and Mbx1p associated promoter DNAs in fission yeast, thereby revealing both common but also perhaps distinct genome-wide promoter targets. Such studies could also provide insight into the intricate interplay between these factors and the Plo1p kinase that drives M-G1 phase specific gene expression. These experiments are currently being undertaken in collaboration with Dr. Jürg Bähler's group (Wellcome Centre, Hinxton).

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Appendix I: Oligonucleotides

Lab number

- GO 46 5'- CAC GCC TGG CGG ATC TG-3'
- GO 47 5'- CTA AAC TCA CAA ATT AGA GC-3'
- GO 398 5'- GCA ACG GTT GCT AGG GAC-3'
- GO 400 5'- GTT GCT AGG GAC GGT TGT C-3'
- GO 568 5'- GGG GGA TCC GAA TGA CTG TTC GCA GAC TC-3'
- GO 569 5'- GGG CTC TCG AGC ACT ACT TTT AAC ATT-3'
- GO 570 5'- CCG CCC CGG GGA TGA ATT TTA ATT CTA CTA ACC CT-3'
- GO 571 5'- CCCTCTCGAG CTTAGAATAG TGTTGAAGTTTGA CG-3'
- GO 572 5'- GCG CCC CGG GGA TGG ATA TTA ATC CTC CTC CT-3'
- GO 573 5'- GGGCT CTCGAG CTTAAGGGG CATTTCG TTCAAT-3'
- GO 588 5'- TCG AGT TCC TGG TAG TCG ATT TCA GGA AAA GAG TAA ACA TGT TTG TTT
ACT GCG ACT ACT GCG GTG ACA ACC GTC CCT AGC AAC AAC CAA CTA
TCT GAG TGC ACA ATA C-3'
- GO 589 5'- TCG AGT ATT GTG CAC TCA GAT AGT TGG TTG TTG CTA GGG ACG GTT GTC
ACC GCA GTA GTC GCA GTA AAC AAA CAT GTT TAC TCT TTT CCT GAA ATC
GAC TAC CAG GAA C-3'
- GO 590 5'- TCG AGT ATT GTG CAC TCA GAT AGA AGG TTG TTG CTA GGG ACG GTT GTC
ACC GCA GTA GTC GCA GTA AAC AAA CAT GTT TAC TCT TTT CCT GAA ATC
GAC TAC CAG GAA C-3'
- GO 591 5'- TCG AGT TCC TGG TAG TCG ATT TCA GGA AAA GAG TAA ACA TGT TTG TTT
ACT GCG ACT ACT GCG GTG ACA ACC GTC CCT AGC AAC AAC CTT CTA TCT
GAG TGC ACA ATA C-3'
- GO 701 5'- TCG AGT ATT GTG CAC TCA GAT AGG CAA CGG TTG CTA GGG ACG GTT GTC
ACC GCA GTA GTC GCA GTA AAC AAA CAG TGG GGT TCT TTT CCT GAA ATC
GAC TAC CAG GAA C-3'
- GO 702 5'- TCG AGT TCC TGG TAG TCG ATT TCA GGA AAA GAA CCC CAC TGT TTG TTT
ACT GCG ACT ACT GCG GTG ACA ACC GTC CCT AGC AAC CGT TGC CTA TCT
GAG TGC ACA ATA C-3'

GO 703 5'- TCG AGT ATT GTG CAC TCA GAT AGT TGG TTG TTG CTA GGG ACG GTT GTC
ACC GCA GTA GTC GCA GTA AAC AAA CAG TGG GGT TCT TTT CCT GAA ATC
GAC TAC CAG GAA C-3'

GO 704 5'- TCG AGT TCC TGG TAG TCG ATT TCA GGA AAA GAA CCC CAC TGT TTG TTT
ACT GCG ACT ACT GCG GTG ACA ACC GTC CCT AGC AAC AAC CAA CTA
TCT GAG TGC ACA ATA C-3'

GO 708 5'- GGCAGTCTAAGAAAGGATCGAA-3'

GO 709 5'- TACTGCGACTACTGCGGTGA-3'

GO 710 5'- CAACGATAACAAAGACGCATT-3'

GO 711 5'- AGCAATTGTGCTGTTGGTGT-3'

GO 712 5'- CGAATTTACTGAAATTTTTGCAC-3'

GO 713 5'- CACTCGCCATTTGGTATTTG-3'

GO 714 5'- TGCCGATCGTATGCAAAAGG-3'

GO 715 5'- CCGCTCTCATCATACTCTTG-3'

