PERIODIC GENE EXPRESSION PROGRAM OF THE FISSION YEAST CELL CYCLE

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Declaration

I hereby declare that my dissertation contains material that has not been submitted for a degree or diploma or any other qualification at any other university. This thesis describes my own work and does not include the work that has been done in collaboration, except when specifically indicated in the text.

Gabriella Rustici

26/04/2004

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ABSTRACT

In every cell, thousands of genes and their protein products function in a complicated and orchestrated way that creates life. However, traditional methods in molecular biology and genetics normally work on a 'one gene in one experiment' basis. In recent years, a new technology, called DNA microarray, has attracted tremendous interest among biologists. This technology allows monitoring the expression levels of every gene in a single experiment so that researchers can obtain a global picture of the activity and interactions of thousands of genes simultaneously.

The overall objective of this thesis was to use DNA microarrays containing all the genes of the fission yeast *Schizosaccharomyces pombe* to analyse transcriptional profiles of the entire gene set during the cell cycle. Cell cycle events form the basis of growth and proliferation of all cells, and fission yeast is a valuable model organism to study cell cycle regulation. Two different standard methods were used to synchronise cells in the cell cycle. mRNA was extracted from different cell cycle stages of wild-type and mutant cells, labelled with fluorescent markers, and hybridised to the microarrays. Hybridisation was then quantified with a confocal scanner, and data evaluated using a wide range of computational methods. This work provides for the first time a genome-wide overview of genes that are periodically expressed during the fission yeast cell cycle. Several deletion mutants of well known as well as less characterised or putative transcriptional regulators have also been used with the purpose of clarifying the mechanisms that regulate gene expression during the cell cycle. Clustering of genes that are co-expressed under various conditions helped to define new consensus sequence motifs required for particular patterns of transcriptional regulation. Conservation of periodic gene transcription through evolution is discussed with respect to the *Saccharomyces cerevisiae* and human orthologues to the fission yeast periodic genes.

This research forms a basic dataset for future functional genomics approaches in fission yeast and other organisms and provides a valuable framework to characterise unknown genes in more detail using the whole range of genetics, cell biological, and biochemical methods available in fission yeast.

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1. INTRODUCTION

This introduction will first describe the general properties of the eukaryotic cell cycle and review what is known about this process in the two model organisms budding yeast *Saccharomyces cerevisiae*, fission yeast *Schizosaccharomyces pombe* and in multicellular eukaryotes. The second part will focus on the role of transcriptional regulation during the cell cycle and present the use of DNA microarray technology as a global approach to the study of this complex mechanism.

1.1 CELL CYCLE

1.1.1 General overview

Cells reproduce themselves during the cell cycle, which is defined as the series of events that occurs from the birth of the cell to its subsequent division into two new cells. During the cell cycle, all components necessary for survival, most notably the genome, must be doubled in amount and equally distributed to the two newly formed cells at division. The cell cycle has been the subject of investigation over the last 150 years since the "cell theory" was first formulated in 1858 by Virchow in his book *Cellularpathologie*. He was the first one to clearly state that cells form the fundamental structural and functional units of all living organisms and that a new cell can only arise from a pre-existing one.

A century of studies has since revealed that the basic processes and control mechanisms involved in this process are universal in eukaryotes and has led to the view of the cell cycle as a highly regulated developmental sequence that brings about the reproduction of the cell. Most cell cycle research has focused on identifying the components of the cell cycle machinery involved in its control and progression.

In all eukaryotic cells, key regulatory steps are responsible for deciding if the cell will keep dividing or will enter a quiescent state (called stationary phase or G_0) or undergo differentiation, depending on the environmental and nutritional conditions as well as external signals in mammalian cells. Once the cell commits to a new round of DNA synthesis and mitosis, the process is irreversible and the cell cycle machinery is responsible for checking that all ongoing processes have been completed before moving to the next stage.

If any abnormality (unreplicated DNA, damaged DNA, insufficient cell growth) is detected, the cycle will pause and progression will be restored only when the situation has reverted to normal. Many diseases, most notably cancer, have been linked with the aberrant behaviour of some of the protein complexes that drive the cell cycle. Molecules involved in G1/S transition appear to be deregulated in most human tumors (Malumbres M. and Carnero A., 2003) and cardiovascular diseases (Boehm M. and Nabel E.G., 2003) and unsuccessful mitosis can be detected in human neurodegenerative diseases (Alzheimer) (Vincent I. *et al.*, 2003), just to mention few examples. This shows the great medical importance of cell cycle research. A better understanding of this process is therefore needed especially to identify new useful drug targets and better strategies for the treatment of these diseases.

1.1.2 Eukaryotic cell cycle

The eukaryotic cell cycle is usually divided into four separate phases called G1, S, G2 and M, the most crucial events being nuclear and cell division (Fig. 1.1).

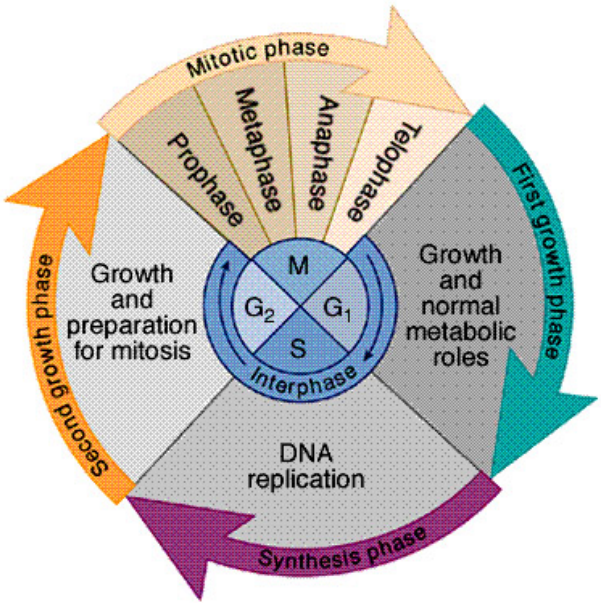


Fig. 1.1 Schematic representation of the eukaryotic cell cycle.
From http://www.bmb.psu.edu/courses/biotc489/notes/biointeract.htm

At the beginning of mitosis, in prophase, the DNA condenses into chromosomes, and the cell's microtubules are rearranged to form the mitotic spindle that will provide a

platform for chromosome separation. As mitosis progresses, the cell pauses in a state called metaphase, in which the duplicated chromosomes are aligned on the mitotic spindle, ready for segregation. The next stage called anaphase starts when the chromosomes move to the poles of the spindle, where they decondense and reform the intact nuclei. The cell then divides in two by a process called cytokinesis (Fig. 1.2).

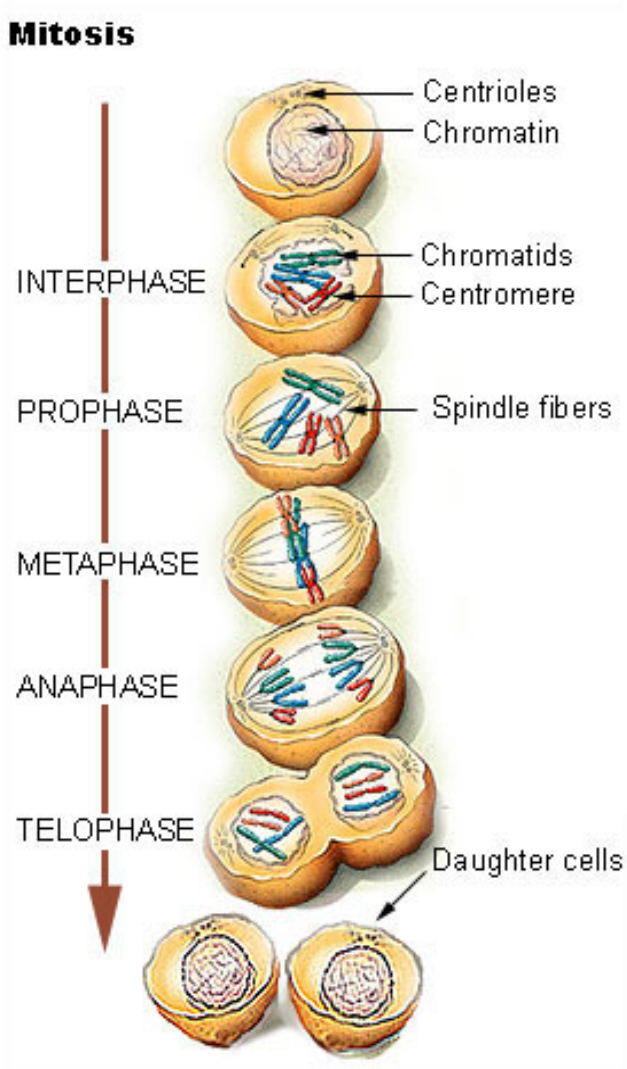


Fig. 1.2 Mitotic division and its phases.
From http://training.seer.cancer.gov/module_anatomy/unit2_1_cell_functions_2.htm

The other, much longer part of the cycle is known as interphase and includes G1, S and G2. DNA replication takes place during a relatively small portion of interphase, called the S (Synthesis) phase. The interval between M and S is called G1 (Gap1) phase, and the interval between S and M is called G2 (Gap2) phase.

G1 and G2 provide extra time for cell growth: the cell requires time to double its mass before dividing. If not, it would become smaller and smaller after each round of division. During G1, the cell "measures" its own size and only when a critical mass has been reached, commits to DNA replication and completion of a division cycle. Similarly, G2 phase provides a safe gap, allowing the cell to ensure that DNA replication is complete before entry into mitosis. In addition, if cells in G1 have not yet committed themselves to DNA replication, they can pause in a specialized resting state, called G0, where they can remain until the environmental conditions are favourable to resume proliferation.

The molecular machinery responsible of running the cell cycle is a large one and involves many components. For example, DNA replication on its own requires primases and DNA polymerases to synthesise the DNA strand complementary to a pre-existing template, topoisomerases and helicases to unwind the DNA strands and ligases to link strands together. Such complex machinery requires an accurate control. The strategy that has been evolved to achieve the tight regulation of the cell cycle events can be explained by two concepts. The first is the existence of key molecular complexes driving cell cycle progression, called cyclin-dependent kinases (CDKs). The second is that of "checkpoints", which can be defined as specific points when the cell makes sure that all the ongoing processes are completed before progressing to the next stage. I shall first consider the CDKs.

1.1.3 Cell cycle control mechanisms: cyclin-dependent kinases

The process that led to the identification of the key molecules involved in orchestrating cell cycle progression has benefited from studies carried out in different organisms. First came the discovery in yeast of cell division cycle (cdc) mutants whose characterisation allowed to identify genes which, when mutated, make the cell unable to divide and are therefore required for essential cell cycle events (Hartwell L.H. *et al.*, 1970; Nurse P. *et al.*, 1976). The product encoded by one of those genes was later identified in fission yeast as the protein kinase cdc2p (Nurse P. and Bissett Y., 1981).

At the same time, a protein-like substance named maturation-promoting factor (MPF) was isolated in *Xenopus* egg cytoplasm showing chromosome condensation activity (Wasserman W.J. and Masui Y., 1976). This was later on shown to be a complex

made of a protein kinase and a cyclin (Lohka M.J. *et al.*, 1988). In addition, a cyclin was discovered in sea urchin embryos, a protein that is synthesised and destroyed in correspondence with cell cycle division in blastomeres (Evans T. *et al.*, 1983).

These protein complexes are formed from two basic types of components, a protein kinase subunit (called CDK) with a catalytic function, and an activating protein called cyclin with a regulatory function (Cross F., 1995; Wuarin J. and Nurse P., 1996; Stern B. and Nurse P., 1996; Nurse, 1997).

CDK activity is tightly regulated through several mechanisms: (1) binding by specific cyclin cofactors, (2) binding by CDK inhibitors, and (3) inhibitory or activating phosphorylation (Tyers M. and Jorgensen P., 2000).

Progression through the cycle is achieved through alternation of a state of low CDK activity in G1 phase to a state of high CDK activity in S, G2 and M phases. S-phase is initiated when protein kinase activity increases from a very low to a moderate level, and a further increase of activity to a high level initiates mitosis (Fisher D.L. and Nurse P., 1996). Inactivation of the kinase activity at the end of mitosis resets the cell for a new cell cycle. The alternation between those two CDK states guarantees that DNA replication and chromosome segregation will occur only once per division cycle.

Protein kinases are present throughout the entire cycle, and they need to be activated at specific points and inactivated once their action is not required any longer. Such a tight regulation is achieved by collaboration with different cyclin partners. These proteins do not have enzymatic activity by themselves but by binding to their partner kinase they activate the kinase. The CDK subunit recognises and binds to a specific domain, called cyclin box, present in every cyclin (Morgan D.O., 1997). Cyclin concentration oscillates during the cycle and this is achieved by combining periodic gene transcription and protein degradation (Tyers M. and Jorgensen P., 2000). Cyclin degradation depends on a sequence located near the amino-terminus called the destruction box, which targets cyclins to the ubiquitination-dependent proteolytic pathway (Morgan D.O., 1997)**.** A multiprotein complex, called anaphase-promoting complex (APC), contains an ubiquitin protein ligase that catalyses the ligation of several ubiquitin molecules to a cyclin, targeting it to the proteasomes for degradation. Cyclin degradation results in CDK inactivation.

Even when the CDK-cyclin complex is formed it still requires further activation to be fully functional. Several kinases and phosphatases are responsible for this regulation

and some of them are also effectors of the checkpoint cascade, linking the cell cycle machine to such important signalling pathways. CDK activation is also achieved by phosphorylation of a threonine residue of the CDK subunit operated by a CDK-activating kinase (CAK) or by removing an inhibitory phosphorylation operated by a CDK inhibitor (CKI) at a tyrosine residue.

1.1.4 Cell cycle control mechanisms: checkpoints

The definition of checkpoints in yeast was proposed for the first time in 1989 by Hartwell and Weinert (Hartwell L.H. and Weinert T.A., 1989) as a surveillance system monitoring the status of the cell and able to arrest the cycle if abnormalities are detected. Since then, many of the components involved in this process have been identified and assigned to four different categories: signals, sensors, transducers and receivers. Signals, which activate the checkpoint, are detected by sensors, that are responsible for monitoring cell size, status of the chromosomes or of the mitotic spindle. If any anomaly is present, the sensor will detect it and in turn activate a signalling cascade involving first the transducers and then the receivers. Transducers transmit and amplify the checkpoint signal to the receivers, which are the downstream targets of the checkpoint. Receivers often are proteins involved in regulating cell cycle progression and therefore responsible for arresting the cell cycle at different stages as summarised in Table 1.1.

These checkpoints help to coordinate cell cycle events, because if the order of events is incorrect then an incomplete set of genetic information is transmitted to the newly formed cell, which may lead to cancer in higher eukaryotes (Hunter T. *et al.*, 1994; Hall M. and Peters G., 1996).

Table 1.1 Yeast checkpoints

Name of checkpoint	Defect	Arrest point(s)
S-M	Unreplicated DNA	S
DNA damage	Damaged DNA	G1/S, G2/M
Intra-S damage	Damaged DNA	Extended S
Re-replication	Uncompleted M	S onset
Spindle assembly (SAC)	Defective spindle	Metaphase/anaphase
Spindle orientation (SOC)	Spindle misorientation	M
Morphogenesis	Disorganised actin cytoskeleton	G2

Cell size	Smaller cell size	G1
Meiotic prophase	Incomplete recombination	Meiosis I

The S-M and DNA damage checkpoints (Murakami H. and Nurse P., 2000; Nurse P., 1997) ensure the dependence of mitosis on completion of DNA replication and on undamaged DNA, respectively. If the cell was forced to undergo mitosis before DNA replication was complete or DNA damage repaired, it would pass on broken or incomplete sets of chromosomes to its daughters. The DNA replication checkpoint is also active in meiosis together with another checkpoint responsible for detecting defects in recombination during meiotic prophase (Murakami H. and Nurse P., 2000).

The Re-replication checkpoint is responsible for DNA replication taking place only once per cycle (Stern B. and Nurse P., 1996), whereas the Intra-S checkpoint slows down DNA synthesis in response to DNA damage caused by Ionising Radiation (IR), UV or the alkylating agent methylmethane sulfonate (MMS) (Carr A. and Caspari T., 2004).

The spindle orientation checkpoint (SOC) delays anaphase onset when the actin cytoskeleton is not properly oriented (Gachet Y. *et al.*, 2001) and the spindle assembly checkpoint (SAC) delays mitosis when the mitotic spindle is not properly organised or chromosomes are detached from it (Amon A., 1999). If the cell progresses into anaphase and starts to divide before all the chromosomes are aligned on the mitotic spindle, the chromosomes will not be allocated equally between the daughter cells.

The G1 checkpoint, which senses cell size, is called START or Restriction point (R) in mammals. If the cell is too small, the cycle will pause until the cell reaches the right size needed to progress through S phase. In addition, START is probably one of the most crucial points in cell cycle progression and at least two distinct control elements are known to be required for passing it and initiate the cell cycle: cyclin-dependent kinases (CDKs) which drive onset and progression of S phase and transcription factors that activate the G1/S specific wave of gene transcription whose expression is essential to complete DNA synthesis. The rest of this introduction will describe the role of CDKs, checkpoints and transcriptional regulation in governing the cell cycle in yeast and higher eukaryotes.

1.2 YEAST CELL CYCLE

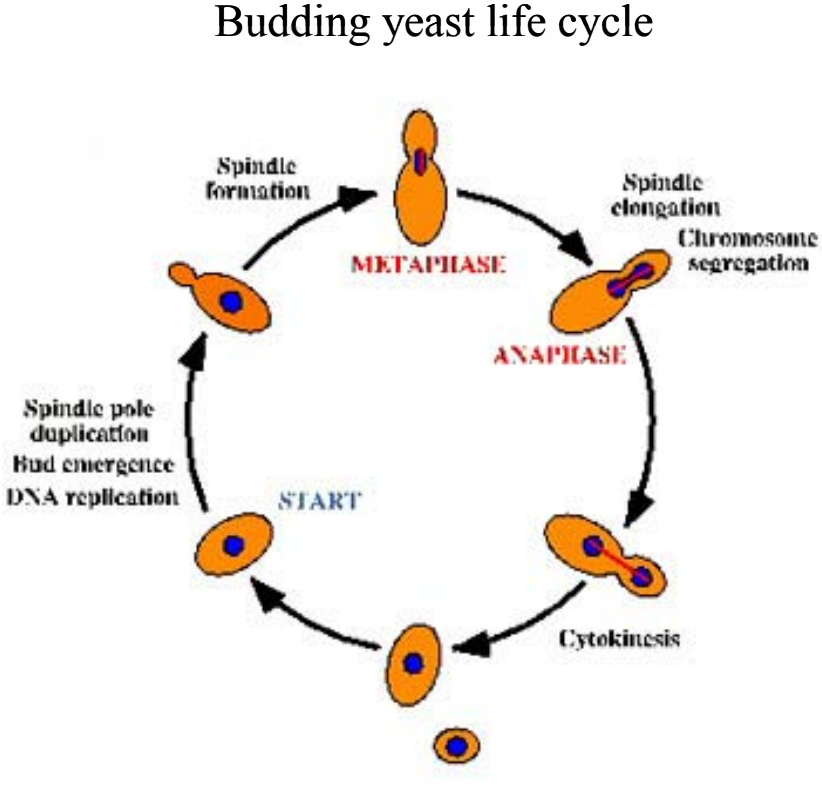
1.2.1 General overview

Yeasts are unicellular fungi – a large heterogeneous group of eukaryotic organisms. They are ideal for genetic studies of eukaryotic cell biology because they reproduce almost as rapidly as bacteria and have a genome size less than $1/100^{th}$ that of mammals. They are very well suited for identifying, cloning and characterizing the genes involved in controlling the cell cycle. The two major yeast model organisms are the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*. Although the evolutionary lineages leading to budding and fission yeasts diverged many hundreds of millions of years ago (Heckman D.S. *et al.*, 2001; Sipiczki M., 2000), the two organisms have similar life cycles (Fig. 1.3).

S. cerevisiae has a long and distinguished experimental history; it was the first eukaryote to have its genome sequenced (Goffeau A. *et al.*, 1997) and, because of its well understood biology, it has also become a common model for molecular biology. *S. pombe* is evolutionary distant to budding yeast and has dozens of genes present in multicellular organisms (including disease genes) but not in budding yeast. A number of the key cell cycle regulatory molecules now being extensively studied in higher eukaryotes were first identified and characterized in fission yeast, and it is clear that many of the regulatory networks elucidated through genetic analysis in *S. pombe* are conserved in multicellular eukaryotes as well (Forsburg S.L. and Nurse P., 1991).

As in higher eukaryotes, the cell cycle processes of both yeasts can be divided into four discrete phases (G1, S, G2 and M) with the fission yeast cell cycle being characterised by relatively short G1 and S phases compared to budding yeast. Due to the differences in the length of cell cycle phases, the main control step takes place at the G1/S transition in budding yeast and at the G2/M transition in fission yeast (Fig. 1.3A and B).

Budding yeast life cycle



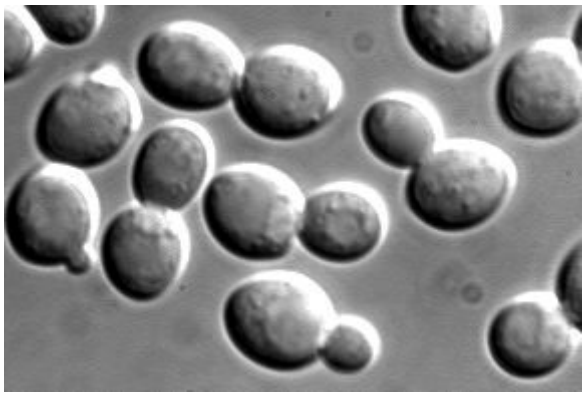


Fig. 1.3A Life cycles of both yeasts – Budding yeast.

Adapted from Vanderbilt Medical Center, Gould Lab webpage:
http://www.mc.vanderbilt.edu/vumcdept/cellbio/gould/html/news.html.
The image of budding yeast was taken from the University of Winnipeg, Simmons lab webpage:
http://io.uwinnipeg.ca/~simmons/2152web/2152/fungi2a.htm

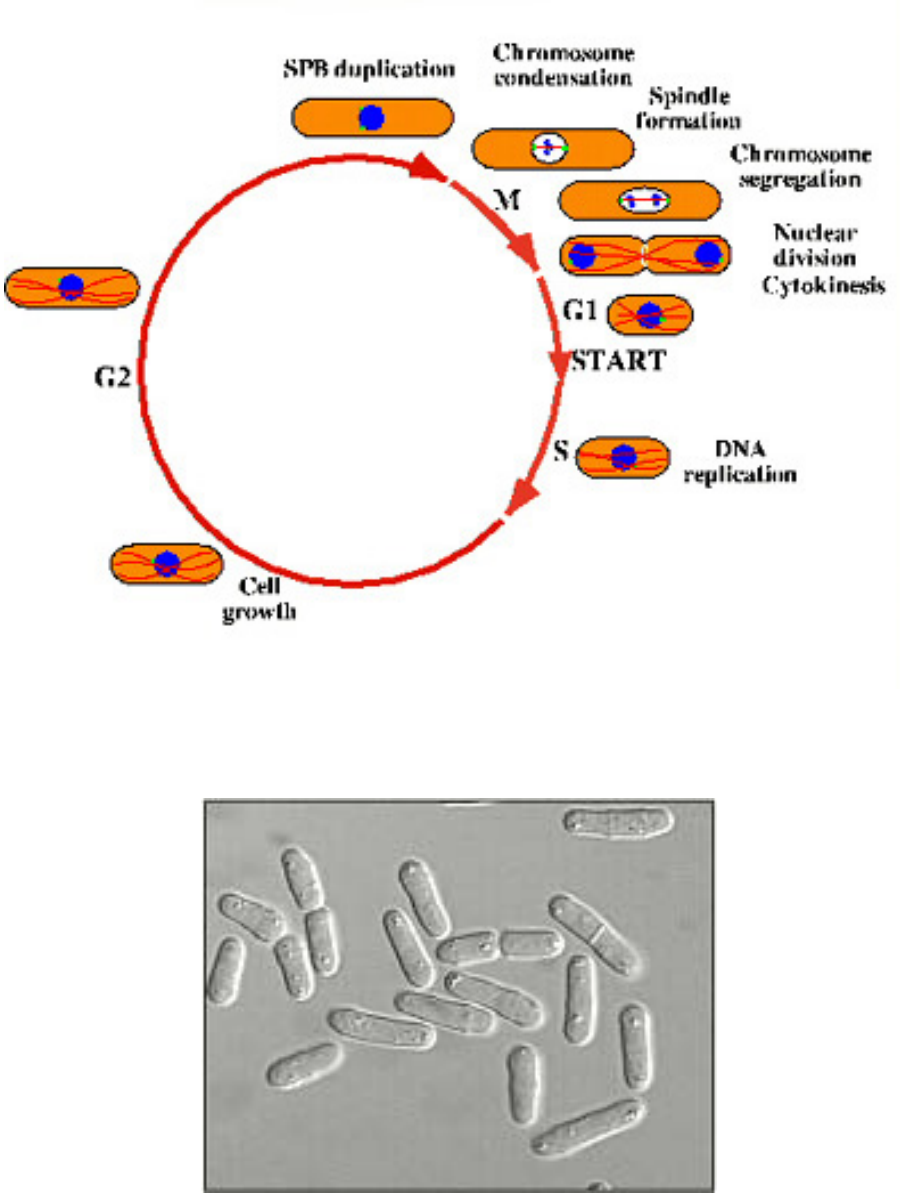


Fig. 1.3B Life cycles of both yeasts – Fission yeast.
Adapted from Vanderbilt Medical Center, Gould Lab webpage: http://www.mc.vanderbilt.edu/vumcdept/cellbio/gould/html/news.html.

Another important difference in their life cycle is that *S. cerevisiae* spends most of the time in a diploid state since the cells can mate under all growth conditions whereas *S. pombe* is haploid since mating is immediately followed by meiosis.

In both organisms cell cycle progression is driven by a single CDK and its cyclin partners. This section of the introduction will describe all the major players involved in the budding and fission yeasts' cell cycles and their counterparts in higher eukaryotes.

1.2.2 Budding yeast cell cycle

Five different CDKs are encoded by the *S. cerevisiae* genome. The most important one involved in cell cycle regulation is Cdc28p that acts in conjunction with nine different cyclins belonging to two families. These are three G1 cyclins, Cln1-3p, which function at START, and six B-type cyclins, Clb1-6p, which function in S, G2 and M phase (Nasmyth K., 1996).

The three G1 cyclins have different functions. Cln3p is needed to promote the activation of START-dependent transcription (regulated by the transcription factor complexes SBF and MBF as described later on), whereas Cln1p and Cln2p act downstream of Cln3p as effectors required to trigger START-regulated events such as budding and DNA replication. The genes coding for cyclins are transcribed in a periodic manner: the *CLN1*/*2* peak of expression coincides with START and is Cln3p-dependent. *CLN3* itself has a periodic behaviour with expression peaking at the M-G1 transition.

Similarly, of the six B-type cyclins, Clb5p/Clb6p governs entry into S phase whereas Clb1p-Clb4p regulates entry into mitosis. Clb5p and Clb2p play the major role and are supported in this by the other cyclin partners (Tyers M. and Jorgensen P., 2000). The peak of expression of their genes again coincides with the time their products are required: *CLB5*/*6* peak in late G1, *CLB3*/*4* in early G2 and *CLB1*/*2* in late G2.

Two proteins are responsible for inhibiting the kinase complexes, Sic1p and Far1p. Sic1p is specific for Clb-Cdc28p, while Far1p is specific for Cln-Cdc28p. In G1, the inhibitory effect of Sic1p (present in M and G1), together with proteolytic degradation of the cyclins operated by the APC complex, is responsible for switching off the kinase activity and allowing formation of the pre-replicative complexes (containing origin-recognition complex proteins or ORC and Cdc6p). Once these complexes are formed and bound to the DNA, Sic1p is inactivated by phosphorylation operated by Clb-Cdc28p itself, and kinase activity rises again allowing the MCM proteins to associate with the chromatin and DNA replication to start. Far1p acts as a repressor of *CLN1*/*2* expression, inhibiting their regulator Cln3p-Cdc28p and is inactivated by phosphorylation by Cln-

Cdc28p complexes similarly as Sic1p. If we imagine progressing through the budding yeast cell cycle then the series of events that we would witness is shown in Fig. 1.4. In early G1, Clb-Cdc28p activity is kept low because of the inhibitory role of Sic1p, low transcription of the Clb cyclin genes and degradation of the Clb proteins already synthesized. While cell size increases, Cln3p-Cdc28p activates *CLB5*/*6* transcription (mediated by the transcription factor MBF) causing S phase onset and *CLN1*/*2* transcription (mediated by the transcription factor SBF) leading to the accumulation of their products which results in Sic1p proteolysis, the factor responsible for Clb degradation. The cell is now replicating its DNA and the concentration of Clb cyclins starts to increase further once the Sic1p inhibitory effect has been removed (Nasmyth K., 1996).

Passage to G2 is characterised by reduction of Cln1p-Cdc28p and Cln2p-Cdc28p as a consequence of a drop in *CLN1*/*2* transcription caused by Clb-Cdc28p kinases. The CDK inhibitor Far1p also contributes to a reduction in *CLN1*/*2* transcription, probably by inhibition of Cln3p-Cdc28p activity. Sic1p is still degraded whereas the Clb cyclins are not targeted by the APC. The cell is now in G2 where the major players are the Clb-Cdc28p complexes and it can then enter M phase.

The end of mitosis is marked by an increase in *SIC1* transcription (mediated by the transcription factor Swi5p) and consequent inhibition of the Clb-Cdc28p complexes by Sic1p. At the same time the APC is activated (by Cdc20p and Cdh1p) and the Clb cyclins are degraded. At this stage the Cln-Cdc28p activity has the chance of increasing again thus driving the cell into a new cycle.

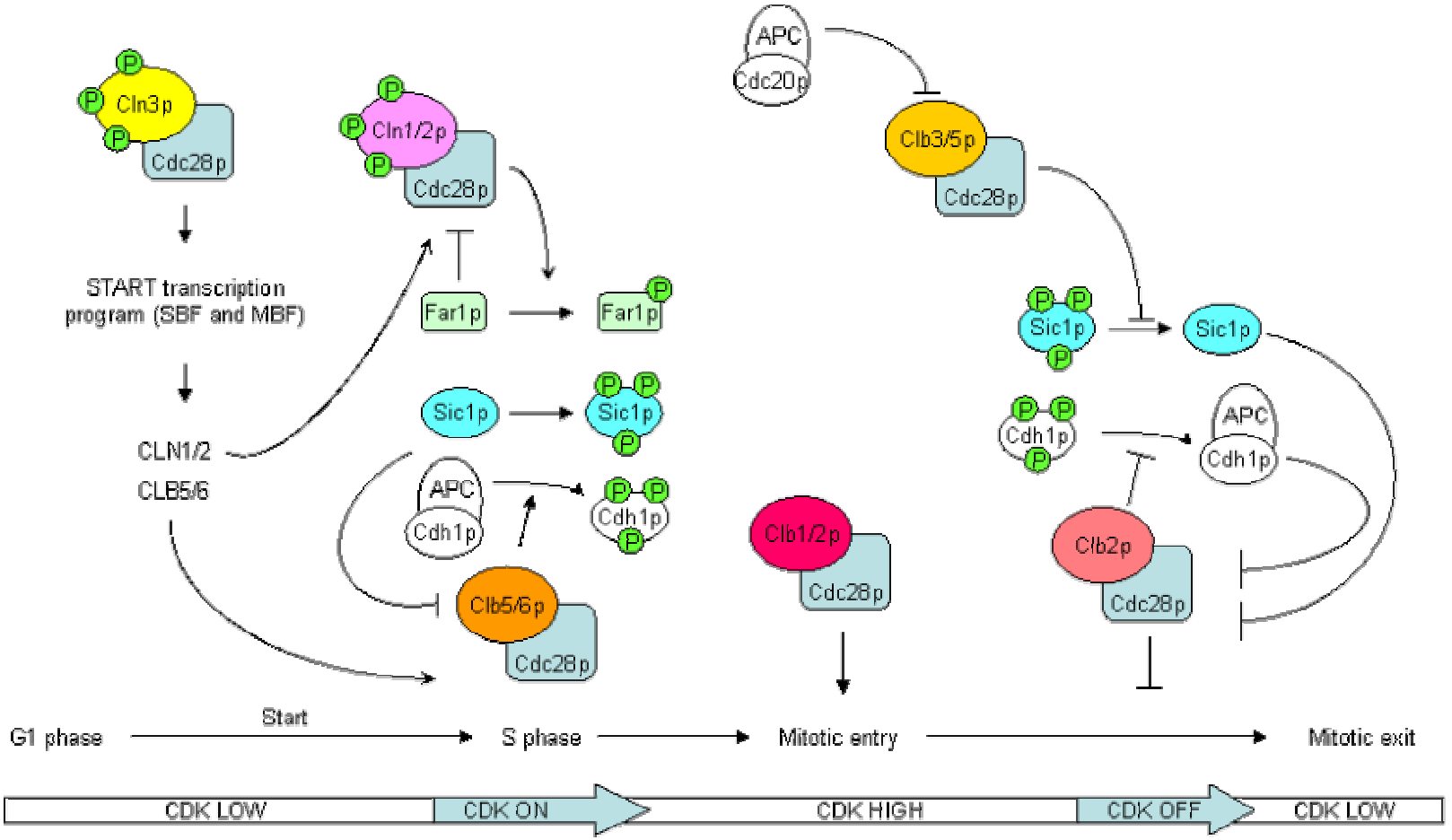


Fig. 1.4 **Central roles of CDK complexes in driving the budding yeast cell cycle.** Arrows indicate activation, blunt arrows inhibition and P stands for phosphorylation. Adapted from (Tyers M. and Jorgensen P., 2000).

1.2.3 Budding yeast checkpoints

The S-phase/DNA-damage checkpoint is a complex signalling pathway responsible for blocking or delaying cell cycle progression or DNA replication when the DNA is damaged. The proteins involved in this response are well conserved in eukaryotes and a unified model has been proposed for this checkpoint (Melo J. and Toczyski D., 2002). Several of the proteins involved in eukaryotic S-phase/DNA damage checkpoint can be found in the following table:

Table 1.2 Proteins involved in the S-phase/DNA damage checkpoint in yeast and mammals

Protein function	***S. cerevisiae***	***S. pombe***	**Mammals**
ATM/ATR kinases	Mec1 Tel1	Rad3 Tel1	ATR ATM
ATR-interacting proteins	Ddc2	Rad26	ATRIP
RFC-like proteins	Rad24	Rad17	Rad17
PCNA-like proteins	Ddc1 Rad17 Mec3	Rad9 Rad1 Hus1	Rad9 Rad1 Hus1
Mediators	Rad9 Mrc1	Crb2 Mrc1	BRCA1 Claspin
Effector kinases	Rad53 Chk1	Cds1 Chk1	Chk2 Chk1

Here I will briefly describe this model in budding yeast. The DNA damage can be detected by either Mec1p (related to human ATR) or Tel1p (related to human ATM). Mec1p responds to UV damage, double-strand breaks and replication blocks whereas Tel1p to double-strand breaks only. The sensing mechanism still remains unknown and hypotheses have been made that the cascade is activated by detection of abnormal DNA structures (Chrispell Forbes K. and Enoch T., 2000).

Before activating the signalling cascade that will result in a cell cycle arrest, these proteins need some additional factors to be loaded at the site of damage (Longhese M.P. *et al.*, 2003). These complexes in budding yeast are Rad24p and Ddc1p-Rad17p-Mec3p. The structural similarity of these proteins to others involved in DNA binding suggests a

model where Rad24p first binds to double-strand breaks then allows the loading of Ddc1p-Rad17p-Mec3p and then activates Mec1p kinase (bound to its partner Ddc2p). This kinase phosphorylates Rad9p (in response to DNA damage), Mrc1p (in response to DNA replication stress) or Chk1p. Phosphorylated Rad9p binds to Rad53p that inactivates Cdc5p, blocking cells in metaphase. Phosphorylated Chk1p in turn causes phosphorylation of Pds1p, an anaphase inhibitor, resulting again in a metaphase arrest (Murakami H. and Nurse P., 2000).

In budding yeast, the cell cycle arrests in metaphase-anaphase as a consequence of DNA damage whereas in fission yeast and higher eukaryotes the cycle is blocked at the G2-M transition. As described below (section 1.2.5) the G2/M arrest is achieved by an inhibitory phosphorylation of the CDK driving cell cycle progression. *CDC28* in *S. cerevisiae* is not involved in the DNA damage and DNA replication checkpoint control.

During mitosis the spindle checkpoint is responsible for halting the cycle when spindle defects or unattached chromosomes are detected. In budding yeast, the pathway is activated by the protein kinase Mps1p that phosphorylates Mad1p in a Bub1p, Bub3p and Mads2p dependent manner. Several of these proteins have been shown to interact with each other, and the most accepted model is that they form a complex that in turn interacts with Cdc20p (in a Mad2p-dependent manner), the activator of the APC. The result of this interaction is the inactivation of the APC ubiquitination activity. When APC is active, it is responsible for degradation of Pds1p, the inhibitor of sister-chromatid separation. Until Pds1p is present in the cell, sister-chromatid separation is not going to take place. Only when the spindle defects will be repaired, Mad2p will dissociate from Cdc20p leading to APC activation and Pds1p degradation, allowing the cycle to progress again (Amon A., 1999; Chrispell Forbes K. and Enoch T., 2000).

1.2.4 Fission yeast cell cycle

As in budding yeast, a single CDK encoded by *cdc2*, is directly involved in cell cycle regulation, controlling both onset of S-phase and M-phase (Fig. 1.5). Cdc2p activity is low in G1, moderate during S and G2 and high during M. Cdc2p is known to associate with four different cyclins: cdc13p, cig1p, cig2p and puc1p. Therefore, the cell cycle specific activity of cdc2p is determined by: (1) its associated cyclin, (2) inhibitory phosphorylation operated by the wee1p and mik1p kinases, which is removed by the

cdc25p phosphatase and (3) the CDK inhibitors rum1p (Sic1p analogue) and members of the Anaphase Promoting Complex (APC), ste9p and slp1p.

Cdc13p is an essential B-type cyclin in *S. pombe* (Fisher D.L. and Nurse P., 1996). During S phase, cdc13p begins to accumulate, due to a reduction in the concentration of its inhibitor rum1p (Benito J. *et al.*, 1998). Despite the formation of the cdc13p/cdc2p complex, its activity remains low in G2 phase because of the inhibition carried out by the tyrosine kinases wee1p and mik1p through phosphorylation of cdc2p. Activation during mitosis onset is due to an increase of the tyrosine phosphatase cdc25p (Russell P. and Nurse P., 1986), antagonist of protein kinases wee1p (Russell P. and Nurse P., 1987; Featherstone C. and Russell P., 1991) and mik1p (Lundgren K. *et al.*, 1991; Lee M.S. *et al.*, 1994). Cdc25p activity exceeds wee1p/mik1p activity causing dephosphorylation of cdc2p and therefore activation of the cdc13p/cdc2p complex. To exit mitosis, cdc13p is degraded by proteolysis and its removal results in very low level of CDK activity, preparing the cell for the next cycle.

The cig2p/cdc2p activity appears at the onset of S phase and falls after progression into S, due to cig2p proteolysis carried out by ubiquitination operated by the SCF (Skp1-Cullin-1-F-box) in G2 and M phases and by the APC in anaphase and G1 phase (Yamano H. *et al.*, 2004). Cig2p abundance is also controlled at the transcriptional level, with *cig2* gene expression peaking at G1/S.

Cig2p/cdc2p also appears to be involved in regulating the MBF transcription factor phosphorylating one of its components, res1p (Ayte J. *et al.*, 2001). This phosphorylation could either result in degradation of MBF via ubiquitination or in dissociation of res1p and res2p with loss of MBF-specific binding activity.

The functions of the other two complexes cig1p/cdc2p and puc1p/cdc2p are less understood. One of the roles of cig1p/cdc2p seems to be to relieve inhibitory effect of rum1p over cdc2p/cig2p and cdc2p/cdc13p, phosphorylating rum1p and therefore targeting it for degradation through the proteasome-dependent proteolytic pathway (Benito J. *et al.*, 1998). Puc1p/cdc2p has a specific G1 role and it is probably involved in the phosphorylation/inactivation of the CDK inhibitors, rum1p and ste9p (Martin-Castellanos C. *et al.*, 2000). Ste9p is responsible for degradation of the mitotic cyclins cdc13p and cig1p. CDK phosphorylation of ste9p promotes its degradation and prevents its interaction with the APC (Blanco M.A. *et al.*, 2000).

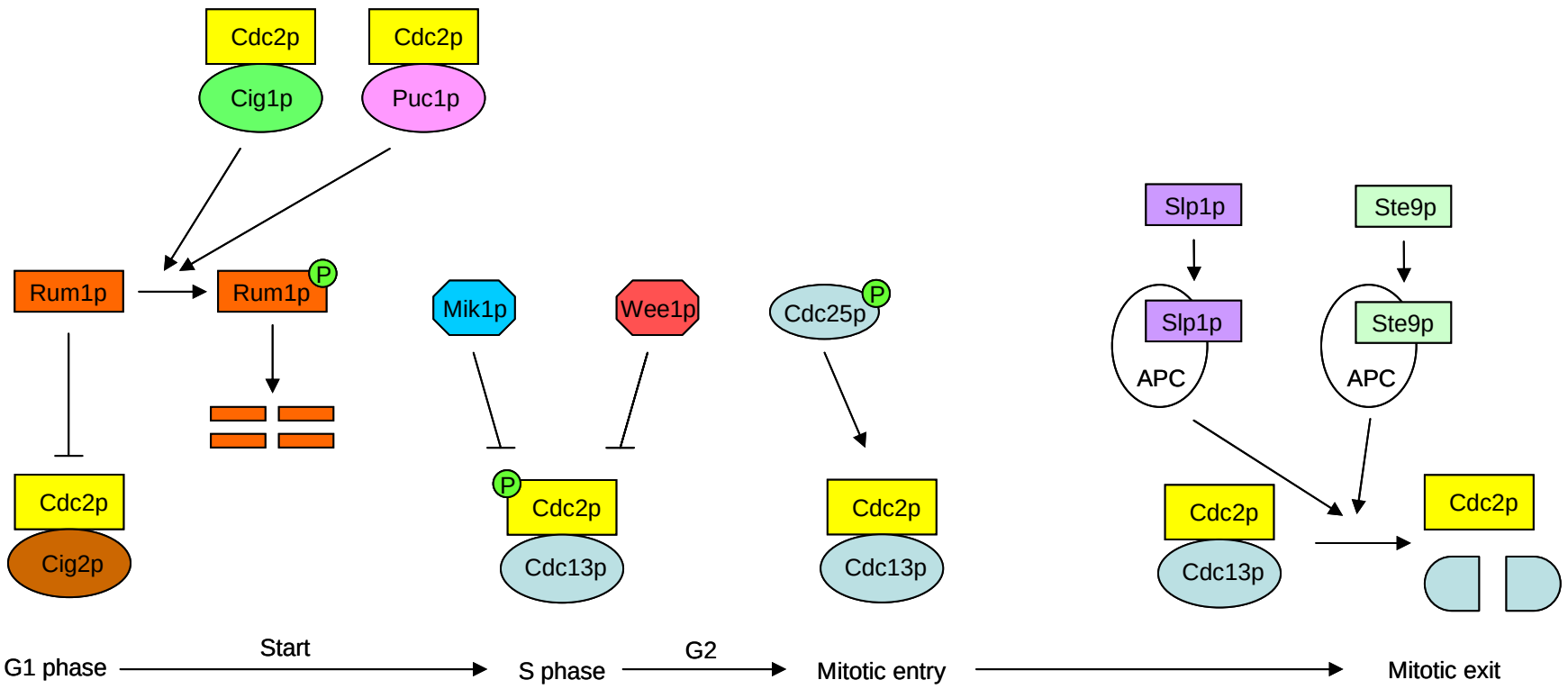


Fig. 1.5 Schematic representation of regulatory events during the fission yeast cell cycle.

Arrows indicate activation, blunt arrows indicate inhibition and P indicates phosphorylation.

1.2.5 Fission yeast checkpoints

If the cell cycle is perturbed by a block of DNA replication or by unrepaired DNA damage, then mitosis is prevented until the perturbation is corrected. This means that the replication and damage status of DNA is monitored and that this information is communicated by the regulatory pathway to mitotic cell cycle regulators leading to a block of mitosis. The components, identified so far, involved in this checkpoint in *S. pombe* are rad3p, rad26p, rad17p, rad1p, rad9p, hus1p, cut5p, crb2p, chk1p, mrc1p and cds1p.

First of all, the DNA damage needs to be sensed. Despite the fact that the sensing mechanism has not been fully elucidated, rad26p is known to be phosphorylated by rad3p in response to DNA damage as well as rad9p and hus1p. Rad9p, rad1p and hus1p physically bind to the damaged chromatin and rad17p appears to be required for the loading of this complex onto the DNA. Rad17p itself forms a complex with the Replication Factor C-like proteins (Green C.M. *et al.*, 2000).

Once DNA damage is detected, a signal needs to be sent to the cell cycle control machinery in order to stop cell cycle progression until the DNA replication can be restored. The link between the Rad checkpoint proteins and the CDK cdc2p is provided by two protein kinases, chk1p and cds1p. Chk1p is phosphorylated by crb2p (probably acting in a complex with cut5p) in response to DNA damage and acts by phosphorylating wee1p/mik1p (activating them) and cdc25p (inhibiting it). Mik1p appears to play a more important role than wee1p in the checkpoint response (Rhind N. and Russell P., 2001). This results in the inhibition of cdc2p/cdc13p complex and therefore in a temporary cell cycle G2-arrest that will last until the DNA is repaired or DNA replication restarted. Cds1p acts in the same way (via mrc1p activation) but seems to be specific for DNA replication blocks whereas chk1p is primarily involved in DNA damage checkpoints.

The spindle checkpoint operates in fission yeast in a very similar way to the one described for budding yeast (section 1.2.3). Interaction between mad2p and the APC slp1p causes stabilisation of cut2p and therefore prevents cut1p (bound to cut2p) from promoting chromatid separation (Chrispell Forbes K. and Enoch T., 2000).

1.3 CONSERVED CELL CYCLE MECHANISMS IN EUKARYOTES

CDKs are widely conserved among eukaryotes, from yeast to human. In both the budding and the fission yeast, a single CDK (Cdc28p and cdc2p, respectively) is responsible for catalysing all major cell cycle transitions. In higher eukaryotes, there has been an expansion in the number of CDKs that regulate the cell cycle, with up to five CDKs in humans and mice. This expansion allowed the specialisation of CDKs for particular cell cycle transitions in mammals: CDK4, CDK6 and CDK3 regulate G1 phase progression and entry into S phase; CDK2 is required for entry into S phase and DNA replication; and CDK1 is required for mitosis.

CDK4 and 6 are activated in response to growth factors and act in collaboration with cyclin D in promoting the transcription of proteins required for G1 and S phase. CDK1 and CDK2 appear to be the functional homologues to yeast cdc2p/Cdc28p and therefore are responsible for driving the cell cycle. CDK2 interacts with cyclin E at the beginning of S phase to trigger DNA synthesis, and then binds cyclin A throughout S phase. Both cyclin E/CDK2 and cyclin A/CDK2 activities are essential for initiation and completion of DNA replication and for ensuring that replication takes place only once in each cell cycle. Mitosis is then initiated by CDK1 in association with cyclin A and B. CDK3 is very closely related to CDK1 and 2 but its cyclin partner has not been identified yet. Similarly to what was described for yeasts (section 1.1.4), destruction of mitotic cyclins is essential for cell cycle progression and is again mediated by the APC.

CDK-cyclin complexes are negatively regulated by inhibitors belonging to two separate families: Ink4 family, which specifically inhibits CDK4 complexes and Cip/Kip family that targets CDK2-containing complexes.

Similarly to what has been described in yeast, CDKs are both positively and negatively regulated by phosphorylation. Inhibitory phosphorylation is catalysed by both a nuclear kinase, WEE1 and a cytoplasmic protein, MYT1. Inhibition is removed by members of the phosphatase family CDC25. In mammalian cells there are three forms of CDC25: CDC25A which specifically dephosphorylates cyclin E-CDK2 complexes early in the cycle and CDC25B/C which both function at the G2/M transition.

Cell cycle progression is tightly controlled in higher eukaryotes by a checkpoint system. If DNA is damaged, cell cycle will be delayed in G1 or G2 until the damage is repaired. ATM and ATR (Table 1.2) control the DNA damage response in mammalian

cells activating p53. Phosphorylation of p53 drives transcription of the CDK inhibitor p21, resulting in G1 arrest and transcription of 14-3-3 proteins (mammalian homologues to *S. pombe* Rad24 and Rad25), responsible for G2 arrest. ATM and ATR also phosphorylate the kinases Chk1 and Chk2. Chk2 activates p53 and both Chk1/Chk2 inhibit CDC25, causing a delay in mitosis onset. Both kinases also delay DNA replication targeting CDC25A for ubiquitin-degradation.

Such a high degree of conservation of cell cycle and checkpoint control in eukaryotic organisms underlies, once again, the fundamental importance of these pathways for the survival of the cell.

1.4 CELL CYCLE CONTROL OF GENE EXPRESSION

1.4.1 General overview

As discussed, progression through the cell cycle from yeast to human is the result of a series of events driven by CDK activity, including changes in gene expression. Periodic gene transcription of the cyclin and of many other genes involved in DNA replication is crucial for completion of the cycle. Changes in mRNA level of expression are normally due to transcription factors that specifically bind to promoter sequences of their target genes activating their transcription at certain stages of the cycle.

The first example of transcripts whose concentration was found to oscillate as a function of the cell cycle were the *Saccharomyces cerevisiae* histone mRNAs (Hereford L.M. *et al.*, 1981). Histone synthesis occurs specifically during S phase, which ensures that protein supply coincides with demand as chromosomal DNA is replicated. Other examples of cell cycle-regulated genes include those encoding most cyclins and many of the enzymes required for DNA synthesis. The expression of several genes fluctuates in a phase-dependent manner during the interphase period between mitoses.

Since their discovery, finding and studying genes whose transcription is cell cycle-regulated has been a challenging task for molecular biologists. By 1998, hundreds of cell cycle regulated genes were known in various organisms, but there were still many gaps: many genes and mechanisms important to the cell cycle regulation were unknown.

The development of the microarray technology (Duggan D.J. *et al.*, 1999; Brown P.O. and Botstein D., 1999; Ferea T.L. and Brown P.O., 1999; Lockhart D.J. and Winzeler E.A., 2000) which allows investigating the complete gene expression profile of an organism, made it possible to dissect the complex regulatory network that drives cell division and identify many of the proteins involved in its control (see section 1.5)**.**

1.4.2 Transcriptional cell cycle regulation in budding yeast

Three major types of cell cycle transcription factor complexes are known in *S. cerevisiae*: MBF / SBF, forkheads and Ace2p / Swi5p.

MBF (*Mlu*I binding factor) and SBF (SCB binding factor) operate at START (G1-S transition). The existence of such factors was first proposed when it was noticed that

most of the genes transiently transcribed during late G1 contained in their promoters one of two different UAS sequences, later named SCB element (Swi4/Swi6 cell cycle box - CACGAAA) and MCB element (*Mlu*I cell cycle box - ACGCGTNA) (Lowndes N.F. *et al.*, 1992). Further characterisation revealed that those sequences are specifically recognised by two different heterodimeric complexes that contain a common subunit, Swi6p, which is unable to bind DNA on its own and therefore requires an additional partner (Dirick L. *et al.*, 1992).

SBF is formed by Swi6p and Swi4p (Andrews B.J. and Herskowitz I., 1989) and preferentially binds to SCB elements (Primig M. *et al.*, 1992) whereas MBF contains Swi6p and Mbp1p (Koch C. *et al.*, 1993) and preferentially binds to MCB elements, although the distinction between SBF- and MBF-controlled genes is not possible based on the promoter sequences alone. Swi4p/Mbp1p DNA binding capacity is due to the presence of ankyrin repeats in the central part of those proteins, a characteristic feature of these transcription factor complexes conserved across yeast species (Koch C. *et al.*, 1993).

The precise mechanisms responsible for the regulation of MBF and SBF still remain unclear. Their activity is regulated at different levels: *SWI4* expression peaks at the M-G1 transition and Swi6p appears to be localised to the nucleus during S and to the cytoplasm during M but this is not enough to explain the G1 specific activation. Both transcription factors bind to the promoter region of their target genes during G1/S, before transcription is switched on (Koch C. *et al.*, 1996). A specific factor must therefore be responsible for their activation. Cln3p, in association with Cdc28p, has been proposed to be involved in such regulation (Koch C. *et al.*, 1996), and a recent study (Wijnen H. *et al.*, 2002) has proposed a similar model for Cln3p as for CDKs in mammals in activating E2F transcription factor. According to this view, Cln3 would interact with a Swi6p-specific inhibitor (still uncharacterised) therefore allowing MBF and SBF to activate transcription.

An alternative pathway for MBF regulation, functioning in parallel with the one just described, involves Stb1p; Cln-Cdc28p would in this case phosphorylate Stb1p causing its dissociation from MBF and therefore resulting in a down-regulation of MBF-dependent transcription (Costanzo M. *et al.*, 2003). In this case Stb1p would function as an activator of MBF-dependent transcription. Previous studies also demonstrated that the

SCB elements are not occupied by SBF in G2 and M and that the Clb1p-Clb4p kinase activity is responsible for this repression (Amon A. *et al.*, 1993).

Forkheads operate at the G2-M transition, and the characterisation of the components present in this complex was only completed very recently (Zhu G. *et al.*, 2000; Pic A. *et al.*, 2000; Kumar R. *et al.*, 2000; Koranda M. *et al.*, 2000; Jorgensen P. and Tyers M., 2000). It is formed by the forkhead-like transcription factors Fkh1p and Fkh2p, a MADS-box transcription factor Mcm1p and a transcriptional activator Ndd1p. It is the first example of an eukaryotic transcription factor complex containing both a MADS-box and a forkhead protein and the role of forkheads appears to be conserved from yeast to human (Alvarez B. *et al.*, 2001).

Fkh1p and Fkh2p seem to have overlapping roles in controlling G2-M transcription. Fkh2p cannot bind the DNA in the absence of Mcm1p (Kumar R. *et al.*, 2000). This two-element complex is bound to the promoter sequence of its targets throughout the cycle, and only the binding of an additional factor, Ndd1p, activates gene transcription (Koranda M. *et al.*, 2000; Jorgensen P. and Tyers M., 2000). Fkh1p is also able to bind various genes without any additional factor (Simon I. *et al.*, 2001). It is still unknown what activates members of the forkhead family of transcription factors; Fkh2p appears to be phosphorylated during the cell cycle (Pic A. *et al.*, 2000), and this might be an indication of a CDK regulation.

Ace2p and Swi5p are active in late M and early G1. Swi5p was first identified as specific activator of the HO gene, and Ace2p isolated afterwards because of its homology to Swi5p (Dohrmann P.R. *et al.*, 1992). There are many similarities between those two factors: their zinc-finger domains are 83% identical, *SWI5* and *ACE2* show a periodic behaviour peaking in M, their proteins are localised in the cytoplasm until M and then moved to the nucleus and their target genes peak in G1. Despite those similarities each factor activates a range of different genes, and this is due to a specific part of the proteins showing a promoter-specific activation (McBride H.J. *et al.*, 1999). Among the Swi5p targets is *SIC1* that is responsible for mitotic exit (Toyn J.H. *et al.*, 1997)

All the information collected in decades of research using traditional genetic approaches have been now integrated through the data collected using microarrays. The result has been the creation of a much more refined model for cell cycle control in *S. cerevisiae* (Futcher B., 2002). Two microarray studies of the budding yeast cell cycle have identified hundreds of periodic genes (Spellman P.T. *et al.*, 1998; Cho R.J. *et al.*,

1998) and assigned them to specific phases of the cycle (Spellman P.T. *et al.*, 1998). Cho *et al.* (1998) and Spellman *et al.* (1998) identified 416 and 800 periodic genes, respectively. Here I will discuss in more details the results of the Spellman study because they were analysed by the authors in more depth compared to the Cho dataset.

