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Transcriptional Regulation at the G2/M Transition in the Budding Yeast, *Saccharomyces cerevisiae*

by
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Declaration

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Abstract

The eukaryotic cell cycle is a highly ordered and tightly regulated process through which a cell replicates its genetic material, segregates its sister chromatids and divides into two genetically identical cells. Regulation and ordering of the cell cycle is through cyclin dependent kinases (cdk). The cdk is a protein complex that is comprised of a catalytic subunit (cdk) and a regulatory subunit (cyclin) which, as their name indicates, oscillate throughout the cell cycle giving specificity to the cdk for different phases of cell division. This oscillation is controlled, at least in part, through gene regulation.

Microarray analysis performed on the budding yeast, *Saccharomyces cerevisiae*, revealed several waves or "clusters" of transcriptional activity associated with cell cycle progression. One of these "clusters", the "CLB2 cluster", is comprised of 35 genes that are important for the G2/M transition and mitotic progression. Previous work on the upstream activating sequences of two "CLB2 cluster" members, *CLB2* and *SWI5*, revealed the binding of an Mcm1p homodimer and, until recently, an unidentified activity SFF (*SWI5* Factor). Recently in our laboratory an activity that followed SFF binding *in vitro* was purified and identified as Fkh2p.

In this thesis the biochemical and genetic characterisation of Fkh2p identifies it as a major component of SFF. Firstly, Fkh2p has been shown to bind DNA in an Mcm1p

dependent manner and that the Fkh2p DNA binding domain is essential for this interaction. Furthermore, the protein interaction domain of Mcm1p has been demonstrated to be essential for ternary complex formation. Through deletion studies, Fkh2p, along with a functionally redundant protein Fkh1p, have been shown to control the periodic expression of the "CLB2 cluster" genes. Furthermore, the functional characterisation of Fkh2p domains reveals an important role for both the Forkhead associated domain and the C-terminus. Finally, Ndd1p, another protein important for mitotic progression, is shown to be important for "CLB2 cluster" regulation by de-repressing Fkh2p and activating gene expression. In addition, the role of cdk activity is shown to act through the "CLB2 cluster" upstream activating sequences, possibly through Ndd1p.

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Chapter 1:

INTRODUCTION

1.0 Introduction

1.1 The Cell Cycle

The eukaryotic cell cycle is a highly ordered and tightly regulated process through which a cell replicates its genetic material and divides into two genetically identical cells. There are four phases of the cell cycle that occur in a conserved order ensuring the cell replicates its genetic material once before it is segregated and the cell divides. S-phase is where the DNA is replicated whereas M-phase divides the duplicated genome into two genetically identical pools before the cell divides. These two phases are split by two gap phases G1 and G2. During G1, the cell makes the irreversible decision to enter into another cell cycle based on internal and external signals, whereas the G2-phase allows the cell to check the integrity of the DNA after replication and prepare for cell division (figure 1.1). This ordering of the cell cycle is tightly regulated by cyclin dependent kinases (cdk's).

1.2 CDK's and the budding yeast cell cycle

Cdk's are proline-directed kinases that phosphorylate serine and threonine residues at S/T-P-X-R/K motifs (where X is any amino acid) (Langan *et.al.*, 1989). In the budding yeast *Saccharomyces cerevisiae*, there are five identified members of the cdk family, Cdc28p, Pho85p, Kin28p Ssn[a] and Ctk1 (Hartwell, 1974, Cismowski *et.al.*, 1995, Kuchin *et.al.*, 1995 and Sterner *et.al.*, 1995). Of these five family members, Cdc28p is

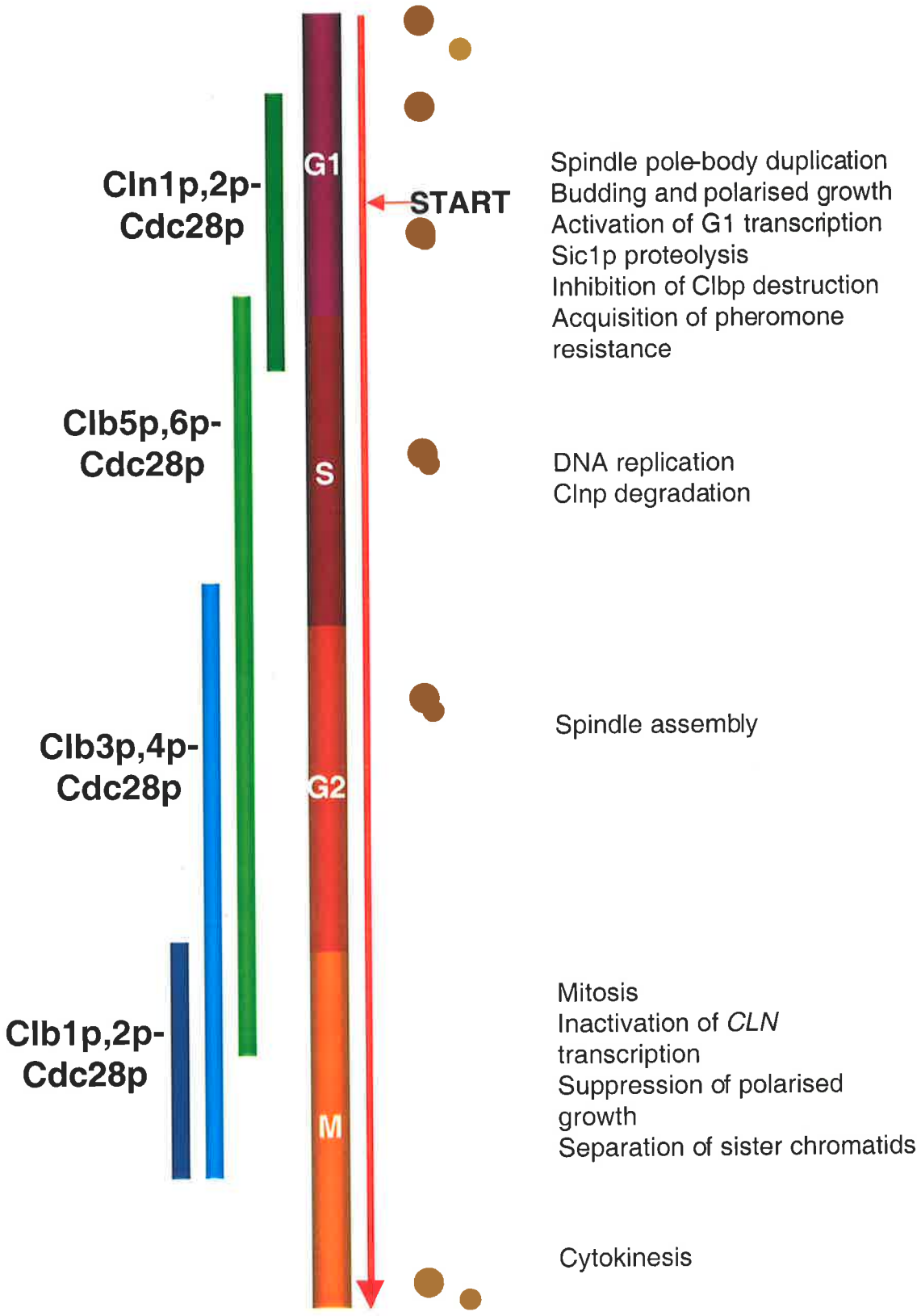


Figure 1.1: Schematic diagram of Cdk activity through the budding yeast cell cycle. Cell cycle progression is influenced by subsequent waves of Cdk activity. Roles for the different Cdk activity and cell budding are indicated.

the only one identified as essential for cell cycle progression (Hartwell, 1974) and whose kinase activity is cell cycle regulated (Reed *et.al.*, 1985 and Wittenburg and Reed, 1989). Furthermore, different *cdc28* temperature sensitive mutants (*ts*) exhibit cell cycle arrest in different phases of the cell cycle (Mendenhall *et.al.*, 1988, Reed and Wittenburg, 1990 and Surana *et.al.,* 1991). For example, *cdc28-4*^{ts}, along with other *ts* mutants, arrest in G1 prior to START (Mendenhall *et.al.,* 1988) and when allowed progress through START at the permissive temperature they arrested in G2 (Reed and Wittenburg, 1990). *cdc28-1n*^{ts} is another *ts* mutant that primarily exhibits a G2 arrest at the restrictive temperature (Surana *et.al.*, 1991). Cdc28p levels are constitutive throughout the cell cycle (Mendenhall et.al., 1987) so the regulation of its activity has been attributed to post-translational modifications.

1.2.1 Regulatory subunit, Cyclin

Through microarray analysis, several overlapping waves of transcription over the cell cycle have been identified and the genes represented in these waves have been designated as "clusters" (Spellman *et.al.*, 1998). Cyclins are members of different clusters and are reliant on each successive wave of transcription (figure 1.1 and 1.2) and gives specificity to the cdk for specific phases of the cell cycle. In the budding yeast *Saccharomyces cerevisiae*, there are nine Cdc28p-binding cyclins which are divided into two main groups; G1 cyclins (*CLN's 1 - 3*) (Tyers and Futcher, 1993 and Wittenburg *et.al.*, 1990) and B-type cyclins (*CLB's 1-6*) (Schwob and Nasmyth, 1993

and Surana *et.al.*, 1991). The binding of its cyclin partner produces a conformational change within Cdc28p that facilitates its activation (Jeffery *et.al.,* 1995).