Appendix II: Strains

Schizosaccharomyces pombe* strains

Lab number

GG 217	h ⁻	972	
GG 218	h ⁺	975	
GG 308	h ⁻	<i>cdc25-22</i>	
GG 313	h ⁺	<i>sep1-3HA:kanR</i>	
GG 504	h ⁻	<i>mbx1-13myc:kanR ade6-M210 his7-366</i>	
GG 507	h ⁻	<i>fkh2-13myc:kanR ade6-M210</i>	
GG 511	h ⁻	<i>fkh2::kanR</i>	
GG 513	h ⁻	<i>mbx1::kanR ade6-M210 his7-366</i>	
GG 515	h ⁻	<i>sep1::ura4⁺</i>	
GG 527	h ⁻	<i>mbx1-13myc:kanR ade6-M210 his7-366 pIRT2u:mbx1⁺</i>	
GG 539	h ⁺	<i>fkh2-3HA:kanR pUR19:fkh2⁺</i>	
GG 555	h ⁻	<i>fkh2-3HA:kanR plo1-ts35</i>	
GG 558	h ⁺	<i>fkh2-3HA:kanR</i>	
GG 704	h ⁺	<i>leu1:nmt41:plo1-3HA plo1::his3⁺ his3-D1 ade6-</i>	
GG 743	h ⁺	<i>mbx1-13myc:kanR cdc25-22</i>	
GG 745	h ⁺	<i>fkh2-3HA:kanR cdc25-22</i>	
GG 756	h ⁻	<i>fkh2::kanR cdc25-22</i>	Buck et al (2004) JCS 117: 5623
GG 767	h ⁻	<i>sep1-13myc:kanR</i>	
GG 776	h ⁺	<i>sep1::kanR cdc25-22</i>	Buck et al (2004) JCS 117: 5623
GG 1030	h ⁺	<i>sep1-3HA:kanR fkh2-13myc:kanR ade6-M210</i>	
GG 1032	h ⁻	<i>sep1-3HA:kanR mbx1-13myc:kanR his7-366</i>	
GG 1035	h ⁻	<i>mbx1-13myc:kanR fkh2-3HA:kanR his7-366</i>	
GG 1040	h ⁻	<i>sep1::ura4⁺ fkh2-3HA:kanR</i>	
GG 1044	h ⁺	<i>fkh2::kanR sep1-3HA:kanR</i>	
GG 1045	h ⁺	<i>fkh2::kanR sep1-3HA:kanR</i>	
GG 1047	h ^{-/+}	<i>mbx1::kanR fkh2-13myc:kanR ade6-M210</i>	
GG 1051	h ⁻	<i>fkh2::kanR sep1-3HA:kanR</i>	
GG 1052	h ⁻	<i>fkh2::kanR sep1-3HA:kanR</i>	
GG 1055	h ⁻	<i>fkh2::kanR sep1-3HA:kanR</i>	
GG 1060	h ⁻	<i>fkh2::kanR sep1-3HA:kanR</i>	
GG 1069	h ^{-/+}	<i>mbx1::kanR his7-366</i>	
GG 1097	h ⁺	<i>leu1:nmt41:plo1-HA plo1::his3⁺ cdc25-22 his3-D1</i>	
GG 1110	h ⁻	<i>plo1-3HA:kanR</i>	
GG 1118	h ⁺	<i>sep1-13myc:kanR cdc25-22</i>	

*All were *leu1-32 ura4-D18* unless otherwise stated. *ade6-* is either *ade6-M210* or *ade6-M216*

Bacterial strains

Lab number

GB 4	DH5 α F ⁻ omp ThsdS (r _B ⁻ m _B ⁻) dcm ⁺ Tet ^r ga λ (DE3) endA Hte [argU ileW leuW Cam ^r]
GB 455	BL21 DE3 codon (+)-RIL supE44 Δ lacU169 (ϕ 80lacZ Δ M15) hsdR17recA1endA1 byrA96thi1relA1

Bacterial vectors

Lab number

GB 10	pSP Δ 178	Lowndes <i>et al</i> (1992) <i>Nature</i> 355 : 449
GB 12	pET-28-a(+)	
GB 159	pGEX-KG	Guan and Dixon (1991) <i>Anal. Biochem.</i> 192 : 262
GB 194	pSP Δ 178.15UAS	Anderson <i>et al</i> (2002) <i>EMBO J</i> 21 : 5745
GB 329	pBTM116- <i>plo1</i> ⁺	Reynolds and Ohkura (2002) <i>J. Cell Sci.</i> 116 : 1377
GB 331	pACT2	
GB 332	pBTM116	
GB 333	pBTM116- <i>plo1.K69R</i>	Reynolds and Ohkura (2002) <i>J. Cell Sci.</i> 116 : 1377
GB 334	pBTM116- <i>plo1.472-684</i>	Reynolds and Ohkura (2002) <i>J. Cell Sci.</i> 116 : 1377
GB 335	pBTM116- <i>plo1.DHK625AAA</i>	Reynolds and Ohkura (2002) <i>J. Cell Sci.</i> 116 : 1377
GB 344	pSP Δ 178.15UAS.MUT	Buck <i>et al</i> (2004) <i>J. Cell Sci.</i> 117 : 5623
GB 403	pCR2.1- <i>fkh2</i> ⁺	
GB 417	pET-28-c (+)- <i>fkh2</i> ⁺	
GB 425	pET-28-a (+)- <i>sep1</i> ⁺	
GB 438	pACT2- <i>fkh2</i> ⁺	
GB 441	pGEX-KG- <i>sep1</i> ⁺	
GB 442	pACT2- <i>sep1</i> ⁺	
GB 444	pCR2.1- <i>sep1</i> ⁺	
GB 445	pCR2.1- <i>mbx1</i> ⁺	
GB 447	pACT2- <i>mbx1</i> ⁺	
GB 448	pGEX-KG- <i>mbx1</i> ⁺	
GB 469	pSP Δ 178.15UAS.MUT2	
GB 471	pSP Δ 178.15UAS.MUT4	
GB 475	pSP Δ 178.15UAS.MUT3	
GB 520	pGAD GH- <i>sck1</i> ⁺	Reynolds and Ohkura (2002) <i>J. Cell Sci.</i> 116 : 1377

Saccharomyces cerevisiae strains

Lab number

GGBY 138	CTY105d (lexA)	
MATa	URA3::lexA-lacZ met- his3 ade2 trp1 leu2 gal4-D gal80-D	Reynolds and Ohkura (2002) <i>J. Cell Sci.</i> 116 : 1377

Appendix III: Media

Fission yeast media

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

Vitamins	ml/EMM litre
Vitamins	1
Trace	0.1

Vitamins	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.

Adenine	225 µg/ml in EMM
Uracil	225 µg/ml in EMM

Amino acids

Leucine	150 µg/ml in EMM
Histidine	150 µg/ml in EMM

For uracil and adenine prepare a 7.5 g/l stock solution in dH₂O, while for leucine and histidine prepare a 3.75 g/l stock solution in dH₂O. Autoclave stock solutions to sterilise.