The 800 periodic genes identified by Spellman *et al.* were classified using a hierachical clustering algorithm (Eisen M.B. and Brown P.O., 1999) and their transcriptional regulation has been investigated by combining expression data together with transcription factor binding site search. In addition, the role of the cyclins Cln3p and Clb2p in controlling progression through the cycle was investigated looking at the expression profile of strains where Cln3p or Clb2p were induced. With this approach 363 genes were successfully assigned to a specific cell cycle stage, and the regulation understood to some degree for around 500 genes. The remaining 300 had smaller changes in expression and did not have good binding sites making it difficult to classify them. The results of this classification are summarised in the following table:

Table 1.3 Clusters of periodic genes in *S. cerevisiae* according to Spellman *et al.*, (1998)

Cluster	No of genes	Binding site	Regulator	Peak of expression	CLN3 effect	CLB2 effect
CLN2	119	ACGCGT	MBF, SBF	G1	I	R
Y	26	Unknown	Unknown	G1	-	-
FKS1	92	ACRMSAAA	SBF (MBF?)	G1	I	R
Histone	10	ATGCGAAR	Unknown	S	I	No effect
MET	20	AAACTGTGG	Met31p, Met32p	S	Some I	?
CLB2	35	MCM1 + SFF	Mcm1p + SFF	M	R	I
MCM	34	MCM1	Mcm1p	M/G1	R	I
SIC1	27	RRCCAGCR	Swi5p/Ace2p	M/G1	Maybe R	No effect

I: Induced, R: Repressed

The G1 clusters are:

1. “CLN2” which is regulated by MBF/SBF, includes mostly genes involved in DNA replication and the cyclin genes *CLN1*, *CLN2* and *CLB6* and appears to be induced by *CLN3* and repressed by *CLB2*.
2. “Y” which includes open reading frames located in Y elements at the chromosome ends. Nothing is known about their regulation or functional significance.
3. “FKS1” is represented by cell wall synthesis genes, the majority containing an SBF motif. They are induced by *CLN3* and repressed by *CLB2*.

The S and M clusters are:

1. “Histone” which appears to be induced by *CLN3*.
2. “MET” formed by genes involved in methionine biosynthesis, some of them induced by *CLN3* and most of them containing a binding site for Met31p/Met32p (Blaiseau P.L. *et al.*, 1997).
3. “CLB2” containing genes involved in mitosis and induced by *CLB2* and repressed by *CLN3*. Most of the components of this group (including *CLB1*, *CLB2*, *SWI5* and *BUD4*) have binding sites for Mcm1p/Fkh2p/Ndd1p.

The M/G1 clusters are:

1. “MCM” which includes genes involved in DNA replication (including the six MCM genes) that appear to be induced by *CLB2* and repressed by *CLN3*. The majority contain a binding site for Mcm1p and some an additional site for Mcm1p/Fkh2p/Ndd1p.
2. “SIC1” is regulated by Swi5p and Ace2p, some of the genes are repressed by *CLN3* whereas *CLB2* seems to have no effect on their regulation.

This was the first attempt made to clarify the transcriptional regulatory network in budding yeast. Several studies that followed have contributed to dissecting it in much more detail through the use of new experimental techniques such as crosslinking chromatin immunoprecipitation (ChIP) combined with DNA microarrays (ChIP-chip) (Simon I. *et al.*, 2001; Iyer V.R. *et al.*, 2001) and refined computational methods for the identification of transcription factor binding sites.

The model now proposed for the *S. cerevisiae* cell cycle is built around nine transcriptional regulators (Mbp1p, Swi4p, Swi6p, Mcm1p, Fkh2p, Fkh1p, Ndd1p, Swi5p and Ace2p) and two CDK complexes (Cln3p/Cdc28p and Clb2p/Cdc28p) as shown in Fig. 1.6.

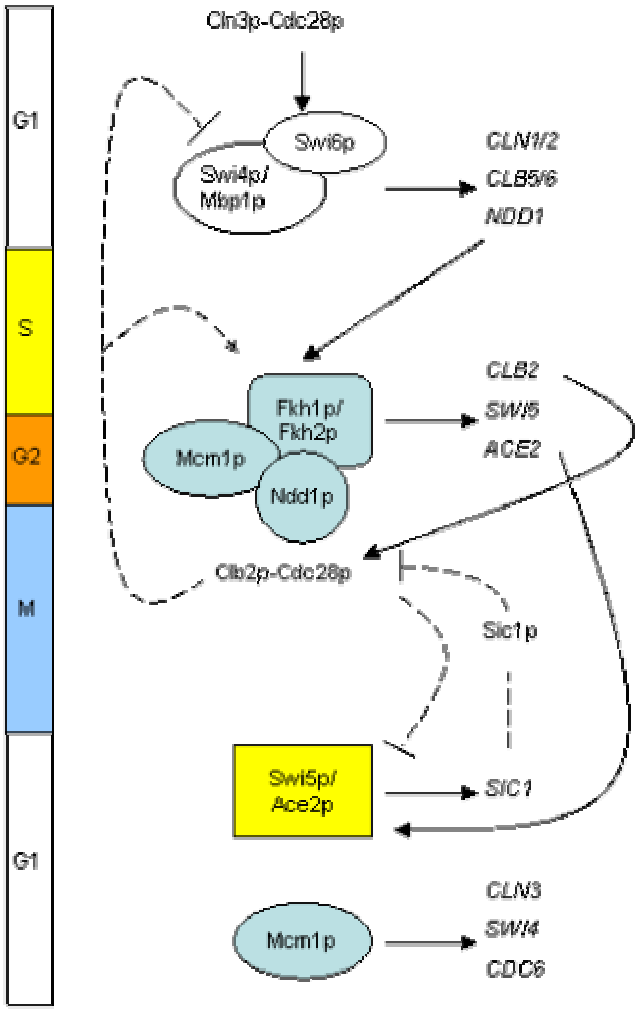


Fig 1.6. Serial regulation of gene transcription in *S. cerevisiae*.
Arrows indicate activation and blunt arrows inhibition. Adapted from (Tyers M. and Jorgensen P., 2000).

Simon *et al.* (2001) showed how a transcriptional regulator that is active in one specific cell cycle stage is responsible for the regulation of the transcriptional regulator that is active in the next stage of the cycle. Therefore, Cln3p-Cdc28p activates MBF

(Mbp1p/Swi6p) and SBF (Swi4p/Swi6p) in G1, which in turn switch on the transcription of the "CLN2" genes including the cyclin genes *CLN1/2* and *CLB5*/*6* (that drive the cycle through S phase) and the transcriptional activator *NDD1* (which is the limiting component of the complex activating G2/M transcription). SBF alone also participates in *CLB2* regulation at a later stage inducing its expression that as a result inhibits *CLN1/2* expression committing the cell to mitosis.

Mcm1p/Fkh2p/Ndd1p regulates the "CLB2" cluster in G2/M including *CLB2* that is responsible for the mitosis onset and *SWI5*/*ACE2* that regulate M/G1 genes. It is also responsible for the regulation of *CDC20* that encodes an APC activator and is therefore involved in mitosis exit. Mcm1p alone has been identified by McInerny *et al*. (1997) as a regulator of a subset of genes involved in pre-replication complex formation and in mating as well as *SWI4*, linking it to the regulation of SBF. This regulation does not involve forkheads. Similarly, Fkh1p alone was found to be able to bind several genes involved in chromatin structure and its regulation as well as the *CLB4* cyclin gene. Ace2p and Swi5p are regulators of M/G1 genes including *SIC1*, a specific Clb-Cdc28p inhibitor responsible for mitosis exit. Ace2p, Swi5p and Mcm1p are all able to bind *CLN3* and activate it. Exit from mitosis and increase of Cln3p concentration will prepare the cell for a new cycle.

It is now clear that partially redundant transcription factors have an overlapping set of genes that they control as well as specific ones for each factor (Simon I. *et al.*, 2001). This has been definitely shown by Iyer *et al.* (2001) for SBF and MBF, where SBF controls genes involved mainly in membrane/cell-wall formation and bud growth, and MBF controls genes for DNA replication.

In budding yeast, the transcriptional regulatory network that governs the cell cycle can be considered as a cycle itself, where all the transcription factors involved are progressively activated in a cascade. Some of those factors also appear to be able to inhibit their activators with a negative feedback resulting in a transition to the next cell cycle stage.

1.4.3 Transcriptional cell cycle regulation in fission yeast

More than thirty periodically expressed genes have been identified in fission yeast but far less transcription factors are known. In *S. pombe* the G1-S transition is characterised by a

transient peak of expression of a subset of genes: *cig2* (Connolly T. and Beach D., 1994), as well as *cdc18* (Kelly T.J. *et al.*, 1993), *cdc22* (Gordon C. and Fantes P., 1986), *cdt1* (Hofmann J.F. and Beach D., 1994), *cdt2* (Hofmann J.F. and Beach D., 1994; Obara-Ishihara T. and Okayama H., 1994), *mik1* (Christensen P.U. *et al.*, 2000; Ng S.S. *et al.*, 2001; Baber-Furnari B.A. *et al.*, 2000), *rad21* (Birkenbihl R.P. and Subramani S., 1995), *ste6* (Maqbool Z. *et al.*, 2003), *ssb1* (Parker A.E. *et al.*, 1997), *ste9* (Tournier S. and Millar J.B., 2000) and *suc22* (Harris P. *et al.*, 1996; Fernandez Sarabia M.J. *et al.*, 1993). Promoter sequences of those genes all contain MCB (*Mlu*I cell cycle box - ACGCGTNA) elements that are the binding sites for a transcription factor complex named DSC1 (DNA synthesis control) or MBF (*Mlu*I binding factor).

This complex contains the products of *cdc10*, *res1* and *res2* genes and is activated by rep2p (Lowndes N.F. *et al.*, 1992; Caligiuri M. and Beach D., 1993; Tanaka K. *et al.*, 1992; Zhu Y. *et al.*, 1994; Miyamoto M. *et al.*, 1994; Nakashima N. *et al.*, 1995; Zhu Y. *et al.*, 1997). Cdc10p, res1p and res2p are homologous to *S. cerevisiae* Swi6p, Swi4p and Mbp1. Their functional domains appear to be highly conserved: the carboxy-terminal region in cdc10p/Swi6p, the amino-terminal region in res1p/res2p/Swi4p/Mbp1p (representing the DNA binding domain) and several copies of an ankyrin motif present in the central part of all of these proteins (Ayte J. *et al.*, 1995; Sturm S. and Okayama H., 1996).

Cdc10p does not bind DNA directly and relies on res1p and res2p for this activity (Whitehall S. *et al.*, 1999). Cdc10p can form a heteromeric complex with res1p (Caligiuri M. and Beach D., 1993) or res2p (Zhu Y. *et al.*, 1994). Since both cdc10p/res1p and cdc10p/res2p recognise MCB elements, an overlapping role has been originally suggested for them, with cdc10p/res1p acting mainly at START and cdc10p/res2p controlling pre-meiotic S phase (Miyamoto M. *et al.*, 1994).

Different Res-protein domains appear to be required for cell cycle-dependent transcription: in the case of res2p, the extreme carboxy-terminal (C) and a domain including the amino-terminal ankyrin repeat are important for transcription activation whereas the C-terminus alone is responsible for rep2p binding (Whitehall S. *et al.*, 1999). The most important domain in res1p corresponds to a region overlapping the amino-terminal ankyrin repeat, and no specific function seems to be associated with the C-terminus (Whitehall S. *et al.*, 1999).

Whitehall *et al*. (1999) have modified the previous model proving that cdc10p, res1p and res2p are present in the MBF complex throughout the entire cell cycle in an inactive state (Whitehall S. *et al.*, 1999) and that transcriptional activation occurs when rep2p, a zinc-finger protein, specifically binds res2p (Nakashima N. *et al.*, 1995; Tahara S. *et al.*, 1998). Another zinc-finger protein, rep1p, was found to be involved in controlling initiation of pre-meiotic DNA synthesis interacting with cdc10p/res1p and cdc10p/res2p (Sugiyama A. *et al.*, 1994) and its role has always been thought to be meiosis specific until recently when White *et al.* (White S. *et al.*, 2001) suggested that rep1p might have an equally important role to rep2 in mitosis. This hypothesis requires further investigation.

As explained in section 1.3.2, in budding yeast the CDK Cln3p/Cdc28p is responsible for regulating the MBF/SBF complexes. Its fission yeast counterpart, cdc2p, and its cyclin partners surprisingly appear not to be involved in such regulation (Baum B. *et al.*, 1997). Tanaka *et al*. (Tanaka K. and Okayama H., 2000) showed that cdc10p/res2p is instead activated by the Pcl-like cyclin pas1p and its kinase partner pef1p, proposing a link between transcriptional regulation and progression through the cell cycle in *S. pombe*. Such a link between a CDK activity and MBF regulation has never been found in fission yeast and this hypothesis requires more evidence.

Another transcription factor named sep1p, belonging to the forkhead family along with *S. cerevisiae* Fkh1p and Fkh2p, has been identified during the characterisation of a mutant showing severe septation defects (Ribar B. *et al.*, 1997; Ribar B. *et al.*, 1999)**.** It is responsible for periodic transcription of *cdc15* whose product is involved in the formation of the actin ring during mitosis (Zilahi E. *et al.*, 2000) and whose peak of expression can be detected just before septation (Fankhauser C. *et al.*, 1995). Deletion of *sep1* results in loss of *cdc15* periodicity. No other sep1p targets have been identified so far, nor has the mechanism of its activity or regulation been clarified. The only other fission yeast forkhead transcription factor reported in the literature is mei4p, which has a meiosis specific role (Horie S. *et al.*, 1998).

The promoter sequence GNAACg/a that confers periodicity to cdc15 has been identified and shown to be present in a subset of genes all showing a peak of expression at the M/G1 transition (Anderson M. *et al.*, 2002). Those genes are: *cdc19*, *dmf1*, *fin1*, *plo1*, *ppb1*, *spo12* and *sid2*. The plo1p kinase is involved in controlling the expression of those genes and probably acts indirectly through a still uncharacterised transcription

factor named PBF by Anderson *et al*. (2002). The homologous *S. cerevisiae* genes *CDC5* (*plo1*), *DBF2* (*sid2*), *SPO12* (*spo12*) and *MCM2* (*cdc19*) that also show a periodic behaviour in budding yeast are under the control of Mcm1p/Fkh2p/Ndd1p forkhead-type transcription factor complex. The *S. pombe* forkhead sep1p protein might not be part of the putative PBF transcription factor (Anderson M. *et al.*, 2002).

The study of another mutant showing a cell separation defect has led to the identification of another transcription factor in *S. pombe*. Cells lacking the *eng1* gene are able to form a septum between mother and daughter cell at the end of mitosis but they cannot separate (Martin-Cuadrado A.B. *et al.*, 2003). Eng1p has a β-glucanase activity responsible for digesting the primary septum that allows cell separation as result of cytokinesis. In wild type cells, *eng1* mRNA shows a periodic behaviour peaking before septation. This periodicity is abolished in mutants lacking the *ace2* gene. *S. pombe* ace2p has been identified based on similarity with *S. cerevisiae* Ace2p, which in budding yeast acts as transcription factor responsible for the activation of the M/G1 wave of transcription, also including the β-glucanase *ENG1*.

1.4.4 Transcriptional cell cycle regulation in humans

Since the sequence and function of the MBF factor components are conserved between the two distantly related yeasts, homologues might also be expected in higher eukaryotes. However, only a functional homologue to MBF could be identified in metazoans, the E2F protein family, whose members show very little homology at the protein level with Swi4p/Swi6p. Functional homology relies on the fact that both complexes regulate gene transcription at the G1/S boundary, they bind a core consensus sequence CGCG, and they have a similar folding pattern (Breeden L.L., 2003).

E2F acts as a heterodimer binding to a member of the pocket protein family. In mammals at least six E2F (E2F1 to E2F6) proteins have been identified, together with three pocket proteins, pRB – product of the *retinoblastoma susceptibility* gene, p107 and p130. Different forms of E2F preferentially bind to certain pocket proteins and different heterodimers are formed at different stages of the cycle (Stevaux O. and Dyson N.J., 2002). E2F binds to p130 in G0, to p107 and pRB in early G1 and is found free in S phase. When bound to E2F, the pocket proteins have an inhibitory role. The inhibition needs to be removed for the cell to progress through the cycle.

Most changes in the E2F state take place at the G1/S transition, called 'Restriction point' (R) in mammalian cells. E2F is regulated by pRB which acts as a transcriptional inactivator. pRB is a negative regulator of cellular proliferation and it is functionally inactived in the majority of human tumors (Wang J.Y. *et al.*, 1994; Harbour J.W. and Dean D.C., 2000; Nevins J.R., 2001; Sherr C.J. and McCormick F., 2002). At START the CDK4/cyclin D and CDK6/cyclin E complexes phosphorylate the pocket protein bound to E2F causing its dissociation from the transcription factor that can therefore activate expression of its targets (McGowan C.H., 2003). One of its targets is the gene encoding cyclin E that forms complexes with CDK2, which are responsible for further phosphorylating the pocket proteins and keeping them in an inactive state. At the end of S phase, cyclin A substitutes for cyclin E in forming a complex with CDK2, resulting in E2F phosphorylation and downregulation of the E2F-dependent transcription.

E2F targets have recently been shown to be involved in several processes, beyond G1/S transition. They range from genes involved in triggering S-phase (Cyclin E and CDK2), in assembling the pre-replicative complex (ORC and MCM proteins) and in DNA synthesis (polymerase α) as well as genes involved in DNA repair (Stevaux O. and Dyson N.J., 2002). Surprisingly, E2F seems to also regulate genes with a mitotic role (Ishida S. *et al.*, 2001) and is itself a target of ATM (responsible for its activation), involved in response to DNA damage as described in session 1.2.5.

Similarly, another function that appears to be conserved from yeast to mammals is the one exerted by the forkhead family of transcription factors or forkhead box factors (FOX) in controlling mitosis. Within the large family of FOX factors in humans, the FOXO group is responsible for many fundamental cell processes, including cell cycle progression and DNA repair. This group includes FOXO1 (formerly known as FKHR), FOXO3a (FKHR-L1) and FOXO4 (AFX) (Burgering B.M. and Kops G.J., 2002). Their activity is regulated by the phosphatidyl-inositol 3OH kinase (PI(3)K)/protein kinase B (PKB) pathway. PKB phosphorylates FOXO members inhibiting their transcriptional activity at the G1 phase of the cycle. Beyond this stage PKB activity goes down allowing FOXO members activities to increase, in correspondence with G2/M, allowing mitosis execution (Alvarez B. *et al.*, 2001). The FOXO targets identified so far (based on homology with the budding yeast identified targets) are cyclin B and PLK, which are both involved in mitosis progression (Glover D.M. *et al.*, 1998).

Several microarray studies investigating gene expression in the human cell cycle have been published so far (Whitfield M.L. *et al.*, 2002; Cho R.J. *et al.*, 2001; Crawford D.F. and Piwnica-Worms H., 2001; Iyer V.R. *et al.*, 1999), as well as in mouse cells (Ishida S. *et al.*, 2001).

Whitfield *et al*. (2002) identified 874 periodic genes in the HeLa cancer cell line and have clustered them according to the cell cycle phase coincident with their peak of expression**.** They have also shown a good correlation between the timing of gene expression and the biological function of genes, as summarised in the following table.

Table 1.4 Periodic gene clusters in HeLa cells according to Whitfield *et al*. (2002)

Cell cycle phase	Cluster	Biological function	Genes
G1	Early DNA replication	DNA replication	CDC6, MCM2-6, DNA pol δ3, ORC1L
		DNA packaging	CHAF1A, CHAF1B, PCNA
		DNA repair	MSH2, FEN1, PCNA
		Cell cycle control	Cyclin E1, E2F1, CDC25A, Cyclin E2
S	Late DNA replication	Nucleotide metabolism	
		DNA replication	DNA polα and θ
		DNA repair and DNA recombination	RAD54, RAD 51
	Histone	Chromatin assembly/disassembly	H2A, H2B, H4, H1, SLBP, NPAT
G2	Tubulin		α-/β-tubulins (TUBA1-3, TUBB, TUBB2), BUB3
G2/M		Mitotic spindle organisation	Kinesins (KNSL1-6), TTK, Cyclin A2, CDC2, CKS1, Cylcin F, ESP1
G2/M		Mitotic spindle checkpoint	Cyclin B2, BUB1, BUB1B, CDC20, CENPE
		Centrosome duplication	STK15, PLK1, NEK2
M/G1		Actin cytoskeleton remodelling	KRAS2
		Cell adhesion	p120, vinculin
		Chromosome architecture	RAD21

Many of the periodic genes identified in this study have been previously reported as cyclic by Ishida *et al*. (2001) (Ishida S. *et al.*, 2001) and by Crawford and Piwnica-Worms (2001) (Crawford D.F. and Piwnica-Worms H., 2001). The Ishida study was performed on mouse embryo fibroblasts and aimed to identify most of the E2F-dependent genes, whereas Crawford and Piwnica-Worms studied the S-G2 specific transcription in HeLa cells.

Comparison with the other two studies by Iyer *et al*. (1999) and Cho *et al*. (2001) did not give equally good results. In the case of Iyer *et al*., the authors themselves recognised that most of the gene changes on the arrays are due to a wound-healing response of the fibroblasts, making it difficult to clearly identify the cell cycle response. Cho *et al.,* (2001) identified 731 periodic genes using human fibroblasts synchronised with a double-thymidine block protocol (Cho R.J. *et al.*, 2001). The overlap with the Whitfield study is only 96 genes, and this is probably due to the many differences in terms of cell lineage, microarray technology and analysis methods between the two studies and also the different degree of synchrony achieved in the different experiments.

1.5 CELL CYCLE RESEARCH USING DNA MICROARRAYS

With an increasing number of organisms now being sequenced, new methodologies are required to make sense of this huge amount of information and to complement more traditional 'gene by gene' approaches. DNA microarrays are increasingly popular tools for studying global gene expression, and the model organisms have been the first for which comprehensive genome-wide surveys of cell cycle regulated genes have been performed, including yeast (Cho R.J. *et al.*, 1998; Spellman P.T. *et al.*, 1998), bacteria (Laub M.T. *et al.*, 2000; Wei Y. *et al.*, 2001), protozoa (Bozdech Z. *et al.*, 2003), plants (Hennig L. *et al.*, 2003; Menges M. *et al.*, 2002) and humans (Cho R.J. *et al.*, 2001; Whitfield M.L. *et al.*, 2002).

In this study spotted arrays were used in two-color hybridisation experiments, as pioneered by Pat Brown and colleagues at Stanford University. Briefly, arrays of thousands of discrete DNA sequences are printed on a glass microscope slide using a robotic "arrayer". To compare the relative abundance of each of these gene sequences in two DNA or RNA samples (for example a 'test' cell state and a 'reference' cell state) the two samples are first labelled using different fluorescent dyes (say, a red dye and a green dye). They are then mixed and hybridised to the arrayed DNA spots. After hybridisation, fluorescence measurements are made with a confocal laser scanner that illuminates each DNA spot and measures fluorescence for each dye separately; these measurements are used to determine the ratio and, in turn, the relative abundance of the sequence of two mRNA or DNA samples for each gene. Computational methods are then applied to the microarray data to identify co-regulated clusters of genes, and motif-finding algorithms are used to find promoter elements characteristic of each cluster (Brown P.O. and Botstein D., 1999; Duggan D.J. *et al.*, 1999).

The research I have performed is inspired by that done with budding yeast (Cho R.J. *et al.*, 1998; Spellman P.T. *et al.*, 1998), as described in the Introduction, section 1.4.2. My work has provided a global picture of the expression profile during the fission yeast cell cycle. This will serve as a framework for the identification and further characterization of previously unknown genes that play a key role in the regulation of the cell cycle. In addition, since fission yeast and budding yeast are only distantly related, complementary studies in the two yeasts have proven to be very fruitful in understanding cell cycle regulation (Forsburg S.L., 1999). Both differences and similarities in the

regulation of gene expression between the two yeasts will be insightful to learn more about transcriptional control of the cell cycle and gene function in eukaryotes.

2. MATERIALS AND METHODS

This chapter contains all the materials and methods utilised to generate the results presented in chapters 3, 4 and 5. All strains used are listed in Appendix I, all buffers, solutions and media in Appendix II, all primers in Appendix III, and a table summarizing the conditions for all the different experiments in Appendix IV.

2.1 FISSION YEAST GROWTH AND MAINTENANCE

2.1.1 Propagation and storage of fission yeast strains

All strains used in this thesis (see Appendix I) were maintained in solid or liquid Yeast Extract (YE). Before conducting any experiment, the mating type and phenotype of each strain have been checked (Moreno S. *et al.*, 1991). When used for an experiment all strains were grown in liquid Edinburgh Minimal Media (EMM) (unless specified - see Appendix IV) supplemented with the required amino acids (225 mg/L), at the temperature specified in the description of each experiment (see Appendix IV) and shaken at 170 rpm. Cells were normally grown to optical density (OD_{595}) of 0.25 -0.3 corresponding to 5 x 10^6 – 6.5 x 10^6 cells/ml. For long term storage strains were stored at -70°C in 50% glycerol - 50 % YE.

2.1.2 Experimental conditions

Elutriation and 'block and release' experiments were performed as described in section 2.3.1. The *nda3* experiment was carried out as described by Baum *et al.* (1998) collecting samples at 2, 4 and 6 hours after the temperature shift to 20 ºC. A zero hour timepoint collected before the temperature shift was used as reference. The hydroxyurea (HU) experiment was done according to Baum *et al.* (1997) collecting samples at 3 and 4 hours during the HU block (11mM HU) and 1 and 2 hours after the release from the block. A zero hour timepoint collected before HU addition was used as reference. The cdc10Δ::ura4 experiment was carried out as described by Ayte *et al*. (1995) collecting samples at 2, 4 and 6 hours after addition of thiamine (2 μM). A zero hour timepoint collected before thiamine addition was used as reference. Overexpression experiments

were carried out as explained in section 2.2.3. For all deletion mutants, cells were grown to OD_{595} of 0.2-0.3 and one single timepoint collected. Wild type cells grown in the same conditions were used as reference. More details concerning the experimental conditions for each mutant can be found in Appendix IVb.

2.2 FISSION YEAST MOLECULAR GENETICS

2.2.1 PCR-based gene deletions

A PCR-based approach was used for specific *S. pombe* gene deletions (Bahler J. *et al.*, 1998). The PCR primers used (for *ace2*, *fhl1*, *meu3* and *meu19* genes) were 100 nucleotides long (see Appendix III for the list of primers used); each primer contained at the 5'-end a gene-specific sequence and at the 3'-end a stretch of sequence homologous to the plasmid multiple cloning site. DNA fragments were amplified using the Advantage 2 polymerase mix (BD Biosciences) and the plasmid pFA6a-kanMX6 as template. A detailed map of the plasmid is shown in Fig. 2.1.

Fig. 2.1 Map of the pFA6a-kanMX6 plasmid, ~ 1.6 Kb size.

PCR reactions were performed in 96-well plates (Corning) and the reaction mix was prepared as follows: 2 µl pFA6a-kanMX6 template, 5 µl 10X Advantage PCR buffer, 1 µl 10 mM dNTPs, 1 µl 60 µM Forward Primer, 1 µl 60 µM Reverse Primer, 1 µl 50 X Advantage 2 DNA polymerase and 40 µl H_2O. The programme used for the amplification consists of 20 cycles at 95°C for 1 min, 55°C for 1 min and 68°C for 2 min. The PCR products were then checked on 1% agarose gels before proceeding. The product of the PCR reaction, of approximately 1.6 kb in size, is the kan^r cassette flanked by a gene-specific sequence. When *S. pombe* is transformed with such a construct, due to

homologous recombination, the kan^r cassette replaces the gene whose flanking sequences are next to the kan^r gene, resulting in a specific gene deletion. Once checked on agarose gel, the products from four PCR reactions were pooled, extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with 3 volumes of 100% ethanol and dissolved in 20 µl of TE (pH 8). This concentrated DNA was used directly for the transformation as described in section 2.2.3.

2.2.2 Cloning for overexpression

Overexpression strains were constructed using a multistep procedure. The genes were first amplified by PCR, then cloned into the pPCR-Script Cam SK(+)vector (Stratagene), re-cloned into the pREP3X expression vector and then transformed into *S. pombe* cells.

PCR reaction

PCR reactions with gene specific primers (for *ace2*, *fhl1*, *fkh2* and *sep1* genes – Appendix III) were used to amplify the gene of interest using the corresponding cosmid DNA as template (Wood V. *et al.*, 2002). All the forward primers contain an *Xho* I restriction site for the following cloning step. PCR reactions were performed with 1 µl of cosmid DNA, 1 µl of 60 µM forward primer, 1 µl of 60 µM reverse primer, 1 µl of dNTPs mix, 1 µl of 50 X Advantage 2 DNA polymerase and 40 µl of water. The programme used consists of 25 cycles at 95°C for 1 min, 55°C for 1 min and 68°C for 2 min.

Preparation of the insert

The first cloning step was done using the PCR-Script Cam Cloning Kit (Stratagene). In order to separate the PCR product from primers, unincorporated nucleotides, buffer components and enzymes, the amplified fragment was purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions and treated with Pfu DNA polymerase (Stratagene) to generate a blunt-end insert for the first step of cloning. The reaction was prepared by adding 10 µl of the purified PCR product, 1 µl of 10 mM dNTPs, 1.3 µl of 10 X polishing buffer and 1 µl of cloned Pfu DNA polymerase (0.5 U)

and incubated for 30 min at 72°C. The fragment was then directly used for the ligation reaction.

Ligation reaction into pPCR-Script vector

The vector used at this stage was the pPCR-Script Cam SK(+) (Fig. 2.2).

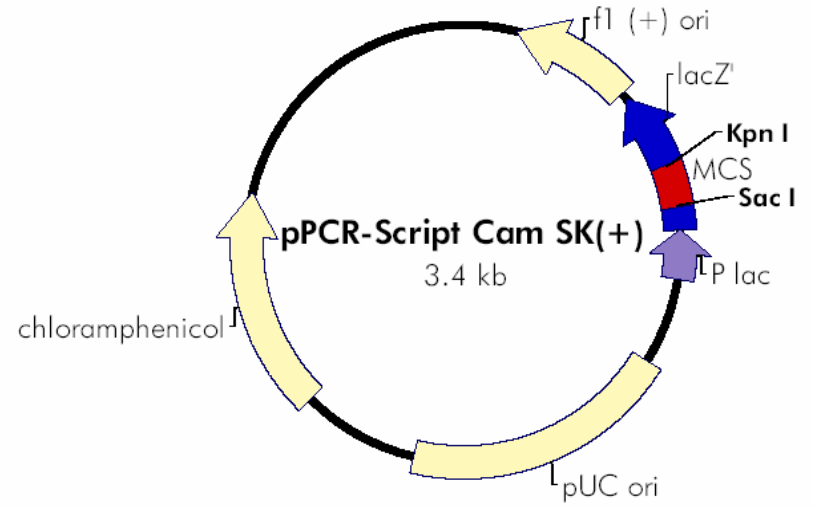


Fig. 2.2 Map of the pPCR-Script Cam SK(+) vector.

Ligation reactions were performed overnight at 15°C with 4 µl of the blunt-ended PCR product, 0.9 µl of the pPCR-Script Cam SK(+) vector, 1 µl of PCR-script 10X buffer, 0.5 µl of 10 mM rATP, 0.9 µl of *Srf*I restriction enzyme (5 U/ µl), 0.9 µl of T4 DNA ligase (4 U/ µl) and 1.8 µl of distilled water. After the overnight incubation the ligation reaction was heated up to 65°C for 10 minutes to inactivate the enzyme and stored on ice until transformation.

E. coli transformation

Transformations were carried out using XL10-Gold Ultracompetent *E. coli* cells (Invitrogen). Before starting the procedure, 1.6 µl of β-mercaptoethanol was added to 40 µl of competent cells to disrupt the membrane and facilitating plasmid DNA uptake. Cells

were then incubated on ice for 10 min, swirling gently every 2 minutes and then 2 µl of ligation mix was added to the cells. The cell/ligation mix was placed on ice for 30 minutes before being heat shocked for 30 seconds at 42°C and immediately quenched on ice for 2 minutes. 450 µl of pre-warmed LB medium was added to each sample and the samples incubated for 1 hour at 37°C with shaking. Each transformation was plated on LB plates supplemented with chloramphenicol (50 mg/ml) and the plates were incubated overnight at 37°C. The following morning, transformed colonies were picked into 2 ml of LB/chloramphenicol medium and the clones grown overnight at 37°C for plasmid DNA minipreps.

Plasmid DNA minipreps

Plasmid DNA was extracted from the overnight culture using the QIAprep Spin Miniprep Kit (Qiagen) and checked by restriction digest for the presence of an insert. 20U of *BamH*I and *Xho*I were used per reaction containing 5 µl of plasmid DNA, 1.5 µl of 10X buffer and distilled water to a final volume of 15 µl. Reactions were incubated for 4 hours at 37°C and the digestion product run out on a 1% agarose gel. Clones were selected among those carrying the insert for the next cloning step.

Preparation of the insert and ligation reaction into pREP-3X vector

The insert cloned into the pPCR-Script Cam SK(+) vector was then transferred to an expression vector, the pREP-3X (Fig. 2.3), capable of being expressed in *S. pombe* cells. The pREP-3X vector contains the inducible *nmt1* promoter (Maundrell K., 1993). Presence of thiamine (15 µM) in the medium represses the promoter. Once thiamine is removed, the promoter gets activated resulting in the transcription of the gene cloned downstream.

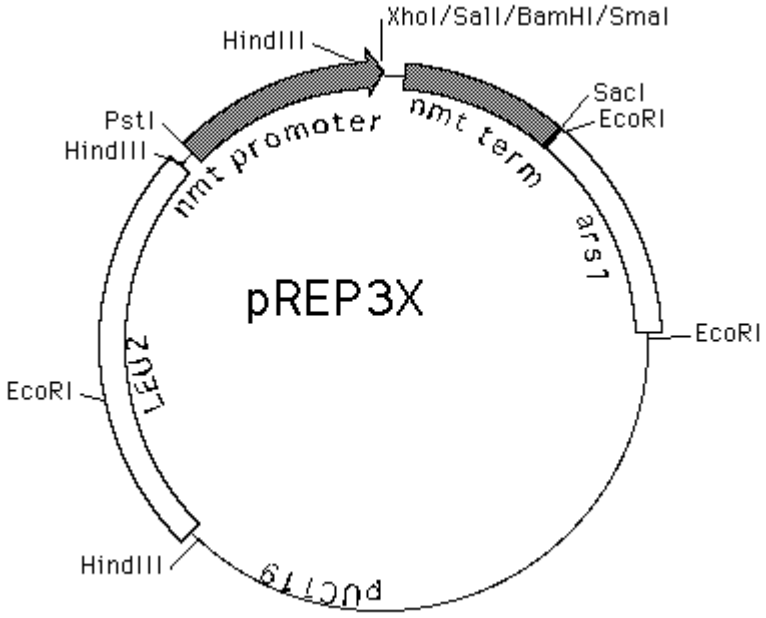


Fig. 2.3 Map of the pREP-3X expression vector

Plasmid DNA from selected clones (20 µl) and pREP-3X DNA (5 µl) were digested using *BamH*I and *Xho*I as described above (doubling the reaction volume). After incubation the samples were loaded onto 1% agarose gels made with 1X TAE and crystal violet (10 µg/ml). This DNA dye is very useful for isolating DNA fragments without exposing the nucleic acid to UV light, as normally occurs when ethidium bromide is used as a marker. This should improve cloning efficiency since the DNA damaging effect of UV light is removed (Hartman P.S., 1991).

Linearised plasmid and insert bands were cut out and purified using GFX PCR DNA and Gel Purification Kit (Amersham Pharmacia Biotech), eluting with 30 µl of elution buffer. The purified pREP-3X (2 µl) and the insert (2 µl) were then ligated with 1.5 µl of 10X ligase buffer, 1 µl of T4 DNA ligase (4 U/µl, Stratagene) and 8.5 µl of water. Ligation was carried out overnight at 15°C.

Second E. coli transformation

The ligation reaction was then used to transform ElectroMAX DH10B cells (Invitrogen) by electroporation. 2 µl of each ligation was placed in a microcentrifuge tube, 20 µl of competent cells added and the mixture pipetted into a chilled 0.1 cm *E. coli* Pulser

Cuvette (Biorad). Samples were electroporated using a Biorad GenePulser II electroporator at 2.0 kV. 1 ml of SOC medium was then added, the solution transferred to a Falcon tube and incubated at 37°C for one hour. Transformations were then plated onto LB plates supplemented with ampicillin (50 μg/ml) and plates incubated overnight at 37°C. Transformed colonies were picked into 2 ml of LB/ampicillin medium and the clones grown overnight at 37°C.

Plasmid DNA minipreps

As previously described, plasmid DNA was then extracted from an overnight culture using the QIAprep Spin Miniprep Kit (Qiagen) and checked by *BamH* I and *Xho* I restriction digest for the presence of an insert.

Plasmid DNA was again extracted from the selected clones using the QIAprep Spin Miniprep Kit (Qiagen) and transformed into *leu1-32 h*$^-$ *S. pombe* cells using the lithium acetate procedure.

2.2.3 Yeast transformation (Lithium acetate procedure)

S. pombe cells were transformed as described in Bähler *et al*. (1998). Cells were grown at 30°C in YE medium to a concentration of ~ 10^7 cells/ml. Around 20 ml of this culture were used for each transformation. Cells were washed once with an equal amount of water, the cell pellet was resuspended in 1 ml of water, washed once with 1 ml of LiAc/TE. The cell pellet was then resuspended in LiAc/TE at 2 X 10^9 cells/ml. 100 μl of the concentrated cells were mixed with 2 μl sheared herring testis DNA and 10 μl of the transforming DNA. After 10 min of incubation at room temperature, 260 μl of 40% PEG/LiAc/TE were added. The reaction was gently mixed and incubated at 30°C for 1 hour. After this second incubation 43 μl of DMSO were added and the cells heat shocked for 5 min at 42°C. Transformed cells were then washed once with 1 ml of water, resuspended in 500 μl of water and plated onto two YE plates (250 μl/plate). These plates were incubated at 30°C for 18 hours.

If the plasmid used for transformation was the pFA6a-kanMX6 (as described in section 2.2.1 for the PCR–based gene deletion experiments) colonies were checked for kanamicin resistance by replica plating them onto YE plates containing 100 mg/l G418

Geneticin (Life Technologies). The replica plates were incubated at 30°C for 2-3 days and large colonies were restreaked onto fresh YE plates containing G418. G418-resistant colonies were checked by colony PCR for integration of the DNA fragment. The PCR reaction mix was prepared as described above and a small amount of each colony used directly as template, resuspending it in the PCR reaction mix already aliquoted in 96-well plates (Corning). The primers used are listed in appendix III. The programme used for the amplification consists of 35 cycles of 95°C for 30 sec, 55°C for 45 sec minus 0.3°C per cycle and 72°C for 45 sec.

For the overexpression experiments (section 2.2.2) transformations were plated onto EMM plates containing thiamine (15 μM) (when the *nmt1* promoter is inactive). To induce overexpression, selected strains were then plated on EMM plates without thiamine (when the *nmt1* promoter is active) and grown for 18 hours at 32ºC for full induction. Similarly, liquid cultures were first prepared growing the strains for 12/16 hours in EMM/thi. Cells were then washed twice with EMM and grown for 18 hours in EMM before collecting a 50 ml sample. Cells carrying the pREP-3X control vector were grown and treated in the same conditions as reference.

2.2.4 Construction of double mutant strains

Double mutant strains *ace2Δ sep1Δ* and *fhl1Δ sep1Δ* were constructed as follows. *Ace2* and *fhl1* were deleted in a *sep1::ura4 ade6-M210/M216 ura4-D18 leu1-32* diploid strain (kindly provided by M. Sipiczki) using the PCR-based approach described in section 2.2.1 followed by *S. pombe* cell transformation as explained in section 2.2.3. Primers used for the deletion are listed in Appendix III. Kanamicin-resistant colonies were sporulated on EMM without nitrogen for 1/2 days at 25°C and tetrad analysis carried out on the meiotic progeny using the Singer MSM System (Singer Instruments). For each strain, several asci produced from sporulation were individually picked using a micromanipulator and placed in a line about 3-5 mm apart on YE plates. The ascus walls were left to break down at 32°C for 2/3 hours and each ascus was then micromanipulated to give a line of four isolated spores, separated by about 3-5mm. Spores were incubated until colonies form at the appropriate temperature for the strain. Colonies carrying the double mutation were then identified by colony PCR and by replica plating on selective medium, as previously explained.

2.3 FISSION YEAST PHYSIOLOGY

2.3.1 Synchronized cultures

Elutriation

In the elutriation process, cells are pumped into a specially designed centrifugal chamber (Beckman), where they are subjected to two opposing forces, the centrifugal force generated by the rotor and the counterflow of the fluid that is pumped in the opposite direction. As a result, each cell migrates to a zone where the two forces are balanced, forming a gradient of cell size along the chamber, as shown in Fig. 2.4.

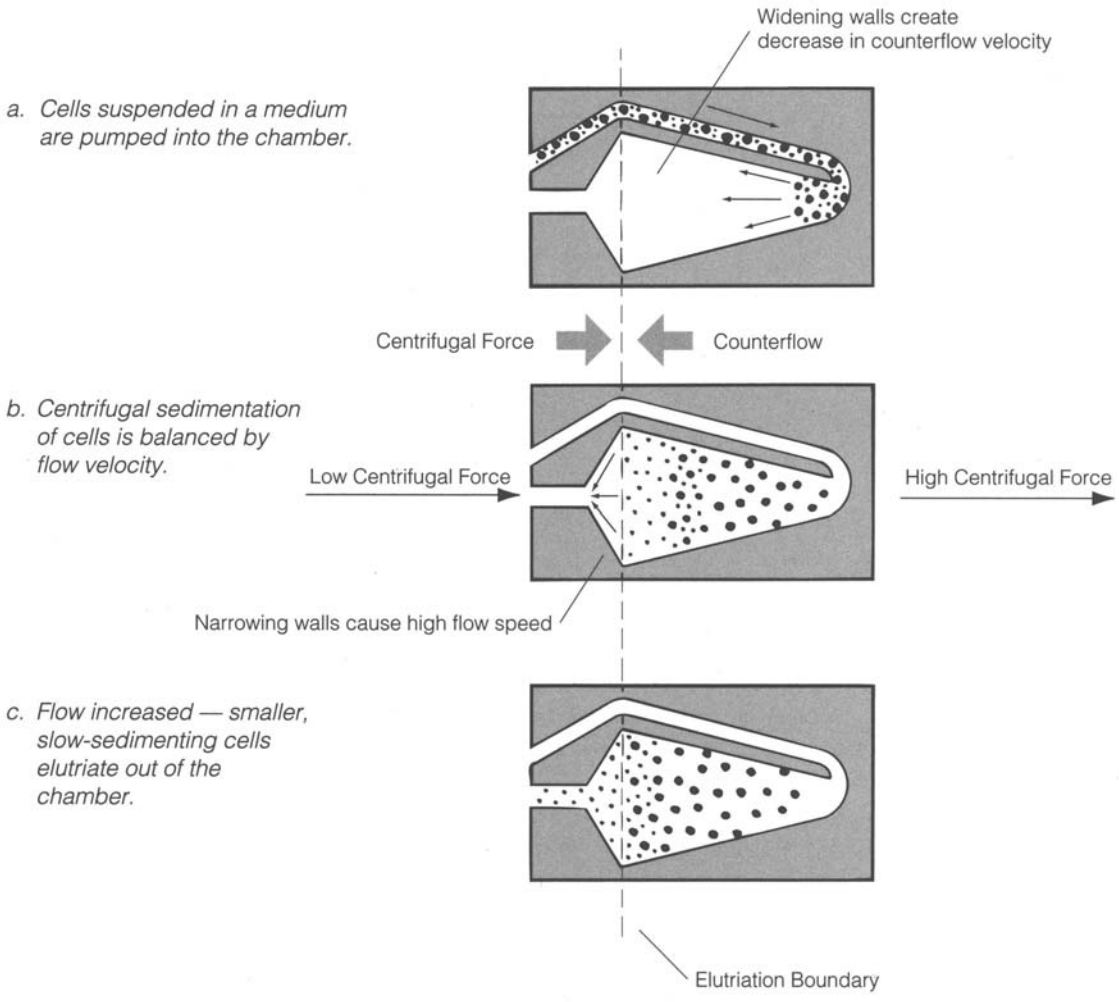


Fig. 2.4 The elutriation process.

From "The JE 5.0 Elutriation System – instruction manual", Beckman Coulter

The smallest, slowest sedimenting cells are held at the top of the chamber and can be specifically eluted by a small increase in the flow rate. This yields a homogeneous population of cells at the beginning of the cell cycle that will progress into division in a synchronous manner.

Five litres of wild-type fission yeast strain *972h*$^{-}$ were grown in minimal medium (EMM) at 30ºC to a cell density of around 5 X 10^6 cells/ml. This volume was loaded into a dual-chamber elutriation chamber (Beckman Instruments) in a Beckman J-6 M/E centrifuge equipped with a JE-5.0 elutriation rotor. A control sample of 300 ml was collected before starting the loading procedure. During the loading step the medium in which the cells were grown was collected and was referred to as conditioning medium. The elution was achieved using minimal medium (containing 20% of conditioning medium, 0.005% YE) pre-warmed to 30ºC. Small cells (650 ml at 2X10^6 cells/ml) were collected and grown at 30ºC for an hour to allow recovery. After that, samples were taken every 15 min for the next 6-7 h in order to cover two full cell cycles, with independent samples for DAPI and Calcofluor staining (1 ml), FACS analysis (1 ml), cell counting (1.4 ml) and RNA preparation (25 ml).

Arresting cells with temperature-sensitive mutants

Temperature-sensitive cell cycle mutants can be used to block cells at a particular point in the cell cycle. On return to the permissive temperature, cells will then proceed synchronously through the subsequent phases of the cycle. The temperature-sensitive strains used in this study are: *cdc25-22* which arrests in late G2 when shifted at the restrictive temperature, *cdc10-129* which arrests in G1 and *nda3-KM311*, a β-tubulin mutant that arrests in metaphase.

The *cdc25-22 h-* strain was grown to an OD_{600} of 0.15-0.2 in EMM medium at 25°C shaking at 170-200 rpm. The culture was then shifted to a 36°C water bath and held at that temperature for 3.5 hours. By this time cell density reached an OD_{600} value of 0.3-0.5. The cells were then released from the cdc25 arrest by shifting the culture to a 25°C water bath. 25 ml samples were taken every 15 min for 360 min after the shift. A control sample of 300 ml was collected before the temperature shift. Synchrony and progression through the cell cycle were monitored by FACS analysis (to measure DNA replication),

by DAPI staining of DNA (to determine mitotic cells), by cell counting and by Calcofluor staining of the cell wall (to determine cell division septa).

Two experiments, one using *cdc25-22* and another using *cdc10-129* were performed combining the two synchronization methods. Cells were first elutriated as explained above and subsequently blocked at 36ºC for 2.5 hours. Samples were collected every 15 minutes during the block and after the release following one full cell division. The *nda3-KM311* strain was grown at 32ºC for 12/16 hours and then shifted to 20ºC. Samples were collected at 2, 4 and 6 hours after the shift. A reference sample was collected before the shift to 20ºC. A wild type *972 h-* culture was grown in parallel and a single timepoint collected 6 hours after the shift to the restrictive temperature.

2.3.2 Cell number measurement

For cell counting cells (1.4 ml) were fixed by adding 2.8 ml of formal saline solution. Samples were diluted between 20X to 100X in ISOTON II (Beckman Coulter) in order to get an average count of 25000. Cell number was measured using a Beckman Coulter Z Series.

2.3.3 DNA content measurement

DNA content determination samples were prepared using propidium iodide staining as previously described (Sazer S. and Sherwood S.W., 1990). 500 μl of the ethanol fixed cells are washed in 1 ml of 10mM EDTA, cells are centrifuged for 1 min at 14000 rpm, the supernatant discarded and the pellet resuspended in 500 μl of 10 mM EDTA containing RNase A at 0.1 mg/ml final concentration. Cells are incubated at 37°C for at least 2 hours. After incubation 500 μl of 10 mM EDTA containing propidium iodine (4 μg/ml) are added to each sample. DNA content is measured using the Becton Dickinson FACScan.

2.4 FISSION YEAST MICROSPOPY

DAPI and Calcofluor staining were performed as described in Moreno *et al.* (1991). A separate 1 ml aliquot was taken at each time point of the time course experiments, cells were centrifuged for 15 sec at 14000 rpm and the pellets resuspended in 1 ml of cold 70% ethanol. Alternatively cells were fixed using a formaldehyde solution. For this fixation 10 µl of formaldehyde were added to 900 µl of sample.

2.4.1 DAPI staining

For DAPI staining, 50 µl of the ethanol fixed cells were washed in 1 ml of water, centrifuged for 1 min at 14000 rpm and the pellet resuspended in water containing DAPI at 1 µg/µl final concentration. Cells were then placed on a glass slide and visualized by fluorescence microscopy, using a Zeiss microscope.

2.4.2 Calcofluor staining

For Calcofluor staining, formaldehyde-fixed cells were centrifuged for 1 min at 14000 rpm, the supernatant discarded and the pellet resuspended in 10 µl of PBS. 1-2 µl of a Calcofluor saturated solution was added to 5 µl of sample and cells visualized using a Zeiss microscope.

2.5 MICROARRAY EXPERIMENTS

General overview

The microarrays used in this study were DNA arrays. They were fabricated by printing on a glass microscope slide many gene-specific probes obtained by PCR amplification from *S. pombe* genomic DNA. After fixation of the DNA to the glass surface, the slides were then hybridized with fluorescently tagged cDNA synthesized from total RNA of sample and reference. The fluorescent signal was then detected using a laser scanner, an image

acquired for each slide and subsequently processed to extract the data and analyze them (see Fig. 2.5).

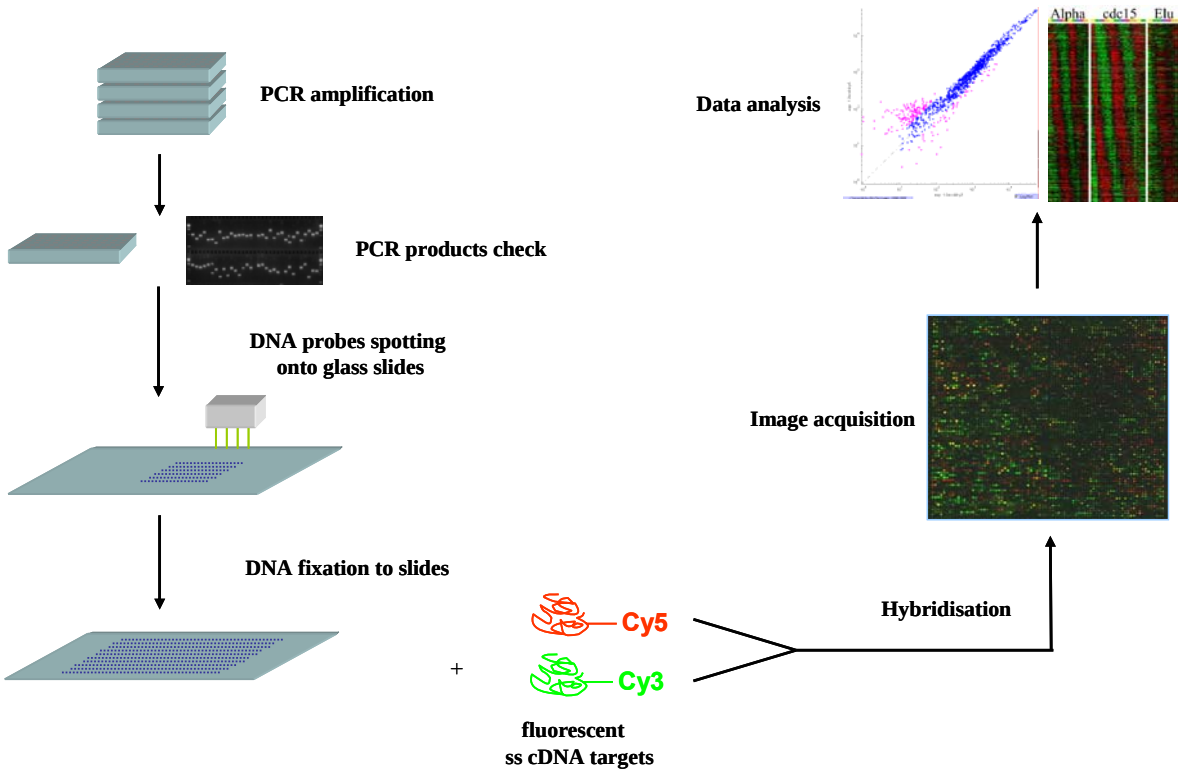


Fig. 2.5 **Overview of a typical microarray experiment**

2.5.1 Microarray fabrication

Each array contains probes for all the known *S. pombe* ORFs (the number of which varies between 4900 and 5270 depending on the generation of arrays), for 11 mitochondrial genes, 19 pseudogenes, several RNA genes, 114 hypothetical ORFs, 33 large introns as well as several control spots such as 22 *S. cerevisiae* genes, markers and tagging sequences (Kan-MX, GFP, GST, Myc, etc) and five *Bacillus subtilis* genes (lysA, pheB, dapB, thrB and trpC).

Two rounds of PCR amplification were performed for each array element: a first round using gene-specific primers with *S. pombe* genomic DNA, and a second round using a combination of a gene-specific reverse primer and a universal forward primer with the product of the first amplification. The universal primer contains a 5'-amino

modification that allows covalent attachment of the DNA to the glass slide. Array elements were printed onto amine-binding slides (Codelink, Amersham) using a MicroGrid II Total Array System (Biorobotics) at The Sanger Institute Microarray Facility. All elements were printed in duplicate, in separate halves of the slide, for a total number of 13000 spots. More details concerning microarray fabrication, printing and post-processing of the slides can be found in Lyne *et al*. (2003) or on the lab website (http://www.sanger.ac.uk/PostGenomics/S_pombe/).

2.5.2 RNA preparation

S. pombe total RNA was isolated according to Jang *et al.* (1995) with minor modifications. 25 ml aliquots of yeast culture were collected during a time course experiment every 15 min, harvested by centrifugation and frozen at -70ºC. Cells were then thawed on ice and resuspended in 1 ml of DEPC-treated water and pelleted by centrifugation. 750 µl of TES extraction buffer was added to the pelleted cells, cells resuspended and 750 µl of acidic phenol-chloroform (5:1, Sigma) added. The mixture was vortexed for 10 sec and incubated for 1 h at 65°C, vortexing for 10 sec every 10 min. The mixture was then rapidly chilled on ice and centrifuged for 20 min at 14000 rpm in a microfuge at 4°C to separate the aqueous and organic phases. The aqueous phase was transferred to a phase-lock tube (Eppendorf) and extracted with 700 µl of acidic phenol-chloroform. After centrifugation, a third extraction was similarly carried out with 700 µl of chloroform/isoamyl alcohol (25:1, Sigma). The aqueous phase collected after the centrifugation was precipitated in 2.5 volumes of absolute ethanol and 1/20th of 3M Na acetate pH 5.2. Afterwards the RNA pellet was washed with ice-cold 70% ethanol and air-dried to evaporate the residual ethanol. The pellet was resuspended in 100 µl of DEPC-treated water, the OD was measured and 2 µl checked on a 1% agarose gel.