The crystal structure of human Cdk2 has been determined and is used as a model for other cdk's such as the budding yeast Cdc28p. Other monomeric kinases such as the cyclic AMP –dependent protein kinase (cAMP) have a bilobed structure with the catalytic core lying between these two lobes (Knighton *et.al.*, 1991). Cdk kinases also possess this bilobed structure, however in its monomeric form there is a displacement of one lobe in relation to the other. This displacement results in a misalignment of key catalytic residues involved in the kinase reaction and covers the substrate-binding site of Cdk2 and has been attributed to the inactivity of the monomeric form (de Bondt *et.al.*, 1993). The binding of cyclin partners results in conformational changes which align the two cdk lobes opening the substrate binding site and aligning the catalytic residues (Jeffery *et.al.*, 1995).

CLN1 and *CLN2* were first isolated as high copy number suppressors of the *cdc28-4*^{ts} mutant (Hadwiger *et.al.*, 1989). The levels of *CLN1* and *CLN2* oscillate with peak expression in G1, just before START (figure 1.2) and Cln1p and Cln2p have been shown to interact with and activate Cdc28p through co-immunoprecipitation experiments (Wittenburg *et.al.,* 1990). Single deletion of the *CLN's* has little or no effect on cell cycle but double deletion lengthens a cells G1 phase and causes an aberrant phenotype

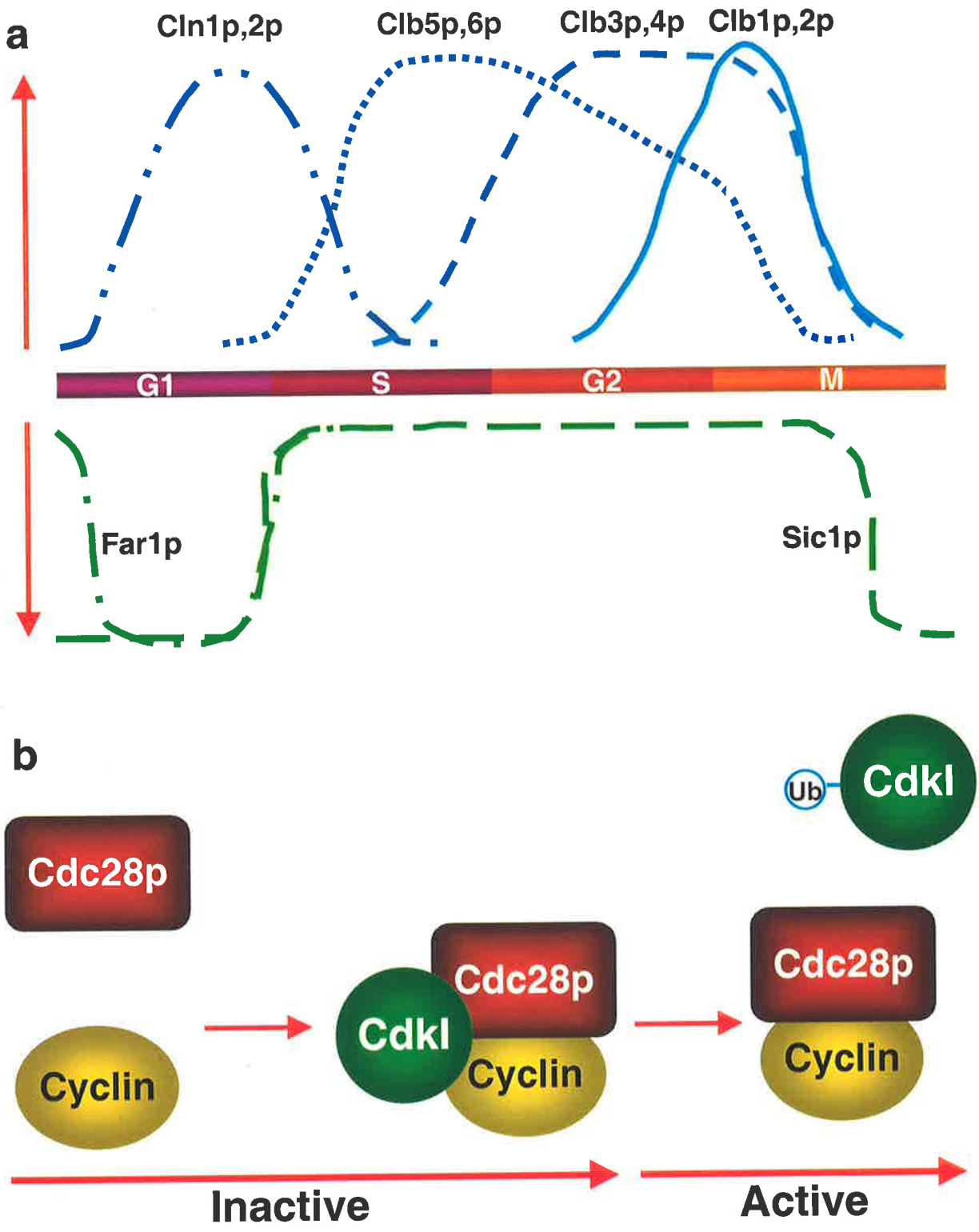


Figure 1.2: Schematic diagram of cyclin expression, CdkI activity and active Cdk complex. **a** The expression of cyclins and CdkI over the cell cycle where Cln1p, 2p are G1 cyclins, Clb1p-6p are B-type cyclins and Far1p and Sic1p are Cdk inhibitors. **b** Active Cdk requires cyclin binding. The binding of CdkI inhibits the Cdk/Cyclin complex. Its release and degradation allows Cdk activation.

whereas the construction of a stable Cln2p accelerates entry into S-phase (Hadwiger *et.al.,* 1989). These experiments show an importance for Cln1p, 2p in G1 events and they have been shown to control budding, the cell cycle, spindle pole body duplication and initiating DNA replication. *CLN3*, another *CLN*, is not highly periodic with only a slight increase in levels just prior to G1 (Tyers *et.al.,* 1993) and has been implicated in activating *CLN1* and *CLN2* expression (Stuart and Wittenburg, 1995).

The six B-type cyclins are paired into three groups based on transcription profile; *CLB1,2, CLB3,4* and *CLB5,6*. *CLB5,6* are co-expressed with *CLN1,2* (figure 1.2) and overexpression of these *CLB's* can suppress a *cln* delete lethality indicating an overlapping role in initiating entry into the cell cycle (Schwob and Nasmyth, 1993 and Basco *et.al.,* 1995). Even though *CLB5,6* are expressed along with *CLN1,2* in wild-type cells, they are held inactive by the presence on the Clbp-kinase inhibitor Sic1p until Clnp kinases initiate Sic1p degradation (Schwob *et.al.,* 1994). There are three primary roles for Clb5p,6p activity; the correct firing of origins of replication, prevention of re-initiation of replication origins and the negative regulation of Clnp kinase activity (Schwob *et.al*, 1994, Dahmann *et.al.*, 1995 and Basco *et.al.*, 1995). Cells lacking Clb5p have longer S-phases and those that are *Δclb5, Δclb6* double delete have a delay in S-phase initiation (Schwob and Nasmyth, 1993) indicating an important role for these kinases in S-phase control.

CLB3,4 were identified as high copy number suppressors of the *cdc28-1n*^{ts} mutant (defective in G2 function) (Surana *et.al.*, 1991). The transcript levels of these two cyclins begin to increase in S-phase as Clnp's begin to disappear and remain high until late anaphase (Richardson *et.al.*, 1992) (figure 1.2). These two cyclins contain a destruction box targeting them for APC dependent destruction in anaphase (Fitch *et.al.*, 1992). *CLB3, 4* are not essential (Richardson *et.al.*, 1992 and Schwob and Nasmyth, 1993) but deleting them in conjunction with *clb5* is inviable (cannot make spindles) and with *clb5,6* deletes has difficulty initiating S-phase (Schwob and Nasmyth, 1993). Therefore, it has been proposed that Clb3p,4p play an important role in spindle formation (although Clb1p, 2p, can accomplish this role) (Richardson *et.al.*, 1992).

CLB1,2 were cloned along with *CLB3,4* as high copy suppressors of the *cdc28-1n*^{ts} mutation (Surana *et.al.*, 1991) and like Clb3p, 4p, they contain destruction boxes used to target them for destruction at anaphase (Fitch *et.al.*, 1992). Deletion of the Clb2p destruction box impairs a cell's ability to exit mitosis due to elevated Clb2p-Cdk kinase activity (Surana *et.al.*, 1993, Wasch and Cross, 2002). The transcripts of these two genes begin to accumulate during G2 and peak in mitosis (Surana *et.al.*, 1991) (figure 1.2) which is indicative of a role in mitotic control. Supporting this, deletion of *clb2* in combination with either *clb1,3,4* is lethal with an arrest phenotype prior to mitosis (Surana *et.al.*, 1991). Interestingly, the single deletion of *clb2* or the triple *clb1,3,4* deletions are viable (Surana *et.al.*, 1991 and Amon et.al., 1993). This indicates that

Clb2p has the major role in mitosis, which is supported by its role in spindle elongation and negative regulation of bud emergence (Lew and Reed, 1993). Importantly for cell cycle timing, Clb2p kinase activity inhibits Swi4-dependent Binding Factor (SBF) activity, inhibiting Cln1p,2p production and inhibiting the cells from entering another round of the cell cycle until sister chromatids have been separated and cell division has occurred (Amon *et.al.,* 1993).