YE	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 of YE.

ME media	g/300 ml
Malt extract	9
Bacto agar	6

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

Budding yeast media

SD-Trp-Leu-His-Ura	g/litre
Yeast Nitrogen base w/o amino acids	6.7
CSM-Trp-Leu-His-Ura	0.61
D-Glucose	20

pH to 5.8 and autoclave. To make solid media, add 8 g of Bacto agar in every 400 ml of SD-Trp-Leu-His-Ura.

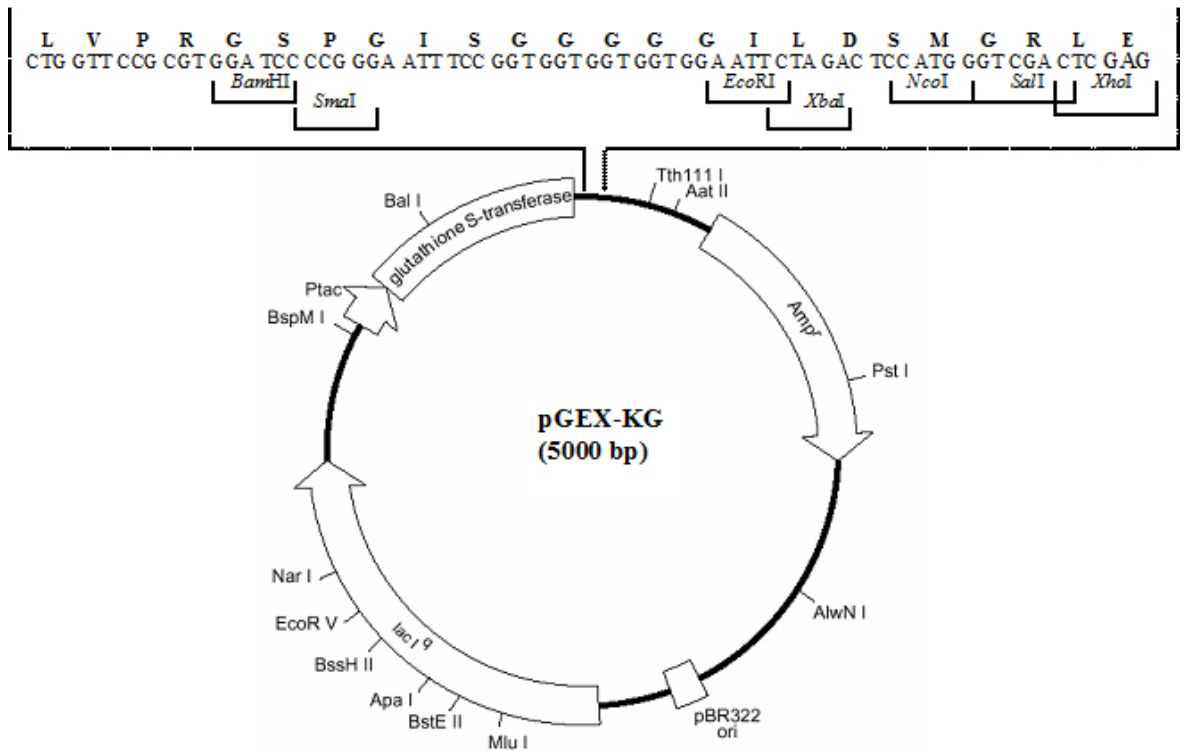
Uracil	35 µg/ml
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Amino acids

Tryptophan	80 µg/ml
Leucine	80 µg/ml
Histidine	20 µg/ml

Whenever necessary, SD-Trp-Leu-His-Ura media was supplemented with one or more amino acids and/or uracil from 7.5 g/l or 3.75 g/l stock solutions.

Appendix IV: Vectors



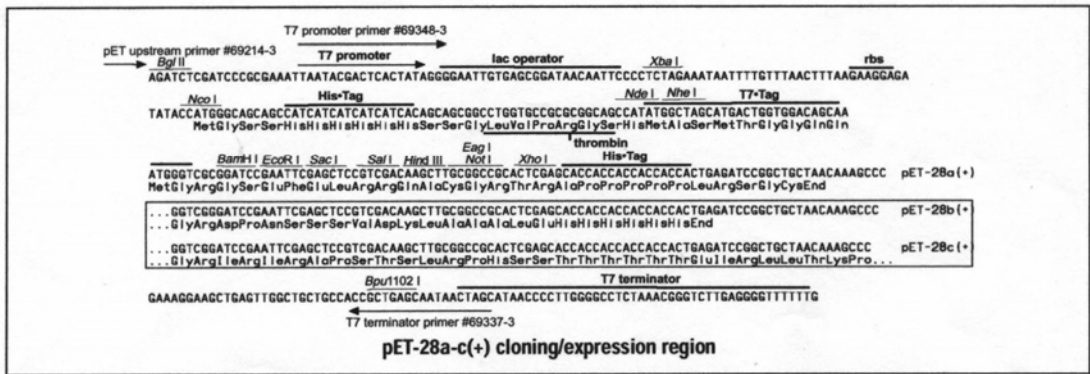
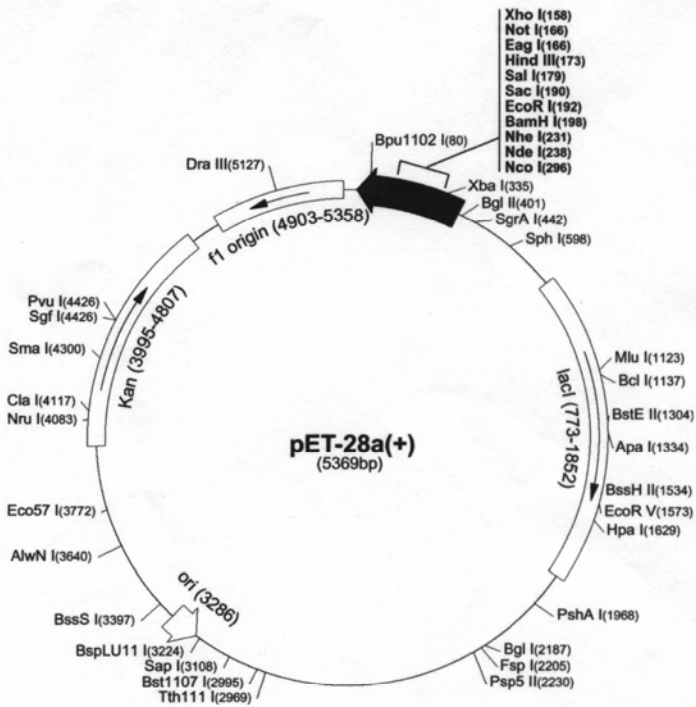
Plasmid map of the pGEX-KG cloning and expression vector