2.5.3 Labelling protocol for total RNA

Total RNA (10-20 µg) and 2 µg of anchored oligo-dT/random primers were combined in a total volume of 16.4 µl. RNA/primer mixtures were heated to 70°C for 10 min and then cooled on ice for 1 minute. 2 µl of 25 mM Cy3- or Cy5-conjugated dCTP (NEN), 3 µl of 0.1 M DTT, 6 µl of first-strand buffer (GibcoBRL), 0.6 µl of dNTPs (25 mM each of

dATP, dTTP, and dGTP and 10 mM dCTP), and 2 µl of Superscript II reverse transcriptase (Gibco BRL) were added. Each sample was then incubated at 42°C for 1.5 hours to generate Cy-labelled cDNA. Starting RNA was hydrolysed by addition of 1.5 µl of 1 M NaOH and incubation at 70°C for 15 min. Samples were neutralized by addition of 1.5 µl of 1 M HCl. Labelled cDNA was separated from unbound fluorochromes by separation in an AutoSeq G-50 column according to the manufacturer's instructions (Amersham). After ethanol precipitation, the probe was resuspended in 27 µl/reaction of hybridisation buffer and 3 µl/reaction of polyA DNA (2 µg/µl, Sigma). The hybridisation mixture was then denatured at 100°C for 5 min, cooled down at room temperature for 10 min and hybridised to a microarray.

2.5.4 Hybridisation and posthybridisation

The microarray was covered by a 25X60 mm coverslip (Menzel-Glaser) and placed in a hybridisation chamber. 2 ml of 15X SSC were placed inside the hybridisation chamber before sealing, and the chamber placed in a 49°C oven. The microarrays were hybridised for 12-16 h. Microarrays were removed from the chambers and placed in standard histochemistry slide holders where they were washed at room temperature once for 5 min in washing solution 1, twice for 15 min in washing solution 2 and once for 5 min in washing solution 3 (see appendix II for details on washing solutions). Slides were then dried by spinning for 2 min at 1200 rpm.

2.5.5 Image acquisition

Slides were scanned using GenePix 4000B (Axon Instruments), a confocal scanner that uses two lasers operating at 635 nm and 532 nm to excite Cy5 and Cy3, respectively. The scanner uses a simultaneous dual-laser scanning system to produce a ratio image in real time. Each image was saved as a separate 16-bit TIFF file. These images were analyzed to calculate the relative expression levels of each gene and to identify differentially expressed genes.

2.6 MICROARRAY DATA ANALYSIS

2.6.1 Image processing

For image processing GenePix Pro 3.0-5.0 software (Axon Instruments, Inc.) was used. It applies a thresholding algorithm to separate spots from background, allowing a grid to be laid across the spots. Having found a grid, spots are found within each grid element, local background is calculated, background-subtracted and integrated intensities are calculated in both the Cy3 and Cy5 channels. GenePix Pro 3.0-5.0 computes a large number of different ratio quantities, each of which provides different insight into the raw data. They can be divided into three different subgroups: those that are ratios of quantities derived from whole features (such as 'ratio of medians' and 'ratio of means'), those that are derived from pixel-by-pixel ratios of intensities (such as 'median of ratios' and 'mean of ratios') and quality factors.

2.6.2 Data normalization and evaluation

Following image processing, the data generated for the arrayed genes must be further analysed before differentially expressed genes can be identified. Various computer programs are required to organize and evaluate the data. The first step in this process is the normalization of the relative fluorescence intensities in each of the two scanned channels. Normalization is necessary to adjust for differences in labelling and detection efficiencies for the fluorescent labels and for differences in the quantity of starting RNA from the two samples examined in the assay. These problems can cause a systematic shift in the average ratio of Cy5 to Cy3, and the intensities must be rescaled before an experiment can be properly analysed.

In this thesis, normalisation was performed using a Perl script developed by our group (Lyne R. *et al.*, 2003) that uses as imput file the GenePix result files. The script has two main functions: it first selects the spots on the array that are above certain cut-off parameters and then performs the normalisation on those selected spots. Only the spots that have >50% of pixels that are >2 standard deviation (SD) above median local background signal in one or both channels will pass the first screening. If a spot has

>95% of pixels that are > 2SD above local background in only one channel it will also be kept for the normalisation step. All discarded spots were flagged 'absent'. The normalisation is performed locally, using a sliding window including 400 spots as minimum number of features. The normalisation factor is calculated for each gene individually assuming that the medial signal ratio of all the measurable spots within the window is equal to 1. The resulting normalisation factor will then be used to correct the signal ratio for the spot on which the window was centred.

In addition, for the time course experiments, a second normalisation step was carried out using the 'per gene' normalisation option in GeneSpring. For each gene, it divides the value for each timepoint by the median of the value of all timepoints, thus centring all data to 1.

2.6.3 Identification of periodic genes

Identification of periodic genes in large datasets as obtained using microarrays is not an easy task. Spellman *et al*. (1998) pioneered this field publishing one of the first studies where periodic gene transcription in budding yeast was extensively investigated using microarrays. In the original paper, cell cycle regulated genes were identified using the Fourier transform. Since its publication, the data have been re-analysed in many different ways using increasingly sophisticated statistical methods (Cooper S. and Shedden K., 2003).

Since then several microarray studies focusing on periodic transcription have used different statistical approaches to tackle the same issue: Fourier transform (Whitfield M.L. *et al.*, 2002), fast Fourier transform [FFT - (Bozdech Z. *et al.*, 2003)], discrete cosine transform [DCT - (Laub M.T. *et al.*, 2000)], supervised clustering (Cho R.J. *et al.*, 2001) and hierarchical/K-means clustering (Ishida S. *et al.*, 2001).

It should be mentioned that a purely statistical approach to the identification of periodic genes will not always be the best option. This is because such an approach requires a relatively high number of repeats and the arbitrary choice of a cut-off point below which the data is unreliable (Cooper S. and Shedden K., 2003). For this reason, in this thesis the identification of periodic genes was done combining a simple autocorrelation approach, visual inspection and subsequently validated with a more sophisticated statistical analysis using fast Fourier transform. The traditional clustering

algorithms (Eisen M.B. *et al.*, 1998; Sherlock G., 2000) are based on a correlation algorithm. All single expression profiles in a dataset are compared to each other and a correlation score is assigned to each pair. The members of the pair with the highest correlation score will be joined to form a node of the cluster, and the process will be repeated until all profiles have been assigned to a node.

In the majority of the timecourse experiments done for this thesis two full cell cycle divisions have been followed, allowing the assumption that a gene behaving periodically would have reached a peak of expression in the first cycle and another peak in the second cycle. The first-cycle profile was then compared to the second-cycle profile and an autocorrelation score assigned according to the similarity between the two. An average autocorrelation score was calculated for each gene across three elutriation and two *cdc25-22* 'block and release' experiments, and genes were ranked according to the average autocorrelation value.

In parallel, a fast Fourier transform analysis (FFT) was run on the same dataset as well as on the randomized dataset after 100000 permutations of the expression signals. Randomisation is very important to determine whether the cyclic gene behavior arises from chance arrangements in the measurements or has a real biological significance. Part of this was done in collaboration with Pietro Lio in Alvis Brazma's group at the European Bioinformatics Institute.

For each gene a P-value (*P*) was calculated comparing the result of the FFT analysis performed on the real data with the one obtained from the randomized data. Only genes with a $P < 0.01$ were considered for further analysis. Among those only the ones with a fold change > 1.5 were again visually inspected to confirm periodicity.

Information concerning the biological function of each periodic gene was derived from the following databases: the PombePD (www.incyte.com/control/tools/proteome) and the *S. pombe* GeneDB (www.genedb.org/genedb/pombe/index.jsp). When a more detailed description of the gene function was needed, one of the following text books was consulted: The Molecular and Cellular Biology of the Yeast *Saccharomyces* (1997), The Yeast Nucleus (2000) and The Molecular Biology of *Schizosaccharomyces pombe* (2004). On many occasions, specific research papers were also used but references to them were omitted in the text (especially in Chapter 3, section 3.4).

Identification of genes differentially expressed in gene deletion and overexpression experiments was achieved by combining two methods. First, an intensity-

dependent standard Z-score was calculated by determining the local mean and standard deviation (SD) within a sliding window of 1000 genes (Yang I.V. *et al.*, 2002). Genes with ≥ 2 SD above the local mean in repeated experiments were considered differentially expressed. When at least three or more repeats were available for each experiment, SAM (Significance Analysis of Microarrays) was used to confirm differential expression (Tusher V.G. *et al.*, 2001).

2.6.4 Clustering periodic genes

The simplest example of classification is a case where genes are grouped according to the time of their highest peak of expression which can be easily calculated from the Fourier algorithm. Genes can also be grouped together by various clustering algorithms, based on the similarity between their expression profiles under different conditions (Sherlock G., 2000; Eisen M.B. *et al.*, 1998). This helps to suggest functions of unknown genes that are co-expressed together with genes of known function ('guilt by association'). Moreover, genes that are co-expressed can be checked for regulatory promoter sequence motifs required for the binding of transcription factors (Vilo J. and Kivinen K., 2001; Brazma A. and Vilo J., 2000).

To group genes based on the similarity in their expression profiles, we used the Gaussian mixture model with Pearson coefficient in ArrayMiner 5 (Optimal Design - (http://www.optimaldesign.com/Download/ArrayMiner/AM2whitepaper.pdf). Clustering was also performed using the K-means algorithm (Sherlock G., 2000) in GeneSpring 6 (Silicon Genetics).

2.6.5 Identification of promoter motifs

It seems reasonable to expect that genes that are co-expressed (i.e. that have very similar expression profiles) might also be co-regulated, meaning that they share some common regulatory mechanisms such as being targets of the same transcription factor. Transcription factors act by binding to a sequence-specific site in the DNA and influencing the expression of the downstream gene. These binding sites are located in promoter regions that in yeast are normally less than 1000 bp upstream of the transcription starting site.

Once a cluster of co-expressed genes has been identified, the upstream regions of those genes can be scanned by looking for common regulatory elements. Several programmes are available to perform such analysis, within microarray analysis packages such as GeneSpring as well as other applications. In this thesis the pattern discovery tool SPEXS (http://ep.ebi.ac.uk/EP/SPEXS) (Vilo J. *et al.*, 2000) was used and the analysis was run at the European Bioinformatics Institute by Katja Kivinen in Alvis Brazma's group. The up- and downstream intergenic regions (600bp and 400bp, respectively) for all coding sequences were extracted from genomic sequences from the Sanger Institute (ftp://ftp.sanger.ac.uk/pub/yeast/pombe/). 19 genes were discarded (transposons, telomeric duplications, or very short sequences). We grouped genes in overlapping clusters using a sliding window (40 and 60 genes) along the phase-ranked gene list of periodic genes, shifting the window by 10 genes at a time. Alternatively, we used lists of genes dependent on Ace2p or MBF. To estimate the expected number of pattern occurrences, we used all intergenic regions. We defined cluster-specific significance thresholds from randomized sets of sequences of respective sizes. We required that the pattern occurred in at least six sequences in the cluster, that its binomial probability was smaller than the significance threshold, and that it occurred in the cluster at least twice as often as expected using these criteria. No significant motifs in downstream regions were found. All patterns passing the criteria were grouped by similarity, and the best one in each group was reported. Putative regulatory patterns and locations were visualised using PATMATCH and SEQLOGO tools in Expression Profiler (http://ep.ebi.ac.uk/EP/). For sequence logos, we introduced pattern variations by allowing one wildcard, searched for matches to this approximate pattern and calculated the number of each base occurrence for each position. The letter sizes were rescaled according to the information content in that position. For MCB and Ace2 motif logos, we used the lists of MCB- and Ace2p-dependent genes instead of sliding windows.

2.6.6 Comparison between fission and budding yeasts

Gene lists of *S. cerevisiae* cell cycle regulated genes were downloaded from the accompanying websites of Spellman *et al.* (1998) and Cho *et al.* (1998). These lists were imported into GeneSpring (Silicon Genetics) and the overlapping genes between the two datasets identified. For each comparison a P-value (*P*) was calculated using the

hypergeometric distribution (Fig. 2.6). This equation calculates the probability of overlap corresponding to *k* or more genes between a gene list of *n* genes compared to a gene lists of *m* genes, when randomly sampled from a universe of *u* genes.

$$\frac{1}{\binom{u}{m}} \sum_{i=k}^{n} \binom{m}{i} \binom{u-m}{n-i}$$

Fig. 2.6 Hypergeometric distribution.

This formula was used to calculate the P-value associated with all gene lists comparisons

For each comparison the size of the overlap expected by chance (x) was also calculated using the following formula where y and z represent the size of the two lists compared and T the total number of *S. pombe* genes with a *S. cerevisiae* ortholog:

$$x = \frac{(y)(z)}{T}$$

The resulting core set of genes in common between the two budding yeast studies was used for comparison between the two yeast models. Genes with a prospective *S. pombe* ortholog were determined using a table of curated orthologs created by Val Wood at the Sanger Institute. The total number of orthologs included in the analysis was 2981. Genes were translated into *S. cerevisiae* homologs using this table and lists comparison was performed with Genespring. A comparison was also done with the list of human genes reported as periodic in the literature, using traditional genetic approaches.

3. PERIODIC GENE EXPRESSION DURING THE MITOTIC CELL CYCLE IN FISSION YEAST

This chapter will provide a global overview of the periodic expression profiles during the fission yeast cell cycle. Two different synchronisation methods were used in order to study changes in gene expression as a function of time: centrifugal elutriation and temperature sensitive mutants. The results presented in this chapter were derived from the following eight timecourse experiments: three wild type elutriations, two *cdc25* 'block and release', one *cdc25* experiment done combining elutriation and 'block and release', one *cdc10* experiment again done by combining the two synchronisation methods and one *sep1Δ cdc25* 'block and release'.

3.1 Experimental overview

One aim of this thesis was to identify genes showing a periodic behaviour during the mitotic cell cycle in fission yeast using DNA microarrays. In order to achieve this, the relative abundance of mRNAs was measured as a function of time in cultures synchronised using two different methods, and samples were collected in order to cover two full cell cycles in most experiments. The first method used to obtain a synchronous culture was centrifugal elutriation and the second one was temperature-sensitive mutants. For each timepoint, the gene behaviour in the synchronous population was compared to a reference sample consisting of an asynchronous population of the same strain growing under the same conditions. For each synchronisation method used, at least one experiment has been hybridised with a dye swap. More detailed information on the experimental conditions for all timecourses can be found in Appendix IVa.

When comparing the outcome of several cell cycle microarray studies done in different human cell lines (Cho R.J. *et al.*, 2001; Whitfield M.L. *et al.*, 2002; Iyer V.R. *et al.*, 1999), the overlap between them is poor, and the main reason for this is probably the difference between experimental conditions, especially the different methods used for synchronizing cells. Similarly, the overlap between the two budding yeast studies is relatively small (Cho R.J. *et al.*, 1998; Spellman P.T. *et al.*, 1998). Such differences between studies conducted in similar ways have raised quite a lot of scepticism concerning the validity of the synchronization techniques themselves, claiming that

synchrony cannot be really achieved using traditional whole-culture methods, meaning those experiments where an entire culture of growing cells is used to produce a synchronous cell population (temperature sensitive mutants) (Cooper S. and Shedden K., 2003). From this point of view selective synchronization methods (elutriation) where only a small fraction of a culture is used to obtain a synchronized population are preferable.

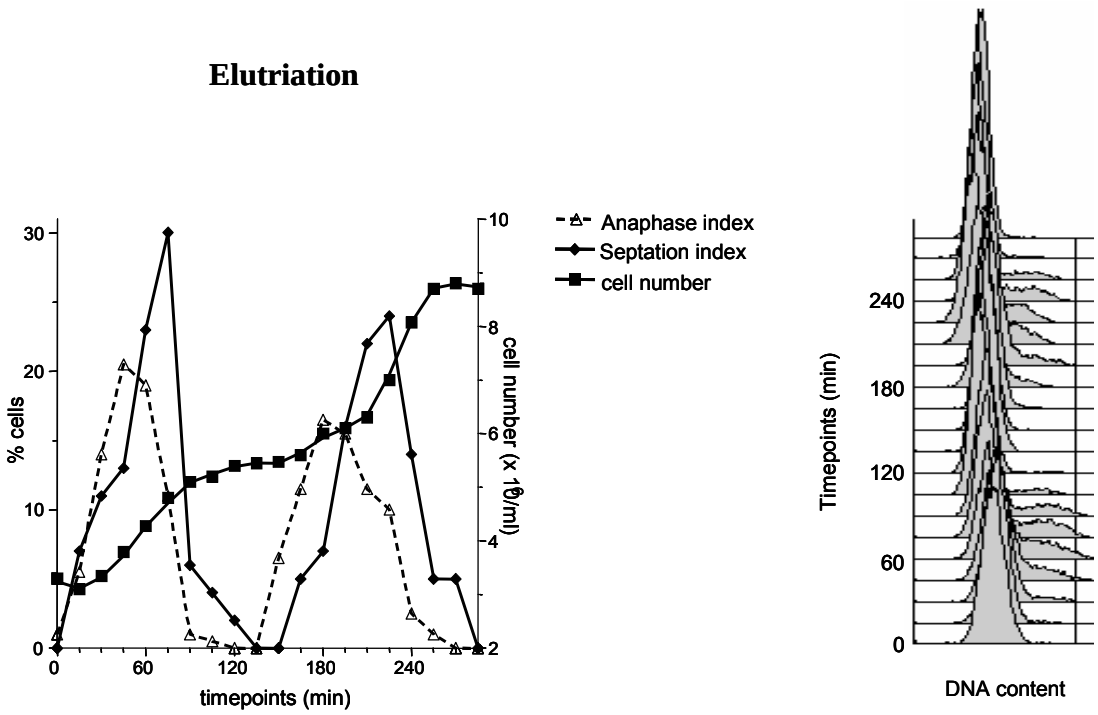
For these reasons, in this thesis both synchronization methods (whole-culture/temperature sensitive mutants and selective/elutriation) were used, and synchrony was assessed by measuring different parameters for each timepoint collected: number of septated cells (named septation index), number of nuclei (DAPI index), DNA content (FACS index) and cell number. The results of these measurements are shown in Figure 3.1A for one elutriation experiment and in Figure 3.1B for one *cdc25* 'block and release' experiment. Additional results for the six remaining timecourses can be found in Appendix V (Fig. V.1-V.3).

In a typical elutriation experiment, nuclear division (Fig. 3.1, DAPI and FACS index) takes place about 1 hour 45 minutes after elutriation (including a 1 hour recovery period for cells). Cell division (Fig. 3.1, septation index) takes place 30 minutes after nuclear division, as also confirmed by the doubling of cell number that can be seen immediately after the peak of septation (Fig. 3.1, cell number). The period between first and second division is normally around 135 minutes in EMM medium at 30°C. In a typical *cdc25* 'block and release' experiment, nuclear division takes place 30 minutes after cells have been shifted back to the permissive temperature, and septation immediately follows at 45 minutes, together with the expected doubling of cell number. The gap between the two divisions is around 120 minutes, in EMM at 25°C. The process of cell division appears to be faster in *cdc25*; because the size of the *cdc25* cell is bigger than the wild type, G2 is therefore shorter because the cell size required to enter mitosis is reached earlier in the cycle.

An apparently higher degree of synchrony was normally achieved in the *cdc25* 'block and release' experiments (60%-70% of septated cells) compared to the elutriations (30% of septated cells). However, much of this difference could be due to extended duration of septation in cdc25 'block and release' experiments; according to the gene expression profiles, there was very little difference in synchrony between the two methods. Despite the fact that temperature sensitive mutants give better synchrony, it is important to consider that a temperature shift likely introduces more artefacts due to the

heat shock involved compared to the moderate mechanical stress experienced during elutriation. For this reason, elutriation is probably the more physiological method for achieving synchrony.

A



B

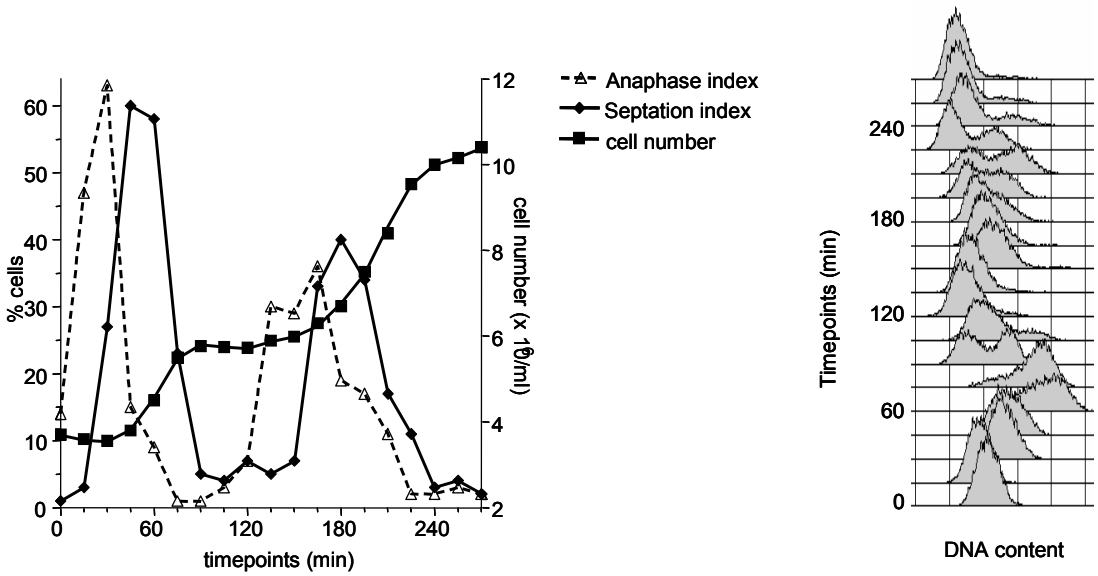


Fig. 3.1 Parameters defining cell cycle synchrony.

Panel A, the left hand side graph shows DAPI index, septation index and cell counting whereas the right hand side graph shows the FACS profile for a typical elutriation experiment. Panel B, shows the same results as panel A for a typical *cdc25* 'block and release' experiment.

3.2 Identification of periodic genes

Genes were ranked according to an average autocorrelation score calculated from the data obtained from three elutriation and three *cdc25-22* 'block and release' experiments (including the *sep1::ura4 cdc25-22* experiment), as described in Materials and Methods. The top 2500 genes were then visually inspected to confirm or reject the periodicity, comparing the profiles with the ones from the *cdc25-22* and *cdc10-129* 'block & release' experiments in which synchronisation was achieved by combining elutriation and temperature shift. In these particular experiments, samples were collected throughout the block and after the release only for one cycle, making it impossible to use these data for the autocorrelation analysis. Around 250 genes were judged to be periodic after this inspection.

These results were compared with the output of the fast Fourier transform and randomisation analysis carried out at the EBI. Following this approach, around 1000 genes had a P value < 0.01 and among these around 800 showed a fold change > 1.5 in all three of the following experiments: two elutriations and one *cdc25* 'block and release'. After visual inspection ~ 400 genes were discarded because their periodicity was not consistent across all experiments or with the duration of the cell cycle. This clearly shows the limitations of an automatic approach for the discovery of periodic genes and underlines again the importance of visually inspecting the dataset to validate the output of any statistical methodology.

From the comparison of the gene lists obtained from these two independent approaches, a total of 407 periodically expressed genes were identified among the 5119 *S. pombe* genes investigated, corresponding to ~ 8% of the total gene number. 136 genes showed a change in expression > 2 fold in the elutriation experiments, and we will refer to them as 'high amplitude' genes. The other 271 showed changes between 1.5 and 2-fold and we will refer to them as 'low amplitude' genes. A complete list of genes can be found in Appendix VI, together with a description of their biological function. The table also

contains information concerning the motifs found in the promoter region of each gene, as will be discussed in chapter 4.

3.3 Clustering of periodic genes

After identification, the 407 genes were classified using a clustering algorithm according to similarities in their expression profiles. Clustering was performed independently using two different methods: ArrayMiner (http://www.optimaldesign.com/Download/ArrayMiner/AM2whitepaper.pdf) and K-means [Materials and Methods – section 2.6.4; (Sherlock G., 2000)]. The ArrayMiner classification resulted in biologically more coherent clusters than the K-means approach in GeneSpring.

Traditional clustering algorithms treat genes as vectors and measure the distance between the two vectors with a distance metric such as Pearson correlation or Euclidean distance (Sherlock G., 2000). This measure reflects the degree of similarity between the two expression profiles and it therefore makes sense to group together those genes that are closest to each other in space. This is an example of *hierarchical clustering*. In the *K-means clustering* method instead, genes are divided from the beginning into an arbitrarily assigned number of clusters. At this stage, the distance between each gene and the centre of the cluster it belongs to are calculated and genes eventually reassigned to a closest cluster. Both methods have their weaknesses. Outliers can represent both meaningful data as well as artefacts created by the experimental procedure. Existing clustering methods will assign them to a cluster, very often altering the structure of the classification. Having to choose from the start the number of clusters is also problematic, especially when little is known about the biological function of each class.

The ArrayMiner non-hierarchical algorithm is based on the assumption that clustering should have the ultimate goal of grouping together genes with distinct biological functions. Therefore the aim is to obtain a distribution of clusters as close as possible to the distribution of the data, modelling the data with a number of Gaussian distributions that best fit the dataset. Outliers are detected and considered as a *uniform distribution* that is competing with the Gaussians. The non-hierarchical approach takes into account the cluster variance and the clusters created remain stable despite the level of detail achieved with the classification. Once a cluster has been identified, increasing the

total number of clusters will result in identifying a subset of smaller clusters within the existing one.

Genes were clustered independently for each of the five experiments into five separate classes. In ambiguous cases, genes have been assigned manually to a cluster when possible or left unclassified if the expression profiles were inconclusive. Ultimately cluster 4 and 5 were merged into one, because separation into two clusters was not consistent across different experiments.

Clusters were assigned to cell cycle phases as follows: cluster 1 corresponding to mitosis, cluster 2 to M/G1 phase (cytokinesis and cell separation), cluster 3 to S phase (DNA replication) and cluster 4 to G2 phase. While this assignment is arbitrary to some degree, especially considering genes at the boundary between two clusters, a good correlation can be found in general between the biological function of the genes and the stage of the cycle when peak of expression occurs. This will be discussed in more detail in the next section. The majority of the 'high amplitude' genes are members of clusters 1, 2 and 3 whereas most cluster 4 genes are only weakly regulated. Fig. 3.2A shows the 407 periodic genes classified into four main clusters for one elutriation experiment and Fig. 3.2B for one *cdc25* 'block and release' experiment. Fig. 3.2C and D show the same clustering for the 136 'high amplitude genes only. Additional figures showing the four clusters in a 3-dimensional view can be found in Appendix V (Fig. V.4-V.5). The purpose of this representation is to show how clusters are separated after projection from the multidimensional expression space into a 2-D space.

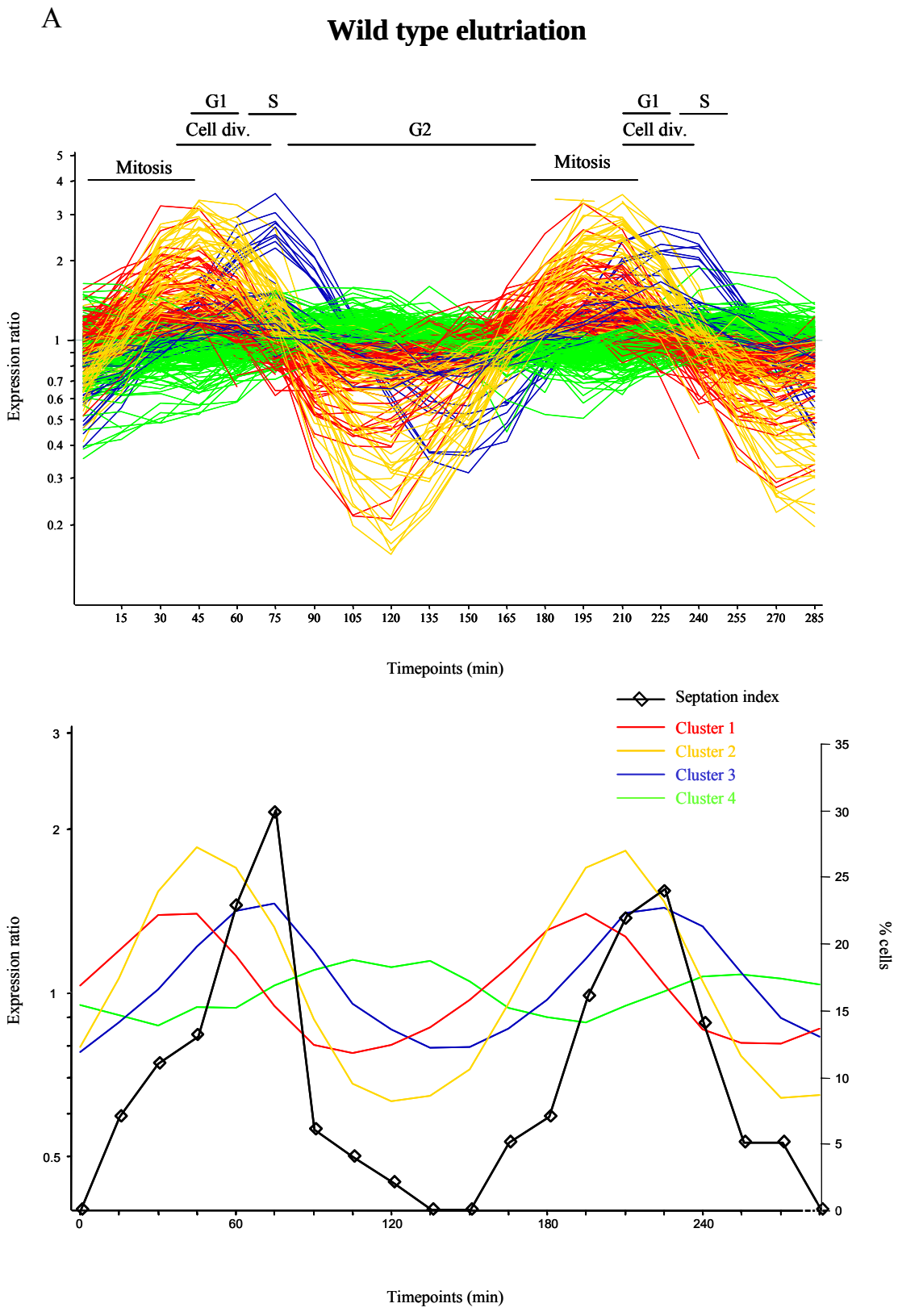


Fig. 3.2 - continues

B *cdc25* 'block and release'

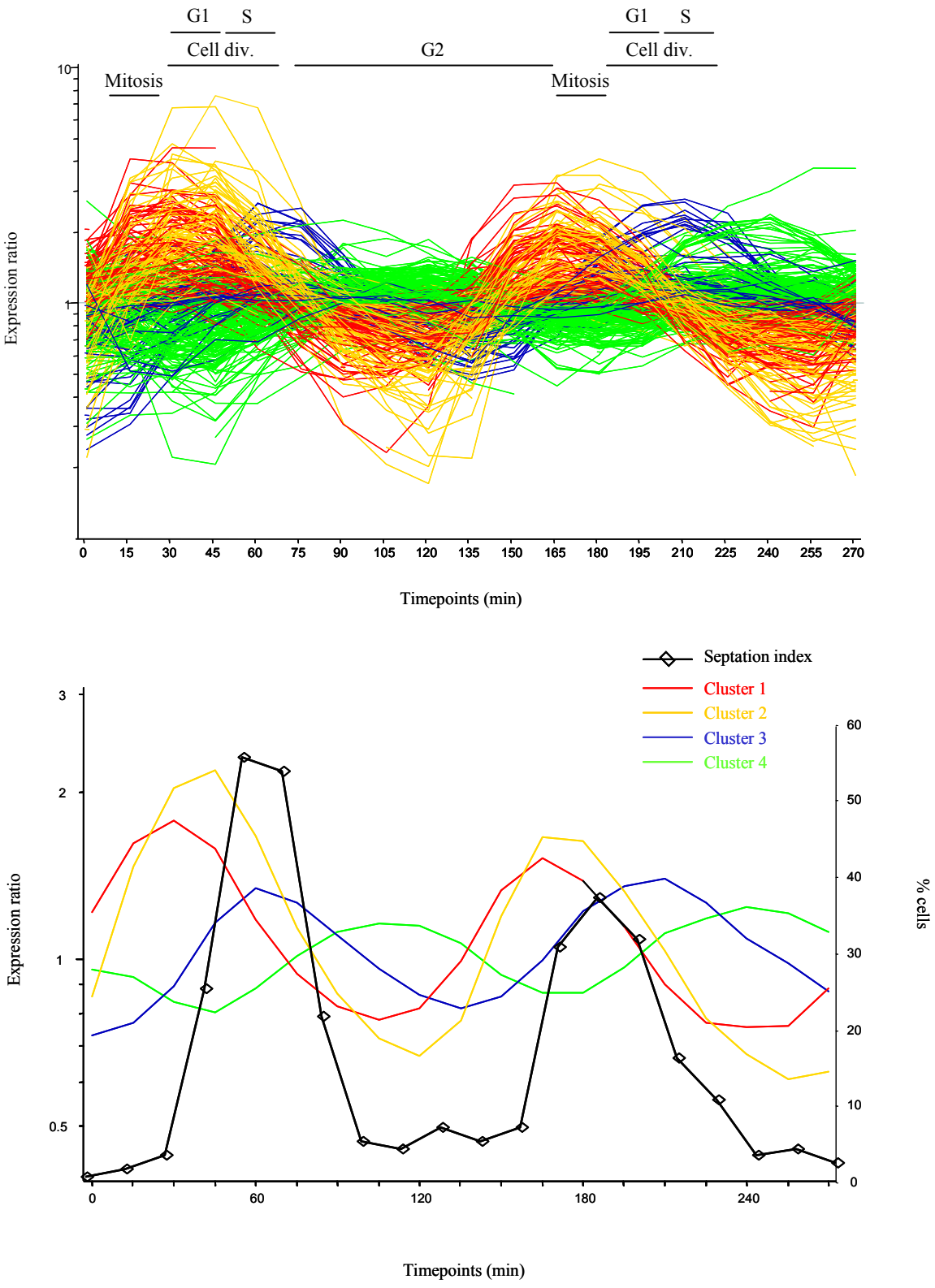


Fig. 3.2 - continues

C

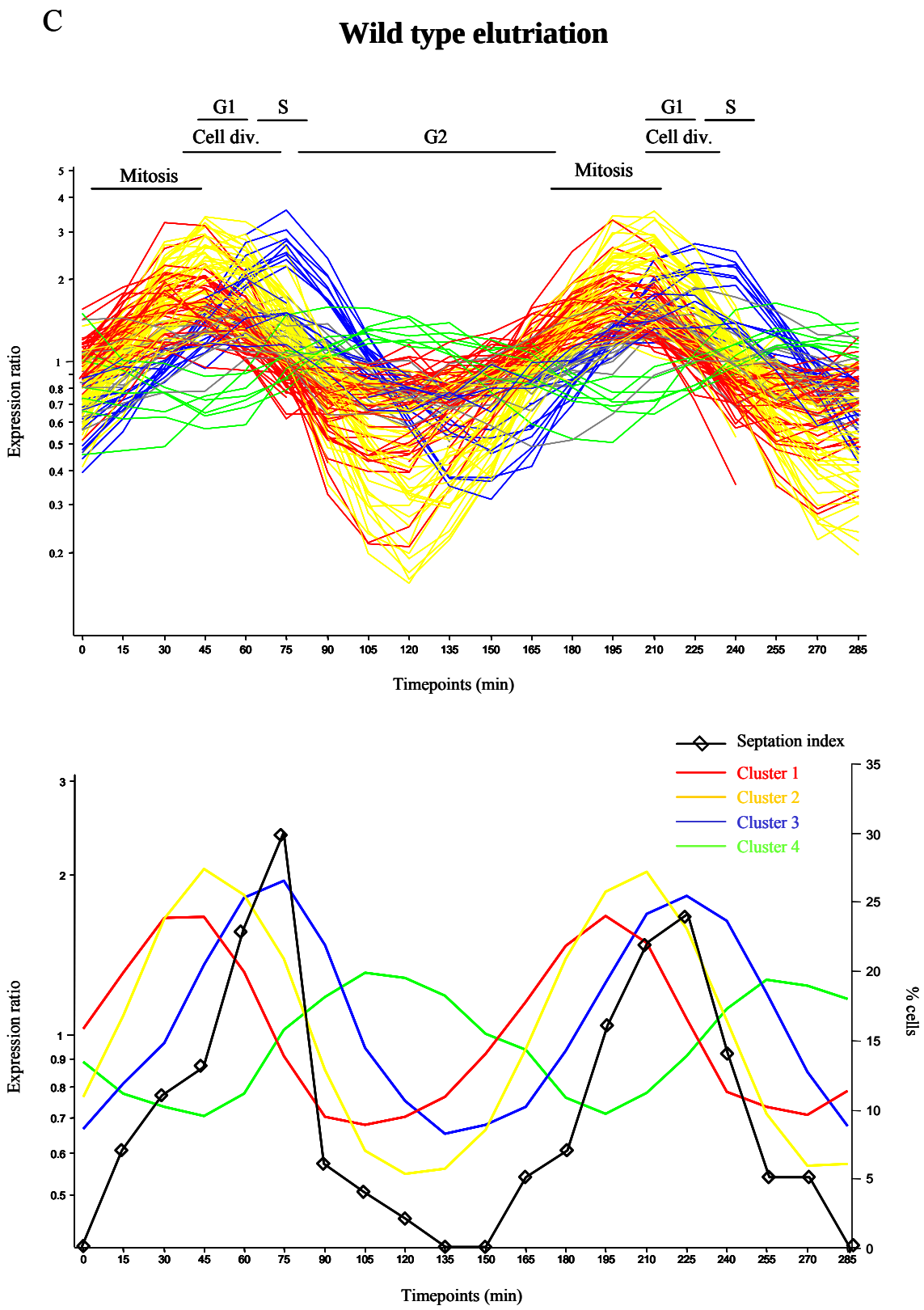


Fig. 3.2 - continues

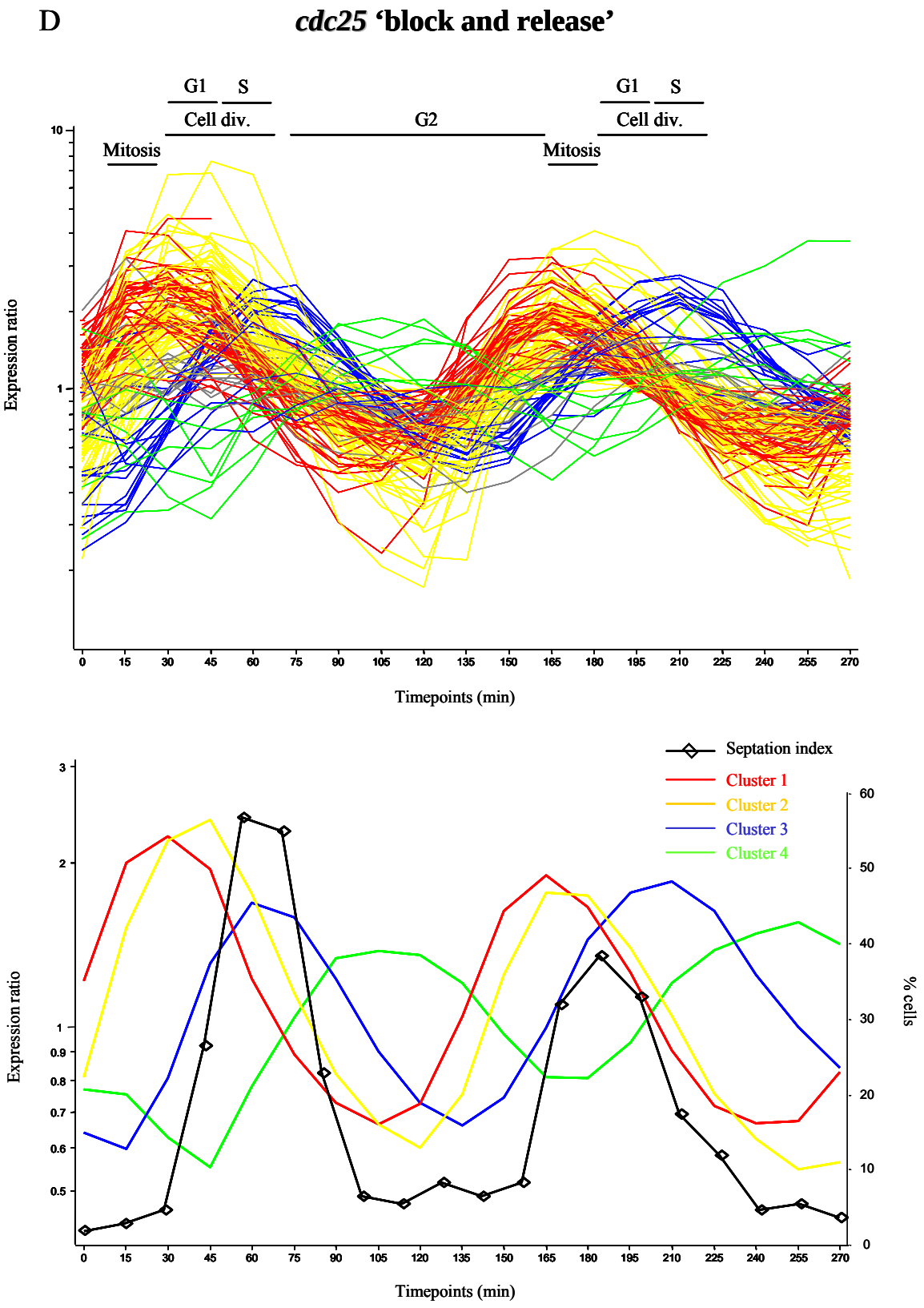


Fig. 3.2 **Clustering of cell cycle regulated genes in *S. pombe*.**

Panel A/C, elutriation experiment (2201). Panel B/D, *cdc25* 'block and release' experiment (2002). In all panels the top graph shows all 407 periodic genes (panel A and B) or the 136 high amplitude genes only (panel C and D) grouped into four main clusters whereas the bottom graph shows the average expression profiles of all genes in each class together with the septation index for the same experiment.

Several fission yeast genes had been previously described as periodic, using traditional molecular genetic approaches. A complete list of the 35 already known cell cycle regulated genes can be found in Table 3.1. 28 of those genes were confirmed to be periodic by our data. Among the seven genes missing, *mid1*, *ppb1*, *res2* and *suc22* showed a weak periodicity, especially in the *cdc25* 'block and release' experiments, but this was insufficient to be included among the periodic genes according to our criteria. It should be mentioned that in two cases (*cdc19* and *ppb1*) results in the original studies concerning the actual periodicity of those genes contradict each other (Anderson M. *et al.*, 2002; Forsburg S.L. and Nurse P., 1994; Plochocka-Zulinska D. *et al.*, 1995). For *res2*, only a marginal periodicity was reported in the original paper (Obara-Ishihara T. and Okayama H., 1994). *Suc22* encodes two separate transcripts: a large one that is weakly expressed and shows a periodic behaviour and a small one, which is much more abundant and continuously expressed through the cycle (Harris P. *et al.*, 1996; Fernandez Sarabia M.J. *et al.*, 1993). In the microarray experiment, the small abundant transcript probably hybridises more efficiently than the large one, explaining why the periodic behaviour of the gene could not be detected. *Cdc19*, *cmk1* and *rrg1* were not periodic under any conditions in study.

All the genes previously reported as periodic are members of the first three clusters, showing a good correlation between the peak of expression in our experiments and the one reported in the literature (see table 3.1 for reference to original publications). None of the genes included in the fourth cluster had been shown before to be periodic.

Table 3.1 Genes previously reported as cell cycle regulated in *S. pombe*

Gene Name	Expression peak	Cluster	References
cdc15	M	1	(Fankhauser C. *et al.*, 1995)
cdc18	G1/S	2	(Kelly T.J. *et al.*, 1993)
cdc19?	M/G1?	ND	(Anderson M. *et al.*, 2002; Forsburg S.L. and Nurse P., 1994)
cdc22	G1/S	2	(Gordon C. and Fantes P., 1986)
cdc25	M	1	(Moreno S. *et al.*, 1990)
cdt1	G1	2	(Hofmann J.F. and Beach D., 1994)
cdt2	G1	2	(Hofmann J.F. and Beach D., 1994)
cig2	G1/S	2	(Connolly T. and Beach D., 1994; Obara-Ishihara T. and Okayama H., 1994)
cmk1	G1/S	ND	(Rasmussen C.D., 2000)
cnp1	G1/S	2	(Takahashi K. *et al.*, 2000)
dfp1	G1/S	2	(Brown G.W. and Kelly T.J., 1999)
fin1	as *cdt1*	2	(Krien M.J. *et al.*, 2002)
eng1	G1/S	2	(Martin-Cuadrado A.B. *et al.*, 2003)
hht1	S	3	(Takahashi K. *et al.*, 2000; Matsumoto S. and Yanagida M., 1985)
hta1	S	3	(Aves S.J. *et al.*, 1985)
htb1	S	3	(Matsumoto S. *et al.*, 1987)
mid1	M/G1	ND	(Anderson M. *et al.*, 2002)
mid2	M	2	(Tasto J.J. *et al.*, 2003)
mik1	G1/S	2	(Christensen P.U. *et al.*, 2000; Ng S.S. *et al.*, 2001; Baber-Furnari B.A. *et al.*, 2000)
mrc1	as *cdc18*	2	(Tanaka K. and Russell P., 2001)
pht1	S	3	(Carr A.M. *et al.*, 1994; Durkacz B.W. *et al.*, 1986)
plo1	M/G1	1	(Anderson M. *et al.*, 2002)
ppb1?	S or M/G1?	ND	(Anderson M. *et al.*, 2002); Plochocka-Zulinska D. *et al.*, 1995)
rad21	G1/S	2	(Birkenbihl R.P. and Subramani S., 1995)
res2?	G1/S?	ND	(Obara-Ishihara T. and Okayama H., 1994)
rhp51	before *cdc22*	1	(Jang Y.K. *et al.*, 1996)
rph1	G1/S	2	(Tanaka H. *et al.*, 2002)
rrg1	G2/M	ND	(Kim M.J. *et al.*, 2002)
rum1	end G2	1	(Benito J. *et al.*, 1998)
sid2	M/G1	1	(Anderson M. *et al.*, 2002)
slp1	M	1	(Yamada H.Y. *et al.*, 2000)
spo12	as *cdc15*	1	(Samuel J.M. *et al.*, 2000)
ssb1	as *cdc22*	2	(Parker A.E. *et al.*, 1997)
ste9	as *cdc18*	2	(Tournier S. and Millar J.B., 2000)
(*suc22*)	G1/S (transcript dep.)	ND	(Harris P. *et al.*, 1996; Fernandez Sarabia M.J. *et al.*, 1993)

3.4 Biological function of genes in four clusters

This section describes each of the four waves of transcription focusing on the biological function of its members. For each cluster, genes are grouped in tables (see below) according to the biological process when their function is performed. It should here be

mentioned that this grouping based on function is not rigorous and was here adopted only to facilitate the presentation of the results. Fig. 3.3 shows the expression profiles of all genes in each cluster.

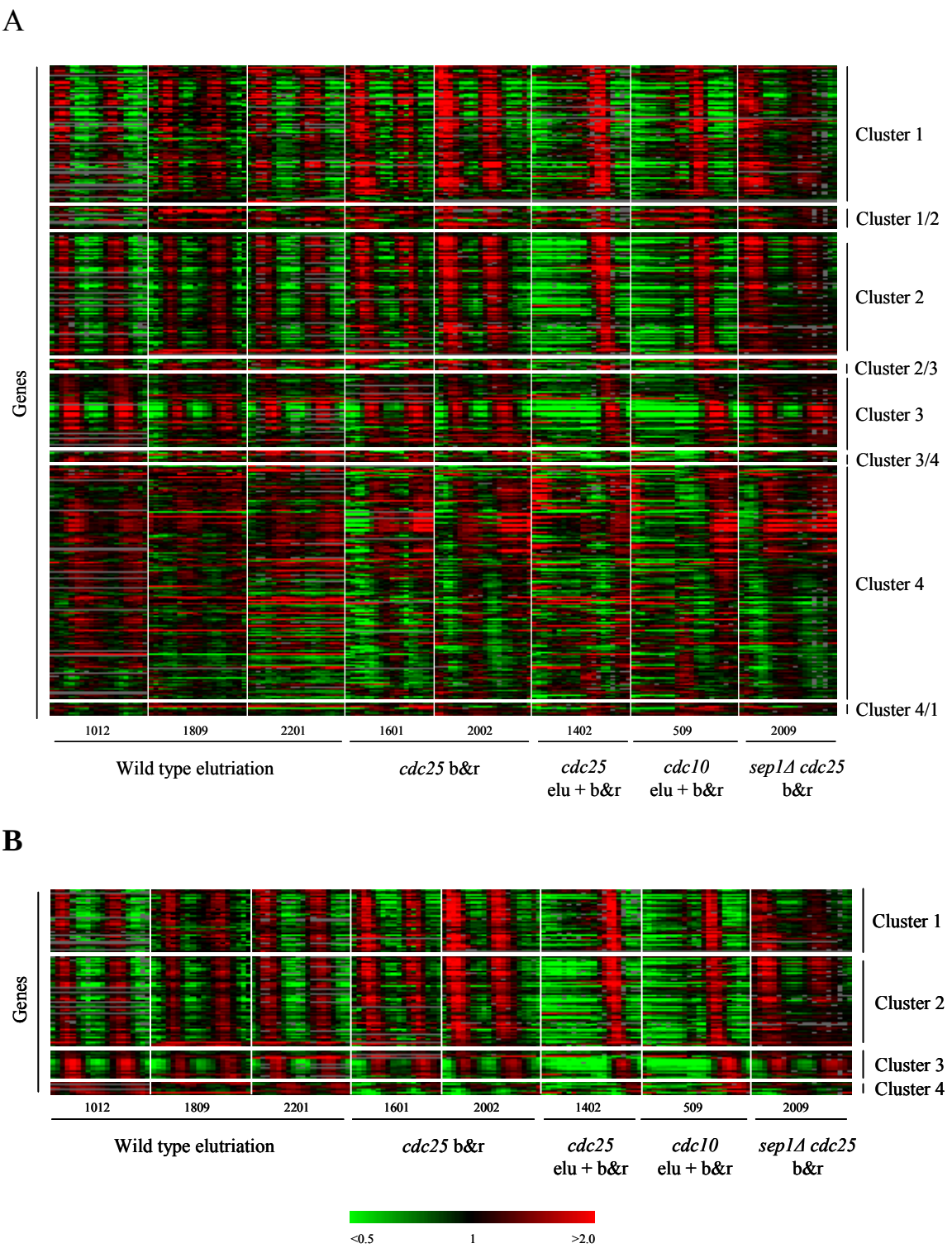


Fig. 3.3 **Cell cycle regulated genes in *S. pombe* and their classification.**

Rows represent the profiles of the 407 periodic genes (panel A) and of the 136 'high amplitude' genes only (panel B) ordered by the time of their peak of expression. Columns represent synchronised experimental samples (8 timecourses of 18-22 timepoints collected at 15 min intervals – 161 timepoints in total). Red: induced expression; green: repressed expression; grey: no data. Classification of genes into 4 major clusters is also shown. Smaller clusters of unclassified genes are also shown (clusters named 1/2, 2/3, 3/4 and 4/1).

Cluster 1

This cluster includes 87 genes, 40 of which are 'high amplitude' genes, showing a peak of expression coincident with mitosis. It is possible to identify subgroups of genes involved in the same biological process.

Many of the events happening during mitosis involve dramatic changes to the cytoskeleton due to rearrangement of the microtubules to form the mitotic spindle as well as nuclear changes like chromosome condensation, segregation and separation (Su S.S.Y. and Yanagida M., 1997). This subset of genes includes: *klp5* and *klp6* (kinesin motors that influence microtubule dynamics), *myo3* (encoding a myosin, which interacts with actin), several genes involved in sister chromatid cohesion (*pds5* – for the establishment of cohesion and *psc3* – encoding a mitotic cohesion subunit) and chromosome segregation (*dis1* which acts in collaboration with klp5p and klp6p) as well as an essential component of the spindle pole body (*sad1*).

Other genes encode proteins involved in cytokinesis and cell separation. In fission yeast the final stage of cell division is characterised by the formation of a septum which will then be digested to allow cell separation (Su S.S.Y. and Yanagida M., 1997). It is not surprising to find expressed at this stage *plo1* (encoding a mitotic regulatory kinase and an inducer of septum formation and cytokinesis), *cdc15* (regulator of septum formation), *imp2* (cdc15p-like protein), *sid2* (protein kinase responsible for triggering septation) and *mob1* (whose gene product binds to sid2p). Many genes involved in metabolic pathways and cell wall biosynthesis are also highly induced at this stage, possibly reflecting the *de novo* synthesis of cell wall and membrane concomitant to cytokinesis.

Several genes involved in cell cycle regulation can also be found in this cluster: *ace2* (transcriptional regulator), *ark1* (encoding the Aurora kinase required for chromosome condensation) as well as genes encoding key regulatory molecules involved

in the regulation of the cyclin-dependent kinase (CDK) cdc2p such as cdc13p (cyclin partner of cdc2p), cdc25p (cdc2p-cdc13p activator), rum1p (cdc2p inhibitor), crk1p and csk1p (cdc2 activators) and cdr1p (indirect activator of cdc2p). *Cdc2* itself is member of cluster 4, as described later.

In addition the gene SPBC16G5.15c, whose function is still uncharacterised, encodes a protein containing a forkhead binding domain typical of a highly conserved class of transcription factors. Two smaller groups, one of meiotic genes [including *mus81* coding for a nuclease and *meu16*, a non coding RNA potentially involved in meiosis regulation (Watanabe T. *et al.*, 2001)] and a second one of genes involved in DNA repair (including *slp1*, component of the spindle pole body checkpoint) are also included. This last group also includes *cdc20*, encoding DNA polymerase epsilon, which had been previously reported as non periodic (Sugino A. *et al.*, 1998).

18 genes are coding for proteins whose functions are still poorly characterised (e. g. domains identified in the protein or function suggested based on similarity with other known proteins).