It is the regulation of Cyclins at the level of transcription and their subsequent association with the Cdk (the rate-limiting step in cell cycle progression) that brings order to the cell cycle.

1.2.2 Regulation of Cdc28p by phosphorylation

Other than cyclins, the phosphorylation of Cdc28p is important for Cdk regulation in both a stimulatory and inhibitory fashion. The major site of activating phosphorylation is T169 (Cismowski *et.al.*, 1995 and Deshaies and Kirschner 1995) which results in a conformational change in the Cdk structure opening the substrate binding site and optimising the interaction with its cyclin binding partner (Russo *et.al.,* 1996). Phosphorylation of monomeric and complexed Cdc28p occurs through cdk activating kinase 1 (*CAK1*) (Espinoza *et.al.*, 1996, Kaldis *et.al.,* 1996 and Thuret *et.al.*, 1996 and Cross and Levine, 1998) (figure 1.3). Experiments using different *ts* mutants of Cak1p generate different arrest phenotypes indicating the presence of Cak1p at the various stages of the cell cycle is important for progression (Thuret *et.al.*, 1996, Kaldis *et.al.*, 1996, Chun and Goebl, 1997 and Sutton and Freiman 1997). *CAK1* has been shown to

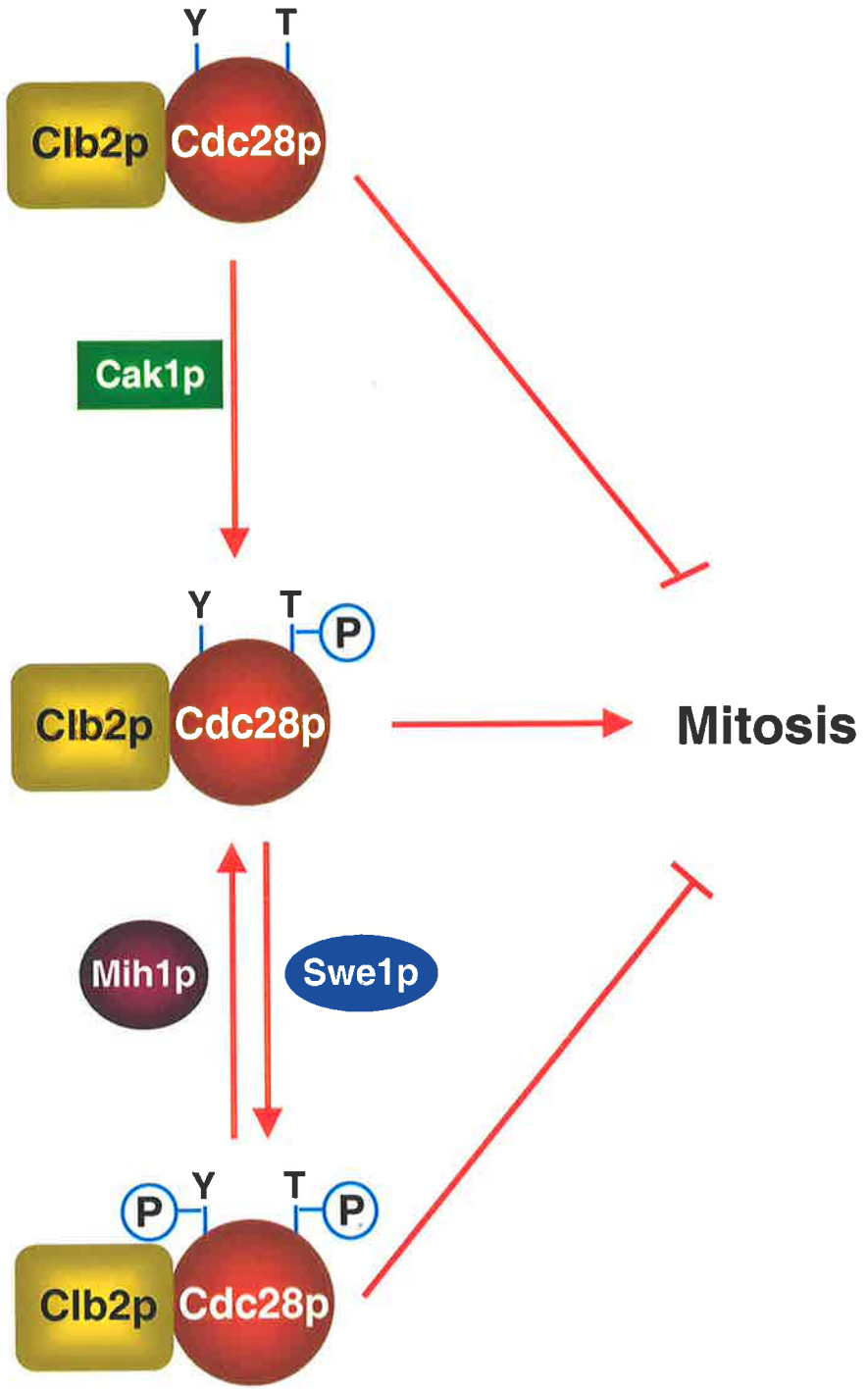


Figure 1.3: Cdk activity is controlled by phosphorylation. At the G2-M transition, the mitotic Cdk activity is regulated by phosphorylation events. Phosphorylation at T169 is activating, conversely phosphorylation at Y19 is inhibitory.

be an essential gene with its only essential role being Cdc28p phosphorylation through the creation of a *cdc28-T169E* mutant that no longer requires Cak1p for viability (Cross and Levine, 1998). In fact, Cak1p activity appears to be constitutive throughout the cell cycle because there is no noticeable change to the T169 phosphorylation state of Cdc28p (Hardwiger and Reed, 1988 and Amon *et.al.*, 1992). ***This indicates the phosphorylation of T169 is not the rate-limiting step of Cdc28p activation but is reliant on cyclin association*** and removal of inhibitory phosphorylation. This latter phosphorylation occurs on tyrosine 19 (Y19) of Cdc28p during S and G2 phases of the cell cycle and is proposed to control the timing of entry into mitosis (Amon *et.al.*, 1992, Sorger and Murray, 1992 and Lim *et.al.*, 1996) and plays a secondary role in checkpoint control when bud formation is delayed (Lew and Reed, 1995).

Y19 is phosphorylated by Swe1p, a protein kinase similar to *wee1* in *S. pombe*, which is specific for Clb2p/Cdc28p complexes (mitotic cdk's) (Booher *et.al.*, 1993). Swe1p overexpression arrests cells prior to mitosis with short mitotic spindles whereas its deletion has little or no effect on the cell cycle (Booher *et.al.*, 1993) and in addition, construction of a *cdc28-Y19F* allele suppresses the requirement for Swe1p (Lim *et.al.*, 1996). This action of Swe1p is opposed by the protein phosphatase Mih1p (Russel *et.al.*, 1989) (figure 1.3).

Therefore, two phosphorylation events on Cdc28p are important for mitotic entry. First, the essential activation of Clb2p/Cdc28p by T169 phosphorylation and secondly, the inhibitory phosphorylation of Y19 by Swe1. Progression occurs only when the inhibitory phosphorylation of Y19 is removed by the phosphatase Mih1p (figure 1.3). In addition, phosphorylation at T169 is important for optimal cyclin-Cdk activity throughout the cell cycle.

1.2.5 Cdk Inhibitors

In the budding yeast there are two main Cdk inhibitors that regulate Cdk activity. Far1p, first discovered as essential for pheromone-directed cell cycle arrest (Chang and Herskowitz, 1990), has been shown to be an inhibitor of Clnp-Cdc28 kinase activity (Peter and Herskowitz, 1994, Tyers and Futcher, 1993 and Jeoung *et.al.,* 1998). Peak expression of Far1p occurs in G1 after which it is degraded early in S-phase (McKinney *et.al.,* 1993) (figure 1.2). Furthermore, Far1p has been found to bind to Cln1,2-Cdc28p Cdk complexes and when deleted, cells have a shorter G1 phase (McKinney and Cross, 1995), which is indicative of a role in G1 control, in particular, cell cycle entry. Sic1p is another Cdk inhibitor isolated as a substrate of Cdc28p and inhibits Clbp-Cdc28p kinase activity (Mendenhall, 1993, Schwob *et.al.,* 1994). It is cell cycle regulated with peak expression in G1 and is directed for destruction by Clnp-Cdk activity (Mendenhall *et.al.*, 1987) (figure 1.2). There seems to be two roles for Sic1p. First, inhibition of Clb5p,6p-Cdc28p kinase until the initiation of S-phase and spindle pole body duplication (Schwob *et.al.*, 1994) and secondly, in helping the cells to exit mitosis by

inhibiting Clb1,2p-Cdc28p kinase activity (Toyn *et.al.,* 1996). So it seems that both these inhibitors operate in G1 to regulate Cdk kinase activity so the cell cycle will progress when ready and in the correct order (figure 1.2).