The pGEX-KG vector carries an N-terminal GST-tag sequence followed by a thrombin cleavage site and unique restriction sites are shown on the circle map. The single-letter symbols of amino acids in-frame with the GST-tag are indicated above the nucleotide sequence together with unique restriction sites within the multiple cloning site. The locations of genes for ampicillin resistance (Amp^R) and Lac repressor (LacI^q) are indicated (adapted from Guan and Dixon, 1991).

pET-28a(+) sequence landmarks

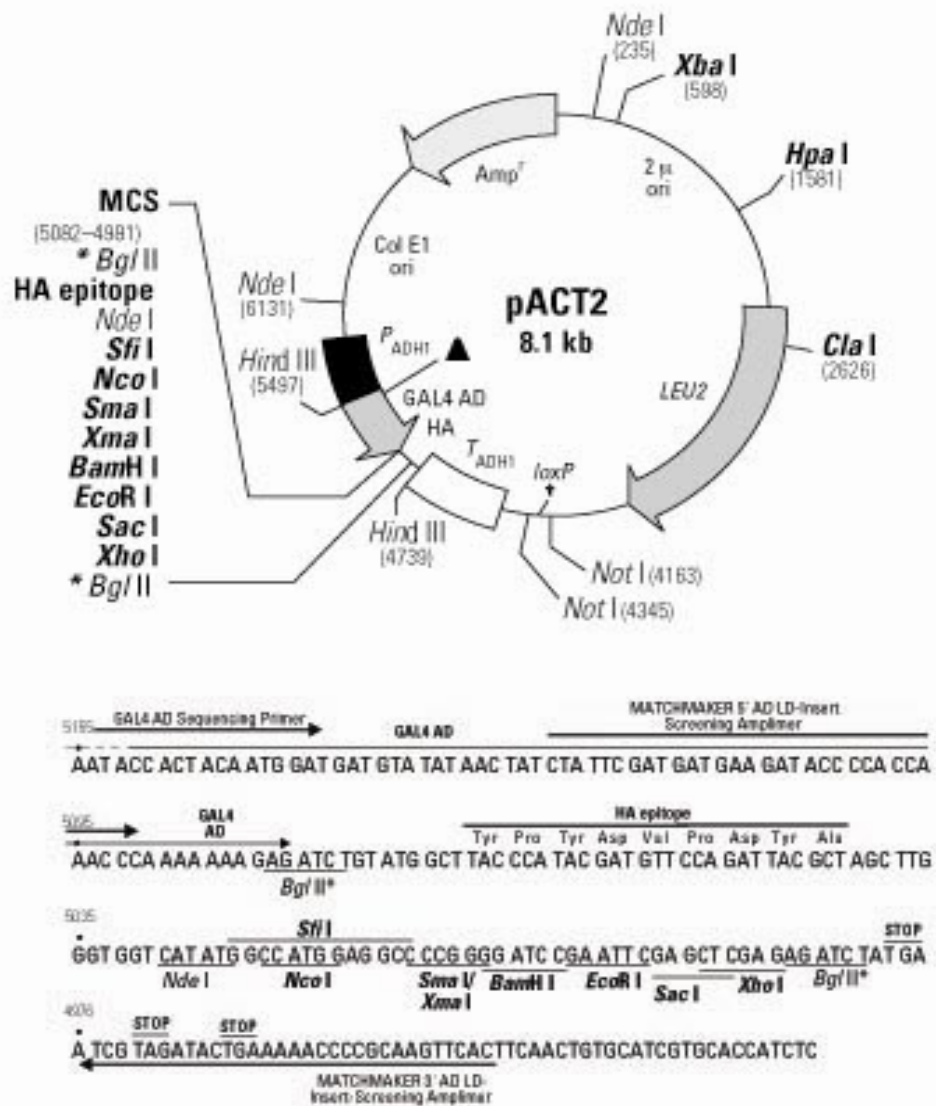
T7 promoter	370-386
T7 transcription start	369
His•Tag coding sequence	270-287
T7•Tag coding sequence	207-239
Multiple cloning sites (<i>Bam</i> H I - <i>Xho</i> I)	158-203
His•Tag coding sequence	140-157
T7 terminator	26-72
<i>lac</i> I coding sequence	773-1852
pBR322 origin	3286
Kan coding sequence	3995-4807
f1 origin	4903-5358

The maps for pET-28b(+) and pET-28c(+) are the same as pET-28a(+) (shown) with the following exceptions: pET-28b(+) is a 5368bp plasmid; subtract 1bp from each site beyond *Bam*H I at 198. pET-28c(+) is a 5367bp plasmid; subtract 2bp from each site beyond *Bam*H I at 198.