Table 3.2 Selected cluster 1 members and their biological function

Biological names	Systematic names	Gene description
Cytokinesis and cell separation		
cdc15; rng1	SPAC20G8.05c	Protein involved in cytokinesis
etd1	SPAC1006.08	Protein required for cytokinesis
imp2	SPAC13F4.08c; SPBC11C11.02	Protein required for medial ring disassembly after cytokinesis
mac1	SPAC13G7.04c	Transmembrane protein involved in cell separation
mob1	SPBC428.13c	Protein involved in regulation of cytokinesis
myo3; myp2	SPAC4A8.05c	Myosin-3 isoform, heavy chain (Type II myosin)
plo1	SPAC23C11.16	Polo kinase involved in regulation of mitosis and cytokinesis
rho4	SPAC16A10.04	Rho protein involved in regulation of cytoskeleton, cytokinesis, and cell wall integrity
sid2; pld5	SPAC24B11.11c	Protein kinase involved in regulation of cytokinesis
spn2	SPAC821.06	Septin homolog, involved in cell separation
spn7; mde8	SPBC21.08c; SPBC19F8.01c	Septin homolog, involved in cell separation
Cell cycle control genes		
ace2	SPAC6G10.12c	Zinc finger transcription factor
apc15; apc16	SPBC83.04	Component of APC/cyclosome complex
ark1; sex1	SPCC330.16; SPCC320.13c	Aurora kinase involved in regulation of mitosis
cdc13	SPAC19G10.09C; SPBC582.03	Cyclin that promotes entry into mitosis from G2 phase, forms complex with Cdc2
cdc25; sal2	SPAC24H6.05	Tyrosine phosphatase that activates Cdc2p kinase,

		involved in G2/M transition and DNA damage checkpoints
cdr1; nim1	SPAC644.06c	Protein kinase involved in regulation of mitosis
crk1; mcs6; mop1	SPBC19F8.07	Cyclin-dependent kinase activating kinase (CAK) involved in activating Cdc2p kinase, putative transcription initiation factor TFIIH subunit
csk1	SPAC1D4.06c	Cyclin-dependent kinase activating kinase (CAK) involved in activating Cdc2p (activity partially redundant with Mcs6p-Mcs2p complex)
rum1	SPBC32F12.09	Inhibitor of the Cdc2p cyclin-dependent kinase complex
wis3; spo12	SPAC3F10.15c	Protein likely to play role in regulating cell cycle progression, possibly at G2 to M phase transition
	SPBC16G5.15c	Fork head protein type transcription factor
DNA repair and checkpoints		
cdc20; pol2	SPBC25H2.13c	DNA polymerase epsilon catalytic subunit
msh6	SPCC285.16c	Protein involved in mismatch repair (mutS family)
rhp51; rad51	SPAC644.14C	Required for DNA repair and meiotic recombination
slp1	SPAC821.08c	WD-domain protein of the spindle defect checkpoint and APC activator
Cell wall biosynthesis and maintenance		
chs2	SPBC1734.17; SPBC1709.01	Member of chitin synthase family, involved in cell wall maintenance
sso1; psy1	SPCC825.03c	Syntaxin-like component of the plasma membrane docking/fusion complex
	SPAC23H4.19; SPAC1705.03c	Putative cell wall biogenesis protein
	SPBC1198.07c	Putative glycosylphosphatidylinositol (GPI)-anchored protein involved in cell wall biosynthesis
	SPAC11E3.13c	Member of glycolipid anchored surface protein (GAS1) family, possible involvement in cell wall maintenance
	SPAC19B12.02c	Protein with high similarity to 1,3-beta-glucanosyltransferase, member of glycolipid anchored surface protein (GAS1) family
Chromosome segregation and chromatidin cohesion		
dis1	SPCC736.14	Microtubule-associated protein required for chromosome segregation (functions with Klp5p and Klp6p in kinetochore-spindle attachment)
klp6	SPBC1685.15c; SPBC649.01c	Kinesin motor protein; KIP3 subfamily
klp5	SPBC2F12.13	Kinesin motor protein; KIP3 subfamily
pds5	SPAC110.02	Protein required for maintenance of sister chromatid cohesion
psc3	SPAC17H9.20; SPAC607.01	Cohesin complex component, required for sister chromatid cohesion and normal mitosis
sad1; sta1	SPBC16H5.01c; SPBC12D12.01	Spindle pole body associated protein
top1	SPBC1703.14c	DNA topoisomerase I, involved in chromatin organisation
Meiosis		
mde6	SPAC15A10.10	Protein likely to play a role in meiosis or sporulation, requires Mei4p for transcriptional activation
meu16		Non-coding RNA
mus81	SPCC4G3.05c	Holliday junction resolvase subunit
ste7	SPAC23E2.03c	Protein required for mating and meiosis

Metabolic genes		
cid13	SPAC821.04c	Cytoplasmic poly(A) polymerase involved in regulation of ribonucleotide reductase (suc22) mRNA, TRF family of nucleotidyltransferases
	SPBC646.06c	Member of glycosyl hydrolase family 71, putative glucanase
	SPAC589.09	Protein containing a CRAL-TRIO domain, putative phosphatidylinositol metabolism
	SPCC576.02	Member of aspartate and glutamate racemases family
	SPAC30D11.01c; SPAC56F8.01	Member of glycosyl hydrolases family 31, involved in carbohydrate metabolism
	SPAC13G6.03	Member of type I phosphodiesterase or nucleotide pyrophosphatase family
	SPAC13C5.05c	Member of phosphoglucomutase or phosphomannomutase C-terminal domain containing family
	SPBC27B12.06	Protein with possible role in glycosylphosphatidylinositol biosynthesis
	SPBPB2B2.09c	Member of the ketopantoate reductase PanE or ApbA family, involved in thiamine biosynthesis
	SPCC757.12	Protein containing an alpha amylase N-terminal catalytic domain

Cluster 2

75 genes are members of this cluster, 58 of which are regulated with 'high amplitude' and they are induced around anaphase and cytokinesis, which in *S. pombe* corresponds to G1 phase. Many of them are involved in DNA replication initiation like *cdc18*, *cdt1* and *dfp1* (essential factors interacting with the pre-replicative complex), *cdc22* (coding for the large subunit of ribonucleotide reductase), *cdt2* (potentially involved in the formation of protein complexes required for DNA replication (Yoshida S.H. *et al.*, 2003) and *ssb1* (encoding for Replication Protein A, involved in DNA replication, recombination and repair). Two polymerase subunits are also expressed (*pol1* – alpha subunit and *cdm1* – delta subunit). *Pol1* was previously reported as not cell cycle regulated (Park H. *et al.*, 1993).

In *S. pombe*, septation does not need to be completed for DNA replication to start, and the two processes are partially overlapping due to a very short G1 phase. Therefore, this cluster also includes genes required for cell separation: *eng1* and *exg1* (glucanases involved in septum digestion), *cdc4* and *klp8* (involved in actin and microtubule rearrangements). Similarly, chromosome segregation genes like *fin1* (kinase promoting chromatin condensation), *rad21* (mitotic cohesion subunit), *ams2* (chromatin binding

protein) and *cnp1* (CeNtromere Protein-A-like, histone H3 variant) as well as sister chromatid cohesion genes (*cut2*, *eso1* and *psm3*) are also present.

Some other well characterised genes involved in cell cycle progression are also expressed at this stage: *cdc10* and *rep2* (encoding a component and a regulator of the MBF transcription factor), *ste9* (Anaphase Promoting Complex regulator), *cig2* (cdc2p cyclin partner) and *mik1* (cdc2p inhibitor), and *mrc1* (DNA damage checkpoint protein).

Another non-coding RNA, *meu19*, with a putative meiotic regulatory role, is found in the cluster. Among the members of this cluster with an unknown function, SPBC21B10.13c is worth mentioning, which encodes a protein containing a homeobox domain, a domain frequently found in transcription factors (Gehring W.J. *et al.*, 1994). Its budding yeast homologue, *YOX1*, encodes a homeodomain protein which acts as a transcriptional repressor, restricting expression of a subset of genes to M/G1 (Pramila T. *et al.*, 2002). *YOX1* itself is periodic in *S. cerevisiae*. SPBC21B10.13c might also have a regulatory role in meiosis (Mata J. *et al.*, 2002). 32 genes code for proteins with unknown function.

Table 3.3 Selected cluster 2 members and their biological function

Biological names	Systematic names	Gene description
Cell cycle control genes		
cdc10	SPBC336.12c	Component of MBF transcriptional activation complex involved in control of START
cig2; cyc17	SPAPB2B4.03	Major G1/S-phase cyclin, promotes onset of S phase
mik1	SPBC660.14	Protein kinase that inhibits Cdc2p kinase
	SPBC21B10.13c; SPAC21B10.13c	Homeobox domain (homeodomain) protein, putative transcription factor
rep2	SPBC2F12.11c	Zinc finger transcriptional activator, MBF transcriptional complex
ste9; srw1	SPAC144.13c	Protein required for mating and sporulation, may regulate anaphase promoting complex
DNA replication		
cdc18	SPBC14C8.07c	Protein that couples cell cycle signals to DNA replication machinery and induces replication
cdc22	SPAC1F7.05	Ribonucleoside-diphosphate reductase large chain, likely required for initiation of DNA replication
cdm1	SPBC12D12.02c	DNA polymerase delta subunit
cdt1	SPBC428.18	Protein that coordinates completion of S phase with onset of mitosis
cdt2	SPAC17H9.19c	Protein required for DNA replication
dfp1; him1; rad35	SPCC550.13	Regulatory subunit of the Hsk1p-Dfp1p kinase complex involved in S phase initiation
mrc1; huc1	SPAC694.06c	Protein required for DNA replication checkpoint
pol1; swi7	SPAC3H5.06c	DNA polymerase alpha catalytic subunit

rph1; pfh1; pif1	SPBC887.14c	ATP-dependent DNA helicase involved in telomere maintenance, DNA replication, and DNA repair
ssb1; rad11	SPBC660.13c	Single-stranded DNA-binding protein subunit, required for DNA replication
DNA repair and checkpoint		
mrc1; huc1	SPAC694.06c	Protein required for DNA replication checkpoint
Cell wall biosynthesis		
bgs4; cwg1	SPCC1840.02c	Putative 1,3-beta-glucan synthase component, cell wall synthesis
Meiosis		
meu19		Non-coding RNA
Cytokinesis and cell separation		
cdc4	SPAP8A3.08	EF-hand component of actomyosin contractile ring, required for cytokinesis
chs5	SPAC6G9.12	Protein with fibronectin domain involved in cell surface binding, and BRCT domain found in checkpoint proteins, similar to chitin synthase
eng1	SPAC821.09	Endo-beta-1,3-glucanase required for cell separation
exg1	SPBC1105.05	Putative exo-beta-1,3-glucanase
klp8	SPAC144.14	Protein containing a kinesin motor domain
mid2	SPAPYUG7.03c	Protein required for septin function and stability during cytokinesis
par2; pbp2	SPAC6F12.12	Protein phosphatase PP2A, B' regulatory subunit, required for cytokinesis, morphogenesis, and stress tolerance
pob1	SPBC1289.04c	Protein required for cell polarity and cell separation
	SPBC1289.01c; SPBC1539.11c	Unknown function, putative involvement in chitin biosynthesis
	SPBC3E7.12c	Unknown function, possible role in regulation of chitin synthase
	SPCC1322.10	Unknown function, similar to cell-surface proteins and proteoglycans
	SPAC14C4.09	Unknown function, putative glucanase
Chromosome segregation and chromatid cohesion		
ams2	SPCC4F11.01; SPCC290.04	Protein that binds binds chromatin at centromere and is involved in chromosome segregation
cnp1; sim2	SPBC1105.17	CENP-A-like protein, histone H3 variant specific to inner centromeres and required for chromosome segregation
cut2	SPBC1815.02c; SPBC14C8.01c	Securin; required for sister chromatid separation
eso1; eco1	SPBC16A3.11	DNA polymerase eta, involved in sister chromatid cohesion
fin1	SPAC19E9.02	NimA family kinase; regulates spindle formation and recruitment of Plo1p to SPB, promotes chromatin condensation
psm3; smc3	SPAC10F6.09c	Cohesin complex subunit, involved in sister chromatid cohesion and progression through mitosis
rad21	SPCC338.17c	Cohesin complex subunit, double-strand-break repair protein
Metabolic genes		
	SPCC1322.04	Putative UTP-glucose-1-phosphate uridylyltransferase
	SPBC32F12.10	Protein with phosphoglucomutase or phosphomannomutase C-terminal domain

	SPBPB2B2.13	Protein similar to galactokinase

Cluster 3

This cluster contains 46 genes expressed during DNA replication, which overlaps with septation and cell separation in rapidly growing fission yeast cells. 18 genes are 'high amplitude'.

All histone genes peak during S phase as expected, and represent the tightest subcluster within cluster 3. Other interesting genes are: *rad25*, whose product is responsible for sequestering cdc25p to the cytoplasm causing a G2/M arrest in response to DNA damage and *pas1*, encoding a cyclin partner for the Pef1p kinase complex possibly involved in the regulation of MBF transcription factor (Tanaka K. and Okayama H., 2000).

Proteins with unknown function are encoded by 16 members of this cluster; among those are several proteins containing some well known domains such as zinc fingers, HMG-box and GTPase activation. The non coding RNA *prl36* is also member of this cluster.

Table 3.4 Selected cluster 3 members and their biological function

Biological names	Systematic names	Gene description
Histones		
hht1	SPAC1834.04	Histone H3.1
hht2	SPBC8D2.04	Histone H3.2
hht3; clo5	SPBC1105.11c	Histone H3.3
hhf1; ams1	SPAC1834.03c	Histone H4.1
hhf2; ams3	SPBC8D2.03c	Protein similar to histone H4.1, contains a core histone domain
hhf3; ams4	SPBC1105.12	Histone 4.3
hta1	SPCC622.08c	Histone H2A-alpha
hta2	SPAC19G12.06c	Histone H2A-beta
htb1	SPCC622.09	Histone H2B-alpha
pht1	SPBC11B10.10c	Histone H2A variant
Cell cycle checkpoints		
rad25	SPAC17A2.13c	14-3-3- protein involved in DNA damage checkpoint control
Meiosis		
pas1	SPAC57A10.01; SPAC19E9.03	Cyclin involved in regulation of mating, interacts with Pef1p and Cdc2p kinases
Metabolic genes and others		

	SPBC1348.10c; SPAC1348.10c	Member of lysophospholipase catalytic domain family, putative lysophospholipase precursor
	SPBC21B10.09; SPAC21B10.09	Protein similar to acetyl-CoA transporter
	SPAC977.09c	Member of lysophospholipase catalytic domain containing family, similar to phospholipase B, which deacylates phosphatidylinositol
	SPCC306.08c	Malate dehydrogenase, mitochondrial precursor
	SPCC1906.01	Mannose-1-phosphate guanyltransferase
prl36		Non coding RNA

Cluster 4

This cluster contains 147 genes, peaking at different times during G2, only 7 of which belong to the 'high amplitude' subgroup. It is the largest and most heterogeneous cluster of the four, and only 20% of its members have a characterised function. It contains 68 genes encoding proteins with unknown functions or with only an identified domain.

Cdc2, encoding the kinase responsible for driving cell cycle progression and *cig1*, one of cdc2p cyclin partners, both peak in G2, together with *spd1*, an S-phase inhibitor through association with cdc2p. Many genes encoding membrane transporters or proteins involved in ribosome biogenesis and RNA processing are also transcribed at this stage, reflecting the actively growing state of the cell in G2 phase.

Another subcluster is represented by several tf2-type transposon elements (9 genes). It is also interesting to notice the presence of several stress genes, including the transcription factor *pcr1*, involved in regulating meiosis and stress response. At least 50 genes belonging to this cluster, most of them still uncharacterised, are known to be induced in response to stress (Chen D. *et al.*, 2003).

Table 3.5 Selected cluster 4 members and their biological function

Biological names	**Systematic names**	**Gene description**
Cell cycle control		
cdc2; swo2	SPBC11B10.09	Cyclin-dependent kinase, regulates cell cycle transitions G1/S and G2/M
cig1	SPCC645.01; SPCC4E9.02	B-type cyclin involved in G1 to S phase transition
spd1	SPAC29B12.03	Negative regulator of S phase
Transporters		
mae1	SPAPB8E5.03	Malate transporter
sst1	SPAC521.04c	Member of sodium or calcium exchanger protein family of

		membrane transporters
	SPAP7G5.06	Protein similar to amino acid permease, a proton symport transporter for all naturally-occurring L-amino acids
	SPAC1039.01	Member of amino acid permease family of membrane transporters
	SPCC126.09	Member of ZIP zinc transporter family, possible metal transporter and vacuolar membrane protein
	SPAC139.02c	Probable mitochondrial oxaloacetate transporter
	SPBC16D10.06	Member of ZIP zinc transporter family
	SPBC29A3.01	Member of P-type ATPase, similar to copper-transporting ATPase
	SPAC212.10	Pseudogene; malic acid transport protein; truncated C at terminal
	SPCC548.06c	Protein similar to putative H+-glucose symporter involved in glucose transport
	SPAC9.10	Member of amino acid permease family of membrane transporters
	SPAC869.02c	Member of globin family of oxygen transporters, similar to flavohemoglobin that protects from stress
	SPAPB24D3.09c	Protein with ABC transporter domains, similar to brefeldin A resistance protein involved in multidrug resistance
	SPBC1271.10c	Protein similar to membrane transporter
	SPCC794.03	Member of amino acid permease family of membrane transporters
	SPCC31H12.01; SPCC1183.11	Member of mechanosensitive ion channel family
	SPCC794.11c	Protein with actin binding domain, possible role in formation of clathrin coats at the Golgi and endosomes
Transposons		
TF2-1		Retrotransposable element; tf2-type transposon
TF2-10		Retrotransposable element; tf2-type transposon
TF2-2		Retrotransposable element; tf2-type transposon
TF2-3; TF2-4		Retrotransposable element; tf2-type transposon
TF2-5		Retrotransposable element; tf2-type transposon
TF2-6		Retrotransposable element; tf2-type transposon
TF2-7		Retrotransposable element; tf2-type transposon
TF2-8		Retrotransposable element; tf2-type transposon
TF2-9		Retrotransposable element; tf2-type transposon
Stress genes		
trx1; trx2	SPAC7D4.07c	Putative thioredoxin involved inresponse to heavy metals
pcr1; mts2	SPAC21E11.03c	Transcription factor that plays roles in mating, meiosis and stress response
rds1	SPAC343.12	Stress response protein
ssp1	SPCC297.03	Protein kinase that mediates rapid osmotic stress response at cell surface
uvi15	SPBC649.04	Protein essential for stationary phase survival, induced by stress
Metabolic genes		
arg5	SPBC56F2.09c	Protein similar to amidotransferase small subunit of carbamoylphosphate synthetase
dak1; dak2	SPAC977.16c	Dihydroxyacetone kinase, isoenzyme II
gpd2	SPAC23D3.04c	Glycerol-3-phosphate dehydrogenase
gmh2	SPAC5H10.13c	Protein similar to alpha-1,2-galactosyltransferase
gps2	SPBC365.14c	Putative UDP-glucose 4-epimerase involved in UDP-galactose synthesis and protein glycosylation

	SPBC119.10	Asparagine synthetase
	SPCC1827.06c	Aspartate semialdehyde dehydrogenase
	SPAC5H10.06c	Protein similar to alcohol dehydrogenase IV, which is involved in carbohydrate metabolism
	SPBC8E4.03	Protein with arginase family domain, similar to agmatine ureohydrolase
	SPAC19G12.09	Protein with aldo-keto reductase family domain, similar to aldehyde reductase
RNA processing and ribosome biogenesis		
	SPBC13G1.09	Member of bystin family, possible role in 35S pre-rRNA processing into 18S rRNA
	SPBC17D1.06; SPCC17D1.06	Member of the DEAD or DEAH box ATP-dependent RNA helicase
	SPAC2C4.18; SPAC25G10.01	Protein with RNA recognition motif, possible splicing factor that activates pre-mRNA splicing
csx1	SPAC17A2.09c	Protein containing three RNA recognition motifs, similar to U1 snRNA-associated protein
	SPBP8B7.15c	Protein similar to Polyadenylation Factor I complex component required for mRNA cleavage and polyadenylation
	SPCP1E11.08	Protein similar to nuclear protein involved in ribosome biogenesis
	SPAC1486.09	Protein similar to protein that functions in 20S proteasome maturation and 26S proteasome assembly
	SPAC823.03; SPAC1E11.03	Protein with kinase domain, similar to CDC-like kinase 2, which may regulate mRNA splicing
	SPAC23H4.15	Member of DUF663 protein of unknown function family, possible role in rRNA processing and 40S ribosomal subunit biogenesis
	SPAC31A2.07c	Putative RNA helicase, possible role in ribosome biogenesis
	SPCC1494.06c	Member of the DEAD or DEAH box ATP-dependent RNA helicase, possible role in rRNA processing
	SPCC1682.08c	Protein containing six Pumilio-family RNA binding domains, possible role in mRNA metabolism
	SPAC16C9.03	Possible role in nuclear export of 60S ribosomal subunits
Cell wall biosynthesis and maintenance		
bgl2	SPAC26H5.08c	Protein similar to beta-glucosidase, a cell wall endo-beta-1,3-glucanase
psu1	SPAC1002.13c	Protein required for cell wall integrity, member of SUN protein family
	SPBC11C11.05	Member of yeast cell wall synthesis protein KRE9 or KNH1 family

Unclassified genes

48 genes could not be assigned to a specific cluster (Table 3.5). Regardless of the clustering method used, classification is somewhat arbitrary and this becomes more evident when looking at the genes at the boundary of each cluster where assignment becomes difficult. For all the unclassified genes, Arrayminer gives an estimate of the

closest cluster they could belong to. Taking this into account and also considering the timing of peak of expression of each gene, some of these 48 genes have been assigned to smaller clusters named 1/2, 2/3, 3/4 and 4/1 (Fig. 3.3).

Among the genes peaking in M and G1 (Fig. 3.3 - cluster 1/2) is *hsk1*, encoding a factor responsible for DNA replication initiation in association with its partner dfp1p (gene member of cluster 2). *Mfm2*, *spk1* and *byr2* are all involved in mating, sporulation and the pheromone signalling pathway. Another essential meiotic gene expressed at this boundary is *mei2*, which encodes an RNA binding protein crucial for initiation of premeiotic DNA synthesis and meiosis I.

The genes assigned to the G2/M boundary (Fig. 3.3 - cluster 4/1) include a sulphate transporter family member and several enzymes involved in different metabolic pathways, similar functions to most of the previously characterised cluster 4 members. *Sim4*, involved in chromosome segregation it is also part of this group.

The majority of the genes peaking at either G1/S (Fig. 3.3 - cluster 2/3) or S/G2 (Fig. 3.3 - cluster 3/4) do not have a well characterised function except *fim1*, a fimbrin coding gene, which may be involved in polarised growth, assigned to cluster 3/4.

Table 3.6 Selected unclassified genes and their biological function

Biological names	Systematic names	Gene description
Cluster 1/2 (M-G1 genes)		
vip1	SPAC10F6.06	Protein containing an RNA recognition motif
prl3		Non-coding RNA
hsk1	SPBC776.12c	Protein kinase of the Hsk1p-Dfp1p complex involved in S phase initiation
mfm2	SPAC513.03	Precursor polypeptide for mating pheromone M factor produced by h- cells
spk1	SPAC31G5.09c	MAP kinase (MAPK) acting in the mating and sporulation pathways
byr2; ste8	SPBC2F12.01; SPBC1D7.05	MAP kinase kinase kinase acting upstream of MAPKK Byr1p and MAP kinase Spk1p in pheromone signaling pathway
	SPAC1006.06	Protein with RhoGEF domain, similar to Rho GDP-GTP exchange factor activated by cell wall defects
	SPAC12G12.06c	Probable RNA 3'-terminal phosphate cyclase
	SPBC1683.07	Protein similar to alpha-glucosidase
hri1	SPAC20G4.03c	Translation initiation factor 2 alpha kinase, may play role in negative regulation of eIF2alpha in response to stress
mei2	SPAC27D7.03c	RNA-binding protein involved in meiosis
spm1; pmk1	SPBC119.08	MAP kinase involved in maintenance of cell wall integrity
	SPCC965.06	Protein similar to potassium voltage-gated channel
cnd2	SPCC306.03c	Subunit of condensin complex involved in chromosome condensation

isp6; prb1	SPAC4A8.04	Putative subtilase-type proteinase, role in sexual differentiation+E49
Cluster 4/1 (G2/M genes)		
	SPAC869.05c	Member of sulfate transporter family, similar to sulfate permease
	SPAC1002.17c	Protein with phosphoribosyl transferase domain, possible role in pyrimidine salvage pathway
	SPCC16C4.06c	Protein with tRNA pseudouridine synthase domains
sim4	SPBC18E5.03c	Centromere-associated protein required for chromosome segregation and silencing
	SPBC19G7.07c	Member of PPR repeat containing family
	SPCC330.15c; SPCC320.14	Member of pyridoxal phosphate dependent enzyme family, similar to racemase that catalyzes the racemisation of L-serine to D-serine
Cluster 2/3 (G1/S genes)		
	SPCC553.07c	Member of impB, mucB or samB family, possible role as translesion DNA repair polymerase
	SPBC800.11	Protein with inosine-uridine preferring nucleoside hydrolase domain
	SPBC409.22c; SPBC1306.01c	Protein with elongation factor Tu GTP binding domain, similar to mitochondrial translation elongation factor G
	SPAC17G6.03	Protein with calcineurin-like phosphoesterase domain
	SPBC21B10.07; SPAC21B10.07	Protein with glycosyl hydrolase family 16 domain
	SPAC2E1P3.04	Protein with possible role in detoxifying extracellular amines and nitrogen metabolism
	SPAC29A4.05	Protein similar to calmodulin 1, which regulates the calcium-dependent activity of enzymes including phosphatases
Cluster 3/4 (S/G2 genes)		
	SPAC631.02	Protein with two bromodomains, which interact with acetylated lysine
	SPBC1271.09	Member of sugar (and other) transporter family, possible role in inositol metabolism
	SPCC364.07; SPCC4G3.01	Protein similar to 3-phosphoglycerate dehydrogenase, which catalyzes first step in synthesis of serine
	SPAC3A11.10c	Member of Rnal dipeptidase family, zinc-dependent metalloproteinases that hydrolyze various dipeptides
	SPAC664.03	Member of Paf1 family, components of RNA polymerase II associated complexes
pro1	SPAC821.11	Protein similar to gamma-glutamyl phosphate reductase involved in proline biosynthesis
fim1	SPBC1778.06c	Fimbrin, role in actin organization during medial ring formation and polarized growth
	SPBPB21E7.09; SPAPB21E7.09	Protein similar to L-asparaginase II

4. TRANSCRIPTIONAL REGULATION OF PERIODICALLY EXPRESSED GENES IN FISSION YEAST

This chapter will provide an insight into the transcriptional regulatory network that governs periodic gene transcription in fission yeast. Several deletion mutants of well known, as well as less characterised or putative, transcriptional regulators have been used with the purpose of clarifying the mechanisms that regulate gene expression during the cell cycle.

4.1 Experimental overview

Gene deletion mutants represent a powerful tool to investigate gene function. The first indication of a gene's role often comes from the phenotype of the mutant strain. Ultimately, expression profiling of a gene deletion using microarrays can reveal the relationship between a transcription factor and potential targets. If a gene is responsible for regulating a specific wave of transcription, its deletion will have an effect on the expression profile of its targets. In unsynchronised cells this should lead to higher (in the case the gene acts as repressor) or lower expression (in the case of an activator) compared to wild type cells. Overexpression of the same gene similarly should lead to higher (activator) or lower (repressor) levels of expression of its targets. In reality transcriptional regulatory networks are much more complicated and transcription factors are often interdependent making the understanding of regulation a less straightforward process.

Details concerning the experimental conditions can be found in Appendix IVb. At least two independent biological experiments were carried out for each mutant and a technical replicate (microarray hybridisation with a dye swap) was performed in most cases. Asynchronous mutants were compared to asynchronous wild type cells and differentially expressed genes identified by combining two methods as described in Materials and Methods, section 2.6.3. Overexpression strains transformed with the pREP-3X vector carrying the gene of interest, were compared to a control strain transformed with the pREP-3X vector only.

4.2 Sep1p-dependent regulation

Sep1p, a member of the forkhead family of transcription factors (Ribar B. *et al.*, 1997; Ribar B. *et al.*, 1999), regulates periodic expression of *cdc15*, which encodes a protein involved in cytokinesis and septation (Zilahi E. *et al.*, 2000). *Sep1* mRNA levels are constant throughout the cycle whereas *cdc15* levels peak before septation. In a *sep1* deletion (*sep1Δ*), *cdc15* periodicity is lost (Zilahi E. *et al.*, 2000). *Sep1Δ* displays a distinctive phenotype (Fig. 4.1B) with very elongated, multiseptated and branched cells due to the septation defect. The *S. cerevisiae* homologues, *FKH1* and *FKH2*, are also involved in regulation of cell cycle periodic transcription (see Introduction).

Two *sep1Δ* mutants (in different backgrounds) have been used to investigate the role of sep1p in regulating gene transcription: *sep1*Δ and *sep1*Δ *cdc25*. In parallel, the *pREP3X-sep1* strain, overexpressing sep1p, has also been used (sep1p OE). The overexpression strain did not show any particular phenotype (Fig. 4.2C).

Transcription levels were analysed in asynchronous *sep1*Δ cells versus wild type cells. 67 genes were found to be expressed at lower level and around 70% (45 genes) of these were cell cycle regulated (Table 4.1). The same subset of 45 genes was also found to be induced in cells overexpressing *sep1* (compared to cells transformed with the expression vector only), confirming their dependency on sep1p (Fig. 4.3A). The majority of the 45 sep1p-dependent periodic genes were members of either cluster 1 (15 genes) or 2 (26 genes). Therefore sep1p must be involved in the regulation of both waves of transcription.

Cdc15 is found in this group, as expected, together with several other genes encoding proteins involved in cytokinesis (SPAC14C4.09, *eng1*, *etd1*, *exg1*, *mid2* and *plo1*) and cell wall maintenance (*chs2*) or synthesis (SPAC23H4.19 and SPBC3E7.12c); this correlates well with the *sep1Δ* phenotype. The transcription factor gene *ace2* was also found in this group.

As described in section 1.3.2 of the introduction, in budding yeast the forkhead-type transcription factor complex Mcm1p/Fkh2p/Ndd1p is responsible for activating the G2/M wave of transcription which includes another transcription factor, Ace2p (Simon I. *et al.*, 2001). Since in fission yeast, *ace2* (homologous to *ACE2* in bidding yeast) also appears to be a forkhead/sep1p-dependent gene, an investigation into the role of ace2p in

transcriptional regulation was performed, particularly focusing on a possible interaction between the two transcription factors sep1p and ace2p.

Table 4.1 Sep1p-dependent periodic genes

Biological/systematic name	Gene description	Cluster
ace2	Zinc finger transcription factor	1
bet1 [a, b]	Member of SNARE domain containing family	1
SPAC19B12.02c [a, b]	Protein with high similarity to 1,3-beta-glucanosyltransferase, member of glycolipid anchored surface protein (GAS1) family	1
SPAC23H4.19 [a, b]	Putative cell wall biogenesis protein	1
SPBC27.05 [a, b]	Unknown function	1
SPAC3F10.15c [a, b]	Protein likely to play role in regulating cell cycle progression, possibly at G2 to M phase transition	1
SPBC4F6.12 [a, b]	LIM domain protein, low similarity to paxillin focal adhesion protein that regulates integrin or growth factor-mediated responses	1
SPCC757.12 [a, b]	Protein containing an alpha amylase N-terminal catalytic domain	1
cdc15 [a, b]	Protein involved in cytokinesis	1
chs2 [a]	Member of chitin synthase family, involved in cell wall maintenance	1
etd1 [a]	Protein required for cytokinesis	1
klp5 [a, b]	Kinesin motor protein; KIP3 subfamily	1
myo3: myp2 [a, b]	Myosin-3 isoform, heavy chain (Type II myosin)	1
plo1 [a, b]	Polo kinase involved in regulation of mitosis and cytokinesis	1
slp1 [a]	WD-domain protein of the spindle defect checkpoint and APC activator	1
SPBC1709.12	Unknown function	2
SPCC18.01c	Member of SUN family, contains predicted N-terminal signal sequence	2
SPAC22G7.02 [a]	Unknown function	2
SPBC27.04 [a, b]	Unknown function	2
SPBC2A9.07c	Unknown function	2
SPAC2E1P5.03	Protein containing a DnaJ domain, which mediates interaction with heat shock proteins	2
SPBC31F10.17c [a]	Unknown function	2
SPBC32F12.10 [a, b]	Protein with phosphoglucomutase or phosphomannomutase C-terminal domain	2
SPAC343.20	Unknown function	2
SPBC3E7.12c	Unknown function, possible role in regulation of chitin synthase	2
SPAC644.05c [a]	Protein similar to dUTP pyrophosphatase, which maintains dUTP at low levels to prevent misincorporation into DNA	2
SPBC651.04	Unknown function	2
cdm1	DNA polymerase delta subunit	2
eng1	Endo-beta-1,3-glucanase required for cell separation	2
exg1 [a]	Putative exo-beta-1,3-glucanase	2
klp8 [a]	Protein containing a kinesin motor domain	2
meu19 [a, b]	Non-coding RNA	2
mid2	Protein required for septin function and stability during cytokinesis	2
SPBPB2B2.13 [b]	Protein similar to galactokinase, which catalyzes first step in	2

	galactose metabolism	
SPAPJ760.03c	Unknown function	2
rad21 [a]	Cohesin complex subunit, double-strand-break repair protein	2
rgf3	Protein containing a pleckstrin homology (PH) and a RhoGEF (GTPase exchange factor) domain	2
rpc17	Unknown function	2
SPCC1322.10	Unknown function, similar to cell-surface proteins and proteoglycans	2
SPAC14C4.09	Unknown function, putative glucanase	2
SPAC19G12.17c	Unknown function, similarity to podocalyxin like, a transmembrane sialomucin important for lymphocyte adhesion and homing	2
SPBPJ4664.02	Unknown function, possible cell surface glycoprotein	3
SPAP7G5.06 [a]	Protein similar to amino acid permease, a proton symport transporter for all naturally-occurring L-amino acids	4
SPBC1271.09 [a, b]	Member of sugar (and other) transporter family, possible role in inositol metabolism	N(3,4)
SPCC965.06	Protein similar to potassium voltage-gated channel	N(1,2,3)

[a] sep1p-dependent genes only

[b] sep1p-dependent genes upregulated in fkh2Δ

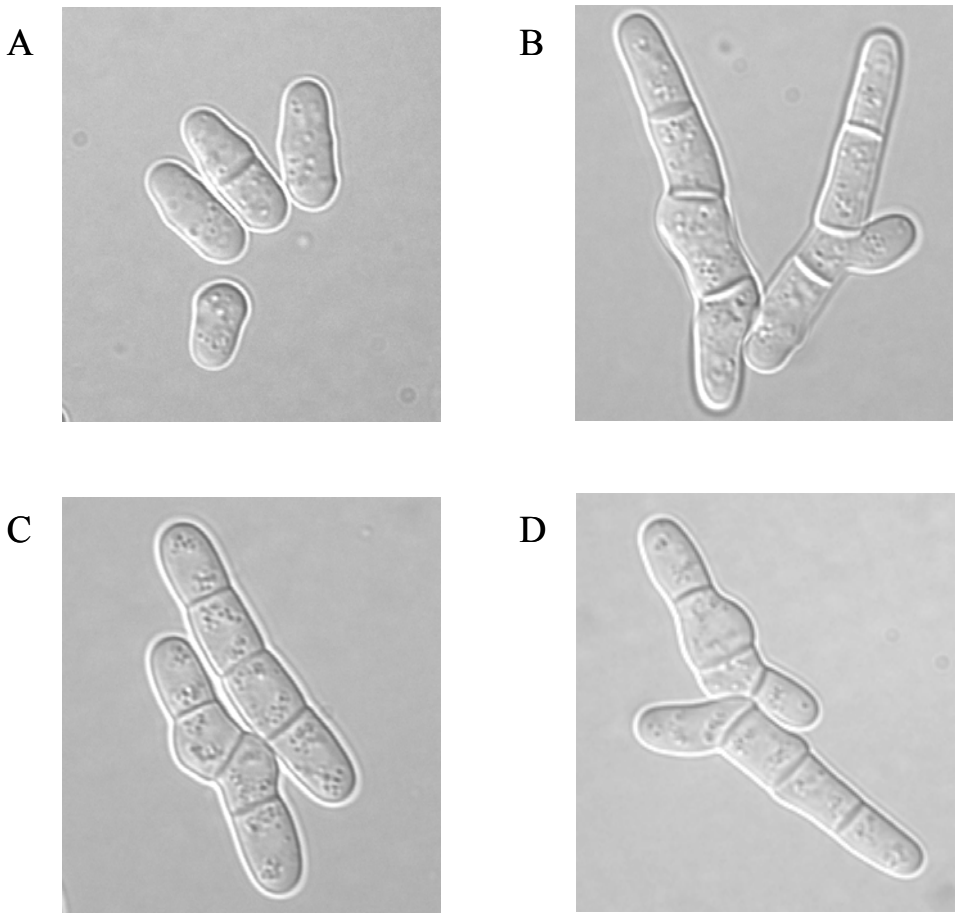


Fig. 4.1 Microscopic appearance of wild type (A), *sep1*Δ (B), *ace2*Δ (C) and *ace2*Δ *sep1*Δ (D) mutant cells. Photographs of differential interference contrast microscopy (DIC).

4.3 Ace2p-dependent transcription

Ace2p, a transcriptional regulator containing a zinc finger domain, has been identified based on homology with *S. cerevisiae* Ace2p, which regulates a group of genes involved in cell separation, including *ENG1*, encoding for a glucanase required for septum digestion at cytokinesis. Similarly in fission yeast, ace2p regulates periodic expression of *eng1* (cluster 2 in this study) and in *ace2Δ* periodic accumulation of *eng1* is lost (Martin-Cuadrado A.B. *et al.*, 2003). Based on the literature and on what was found in this study so far, in fission yeast sep1p regulates expression of *ace2*, a member of cluster 1, which in turn regulates *eng1*, a member of cluster 2. It is reasonable to assume that other genes in cluster 2 are also regulated by ace2p.

An *ace2Δ* and an ace2p OE strain were prepared and their expression profile investigated using microarrays. The phenotype of the *ace2Δ* strain is very similar to the *sep1Δ* strain with cells showing septation defects but cells are less elongated than in *sep1Δ* (Fig. 4.1C). The ace2p OE instead is characterised by round cells (Fig. 4.2B).

23 periodic genes were found to be expressed at lower levels in asynchronous *ace2Δ* versus wild type (Table 4.2), and the same subset of genes were highly induced in ace2p OE. Five additional periodic genes (Table 4.2 – genes marked with [a]) were found to be highly induced in ace2p OE but only slightly downregulated in *ace2Δ*, below the criteria applied to define downregulated genes according to the method used. 23 of these genes belong to cluster 2, as does *eng1*, the only ace2p target known before.

74% of the ace2p-dependent genes are also sep1-dependent genes and most of the overlapping targets are members of cluster 2. The level of induction of those genes in the ace2p OE appears to be higher compared to sep1p OE (Fig. 4.3A), suggesting that ace2 is the main regulator of their expression. Their promoter sequences also contain a binding site (5'-CCAGCC-3' that was here named 'Ace2' –Appendix VI) very similar to the one identified in budding yeast for Ace2p (5'-RRCCAGCR-3'), illustrating once more how conserved the ace2 regulation mechanism is between the two yeasts. Ace2p is therefore responsible for regulating periodic expression of a subset of genes, all members of cluster

2. These genes are only indirect targets of sep1p since sep1p is the regulator of *ace2* periodic expression.

Table 4.2 Ace2p-dependent periodic genes

Biological/systematic name	Gene description	Cluster
SPAC1071.09c[a]	Protein containing a DnaJ domain, which mediates interaction with heat shock proteins	2
SPBC1289.01c[a]	Unknown function, putative involvement in chitin biosynthesis	2
SPCC1322.10	Unknown function, similar to cell-surface proteins and proteoglycans	2
SPAC14C4.09	Unknown function, putative glucanase	2
SPBC1709.12	Unknown function	2
SPCC18.01c	Member of SUN family, contains predicted N-terminal signal sequence	2
SPAC19G12.17c	Unknown function, similarity to podocalyxin like, a transmembrane sialomucin important for lymphocyte adhesion and homing	2
SPBC2A9.07c	Unknown function	2
SPBC2A9.13	Unknown function	2
SPAC2E1P5.03	Protein containing a DnaJ domain, which mediates interaction with heat shock proteins	2
SPBC31F10.17c[a]	Unknown function	2
SPAC343.20	Unknown function	2
SPBC3E7.12c	Unknown function, possible role in regulation of chitin synthase	2
SPBC651.04	Unknown function	2
cdm1	DNA polymerase delta subunit	2
cut2[a]	Securin; required for sister chromatid separation	2
eng1	Endo-beta-1,3-glucanase required for cell separation	2
klp8[a]	Protein containing a kinesin motor domain	2
mid2	Protein required for septin function and stability during cytokinesis	2
par2	Protein phosphatase PP2A, B' regulatory subunit, required for cytokinesis, morphogenesis, and stress tolerance	2
SPAPJ760.03c	Unknown function	2
rgf3	Protein containing a pleckstrin homology (PH) and a RhoGEF (GTPase exchange factor) domain	2
rpc17	Unknown function	2
SPBC28F2.11	Protein with a high mobility HMG-box domain	3
SPBPJ4664.02	Unknown function, possible cell surface glycoprotein	3
SPCC965.06	Protein similar to potassium voltage-gated channel	N(1,2,3)
SPBC800.11	Protein with inosine-uridine preferring nucleoside hydrolase domain	N(2,3)

[a] ace2p-dependent genes highly induced in *Ace2p OE*

Looking back to the list of sep1p-dependent genes that do not appear to be ace2p-dependent, the majority of them are members of cluster 1 (Table 4.2 - genes marked with [a]). Several cluster 1 genes are enriched for a promoter motif (5'-TGTTTAC-3', called 'FLEX' –Appendix VI) similar to the conserved binding sites reported for forkhead

proteins (Alvarez B. *et al.*, 2001; Zhu G. *et al.*, 2000; Horie S. *et al.*, 1998). The overlap between the sep1p-dependent genes and cluster 1 genes containing a forkhead binding site in their promoter sequence is statistically significant ($P \sim 10^{-16}$).

This same set of sep1-dependent genes was slightly induced in *ace2Δ* and downregulated in ace2p OE as if ace2p was acting as an inhibitor of their expression (Fig. 4.3A).

From the results presented so far, it can be concluded that sep1p and ace2p are members of the same transcriptional cascade whereby sep1p directly regulates transcription of cluster 1 members, including *ace2*, which in turn activates transcription of cluster 2 members and possibly inhibits transcription of sep1p/cluster 1 targets (Fig. 6.1).

A double mutant strain *ace2*Δ *sep1*Δ was also constructed in this study. Since the mechanism of regulation proposed here for sep1p and ace2p implies that ace2p is acting downstream of sep1p, this double mutant is expected to show a phenotype and expression profile similar to the one of sep1p. As can be seen in Fig. 4.1D, cells have a very similar morphology to *sep1*Δ, slightly less elongated and they tend to form bigger clumps of cells, due to the separation defect. No major differences were found between the expression profile of the *ace2*Δ *sep1*Δ double mutant and the single *sep1*Δ mutant. The same genes found to be downregulated in *sep1*Δ showed the same pattern in the *ace2*Δ *sep1*Δ double mutant (Fig. 4.3A). This is consistent with our model for the sep1p/ace2p regulatory cascade.

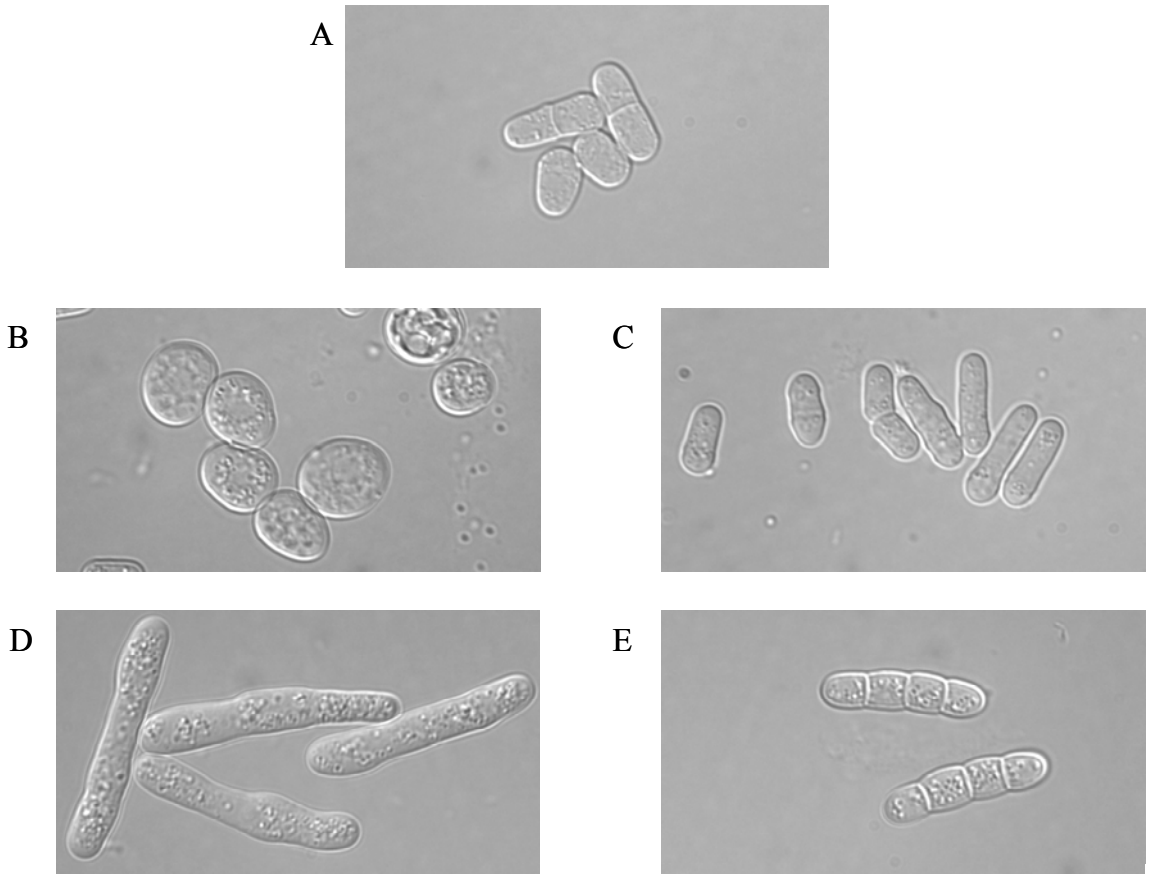


Fig. 4.2 Microscopic appearance of *leu1-32 h*$^-$ overexpressing the following vectors: *pREP3X* only (A), *pREP3X-ace2* (B), *pREP3X-sep1* (C), *pREP3X-fkh2* (D) and *pREP3X-fhl1* (E). DIC photographs are shown.

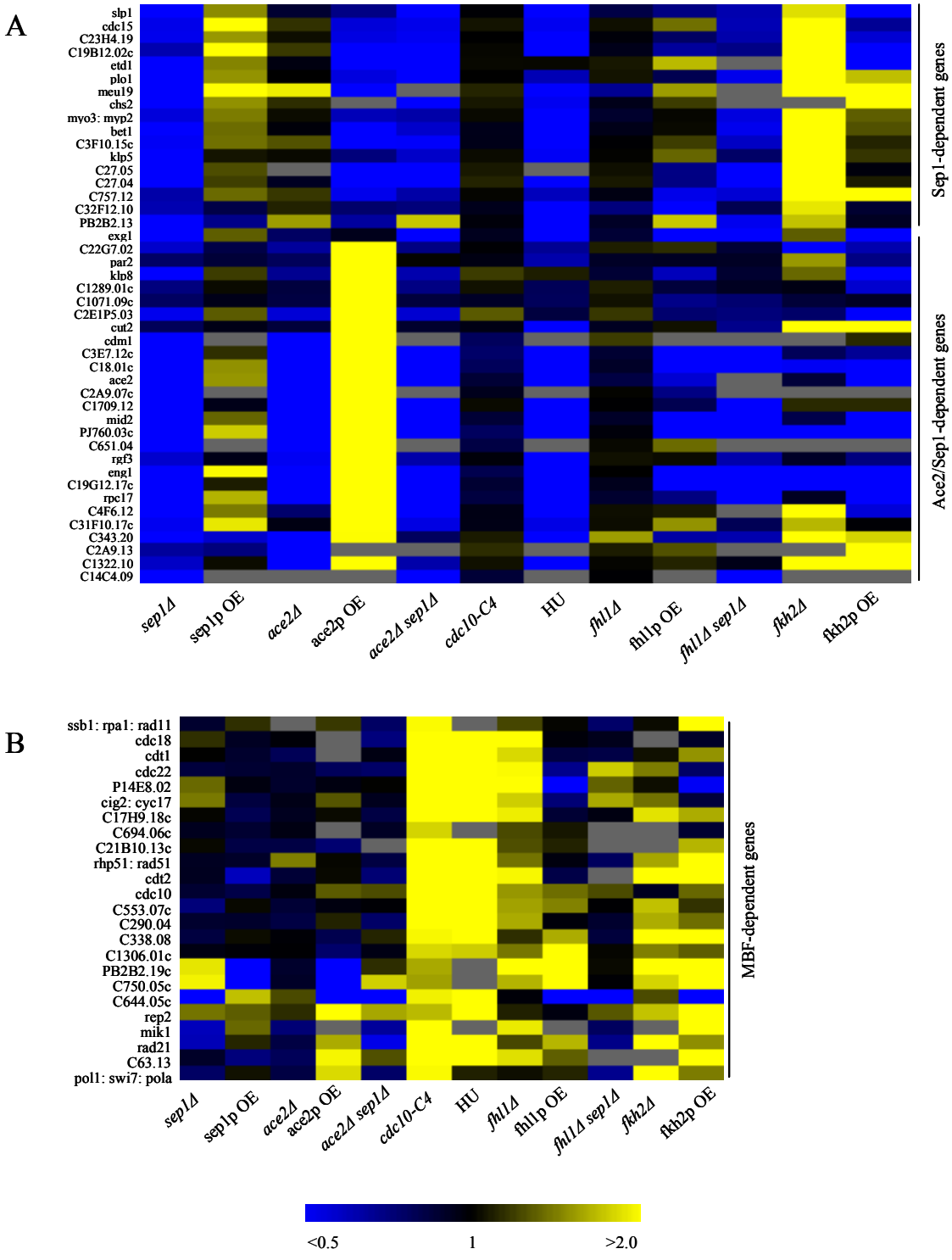


Fig. 4.3 Transcriptional regulation of selected cluster 1 and 2 genes.

Hierarchical clustering of genes based on their expression level in the following experiments: *sep1* deletion (*sep1Δ*), sep1p overexpression (sep1p OE), *ace2* deletion (a*ce2Δ*), ace2p overexpression (ace2p OE), *ace2Δ sep1Δ* double deletion (*ace2Δ sep1Δ*), *cdc10-C4* cells, S-phase 3 hours hydroxyurea (HU) arrest, *fhl1* deletion (*fhl1Δ*), fhl1p overexpression (fhl1p OE), *fhl1Δ sep1Δ* double deletion (*fhl1Δ sep1Δ*), *fkh2* deletion (*fkh2Δ*) and fkh2p overexpression

(fkh2p OE). Panel A shows the behaviour of sep1p- and sep1p/ace2p-dependent genes, panel B of MBF-dependent genes. Clusters were obtained in GeneSpring using Pearson correlation as the distance measurement.

4.4 Other fission yeast forkhead genes

In budding yeast, at least two forkhead genes are involved in regulation of G2/M periodic transcription, *FKH1* and *FKH2*, with overlapping and distinct functions (Hollenhorst P.C. *et al.*, 2000; Kumar R. *et al.*, 2000). The role of other fission yeast forkhead genes was therefore investigated in order to unmask possible roles of them in regulating gene transcription.

Excluding *sep1*, three other fission yeast genes encode proteins containing a forkhead motif: *mei4*, encoding a meiosis-specific transcription factor; SPAC1141.08 (hereafter referred as *fhl1*), whose protein shows similarity to budding yeast transcriptional activator Fhl1p, and SPBC16G5.15c (hereafter referred as *fkh2*), whose protein shows similarity to budding yeast Fkh2p. In order to investigate the function of the last two genes, the expression profiles of two gene deletions (*fhl1Δ* and *fkh2Δ*) and two overexpression strains (fhl1p OE and fkh2p OE) were analysed. *Mei4* was not investigated any further because of its presumed specific role in meiosis. Only *fkh2* was cell cycle regulated in this study, belonging to cluster 1.

Fhl1Δ did not show any particular phenotype (Fig. 4.4C); on the contrary *fkh2Δ* (strain kindly provided by Dr. Brian Morgan) showed a severe phenotype with elongated, branched cells (Fig. 4.4B) and slow growth rate (4.5-5 hours at 30°C in YE). The fhl1p OE phenotype was characterised by multiseptated cells (Fig. 4.2E) and fkh2p OE by very elongated and sometimes branched cells (Fig. 4.2D).

In *fhl1Δ* cells very few genes were found to be expressed at lower levels and no correlation was found between this short list and the genes upregulated in fhl1p OE. Although, several sep1p-dependent genes appear expressed at higher levels in fhl1p OE (Fig. 4.3A), suggesting a regulatory role of fhl1p on a subset of sep1p targets. This could account for the septation defect of the cells overexpressing fhl1p. In *fkh2Δ* cells at least 100 genes appeared to be upregulated and a large portion of them was represented by stress genes (Chen D. *et al.*, 2003), probably reflecting the state of sickness of the strain itself.

16 sep1p-dependent genes (Table 4.1 – genes marked with [b]) were found among the upregulated genes in *fkh2Δ* (Fig. 4.3A). This, together with the septation defect of the deletion, could suggest a possible role of fkh2p in regulating gene expression with a possible negative function on the expression of sep1p-dependent genes. This would once again draw a correlation with the budding yeast regulatory mechanism since Fkh2p has also negative transcriptional roles in *S. cerevisiae* (Koranda M. *et al.*, 2000). This hypothesis requires further work to be proved. It has to be mentioned here that most of these 16 genes were not downregulated in the fkh2p OE.

A double mutant strain *fhl1Δ sep1Δ* was also made and analysed in order to find out if any cumulative effect could result from the deletion of both genes. The phenotype of the strains once again resembles the one of the *sep1Δ* cells (Fig. 4.4D) with multiseptated cells. Similarly, the expression profile did not present any difference when compared with the one obtained for the single mutant *sep1Δ* (Fig. 4.3A).

Based on these data, no obvious role in regulating periodic gene expression during the fission yeast cell cycle could be assigned to fhl1p. Further work is needed to clarify its potential role in regulating some of the sep1p targets. Fkh2p instead could play an inhibitory role on the expression of some sep1p-dependent genes.

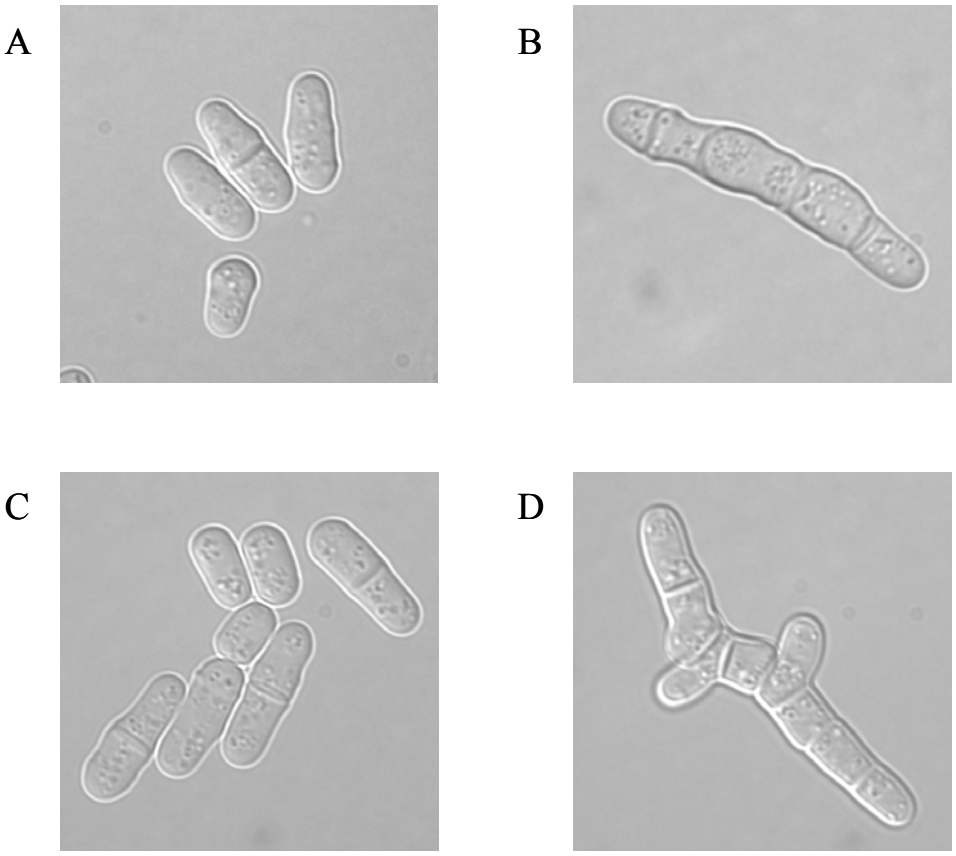


Fig. 4.4 Microscopic appearance of wild type (A), *fkh2*Δ (B), *fhl1*Δ (C) and *fhl1*Δ *sep1*Δ (D) mutant cells. DIC photographs are shown.