1.3 Cell cycle Progression

A theory was put forward by Nasmyth (1996) stating that the ordering of the cell cycle is controlled by the levels of B-type cyclin cdk activity. At the beginning of S-phase, B-type cyclin-cdk activity initiates DNA replication by firing the origins of replication, releasing the complexes needed for initiation and inhibiting them from reforming. Therefore, re-replication cannot occur until the end of mitosis when B-type cyclins are destroyed and the reformation of ORC (origin of replication complexes) can occur in preparation of another round of cell cycle. This dual role for B-type cyclins in initiating and inhibiting replication ensures the cells only go through one round of DNA synthesis and chromatid segregation per cell cycle. The production of B-type cyclins is controlled at the level of transcription and is first initiated by Clnp-Cdk activity in G1. Microarray analysis revealed the "G1 cluster" was comprised of those genes required for entry into and passage through S-phase (Spellman *et.al.*, 1998). These genes include the major Cdc28p regulators *CLN1*, *CLN2* (Wittenburg *et.al.,* 1990) and *CLB5*, *CLB6* (Schwob and Nasmyth, 1993) and other genes that are necessary for DNA replication.

1.3.2 G1 to S-phase Transition

1.3.2.1 G1 to S-phase Transcriptional Control

In G1, Cln3p-Cdk activity starts to accumulate and when it reaches a threshold of activity initiates late G1 transcription through the activation of the transcription factor complex Swi4p dependent Binding Factor (SBF) and Mlu1p Binding Factor (MBF) (Dirick *et.al.*, 1995) (figure 1.4). The initial activation of SBF through Cln3p-Cdk activity produces *CLN1,2*, the G1 cyclins, which establish a positive feedback loop to enhance the SBF and MBF activity until it is switched off via proteolysis (Koch *et.al.*, 1996, Dirick *et.al.*, 1995, Schwob *et.al.*, 1994, Dirick and Nasmyth, 1991 and Nasmyth and Dirick, 1991). Furthermore, SBF and MBF activity produces Clb5p,6p along with the genes necessary for DNA replication and inhibits the cells ability to exit the cell cycle in response to pheromones (Oehlen and Cross, 1994, Dirick *et.al.*, 1995 and Schwob *et.al.*, 1994).

SBF and MBF are protein complexes that share one factor, Swi6p, and contain a specific DNA binding partner. In the case of SBF the binding partner is Swi4p and for MBF, Mbp1p (Primig *et.al.*, 1992 and Koch *et.al.*, 1993). These complexes bind DNA elements known as the SCB (Swi4p/Swi6p dependent cell cycle box) (Sidorova and Breeden, 1993 and Primig *et.al.*, 1992) and MCB (*Mlu*I cell cycle box) (McIntosh *et.al.*, 1991) but binding alone does not initiate transcription. Studies have shown MBF is constitutively bound throughout the cell cycle but only activates genes in S-phase

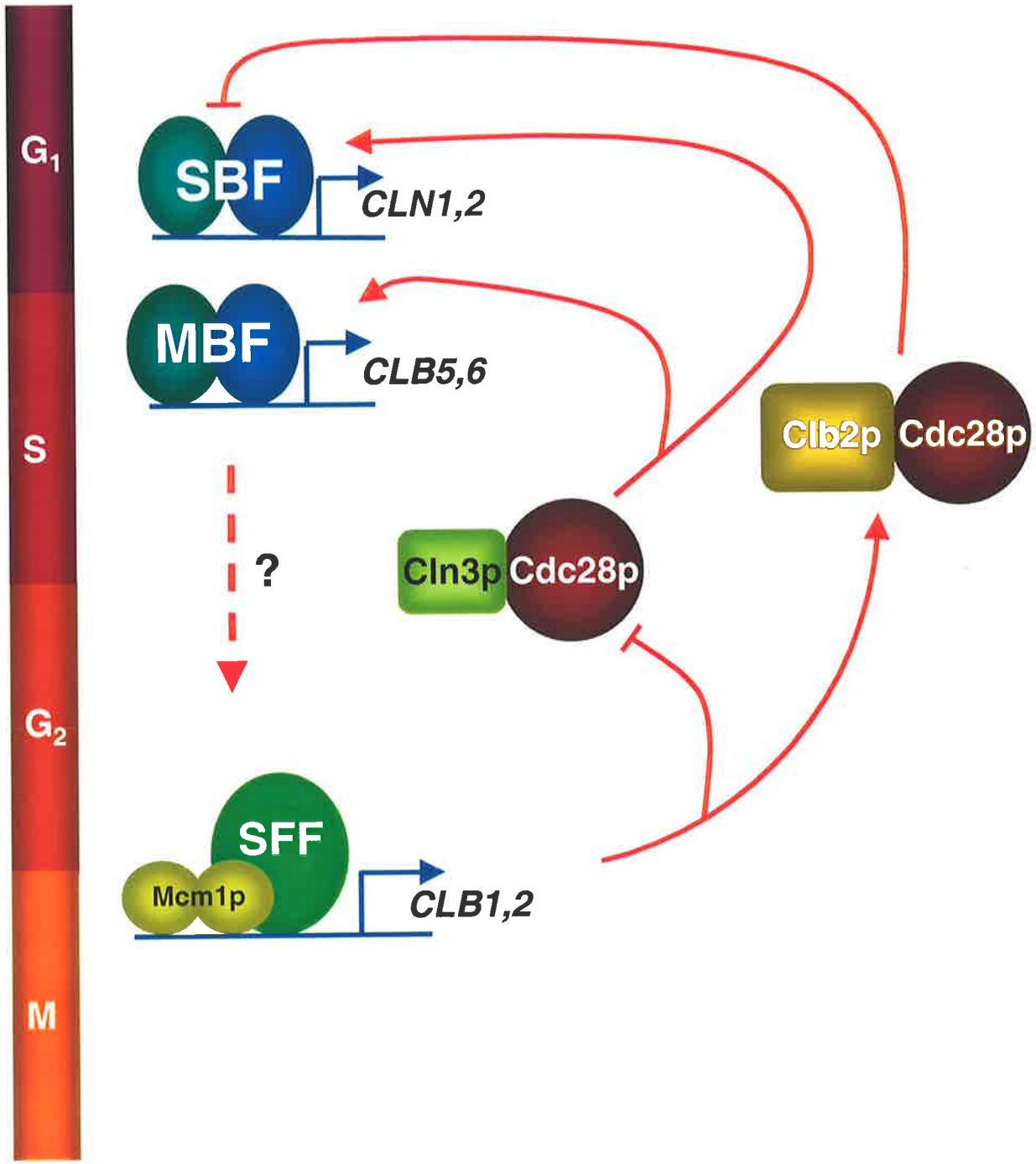


Figure 1.4: Cdk activity regulates cell cycle transcription. Activation of the "G1 cluster" via SBF and MBF occurs through Cln3p/Cdc28p activity. This activation is repressed by Clb2p/Cdc28p switching off SBF activated transcription.

whereas SBF is only bound prior to activation of genes but its continual presence does not eradicate periodic expression (Dirick *et.al.,* 1992 Koch *et.al.,* 1996 and Breeden and Mikesell, 1994). This indicates another mode of transcriptional activation, which is determined by the presence of Cdc28p complexes.

As the cells progress through the cell cycle SBF is repressed by Clb2p/Cdc28p activity through sequestration and subsequent phosphorylation of Swi4p ensuring cells do not traverse Start and prematurely enter another round of cell division (Koch *et.al.*, 1996 and Amon *et.al.*, 1993) (figure 1.4). MBF controlled promoters are not inhibited by Clb2p/Cdc28p but cells are unable to enter into S phase without SBF activity (Amon *et.al.*, 1993).

1.3.2.2 S-phase Proteolysis

Along with transcriptional regulation at the G1-S transition, proteolysis works to regulate Clnp- and Clbp-Cdk activity through the regulation of Cdk inhibitors and cyclin binding partners. The proteins that are involved in Start proteolysis contain PEST-like sequences that are regions rich in proline (P), glutamic acid (E), serine (S) and threonine (T) and hence, contain many (T/S)-P motifs which is the minimal requirement for Cdk phosphorylation. Studies of Sic1p (Verma *et.al.*, 1997 and Schwob *et.al.,* 1994), Far1p (McKinney *et.al.,* 1993, Peter *et.al.*, 1993 and Henchoz 1997) and Cln2p (Deshaies *et.al.*, 1995, Barral *et.al.*, 1995 and Lanker *et.al.*, 1996)

proteolysis have all shown links between cdk phosphorylation, ubiquitination and proteolysis.

In ubiquitination, a single ubiquitin is attached to E1 (ubiquitin-activating enzyme) in an ATP dependent manner. The ubiquitin is then transferred to E2 (ubiquitin conjugating enzyme) from where it can be covalently bound to the target protein on a lysine residue. In some cases (especially Start and Anaphase proteolysis) a third enzyme (E3) is needed. E3 is a ubiquitin ligase which not only gives specificity to E2 for substrates, but also participates actively in the ubiquitin reaction (review Ciechanover and Schwartz, 1998). In the budding yeast, E1 is Uba1p (McGrath *et.al.,* 1991) and the essential E2 is Cdc34p (Goebl et.al., 1988), although there are 12 other potential E2 genes and in S-phase proteolysis, the E3 is a protein complex known as SCF. The SCF protein complex is comprised of common subunits **S**kp1p and **C**dc53p and a third variable subunit known as **F** box proteins (Bai *et.al.*, 1996) which determines substrate specificity (Patton *et.al.* **(a),** 1998 and Skowyra *et.al.,* 1997). The F-box family of proteins contain a conserved motif of approximately 44 amino acids and in *S. cerevisiae,* there are 17 proteins that contain the F-box, of which two (Cdc4p and Grr1p) have been shown to play important roles in Cdc28p regulation (Bai *et.al.*, 1996 and Patton *et.al.*, 1998 (**b**)). SCF complexed with a WD repeat protein, Cdc4p (SCF^{Cdc4}) recognises Sic1p, Far1p and Cdc6p and targets them for degradation (Bai *et.al.,* 1996, Skowyra *et.al.*, 1997, McKinney *et.al.*, 1995, Henchoz *et.al.,* 1997 and

Piatti *et.al.*, 1996) and in the cases of Sic1p and Far1p, the interaction with SCF^{Cdc4} is reliant on prior Cdk phosphorylation (Skowyra *et.al.*, 1997 and Henchoz *et.al.*, 1997). Grr1p, when complexed with SCF (SCF^{Grr1}), targets Cln1p and Cln2p for degradation and, as for Cdc4p, this interaction is dependent on Cdk phosphorylation of the target proteins (Barral *et.al.,* 1995, Skowyra *et.al.*, 1997 and Willems *et.al.,* 1996). Other F-box proteins are proposed to be involved in Cdc28p regulation but are yet to be identified.