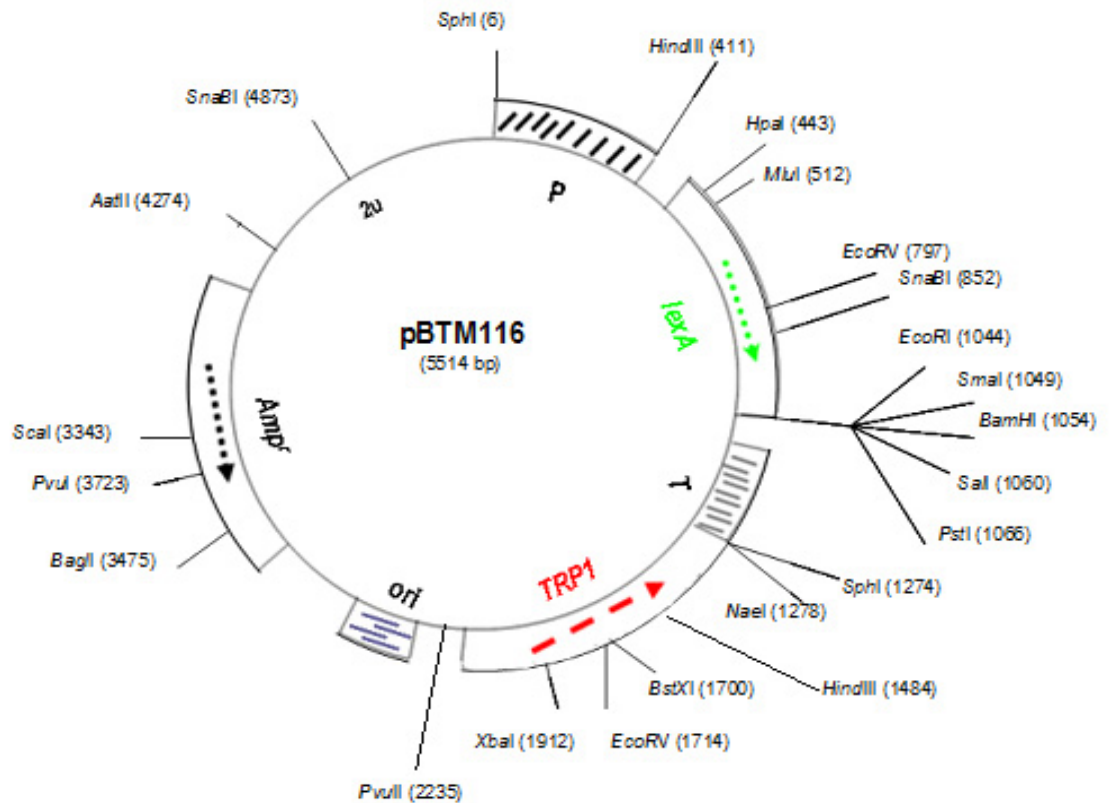
Plasmid map of the pET-28a-c (+) cloning and expression vector system

The pET-28a-c (+) vectors carry an N-terminal His-Tag®/thrombin/T7-Tag® configuration plus an optional C-terminal His-Tag sequence. Unique restriction sites are shown on the circle map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown in more detail in the box (adapted from www.novagen.com).



Plasmid map of the pACT2 AD cloning and expression vector

pACT2 is a shuttle vector that propagates in both *E. coli* and *S. cerevisiae* and carries the *bla* gene as an *E. coli* ampicillin resistant marker and the *LEU2* gene as a budding yeast nutritional marker. pACT2 contains DNA sequence encoding the GAL4 AD (amino acids 768–881), an HA epitope tag and a downstream mu**l**tiple cl**o**n**i**ng si**t**e (MCS), allowing expression of the gene of interest as a GAL4 AD-fusion protein from the constitutive *ADH1* promoter (P); transcription is terminated at the *ADH1* transcription termination signal (T) (adapted from www.clontech.com). The cloning/expression region containing the MCS is shown below the map; unique restriction sites are in **bold**. * denotes that the *Bgl* II site is not unique.