4.5 Cdc10p-dependent transcription

As presented in section 3.5, cluster 2 includes several genes known to be regulated by the MBF transcription factor complex, whose components are cdc10p, res1p, res2p and the regulator rep2p (see Introduction). *Cdc10* and *rep2* were identified as periodic in this study and assigned to cluster 2, although the change in *cdc10* expression was of low amplitude.

To further investigate the role of cdc10p in regulating gene transcription, the transcription profile of a well characterised *cdc10* mutant, *cdc10-C4*, was analysed. In this mutant cdc10p is truncated by 61 aminoacids before the C-terminus resulting in a protein that retains some activity when grown at low temperature and looses it at high temperature. As a consequence of the loss of function cdc10p targets are expressed at very high levels throughout the cell cycle (McInerny C.J. *et al.*, 1995).

62 genes were expressed at high levels in *cdc10-C4* cells compared to wild type and 32 of them were periodic genes, most of them members of cluster 2 (Table 4.3 and Fig. 4.3B). Among those were included nine of the ten previously characterised MBF targets (Table 4.3 – genes marked with [a]). The promoter regions of these genes were also enriched for regulatory sequences (MCB1, 5'-AACGCG-3' and MCB2, 5'-CGCGNCGCG-3') similar to the consensus MCB sequence (5'-ACGCGT-3') recognised by MBF in budding yeast and to the E2F transcription factor site in mammalian genes (5'-TTTTGCGCG-3' or 5'-CGCGCAAAA-3') (McIntosh E.M., 1993). Cdc10p is therefore responsible for the regulation of a subset of the cluster 2 genes.

The changes in gene expression in a *cdc10* deletion were also studied. Since *cdc10* is an essential gene, its deletion results in an unviable strain. In the mutant *cdc10Δ* used in this thesis, the *cdc10* mutation is rescued by the overexpression of the res1p N-terminus (1 to 192 aa) (Ayte J. *et al.*, 1995). When cells are grown in normal conditions, res1p directly binds to the MBF targets, substituting cdc10p and allowing normal expression of MBF targets. When thiamine is added to the media, overexpression of rep1p is switched off resulting in the development of the *cdc* phenotype. Levels of *cdc18* decrease by 50% within 2 hours after thiamine addition. Microarray analysis of this strain was not conclusive. Despite a general tendency to a reduction in the expression levels of the MBF-dependent genes so far identified, the changes were not as dramatic as expected and some of the targets were not affected at all by thiamine addition. Some of the known targets such as *cdc18* and *cdc22* decreased within 4 hours, after thiamine was added but their concentration started to increase again after 6 hours.

Similarly the *nda3-KM311* strain was used to better understand the MBF mechanism of regulation. It has been reported that in this strain MBF targets are already transcribed in mitotic cells during a metaphase block (Baum B. *et al.*, 1998). When cells are shifted to the restrictive temperature, the MBF targets accumulate as a result of a metaphase block caused by the mutation in β-tubulin which results in spindle anomalies and therefore in cell cycle arrest at the metaphase-anaphase transition (Hiraoka Y. *et al.*, 1984), (Baum B. *et al.*, 1998). Again the microarray analysis of this strain was not conclusive. Despite the level of some MBF targets increasing, the changes in gene expression were relatively low. *Cig2* increased in this experiment more than *cdc18* and *cdc22*, contrary to what was reported (Baum B. *et al.*, 1998). Therefore, we could not confirm previously published data and both *cdc10Δ* and *nda3* experiments could not be

used to validate the results obtained with the other mutants. It cannot be excluded that this was due to experimental errors.

Based on what has been discussed so far, both cdc10p and ace2p seem to regulate genes in cluster 2 but no overlap was seen between the MBF-dependent and the ace2p-dependent genes. Additional experiments have been performed with the intent of better understanding the relationship between MBF, sep1p and ace2p and the results will be presented in the next section.

Table 4.3 MBF-dependent periodic genes

Biological/systematic name	Gene description	Cluster	Motifs
psc3	Cohesin complex component, required for sister chromatid cohesion and normal mitosis	1	MCB1
rhp51: rad51 [a]	Required for DNA repair and meiotic recombination	1	MCB1
ams2	Protein that binds binds chromatin at centromere and is involved in chromosome segregation	2	MCB 1
SPAC17H9.18c	Unknown function	2	FLEX
SPBC21B10.13c	Homeobox domain (homeodomain) protein, putative transcription factor	2	
SPCC63.13	Protein containing a DnaJ domain, which mediates interaction with heat shock proteins	2	
SPAC644.05c	Protein similar to dUTP pyrophosphatase, which maintains dUTP at low levels to prevent misincorporation into DNA	2	MCB 1, MCB 2
cdc10	Component of MBF transcriptional activation complex involved in control of START	2	
cdc18 [a]	Protein that couples cell cycle signals to DNA replication machinery and induces replication	2	MCB 1, MCB 2
cdc22 [a]	Ribonucleoside-diphosphate reductase large chain, likely required for initiation of DNA replication	2	MCB 1, MCB 2
cdt1 [a]	Protein that coordinates completion of S phase with onset of mitosis	2	MCB 1, MCB 2
cdt2 [a]	Protein required for DNA replication	2	MCB 1, MCB 2
cig2: cyc17 [a]	Major G1/S-phase cyclin, promotes onset of S phase	2	FLEX
cnp1	CENP-A-like protein, histone H3 variant specific to inner centromeres and required for chromosome segregation	2	FLEX, MCB2
eso1	DNA polymerase eta, involved in sister chromatid cohesion	2	FLEX, MCB1
mrc1	Protein required for DNA replication checkpoint	2	MCB 1, MCB 2
mik1 [a]	Protein kinase that inhibits Cdc2p kinase	2	FLEX, MCB1, MCB2
pol1: swi7: pola	DNA polymerase alpha catalytic subunit	2	FLEX, histone
rad21 [a]	Cohesin complex subunit, double-strand-break repair protein	2	
rep2	Zinc finger transcriptional activator, MBF transcriptional complex	2	

ssb1: rpa1: rad11 [a]	Single-stranded DNA-binding protein subunit, required for DNA replication	2	MCB1, MCB2, Novel 1
SPAP14E8.02	Unknown function	2	MCB 1, MCB 2
SPBC16D10.06	Member of ZIP zinc transporter family	4	
SPBC25B2.08	Unknown function	4	FLEX
SPAC869.02c	Member of globin family of oxygen transporters, similar to flavohemoglobin that protects from stress	4	
SPBC1683.07	Protein similar to alpha-glucosidase	N(1,2)	Ace2
SPCC338.08	Unknown function	N(1,2)	
SPBC428.17c	Unknown function	N(1,4)	
SPBC1306.01c	Protein with elongation factor Tu GTP binding domain, similar to mitochondrial translation elongation factor G	N(2,3)	
SPCC553.07c	Member of impB, mucB or samB family, possible role as translesion DNA repair polymerase	N(2,3)	
CSPA750.05c	Telomeric protein of unknown function, highly similar to S. pombe SPAC977.01, SPAC1348.02 and SPBPB2B2.19c	N(2,3)	
SPBPB2B2.19c	Telomeric protein of unknown function, highly similar to S. pombe SPAC977.01, SPAC1348.02 and SPAC750.05C	N(2,3)	

[a] previously known cdc10p targets

4.6 Additional experiments addressing regulation by sep1p, ace2p and cdc10p

The roles of MBF, sep1p and ace2p transcription factors were also investigated in a *sep1Δ cdc25* strain in cells synchronised in a 'block and release' experiment. Sep1p-dependent and ace2p-dependent genes still maintained their periodic behaviour but the amplitude of the expression was reduced when compared to the profiles of the same genes in wild type elutriated cells or in a *cdc25* 'block and release' experiment (Fig. 4.5). The expression amplitude of the MBF-dependent targets was not affected but a delay of 30 min in the second peak of expression of those genes could be observed when compared with a typical *cdc25* 'block and release' experiment.

The relative timing of the peaks of expression for MBF-dependent and ace2p-dependent genes seems to be different (especially in the first cycle) when the two synchronisation methods were compared. They are coincident when elutriation is used whereas the MBF-regulated wave seems to precede the ace2p-regulated one when temperature sensitive mutants are used. This is consistent with the timing of the cell cycle

events in *cdc25* cells where DNA replication occurs earlier than in wild type elutriated cells as shown in Fig. 3.2.

Despite their peaking at nearly the same time, those two sets of genes clearly show differences in the mechanism of their regulation. This was confirmed also by the different response in their behaviour when wild type cells were synchronised using hydroxyurea (HU). This drug inhibits DNA replication causing cell cycle arrest in S-phase. As a consequence of the HU treatment, sep1p- and ace2p-dependent genes were repressed whereas MBF-dependent genes were strongly induced (Fig. 4.3A and B).

A further attempt was made to temporally separate the MBF and ace2p-waves using *cig1*Δ *cig2*Δ *puc1*Δ strain synchronised by elutriation. Fission yeast cells lacking the three G1 cyclins cig1p, cig2p and puc1p have an extended G1 phase compared to wild type cells in which G1 is relatively short and difficult to study (Martin-Castellanos C. *et al.*, 2000). MBF and ace2p waves still seemed coincident in this experiment (Fig. 4.6). It is worth mentioning that elutriating this strain was more difficult than any of the other mutants. Despite it being repeated several times (3 independent attempts), interpretation of the FACS profile was difficult, making it hard to establish how good cell synchrony was and therefore judge the validity of the experiment.

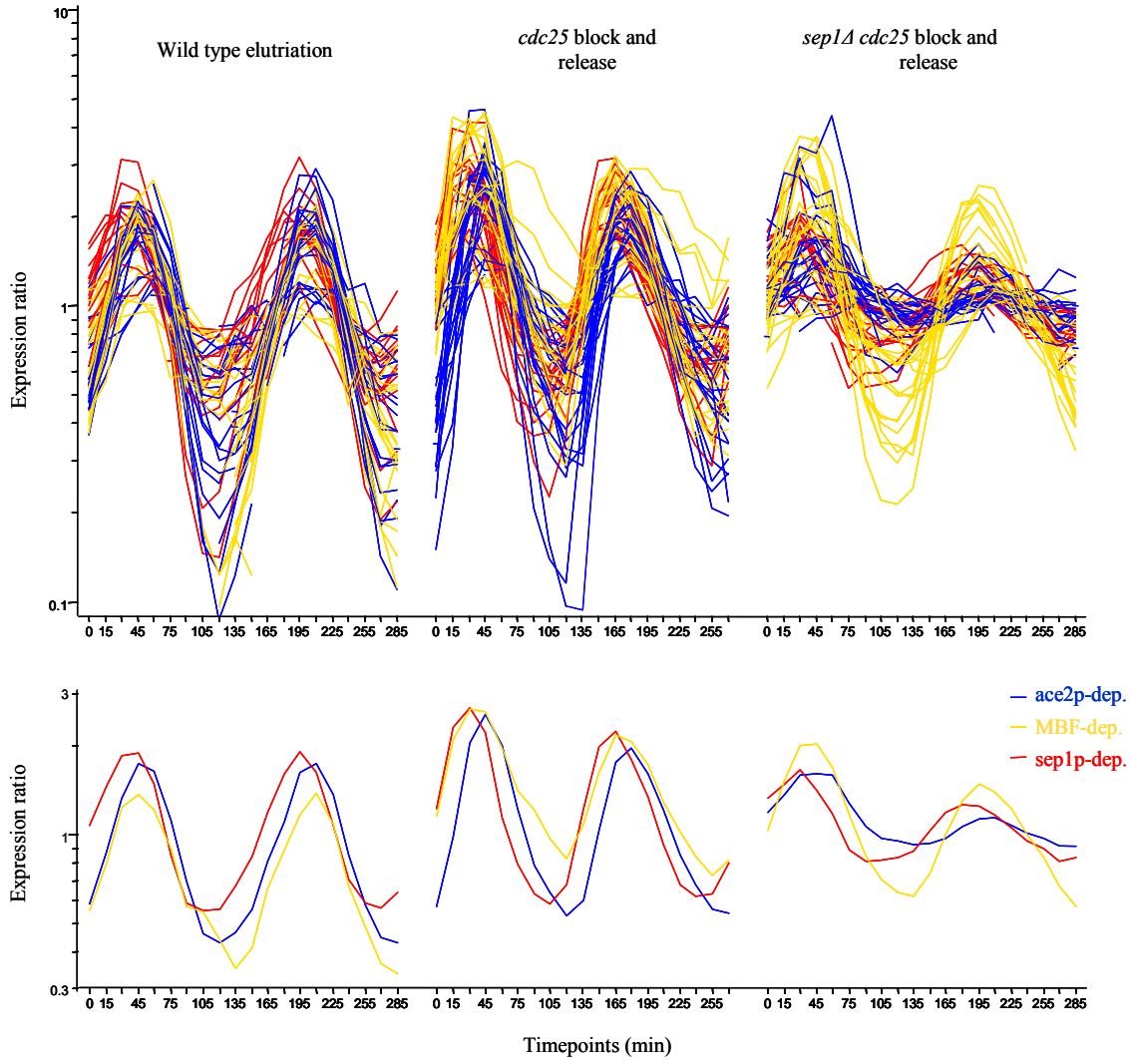


Fig. 4.5 MBF, ace2p and sep1p transcriptional regulation of cluster 1 and 2 genes in a *sep1Δ cdc25* 'block and release' experiment.

Expression profiles of MBF-, ace2- and sep1-dependent genes are shown (from right to left) in a wild type elutriation, in a *cdc25* 'block and release' and in a *sep1Δ cdc25* 'block and release' experiment (top graphs). The bottom graphs show the average profiles for the same subset of genes. Red: sep1p-dependent; yellow: MBF-dependent; blue: ace2p-dependent.

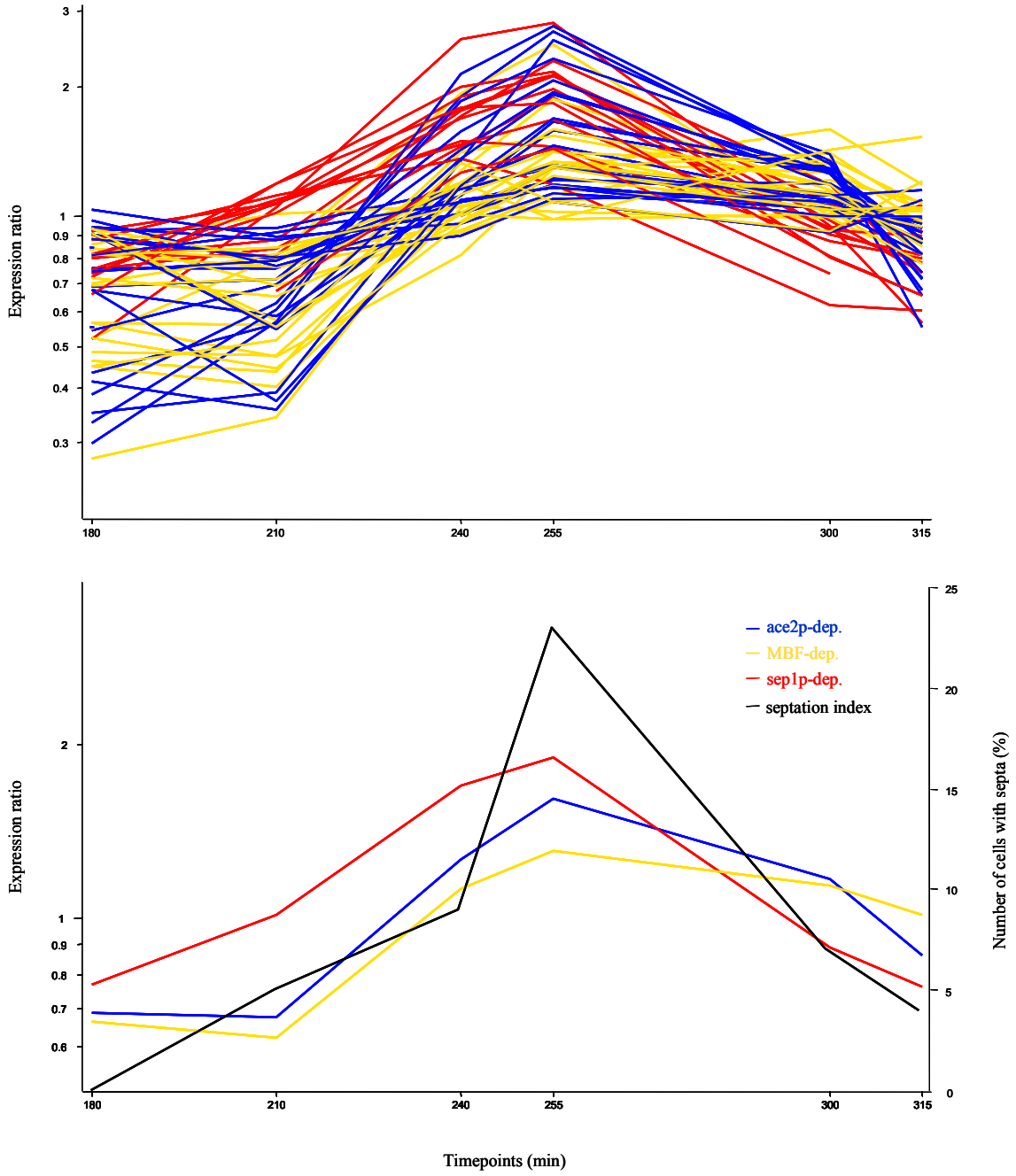


Fig. 4.6 MBF, ace2p and sep1p transcriptional regulation of cluster 1 and 2 genes in a *cig1*Δ *cig2*Δ *puc1*Δ elutriation experiment.

Red: sep1p-dependent; yellow: MBF-dependent; blue: ace2p-dependent; black: septation index. Only selected timepoints around the septation peak have been hybridised.

4.7 Studies with additional potential regulatory genes

Meu3 and *meu19*

Five non-coding RNAs are transcribed by *meu3*, *meu11*, *meu16*, *meu19* and *meu20* genes. It has been speculated that these RNAs might have a regulatory role in meiosis, similarly to meiRNA which specifically binds to mei2p allowing premeiotic DNA synthesis and meiosis I onset (Watanabe T. *et al.*, 2001).

Out of those three genes, only *meu19* was found to be periodically expressed during the cell cycle (member of cluster 2). *Meu3* was periodic in the preliminary data and subsequently removed from the periodic genes. *Meu3* and *meu19* are twin genes; they share an identical 5' half sequence, differ in the 3' half sequence and are located in distinct genomic areas. A *meu3Δ* and a *meu19Δ* were constructed in this study with the intention of investigating a potential regulatory role of these genes in mitosis. *Meu3Δ* did not show any particular phenotype and isolation of *meu19Δ* haploid cells was particularly difficult (Fig. 4.7C and D). None of the periodic genes appeared to be affected by the absence of *meu3* or *meu19*. Based on the results of those experiments, a function of those genes in regulating periodic gene expression is unlikely.

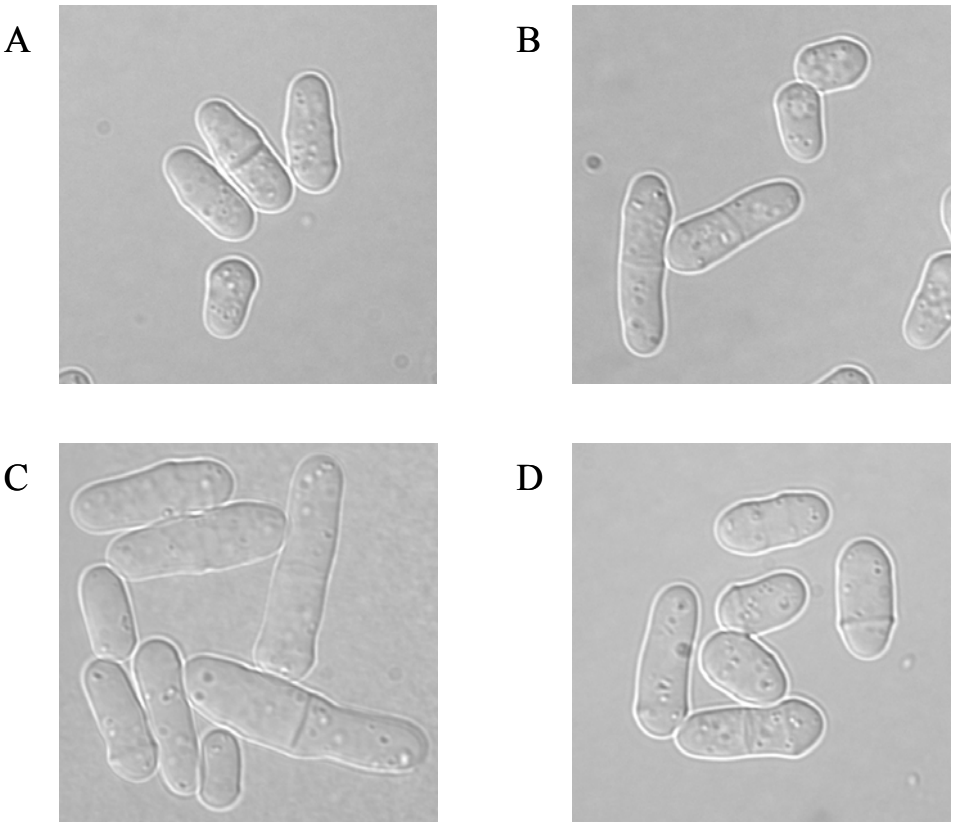


Fig. 4.7 Microscopic appearance of wild type *972 h*$^-$ (A), SPBC19G7.06Δ (B), *meu19*Δ (C) and *meu3*Δ (D). DIC photographs are shown. Information about SPBC19G7.06Δ can be found in the next section.

MADS-box gene

In budding yeast, the transcription factor complex that regulates expression of G2/M is composed of a forkhead-type protein, Fkh2p, a MADS-box protein, Mcm1p and an activator, Ndd1p. It is a clear example of a regulatory complex containing two different kinds of transcription factors, forkheads and MADS-box proteins interacting with each other.

MADS-box proteins are a highly conserved family of transcription factors involved in many biological functions (Messenguy F. and Dubois E., 2003). Two proteins containing a MADS-box motif have been identified in fission yeast: map1p that is involved in transcriptional activation of mating type-specific genes and SPBC19G7.06 encoded protein whose function is still unknown. A strain with SPBC19G7.06 deleted (kindly provided by Dr. J. Millar) was analysed to investigate the potential role of this protein in regulating gene expression. This deletion did not show any particular phenotype (Fig. 4.7B). SPBC19G7.06 expression is not cell cycle regulated.

Few periodic genes (12) were expressed at lower levels in this mutant strain compared to wild type (Table 4.4). Interestingly, a few of them are members of cluster 2 and contain a FLEX binding site, a typical forkhead-binding motif. Interaction between forkhead-type transcription factors and MADS-box has never been reported in *S. pombe.* Although, considering the significant degree of conservation of the forkhead regulation mechanism between the two yeasts and the function of some of the genes downregulated in the SPBC19G7.06 deletion (glucanases and chitin synthase involved in cell separation), a possible interaction between the two factors is possible. Further analysis will be needed to validate this theory.

Table 4.4 Downregulated genes in SPBC19G7.06 deletion

Biological/systematic name	Gene description	Cluster	Motifs
chs2[b]	Member of chitin synthase family, involved in cell wall maintenance	1	MCB 1

C14C4.09[a]	Unknown function, putative glucanase	2	
C18.01c [a]	Member of SUN family, contains predicted N-terminal signal sequence	2	Ace2, FLEX
C965.14c	Member of cytidine and deoxycytidylate deaminase zinc-binding region family	2	FLEX
eng1 [a]	Endo-beta-1,3-glucanase required for cell separation	2	FLEX
PJ760.03c [a]	Unknown function	2	Ace2, FLEX
C19C7.04c	Unknown function	3	
PJ4664.02 [b]	Unknown function, possible cell surface glycoprotein	3	
bgl2	Protein similar to beta-glucosidase, a cell wall endo-beta-1,3-glucanase	4	
C27D7.09c	Unknown function	4	
C27D7.11c	Unknown function	4	Novel 3
nrd1	Protein containing four RNA recognition motifs	4	

[a] ace2p-dependent genes

[b] sep1p-dependent genes

Cluster 4 regulation

It has been mentioned before that several cluster 4 members are genes involved in stress response including the transcription factor pcr1p which plays a role in mating, meiosis and stress response (Watanabe Y. and Yamamoto M., 1996). Pcr1p forms a complex with atf1p, another transcription factor that controls expression of most genes involved in stress defense (Chen D. *et al.*, 2003; Toone W.M. and Jones N., 1998; Takeda T. *et al.*, 1995; Wilkinson M.G. *et al.*, 1996). Another transcription factor, prr1p, is involved in response to oxidative stress and to elevated salt concentrations as well as sexual differentiation, in a pathway that presumably functions in parallel with atf1p (Ohmiya R. *et al.*, 1999; Ohmiya R. *et al.*, 2000; Greenall A. *et al.*, 2002). *Atf1* and *prr1* were not among the 407 cell cycle regulated genes. Since cluster 4 is enriched with stress related genes, it was interesting to investigate a possible involvement of any of these transcriptional regulators (pcr1p, prr1p and atf1p) in controlling the cluster 4/G2 wave of transcription in fission yeast. For this purpose, the expression profiles of *atf1Δ*, *pcr1Δ* and *prr1Δ* have been analysed. *Atf1Δ* and *pcr1Δ* showed a very similar expression profile; most of the genes that require atf1p for their basal level of expression (Chen D. *et al.*, 2003) appeared repressed in both deletions as expected. Similarly, most of the atf1p-repressed genes from the same study were induced in *atf1Δ* and *pcr1Δ*. All the prr1p identified targets (*ste11*, *mam2* and *mei2*) were highly repressed in *prr1Δ*.

In all three deletions some of the cluster 4 members were expressed at low levels but most of the members of this cluster were not affected. Atf1p, pcr1p and prr1p may be involved in the regulation of some cluster 4 members but this does not mean they are involved in controlling their periodic expression. The only way to verify this would be to perform a timecourse experiment for each deletion and observe if any change occurs in the expression of cluster 4 members.

4.8 Potential regulatory promoter motifs

The search for regulatory sequences in the upstream region of the periodically expressed genes identified several motifs, some of them already known as well us some novel ones. All the motifs identified are listed in Table 4.5. The most significant sequence patterns are shown together with the cluster they are most associated with, indicated by the significance (*P* value) of the overlap between genes in the cluster and genes with the given motif among all genes in the genome.

As described before, MBF-dependent genes in cluster 2 were enriched with two motifs, named MCB1 and MCB2, very similar to the MCB motif found in the upstream sequence of well characterised fission yeast MBF targets. Similar sequences are recognised by MBF in *S. cerevisiae* and by E2F in mammals.

A common motif named ACE2 was found in this study in the promoter region of ace2p-dependent genes in cluster 2. This motif shows similarities to the Ace2 consensus sequence recognized by the homologous budding yeast transcription factor.

Several sep1p-dependent genes as well as several other cluster 1 members contained a FLEX promoter sequence, a well known target for forkhead proteins like mei4p. It is the first time a link with the cell cycle is proposed for this motif which until now has been only investigated in relation to meiosis. Other forkhead/MADS box binding motifs have been identified in mammals and *S. cerevisiae* (Maher M. *et al.*, 1995; Messenguy F. and Dubois E., 2003), as an independent transcriptional regulator as well as in combination with Ndd1p/Fkh2p. No motif similar to Mcm1 has been found in fission yeast in this study.

Two novel motifs, named Novel 1 and 2, were often found in combination with forkhead motifs, in particular among cluster 1 members without overlapping with sep1p-dependent genes.

In cluster 3, the nine histone genes, as well as some other non-histone genes (5) in cluster 2 and 3, have in common the Histone motif (Matsumoto S. and Yanagida M., 1985). This motif is not conserved between the two yeasts and not much has been elucidated concerning the regulation of the histone genes. In mammalian cells, histone gene expression is controlled largely at the post-transcriptional level involving mRNA 3'-end formation and RNA stability (Marzluff W.F. and Duronio R.J., 2002).

In cluster 4, only one motif was found, named Novel 3, but the majority of the genes in this cluster do not share any common regulatory sequence. This raises the possibility that these genes might be regulated at a different level, possibly RNA stability.

Table 4.5 Potential regulatory promoter motifs

Motif name	Sequence pattern	Associated cluster (*P* value)/gene list
FLEX	5'-TGTTTAC-3'	1 (<1e-40)
Novel 1	5'-GTTGNCATG-3'	1 (6.1e-07)
Novel 2	5'-TTGCATTTNC-3'	1 (2.0e-05)
MCB 1	5'-AACGCG-3'	2 (1.6e-27)
MCB 2	5'-CGCGNCGCG-3'	2 (2.0e-19)
MCB 2	5'-CGCGNCGCG-3'	MBF-dep.
Ace2	5'-CCAGCC-3'	2 (3.5e-18)
Ace2	5'-ACCAGCCNT-3'	Ace2p-dep.
Histone	5'-AACNCTAAC-3'	3 (4.5e-15)
Novel 3	5'-ACCNCGC-3'	4 (5.2e-11)

N = either A, C, G or T

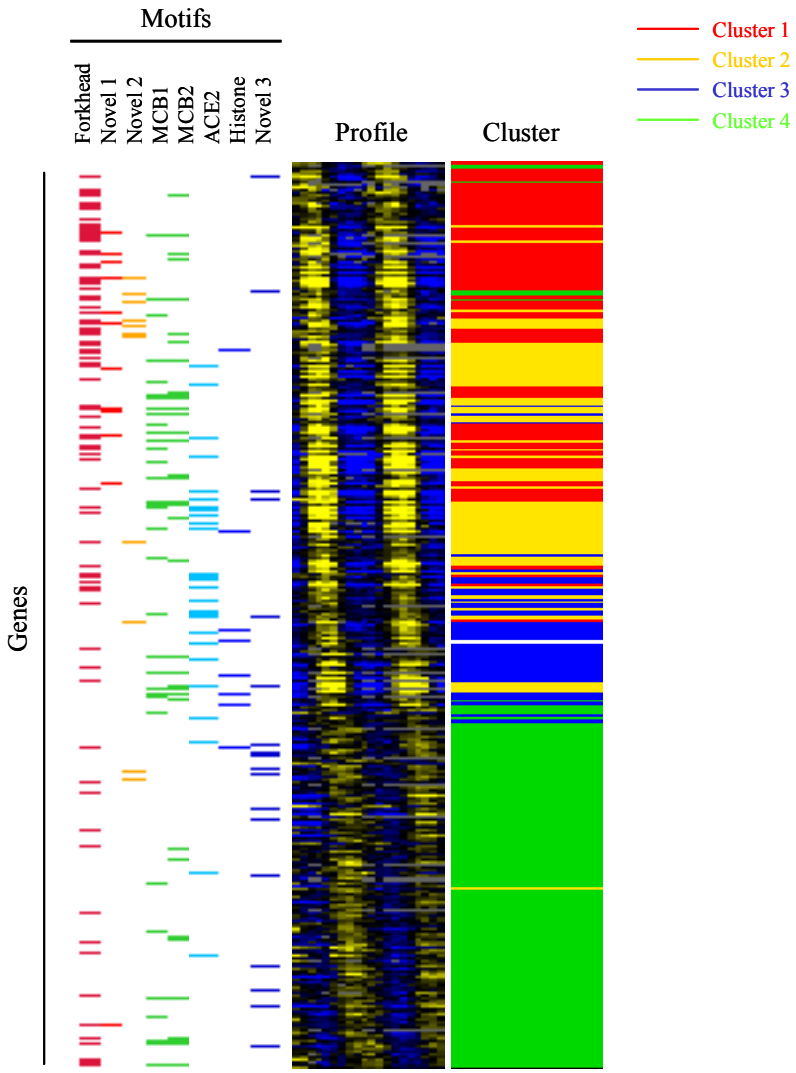


Fig. 4.8 Identification of potential regulatory promoter motifs.

The left hand side graph shows the presence of the eight motifs (indicated by colour bars – the colours chosen have no correlation with the cluster assignment). The middle graph shows the corresponding expression profiles for one elutriation experiment (2201) and the right hand side cluster assignment of the genes.

5. CONSERVATION OF CELL CYCLE REGULATED GENE EXPRESSION

This chapter will focus on the conservation of periodic gene transcription through evolution. The fission yeast cell cycle program of gene expression compiled for this thesis has been compared with the one described for budding yeast (Cho R.J. *et al.*, 1998), (Spellman P.T. *et al.*, 1998) in order to identify a core set of genes whose function and regulation are conserved across yeast species. A similar comparison has been done, to a lesser extent, with the human cell cycle (Cho R.J. *et al.*, 2001; Whitfield M.L. *et al.*, 2002).

5.1 Fission yeast and budding yeast: what is conserved?

Fission yeast and budding yeast are only distantly related and they represent a good complementary system for the identification of conserved mechanisms among eukaryotes.

Two budding yeast microarray studies of cell cycle gene expression identified ~ 400 (Cho R.J. *et al.*, 1998) and 800 (Spellman P.T. *et al.*, 1998) periodic genes. For budding yeast two gene lists were used: the complete Spellman list of periodic genes and a second list of 301 genes, which were periodic in both the Spellman and Cho datasets. For fission yeast, the analysis was performed using both the complete list of 407 periodic genes as well as the list of 136 'high amplitude' periodic genes. Homologous *S. cerevisiae* genes were identified using a curated ortholog table including a total number of 2981 genes (Materials and methods, section 2.6.6). For each list, only genes with an ortholog were included in the analysis (Table 5.1 – the numbers in parentheses refer to the number of ortholog genes within each list).

The overlap of periodic genes was surprisingly small yet statistically highly significant. Table 5.1 shows the *P*-values associated with the different gene list comparisons, together with the sizes of the overlaps expected by chance for the same comparisons (calculated based on the size of the gene lists used and the total number of *S. pombe* genes with a *S. cerevisiae* ortholog as explained in section 2.6.6). The best results were obtained for the comparisons where the 'high amplitude' gene lists were used (Table 5.1- comparisons in bold), against the all periodic Spellman genes (P = 4.3e-32) and

against the Spellman/Cho overlap (*P* = 2.33e-35) respectively. The number of conserved cell cycle regulated genes increased when the lists of all periodic *S. pombe* genes and all periodic Spellman genes were compared but the significance was drastically reduced (*P* = 2.0e-22). Among the 'high amplitude' genes, 87 had a budding yeast ortholog and 42 of them were also periodic in both budding yeast studies (Table 5.2). This is the most conservative list of periodic/ortholog genes in the two yeasts. The additional 13 genes that were periodic in fission yeast ('high amplitude' *S. pombe* genes) and in the Spellman study are also listed in Table 5.2.

In addition, the five clusters of *S. pombe* cell cycle regulated genes were compared with the five clusters of *S. cerevisiae* periodic genes, as identified by Spellman *et al.* (1998); only the *S. pombe* 'high amplitude' genes were used for this comparison. Despite the many differences in the life cycles of the two yeasts, a significant number of orthologs was regulated at corresponding cell cycle phases in the two model organisms. Fission yeast clusters 1 to 4 overlapped significantly with the budding yeast G2/M (*P* = 1.0e-08), G1 (*P* = 8.3e-10), S (*P* = 5.4e-17) and S/G2 (*P* = 1.6e-03) phase genes, respectively. *S. pombe* cluster 4 and *S. cerevisiae* S/G2 genes showed a weaker overlap, probably as consequence of the fact that this cluster in fission yeast represents quite an heterogeneous group of genes, expressed over a relatively long G2 phase. *S. cerevisiae* M/G1 cluster showed a weak overlap with *S. pombe* cluster 2.

Table 5.1 Overlap of periodic genes between *S. pombe* and *S. cerevisiae*

***S. pombe* (ortholog number)**	***S. cerevisiae* (ortholog number)**	**Ortholog number periodic in both yeasts**	**Expected overlap in gene number**	***P*-value**
All periodic genes (252)	Spellman (322)	81	27.2	2.0e-22
All periodic genes (252)	Cho and Spellman (137)	55	11.6	7.7e-26
'High amplitude' periodic (87)	**Spellman (322)**	**54**	**9.4**	**4.3e-32**
'High amplitude' periodic (87)	**Cho and Spellman (137)**	**42**	**4.0**	**2.33-35**
'High amplitude' cluster 1 (28)	Spellman: G1 (127)	9	1.2	1.2e-06
'High amplitude' cluster 1 (28)	Spellman: G2/M (74)	9	0.7	1.0e-08

'High amplitude' cluster 2 (36)	Spellman: G1 (127)	13	1.5	8.3e-10
'High amplitude' cluster 2 (36)	Spellman: M/G1 (33)	3	0.4	6.9e-03
'High amplitude' cluster 3 (14)	Spellman: S (40)	10	0.2	5.4e-17
'High amplitude' cluster 4 (4)	Spellman: S/G2 (50)	2	0.1	1.6e-03

Table 5.2 Core set of periodically expressed genes in fission and budding yeasts

***S. pombe* ortholog**	***S. cerevisiae* ortholog**	**Function**
DNA replication		
pol1	*POL1*	DNA polymerase α
cdc20	*POL2*	DNA polymerase ε
ssb1	*RFA1*	Single-stranded DNA-binding protein
cdc18	*CDC6*	Regulator of DNA replication initiation
mrc1	*MRC1*	DNA replication checkpoint protein
cdc22	*RNR1*	Ribonucleotide reductase
psm3	*SMC3*	Cohesin
rad21	*MCD1*	Cohesin
pht1	*HTZ1*	Histone variant
hta1, hta2	*HTA1, HTA2*	Histone H2A
htb1	*HTB1, HTB2*	Histone H2B
hht1, hht2, hht3	*HHT1, HHT2*	Histone H3
hhf1, hhf2, hhf3	*HHF1, HHF2*	Histone H4
Mitosis and cell division		
plo1	*CDC5*	Polo kinase
ark1	*IPL1*	Aurora kinase
fin1	*KIN3*	NimA kinase
cut2	*PDS1*	Securin (sister chromatid separation)[a]
slp1	*CDC20*	Activator of anaphase promoting complex
wis3	*SPO12*	Putative cell-cycle regulator
klp5, klp6, klp8	*KAR3, KIP1*	Kinesin microtubule motor[b]
mob1	*MOB1*	Protein involved in mitotic exit/septation
sid2	*DBF2*	Kinase involved in mitotic exit/septation
myo3	*MYO1*	Myosin II heavy chain
mid2	*BUD4*	Protein involved in cytokinesis
ace2	*ACE2*	Transcription factor
imp2	*HOF1*	Protein involved in cell division
chs2	*CHS2*	Protein involved in septum formation
eng1	*DSE4*	Glucanase for cell separation
mac1	*TOS7*	Putative role in cell separation (*S. pombe*)
Others		
rum1	*SIC1*	Inhibitor of cyclin-dependent kinase[a]
mik1	*SWE1*	Kinase inhibiting cyclin-dependent kinase
cig2	*CLB1-CLB6*	B-type cyclin[b]
msh6	*MSH6*	Mismatch-repair protein

rhp51	*RAD51*	DNA repair protein
SPBC32F12.10	*PGM1*	Phosphoglucomutase, carbohydrate metabolism
SPAP14E8.02	*TOS4*	Unknown function
Additional conserved genes from the comparison between 'high amplitude' periodic and Spellman		
cfh1, cfh2, cfh3, cfh4	*SKT5*	Protoplast regeneration and killer toxin resistance protein
psu1	*SIM1*	Essential protein required for cell wall integrity, member of the SUN protein family
SPCC1322.04	*UGP1*	UDP-glucose pyrophosphorylase (UTP-glucose-1-P uridylyltransferase)
SPAP7G5.06	*GAP1*	Protein with high similarity to general amino acid permease
exg1	*EXG1*	Exo-beta-1,3-glucanase
SPBC1198.07	*DFG5*	Putative glycosylphosphatidylinositol (GPI)-anchored protein
SPBC19C7.04	YMR295C	Unknown function
SPAC19B12.02	*GAS1*	Protein with high similarity to 1,3-beta-glucanosyltransferase
SPCC338.12	*PBI2*	Member of the subtilisin N-terminal region containing family
psy1	*SSO1*	Syntaxin-like protein component of the plasma membrane docking/fusion complex

[a] Proteins encoded by these genes show little sequence homology but are functional homologs
[b] Protein families with various functions

5.2 Conserved genes across yeast species and their function

Among the genes whose periodic behaviour has been conserved in the two yeasts, two subsets of genes were identified: a group involved in DNA replication and another one involved in mitosis and/or cell division. The histone genes are a prominent group among those one whose function is linked to DNA replication. Histone genes show a periodic behaviour that is highly conserved across eukaryotes (Plumb M. *et al.*, 1983; Hereford L.M. *et al.*, 1981, Aves S.J. *et al.*, 1985).

The process of chromosomal DNA replication in yeast involves many components whose function appears conserved through evolution. The initiator is the origin recognition complex (ORC), which binds to replication origins and is required for their firing. The next step requires the binding of an ORC-interacting factor (*S. cerevisiae* Cdc6p/*S. pombe* cdc18p) and of the mini-chromosome maintenance (MCM) protein complex, responsible for the expansion of the unwound DNA at the replication origin. Once the DNA is unwound, short RNA-DNA primers are synthesised by the DNA polymerase α (Pol1p/pol1p) and then elongated by the DNA polymerase ε

(Pol2p/cdc20p). The process is then terminated by the ligation of all DNA fragments. The conservation of this fundamental process is reflected by the periodic behaviour of *CDC6*/*cdc18* and of the DNA polymerase α and ε coding genes, observed in both yeasts. Other genes whose function is directly involved in DNA replication and that were found to be cell-cycle regulated in both yeasts are: *RFA*/*ssb1*, coding for replication protein A whose function is essential for the formation of the replication fork (Wold M.S., 1997) and *RNR1*/*cdc22*, coding for the catalytic subunit of a ribonucleotide reductase which is responsible for catalyzing production of deoxyribonucleotides for DNA synthesis (Fernandez Sarabia M.J. *et al.*, 1993).

Another well conserved process between the two yeasts is the DNA-damage checkpoint, although some differences in the pathways can be identified (Melo J. and Toczyski D., 2002). The adaptor protein Mrc1p/mrc1p has a conserved function in both species within the checkpoint cascade and shows a periodic behaviour in both organisms.

Sister-chromatid cohesion is essential to ensure proper chromosome segregation in M phase and is regulated by two complexes, condensin and cohesin. When cohesins are cleaved, they dissociate from the chromosomes and lead to sister chromatid separation. Two cohesin-coding genes, *SMC3*/*psm3* and *MCD1*/*rad21* showed a conserved periodic behaviour in fission and budding yeast, although some differences have been detected in the dissociation of these proteins from the chromosomes in the two yeasts (Tomonaga T. *et al.*, 2000).

The mitotic cascade of events is activated by a cyclin-dependent kinase and regulated downstream by members of three separate kinase families: the Aurora, Polo and NIMA-related kinases. They are highly conserved in eukaryotes with a single family member in yeasts and multiple-gene families in humans. In yeasts these kinases control several mitotic processes such as spindle formation, CDK-phosphorylation, the anaphase promoting complex which in turns regulates M phase exit, and cytokinesis (Nigg E.A., 1998; O'Connell M.J. *et al.*, 2003; Andrews P.D. *et al.*, 2003). All three kinases in budding (*CDC5*, *IPL1* and *KIN3*) and in fission yeast (*plo1*, *ark1* and *fin1*) have a conserved periodic behaviour.

Microtubules contribute to the formation of the mitotic spindle whose function is to segregate the chromosomes; microtubule rearrangements are mainly due to kinesin motor proteins such as Kar3p and Kip1p in budding yeast and klp5p, klp6p and klp8p in fission yeast, a protein family involved in many different functions. Sister chromatids get

separated when Pds1p/cut2p is degraded by the anaphase promoting complex which is activated by Cdc20p/slp1p. All genes coding for these proteins were found to be periodic.

Cytokinesis is conducted differently in different organisms but the major events appear to be universal. In *S. pombe* and animal cells, an actomyosin ring is formed at the cell equator, a membrane barrier is then synthesised to separate the two cytoplasms and then the two new cells will separate. In *S. pombe* an additional septum is synthesised behind the ring and then enzymatically digested to allow cell separation. In *S. cerevisiae* cells divide by budding. Positioning of the actomyosin ring differs significantly in the two yeasts but the downstream events are more conserved (Guertin D.A. *et al.*, 2002). The myosin II heavy chain protein responsible for contraction of the actomyosin ring (Myo1p/myo3p), the cdc15-like protein Hof1p/imp2p and Bud4p/mid2p, both components of the contractile ring, the Chs2p/csh2p septum component, the transcription factor Ace2p/ace2p, the glucanase Dse4p/eng1p involved in septum digestion, and the kinase Dbf2p/sid2p that regulates septum formation together with Mob1p/mob1p are all encoded by genes that show a conserved periodic behaviour.

As explained in the introduction to this thesis, the engine that drives the cell cycle is a complex formed by a kinase and its cyclin partners, highly conserved across eukaryotes. Three *S. pombe* cyclin coding genes (*cdc13*, *cig1* and *cig2*) appear to be cell cycle regulated and at least eight *S. cerevisiae* genes coding for cyclins are transcriptionally regulated. It should here be mentioned that in the case of cyclin genes it is difficult to establish a one to one relationship between orthologs, probably as a consequence of the duplication events that led in budding yeast to an increase in the number of cyclin genes. Also two of the CDK inhibitors have a conserved pattern of periodic gene expression, *SIC1*/*rum1* and *SWE1*/*mik1*. The gene *RAD51*/*rhp51,* coding for a protein regarded as a key player in homologous recombination and recombinational DNA repair and *MSH6/mhs6*, coding for a member of the eukaryotic DNA mismatch repair system, were found periodic in both model organisms.

5.3 Yeasts and humans: what is conserved?

A similar approach was adopted to compare cell-cycle regulated genes between yeasts and humans. Unfortunately, the lack of a reliable ortholog table for human genes made it impossible to run a systematic comparison as the one done between fission and budding

yeast. Therefore, only hand-curated human gene orthologs to the conserved/periodic fission/budding yeast genes were checked for periodicity. For this comparison, the datasets produced by Whitfield *et al*. (2002) and Cho *et al*. (2001) were considered. However, a statistical reanalysis of both studies revealed that cyclicity of human genes arises from experimental errors and that the reproducibility between experiments was very poor (Shedden K. and Cooper S., 2002; Cooper S. and Shedden K., 2003). Consequently, comparison was limited to those genes that were identified as periodic in human cells using traditional genetic approaches.

Human homologs to yeast genes coding for Aurora (STK6), Polo (SNK) and NIMA-related (NEK2) kinases showed a periodic behaviour underlying how conserved these protein families and their regulation are through evolution. Among the other human homologs that also appeared cell cycle regulated there were POLA (*POL1/pol1* homolog), CDC20 (*CDC20/slp1* homolog), RAD21 (*MCD1/rad21* homolog), RRM1 (*RNR1/cdc22* homolog), CDC6 (*CDC6/cdc18* homolog), which are all involved in DNA replication and have a conserved function across eukaryotes. Similarly, histones and several human cyclin genes (CCNE1 and 2, CCNG2, CCNA2, CCNF, CCNB1 and 2) were cell cycle regulated; periodic expression of these genes is a conserved mechanism of regulation from yeast to humans.

SNAI3 (*ACE2/ace2* homolog), which contains a zinc finger binding domain and acts as a transcriptional repressor, was also found periodic in the human studies. In this case homology is due only to the presence of the zinc finger domain and further studies are needed to investigate a potential functional homology between SNAI3 and ace2-type transcription factors.

Some genes were periodically expressed in fission yeast and humans only, including the protein kinase CDC2 and the protein phosphatase CDC25. Conversely, DNA replication MCM complex genes were periodic in budding yeast and humans only.

Despite the fact that absolute numbers of conserved genes among yeast species and between yeasts and humans are surprisingly small it is interesting that most of them have a well characterised regulatory function associated with basic cell cycle processes such as DNA replication, mitosis and cytokinesis. This differs from the larger list of cell cycle regulated genes and probably reflects the fact that periodic transcription of those genes plays a critical role in driving cell cycle progression.

6. GENERAL DISCUSSION

In this thesis the gene expression program during the fission yeast cell cycle has been investigated. It is the first time that gene expression has been comprehensively studied in vegetatively growing cells of *S. pombe*. These results have provided a useful framework for fission yeast research as well as an interesting point of comparison with other eukaryotic organisms such as budding yeast and humans. Results and their implications are discussed below.

6.1 Cell-cycle periodic genes and their regulation

Conventional methods of RNA measurements have been applied over the last decades to identify cell cycle regulated genes. The availability of the complete genome sequence of an increasing number of organisms and the development of the microarray technology, which enables transcript levels to be measured across the entire genome, has dramatically accelerated the identification of periodic transcripts. It is now possible to obtain a comprehensive picture of how the transcription profiles of all genes vary across the cell cycle in any population of cells that can be synchronised. In this study, fission yeast cells have been synchronised in two independent ways (whole-culture method/temperature sensitive mutants and selective method/elutriation), and microarray analysis of the fission yeast cell cycle was performed. Microarray data were derived from eight timecourse experiments, using microarrays containing > 99.5% of all known and predicted fission yeast genes. 407 genes (out of the 5119 investigated, corresponding to ~8% of the genome) whose expression is periodic were identified using an autocorrelation algorithm combined with visual inspection of each expression profile. The results were validated independently using a Fourier transform approach. Among these genes, 136 showed changes in the levels of expression bigger than 2-fold and they are referred to as ‘high amplitude’ genes (Appendix VI). The remaining 271 showed changes between 1.5 and 2 fold and they are referred to as ‘low amplitude’ genes.

Periodic genes can be grouped into four clusters resulting in four successive waves of transcription (Fig. 3.2), corresponding to different cell cycle phases: cluster 1 to mitosis (including genes involved in chromosome condensation and segregation, cytokinesis and cell separation), cluster 2 to M/G1 (including genes involved in DNA

replication initiation and cell separation), cluster 3 to S (including histones and other DNA replication genes) and cluster 4 to G2 (including several stress related genes). Clusters 1 to 3 occupy only 30% of the cell cycle, as a result of the very short G1 phase in *S. pombe* (MacNeill S.A. and Nurse P., 1997), and they include the majority of fission yeast genes previously characterised as periodic (Table 3.1). The majority (111/136) of the 'high amplitude' genes peaks around mitosis and G1, which consists of an even shorter cell cycle window (~20% of the entire cycle). Cluster 4 is more weakly regulated and covers the majority of the cycle (~70%), corresponding to a long G2 phase. This differs from *S. cerevisiae* where at least twice as many genes are regulated during the cell cycle and periodic transcription is evenly spread throughout the cycle (Cho R.J. *et al.*, 1998; Spellman P.T. *et al.*, 1998). In budding yeast G1 is longer and G2 shorter compared to fission yeast, in which most of the changes are concentrated around a short G1 and little seems to happen during a lengthy G2 phase.

Periodic transcription is usually regulated by transcription factors that can exert either positive or negative roles on their gene targets. Three major transcription factors or complexes (MBF, forkhead and ace2p) are conserved across the two yeasts (Koch C. and Nasmyth K., 1994; Kumar R. *et al.*, 2000; Ribar B. *et al.*, 1997; Martin-Cuadrado A.B. *et al.*, 2003), but there are evident differences between the transcriptional cascades they are part of, reflecting the different strategies adopted by the two species during evolution.

Two transcription factors, the forkhead-type Sep1p (Ribar B. *et al.*, 1997); (Ribar B. *et al.*, 1999); (Zilahi E. *et al.*, 2000) and Ace2p (Martin-Cuadrado A.B. *et al.*, 2003), are part of a cascade that regulates some of the cluster 1 and 2 members, mainly involved in mitosis and cell division. Sep1p controls expression of cluster 1 members, including *ace2*, which in turn regulates cluster 2 members. A third transcription complex (MBF) is the regulator of a different subset of genes also belonging to cluster 2, mainly involved in DNA replication, and it acts in parallel to and independently of Ace2p. This again is in contrast with what has been observed in budding yeast, where periodic gene expression is driven by eight main transcriptional regulators which are all connected through a cascade cycle of serial regulation (Simon I. *et al.*, 2001) (Fig. 6.1). In budding yeast, pairs of transcription factors such as MBF/SBF and Ace2p/Swi5p have similar and overlapping roles, as a consequence of a duplication event of the entire budding yeast genome that resulted in different degrees of divergence of duplicated gene pairs (Wolfe K.H. and Shields D.C., 1997; Kellis M. *et al.*, 2004). In fission yeast only one transcriptional

activator seems to operate at each stage of the cycle. A recent study has proved that gene duplication events in the two yeasts have affected 56 independent gene families, resulting in the adaptation of the two species to their characteristic life cycles (Hughes A.L. and Friedman R., 2003). Some additional transcription factors may still be missing in the transcriptional regulatory network that governs the fission yeast cell cycle. In budding yeast, the forkhead protein Fkh2p is known to have overlapping roles with another forkhead-type protein, Fkh1p, and interacts with a MADS-box transcription factor, Mcm1p. The fission yeast genome encodes for another forkhead gene, *fkh2* that from the results of this study appears to have a possible negative role in regulating some sep1p-dependent genes. The gene SPBC19G7.06, coding for a MADS-box protein, also appears to have a regulatory role on a subset of genes that contain a forkhead binding motif. An involvement of these genes in regulating periodic gene expression in fission yeast is possible.