1.3.2.3 Summary of G1-S Phase Transition

Once the decision has been made to enter another round of cell division, Cln3-Cdk activity activates the production of Cln1p,2p through transcriptional activation of SBF. This in turn sets up a positive feedback loop to keep SBF activity on and initiates the production of SBF and MBF driven genes, including the G1 cyclins, S-phase cyclins and genes necessary for DNA replication. Once Clb5p,6p complex with the Cdk the replication origins fire releasing ORC's which are inhibited from reforming on the DNA by Clb-Cdk activity. Also, the G1 cyclins are phosphorylated and targeted for degradation by the SCF along with the B-type-Cdk inhibitor Sic1p and the Clnp-Cdk inhibitor Far1p allowing the cells to progress through S-phase into G2 without re-replication until B-type-Cdk activity is destroyed at the end of mitosis. This ensures DNA replication occurs only once prior to chomatid segregation.

1.3.3 G2 and Mitosis

M-phase is comprised of two events, mitosis where chromosome condensation and segregation occurs and cytokinesis where the cell divides into two genetically identical cells. In the budding yeast, mitosis is broken down into discreet phases; prophase, metaphase, anaphase and telophase. In prophase, chromosomes condense and the spindle pole body aligns on opposing poles. Metaphase is where the mitotic spindle elongates and the chromosomes align on the metaphase plate ready for sister chromatid separation that occurs in anaphase and then is followed by sister chromatid expansion in telophase. These events, including mitotic entry and exit, are controlled mainly through Cdk activity, in particular by the Clb2p/Cdc28p kinase. A major event at the entry into mitosis is the transcriptional activation of the mitotic cyclins *CLB1,2*, members of a cluster of genes known as the "CLB2 cluster" which share a transcriptional profile. The mechanism that controls this cluster of genes is not fully understood and will be discussed later. To exit mitosis a proteolytic pathway known as "Anaphase proteolysis" is required to inactivate the mitotic- Cdk.

1.3.3.1 Anaphase Proteolysis.

Anaphase proteolysis is a ubiquitin dependent pathway that is vital for cell cycle progression (Zachariae and Nasmyth, 1996, Zachariae *et.al.,* 1996 and Ghislain *et.al.*, 1993). In mitosis, entry via transcriptional activation of the "CLB2 cluster" (refer to next section), sister chromatid separation and inactivation of the mitotic kinase are three important events of which "Anaphase proteolysis" controls two; sister chromatid

segregation and mitotic kinase inactivation (Irniger *et.al.*, 1995). The key protein complex important for ubiquitination of target proteins is the E3 ubiquitin ligase Anaphase Promoting Complex/Cyclosome (APC/C) (Morgan, 1999). At least 10 subunits make up the APC/C (Zachariae and Nasmyth, 1999) which shares a high similarity to SCF, its counterpart in S-phase (Jorgensen and Tyers, 1999). As with the SCF, the APC/C needs the association of co-factors and phosphorylation by the mitotic-Cdk for activation (Visintin *et.al.*, 1997 and Rudner and Murray, 2000). Two of these factors are the WD repeat proteins (like Cdc4p in S-phase) Cdh1p and Cdc20p (Visintin *et.al.*, 1997). The substrates of the APC/C contain a targeting domain known as the destruction box (Cross *et.al.,* 1999 and Jacobson *et.al.*, 2000) and deletion of this domain stabilises these proteins (Wasch and Cross, 2002).

Cdc20p plays a pivotal role at the metaphase/anaphase transition initiating the separation of sister chromatids (Shirayama *et.al.*, 1998 and Shirayama *et.al.,* 1999). Cdc20p is itself cell cycle regulated with peak expression in G2/M putting it at the correct phase of the cell cycle for a role in APC/C dependent degradation (Fang *et.al.,* 1998). During S-phase a cohesion complex (containing Scc1p) assembles on the chromosomes to hold the two sister chromatids together by opposing the forces of the microtubules (Toth *et.al.*, 1999 and Michaelis *et.al.*, 1997). The separation of sister chromatids at the metaphase/anaphase transition seems to be reliant on the disappearance of Scc1p through the proteolytic cleavage at two sites by the separin,

Esp1p (Michaelis *et.al.*, 1997, Uhlmann *et.al.*, 1999). Until this transition, Esp1p is held in a repressive complex by the securin Pds1 and it is the destruction of Pds1p by the APC/C^{cdc20} that indirectly mediates sister chromatid separation (Cohen-Fix *et.al.*, 1996 and Ciosk *et.al.,* 1998). This seems to be the only essential role of the APC/C^{cdc20} at the metaphase/anaphase transition (Shirayama *et.al.,* 1998, Ciosk *et.al.*, 1998 and Yamamoto *et.al.,* 1996), however, another role for the APC/C^{Cdc20} is the degradation of Clb5p (Shirayama *et.al.*, 1999). The presence of Clb5p-Cdk activity keeps Sic1p (CdkI) and APC/C^{Cdh1} inactive until its destruction which triggers the activation of Sic1p and Cdh1p necessary for the inhibition of mitotic-Cdk activity so cells and exit from mitosis (Shirayama *et.al.*, 1999, Visintin *et.al.,* 1998, Zachariae *et.al.*, 1998 and Jaspersen *et.al.*, 1999). Central to this inhibition of mitotic-cdk is the phosphatase, Cdc14p (Visintin *et.al.*, 1998).

During the cell cycle, Cdc14p is sequestered to the nucleolus by a protein complex known as RENT (Visintin *et.al.*, 1999 and Shou *et.al.,* 1999). It is proposed that phosphorylation of RENT releases Cdc14p from the nucleolus and RENT then becomes a target for Cdc14p phosphatase in a negative feedback loop (Shou *et.al.*, 1999). Furthermore, release of Cdc14p from the nucleolus is dependent on the destruction of Pds1p and Cdc14p activity as a phosphatase requires Clb5p destruction, both are controlled by APC/C^{cdc20}-dependent proteolysis (Shirayama *et.al.,* 1999). A GTPase-regulated kinase cascade, known as the Mitotic Exit Network (MEN), is also important

for the release of Cdc14p. MEN is comprised of seven components; a RAS-like GTPase Tem1p, the phosphoprotein phosphatase Cdc14p, four protein kinases (Cdc5p, Cdc15p, Dbf2p and Dbf20p) and the Dbf2p binding protein Mop1p (reviewed by McCollum and Gould, 2001, Jorgensen and Tyers, 1999 and Hoyt, 2000). Temperature sensitive mutants of these components of the MEN pathway arrest late in anaphase with an elongated spindle, segregated chromosomes and high mitotic cdk activity (reviewed by Jorgensen and Tyers, 1999 and Hoyt, 2000). Through epistasis genetic analysis, the order of the cascade was determined with Tem1p acting upstream of the kinase cascade and Cdc14p release as its goal (Lee *et.al.*, 2001). Lte1p, a GDP/GTP exchange factor (a positive regulator) and Bub2p (a negative regulator) were also identified (Lai et.al., 1993) as possible regulators of this pathway. It has been shown through a number of studies the importance of localisation of cascade members for its activation (Li, 1999, Bardin *et.al.,* 2000 and Gruneberg *et.al.*, 2000). The resultant release of Cdc14p at the end of this pathway activates APC/C^{Cdh1} and Sic1p (CdkI) which inactivates mitotic-Cdk activity through degradation of the mitotic cyclin and inhibition respectively (Visintin *et.al.*, 1998) (figure 1.5 b).

Anaphase proteolysis is important for cell cycle progression. First it triggers the separation of sister chromatids at the metaphase/anaphase transition through APC/C^{Cdc20} directed proteolysis. Secondly, it helps the cells exit mitosis through the APC/C^{Cdc20} dependent degradation of Clb5p and the subsequent release of Cdc14p from the

nucleolus which activates APC/C^{Cdh1} directed proteolysis of mitotic cyclins and the inhibition of mitotic-Cdk by Sic1p (CdkI) (figure 1.5 b).