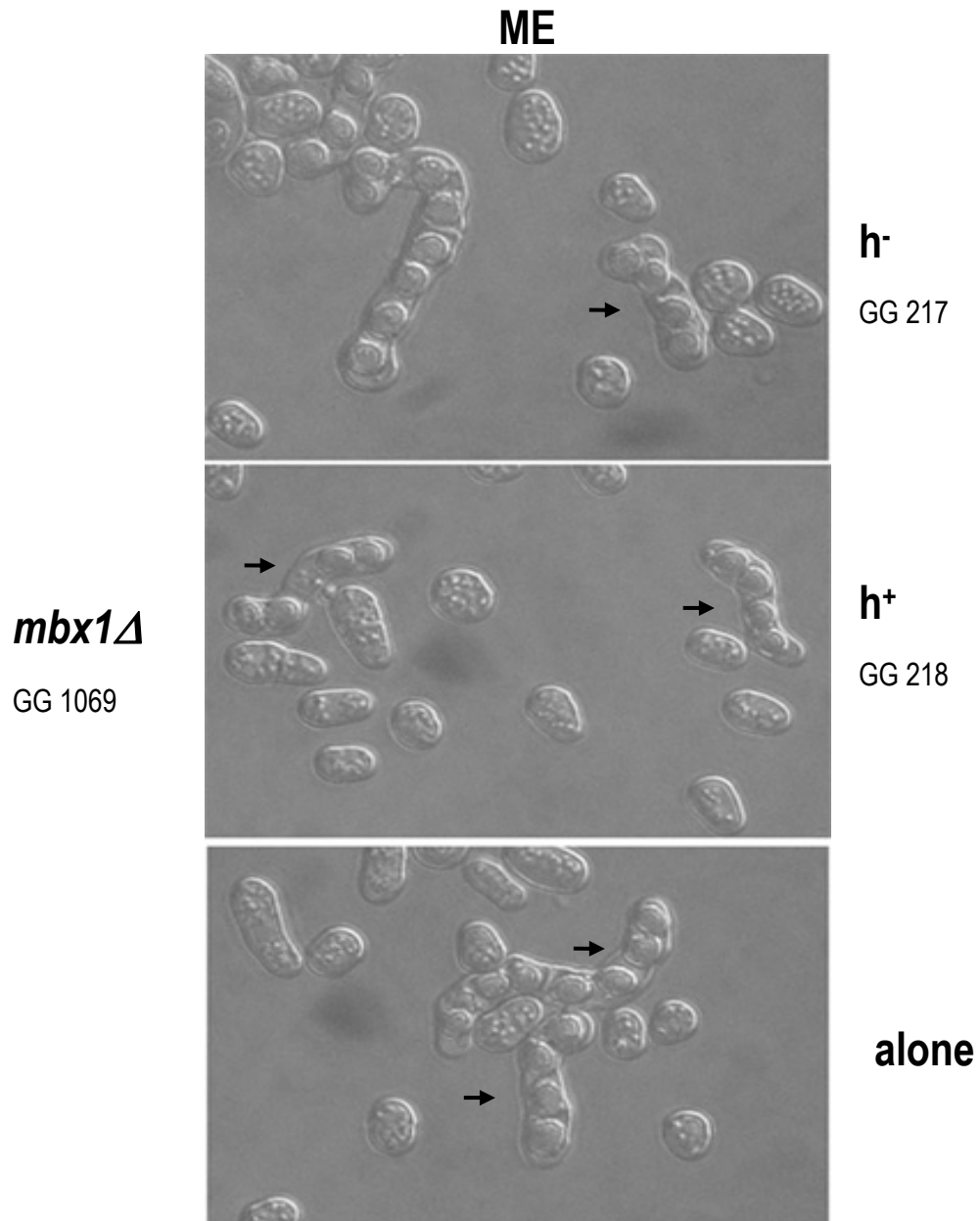
Plasmid map of the pBTM116 cloning and expression vector

pBTM116 is a shuttle vector that propagates in both *E. coli* and *S. cerevisiae* and carries an *E. coli* ampicillin resistant marker and the *TRP1* gene as a budding yeast nutritional marker. pBTM116 contains the complete *lexA* sequence under the control of the *ADH1* promoter. Fusion proteins are constructed following cloning within the following sequence (Paul Bartel and Stanley Fields, State University of New York):

GAA TTC CCG GGG ATC CGT CGA CCT GCA GCC*
EcoRI *SmaI* *BamHI* *SalI* *PstI*

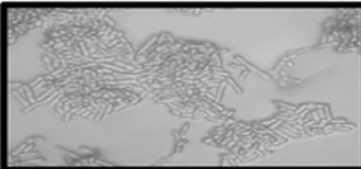
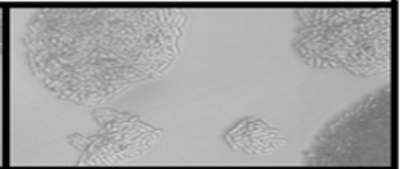
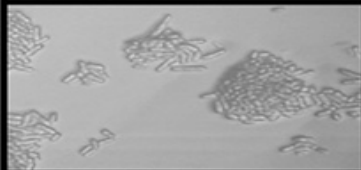
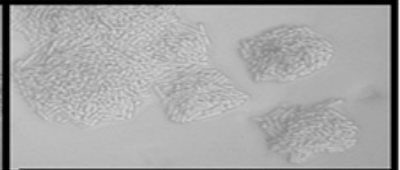
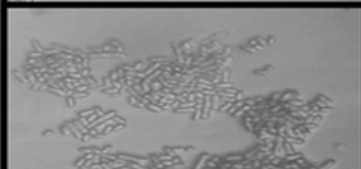
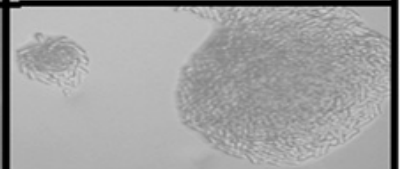
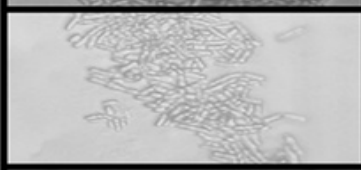
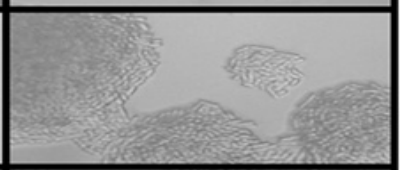
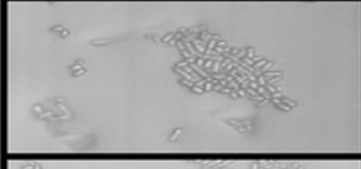
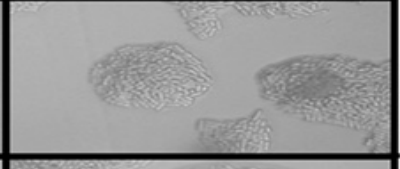
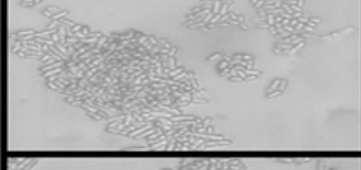
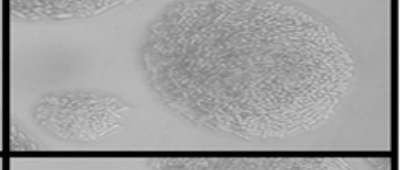