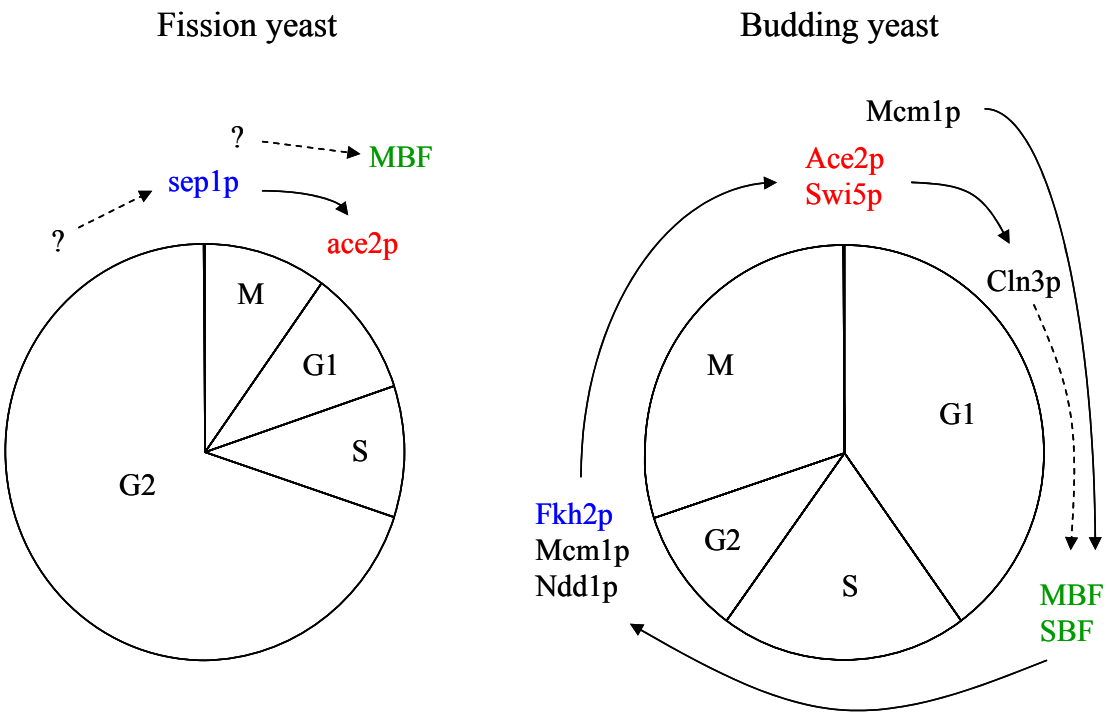


Fig. 6.1 Transcriptional regulation cascade in fission and budding yeast.
Orthologous transcription factor complexes are shown in corresponding colours and approximate cell cycle phases are represented within the cycles. Solid arrows indicate transcriptional regulation, dashed arrows posttranscriptional regulation. Question marks refer to still unidentified posttranscriptional mechanisms which might regulate transcription factors in *S. pombe*.

The part of the cascade from forkhead to ace2p, responsible of regulating M and G1 transcripts, is conserved in the two yeasts. The MBF complex, which operates at G1/S, is also conserved in the two organisms. However, it acts downstream of Ace2p/Swi5p and upstream of forkhead (Fkh2p) in budding yeast (Simon I. *et al.*, 2001; Futcher B., 2002), whereas in fission yeast it acts in parallel with ace2p and independently of forkhead (sep1p). The reason why ace2p and MBF are functioning in fission yeast at the same time is a consequence of the short G1 phase. In fission yeast cell division and initiation of DNA replication are coincident and this is reflected in the overlap of ace2p- and MBF-dependent transcriptional waves.

In *S. pombe*, the transcriptional regulators identified so far are not fully cyclically connected as in *S. cerevisiae*. Around 70% of the entire cycle corresponds to G2 phase were no transcription factors seem to be acting and transcriptional control alone is unlike to be able to bridge such a lengthy phase.

In addition all genes coding for transcriptional regulators in budding yeast are periodically transcribed themselves, peaking ahead of their targets, whereas in fission yeast this appears to be true for *ace2* only. *Cdc10* (component of the MBF complex) also shows a weak periodicity but its peak of expression is coincident with its targets. It is therefore likely that fission yeast has evolved different mechanisms such as post-transcriptional regulation to orchestrate periodic gene expression. A clear example of an *S. pombe* gene whose expression is regulated at the post-transcriptional level is *rum1* (Daga R.R. *et al.*, 2003). *Rum1* mRNA is periodically transcribed and regulated by mRNA stability. Rum1 protein level is also controlled, post-translationally via phosphorylation and ubiquitin-dependent degradation. Certain types of regulation can have advantages compared to others; changes in mRNA half-life are quicker than *de novo* protein synthesis and might be preferred in certain conditions.

The eukaryotic cell cycle is driven by CDK complexes formed by a kinase and its phase-specific cyclin partners (Morgan D.O., 1997). The budding yeast genome encodes at least eight different cyclins that contribute to cell cycle progression and that are regulated at many different levels, including the transcriptional one. The cyclin genes are periodically expressed and the function of the various cyclins coincides with their appearance. In fission yeast, only expression of one cyclin (*cig2*) is strongly cell cycle regulated whereas expression of *cig1* and *cdc13* is weak. This could be the consequence of evolutionary divergence or reflect the fact that *cig1* and *cdc13* regulation might

become more crucial in a particular situation such as changes in the environmental conditions that would cause the cell cycle to slow down until normal conditions are restored. This again underlies the fact that fission yeast might have evolved other mechanisms than transcriptional regulation for controlling cell cycle progression. Posttranslational modifications are good candidates in playing such a crucial role and are well characterised in many cases. Many fission yeast proteins are known to be regulated by phosphorylation and subsequent degradation via ubiquitination or by anaphase promoting complex mediated proteolysis.

Differences in the regulatory mechanisms between the two yeasts are probably linked to the fact that what needs to be preserved in terms of cell cycle regulation is the activity of the gene in itself and this can be achieved regulating it at many different levels. Integration of all different levels of regulation is what ultimately governs the cell cycle machinery and orchestrates cell cycle events, making sure they take place at the right time and in the right order.

6.2 Conservation of periodic transcription across eukaryotes

Comparisons between budding yeast, fission yeast and humans have revealed that conservation of periodic transcription is limited to a restricted core set of genes, around 40 when both yeasts are considered and lower when the comparison is extended to humans. Such a limited level of conservation suggests that transcriptional regulation is not necessarily a universal feature. From the point of view of efficiency, a protein that is needed throughout the entire cycle can be continuously expressed whereas a protein that is needed only once can be synthesised just before being used. Therefore certain genes are expressed only at a particular stage of the cycle when their product is needed. Taking into account the differences in the life cycle of different eukaryotic cells can explain why some genes have lost their periodic behaviour. A more detailed analysis of the individual functions reveals that most of these genes are involved in basic cell cycle processes such as DNA replication, mitosis and cytokinesis (Table 5.2).

Why has this small set of genes maintained a periodic behaviour across eukaryotes? It is possible that periodic transcription of those genes is a limiting step for cell cycle progression. Their regulation might be responsible for ensuring that cell cycle events are taking place in the right order or that the their products are available in large

quantities at a specific stage of the cycle, e.g. the histones during DNA replication. In some cases, it might also be the strategy adopted to ensure that a fresh pool of a given protein is available in order to override previous posttranslational modifications. In conclusion, only a small core-set of genes are universally transcriptionally regulated in eukaryotes and these genes are probably key players in controlling cell cycle progression.

6.3 Cell cycle periodic genes and their behaviour in meiosis

The transcriptional program of meiosis has also been studied in fission yeast using microarrays (Mata J. *et al.*, 2002). Almost half of the genes encoded by the *S. pombe* genome were upregulated at least 2-fold when compared to vegetatively growing cells and 700 genes were induced more than 5-fold.

Are the cell cycle periodic genes also regulated in meiosis? To answer this question the behaviour of the 136 'high amplitude' genes was checked during meiosis. 18 MBF-dependent genes (Table 4.3) were upregulated during premeiotic S-phase, including *cdc18*, *cdc22*, *cdt2*, *cig2*, *rhp51* and *ssb1*, all previously known cdc10p targets. Some genes such as *ams2* and *mik1* show two peaks of expression, one during premeiotic S-phase and a second one during meiotic division. Cdc10p is responsible for the regulation of its targets both during mitotic and meiotic cell cycle (Cunliffe L. *et al.*, 2004). Interestingly, both cdc10p targets and meiotic recombination genes are enriched for MCB elements, but the recombination genes are not regulated during the mitotic cell cycle. This raises the interesting question of how genes carrying the same promoter sequence are distinguished by a transcription factor. Histone genes peak just after the MBF-dependent genes in meiosis as in mitosis.

12 sep1-dependent genes (Table 4.1) were upregulated during the meiotic divisions including *etd1*, *klp5*, *myo3* and *plo1*, all involved in progression through mitosis and cytokinesis. The ace2-dependent genes (Table 4.2) showed a broader spectrum of behaviours: some genes peak at premeiotic S-phase, some others at meiotic division (*cut2, par2, rpc17* and *eng1*) and then immediately decrease whereas some others remain highly expressed.

The 10 genes encoding for the APC components, which are required for progression through mitotic anaphase, were strongly induced during the meiotic divisions

but only *apc1* appeared periodic in mitosis. These genes might have additional functions during meiosis.

6.4 Future work

This work has provided comprehensive gene expression profiles of the fission yeast cell cycle, allowing global identification of genes showing a periodic behaviour.
In order to better understand the mechanisms that govern periodic transcription, future work will include:

- Analysis of the forkhead gene *fkh2*. The main objective of this would be the identification of fkh2p targets and unmasking possible interactions between fkh2p and other transcription factors, most notably sep1p. In budding yeast, Fkh2p interacts with a MADS-box protein and a transcriptional activator. The results presented in Chapter 4 showed (despite the sickness of the strain) that some periodic genes in *fkh2Δ* are highly expressed, suggesting a negative role for this factor in regulating gene transcription.
- Analysis of the MADS-box gene *mbx1*. This is obviously linked to the further characterisation of *fkh2*, as just described. Again the results presented in Chapter 4 suggested a possible role for mbx1p in interacting with forkhead genes, as reported in budding yeast. Further analysis would help clarifying if this interaction between forkhead/MADS-box protein is a conserved mechanism.
- Analysis of cluster 4 regulation. The attempts made so far for the identification of a transcription factor responsible for the regulation of the forth wave of transcription have weakened the possibility of a direct involvement of transcriptional regulators such as atf1p, pcr1p and prr1p (which are part of the stress response cascade) despite the presence in this wave of many stress related genes. Performing a timecourse experiment for each deletion (*atf1Δ*, *pcr1Δ* and *prr1Δ*) would be the only way of excluding their involvement in the regulation of cluster 4 members.
- Chromatin immunoprecipitation (ChIP) combined with DNA microarrays (ChIP-chip). *In vivo* genomic binding sites for a specific transcription factor can be defined by combining chromatin immunoprecipitation and DNA microarrays.

Proteins are crosslinked with formaldehyde to their target sites *in vivo* and the crosslinked DNA subsequently purified by immunoprecipitation using an antibody against the transcription factor. Once the crosslink is reversed, DNA can be amplified, fluorescently labelled and consequently hybridised onto microarrays. For this purpose chromosome tiling arrays that include all intergenic regions as well as all predicted coding sequences in the fission yeast genome would be used. Traditional expression profiling and ChIP-chip experiments are complementary. From expression data it cannot be distinguished if a transcription factor directly regulates its targets or acts indirectly regulating another transcription factor, such in the case of sep1p and ace2p in fission yeast. ChIP-chip instead identifies targets for a specific transcription factor but does not provide any information concerning the type of regulation, if negative, positive or neutral. In addition, when a transcription factor binds to a promoter that is found in between two divergent genes, ChIP-chip would not help in clarifying which of the two genes is under the control of the transcription factor binding to that specific promoter sequence. Combining those two approaches will contribute to improve our understanding of the action of *S. pombe* transcription factors.

- Systematic gene deletion of all 136 open reading frames coding for the 'high amplitude' genes. Comparison with *S. cerevisiae* allowed identification of a core set of about 40 genes which have a conserved periodic behaviour in both yeasts. Many of those genes have a well characterised functions and the effect of their deletion on the cell cycle is already well known. If the systematic deletion was extended to the 136 'high amplitude' genes, this would reveal the degree of importance of each 'high amplitude' gene in regulating cell cycle progression and show the effect of each deletion on the cell cycle.
- The importance of periodic gene expression can also be investigated placing a periodic gene under the control of a constitutive promoter or swapping promoters between periodic genes. Suppressing periodicity or changing the time of expression for a specific gene should reveal the importance of its periodic behaviour.
- Systematic comparison with microarray datasets from other eukaryotic model organisms. This will include comparison of orthologous expression profiles across different genomes and analysing gene regulatory mechanisms in these

organisms. This will allow investigation of the basal gene network responsible for driving the cell cycle in eukaryotes.

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Appendix I: Strains used in this study

Strain	Source
972 h-	Laboratory collection
cdc25-22 h-	Laboratory collection
cdc10-129 h-	Laboratory collection
cdc25-22 sep1Δ::ura4 h-	Matthias Sipiczki
ace2Δ::kanMX6 ade6-M21?h-	This study
ace2Δ::kanMX6 sep1::ura4 ade6-M21? leu1-32 h?	This study
atf1Δ::ura4 ura4-D18 h-	Nic Jones
cdc10-C4 h^+	Paul Nurse
cig1Δ::ura4 cig2Δ::ura4 puc1Δ::ura4	Sergio Moreno
leu1-32 h- pREP3X-fkh2	This study
leu1-32 h- pREP3X-ace2	This study
leu1-32 h- pREP3X-fhl1	This study
leu1-32 h- pREP3X-sep1	This study
mbx1Δ::kanMX6 leu1-32 ura4-D18 ade6-M210 his7-366 h-	Jonathan Miller
meu3Δ::kanMX6 ade6-M21?h-	This study
meu19::kanMX6 ade6-M21?h-	This study
nda3-KM311 h-	Laboratory collection
pcr1Δ::ura4 h-	Nic Jones
prr1Δ::his7 his7? h-	Nic Jones
sep1Δ::ura4 h-	Matthias Sipiczki
SPAC8C9.01Δ::kanMX6 h90 (fhl1)	Laboratory collection
SPAC8C9.01Δ::kanMK6 sep1::ura4 h?	This study
SPBC16G5.15cΔ::ura4 leu1-32 ura4-D18 ade6-M210/216 his7-366 h+/h-	Brian Morgan
SPBC16G5.15cΔ::ura4 leu1-32 ura4-D18 ade6-M21? his7-366 h-	This study

Appendix II: List of buffers, solutions, media and antibiotics

Buffers and stock solutions	Composition
Edinburgh Minimal medium (EMM)	For 1 liter: 3.0 g potassium hydrogen phthalate, 2.2 g Na_2HPO_4, 5.0 g NH_4Cl, 20g D-glucose, 20 ml salts stock (X50), 1 ml vitamin stock (X1000), 0.1 ml mineral stock (X10000)
0.5 M EDTA	For 1 liter: 186.1 g disodium ethylenediaminetetracetate · $2H_2O$
Formal saline	0.9% saline, 3.7% formaldehyde
Freezing mix	YE containing 250 mg of Glutamic Acid, 50% glycerol
Hybridization buffer	5 x SSC, 6 x Denhardt's 60 mM TrisHCl pH 7.6, 0.12% sarkosyl, 48% formamide; filter sterilized
10X LiAc	1M Lithium Acetate, pH 7.5
Luria Bertani medium (LB)	For 1 liter: 10g NaCl, 10 g tryptone, 5 g yeast extract, pH 7.0
Mineral stock (X10000)	For 1 liter: 5.0 g H_3BO_3, 4.0 g $MnSO_4$, 4.0 g $ZnSO_4$ · $7H_2O$, 2.0 g $FeCl_3$ · $6H_2O$, 0.4 g H_2MOO_4 · H_2O, 1.0 g KI, 0.4 g $CuSO_4$ · $5H_2O$, 10 g citric acid
2X Printing buffer	300 mM sodium phosphate, pH 8.5
PEG/LiAc/TE	For 20 ml: 2 ml 10X LiAc, 2 ml 10X TE, filter-sterilized
Phosphate Buffer Saline (PBS)	For 1 liter: 8.0 g NaCl, 0.2 g KH_2PO_4, 1.44 g Na_2HPO_4, 0.2 g KCl, pH 7.4
Salt stock (X50)	For 1 liter: 53.5 g $MgCl_2$ · $6H_2O$, 0.74 g $CaCl_2$ · $2H_20$, 50 g KCl, 20 g Na_2SO_4
SOC medium	For 100 ml: 2.0g Bacto-tryptone, 0.5g Bacto-yeast extract, 1ml 1M NaCl, 0.25ml 1M KCl, 1ml Mg2+ stock (1M MgCl2 . 6H2O, 1M MgSO4 . 7H2O), filter-sterilized, 1ml 2M glucose, filter-sterilized
20X SSC	3 M NaCl, 300 mM Na-citrate, pH 7.0

1X TAE	40 mM Tris-acetate, 1 mM EDTA, pH 7.2
1X TE	10 mM Tris/HCl, pH 8.0, 1 mM EDTA
1X TES	10 mM Tris pH 7.5, 10 mM EDTA pH 8.0, 0.5% SDS
Vitamin stock (1000X)	For 1 liter: 1.0 g Na pantothenate, 10 g nicotinic acid, 10 g inositol, 10 mg biotin
Washing solution 1	2X SSC
Washing solution 2	0.1X SSC, 0.1% SDS
Washing solution 3	0.1X SSC
Yeast Extract (YE)	For 1 liter: 5 g Difco Yeast Extract, 30 g glucose, pH 5.6. Supplements: 250 mg histidine, leucine, adenine, uracil and lysine
Yeast Extract (YE) agar	YE plus 20 l/liter Difco agar

Appendix III: Primers used in this study

PRIMERS for GENE DELETION

Primer name	Sequence
Meu19_F	5’-agttttaacgaccgctacgtagtgtaatgctacaaaataagtctac tcagatattatgcagccgtactcatctgattttgcggatccccgggttaattaa-3’
Meu19_R	5’-gcttactcataatttcttctttaaaaaatcgatgtgacattgtgaatacata aaccgtcaataatctactgctaaaatgcgaattcgagctcgtttaaac-3’
Meu3_F	5’-taacgctaatgtcatacaacaccgtattcgtctgatttgtctacaaaaatgta aacaaacaattattttatcgcattgatcggatccccgggttaattaa-3’
Meu3_R	5’-ctactcattatttcctcattaaaagatcgacttggcactgaaaacaaatgaac cgccgatatcctatattgttacaaagcgaattcgagctcgtttaaac-3’
Ace2_F	5’-atttctctcatcgttctctccttgatttctctacgcattgcactagatactcgcta tcctaagtaaacaagacaatgtcgcggatccccgggttaattaa-3’
Ace2_R	5’-aattgtcacagcattagttcatgtacgatgcttgaatttgaatttatataaaac aaattaaaaacaataatattagtaagaattcgagctcgtttaaac-3’
Fhl1_F	5’-gctttctagtctcattgtgctttcaattggctagaagtttagcttactataaaaaa aggaagtctttgggtcattaaggccggatccccgggttaattaa-3’
Fhl1_R	5’-ccaagttgtgcaatcactgtcaaaaaaagaaaaatgaaacagcttatgcat gctggtaagtgtaattttcggcactaattggaattcgagctcgtttaaac-3’

The underlined sequence represents the sequence homologous to the plasmid multiple cloning site.

PRIMERS for CHECKING GENE DELETIONS

Primer name	Sequence
Ace2_check_R	5’-gttatacacaatactagggtgatg-3’
Meu3_check_R	5’-cgtttcaatattgaaattcttacag-3’
Meu19_check_R	5’-tcgcatctttagattaacatatagg-3’
Kan_check_R	5’-gtcacatcatgcccctgagc-3’

PRIMERS for CLONING - OVEREXPRESSION

Primer name	**Sequence**
C16g5.15 forward	5’-**ctcgag**atgactgttcgcagactcgaaagc-3’
C16g5.15 reverse	5’-gggataagatattaaacaaggtg-3’
C6g10.12c forward	5’-**ctcgag**atgtcgctttcatat-3’
C6g10.12c reverse	5’-tcattagtgctgtctgcgatc-3’
C1142.08 forward	5’-**ctcgag**atgcctgttgcagag-3’
C1142.08 reverse	5’-tcaggtataagaggatgatgtctc-3’
Sep1 forward	5’-**ctcgag**atcttcctcatgaat-3’
Sep1 reverse	5’-ttagaatagtgttgaagtttgac-3’

The sequence in bold corresponds to the *Xho*I restriction site.

Appendix IVa: Time courses experimental conditions

Experiment	Strain	Timepoints	Sample label	Reference	Ref label	Media	Temp (ºC)	Sample OD	Array batch number
Elutriation I	*972 h-*	20	Cy3	Asynchronous *972 h-*	Cy5	EMM	30	0.65	137 & 140
Elutriation II	*972 h-*	20	Cy5	Asynchronous *972 h*	Cy3	EMM	30	0.24	228 & 232
Elutriation III	*972 h-*	20	Cy5	Asynchronous *972 h*	Cy3	EMM	30	0.15	489
cdc25 b&r I	*cdc25-22 h-*	18	Cy3	Asynchronous *cdc25-22 h-*	Cy5	YE	25	0.34	40 & 70
cdc25 b&r I dye swap	*cdc25-22 h-*	18	Cy5	Asynchronous *cdc25-22 h-*	Cy3	YE	25	0.34	40 & 70
cdc25 b&r II	*cdc25-22 h-*	19	Cy5	Asynchronous *cdc25-22 h-*	Cy3	EMM	25	0.19	292
cdc25 elu + b&r	*cdc25-22 h-*	22	Cy5	Asynchronous *cdc25-22 h-*	Cy3	EMM	25	0.22	226, 228 & 232
cdc10 elu + b&r	*cdc10-129 h-*	22	Cy5	Asynchronous *cdc10-129 h-*	Cy3	EMM	25	0.30	331 & 334
cdc10::ura4	*cdc10::ura4 leu1-32 ura4-D18 ade6-M210 + pREP1-HA-res1(1-192)*	3	Cy5	Untreated cdc10::ura4	Cy3	EMM ade	30	0.2	477
cig1Δ cig2Δ puc1Δ elu	*cig1::ura4 cig2::ura4 puc1::ura4*	6	Cy5	Asynchronous *cig1::ura4 cig2::ura4 puc1::ura4 h-*	Cy3	EMM	30	0.15	477
HU b&r	*972 h-*	4	Cy3	Untreated *972 h-*	Cy5	EMM	32	0.08	489
nda3	*nda3-KM311 h-*	3	Cy5	Untreated nda3	Cy3	EMM		0.23	477
sep1 b&r	*cdc25-22 sep1::ura4 h-*	20	Cy5	*cdc25-22 sep1::ura4 h-*	Cy3	EMM	25	0.12	331 & 334
wt b&r	*972 h-*	18	Cy3	*972 h-*	Cy3	EMM	25	0.15	782 & 784

0.5 OD = 1 X 10^7

Appendix IVb: Mutant strains experimental conditions

Experiment	Strain	Sample label	Reference	Ref label	Media	Temp (ºC)	Sample OD	Array batch number
ace2 #1	*ace2Δ::kanMX6 ade6-M21?h-*	Cy5	*972 h-*	Cy3	EMM ade	32	0.22	489-48
ace2 #2		Cy3		Cy5			0.23	489-7
ace2 #3		Cy5		Cy3			0.23	489-14
ace2 #4		Cy5		Cy3			0.24	489-12
ace2 #5		Cy5		Cy3			0.33	668-2
ace2 sep1 #1	*ace2Δ::kanMX6 sep1::ura4 ade6-M21? leu1-32*	Cy5	*972 h-*	Cy3	EMM ura ade leu	30	0.26	668-9
ace2 sep1 #2		Cy5		Cy3			0.32	668-10
atf1 #1	*atf1Δ::ura4 ura4-D18 h-*	Cy5	*972 h-*	Cy3	YE	25	0.18	596-21
atf1 #2		Cy5		Cy3			0.21	599-2
cdc10-C4 #1	*cdc10-C4 leu1-32 h+*	Cy5	*972 h^+*	Cy3	EMM	25	0.23	489-10
cdc10-C4 #2		Cy5		Cy3			0.24	489-11
cdc10-C4 #3		Cy3		Cy5			0.23	334-19
cdc10-C4 #4		Cy5		Cy3			0.24	334-17
fhl1 #1	*SPAC8C9.01Δ::kanMX6 h90*	Cy3	*972 h-*	Cy5	EMM	25	0.23	334-22
fhl1 #2		Cy5		Cy3			0.23	334-11
fhl1 #3		Cy5		Cy3			0.22	668-41
fkh2 #1	*SPBC16G5.15c::ura4 leu1-32 ura4-D18 ade6-M21? his7-366 h-*	Cy5	*972 h-*	Cy3	YE	30	0.20	560-42
fkh2 #2		Cy5		Cy3			0.20	560-45
mbx1 #1	*mbx1::kanMX6 leu1-32 ura4-D18 ade6-M210 his7-366 h-*	Cy5	*972 h-*	Cy3	EMM leu ura ade his	25	0.23	334-18
mbx1 #2		Cy3		Cy5			0.22	334-20

Experiment	Strain	Sample label	Reference	Ref label	Media	Temp (ºC)	Sample OD	Array batch number
meu3 #1	*meu3::kanMX6 ade6-M21?h-*	Cy5	*972 h-*	Cy3	EMM ade	32	0.23	489-49
meu3 #2		Cy3		Cy5			0.23	489-8
meu19 #1	*meu19::kanMX6 ade6-M21?h-*	Cy5	*972 h-*	Cy3	EMM ade	32	0.22	489-50
meu19 #2		Cy3		Cy5			0.25	489-9
pcr1 #1	*pcr1::ura4 h-*	Cy5	*972 h-*	Cy3	YE	25	0.24	489-17
pcr1 #2		Cy5		Cy3			0.21	560-46
prr1 #1	*prr1Δ::his7 his7? h-*	Cy3	*972 h-*	Cy5	EMM	25	0.23	334-21
prr1 #2		Cy5		Cy3			0.22	334-12
sep1 #1	*sep1Δ h-*	Cy5	*972 h-*	Cy3	EMM	25	0.24	489-13
sep1 #2		Cy3		Cy5			0.25	334-23
sep1 #3		Cy5		Cy3			0.24	334-14
sep1 #4		Cy5		Cy3			0.23	668-5
fhl1 sep1 #1	*SPAC8C9.01Δ::kanMK6 sep1::ura4 ade6-M21? leu1-32 h-*	Cy5	*972 h-*	Cy3	EMM ura ade leu	30	0.23	668-39
fhl1 sep1 #1		Cy5		Cy3			0.23	668-27
ace2 OE #1	*leu1-32 h- pREP3X-ace2*	Cy5	*leu1-32 h- pREP3X*	Cy3	EMM, 15 µM thiamine	32	0.06	596-3
ace2 OE #2		Cy5		Cy3			0.12	560-31
fhl1 OE #1	*leu1-32 h- pREP3X-fhl1*	Cy5		Cy3		32	0.03	596-4
fhl1 OE #2		Cy5		Cy3			0.08	560-47
fkh2 OE #1	*leu1-32 h- pREP3X-fkh2*	Cy5		Cy3		32	0.07	596-20
fkh2 OE #2		Cy5		Cy3			0.04	596-10
sep1 OE #1	*leu1-32 h- pREP3X-sep1*	Cy5		Cy3		32	0.23	596-22
sep1 OE #2		Cy5		Cy3			0.17	596-19

0.5 OD = 1 X 10^7

Appendix V: Additional measurements defining cell cycle synchrony in timecourse experiments and additional clustering

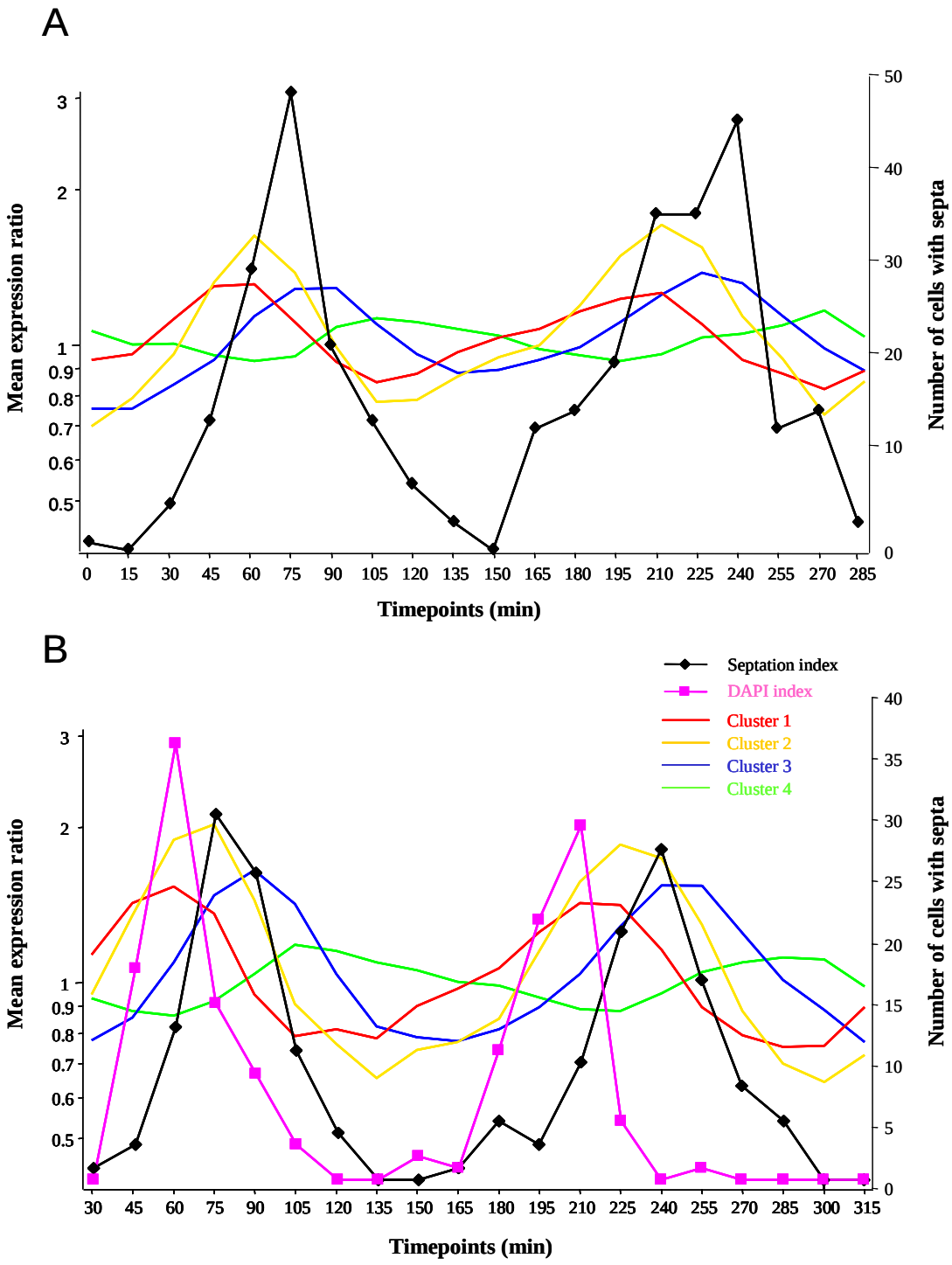

Fig. V.1 Additional measurements for elutriation experiments.

Panel A and B refer to two independent biological experiments, elutriations 1809 and 1012 respectively. For each graph, septation index and average expression profiles for the four clusters are shown. In panel b the DAPI index is also shown

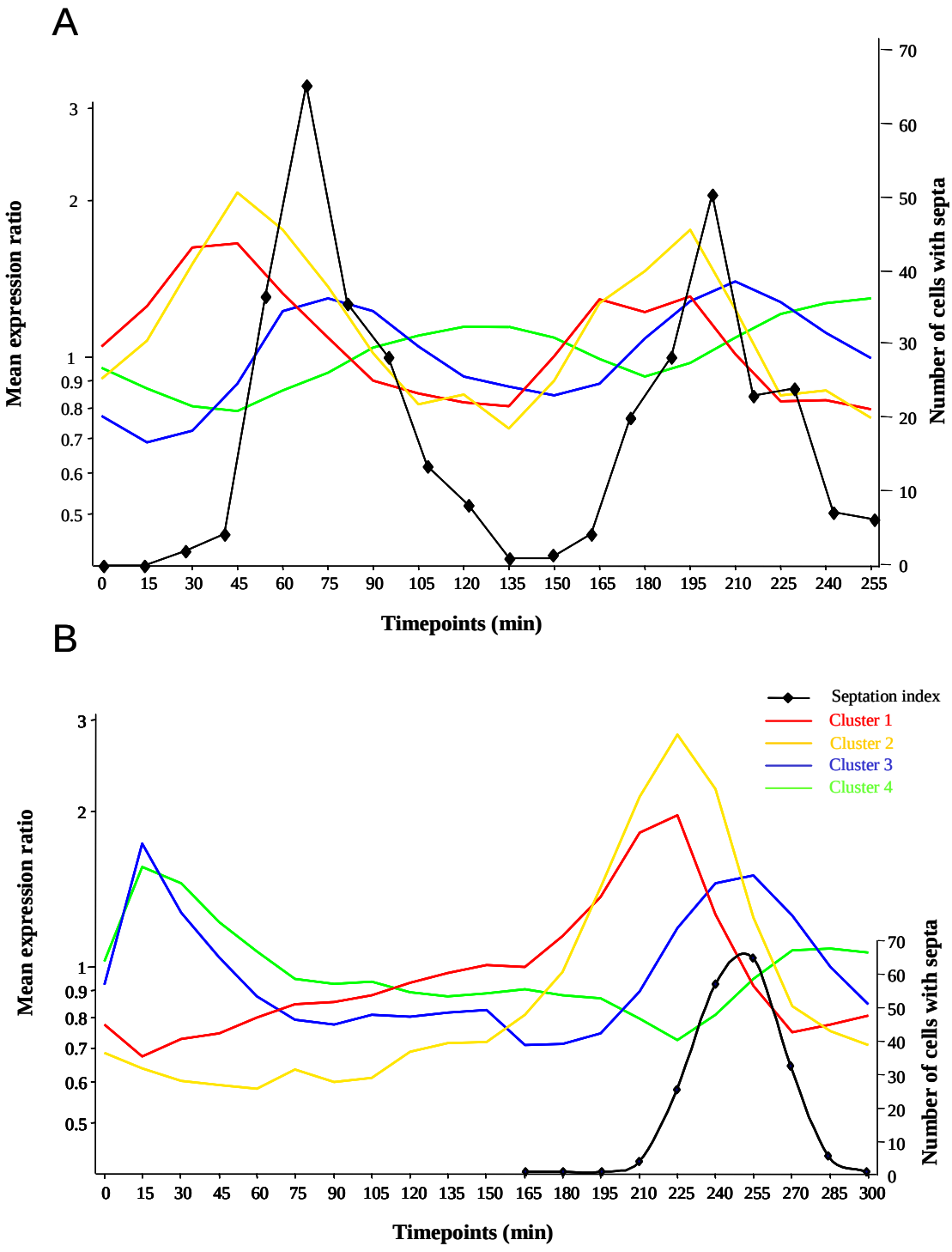

Fig. V.2 Additional measurements for *cdc25* experiments.

Panel A refers to a *cdc25* 'block and release' experiment (1601), panel B refers to a *cdc25* elutriation + 'block and release' (1402). For each graph, septation index and average expression profiles for the four clusters are shown.

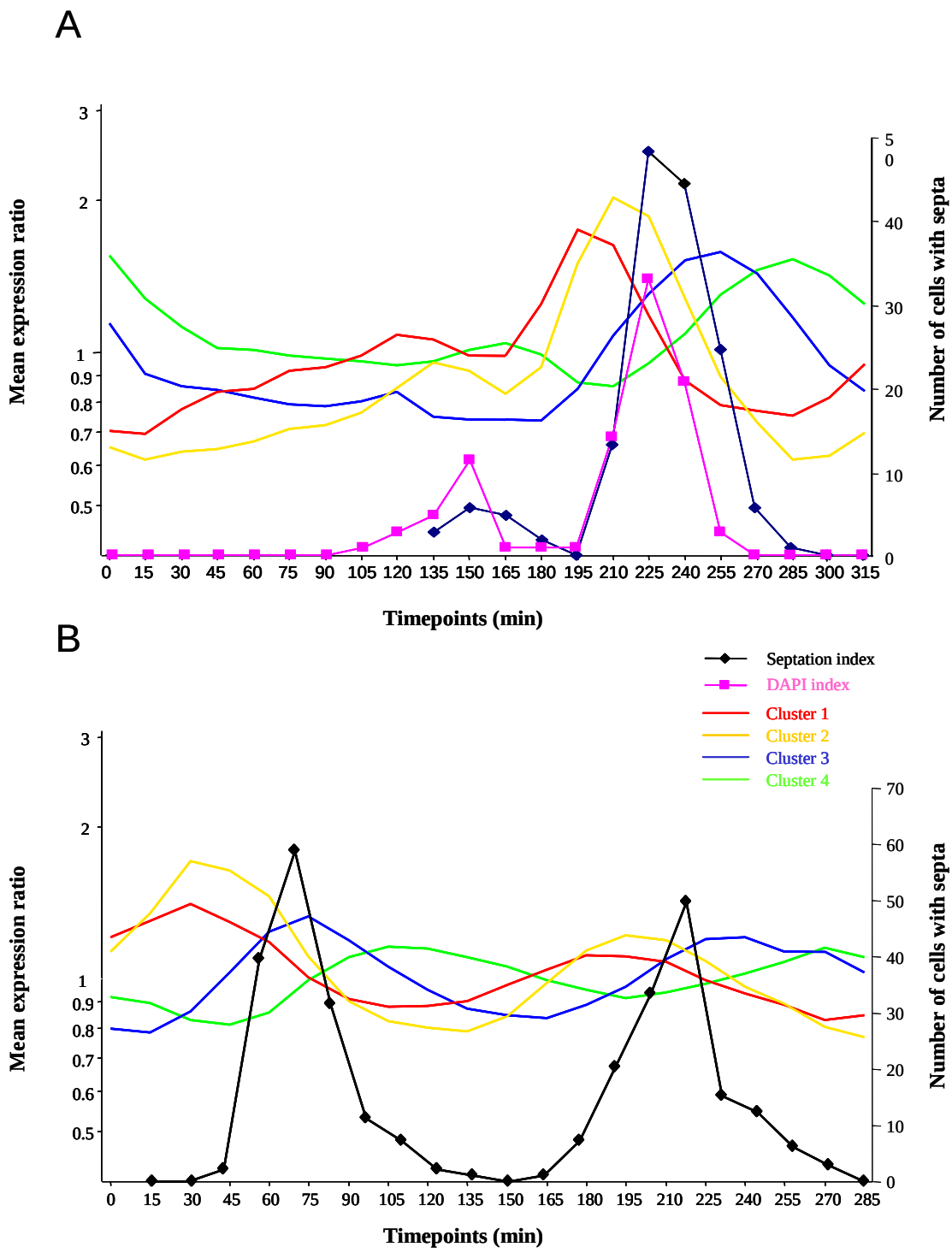

Fig. V.3 Additional measurements for *cdc25* and *cdc10* experiments.

Panel A refers to a *sep1Δ cdc25* 'block and release' experiment (2009), panel B refers to a *cdc10* elutriation + 'block and release' (509). For each graph, septation index and average expression profiles for the four clusters are shown.

Wild type elutriation

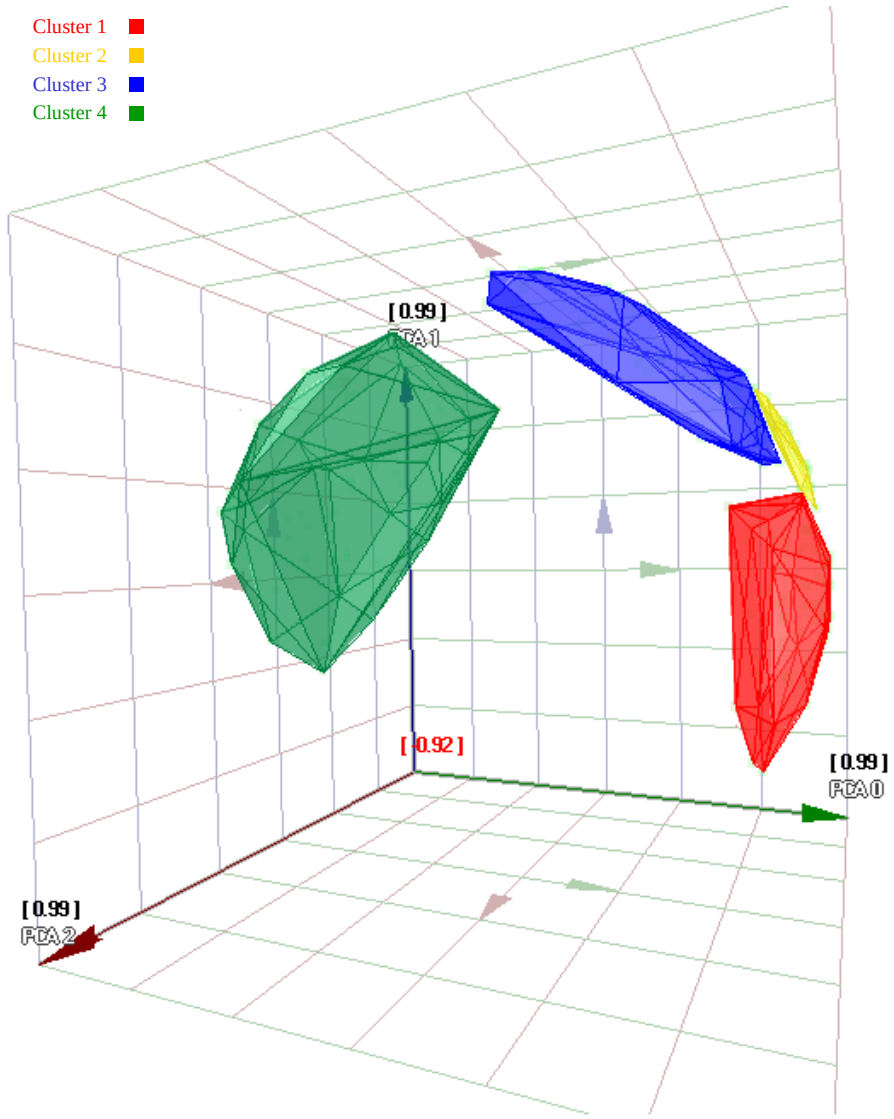

Fig. V.4 3-dimensional representation of the four clusters of cell cycle regulated genes for an elutriation experiment.

Each coloured area represents a cluster of genes. Classification is shown for one elutriation experiment (2201). This graph was obtained using Principal Component Analysis (PCA) in Arrayminer.

cdc25 ‘block and release’

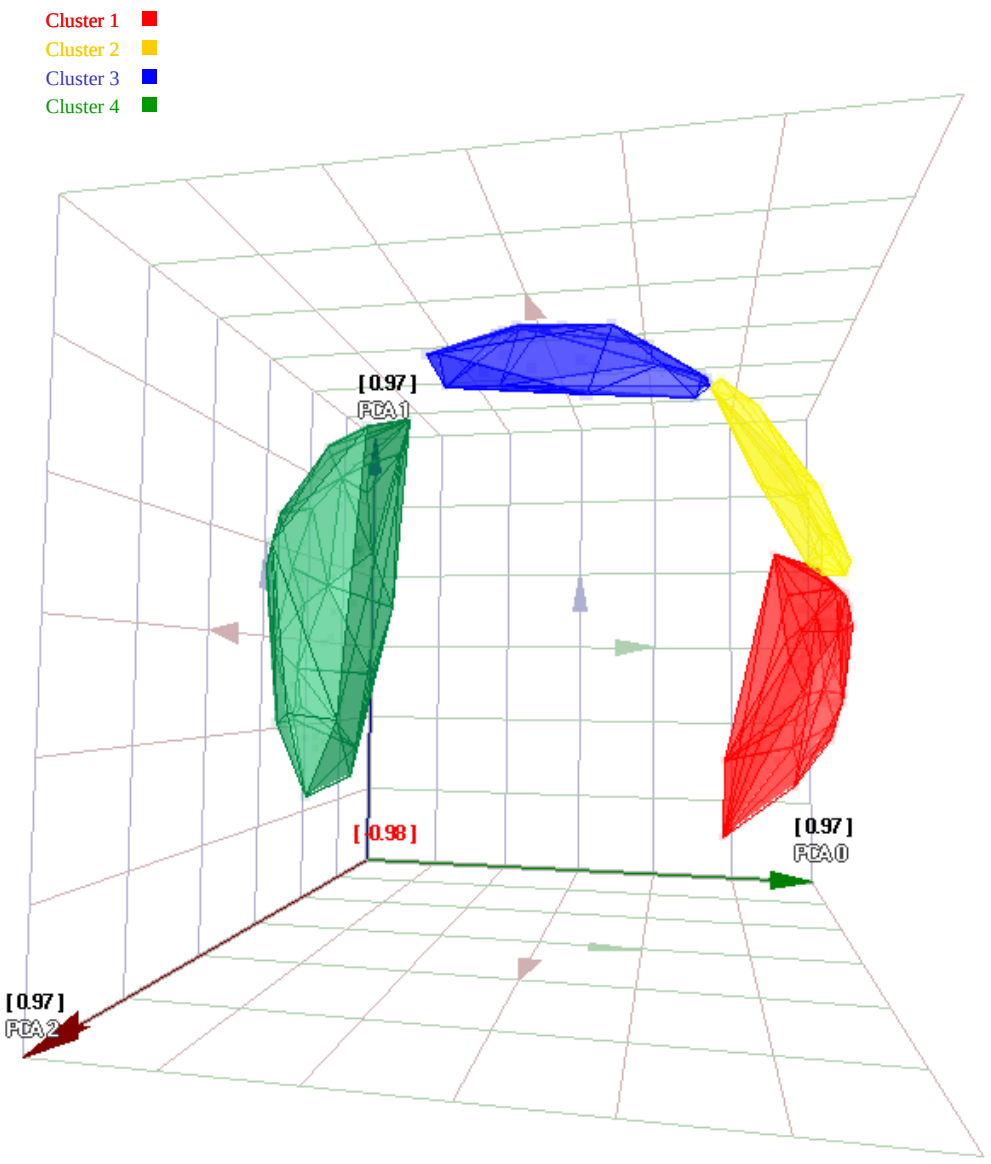

Fig. V.5 3-dimensional representation of the four clusters of cell cycle regulated genes for a *cdc25* ‘block and release’ experiment.

Each coloured area represents a cluster of genes. Classification is shown for one *cdc25* ‘block and release’ experiment (2001). This graph was obtained using Principal Component Analysis (PCA) in Arrayminer.

Appendix VI: List of 407 genes periodically expressed during the cell cycle

Biological names	Systematic names	Gene description [a]	Cluster	Motifs
ste7	SPAC23E2.03c	Protein required for mating and meiosis	1	FLEX
sso1; psy1	SPCC825.03c	Syntaxin-like component of the plasma membrane docking/fusion complex	1	FLEX
slp1	SPAC821.08c	WD-domain protein of the spindle defect checkpoint and APC activator	1	FLEX
sid2; pld5	SPAC24B11.11c	Protein kinase involved in regulation of cytokinesis	1	FLEX
ark1; sex1	SPCC330.16; SPCC320.13c	Aurora kinase involved in regulation of mitosis	1	
rum1	SPBC32F12.09	Inhibitor of the Cdc2p cyclin-dependent kinase complex	1	FLEX, Novel 2
rhp51; rad51	SPAC644.14C	Required for DNA repair and meiotic recombination	1	MCB 1
plo1	SPAC23C11.16	Polo kinase involved in regulation of mitosis and cytokinesis	1	FLEX, Novel 3
myo3; myp2	SPAC4A8.05c	Myosin-3 isoform, heavy chain (Type II myosin)	1	
mus81	SPCC4G3.05c	Holliday junction resolvase subunit	1	
msh6	SPCC285.16c	Protein involved in mismatch repair (mutS family)	1	FLEX
mob1	SPBC428.13c	Protein involved in regulation of cytokinesis	1	FLEX, MCB 1, Novel 2
meu29	SPAC25H1.05	Unknown function	1	Ace2, FLEX
meu16		Non-coding RNA	1	
klp6	SPBC1685.15c; SPBC649.01c	Kinesin motor protein; KIP3 subfamily	1	FLEX
klp5	SPBC2F12.13	Kinesin motor protein; KIP3 subfamily	1	FLEX, MCB 1
imp2	SPAC13F4.08c; SPBC11C11.02	Protein required for medial ring disassembly after cytokinesis	1	FLEX, MCB 1, Novel 1
etd1	SPAC1006.08	Protein required for cytokinesis	1	FLEX, MCB 1
chs2	SPBC1734.17; SPBC1709.01	Member of chitin synthase family, involved in cell wall maintenance	1	MCB 1
cdc20; pol2	SPBC25H2.13c	DNA polymerase epsilon catalytic subunit	1	FLEX, MCB 2
cdc15; rng1	SPAC20G8.05c	Protein involved in cytokinesis	1	FLEX
bet1	SPAC23C4.13	Member of SNARE domain containing family	1	FLEX
apc15; apc16	SPBC83.04	Component of APC/cyclosome complex	1	FLEX

	SPCC757.12	**Protein containing an alpha amylase N-terminal catalytic domain**	**1**	**Novel 2**
ace2	**SPAC6G10.12c**	**Zinc finger transcription factor**	**1**	**FLEX**
	SPAC5D6.02c	**Unknown function**	**1**	
	SPCC576.02	**Member of aspartate and glutamate racemases family**	**1**	**FLEX**
	SPAC30D11.01c; SPAC56F8.01	**Member of glycosyl hydrolases family 31, involved in carbohydrate metabolism**	**1**	
	SPBC4F6.12	**LIM domain protein, low similarity to paxillin focal adhesion protein that regulates integrin or growth factor-mediated responses**	**1**	
wis3; spo12	**SPAC3F10.15c**	**Protein likely to play role in regulating cell cycle progression, possibly at G2 to M phase transition**	**1**	**FLEX, Novel 1**
	SPBC27.05	**Unknown function**	**1**	
	SPAC23H4.19; SPAC1705.03c	**Putative cell wall biogenesis protein**	**1**	**FLEX, Novel 1, Novel 2**
	SPBC19G7.04	**Unknown function, possible transcriptional regulator, may contain HMG box**	**1**	**FLEX**
	SPAC19B12.02c	**Protein with high similarity to 1,3-beta-glucanosyltransferase, member of glycolipid anchored surface protein (GAS1) family**	**1**	**FLEX**
	SPBC16G5.15c	**Fork head protein type transcription factor**	**1**	**FLEX**
rho4	**SPAC16A10.04**	**Rho protein involved in regulation of cytoskeleton, cytokinesis, and cell wall integrity**	**1**	**FLEX, Novel 1**
mde6	**SPAC15A10.10**	**Protein likely to play a role in meiosis or sporulation, requires Mei4p for transcriptional activation**	**1**	**FLEX**
	SPAC15A10.09c	**Unknown function**	**1**	**FLEX, MCB 1, Novel 1**
mac1	**SPAC13G7.04c**	**Transmembrane protein involved in cell separation**	**1**	**FLEX, Novel 2**
	SPAC11E3.13c	**Member of glycolipid anchored surface protein (GAS1) family, possible involvement in cell wall maintenance**	**1**	**MCB 1**
	SPBC1198.07c	**Putative glycosylphosphatidylinositol (GPI)-anchored protein involved in cell wall biosynthesis**	**1**	**FLEX**
top1	SPBC1703.14c	DNA topoisomerase I, involved in chromatin organisation	1	FLEX, Novel 1
spn2	SPAC821.06	Septin homolog, involved in cell separation	1	
sad1; sta1	SPBC16H5.01c; SPBC12D12.01	Spindle pole body associated protein	1	

psc3	SPAC17H9.20; SPAC607.01	Cohesin complex component, required for sister chromatid cohesion and normal mitosis	1	MCB 1
	SPBC32H8.09	Protein containing WD domain G-beta repeat	1	
ndk1	SPAC806.07	Nucleoside diphosphate kinase	1	
dis1	SPCC736.14	Microtubule-associated protein required for chromosome segregation (functions with Klp5p and Klp6p in kinetochore-spindle attachment)	1	
csk1	SPAC1D4.06c	Cyclin-dependent kinase activating kinase (CAK) involved in activating Cdc2p (activity partially redundant with Mcs6p-Mcs2p complex)	1	
crk1; mcs6; mop1	SPBC19F8.07	Cyclin-dependent kinase activating kinase (CAK) involved in activating Cdc2p kinase, putative transcription initiation factor TFIIH subunit	1	
cid13	SPAC821.04c	Cytoplasmic poly(A) polymerase involved in regulation of ribonucleotide reductase (suc22) mRNA, TRF family of nucleotidyltransferases	1	Novel 1
cdr1; nim1	SPAC644.06c	Protein kinase involved in regulation of mitosis	1	
cdc25; sal2	SPAC24H6.05	Tyrosine phosphatase that activates Cdc2p kinase, involved in G2/M transition and DNA damage checkpoints	1	
cdc13	SPAC19G10.09C; SPBC582.03	Cyclin that promotes entry into mitosis from G2 phase, forms complex with Cdc2	1	
aph1	SPCC4G3.02	Diadenosine tetraphosphatase, catalyzes hydrolysis of dinucleoside polyphosphate compounds	1	
	SPBPB2B2.09c	Member of the ketopantoate reductase PanE or ApbA family, involved in thiamine biosynthesis	1	
rps602; rps6	SPAPB1E7.12	Protein with high similarity to ribosomal S6 proteins	1	
	SPAC824.04	Protein containing three WD domains (WD-40 repeat)	1	
	SPAP8A3.11c	Member of GTP1 or OBG family of GTP-binding proteins, similarity to developmentally regulated protein with possible role in neurogenesis	1	
	SPAP27G11.08c	Unknown function	1	FLEX
	SPAC9.11	Unknown function	1	
	SPBC646.06c	Member of glycosyl hydrolase family 71, putative glucanase	1	FLEX, Novel 2
	SPAC589.09	Protein containing a CRAL-TRIO domain, putative	1	

		phosphatidylinositol metabolism		
	SPAC589.02c	Component of mediator subcomplex that may function in negative regulation of transcription	1	
	SPCC4G3.06c	Unknown function, possible mitochondrial ribosomal protein of large subunit	1	FLEX
	SPBC4F6.11c	Unknown function	1	MCB 1
	SPBC4F6.05c	Member of legume-like lectin family	1	
	SPBC428.12c	Putative RNA binding protein	1	
	SPBC1306.02; SPBC4.08	Protein containing eight WD domains (WD-40 repeat), possible role in activated transcription by RNA polymerase II	1	FLEX
	SPBC3H7.13	Member of forkhead associated (FHA) domain family, similarity to S. pombe spindle checkpoint protein Dma1p	1	
pmc2	SPAC2F7.04	Mediator complex component, involved in regulating RNA polymerase II activity	1	
	SPBC27B12.06	Protein with possible role in glycosylphosphatidylinositol biosynthesis	1	FLEX
	SPBC26H8.13c	Unknown function	1	
	SPAC24H6.01c; SPAPB21F2.01	Unknown function, similarity to putative glycerol transporter involved in phospholipid biosynthesis	1	FLEX, MCB 1
	SPAC24C9.05c	Unknown function	1	
	SPAC23G3.04	Unknown function	1	Novel 3
spn7; mde8	SPBC21.08c; SPBC19F8.01c	Septin homolog, involved in cell separation	1	FLEX
	SPBC19C2.10	Protein containing an Src homology 3 (SH3) domain, putative actin binding	1	
spp2; pri2	SPBC17D11.06	DNA primase, large (non-catalytic) subunit	1	
	SPBC1709.13c	Member of SET domain containing family	1	
	SPAC1687.19c	Probable tRNA-guanine transglycosylase	1	FLEX
	SPAC1687.17c	Member of the Der1-like family, putative transmembrane protein with signal peptide	1	FLEX
	SPAC1687.10	Unknown function	1	
	SPBC1685.03	Member of signal peptidases type I family, which cleave signal peptides from secreted proteins	1	