1.3.3.2 G2/M Transcriptional Regulation

At the G2-M transition in *S. cerevisiae*, there are 35 genes necessary for mitotic progression and cytokinesis that share a cell cycle regulated transcription pattern known as the "CLB2 cluster". These include genes such as *CLB1,2* (mitotic cyclins), *SWI5* (transcription factor) and *CDC5* (a polo-kinase). The expression of *CLB2* at the G2/M transition activates the mitotic cdk which establishes a positive feedback loop keeping the "CLB2 cluster" activated and represses G1 transcription through Swi4p sequestration (Amon *et.al.*, 1993) (figure 1.4 and 1.5 a). The best characterised "CLB2 cluster" genes are *SWI5* and *CLB2* whose periodic transcripts have been shown to be dependent on a general transcriptional regulator, Mcm1p (Lydall *et.al.*, 1991, Althoefer *et.al.*, 1995 and Maher *et.al.*, 1995).

1.3.3.2.1 Mcm1p is Essential for G2/M Transcription

Mcm1p is an essential, homodimeric DNA binding protein and a member of the MADS box family of transcription factors (Treisman and Ammerer, 1992). This family of transcription factors is characterised by a conserved 56 amino acid region found in the DNA binding domain (Shore and Sharrocks, 1995). The name is comprised of the initials of the first members of this group; *MCM1*, Arg80, agamous deficiens and serum response factor (SRF) (Schwarz-Sommer *et.al.*, 1990). These transcription factors have a diverse range of biological functions. ArgR1p is involved in gene regulation in

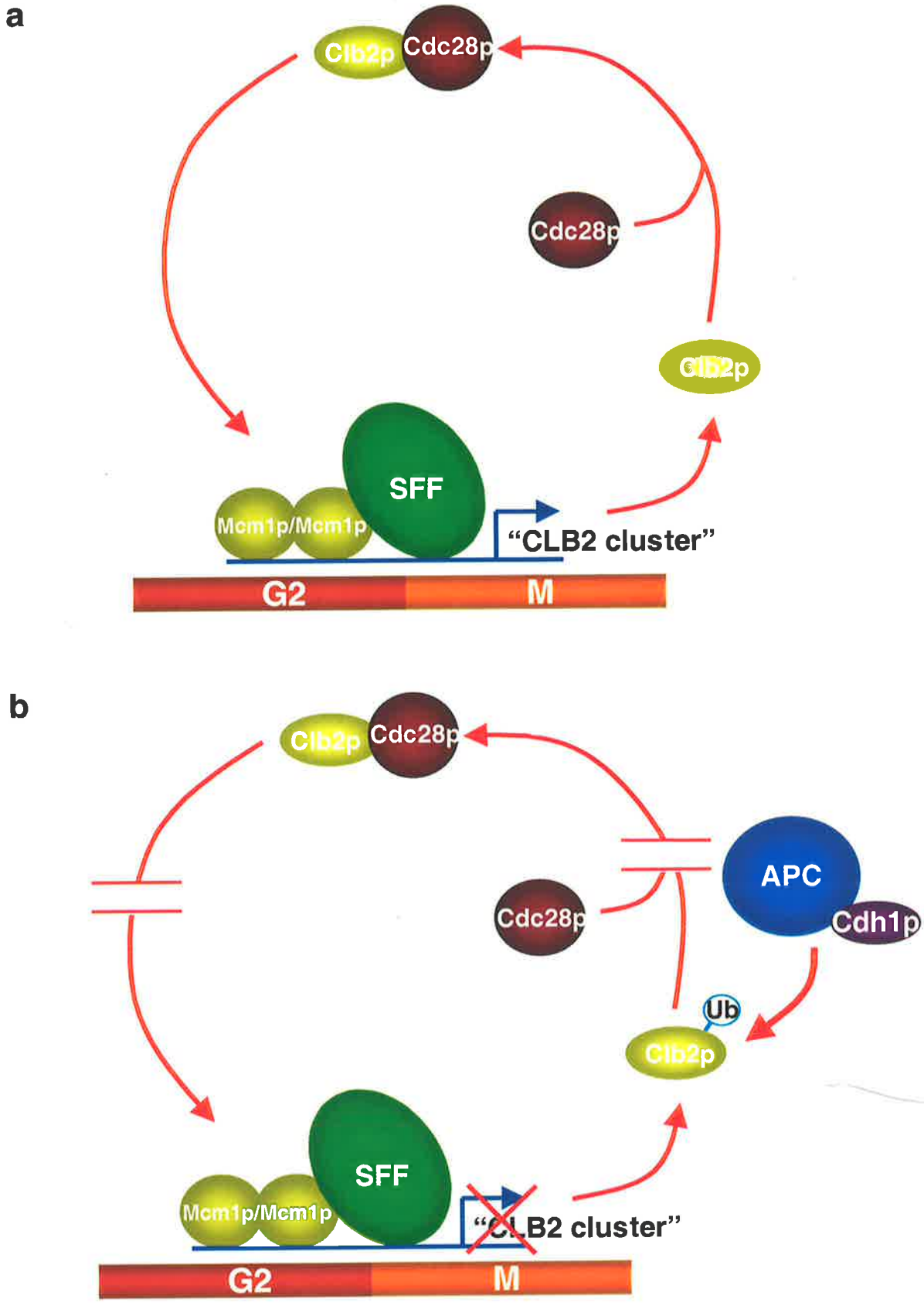


Figure 1.5: The presence of a positive feedback loop at the G2-M transition. **a** The activation of the "CLB2 cluster" promotes Clb2p/Cdc28p kinase activity which establishes a positive feedback loop. **b** The APC^{Cdh1p} acts to degrade Clb2p in anaphase which disrupts the feedback loop and switching off "CLB2 cluster" expression. This then allows the cells to exit mitosis.

arginine metabolism (Dubosis and Messenguy, 1991), SRF regulates immediate-early and muscle specific genes in a variety of higher eukaryotes (Treisman and Ammerer, 1992) and Mcm1p controls genes used in cell type determination, metabolism, minichromosome maintenance, M/G1 and G2/M progression (Treisman and Ammerer, 1992, Messenguy and Dubois, 1993, Passmore *et.al.,* 1989, McInerny *et.al.*, 1997, Lydall *et.al.*, 1991, Althoefer *et.al.,* 1995 and Maher *et.al.*, 1995). Mcm1p's role as a transcriptional regulator is governed by the recruitment of specific co-regulators and co-repressors to specific promoters through protein-protein interactions. In the case of yeast pheromone response, Mcm1p recruits α1 to activate α-specific genes (Bender and Sprague, 1987 and Jarvis *et.al.*, 1989) whereas it interacts with α2 to repress a-specific genes (Smith and Johnson, 1992). In addition, Mcm1p recruits Ste12p in response to secreted pheromones during mating, activating pheromone-responsive genes and arresting cells prior to START (Sprague, 1990). Like most MADS-box family members, Mcm1p binds the DNA as a dimer (Sprague, 1990 and Passmore *et.al.*, 1989) and contains several distinct domains common to this family, mainly the DBD, protein-protein interaction and dimerisation domains (Shore and Sharrocks, 1995). Although Mcm1p is 286 amino acids in length, it only requires the first 96 amino-terminal amino acids for essential functions (Primig *et.al.*, 1991, Christ and Tye, 1991 and Bruhn *et.al.*, 1992). These 96 amino acids contain the necessary sequences for homodimerisation, DNA binding and recruitment of accessory proteins.

Of the 35 "CLB2 cluster" genes, 26 have recognisable Mcm1p binding elements (Spellman *et.al.*, 1998) (figure 1.6 b) and it has been shown through DNA-footprinting that these sites in the *SWI5* and *CLB2* upstream activating sequences (UAS's) are occupied constitutively by Mcm1p (Althoefer *et.al.,* 1995). A Mcm1p-VP-16 fusion protein can rescue the essential nature of Mcm1p and has been shown to activate "CLB2 cluster" genes and specifically operate through the $CLB2_{UAS}$ (Maher *et.al.,* 1995 and Althoefer *et.al.,* 1995). For the $SWI5_{UAS}$, there is one Mcm1p site whereas the $CLB2_{UAS}$ contains three sites spaced over 250bp that is necessary for optimal promoter activity, although a single site can function with reduced activity *in vivo* (Lydall *et.al.*, 1991 and Loy *et.al.*, 1999). ***Studies using the SWI5 and CLB2 UAS have revealed the presence of another unidentified factor that binds cooperatively and is reliant on Mcm1p called SWI5 factor (SFF)*** (Lydall *et.al.*, 1991, Maher *et.al.*, 1995 and Althoefer *et.al.*, 1995).