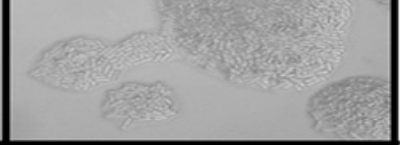
* triplets represent the reading frame relative to *lexA*

Appendix V: Aberrant mating of *mbx1* Δ cells



***mbx1* Δ cells are able to mate with either h⁻ or h⁺ cells, and themselves.** *mbx1* Δ cells alone or mixed with either h⁺ (GG 218) or h⁻ (GG 217) wild-type *S. pombe* cells were plated on solid ME medium and incubated for 2-3 days at 25°C to allow mating to take place. Four spore zygotic asci were observed in all cases, showing that *mbx1* Δ cells are able to cross with h⁻ or h⁺ cells, as well as with themselves. The annotation GG refers to the Glasgow laboratory fission yeast collection number. Black arrows denote zygotic asci.

Appendix VI: Differential growth of fkh2Δ cells on rich and minimal media

	YE	EMM
<i>fkh2Δ</i> GG 511		
<i>fkh2Δ sep1-HA</i> GG 1044		
<i>fkh2Δ sep1-HA</i> GG 1045		
<i>fkh2Δ sep1-HA</i> GG 1051		
<i>fkh2Δ sep1-HA</i> GG 1052		
<i>fkh2Δ sep1-HA</i> GG 1055		
<i>fkh2Δ sep1-HA</i> GG 1060		

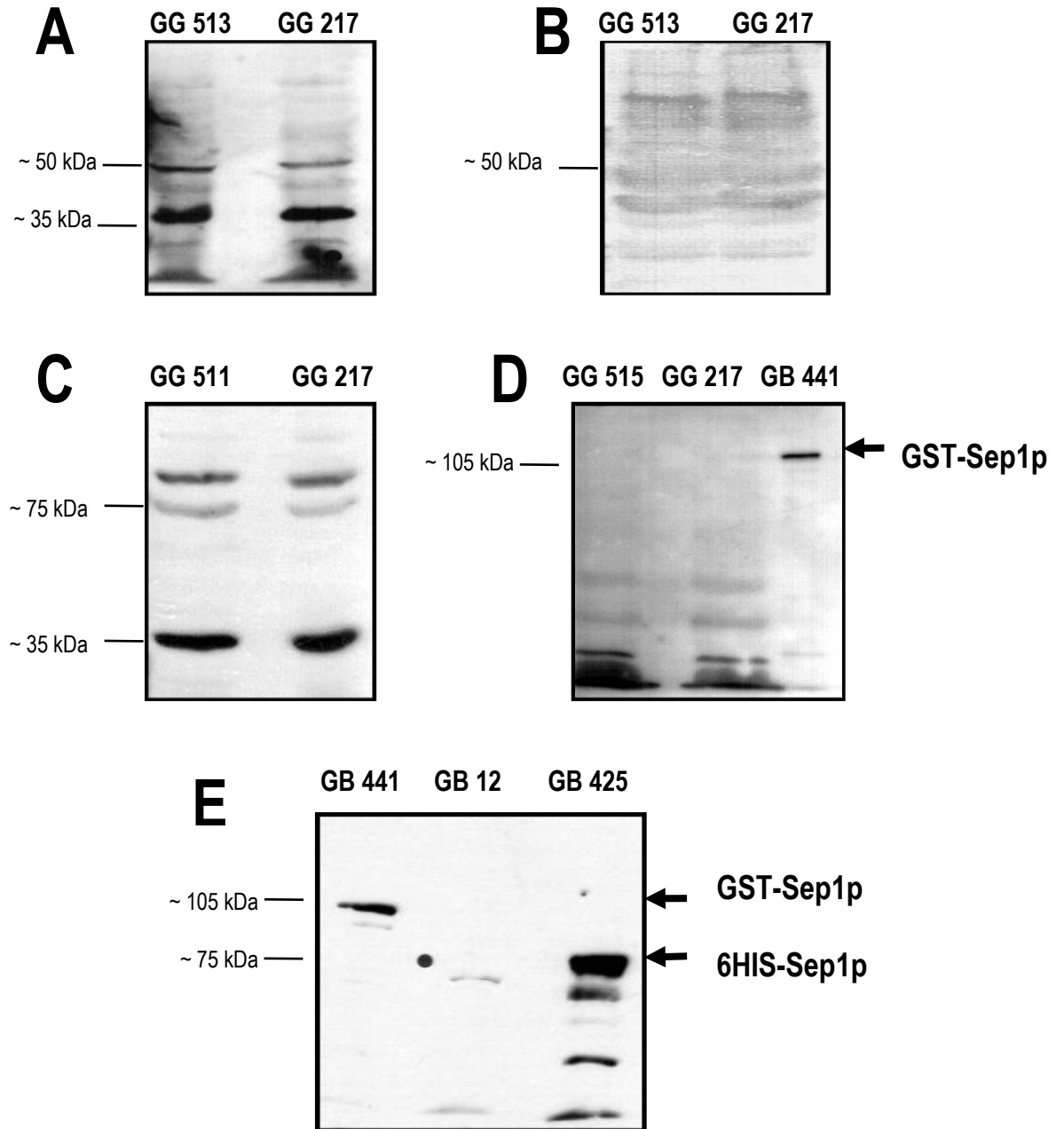
***fkh2Δ* cells display slow growth and septation defects upon growth on rich (YE) but not on minimal (EMM) solid medium.** Various isolates of *fkh2Δ* or *fkh2Δ sep1-HA* cells were grown on either rich (YE) or minimal (EMM) solid media at 25°C. *fkh2Δ* or *fkh2Δ sep1-HA* cells grew more slowly on solid YE than on solid EMM. In addition, these cells formed abnormal colonies and displayed septation defects only when grown on solid YE. The annotation GG refers to the Glasgow laboratory fission yeast collection number.