	SPAC13G7.10	Protein with two Myb-like DNA-binding domains	1	FLEX
	SPAC13G6.03	Member of type I phosphodiesterase or nucleotide pyrophosphatase family	1	
	SPAC13C5.05c	Member of phosphoglucomutase or phosphomannomutase C-terminal domain containing family	1	
pds5	SPAC110.02	Protein required for maintenance of sister chromatid cohesion	1	MCB 1
mik1	**SPBC660.14**	**Protein kinase that inhibits Cdc2p kinase**	**2**	**FLEX, MCB 1, MCB 2**
pol1; swi7	**SPAC3H5.06c**	**DNA polymerase alpha catalytic subunit**	**2**	**FLEX, Histone**
	SPAC644.05c	**Protein similar to dUTP pyrophosphatase, which maintains dUTP at low levels to prevent misincorporation into DNA**	**2**	**MCB 1, MCB 2**
	SPAC1071.09c	**Protein containing a DnaJ domain, which mediates interaction with heat shock proteins**	**2**	
	SPBC1289.01c; SPBC1539.11c	**Unknown function, putative involvement in chitin biosynthesis**	**2**	**MCB 1**
	SPCC1322.04	**Putative UTP-glucose-1-phosphate uridylyltransferase**	**2**	**FLEX**
	SPCC1322.10	**Unknown function, similar to cell-surface proteins and proteoglycans**	**2**	**FLEX**
	SPAC14C4.09	**Unknown function, putative glucanase**	**2**	
	SPBC16A3.07c	**Unknown function**	**2**	**MCB 1, MCB 2**
	SPBC1709.12	**Unknown function**	**2**	**Ace2**
	SPAC17H9.18c	**Unknown function**	**2**	**FLEX**
	SPCC74.07c; SPCC18.01c	**Member of SUN family, contains predicted N-terminal signal sequence**	**2**	**Ace2, FLEX**
	SPCC18.02	**Protein with similarity to synaptic vesicle-associated acetylcholine transporter**	**2**	
	SPAC23A1.01c; SPAC19G12.16c	**Unknown function, similarity to podocalyxin like, a transmembrane sialomucin important for lymphocyte adhesion and homing**	**2**	**Ace2, FLEX, MCB 1**
	SPBC21B10.13c; SPAC21B10.13c	**Homeobox domain (homeodomain) protein, putative transcription factor**	**2**	
	SPAC23H4.01c; SPAP27G11.01	**Unknown function, contains a pleckstrin homology (PH) domain**	**2**	**Ace2, FLEX**
	SPBC27.04	**Unknown function**	**2**	**Novel 1**
ams2	**SPCC4F11.01;**	**Protein that binds binds chromatin at centromere and is**	**2**	**MCB 1**

	SPCC290.04	**involved in chromosome segregation**		
	SPBC2A9.07c	**Unknown function**	**2**	
	SPAC2E1P5.03	**Protein containing a DnaJ domain, which mediates interaction with heat shock proteins**	**2**	**Ace2, MCB 1**
	SPBC31F10.17c	**Unknown function**	**2**	**FLEX, Novel 1**
	SPBC32F12.10	**Protein with phosphoglucomutase or phosphomannomutase C-terminal domain**	**2**	**FLEX**
	SPBC3E7.12c	**Unknown function, possible role in regulation of chitin synthase**	**2**	**Ace2, MCB 1**
rgf3	**SPCC645.06c**	**Protein containing a pleckstrin homology (PH) and a RhoGEF (GTPase exchange factor) domain**	**2**	
	SPBC651.04	**Unknown function**	**2**	**FLEX**
mrc1; huc1	**SPAC694.06c**	**Protein required for DNA replication checkpoint**	**2**	**MCB 1, MCB 2**
	SPBC83.18c	**Protein containing a C2 domain, which may be involved in calcium-dependent phospholipid binding**	**2**	**Ace2**
	SPCC965.14c	**Member of cytidine and deoxycytidylate deaminase zinc-binding region family**	**2**	**FLEX**
cdc18	**SPBC14C8.07c**	**Protein that couples cell cycle signals to DNA replication machinery and induces replication**	**2**	**MCB 1, MCB 2**
cdc22	**SPAC1F7.05**	**Ribonucleoside-diphosphate reductase large chain, likely required for initiation of DNA replication**	**2**	**MCB 1, MCB 2**
cdm1	**SPBC12D12.02c**	**DNA polymerase delta subunit**	**2**	**FLEX, Novel 2**
cdt1	**SPBC428.18**	**Protein that coordinates completion of S phase with onset of mitosis**	**2**	**MCB 1, MCB 2**
cdt2	**SPAC17H9.19c**	**Protein required for DNA replication**	**2**	**MCB 1, MCB 2**
cig2; cyc17	**SPAPB2B4.03**	**Major G1/S-phase cyclin, promotes onset of S phase**	**2**	**FLEX**
cut2	**SPBC1815.02c; SPBC14C8.01c**	**Securin; required for sister chromatid separation**	**2**	**Ace2, FLEX**
dfp1; him1; rad35	**SPCC550.13**	**Regulatory subunit of the Hsk1p-Dfp1p kinase copmlex involved in S phase initiation**	**2**	**FLEX, MCB 1**
eng1	**SPAC821.09**	**Endo-beta-1,3-glucanase required for cell separation**	**2**	**FLEX**
exg1	**SPBC1105.05**	**Putative exo-beta-1,3-glucanase**	**2**	**Novel 2**
fin1	**SPAC19E9.02**	**NimA family kinase; regulates spindle formation and recruitment of Plo1p to SPB, promotes chromatin condensation**	**2**	**FLEX**

klp8	**SPAC144.14**	**Protein containing a kinesin motor domain**	**2**	
meu19		**Non-coding RNA**	**2**	
	SPAP14E8.02	**Unknown function**	**2**	**MCB 1, MCB 2**
par2; pbp2	**SPAC6F12.12**	**Protein phosphatase PP2A, B' regulatory subunit, required for cytokinesis, morphogenesis, and stress tolerance**	**2**	**Ace2**
rep2	**SPBC2F12.11c**	**Zinc finger transcriptional activator, MBF transcriptional complex**	**2**	
rpc17	**SPAPB1E7.10**	**Unknown function**	**2**	
	SPBPB2B2.13	**Protein similar to galactokinase, which catalyzes first step in galactose metabolism**	**2**	
	SPAPJ760.03c	**Unknown function**	**2**	**Ace2, FLEX**
mid2	**SPAPYUG7.03c**	**Protein required for septin function and stability during cytokinesis**	**2**	**Ace2, MCB 1**
rad21	**SPCC338.17c**	**Cohesin complex subunit, double-strand-break repair protein**	**2**	
rph1; pfh1; pif1	**SPBC887.14c**	**ATP-dependent DNA helicase involved in telomere maintenance, DNA replication, and DNA repair**	**2**	
psm3; smc3	**SPAC10F6.09c**	**Cohesin complex subunit, involved in sister chromatid cohesion and progression through mitosis**	**2**	**MCB 2**
ste9; srw1	**SPAC144.13c**	**Protein required for mating and sporulation, may regulate anaphase promoting complex**	**2**	
ssb1; rad11	**SPBC660.13c**	**Single-stranded DNA-binding protein subunit, required for DNA replication**	**2**	**MCB 1, MCB 2, Novel 1**
ulp1	**SPBC19G7.09**	**Ubl2p-specific protease**	**2**	**FLEX, MCB 1**
	SPAC343.20	**Unknown function**	**2**	
	SPCC63.13	**Protein containing a DnaJ domain, which mediates interaction with heat shock proteins**	**2**	
	SPBC839.02; SPBC24E9.02	**Unknown function, contains an N-terminal arrestin (or S-antigen) domain, possibly fungal specific**	**2**	
cdc4	SPAP8A3.08	EF-hand component of actomyosin contractile ring, required for cytokinesis	2	Ace2, Novel 3
cdc10	SPBC336.12c	Component of MBF transcriptional activation complex involved in control of START	2	
bgs4; cwg1	SPCC1840.02c	Putative 1,3-beta-glucan synthase component, cell wall synthesis	2	
	SPAC11E3.10	Unknown function, member of VanZ-like family	2	

	SPAC22F8.04	Unknown function	2	MCB 1
	SPAC22G7.02	Unknown function	2	Ace2, Novel 3
	SPAC27D7.12c	Unknown function	2	MCB 1
	SPBC2A9.13	Unknown function	2	Ace2
	SPAC2F7.14c	Protein similar to 3'-5' exoribonuclease required for 3' processing of ribosomal 5.8S rRNA and component of 3'-5' exosome complex	2	MCB 1
	SPBC32C12.03c	Protein with protein kinase domain, similar to S. pombe Kin1p, a putative serine-threonine protein kinase involved in regulating cell polarity	2	FLEX, MCB 2
	SPCC4F11.03c	Unknown function	2	MCB 1
	SPAC4H3.11c	Unknown function	2	Histone
	SPCC553.12c; SPCC794.13	Unknown functionl	2	MCB 1
	SPCC794.15	Unknown function	2	
	SPBC9B6.07; SPAC9B6.07	Unknown function, possible role in maturation of 25S rRNA	2	
chs5	SPAC6G9.12	Protein with fibronectin domain involved in cell surface binding, and BRCT domain found in checkpoint proteins, similar to chitin synthase	2	FLEX
cnp1; sim2	SPBC1105.17	CENP-A-like protein, histone H3 variant specific to inner centromeres and required for chromosome segregation	2	FLEX, MCB 2
eso1; eco1	SPBC16A3.11	DNA polymerase eta, involved in sister chromatid cohesion	2	FLEX, MCB 1
	SPBPB21E7.10; SPAPB21E7.10	Unknown function	2	
pob1	SPBC1289.04c	Protein required for cell polarity and cell separation	2	Ace2
	SPCC1795.10c	**Unknown function**	**3**	**Ace2**
	SPBC17G9.06c	**Unknown function**	**3**	**Ace2**
	SPBC19C7.04c	**Unknown function**	**3**	
	SPCC338.12	**Unknown function**	**3**	**Ace2, MCB 1, Novel 3**
hht1	**SPAC1834.04**	**Histone H3.1**	**3**	**Histone, MCB 1**
hht2	**SPBC8D2.04**	**Histone H3.2**	**3**	**Histone**
hht3; clo5	**SPBC1105.11c**	**Histone H3.3**	**3**	**Histone**
hhf1; ams1	**SPAC1834.03c**	**Histone H4.1**	**3**	**Histone, MCB 1**

hhf2; ams3	**SPBC8D2.03c**	**Protein similar to histone H4.1, contains a core histone domain**	**3**	**Histone**
hhf3; ams4	**SPBC1105.12**	**Histone 4.3**	**3**	**Histone**
hta1	**SPCC622.08c**	**Histone H2A-alpha**	**3**	**Histone, MCB 1**
hta2	**SPAC19G12.06c**	**Histone H2A-beta**	**3**	**Histone**
htb1	**SPCC622.09**	**Histone H2B-alpha**	**3**	**Histone, MCB 1**
pht1	**SPBC11B10.10c**	**Histone H2A variant**	**3**	
	SPBPJ4664.02	**Unknown function, possible cell surface glycoprotein**	**3**	
prl36		**Non coding RNA**	**3**	
sap1	**SPCC1672.02c**	**DNA-binding protein required for growth and mating type switching**	**3**	
sod2	**SPAC977.10**	**Sodium/proton antiporter**	**3**	
	SPAC1F7.03	Unknown function	3	
	SPCC306.08c	Malate dehydrogenase, mitochondrial precursor	3	
	SPAC1142.02c; SPAC17G6.19c	Unknown function, contains three TPR domains	3	FLEX
	SPBC1105.14	Protein containing two zinc finger DNA binding domains, possible role in proteasome regulation	3	
	SPAC12G12.07c	Unknown function, domain similar to integrin-a cytoplasmic region	3	
	SPBC1348.10c; SPAC1348.10c	Member of lysophospholipase catalytic domain family, putative lysophospholipase precursor	3	MCB 1
	SPAC806.09c; SPAC1639.01c	SUR4 family protein, putative fatty acid elongation protein	3	Ace2
	SPBC16G5.05c	Protein containing MSP domain, possible type II integral ER membrane protein involved in inositol regulation	3	
	SPCC1906.01	Mannose-1-phosphate guanyltransferase	3	
	SPBC19G7.16	Member of IWS1 C terminus containing family, possible roles in in transcription regulation and transcription elongation	3	Novel 3
	SPBC21B10.09; SPAC21B10.09	Protein similar to acetyl-CoA transporter	3	
	SPAC22G7.01c; SPAPJ696.03c	Protein containing metallopeptidase family M24 domain, similar to x-prolyl aminopeptidase	3	
	SPBPJ758.01; SPBC23E6.01c	Protein with RNA recognition motifs, similar to U1 snRNA-associated protein that suppresses splicing defects and mediates	3	

		recombination		
	SPBC28F2.11	Protein with a high mobility HMG-box domain	3	
	SPBC31F10.16	Unknown function	3	Ace2
	SPBC337.20; SPBC1734.04	Protein similar to cis Golgi protein, putative involvement in protein glycosylation in the golgi	3	MCB 1
	SPAC343.13	Member of PET112 family, may be involved in mitochondrial gene translation	3	
	SPAC631.01c	Member of F-actin capping protein beta subunit family	3	
	SPAC6F6.13c	Unknown function, DUF726 domain	3	Ace2
	SPBC83.11	Unknown function, possible role in binding phosphatidylinositol	3	
	SPAC977.09c	Member of lysophospholipase catalytic domain containing family, similar to phospholipase B, which deacylates phosphatidylinositol	3	MCB 1
cam1	SPAC3A12.14	Calmodulin, essential calcium-binding regulatory protein	3	Histone
csx2	SPBC17G9.08c	Protein with putative arf GTPase activation domain and pleckstrin homology domain, similar to GTPase activating protein for ARF	3	Ace2
cyp4	SPBP8B7.25	Protein similar to cyclophilin B, a peptidyl prolyl cis-trans isomerase	3	
pas1	SPAC57A10.01; SPAC19E9.03	Cyclin involved in regulation of mating, interacts with Pef1p and Cdc2p kinases	3	
php5	SPBC3B8.02	CCAAT-binding factor subunit, required for growth on non-fermentable carbon sources	3	
rad25	SPAC17A2.13c	14-3-3- protein involved in DNA damage checkpoint control	3	MCB 1
rer1	SPAC22E12.05c	Protein similar to component of COPII-coated vesicles, member of retention of ER proteins family	3	
spd1	**SPAC29B12.03**	**Negative regulator of S phase**	**4**	
rds1	**SPAC343.12**	**Stress response protein**	**4**	
psu1	**SPAC1002.13c**	**Protein required for cell wall integrity, member of SUN protein family**	**4**	
	SPAC13G6.10c	**Unknown function**	**4**	**MCB 1**
	SPAP7G5.06	**Protein similar to amino acid permease, a proton symport transporter for all naturally-occurring L-amino acids**	**4**	
	SPAC5H10.06c	**Protein similar to alcohol dehydrogenase IV, which is involved in carbohydrate metabolism**	**4**	

	SPAC1039.02	**Unknown function, possible membrane protein**	**4**	
	SPAC1039.01	Member of amino acid permease family of membrane transporters	4	
	SPBC119.10	Asparagine synthetase	4	Novel 3
	SPCC1235.11	Unknown function, member of UPF0041 uncharacterized protein family	4	
	SPCC126.09	Member of ZIP zinc transporter family, possible metal transporter and vacuolar membrane protein	4	
	SPBC1271.07c	Protein containing acetyltransferase (GNAT) domain	4	FLEX
	SPBC1271.08c	Unknown function	4	
	SPAC139.02c	Probable mitochondrial oxaloacetate transporter	4	
	SPBC13G1.09	Member of bystin family, possible role in 35S pre-rRNA processing into 18S rRNA	4	
	SPCC1494.06c	Member of the DEAD or DEAH box ATP-dependent RNA helicase, possible role in rRNA processing	4	
	SPCC1682.08c	Protein containing six Pumilio-family RNA binding domains, possible role in mRNA metabolism	4	
	SPBC16D10.06	Member of ZIP zinc transporter family	4	
	SPCC1739.01; SPCC1906.05	Member of zinc finger family, which bind DNA or RNA	4	
	SPCC1795.12c	Unknown function	4	FLEX
	SPBC17D1.06; SPCC17D1.06	Member of the DEAD or DEAH box ATP-dependent RNA helicase	4	FLEX
pof6	SPCC18.04	Protein involved in cell division, contains F-box domain	4	Novel 3
	SPCC18.05c	Protein containing nine WD domains (WD-40 repeat), possible membrane receptor	4	Novel 3
	SPCC1827.06c	Aspartate semialdehyde dehydrogenase	4	FLEX
rrn3	SPAC18G6.11c	Protein involved in initiation of transcription of rDNA promoter	4	
	SPAC1F12.05	Unknown function	4	
	SPAC212.08c	Telomeric protein of unknown function, possibly S. pombe specific	4	
	SPAC212.10	Pseudogene; malic acid transport protein; truncated C at terminal	4	
	SPAC24B11.10c	Unknown function, possible protoplast regeneration protein that stimulates chitin synthase III activity	4	
	SPBC25B2.08	Unknown function	4	FLEX

	SPAC2C4.18; SPAC25G10.01	Protein with RNA recognition motif, possible splicing factor that activates pre-mRNA splicing	4	
	SPBC29A3.01	Member of P-type ATPase, similar to copper-transporting ATPase	4	
	SPAC2C4.11c	Unknown function	4	
	SPAC323.07c	Member of the MatE family of integral membrane proteins	4	Novel 3
	SPBC365.16	Unknown function	4	
	SPBC3B8.06	Unknown function	4	
	SPCC548.06c	Protein similar to putative H+-glucose symporter involved in glucose transport	4	Novel 3
arg5	SPBC56F2.09c	Protein similar to amidotransferase small subunit of carbamoylphosphate synthetase	4	
	SPCC594.04c	Unknown function	4	
gmh2	SPAC5H10.13c	Protein similar to alpha-1,2-galactosyltransferase	4	MCB 1
	SPAC6B12.07c	Protein with zinc finger and N-terminal domain that may be involved in G protein associated signal transduction	4	
	SPAC6G9.02c	Protein with five Pumilio-family (Puf) RNA binding domains	4	
	SPCC757.11c	Unknown function	4	
ibp1	SPBC24E9.07; SPBC839.07	Protein phosphatase, rhodanese-like domain	4	Novel 3
	SPAC869.02c	Member of globin family of oxygen transporters, similar to flavohemoglobin that protects from stress	4	
	SPBC8E4.03	Protein with arginase family domain, similar to agmatine ureohydrolase	4	
	SPAC9.10	Member of amino acid permease family of membrane transporters	4	
cig1	SPCC645.01; SPCC4E9.02	B-type cyclin involved in G1 to S phase transition	4	FLEX, MCB 1
csx1	SPAC17A2.09c	Protein containing three RNA recognition motifs, similar to U1 snRNA-associated protein	4	
dak1; dak2	SPAC977.16c	Dihydroxyacetone kinase, isoenzyme II	4	Novel 3
gpd2	SPAC23D3.04c	Glycerol-3-phosphate dehydrogenase	4	Novel 3
mae1	SPAPB8E5.03	Malate transporter	4	
	SPCP1E11.08	Protein similar to nuclear protein involved in ribosome biogenesis	4	
	SPBP8B7.15c	Protein similar to Polyadenylation Factor I complex component	4	

		required for mRNA cleavage and polyadenylation		
pac2	SPAC31G5.11	Regulatory protein involved in sexual development via cAMP-independent pathway	4	
	SPAPB24D3.09c	Protein with ABC transporter domains, similar to brefeldin A resistance protein involved in multidrug resistance	4	
	SPBPB2B2.06c	Protein with calcineurin-like phosphoesterase domain	4	
	SPBP7E8.01; SPBPB7E8.01	Unknown function	4	
TF2-1		Retrotransposable element; tf2-type transposon	4	
TF2-10		Retrotransposable element; tf2-type transposon	4	
TF2-2		Retrotransposable element; tf2-type transposon	4	
TF2-3; TF2-4		Retrotransposable element; tf2-type transposon	4	
TF2-5		Retrotransposable element; tf2-type transposon	4	
TF2-6		Retrotransposable element; tf2-type transposon	4	
TF2-7		Retrotransposable element; tf2-type transposon	4	
TF2-8		Retrotransposable element; tf2-type transposon	4	
TF2-9		Retrotransposable element; tf2-type transposon	4	
bgl2	SPAC26H5.08c	Protein similar to beta-glucosidase, a cell wall endo-beta-1,3-glucanase	4	
	SPBC11C11.05	Member of yeast cell wall synthesis protein KRE9 or KNH1 family	4	FLEX
	SPAC11D3.04c	Unknown functionhypothetical protein; sequence orphan; shows expression on microarray	4	
	SPBC1271.10c	Protein similar to membrane transporter	4	
	SPBC12C2.12c; SPBC21D10.03c	Protein similar to glyoxalase I, contains glyoxalase, bleomycin resistance protein or dioxygenase family domains	4	
	SPBC1347.09	Unknown function	4	
	SPAC1486.09	Protein similar to protein that functions in 20S proteasome maturation and 26S proteasome assembly	4	FLEX
	SPCC1494.07	Unknown function	4	Novel 3
	SPCC1494.08c	Unknown function	4	Novel 2
	SPAC14C4.12c	Unknown function, contains SWIRM domain	4	
	SPAC1527.03	Unknown function, contains La domain	4	
	SPAC16C9.03	Possible role in nuclear export of 60S ribosomal subunits	4	

	SPBC16D10.02	Putative DNA-(amino)methyltransferase	4	Novel 3
	SPBC1711.07	Protein containing three WD domains (WD-40 repeat), possible role in ribosome assembly	4	MCB 1
	SPAC17A2.06c	Unknown function, similar to protein involved in vacuolar sorting	4	
	SPBC17D11.08	Unknown function, four WD domains (WD-40 repeats)	4	
	SPCC1827.05c	Protein with RNA recognition motif, similar to nucleolar protein	4	
	SPCC18B5.07c; SPBC18B5.07c	Member of RanBP1 domain containing family, similar to nuclear pore protein (nucleoporin)	4	
	SPCC1919.05	Protein containing nine TPR domains, similar to superkiller 3, which protects cells from RNA viruses	4	
	SPAC19B12.11c	Unknown function, similar to putative nuclear pore protein involved in bud site selection	4	FLEX, Novel 1
	SPAC19G12.09	Protein with aldo-keto reductase family domain, similar to aldehyde reductase	4	
	SPAC1B3.08	Member of PCI (proteasome, COP9-complex and eIF3) or PINT (Proteasome, Int-6, Nip-1 and TRIP-15) domain family	4	MCB 1
	SPAC823.03; SPAC1E11.03	Protein with kinase domain, similar to CDC-like kinase 2, which may regulate mRNA splicing	4	
	SPBC215.13	Unknown function, similar to protein involved in vesicle formation in endoplasmic reticulum	4	MCB 1
	SPAC222.09	Unknown function	4	
	SPAC23H4.15	Member of DUF663 protein of unknown function family, possible role in rRNA processing and 40S ribosomal subunit biogenesis	4	FLEX
	SPBC24C6.10c	Unknown function	4	
	SPBC19F5.05c; SPBC25D12.01c	Unknown function	4	
	SPAC27D7.09c	Unknown function	4	
	SPAC26F1.07	Protein similar to aldehyde reductase that reduces carbonyl-containing substrates and metabolizes xenobiotics	4	
	SPAC27D7.11c	Unknown function	4	Novel 3
	SPAC27F1.06c	Protein with FKBP-type peptidyl-prolyl cis-trans isomerase domain	4	Ace2
	SPBC29A10.08	Glycolipid-anchored surface protein precursor	4	
	SPAC29B12.08	Unknown function	4	MCB 1

	SPAC31A2.07c	Putative RNA helicase, possible role in ribosome biogenesis	4	
	SPAC31G5.02	Possible role in role cell wall organization and biogenesis	4	
	SPCC31H12.01; SPCC1183.11	Member of mechanosensitive ion channel family	4	
	SPAC328.05	Protein containing three RNA recognition motifs, possible role in protein-nucleus export	4	FLEX
gps2	SPBC365.14c	Putative UDP-glucose 4-epimerase involved in UDP-galactose synthesis and protein glycosylation	4	
	SPAC3G9.05	Unknown function, similar to cell polarity and cell fusion protein	4	
	SPBC405.02c; SPBC4C3.01	Unknown function	4	FLEX
	SPCC417.05c	Unknown function, similar to protein that stimulates chitin synthase III activity	4	
	SPBC428.10	Unknown function, similar to cell surface flocculin required for invasive and pseudohyphal growth	4	Novel 3
	SPAC521.02	Unknown function	4	
sst1	SPAC521.04c	Member of sodium or calcium exchanger protein family of membrane transporters	4	
	SPCC553.10	Unknown function	4	FLEX, MCB 1
	SPAC57A10.09c	Protein similar to DNA-binding and DNA-bending protein involved in transcriptional activation, contains HMG family domain	4	
	SPAC637.13c	Unknown function, pleckstrin homology (PH) domain	4	
	SPBC651.01c; SPBC725.18c	Unknown function, similar to a putative nucleolar GTP-binding protein required for ribosomal subunit biogenesis	4	FLEX
	SPBC660.06	Unknown function	4	
	SPAC688.11	Protein with actin binding domains, similar to talin-like protein required for hyphal growth	4	
	SPCC794.03	Member of amino acid permease family of membrane transporters	4	
	SPCC794.11c	Protein with actin binding domain, possible role in formation of clathrin coats at the Golgi and endosomes	4	MCB 1
	SPAC821.03c	Unknown function	4	
	SPAC9.07c	Putative GTP-binding protein	4	
	SPAC9E9.04	Unknown function, contains predicted N-term signal sequence and transmembrane helices	4	FLEX

cbh2	SPBC14F5.12c	DNA binding protein, possible role in chromosome segregation, role in histone tail modifications at centromere	4	
cdc2; swo2	SPBC11B10.09	Cyclin-dependent kinase, regulates cell cycle transitions G1/S and G2/M	4	
cds1	SPCC18B5.11c	Protein kinase involved in unreplicated DNA checkpoint response	4	FLEX
	SPACUNK4.15	Unknown function	4	Ace2
dim1	SPBC336.02	Protein similar to ribosomal RNA adenine dimethylases	4	
git3	SPCC1753.02c	Protein involved in cAMP pathway and required for growth under high osmotic stress	4	
myo52; myp5; myo4	SPCC1919.10c	Class V myosin, involved in polarized cell growth and vacuole fusion	4	FLEX, MCB 1
	SPAC31G5.12c	Unknown function, similar to negative effector of RNA polymerase III	4	
nrd1	SPAC2F7.11	Protein containing four RNA recognition motifs	4	
	SPAPB15E9.01c; SPAPB18E9.06c	Unknown function	4	
	SPAPB18E9.03c	Unknown function	4	
	SPAPB18E9.05c	Unknown function	4	
pcr1; mts2	SPAC21E11.03c	Transcription factor that plays roles in mating, meiosis and stress response	4	
pim1; ptr2; dcd1	SPBC557.03c	GTP-exchange factor (GEF) for Spi1p, required for nucleo-cytoplasmic transport, microtubule function and cytokinesis	4	
pka1; git6	SPBC106.10	Catalytic subunit of the cAMP-dependent protein kinase	4	
rdp1	SPAC1B1.01	Protein containing a C2H2 type zinc finger domain, binds to DNA damage response elements	4	Ace2
ssp1	SPCC297.03	Protein kinase that mediates rapid osmotic stress response at cell surface	4	
thi1; ntf1	SPAC6G10.01; SPAC1486.10	Regulatory protein for thiamine repressible genes, required forsynthesis of thiazole moiety of thiamine	4	MCB 1
	SPAC6F12.03c	Member of SNARE domain containing family	4	
top2	SPBC1A4.03c	DNA topoisomerase II	4	MCB 1
trx1; trx2	SPAC7D4.07c	Putative thioredoxin involved inresponse to heavy metals	4	FLEX
uvi15	SPBC649.04	Protein essential for stationary phase survival, induced by stress	4	Novel 2

vip1	SPAC10F6.06	Protein containing an RNA recognition motif	4	
	SPCC320.02c; SPCC1235.01	Unknown function	N(1)	FLEX, Histone
prl3		Non-coding RNA	N(1)	
hsk1	**SPBC776.12c**	**Protein kinase of the Hsk1p-Dfp1p complex involved in S phase initiation**	**N(1,2)**	
	SPCC338.08	**Unknown function**	**N(1,2)**	
mfm2	**SPAC513.03**	**Precursor polypeptide for mating pheromone M factor produced by h- cells**	**N(1,2)**	
spk1	SPAC31G5.09c	MAP kinase (MAPK) acting in the mating and sporulation pathways	N(1,2)	
byr2; ste8	SPBC2F12.01; SPBC1D7.05	MAP kinase kinase kinase acting upstream of MAPKK Byr1p and MAP kinase Spk1p in pheromone signaling pathway	N(1,2)	
	SPAC1006.06	Protein with RhoGEF domain, similar to Rho GDP-GTP exchange factor activated by cell wall defects	N(1,2)	
	SPAC12G12.06c	Probable RNA 3'-terminal phosphate cyclase	N(1,2)	
	SPCC1322.09	Unknown function	N(1,2)	MCB 2
	SPAC14C4.05c	Unknown function	N(1,2)	FLEX, MCB 2
	SPBC1683.07	Protein similar to alpha-glucosidase	N(1,2)	Ace2
hri1	SPAC20G4.03c	Translation initiation factor 2 alpha kinase, may play role in negative regulation of eIF2alpha in response to stress	N(1,2)	
	SPBC365.01	Unknown function, CRAL-TRIO domain and a CRAL or TRIO N terminus domain	N(1,2)	
din1	SPAC19D5.06c	Unknown function	N(1,2)	Novel 2
mei2	SPAC27D7.03c	RNA-binding protein involved in meiosis	N(1,2)	
spm1; pmk1	SPBC119.08	MAP kinase involved in maintenance of cell wall integrity	N(1,2)	FLEX
	SPCC965.06	Protein similar to potassium voltage-gated channel	N(1,2,3)	Novel 1
cnd2	SPCC306.03c	Subunit of condensin complex involved in chromosome condensation	N(1,3)	
isp6; prb1	SPAC4A8.04	Putative subtilase-type proteinase, role in sexual differentiation+E49	N(1,3,4)	
	SPAC869.05c	**Member of sulfate transporter family, similar to sulfate permease**	**N(1,4)**	
	SPAC1002.17c	Protein with phosphoribosyl transferase domain, possible role in pyrimidine salvage pathway	N(1,4)	

	SPAC1610.04	Unknown function	N(1,4)	
	SPCC16C4.06c	Protein with tRNA pseudouridine synthase domains	N(1,4)	Novel 1
sim4	SPBC18E5.03c	Centromere-associated protein required for chromosome segregation and silencing	N(1,4)	
	SPBC19G7.07c	Member of PPR repeat containing family	N(1,4)	
	SPCC330.15c; SPCC320.14	Member of pyridoxal phosphate dependent enzyme family, similar to racemase that catalyzes the racemisation of L-serine to D-serine	N(1,4)	
	SPBC428.17c	Unknown function	N(1,4)	
	SPCC553.07c	**Member of impB, mucB or samB family, possible role as translesion DNA repair polymerase**	**N(2,3)**	
	SPBC800.11	**Protein with inosine-uridine preferring nucleoside hydrolase domain**	**N(2,3)**	
	SPAC750.05c	**Telomeric protein of unknown function, highly similar to S. pombe SPAC977.01, SPAC1348.02 and SPBPB2B2.19c**	**N(2,3)**	
	SPBC1348.02; SPAC1348.02	**Telomeric protein of unknown function, highly similar to S. pombe SPAC977.01, SPAC750.05C and SPBPB2B2.19c**	**N(2,3)**	
	SPBPB2B2.19c	**Telomeric protein of unknown function, highly similar to S. pombe SPAC977.01, SPAC1348.02 and SPAC750.05C**	**N(2,3)**	**Histone**
	SPAC977.01	**Telomeric protein of unknown function, highly similar to S. pombe SPAC1348.02, SPAC750.05C and SPBPB2B2.19c**	**N(2,3)**	**FLEX**
	SPBC409.22c; SPBC1306.01c	**Protein with elongation factor Tu GTP binding domain, similar to mitochondrial translation elongation factor G**	**N(2,3)**	
	SPAC17G6.03	Protein with calcineurin-like phosphoesterase domain	N(2,3)	
	SPBC18E5.07	Unknown function	N(2,3)	Novel 2
	SPBC21B10.07; SPAC21B10.07	Protein with glycosyl hydrolase family 16 domain	N(2,3)	
	SPAC2E1P3.04	Protein with possible role in detoxifying extracellular amines and nitrogen metabolism	N(2,3)	Ace2, Novel 3
	SPAC29A4.05	Protein similar to calmodulin 1, which regulates the calcium-dependent activity of enzymes including phosphatases	N(2,4)	
	SPAC631.02	**Protein with two bromodomains, which interact with acetylated lysine**	**N(3,4)**	
	SPBC1271.09	**Member of sugar (and other) transporter family, possible role in inositol metabolism**	**N(3,4)**	**Novel 3**

	SPCC364.07; SPCC4G3.01	Protein similar to 3-phosphoglycerate dehydrogenase, which catalyzes first step in synthesis of serine	N(3,4)	
	SPAC3A11.10c	Member of Rnal dipeptidase family, zinc-dependent metalloproteinases that hydrolyze various dipeptides	N(3,4)	
	SPAC664.03	Member of Paf1 family, components of RNA polymerase II associated complexes	N(3,4)	Ace2, FLEX
pro1	SPAC821.11	Protein similar to gamma-glutamyl phosphate reductase involved in proline biosynthesis	N(3,4)	
fim1	SPBC1778.06c	Fimbrin, role in actin organization during medial ring formation and polarized growth	N(3,4)	
	SPBPB21E7.09; SPAPB21E7.09	Protein similar to L-asparaginase II	N(4)	FLEX, MCB 1, Novel 3

[a] Gene descriptions are based on the information in PombePD (www.incyte.com/control/tools/proteome) and *S. pombe* GeneDB (www.genedb.org/genedb/pombe/index.jsp) databases.

Genes are sorted according to the cluster they belong to; genes in bold are of 'high amplitude' and the unclassified genes (N) are listed at the bottom of the list.

Appendix I: Strains used in this study

Strain	Source
972 h-	Laboratory collection
cdc25-22 h-	Laboratory collection
cdc10-129 h-	Laboratory collection
cdc25-22 sep1Δ::ura4 h-	Matthias Sipiczki
ace2Δ::kanMX6 ade6-M21?h-	This study
ace2Δ::kanMX6 sep1::ura4 ade6-M21? leu1-32 h?	This study
atf1Δ::ura4 ura4-D18 h-	Nic Jones
cdc10-C4 h^+	Paul Nurse
cig1Δ::ura4 cig2Δ::ura4 puc1Δ::ura4	Sergio Moreno
leu1-32 h- pREP3X-fkh2	This study
leu1-32 h- pREP3X-ace2	This study
leu1-32 h- pREP3X-fhl1	This study
leu1-32 h- pREP3X-sep1	This study
mbx1Δ::kanMX6 leu1-32 ura4-D18 ade6-M210 his7-366 h-	Jonathan Miller
meu3Δ::kanMX6 ade6-M21?h-	This study
meu19::kanMX6 ade6-M21?h-	This study
nda3-KM311 h-	Laboratory collection
pcr1Δ::ura4 h-	Nic Jones
prr1Δ::his7 his7? h-	Nic Jones
sep1Δ::ura4 h-	Matthias Sipiczki
SPAC8C9.01Δ::kanMX6 h90 (fhl1)	Laboratory collection
SPAC8C9.01Δ::kanMK6 sep1::ura4 h?	This study
SPBC16G5.15cΔ::ura4 leu1-32 ura4-D18 ade6-M210/216 his7-366 h+/h-	Brian Morgan
SPBC16G5.15cΔ::ura4 leu1-32 ura4-D18 ade6-M21? his7-366 h-	This study

Appendix II: List of buffers, solutions, media and antibiotics

Buffers and stock solutions	Composition
Edinburgh Minimal medium (EMM)	For 1 liter: 3.0 g potassium hydrogen phthalate, 2.2 g Na_2HPO_4, 5.0 g NH_4Cl, 20g D-glucose, 20 ml salts stock (X50), 1 ml vitamin stock (X1000), 0.1 ml mineral stock (X10000)
0.5 M EDTA	For 1 liter: 186.1 g disodium ethylenediaminetetracetate · $2H_2O$
Formal saline	0.9% saline, 3.7% formaldehyde
Freezing mix	YE containing 250 mg of Glutamic Acid, 50% glycerol
Hybridization buffer	5 x SSC, 6 x Denhardt's 60 mM TrisHCl pH 7.6, 0.12% sarkosyl, 48% formamide; filter sterilized
10X LiAc	1M Lithium Acetate, pH 7.5
Luria Bertani medium (LB)	For 1 liter: 10g NaCl, 10 g tryptone, 5 g yeast extract, pH 7.0
Mineral stock (X10000)	For 1 liter: 5.0 g H_3BO_3, 4.0 g $MnSO_4$, 4.0 g $ZnSO_4$ · $7H_2O$, 2.0 g $FeCl_3$ · $6H_2O$, 0.4 g H_2MOO_4 · H_2O, 1.0 g KI, 0.4 g $CuSO_4$ · $5H_2O$, 10 g citric acid
2X Printing buffer	300 mM sodium phosphate, pH 8.5
PEG/LiAc/TE	For 20 ml: 2 ml 10X LiAc, 2 ml 10X TE, filter-sterilized
Phosphate Buffer Saline (PBS)	For 1 liter: 8.0 g NaCl, 0.2 g KH_2PO_4, 1.44 g Na_2HPO_4, 0.2 g KCl, pH 7.4
Salt stock (X50)	For 1 liter: 53.5 g $MgCl_2$ · $6H_2O$, 0.74 g $CaCl_2$ · $2H_20$, 50 g KCl, 20 g Na_2SO_4
SOC medium	For 100 ml: 2.0g Bacto-tryptone, 0.5g Bacto-yeast extract, 1ml 1M NaCl, 0.25ml 1M KCl, 1ml Mg2+ stock (1M MgCl2 . 6H2O, 1M MgSO4 . 7H2O), filter-sterilized, 1ml 2M glucose, filter-sterilized
20X SSC	3 M NaCl, 300 mM Na-citrate, pH 7.0

1X TAE	40 mM Tris-acetate, 1 mM EDTA, pH 7.2
1X TE	10 mM Tris/HCl, pH 8.0, 1 mM EDTA
1X TES	10 mM Tris pH 7.5, 10 mM EDTA pH 8.0, 0.5% SDS
Vitamin stock (1000X)	For 1 liter: 1.0 g Na pantothenate, 10 g nicotinic acid, 10 g inositol, 10 mg biotin
Washing solution 1	2X SSC
Washing solution 2	0.1X SSC, 0.1% SDS
Washing solution 3	0.1X SSC
Yeast Extract (YE)	For 1 liter: 5 g Difco Yeast Extract, 30 g glucose, pH 5.6. Supplements: 250 mg histidine, leucine, adenine, uracil and lysine
Yeast Extract (YE) agar	YE plus 20 l/liter Difco agar

Appendix III: Primers used in this study

PRIMERS for GENE DELETION

Primer name	Sequence
Meu19_F	5'-agttttaacgaccgctacgtagtgtaatgctacaaaataagtctac tcagatattatgcagccgtactcatctgattttgcggatccccgggttaattaa-3'
Meu19_R	5'-gcttactcataatttcttctttaaaaaatcgatgtgacattgtgaatacata aaccgtcaataatctactgctaaaatgcgaattcgagctcgtttaaac-3'
Meu3_F	5'-taacgctaatgtcatacaacaccgtattcgtctgatttgtctacaaaaatgta aacaaacaattattttatcgcattgatcggatccccgggttaattaa-3'
Meu3_R	5'-ctactcattatttcctcattaaaagatcgacttggcactgaaaacaaatgaac cgccgatatcctatattgttacaaagcgaattcgagctcgtttaaac-3'
Ace2_F	5'-atttctctcatcgttctctccttgatttctctacgcattgcactagatactcgcta tcctaagtaaacaagacaatgtcgcggatccccgggttaattaa-3'
Ace2_R	5'-aattgtcacagcattagttcatgtacgatgcttgaatttgaatttatataaaac aaattaaaaacaataatattagtaagaattcgagctcgtttaaac-3'
Fhl1_F	5'-gctttctagtctcattgtgctttcaattggctagaagtttagcttactataaaaaa aggaagtctttgggtcattaaggccggatccccgggttaattaa-3'
Fhl1_R	5'-ccaagttgtgcaatcactgtcaaaaaaagaaaaatgaaacagcttatgcat gctggtaagtgtaattttcggcactaattggaattcgagctcgtttaaac-3'

The underlined sequence represents the sequence homologous to the plasmid multiple cloning site.

PRIMERS for CHECKING GENE DELETIONS

Primer name	Sequence
Ace2_check_R	5'-gttatacacaatactagggtgatg-3'
Meu3_check_R	5'-cgtttcaatattgaaattcttacag-3'
Meu19_check_R	5'-tcgcatctttagattaacatatagg-3'
Kan_check_R	5'-gtcacatcatgcccctgagc-3'

PRIMERS for CLONING - OVEREXPRESSION

Primer name	**Sequence**
C16g5.15 forward	5'-**ctcgag**atgactgttcgcagactcgaaagc-3'
C16g5.15 reverse	5'-gggataagatattaaacaaggtg-3'
C6g10.12c forward	5'-**ctcgag**atgtcgctttcatat-3'
C6g10.12c reverse	5'-tcattagtgctgtctgcgatc-3'
C1142.08 forward	5'-**ctcgag**atgcctgttgcagag-3'
C1142.08 reverse	5'-tcaggtataagaggatgatgtctc-3'
Sep1 forward	5'-**ctcgag**atcttcctcatgaat-3'
Sep1 reverse	5'-ttagaatagtgttgaagtttgac-3'

The sequence in bold corresponds to the *Xho*I restriction site.

Appendix IVa: Time courses experimental conditions

Experiment	Strain	Timepoints	Sample label	Reference	Ref label	Media	Temp (ºC)	Sample OD	Array batch number
Elutriation I	*972 h-*	20	Cy3	Asynchronous *972 h-*	Cy5	EMM	30	0.65	137 & 140
Elutriation II	*972 h-*	20	Cy5	Asynchronous *972 h*	Cy3	EMM	30	0.24	228 & 232
Elutriation III	*972 h-*	20	Cy5	Asynchronous *972 h*	Cy3	EMM	30	0.15	489
cdc25 b&r I	*cdc25-22 h-*	18	Cy3	Asynchronous *cdc25-22 h-*	Cy5	YE	25	0.34	40 & 70
cdc25 b&r I dye swap	*cdc25-22 h-*	18	Cy5	Asynchronous *cdc25-22 h-*	Cy3	YE	25	0.34	40 & 70
cdc25 b&r II	*cdc25-22 h-*	19	Cy5	Asynchronous *cdc25-22 h-*	Cy3	EMM	25	0.19	292
cdc25 elu + b&r	*cdc25-22 h-*	22	Cy5	Asynchronous *cdc25-22 h-*	Cy3	EMM	25	0.22	226, 228 & 232
cdc10 elu + b&r	*cdc10-129 h-*	22	Cy5	Asynchronous *cdc10-129 h-*	Cy3	EMM	25	0.30	331 & 334
cdc10::ura4	*cdc10::ura4 leu1-32 ura4-D18 ade6-M210 + pREP1-HA-res1(1-192)*	3	Cy5	Untreated cdc10::ura4	Cy3	EMM ade	30	0.2	477
cig1Δ cig2Δ puc1Δ elu	*cig1::ura4 cig2::ura4 puc1::ura4*	6	Cy5	Asynchronous *cig1::ura4 cig2::ura4 puc1::ura4 h-*	Cy3	EMM	30	0.15	477
HU b&r	*972 h-*	4	Cy3	Untreated *972 h-*	Cy5	EMM	32	0.08	489
nda3	*nda3-KM311 h-*	3	Cy5	Untreated nda3	Cy3	EMM		0.23	477
sep1 b&r	*cdc25-22 sep1::ura4 h-*	20	Cy5	*cdc25-22 sep1::ura4 h-*	Cy3	EMM	25	0.12	331 & 334
wt b&r	*972 h-*	18	Cy3	*972 h-*	Cy3	EMM	25	0.15	782 & 784

0.5 OD = 1 X 10^7

Appendix IVb: Mutant strains experimental conditions

Experiment	Strain	Sample label	Reference	Ref label	Media	Temp (ºC)	Sample OD	Array batch number
ace2 #1	*ace2Δ::kanMX6 ade6-M21?h-*	Cy5	*972 h-*	Cy3	EMM ade	32	0.22	489-48
ace2 #2		Cy3		Cy5			0.23	489-7
ace2 #3		Cy5		Cy3			0.23	489-14
ace2 #4		Cy5		Cy3			0.24	489-12
ace2 #5		Cy5		Cy3			0.33	668-2
ace2 sep1 #1	*ace2Δ::kanMX6 sep1::ura4 ade6-M21? leu1-32*	Cy5	*972 h-*	Cy3	EMM ura ade leu	30	0.26	668-9
ace2 sep1 #2		Cy5		Cy3			0.32	668-10
atf1 #1	*atf1Δ::ura4 ura4-D18 h-*	Cy5	*972 h-*	Cy3	YE	25	0.18	596-21
atf1 #2		Cy5		Cy3			0.21	599-2
cdc10-C4 #1	*cdc10-C4 leu1-32 h+*	Cy5	*972 h$^+$*	Cy3	EMM	25	0.23	489-10
cdc10-C4 #2		Cy5		Cy3			0.24	489-11
cdc10-C4 #3		Cy3		Cy5			0.23	334-19
cdc10-C4 #4		Cy5		Cy3			0.24	334-17
fhl1 #1	*SPAC8C9.01Δ::kanMX6 h90*	Cy3	*972 h-*	Cy5	EMM	25	0.23	334-22
fhl1 #2		Cy5		Cy3			0.23	334-11
fhl1 #3		Cy5		Cy3			0.22	668-41
fkh2 #1	*SPBC16G5.15c::ura4 leu1-32 ura4-D18 ade6-M21? his7-366 h-*	Cy5	*972 h-*	Cy3	YE	30	0.20	560-42
fkh2 #2		Cy5		Cy3			0.20	560-45
mbx1 #1	*mbx1::kanMX6 leu1-32 ura4-D18 ade6-M210 his7-366 h-*	Cy5	*972 h-*	Cy3	EMM leu ura ade his	25	0.23	334-18
mbx1 #2		Cy3		Cy5			0.22	334-20

Experiment	Strain	Sample label	Reference	Ref label	Media	Temp (ºC)	Sample OD	Array batch number
meu3 #1	*meu3::kanMX6 ade6-M21?h-*	Cy5	*972 h-*	Cy3	EMM ade	32	0.23	489-49
meu3 #2		Cy3		Cy5			0.23	489-8
meu19 #1	*meu19::kanMX6 ade6-M21?h-*	Cy5	*972 h-*	Cy3	EMM ade	32	0.22	489-50
meu19 #2		Cy3		Cy5			0.25	489-9
pcr1 #1	*pcr1::ura4 h-*	Cy5	*972 h-*	Cy3	YE	25	0.24	489-17
pcr1 #2		Cy5		Cy3			0.21	560-46
prr1 #1	*prr1Δ::his7 his7? h-*	Cy3	*972 h-*	Cy5	EMM	25	0.23	334-21
prr1 #2		Cy5		Cy3			0.22	334-12
sep1 #1	*sep1Δ h-*	Cy5	*972 h-*	Cy3	EMM	25	0.24	489-13
sep1 #2		Cy3		Cy5			0.25	334-23
sep1 #3		Cy5		Cy3			0.24	334-14
sep1 #4		Cy5		Cy3			0.23	668-5
fhl1 sep1 #1	*SPAC8C9.01Δ::kanMK6 sep1::ura4 ade6-M21? leu1-32 h-*	Cy5	*972 h-*	Cy3	EMM ura ade leu	30	0.23	668-39
fhl1 sep1 #1		Cy5		Cy3			0.23	668-27
ace2 OE #1	*leu1-32 h- pREP3X-ace2*	Cy5	*leu1-32 h- pREP3X*	Cy3	EMM, 15 μM thiamine	32	0.06	596-3
ace2 OE #2		Cy5		Cy3			0.12	560-31
fhl1 OE #1	*leu1-32 h- pREP3X-fhl1*	Cy5		Cy3		32	0.03	596-4
fhl1 OE #2		Cy5		Cy3			0.08	560-47
fkh2 OE #1	*leu1-32 h- pREP3X-fkh2*	Cy5		Cy3		32	0.07	596-20
fkh2 OE #2		Cy5		Cy3			0.04	596-10
sep1 OE #1	*leu1-32 h- pREP3X-sep1*	Cy5		Cy3		32	0.23	596-22
sep1 OE #2		Cy5		Cy3			0.17	596-19

0.5 OD = 1 X 10^7

Appendix V: Additional measurements defining cell cycle synchrony in timecourse experiments and additional clustering

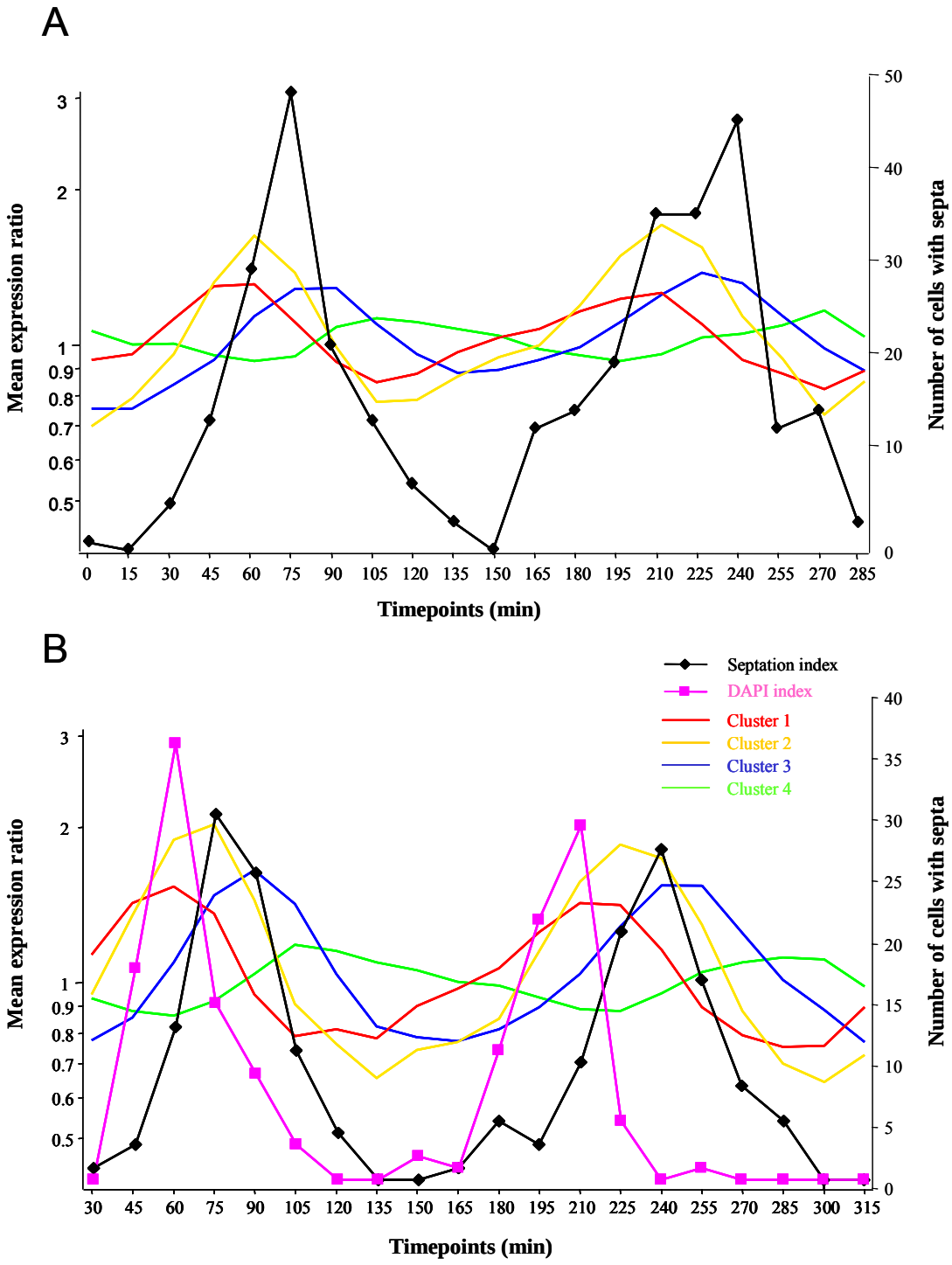

Fig. V.1 Additional measurements for elutriation experiments.

Panel A and B refer to two independent biological experiments, elutriations 1809 and 1012 respectively. For each graph, septation index and average expression profiles for the four clusters are shown. In panel b the DAPI index is also shown

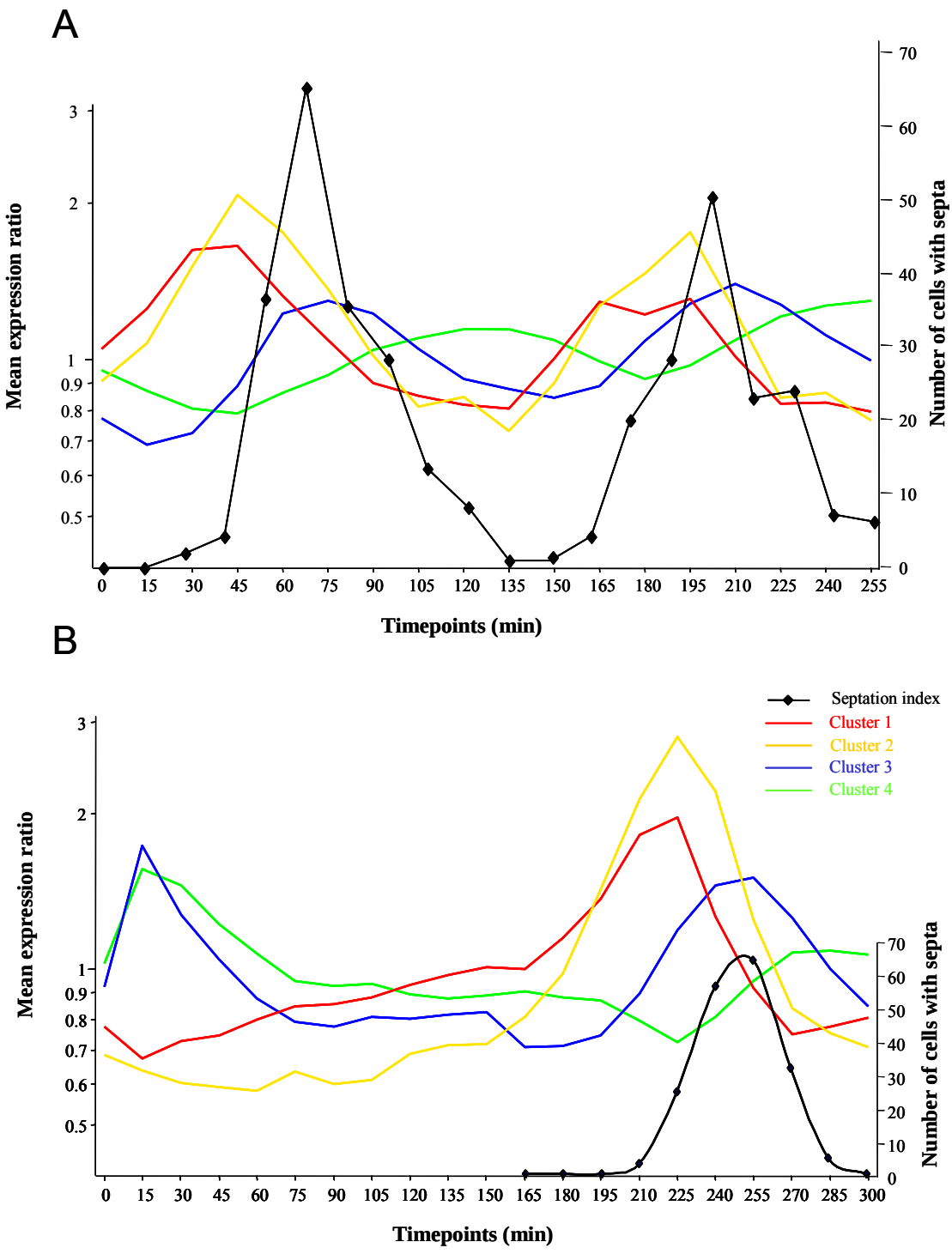

Fig. V.2 Additional measurements for *cdc25* experiments.