1.3.3.2.2 Mcm1p's Co-Factor SFF

Along with Mcm1p, SFF binds to the *SWI5* and *CLB2* UAS's but with a dependency on the presence of Mcm1p (Lydall *et.al.,* 1991 and Althoefer *et.al.*, 1995) (figure 1.5 a). SFF has been suggested to play the regulatory role of the "CLB2 cluster" through the observation that a single base substitution at the SFF response element causes the loss of binding *in vitro* and loss of UAS activity *in vivo* (Lydall *et.al.*, 1991) and the promoters of most of the "CLB2 cluster" contain potential binding sites for Mcm1p and SFF

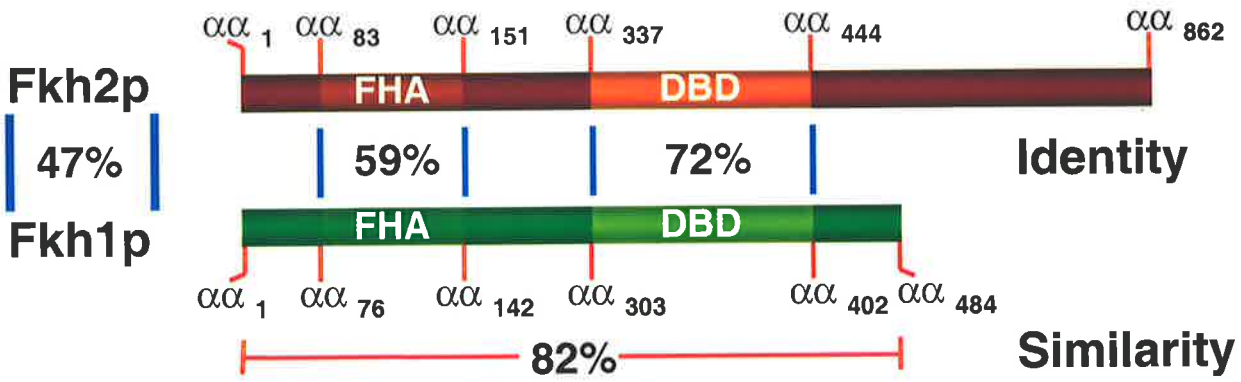
(Spellman *et.al.*, 1998) (figure 1.5 a). As with Mcm1p, DNA-footprinting revealed the SFF binding site was occupied throughout the cell cycle (Althoefer *et.al.*, 1995).

1.3.3.2.3 SFF is Fkh2p

Recently, we were able to purify a biochemical activity that followed that of SFF *in vitro* from yeast through affinity purification and identified it as Forkhead 2 (Fkh2p) through MALDI analysis (Kumar *et.al.*, 2000). Fkh2p is a member of a family of transcription factors that are characterised by their DNA binding domain (DBD) of 110 amino acids, a winged-helix motif (Kaufman and Knochel, 1996). This family has been demonstrated to have roles in cell-type determination, differentiation, development and cell death (Kaufman and Knochel, 1996). The DBD is comprised of 3 α-helices and 3 β-strands and two unordered structures referred to as "wings" where the α-helices and β-strands form the core of the domain with the 2 "wings" flanking them giving a structure that has been referred to as a butterfly (Clark *et.al.*, 1993). Further structural studies of HNF-3γ binding the transthyretin (TTR) promoter has revealed binding primarily in the major groove with one contact with the minor groove (Brennan, 1993).

The forkhead consensus DNA binding site is identical to the reported SFF binding sites found in the *SWI5* and *CLB2* promoters (Kaufman and Knochel, 1996) (figure 1.6 b). In the budding yeast there are four forkhead proteins (Hofmann and Bucher, 1995 and Zhu and Davis, 1998), one of which, Fkh1p, shows an 82% similarity to Fkh2p (figure 1.6 a). The main difference between Fkh1p and Fkh2p is an extension of the carboxyl

a



b

SWI5	-324 ACTTAACCTGTTTAGGAAAAAG__	GTAAACAATAACAA -288
CLB2	-696 TAGCGACCGAATCAGGAAAAG__	GTCAACAACGAAGA -662
CLB1	-521 AACCGCCCAAAGAGGAAAA____	ACATCAACAATCAAGA -487
BUD4	-686 GATGACCCGATTTGGAAAAAG___	GTAAACAACAATGA -552
CDC20	-318 TGATTGCCGAAAGAGGCAAAAC_	GTAAATAGGTTGTT -282
	ECB	SFRE

Figure 1.6: Forkhead proteins and the "CLB2 cluster" binding site. **a** A schematic diagram of Fkh1p and Fkh2p highlighting the conserved DNA binding Domain (DBD), Forkhead associated domain (FHA) and the percent amino acid (αα) identity and similarity. **b** An alignment of the promoter regions of members of the "CLB2 cluster" highlighting the Mcm1p and SFF/Fkhp binding sites.

ECB=Mcm1p binding site and SFRE= SFF binding site.

terminus on Fkh2p that contains six putative cdk phosphorylation sites. Both Fkh1p and Fkh2p contain a second conserved domain known as the forkhead associated domain (FHA) (figure 1.6 a) making them members of a subgroup of Forkhead transcription factors in the budding yeast along with Fhl1p (Hofmann and Bucher, 1995).

1.3.3.2.4 FHA Domains

The FHA domain was first discovered in Forkhead proteins and has become known as a protein-protein interaction domain (Hofmann and Bucher, 1995). This domain recognises phospho-proteins, specifically phosphothreonine (pT) peptides (Durocher *et.al.,* 1999, Durocher *et.al.*, 2000 and Li *et.al.*, 2000) and is associated with proteins involved in signal transduction, transcription, protein transport, DNA repair and protein degradation (Li *et.al.*, 2000 and Durocher *et.al.*, 2000 (b)). Studies involving Rad53p, a yeast protein involved in DNA damage checkpoint, showed the mutation of two conserved residues (R70, H88) abolished the binding of the phospho-protein Rad9p and with a synthetic phospho-peptide (Durocher *et.al.*, 1999). Further to this, a substitution of the pT+3 Asp on the phospho-peptide eradicated binding (Durocher *et.al.*, 1999). This implicates a specific binding consensus sequence for FHA domains in protein-phosphoprotein interactions which have been identified through peptide library screens for different FHA domains (Durocher *et.al.*, 2000 and Liao *et.al.*, 2000). Structural analysis of the $Rad53^{FHA1}$ domain revealed 11 β-strands with the connecting loops

containing the conserved residues that interact with the pT phospho-peptides and not with pS phospho-peptides (Durocher *et.al.*, 2000).

1.3.3.3 Ndd1p, a Possible "CLB2 cluster" Regulator

Ndd1p was first isolated as a high copy suppressor of the *cdc28-1N*^{ts} mutant and was found to have an essential function in mitosis (Loy *et.al.*, 1999). It contains four putative cdk phosphorylation sites, pT180, pT184, pS254 and pT319, and is cell cycle regulated with peak expression just prior to the appearance of the "CLB2 cluster" at the G2/M transition (Loy *et.al.,* 1999). Overexpression studies of Ndd1p have shown an upregulation of "CLB2 cluster" genes without loss of periodic expression (Loy *et.al.*, 1999) implicating Ndd1p as a regulator of "CLB2 cluster" genes at the G2-M transition. Ndd1p cannot bind DNA itself but when fused to a DBD it can activate reporter constructs implicating it as a putative transactivator (Loy *et.al.*, 1999). In addition, Ndd1p has been shown to be recruited to the promoters of members of the "CLB2 cluster" *in vivo* and this recruitment is dependent on the presence of Fkh1p or Fkh2p (Koranda *et.al.*, 2000). However, genetic evidence showing the lethality of a Δ*ndd1* can be rescued by deleting Δ*fkh2* but not Δ*fkh1*, indicates Ndd1p has a role through Fkh2p and not Fkh1p (Koranda *et.al.*, 2000). ***All this evidence taken together implicates Ndd1p as an activator of the "CLB2 cluster" and Fkh2p as a possible represser although the role of Fkh2p and Ndd1p have not yet been elucidated.***

1.3.3.4 Summary of G2/M Transition

At the entry to mitosis Mcm1p and a co-factor SFF are required for the activation of the "CLB2 cluster". Recently SFF activity was purified and identified as Fkh2p, which, along with Ndd1p, may play an important role in mitotic entry. As the Clb1p,2p expression is switched on, a positive feedback loop is established to keep "CLB2 cluster" transcription on. Cells progress through mitosis and align condensed chromosomes on the metaphase plate. At this point the cell activates the APC/C which is directed by Cdc20p to degrade Pds1p and Clb5p to segregate chromosomes and prepare for mitotic exit. The consequential release of Cdc14p promotes Cdh1p dependent APC/C degradation of mitotic cyclins and activation of Sic1p (CdkI) to abolish mitotic-Cdk activity and allow cells to exit mitosis.

1.4 Summary

Nasmyth (1996) proposed that it was the B-type cyclin-Cdk activity that controls the ordering and timing of the cell cycle whose levels are regulated by postranslational events such as proteolysis, transcription of regulators and phosphorylation. Initiation of the cell cycle occurs through Cln-Cdk activity in G1, which regulates transcription in G1 and S-phase, such as the G1 cyclins, S-phase cyclins and genes necessary for DNA replication, via the SBF and MBF. The appearance of the Clb5p,6p-Cdk activity fires the replication origins and the consequential release and phosphorylation of the ORC's by Clb5p,6p-Cdk inhibits reformation of these complexes on the DNA ensuring no re-replication while there are B-type cyclins present. Along with this, Clb5p,6p-Cdk

phosphorylates substrates such as the B-type Cdk inhibitor Sic1p, Cln-Cdk inhibitor Far1 and Cln1p,2p, which target them for degradation. Production of Clb3p,4p occurs during S-phase which influences spindle formation. At the G2/M transition the "CLB2 cluster" are activated in a Mcm1p-SFF dependent fashion by a mechanism which has to be elucidated. As cells progress through mitosis the APC/C is activated by phosphorylation and is directed to substrates through the co-factors Cdc20p and Cdh1p. The result is the separation of sister chromatids and the inactivation of mitotic-Cdk through inhibition and degradation leading to mitotic exit. The inactivation of mitotic-Cdk's allows the ORC's to reform on the DNA in preparation for another round of cell division. Hence, through the regulation and action of the different Cdk activities, the cell progresses through the division cycle in an ordered fashion where DNA replication and cell division occurs only once.