Appendix VII: Positions of PCB and forkhead sequences in promoters of M- G1 transcribed genes

Motif	Gene	Position	sequence
GNAACR	<i>spo12</i> ⁺	-341 : -336	ggcg GTAACA gtaa
GNAACR	<i>spo12</i> ⁺	-29 : -24	tta GCAACA tttg
RTAAACA	<i>spo12</i> ⁺	-311 : -305	gaat ATAACA acagt
TGTTTAY	<i>spo12</i> ⁺	-497 : -491	aatg TGTTTAC tagt
TGTTTAY	<i>spo12</i> ⁺	-147 : -141	tttt TGTTTAT ttac
GNAACR	<i>cdc15</i> ⁺	-138 : -133	atag GCAACG gttg
RTAAACA	<i>cdc15</i> ⁺	-98 : -92	cgca GTAACA aaca
TGTTTAY	<i>cdc15</i> ⁺	-87 : -81	aaca TGTTTAC tctt
GNAACR	<i>plo1</i> ⁺	-14 : -9	gtta GAAACA aata
TGTTTAY	<i>plo1</i> ⁺	-50 : -44	actt TGTTTAC cctc
GNAACR	<i>fkh2</i> ⁺	-208 : -203	aata GCAACG ataa
GNAACR	<i>fkh2</i> ⁺	-85 : -80	gctg GCAACG gaag
RTAAACA	<i>fkh2</i> ⁺	-604 : -598	gtga GTAACA ggtg
RTAAACA	<i>fkh2</i> ⁺	-41 : -35	gtaa ATAACA caca
TGTTTAY	<i>fkh2</i> ⁺	-228 : -222	tgat TGTTTAC aaaa
GNAACR	<i>sid2</i> ⁺	-408 : -403	tata GAAACA tctc
RTAAACA	<i>sid2</i> ⁺	-98 : -92	tgcc GTAACA aaac
TGTTTAY	<i>sid2</i> ⁺	-32 : -26	tgga TGTTTAC gata
GNAACR	<i>slp1</i> ⁺	-289 : -284	aaca GCAACA acaa
RTAAACA	<i>slp1</i> ⁺	-693 : -687	tatt GTAACA aatc
TGTTTAY	<i>slp1</i> ⁺	-780 : -774	ctct TGTTTAT ttac
TGTTTAY	<i>slp1</i> ⁺	-353 : -347	gatt TGTTTAC gaaa
GNAACR	<i>ace2</i> ⁺	-389 : -384	tttg GCAACA aaaag
GNAACR	<i>ace2</i> ⁺	-241 : -236	cata GCAACG gagt
GNAACR	<i>ace2</i> ⁺	-188 : -183	tgct GTAACA aaca
RTAAACA	<i>ace2</i> ⁺	-375 : -369	gtaa ATAACA atcc
RTAAACA	<i>ace2</i> ⁺	-304 : -298	caat GTAACA aata
RTAAACA	<i>ace2</i> ⁺	-220 : -214	tgat ATAACA aata
RTAAACA	<i>ace2</i> ⁺	-12 : -6	ctaa GTAACA agac
TGTTTAY	<i>ace2</i> ⁺	-464 : -458	atcc TGTTTAT tagt
TGTTTAY	<i>ace2</i> ⁺	-355 : -349	tctg TGTTTAC actt

Positions of the PCB and forkhead sequences found within the promoters of *S. pombe* M-G1 transcribed genes. The RSAT web resource was used to search for PCB and forkhead sequences within *spo12*⁺, *cdc15*⁺, *plo1*⁺, *fkh2*⁺, *slp1*⁺, *ace2*⁺ and *sid2*⁺ promoter regions. Red denotes PCB sequences (GNAACR consensus). Forkhead sequences are shown in blue for TGTTTAY and green for RTAAACA. In each case, numbers denote the position of each motif relative to the initiating ATG. R stands for G or A, Y for C or T and N for G, C, T or A.

Appendix VIII: Antibodies



Western blot analysis of (A-D) soluble protein extracts from wild-type *S. pombe* cells and (D-E) bacterially expressed and purified GST-/HIS-fusions of Sep1p to test the specificity of custom made Mbx1p, Fkh2p and Sep1p antibodies. (A-D) *S. pombe* wild-type (GG 217) extracts were analysed by SDS-PAGE, followed by Western blotting, with affinity purified rabbit polyclonal Mbx1p (A), Fkh2p (C), Sep1p (D) antibodies and 3rd bleed sheep polyclonal GST-Mbx1p antisera (B) to detect the respective proteins. *mbx1Δ* (GG 513), *fkh2Δ* (GG 511) and *sep1Δ* (GG 515) fission yeast extracts were used as negative controls. (D-E) Overexpressed and purified GST-/6HIS-Sep1p prepared from BL21 CodonPlus (DE3)-RIL cells transformed with pGEX-KG-*sep1*⁺ (GB 441)/pET-28-a (+)-*sep1*⁺ (GB 425) was analysed by Western blotting with rabbit polyclonal Sep1p antibody. Overexpressed protein fractions prepared from bacterial cells transformed with empty pET-28-a (+) vector (GB 12) were used as negative control. Protein molecular masses are indicated in kDa. Black arrows indicate full-length GST-Sep1p and 6HIS-Sep1p.