Panel A refers to a *cdc25* 'block and release' experiment (1601), panel B refers to a *cdc25* elutriation + 'block and release' (1402). For each graph, septation index and average expression profiles for the four clusters are shown.

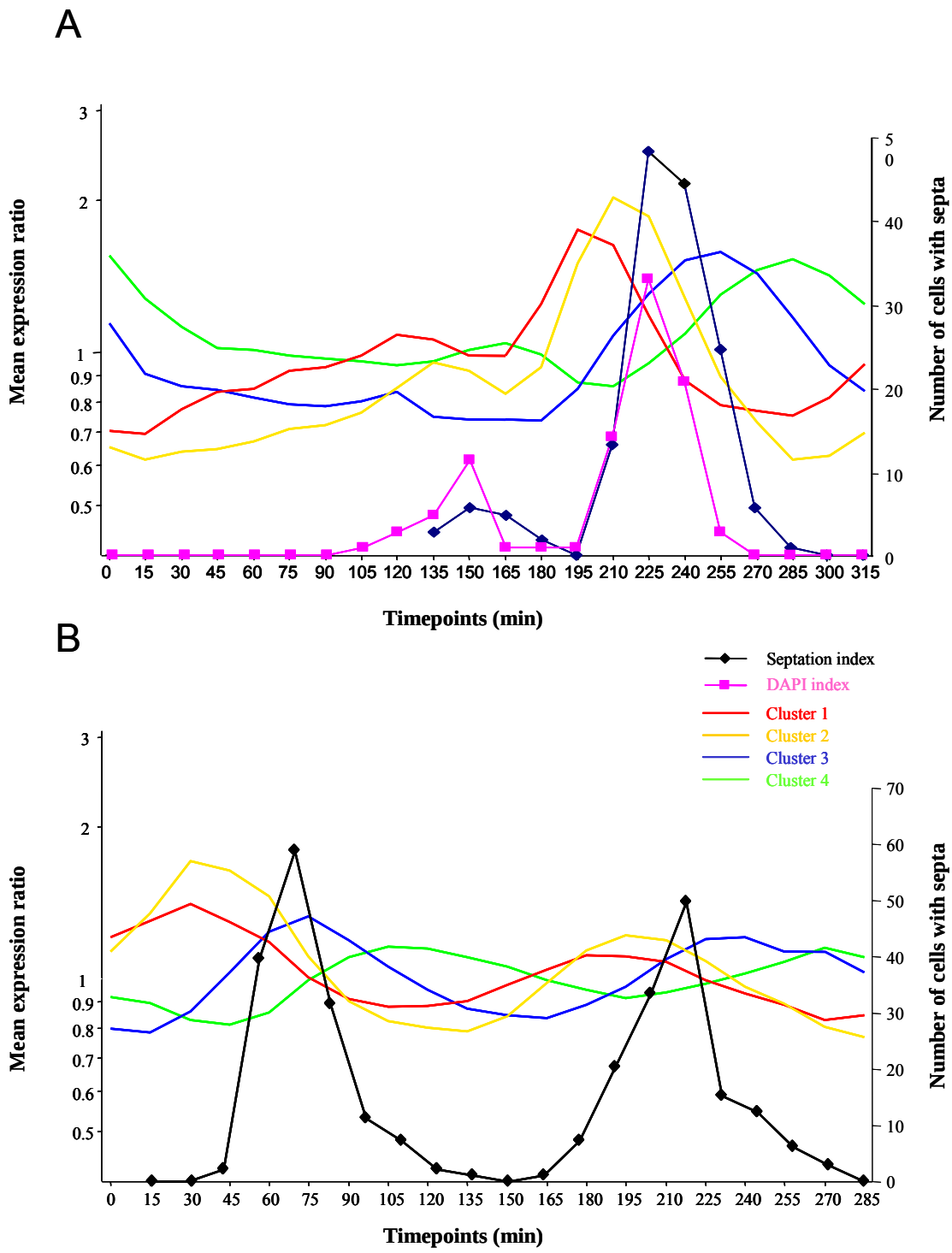

Fig. V.3 Additional measurements for *cdc25* and *cdc10* experiments.

Panel A refers to a *sep1Δ cdc25* 'block and release' experiment (2009), panel B refers to a *cdc10* elutriation + 'block and release' (509). For each graph, septation index and average expression profiles for the four clusters are shown.

Wild type elutriation

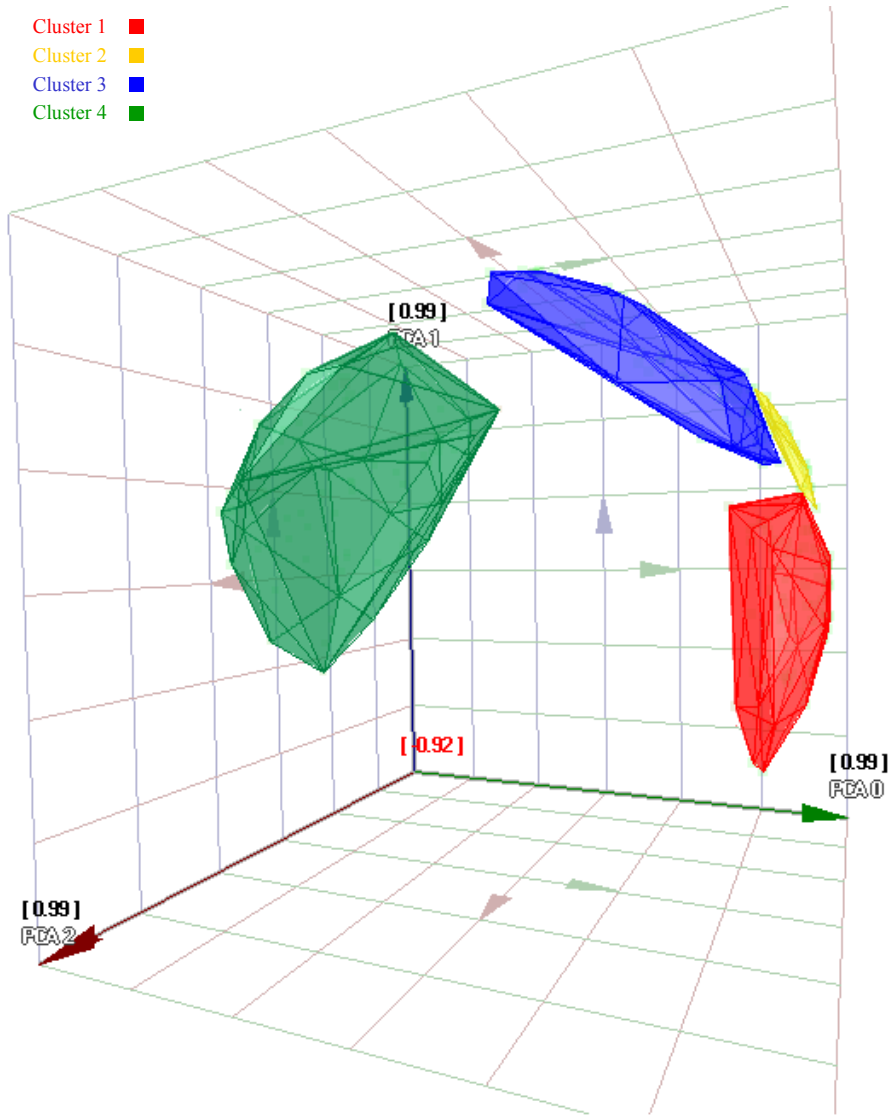

Fig. V.4 3-dimensional representation of the four clusters of cell cycle regulated genes for an elutriation experiment.

Each coloured area represents a cluster of genes. Classification is shown for one elutriation experiment (2201). This graph was obtained using Principal Component Analysis (PCA) in Arrayminer.

cdc25 'block and release'

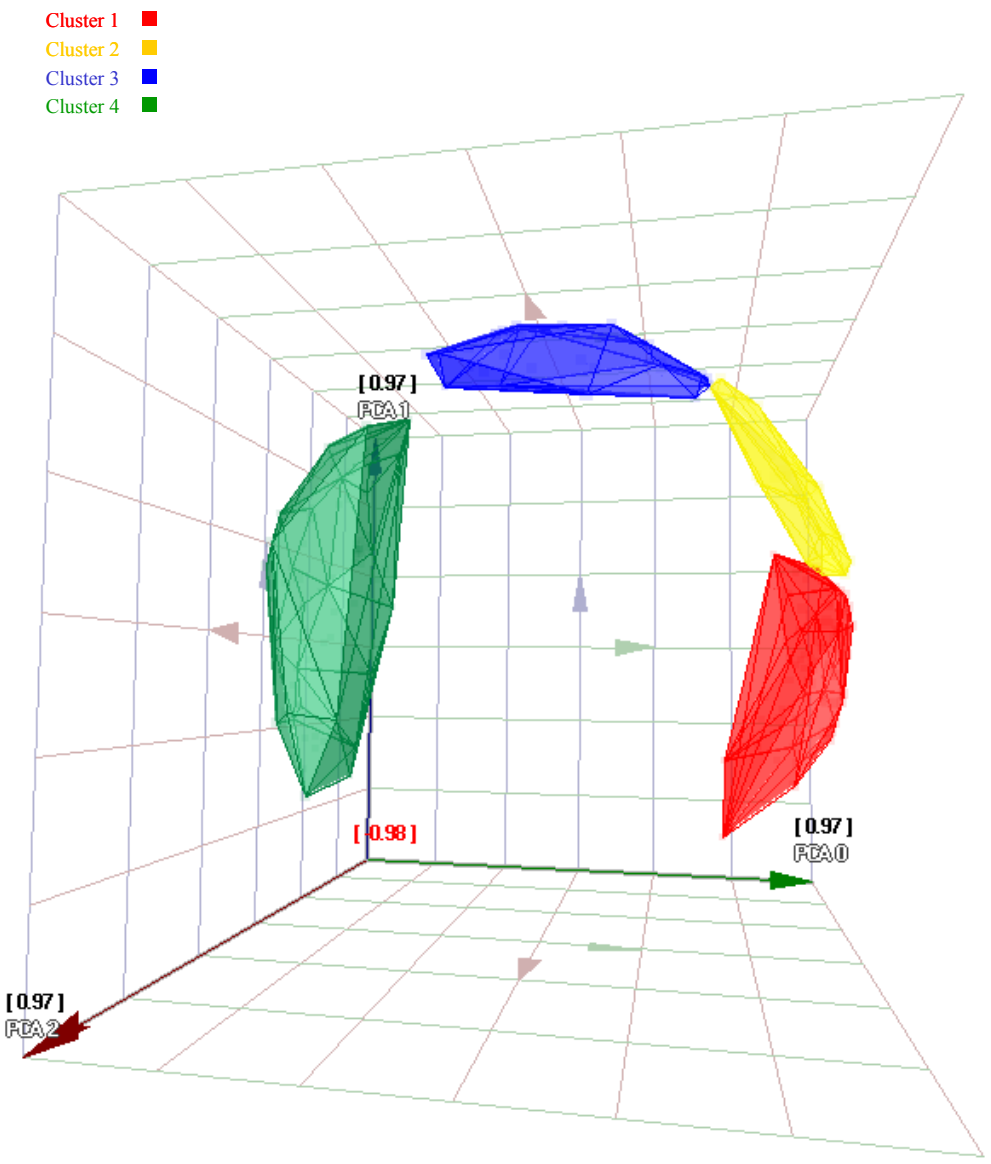

Fig. V.5 3-dimensional representation of the four clusters of cell cycle regulated genes for a *cdc25* 'block and release' experiment.

Each coloured area represents a cluster of genes. Classification is shown for one *cdc25* 'block and release' experiment (2001). This graph was obtained using Principal Component Analysis (PCA) in Arrayminer.

Appendix VI: List of 407 genes periodically expressed during the cell cycle

Biological names	Systematic names	Gene description [a]	Cluster	Motifs
ste7	SPAC23E2.03c	Protein required for mating and meiosis	1	FLEX
sso1; psy1	SPCC825.03c	Syntaxin-like component of the plasma membrane docking/fusion complex	1	FLEX
slp1	SPAC821.08c	WD-domain protein of the spindle defect checkpoint and APC activator	1	FLEX
sid2; pld5	SPAC24B11.11c	Protein kinase involved in regulation of cytokinesis	1	FLEX
ark1; sex1	SPCC330.16; SPCC320.13c	Aurora kinase involved in regulation of mitosis	1	
rum1	SPBC32F12.09	Inhibitor of the Cdc2p cyclin-dependent kinase complex	1	FLEX, Novel 2
rhp51; rad51	SPAC644.14C	Required for DNA repair and meiotic recombination	1	MCB 1
plo1	SPAC23C11.16	Polo kinase involved in regulation of mitosis and cytokinesis	1	FLEX, Novel 3
myo3; myp2	SPAC4A8.05c	Myosin-3 isoform, heavy chain (Type II myosin)	1	
mus81	SPCC4G3.05c	Holliday junction resolvase subunit	1	
msh6	SPCC285.16c	Protein involved in mismatch repair (mutS family)	1	FLEX
mob1	SPBC428.13c	Protein involved in regulation of cytokinesis	1	FLEX, MCB 1, Novel 2
meu29	SPAC25H1.05	Unknown function	1	Ace2, FLEX
meu16		Non-coding RNA	1	
klp6	SPBC1685.15c; SPBC649.01c	Kinesin motor protein; KIP3 subfamily	1	FLEX
klp5	SPBC2F12.13	Kinesin motor protein; KIP3 subfamily	1	FLEX, MCB 1
imp2	SPAC13F4.08c; SPBC11C11.02	Protein required for medial ring disassembly after cytokinesis	1	FLEX, MCB 1, Novel 1
etd1	SPAC1006.08	Protein required for cytokinesis	1	FLEX, MCB 1
chs2	SPBC1734.17; SPBC1709.01	Member of chitin synthase family, involved in cell wall maintenance	1	MCB 1
cdc20; pol2	SPBC25H2.13c	DNA polymerase epsilon catalytic subunit	1	FLEX, MCB 2
cdc15; rng1	SPAC20G8.05c	Protein involved in cytokinesis	1	FLEX
bet1	SPAC23C4.13	Member of SNARE domain containing family	1	FLEX
apc15; apc16	SPBC83.04	Component of APC/cyclosome complex	1	FLEX

	SPCC757.12	**Protein containing an alpha amylase N-terminal catalytic domain**	**1**	**Novel 2**
ace2	**SPAC6G10.12c**	**Zinc finger transcription factor**	**1**	**FLEX**
	SPAC5D6.02c	**Unknown function**	**1**	
	SPCC576.02	**Member of aspartate and glutamate racemases family**	**1**	**FLEX**
	SPAC30D11.01c; SPAC56F8.01	**Member of glycosyl hydrolases family 31, involved in carbohydrate metabolism**	**1**	
	SPBC4F6.12	**LIM domain protein, low similarity to paxillin focal adhesion protein that regulates integrin or growth factor-mediated responses**	**1**	
wis3; spo12	**SPAC3F10.15c**	**Protein likely to play role in regulating cell cycle progression, possibly at G2 to M phase transition**	**1**	**FLEX, Novel 1**
	SPBC27.05	**Unknown function**	**1**	
	SPAC23H4.19; SPAC1705.03c	**Putative cell wall biogenesis protein**	**1**	**FLEX, Novel 1, Novel 2**
	SPBC19G7.04	**Unknown function, possible transcriptional regulator, may contain HMG box**	**1**	**FLEX**
	SPAC19B12.02c	**Protein with high similarity to 1,3-beta-glucanosyltransferase, member of glycolipid anchored surface protein (GAS1) family**	**1**	**FLEX**
	SPBC16G5.15c	**Fork head protein type transcription factor**	**1**	**FLEX**
rho4	**SPAC16A10.04**	**Rho protein involved in regulation of cytoskeleton, cytokinesis, and cell wall integrity**	**1**	**FLEX, Novel 1**
mde6	**SPAC15A10.10**	**Protein likely to play a role in meiosis or sporulation, requires Mei4p for transcriptional activation**	**1**	**FLEX**
	SPAC15A10.09c	**Unknown function**	**1**	**FLEX, MCB 1, Novel 1**
mac1	**SPAC13G7.04c**	**Transmembrane protein involved in cell separation**	**1**	**FLEX, Novel 2**
	SPAC11E3.13c	**Member of glycolipid anchored surface protein (GAS1) family, possible involvement in cell wall maintenance**	**1**	**MCB 1**
	SPBC1198.07c	**Putative glycosylphosphatidylinositol (GPI)-anchored protein involved in cell wall biosynthesis**	**1**	**FLEX**
top1	SPBC1703.14c	DNA topoisomerase I, involved in chromatin organisation	1	FLEX, Novel 1
spn2	SPAC821.06	Septin homolog, involved in cell separation	1	
sad1; sta1	SPBC16H5.01c; SPBC12D12.01	Spindle pole body associated protein	1	

psc3	SPAC17H9.20; SPAC607.01	Cohesin complex component, required for sister chromatid cohesion and normal mitosis	1	MCB 1
	SPBC32H8.09	Protein containing WD domain G-beta repeat	1	
ndk1	SPAC806.07	Nucleoside diphosphate kinase	1	
dis1	SPCC736.14	Microtubule-associated protein required for chromosome segregation (functions with Klp5p and Klp6p in kinetochore-spindle attachment)	1	
csk1	SPAC1D4.06c	Cyclin-dependent kinase activating kinase (CAK) involved in activating Cdc2p (activity partially redundant with Mcs6p-Mcs2p complex)	1	
crk1; mcs6; mop1	SPBC19F8.07	Cyclin-dependent kinase activating kinase (CAK) involved in activating Cdc2p kinase, putative transcription initiation factor TFIIH subunit	1	
cid13	SPAC821.04c	Cytoplasmic poly(A) polymerase involved in regulation of ribonucleotide reductase (suc22) mRNA, TRF family of nucleotidyltransferases	1	Novel 1
cdr1; nim1	SPAC644.06c	Protein kinase involved in regulation of mitosis	1	
cdc25; sal2	SPAC24H6.05	Tyrosine phosphatase that activates Cdc2p kinase, involved in G2/M transition and DNA damage checkpoints	1	
cdc13	SPAC19G10.09C; SPBC582.03	Cyclin that promotes entry into mitosis from G2 phase, forms complex with Cdc2	1	
aph1	SPCC4G3.02	Diadenosine tetraphosphatase, catalyzes hydrolysis of dinucleoside polyphosphate compounds	1	
	SPBPB2B2.09c	Member of the ketopantoate reductase PanE or ApbA family, involved in thiamine biosynthesis	1	
rps602; rps6	SPAPB1E7.12	Protein with high similarity to ribosomal S6 proteins	1	
	SPAC824.04	Protein containing three WD domains (WD-40 repeat)	1	
	SPAP8A3.11c	Member of GTP1 or OBG family of GTP-binding proteins, similarity to developmentally regulated protein with possible role in neurogenesis	1	
	SPAP27G11.08c	Unknown function	1	FLEX
	SPAC9.11	Unknown function	1	
	SPBC646.06c	Member of glycosyl hydrolase family 71, putative glucanase	1	FLEX, Novel 2
	SPAC589.09	Protein containing a CRAL-TRIO domain, putative	1	

		phosphatidylinositol metabolism		
	SPAC589.02c	Component of mediator subcomplex that may function in negative regulation of transcription	1	
	SPCC4G3.06c	Unknown function, possible mitochondrial ribosomal protein of large subunit	1	FLEX
	SPBC4F6.11c	Unknown function	1	MCB 1
	SPBC4F6.05c	Member of legume-like lectin family	1	
	SPBC428.12c	Putative RNA binding protein	1	
	SPBC1306.02; SPBC4.08	Protein containing eight WD domains (WD-40 repeat), possible role in activated transcription by RNA polymerase II	1	FLEX
	SPBC3H7.13	Member of forkhead associated (FHA) domain family, similarity to S. pombe spindle checkpoint protein Dma1p	1	
pmc2	SPAC2F7.04	Mediator complex component, involved in regulating RNA polymerase II activity	1	
	SPBC27B12.06	Protein with possible role in glycosylphosphatidylinositol biosynthesis	1	FLEX
	SPBC26H8.13c	Unknown function	1	
	SPAC24H6.01c; SPAPB21F2.01	Unknown function, similarity to putative glycerol transporter involved in phospholipid biosynthesis	1	FLEX, MCB 1
	SPAC24C9.05c	Unknown function	1	
	SPAC23G3.04	Unknown function	1	Novel 3
spn7; mde8	SPBC21.08c; SPBC19F8.01c	Septin homolog, involved in cell separation	1	FLEX
	SPBC19C2.10	Protein containing an Src homology 3 (SH3) domain, putative actin binding	1	
spp2; pri2	SPBC17D11.06	DNA primase, large (non-catalytic) subunit	1	
	SPBC1709.13c	Member of SET domain containing family	1	
	SPAC1687.19c	Probable tRNA-guanine transglycosylase	1	FLEX
	SPAC1687.17c	Member of the Der1-like family, putative transmembrane protein with signal peptide	1	FLEX
	SPAC1687.10	Unknown function	1	
	SPBC1685.03	Member of signal peptidases type I family, which cleave signal peptides from secreted proteins	1	

	SPAC13G7.10	Protein with two Myb-like DNA-binding domains	1	FLEX
	SPAC13G6.03	Member of type I phosphodiesterase or nucleotide pyrophosphatase family	1	
	SPAC13C5.05c	Member of phosphoglucomutase or phosphomannomutase C-terminal domain containing family	1	
pds5	SPAC110.02	Protein required for maintenance of sister chromatid cohesion	1	MCB 1
mik1	**SPBC660.14**	**Protein kinase that inhibits Cdc2p kinase**	**2**	**FLEX, MCB 1, MCB 2**
pol1; swi7	**SPAC3H5.06c**	**DNA polymerase alpha catalytic subunit**	**2**	**FLEX, Histone**
	SPAC644.05c	**Protein similar to dUTP pyrophosphatase, which maintains dUTP at low levels to prevent misincorporation into DNA**	**2**	**MCB 1, MCB 2**
	SPAC1071.09c	**Protein containing a DnaJ domain, which mediates interaction with heat shock proteins**	**2**	
	SPBC1289.01c; SPBC1539.11c	**Unknown function, putative involvement in chitin biosynthesis**	**2**	**MCB 1**
	SPCC1322.04	**Putative UTP-glucose-1-phosphate uridylyltransferase**	**2**	**FLEX**
	SPCC1322.10	**Unknown function, similar to cell-surface proteins and proteoglycans**	**2**	**FLEX**
	SPAC14C4.09	**Unknown function, putative glucanase**	**2**	
	SPBC16A3.07c	**Unknown function**	**2**	**MCB 1, MCB 2**
	SPBC1709.12	**Unknown function**	**2**	**Ace2**
	SPAC17H9.18c	**Unknown function**	**2**	**FLEX**
	SPCC74.07c; SPCC18.01c	**Member of SUN family, contains predicted N-terminal signal sequence**	**2**	**Ace2, FLEX**
	SPCC18.02	**Protein with similarity to synaptic vesicle-associated acetylcholine transporter**	**2**	
	SPAC23A1.01c; SPAC19G12.16c	**Unknown function, similarity to podocalyxin like, a transmembrane sialomucin important for lymphocyte adhesion and homing**	**2**	**Ace2, FLEX, MCB 1**
	SPBC21B10.13c; SPAC21B10.13c	**Homeobox domain (homeodomain) protein, putative transcription factor**	**2**	
	SPAC23H4.01c; SPAP27G11.01	**Unknown function, contains a pleckstrin homology (PH) domain**	**2**	**Ace2, FLEX**
	SPBC27.04	**Unknown function**	**2**	**Novel 1**
ams2	**SPCC4F11.01;**	**Protein that binds binds chromatin at centromere and is**	**2**	**MCB 1**

	SPCC290.04	**involved in chromosome segregation**		
	SPBC2A9.07c	**Unknown function**	**2**	
	SPAC2E1P5.03	**Protein containing a DnaJ domain, which mediates interaction with heat shock proteins**	**2**	**Ace2, MCB 1**
	SPBC31F10.17c	**Unknown function**	**2**	**FLEX, Novel 1**
	SPBC32F12.10	**Protein with phosphoglucomutase or phosphomannomutase C-terminal domain**	**2**	**FLEX**
	SPBC3E7.12c	**Unknown function, possible role in regulation of chitin synthase**	**2**	**Ace2, MCB 1**
rgf3	**SPCC645.06c**	**Protein containing a pleckstrin homology (PH) and a RhoGEF (GTPase exchange factor) domain**	**2**	
	SPBC651.04	**Unknown function**	**2**	**FLEX**
mrc1; huc1	**SPAC694.06c**	**Protein required for DNA replication checkpoint**	**2**	**MCB 1, MCB 2**
	SPBC83.18c	**Protein containing a C2 domain, which may be involved in calcium-dependent phospholipid binding**	**2**	**Ace2**
	SPCC965.14c	**Member of cytidine and deoxycytidylate deaminase zinc-binding region family**	**2**	**FLEX**
cdc18	**SPBC14C8.07c**	**Protein that couples cell cycle signals to DNA replication machinery and induces replication**	**2**	**MCB 1, MCB 2**
cdc22	**SPAC1F7.05**	**Ribonucleoside-diphosphate reductase large chain, likely required for initiation of DNA replication**	**2**	**MCB 1, MCB 2**
cdm1	**SPBC12D12.02c**	**DNA polymerase delta subunit**	**2**	**FLEX, Novel 2**
cdt1	**SPBC428.18**	**Protein that coordinates completion of S phase with onset of mitosis**	**2**	**MCB 1, MCB 2**
cdt2	**SPAC17H9.19c**	**Protein required for DNA replication**	**2**	**MCB 1, MCB 2**
cig2; cyc17	**SPAPB2B4.03**	**Major G1/S-phase cyclin, promotes onset of S phase**	**2**	**FLEX**
cut2	**SPBC1815.02c; SPBC14C8.01c**	**Securin; required for sister chromatid separation**	**2**	**Ace2, FLEX**
dfp1; him1; rad35	**SPCC550.13**	**Regulatory subunit of the Hsk1p-Dfp1p kinase copmlex involved in S phase initiation**	**2**	**FLEX, MCB 1**
eng1	**SPAC821.09**	**Endo-beta-1,3-glucanase required for cell separation**	**2**	**FLEX**
exg1	**SPBC1105.05**	**Putative exo-beta-1,3-glucanase**	**2**	**Novel 2**
fin1	**SPAC19E9.02**	**NimA family kinase; regulates spindle formation and recruitment of Plo1p to SPB, promotes chromatin condensation**	**2**	**FLEX**

klp8	**SPAC144.14**	**Protein containing a kinesin motor domain**	**2**	
meu19		**Non-coding RNA**	**2**	
	SPAP14E8.02	**Unknown function**	**2**	**MCB 1, MCB 2**
par2; pbp2	**SPAC6F12.12**	**Protein phosphatase PP2A, B' regulatory subunit, required for cytokinesis, morphogenesis, and stress tolerance**	**2**	**Ace2**
rep2	**SPBC2F12.11c**	**Zinc finger transcriptional activator, MBF transcriptional complex**	**2**	
rpc17	**SPAPB1E7.10**	**Unknown function**	**2**	
	SPBPB2B2.13	**Protein similar to galactokinase, which catalyzes first step in galactose metabolism**	**2**	
	SPAPJ760.03c	**Unknown function**	**2**	**Ace2, FLEX**
mid2	**SPAPYUG7.03c**	**Protein required for septin function and stability during cytokinesis**	**2**	**Ace2, MCB 1**
rad21	**SPCC338.17c**	**Cohesin complex subunit, double-strand-break repair protein**	**2**	
rph1; pfh1; pif1	**SPBC887.14c**	**ATP-dependent DNA helicase involved in telomere maintenance, DNA replication, and DNA repair**	**2**	
psm3; smc3	**SPAC10F6.09c**	**Cohesin complex subunit, involved in sister chromatid cohesion and progression through mitosis**	**2**	**MCB 2**
ste9; srw1	**SPAC144.13c**	**Protein required for mating and sporulation, may regulate anaphase promoting complex**	**2**	
ssb1; rad11	**SPBC660.13c**	**Single-stranded DNA-binding protein subunit, required for DNA replication**	**2**	**MCB 1, MCB 2, Novel 1**
ulp1	**SPBC19G7.09**	**Ubl2p-specific protease**	**2**	**FLEX, MCB 1**
	SPAC343.20	**Unknown function**	**2**	
	SPCC63.13	**Protein containing a DnaJ domain, which mediates interaction with heat shock proteins**	**2**	
	SPBC839.02; SPBC24E9.02	**Unknown function, contains an N-terminal arrestin (or S-antigen) domain, possibly fungal specific**	**2**	
cdc4	SPAP8A3.08	EF-hand component of actomyosin contractile ring, required for cytokinesis	2	Ace2, Novel 3
cdc10	SPBC336.12c	Component of MBF transcriptional activation complex involved in control of START	2	
bgs4; cwg1	SPCC1840.02c	Putative 1,3-beta-glucan synthase component, cell wall synthesis	2	
	SPAC11E3.10	Unknown function, member of VanZ-like family	2	

	SPAC22F8.04	Unknown function	2	MCB 1
	SPAC22G7.02	Unknown function	2	Ace2, Novel 3
	SPAC27D7.12c	Unknown function	2	MCB 1
	SPBC2A9.13	Unknown function	2	Ace2
	SPAC2F7.14c	Protein similar to 3'-5' exoribonuclease required for 3' processing of ribosomal 5.8S rRNA and component of 3'-5' exosome complex	2	MCB 1
	SPBC32C12.03c	Protein with protein kinase domain, similar to S. pombe Kin1p, a putative serine-threonine protein kinase involved in regulating cell polarity	2	FLEX, MCB 2
	SPCC4F11.03c	Unknown function	2	MCB 1
	SPAC4H3.11c	Unknown function	2	Histone
	SPCC553.12c; SPCC794.13	Unknown functionl	2	MCB 1
	SPCC794.15	Unknown function	2	
	SPBC9B6.07; SPAC9B6.07	Unknown function, possible role in maturation of 25S rRNA	2	
chs5	SPAC6G9.12	Protein with fibronectin domain involved in cell surface binding, and BRCT domain found in checkpoint proteins, similar to chitin synthase	2	FLEX
cnp1; sim2	SPBC1105.17	CENP-A-like protein, histone H3 variant specific to inner centromeres and required for chromosome segregation	2	FLEX, MCB 2
eso1; eco1	SPBC16A3.11	DNA polymerase eta, involved in sister chromatid cohesion	2	FLEX, MCB 1
	SPBPB21E7.10; SPAPB21E7.10	Unknown function	2	
pob1	SPBC1289.04c	Protein required for cell polarity and cell separation	2	Ace2
	SPCC1795.10c	**Unknown function**	**3**	**Ace2**
	SPBC17G9.06c	**Unknown function**	**3**	**Ace2**
	SPBC19C7.04c	**Unknown function**	**3**	
	SPCC338.12	**Unknown function**	**3**	**Ace2, MCB 1, Novel 3**
hht1	**SPAC1834.04**	**Histone H3.1**	**3**	**Histone, MCB 1**
hht2	**SPBC8D2.04**	**Histone H3.2**	**3**	**Histone**
hht3; clo5	**SPBC1105.11c**	**Histone H3.3**	**3**	**Histone**
hhf1; ams1	**SPAC1834.03c**	**Histone H4.1**	**3**	**Histone, MCB 1**

hhf2; ams3	**SPBC8D2.03c**	**Protein similar to histone H4.1, contains a core histone domain**	**3**	**Histone**
hhf3; ams4	**SPBC1105.12**	**Histone 4.3**	**3**	**Histone**
hta1	**SPCC622.08c**	**Histone H2A-alpha**	**3**	**Histone, MCB 1**
hta2	**SPAC19G12.06c**	**Histone H2A-beta**	**3**	**Histone**
htb1	**SPCC622.09**	**Histone H2B-alpha**	**3**	**Histone, MCB 1**
pht1	**SPBC11B10.10c**	**Histone H2A variant**	**3**	
	SPBPJ4664.02	**Unknown function, possible cell surface glycoprotein**	**3**	
prl36		**Non coding RNA**	**3**	
sap1	**SPCC1672.02c**	**DNA-binding protein required for growth and mating type switching**	**3**	
sod2	**SPAC977.10**	**Sodium/proton antiporter**	**3**	
	SPAC1F7.03	Unknown function	3	
	SPCC306.08c	Malate dehydrogenase, mitochondrial precursor	3	
	SPAC1142.02c; SPAC17G6.19c	Unknown function, contains three TPR domains	3	FLEX
	SPBC1105.14	Protein containing two zinc finger DNA binding domains, possible role in proteasome regulation	3	
	SPAC12G12.07c	Unknown function, domain similar to integrin-a cytoplasmic region	3	
	SPBC1348.10c; SPAC1348.10c	Member of lysophospholipase catalytic domain family, putative lysophospholipase precursor	3	MCB 1
	SPAC806.09c; SPAC1639.01c	SUR4 family protein, putative fatty acid elongation protein	3	Ace2
	SPBC16G5.05c	Protein containing MSP domain, possible type II integral ER membrane protein involved in inositol regulation	3	
	SPCC1906.01	Mannose-1-phosphate guanyltransferase	3	
	SPBC19G7.16	Member of IWS1 C terminus containing family, possible roles in in transcription regulation and transcription elongation	3	Novel 3
	SPBC21B10.09; SPAC21B10.09	Protein similar to acetyl-CoA transporter	3	
	SPAC22G7.01c; SPAPJ696.03c	Protein containing metallopeptidase family M24 domain, similar to x-prolyl aminopeptidase	3	
	SPBPJ758.01; SPBC23E6.01c	Protein with RNA recognition motifs, similar to U1 snRNA-associated protein that suppresses splicing defects and mediates	3	

		recombination		
	SPBC28F2.11	Protein with a high mobility HMG-box domain	3	
	SPBC31F10.16	Unknown function	3	Ace2
	SPBC337.20; SPBC1734.04	Protein similar to cis Golgi protein, putative involvement in protein glycosylation in the golgi	3	MCB 1
	SPAC343.13	Member of PET112 family, may be involved in mitochondrial gene translation	3	
	SPAC631.01c	Member of F-actin capping protein beta subunit family	3	
	SPAC6F6.13c	Unknown function, DUF726 domain	3	Ace2
	SPBC83.11	Unknown function, possible role in binding phosphatidylinositol	3	
	SPAC977.09c	Member of lysophospholipase catalytic domain containing family, similar to phospholipase B, which deacylates phosphatidylinositol	3	MCB 1
cam1	SPAC3A12.14	Calmodulin, essential calcium-binding regulatory protein	3	Histone
csx2	SPBC17G9.08c	Protein with putative arf GTPase activation domain and pleckstrin homology domain, similar to GTPase activating protein for ARF	3	Ace2
cyp4	SPBP8B7.25	Protein similar to cyclophilin B, a peptidyl prolyl cis-trans isomerase	3	
pas1	SPAC57A10.01; SPAC19E9.03	Cyclin involved in regulation of mating, interacts with Pef1p and Cdc2p kinases	3	
php5	SPBC3B8.02	CCAAT-binding factor subunit, required for growth on non-fermentable carbon sources	3	
rad25	SPAC17A2.13c	14-3-3- protein involved in DNA damage checkpoint control	3	MCB 1
rer1	SPAC22E12.05c	Protein similar to component of COPII-coated vesicles, member of retention of ER proteins family	3	
spd1	**SPAC29B12.03**	**Negative regulator of S phase**	**4**	
rds1	**SPAC343.12**	**Stress response protein**	**4**	
psu1	**SPAC1002.13c**	**Protein required for cell wall integrity, member of SUN protein family**	**4**	
	SPAC13G6.10c	**Unknown function**	**4**	**MCB 1**
	SPAP7G5.06	**Protein similar to amino acid permease, a proton symport transporter for all naturally-occurring L-amino acids**	**4**	
	SPAC5H10.06c	**Protein similar to alcohol dehydrogenase IV, which is involved in carbohydrate metabolism**	**4**	

	SPAC1039.02	**Unknown function, possible membrane protein**	**4**	
	SPAC1039.01	Member of amino acid permease family of membrane transporters	4	
	SPBC119.10	Asparagine synthetase	4	Novel 3
	SPCC1235.11	Unknown function, member of UPF0041 uncharacterized protein family	4	
	SPCC126.09	Member of ZIP zinc transporter family, possible metal transporter and vacuolar membrane protein	4	
	SPBC1271.07c	Protein containing acetyltransferase (GNAT) domain	4	FLEX
	SPBC1271.08c	Unknown function	4	
	SPAC139.02c	Probable mitochondrial oxaloacetate transporter	4	
	SPBC13G1.09	Member of bystin family, possible role in 35S pre-rRNA processing into 18S rRNA	4	
	SPCC1494.06c	Member of the DEAD or DEAH box ATP-dependent RNA helicase, possible role in rRNA processing	4	
	SPCC1682.08c	Protein containing six Pumilio-family RNA binding domains, possible role in mRNA metabolism	4	
	SPBC16D10.06	Member of ZIP zinc transporter family	4	
	SPCC1739.01; SPCC1906.05	Member of zinc finger family, which bind DNA or RNA	4	
	SPCC1795.12c	Unknown function	4	FLEX
	SPBC17D1.06; SPCC17D1.06	Member of the DEAD or DEAH box ATP-dependent RNA helicase	4	FLEX
pof6	SPCC18.04	Protein involved in cell division, contains F-box domain	4	Novel 3
	SPCC18.05c	Protein containing nine WD domains (WD-40 repeat), possible membrane receptor	4	Novel 3
	SPCC1827.06c	Aspartate semialdehyde dehydrogenase	4	FLEX
rrn3	SPAC18G6.11c	Protein involved in initiation of transcription of rDNA promoter	4	
	SPAC1F12.05	Unknown function	4	
	SPAC212.08c	Telomeric protein of unknown function, possibly S. pombe specific	4	
	SPAC212.10	Pseudogene; malic acid transport protein; truncated C at terminal	4	
	SPAC24B11.10c	Unknown function, possible protoplast regeneration protein that stimulates chitin synthase III activity	4	
	SPBC25B2.08	Unknown function	4	FLEX

	SPAC2C4.18; SPAC25G10.01	Protein with RNA recognition motif, possible splicing factor that activates pre-mRNA splicing	4	
	SPBC29A3.01	Member of P-type ATPase, similar to copper-transporting ATPase	4	
	SPAC2C4.11c	Unknown function	4	
	SPAC323.07c	Member of the MatE family of integral membrane proteins	4	Novel 3
	SPBC365.16	Unknown function	4	
	SPBC3B8.06	Unknown function	4	
	SPCC548.06c	Protein similar to putative H+-glucose symporter involved in glucose transport	4	Novel 3
arg5	SPBC56F2.09c	Protein similar to amidotransferase small subunit of carbamoylphosphate synthetase	4	
	SPCC594.04c	Unknown function	4	
gmh2	SPAC5H10.13c	Protein similar to alpha-1,2-galactosyltransferase	4	MCB 1
	SPAC6B12.07c	Protein with zinc finger and N-terminal domain that may be involved in G protein associated signal transduction	4	
	SPAC6G9.02c	Protein with five Pumilio-family (Puf) RNA binding domains	4	
	SPCC757.11c	Unknown function	4	
ibp1	SPBC24E9.07; SPBC839.07	Protein phosphatase, rhodanese-like domain	4	Novel 3
	SPAC869.02c	Member of globin family of oxygen transporters, similar to flavohemoglobin that protects from stress	4	
	SPBC8E4.03	Protein with arginase family domain, similar to agmatine ureohydrolase	4	
	SPAC9.10	Member of amino acid permease family of membrane transporters	4	
cig1	SPCC645.01; SPCC4E9.02	B-type cyclin involved in G1 to S phase transition	4	FLEX, MCB 1
csx1	SPAC17A2.09c	Protein containing three RNA recognition motifs, similar to U1 snRNA-associated protein	4	
dak1; dak2	SPAC977.16c	Dihydroxyacetone kinase, isoenzyme II	4	Novel 3
gpd2	SPAC23D3.04c	Glycerol-3-phosphate dehydrogenase	4	Novel 3
mae1	SPAPB8E5.03	Malate transporter	4	
	SPCP1E11.08	Protein similar to nuclear protein involved in ribosome biogenesis	4	
	SPBP8B7.15c	Protein similar to Polyadenylation Factor I complex component	4	

		required for mRNA cleavage and polyadenylation		
pac2	SPAC31G5.11	Regulatory protein involved in sexual development via cAMP-independent pathway	4	
	SPAPB24D3.09c	Protein with ABC transporter domains, similar to brefeldin A resistance protein involved in multidrug resistance	4	
	SPBPB2B2.06c	Protein with calcineurin-like phosphoesterase domain	4	
	SPBP7E8.01; SPBPB7E8.01	Unknown function	4	
TF2-1		Retrotransposable element; tf2-type transposon	4	
TF2-10		Retrotransposable element; tf2-type transposon	4	
TF2-2		Retrotransposable element; tf2-type transposon	4	
TF2-3; TF2-4		Retrotransposable element; tf2-type transposon	4	
TF2-5		Retrotransposable element; tf2-type transposon	4	
TF2-6		Retrotransposable element; tf2-type transposon	4	
TF2-7		Retrotransposable element; tf2-type transposon	4	
TF2-8		Retrotransposable element; tf2-type transposon	4	
TF2-9		Retrotransposable element; tf2-type transposon	4	
bgl2	SPAC26H5.08c	Protein similar to beta-glucosidase, a cell wall endo-beta-1,3-glucanase	4	
	SPBC11C11.05	Member of yeast cell wall synthesis protein KRE9 or KNH1 family	4	FLEX
	SPAC11D3.04c	Unknown functionhypothetical protein; sequence orphan; shows expression on microarray	4	
	SPBC1271.10c	Protein similar to membrane transporter	4	
	SPBC12C2.12c; SPBC21D10.03c	Protein similar to glyoxalase I, contains glyoxalase, bleomycin resistance protein or dioxygenase family domains	4	
	SPBC1347.09	Unknown function	4	
	SPAC1486.09	Protein similar to protein that functions in 20S proteasome maturation and 26S proteasome assembly	4	FLEX
	SPCC1494.07	Unknown function	4	Novel 3
	SPCC1494.08c	Unknown function	4	Novel 2
	SPAC14C4.12c	Unknown function, contains SWIRM domain	4	
	SPAC1527.03	Unknown function, contains La domain	4	
	SPAC16C9.03	Possible role in nuclear export of 60S ribosomal subunits	4	

	SPBC16D10.02	Putative DNA-(amino)methyltransferase	4	Novel 3
	SPBC1711.07	Protein containing three WD domains (WD-40 repeat), possible role in ribosome assembly	4	MCB 1
	SPAC17A2.06c	Unknown function, similar to protein involved in vacuolar sorting	4	
	SPBC17D11.08	Unknown function, four WD domains (WD-40 repeats)	4	
	SPCC1827.05c	Protein with RNA recognition motif, similar to nucleolar protein	4	
	SPCC18B5.07c; SPBC18B5.07c	Member of RanBP1 domain containing family, similar to nuclear pore protein (nucleoporin)	4	
	SPCC1919.05	Protein containing nine TPR domains, similar to superkiller 3, which protects cells from RNA viruses	4	
	SPAC19B12.11c	Unknown function, similar to putative nuclear pore protein involved in bud site selection	4	FLEX, Novel 1
	SPAC19G12.09	Protein with aldo-keto reductase family domain, similar to aldehyde reductase	4	
	SPAC1B3.08	Member of PCI (proteasome, COP9-complex and eIF3) or PINT (Proteasome, Int-6, Nip-1 and TRIP-15) domain family	4	MCB 1
	SPAC823.03; SPAC1E11.03	Protein with kinase domain, similar to CDC-like kinase 2, which may regulate mRNA splicing	4	
	SPBC215.13	Unknown function, similar to protein involved in vesicle formation in endoplasmic reticulum	4	MCB 1
	SPAC222.09	Unknown function	4	
	SPAC23H4.15	Member of DUF663 protein of unknown function family, possible role in rRNA processing and 40S ribosomal subunit biogenesis	4	FLEX
	SPBC24C6.10c	Unknown function	4	
	SPBC19F5.05c; SPBC25D12.01c	Unknown function	4	
	SPAC27D7.09c	Unknown function	4	
	SPAC26F1.07	Protein similar to aldehyde reductase that reduces carbonyl-containing substrates and metabolizes xenobiotics	4	
	SPAC27D7.11c	Unknown function	4	Novel 3
	SPAC27F1.06c	Protein with FKBP-type peptidyl-prolyl cis-trans isomerase domain	4	Ace2
	SPBC29A10.08	Glycolipid-anchored surface protein precursor	4	
	SPAC29B12.08	Unknown function	4	MCB 1

	SPAC31A2.07c	Putative RNA helicase, possible role in ribosome biogenesis	4	
	SPAC31G5.02	Possible role in role cell wall organization and biogenesis	4	
	SPCC31H12.01; SPCC1183.11	Member of mechanosensitive ion channel family	4	
	SPAC328.05	Protein containing three RNA recognition motifs, possible role in protein-nucleus export	4	FLEX
gps2	SPBC365.14c	Putative UDP-glucose 4-epimerase involved in UDP-galactose synthesis and protein glycosylation	4	
	SPAC3G9.05	Unknown function, similar to cell polarity and cell fusion protein	4	
	SPBC405.02c; SPBC4C3.01	Unknown function	4	FLEX
	SPCC417.05c	Unknown function, similar to protein that stimulates chitin synthase III activity	4	
	SPBC428.10	Unknown function, similar to cell surface flocculin required for invasive and pseudohyphal growth	4	Novel 3
	SPAC521.02	Unknown function	4	
sst1	SPAC521.04c	Member of sodium or calcium exchanger protein family of membrane transporters	4	
	SPCC553.10	Unknown function	4	FLEX, MCB 1
	SPAC57A10.09c	Protein similar to DNA-binding and DNA-bending protein involved in transcriptional activation, contains HMG family domain	4	
	SPAC637.13c	Unknown function, pleckstrin homology (PH) domain	4	
	SPBC651.01c; SPBC725.18c	Unknown function, similar to a putative nucleolar GTP-binding protein required for ribosomal subunit biogenesis	4	FLEX
	SPBC660.06	Unknown function	4	
	SPAC688.11	Protein with actin binding domains, similar to talin-like protein required for hyphal growth	4	
	SPCC794.03	Member of amino acid permease family of membrane transporters	4	
	SPCC794.11c	Protein with actin binding domain, possible role in formation of clathrin coats at the Golgi and endosomes	4	MCB 1
	SPAC821.03c	Unknown function	4	
	SPAC9.07c	Putative GTP-binding protein	4	
	SPAC9E9.04	Unknown function, contains predicted N-term signal sequence and transmembrane helices	4	FLEX

cbh2	SPBC14F5.12c	DNA binding protein, possible role in chromosome segregation, role in histone tail modifications at centromere	4	
cdc2; swo2	SPBC11B10.09	Cyclin-dependent kinase, regulates cell cycle transitions G1/S and G2/M	4	
cds1	SPCC18B5.11c	Protein kinase involved in unreplicated DNA checkpoint response	4	FLEX
	SPACUNK4.15	Unknown function	4	Ace2
dim1	SPBC336.02	Protein similar to ribosomal RNA adenine dimethylases	4	
git3	SPCC1753.02c	Protein involved in cAMP pathway and required for growth under high osmotic stress	4	
myo52; myp5; myo4	SPCC1919.10c	Class V myosin, involved in polarized cell growth and vacuole fusion	4	FLEX, MCB 1
	SPAC31G5.12c	Unknown function, similar to negative effector of RNA polymerase III	4	
nrd1	SPAC2F7.11	Protein containing four RNA recognition motifs	4	
	SPAPB15E9.01c; SPAPB18E9.06c	Unknown function	4	
	SPAPB18E9.03c	Unknown function	4	
	SPAPB18E9.05c	Unknown function	4	
pcr1; mts2	SPAC21E11.03c	Transcription factor that plays roles in mating, meiosis and stress response	4	
pim1; ptr2; dcd1	SPBC557.03c	GTP-exchange factor (GEF) for Spi1p, required for nucleo-cytoplasmic transport, microtubule function and cytokinesis	4	
pka1; git6	SPBC106.10	Catalytic subunit of the cAMP-dependent protein kinase	4	
rdp1	SPAC1B1.01	Protein containing a C2H2 type zinc finger domain, binds to DNA damage response elements	4	Ace2
ssp1	SPCC297.03	Protein kinase that mediates rapid osmotic stress response at cell surface	4	
thi1; ntf1	SPAC6G10.01; SPAC1486.10	Regulatory protein for thiamine repressible genes, required forsynthesis of thiazole moiety of thiamine	4	MCB 1
	SPAC6F12.03c	Member of SNARE domain containing family	4	
top2	SPBC1A4.03c	DNA topoisomerase II	4	MCB 1
trx1; trx2	SPAC7D4.07c	Putative thioredoxin involved inresponse to heavy metals	4	FLEX
uvi15	SPBC649.04	Protein essential for stationary phase survival, induced by stress	4	Novel 2

vip1	SPAC10F6.06	Protein containing an RNA recognition motif	4	
	SPCC320.02c; SPCC1235.01	Unknown function	N(1)	FLEX, Histone
prl3		Non-coding RNA	N(1)	
hsk1	**SPBC776.12c**	**Protein kinase of the Hsk1p-Dfp1p complex involved in S phase initiation**	**N(1,2)**	
	SPCC338.08	**Unknown function**	**N(1,2)**	
mfm2	**SPAC513.03**	**Precursor polypeptide for mating pheromone M factor produced by h- cells**	**N(1,2)**	
spk1	SPAC31G5.09c	MAP kinase (MAPK) acting in the mating and sporulation pathways	N(1,2)	
byr2; ste8	SPBC2F12.01; SPBC1D7.05	MAP kinase kinase kinase acting upstream of MAPKK Byr1p and MAP kinase Spk1p in pheromone signaling pathway	N(1,2)	
	SPAC1006.06	Protein with RhoGEF domain, similar to Rho GDP-GTP exchange factor activated by cell wall defects	N(1,2)	
	SPAC12G12.06c	Probable RNA 3'-terminal phosphate cyclase	N(1,2)	
	SPCC1322.09	Unknown function	N(1,2)	MCB 2
	SPAC14C4.05c	Unknown function	N(1,2)	FLEX, MCB 2
	SPBC1683.07	Protein similar to alpha-glucosidase	N(1,2)	Ace2
hri1	SPAC20G4.03c	Translation initiation factor 2 alpha kinase, may play role in negative regulation of eIF2alpha in response to stress	N(1,2)	
	SPBC365.01	Unknown function, CRAL-TRIO domain and a CRAL or TRIO N terminus domain	N(1,2)	
din1	SPAC19D5.06c	Unknown function	N(1,2)	Novel 2
mei2	SPAC27D7.03c	RNA-binding protein involved in meiosis	N(1,2)	
spm1; pmk1	SPBC119.08	MAP kinase involved in maintenance of cell wall integrity	N(1,2)	FLEX
	SPCC965.06	Protein similar to potassium voltage-gated channel	N(1,2,3)	Novel 1
cnd2	SPCC306.03c	Subunit of condensin complex involved in chromosome condensation	N(1,3)	
isp6; prb1	SPAC4A8.04	Putative subtilase-type proteinase, role in sexual differentiation+E49	N(1,3,4)	
	SPAC869.05c	**Member of sulfate transporter family, similar to sulfate permease**	**N(1,4)**	
	SPAC1002.17c	Protein with phosphoribosyl transferase domain, possible role in pyrimidine salvage pathway	N(1,4)	

	SPAC1610.04	Unknown function	N(1,4)	
	SPCC16C4.06c	Protein with tRNA pseudouridine synthase domains	N(1,4)	Novel 1
sim4	SPBC18E5.03c	Centromere-associated protein required for chromosome segregation and silencing	N(1,4)	
	SPBC19G7.07c	Member of PPR repeat containing family	N(1,4)	
	SPCC330.15c; SPCC320.14	Member of pyridoxal phosphate dependent enzyme family, similar to racemase that catalyzes the racemisation of L-serine to D-serine	N(1,4)	
	SPBC428.17c	Unknown function	N(1,4)	
	SPCC553.07c	**Member of impB, mucB or samB family, possible role as translesion DNA repair polymerase**	**N(2,3)**	
	SPBC800.11	**Protein with inosine-uridine preferring nucleoside hydrolase domain**	**N(2,3)**	
	SPAC750.05c	**Telomeric protein of unknown function, highly similar to S. pombe SPAC977.01, SPAC1348.02 and SPBPB2B2.19c**	**N(2,3)**	
	SPBC1348.02; SPAC1348.02	**Telomeric protein of unknown function, highly similar to S. pombe SPAC977.01, SPAC750.05C and SPBPB2B2.19c**	**N(2,3)**	
	SPBPB2B2.19c	**Telomeric protein of unknown function, highly similar to S. pombe SPAC977.01, SPAC1348.02 and SPAC750.05C**	**N(2,3)**	**Histone**
	SPAC977.01	**Telomeric protein of unknown function, highly similar to S. pombe SPAC1348.02, SPAC750.05C and SPBPB2B2.19c**	**N(2,3)**	**FLEX**
	SPBC409.22c; SPBC1306.01c	**Protein with elongation factor Tu GTP binding domain, similar to mitochondrial translation elongation factor G**	**N(2,3)**	
	SPAC17G6.03	Protein with calcineurin-like phosphoesterase domain	N(2,3)	
	SPBC18E5.07	Unknown function	N(2,3)	Novel 2
	SPBC21B10.07; SPAC21B10.07	Protein with glycosyl hydrolase family 16 domain	N(2,3)	
	SPAC2E1P3.04	Protein with possible role in detoxifying extracellular amines and nitrogen metabolism	N(2,3)	Ace2, Novel 3
	SPAC29A4.05	Protein similar to calmodulin 1, which regulates the calcium-dependent activity of enzymes including phosphatases	N(2,4)	
	SPAC631.02	**Protein with two bromodomains, which interact with acetylated lysine**	**N(3,4)**	
	SPBC1271.09	**Member of sugar (and other) transporter family, possible role in inositol metabolism**	**N(3,4)**	**Novel 3**

	SPCC364.07; SPCC4G3.01	Protein similar to 3-phosphoglycerate dehydrogenase, which catalyzes first step in synthesis of serine	N(3,4)	
	SPAC3A11.10c	Member of Rnal dipeptidase family, zinc-dependent metalloproteinases that hydrolyze various dipeptides	N(3,4)	
	SPAC664.03	Member of Paf1 family, components of RNA polymerase II associated complexes	N(3,4)	Ace2, FLEX
pro1	SPAC821.11	Protein similar to gamma-glutamyl phosphate reductase involved in proline biosynthesis	N(3,4)	
fim1	SPBC1778.06c	Fimbrin, role in actin organization during medial ring formation and polarized growth	N(3,4)	
	SPBPB21E7.09; SPAPB21E7.09	Protein similar to L-asparaginase II	N(4)	FLEX, MCB 1, Novel 3

[a] Gene descriptions are based on the information in PombePD (www.incyte.com/control/tools/proteome) and *S. pombe* GeneDB (www.genedb.org/genedb/pombe/index.jsp) databases.

Genes are sorted according to the cluster they belong to; genes in bold are of 'high amplitude' and the unclassified genes (N) are listed at the bottom of the list.