1.5 Aims

An important event that has been discussed is the transcriptional activation at the G2/M transition. The transcription factor complex is comprised of a homodimer of Mcm1p and, until recently, an unidentified factor SFF. We have purified and identified the activity of SFF as Fkh2p. I propose to investigate the role of Fkh2p in the regulation of the "CLB2 cluster". Another protein, Ndd1p, has been implicated in a transcriptional role at the G2/M transition. In this thesis, I will examine the possible role Ndd1p plays in this transcriptional regulation and its association, if any, with Fkh2p.

Chapter 2:

Material and Methods

2.0 MATERIALS AND METHODS

2.1 Abbreviations

Ab	Antibody
bp	Base pairs
C	Celsius
Cdk	Cyclin dependent kinase
DBD	DNA Binding Domain
DNA	Deoxyribonucleic Acid
FACS	Fluorescence Activated Cell Sorting
FHA	Forkhead Associated Domain
g	Gram
HA	Haemagglutinin
hr	Hour
HRP	Horseradish Peroxidase
kb	Kilobase pair
kD	Kilo Dalton
L	Litre
M	Moles per litre
ml	Millilitre
mM	Millimoles per litre

µM	Micromoles per litre
µg	Microgram
µl	Microlitre
mA	milli Amperes
MQ	Milli-Q
mRNA	Messenger RNA
OD	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
rcf	Relative centrifugal fields
RT	Room Temperature
PBS	Phospahte Buffered Saline
PBST	PBS Triton
PEG	Polyethylene glycol
rcf	relative centrifugal force
TBE	Tris Borate EDTA
UV	Ultra Violet
V	Volts

2.2 Materials

2.2.1 Chemicals and Reagents

All chemicals and reagents used were of the highest purity available and were obtained from the following suppliers:

2.2.1.1 Chemicals and Reagents Abbreviations

AMP	Ampicillin	Sigma
APS	Ammonium Persulphate	Sigma
β-Me	β-mercaptoethanol	Sigma
BSA	Bovine Serum Albumin	Sigma
dATP	deoxyadenosine triphosphate	Boeringer Mannheim
dCTP	deoxycytosine triphosphate	Boeringer Mannheim
dGTP	deoxyguanosine triphosphate	Boeringer Mannheim
dTTP	deoxythymidine triphosphate	Boeringer Mannheim
DMSO	Dimethylsulphoxide	Sigma
DTT	1,4-Dithiothreitol	Sigma
EDTA	Ethylenediamine-tetra-acetic Acid	Sigma

EtBr	Ethidium Bromide	Sigma
HCl	Hydrochloric Acid	BDH Chemicals
MOPS	3-[N-Morpholino]propanesulphonic acid	Sigma
NP-40	Nonidet-P 40	Sigma
PMSF	Phenylmethylsulphonylflouride	Sigma
TEMED	N,N,N`,N`-tetramethylethylenediamine	Sigma
Tris	Tris(hydroxymethyl)aminomethane	Sigma
SDS	Sodium Dodecyl Sulphate	Sigma
TEMED	N,N,N',N'-Tetramethyl-Ethenediamine	Sigma
TLCK	N-Tosyl-L-lycinechloromethyl ketone	Sigma
TPCK	Tosyl-L-phenylalaninechloromethyl ketone	Sigma

2.2.1.2 Materials and Reagents

1Kb plus DNA ladder	Life Technologies
Acetic Acid	BDH Chemicals
Acrylamide	BioRad
Amino Acids	Sigma
Ammonium Sulfate	Sigma
Amplify	Pharmacia
Azide	Sigma
Bis-Acrylamide	BioRad
Bromophenol Blue	BioRad

Bradford Reagent	BioRad
Casamino acids	Difco
Chloroform	BDH Chemicals
Ethanol	BDH Chemicals
Formaldehyde	BDH Chemicals
Formamide	BDH Chemicals
Glycine	BDH Chemicals
Glycerol	BDH Chemicals
Glycogen	Boehringer Mannheim
Hepes	Sigma
Isopropanol	BDH Chemicals
Klenow	Pharmacia
Lyticase	Sigma
Lithium Chloride	Sigma
Magnesium Chloride	Sigma
Methanol	BDH Chemicals
PEG4000	BDH Chemicals
PEG8000	Sigma
Phenol	Wako Pure Chemicals
Poly dIdC	Sigma
Ponceau S	Sigma

Potassium Acetate	BDH Chemicals
Rainbow Protein Markers	Amersham
Restriction Enzymes	New England Biolabs
RNAse Inhibitor	Roche
Salmon Sperm DNA	Boehringer Mannheim
Skim Milk Powder	Diploma
Sodium Acetate	BDH Chemicals
Sodium Chloride	Sigma
Sodium Hydroxide	BDH Chemicals
TRIS	Sigma
Triton-X-100	Sigma
ULTRAhyb	Ambion
Yeast Extract	Difco
Yeast Nitrogen Base	Difco

2.2.2 Kits

ECL Detection Kit	Pierce
Megaprime Kit	Amersham
Coupled Reticulocyte Lysate System	Promega
QuickSpin Columns	Boehringer Mannheim
UltraClean GelSpin	MO BIO
UltraClean PCR Purification	MO BIO

2.2.3 Radiochemicals

^{35}S Methionine	Geneworks
[γ-P^{32}] ATP	Geneworks
[γ-P^{32}] CTP	Geneworks

2.2.4 Buffers and Solutions

10x Annealing Buffer	1M NaCl, 100mM Tris pH 8.0, 50mM MgCl
10x DNA/RNA loading Dye	15% Ficoll 400, 0.25% BPB, (0.25% Xylene cyanol for DNA)
10x MOPS	200mM MOPS pH 7.0, 50mM NaOAc, 10mM disodium EDTA
20x SSC	3M NaCl, 300mM sodium citrate
2x SDS Load	20% glycerol, 4%SDS, 100mM Tris-HCl pH 6.8, 1% Bromophenol Blue, 0.5% β-Me
5x Binding Buffer	100mM Tris pH 7.5, 250mM NaCl, 15mM MgCl, 5mM DTT, 25mM spermidine, 250 μg/mL BSA, 100mM EDTA.
Blocking Solution	5% non-fat skim milk powder in PBST
LETS	0.1M LiCl, 0.01M EDTA,

	0.01M Tris-HCL pH 7.4, 2% SDS
Maxam-Gilbert Buffer	0.3M NH_4OAc, 0.1% SDS
Nocodazole (Stock Sol^n)	10mg resuspended in 1ml DMSO
Northern Load Dye	12.5% 10x MOPS, 14.6% 40% formaldehyde, 41.7% formamide, 2.1% EtBr, 29.1% 10x Load Dye
PBS	130mM NaCl, 10mM Na_2HPO_4, 30mM NaH_2PO_4, pH 7.0
PBST	130mM NaCl, 10mM Na_2HPO_4, 30mM NaH_2PO_4, pH 7.0, 0.1% Triton-X-100, H_2O to pH 7.2 with HCl
Phosphate Buffered Saline	130mM NaCl, 10mM Na_2HPO_4, 30mM NaH_2PO_4
Plate Solution	50% PEG4000, 10% LiOAc, 1% 1M Tris-HCl pH 7.5, 0.2% 0.5M EDTA
Ponceau Stain	0.5% Ponceau S, 1% acetic acid in MQ H_2O
Protein Extraction Buffer	0.2M Tris pH 8.0, 0.4M NH_4SO_4, 10mM $MgCl_2$, 1mM EDTA, 10% glycerol, 1mM PMSF, 5mM β-Me, 0.5mM TPCK, 25μM TLCK

Protein Resuspension Buffer	20% glycerol, 20mM Hepes pH 8.0, 5mM EDTA, 1mM PMSF, 5mM β-Me, 0.5mM TPCK, 25μM TLCK
SDS Running Buffer	25mM Tris pH7.0, 250mM glycine, 0.1% SDS
Solution 1	50mM glucose, 10mM EDTA, 25mM Tris pH 8.0
Solution 2	1% SDS, 0.2M sodium hydroxide
Solution 3	250g potassium acetate, 45mL acetic acid, up to1L with H_20
Nothern Stripping Solution	.01% SDS
Wet Transfer Buffer	50mM Tris pH 7.0, 380mM glycine
X-gal Solution	25mg/ml X-gal in dimethylformamide
Z Buffer	60mM disodium hydrogen phosphate, 40mM dihydrogen phosphate, 10mM KCl, 1mM magnesium sulphate, 50mM 2-mercaptoethanol

2.2.5 Plasmids

pBS.KS+ - cloning vector (Stratagene)

pT7plink - pGem2 backbone with T7 promoter (Dalton and Treisman)

pRS316 - CEN6 *URA3* ARS4 (Sikorski and Hieter, 1989)

pRS306 - Integrating *URA3* ARS4 (Sikorski and Hieter, 1989)

pRS303 - Integrating *HIS3* ARS4 (Sikorski and Hieter, 1989)

pRS314 - CEN6 *TRP1* ARS4 (Sikorski and Hieter, 1989)

pRS304 - Integrating *TRP1* ARS4 (Sikorski and Hieter, 1989)

pRS315 - CEN6 *LEU2* ARS4 (Sikorski and Hieter, 1989)

pYIplac204 - Integrating *TRP1* pUC19 MCS (Gietz and Sugino, 1988)

pYIplac211 : Integrating *URA3* pUC19 MCS (Gietz and Sugino, 1988)

pYCplac33 - CEN4 *URA3* pUC19 MCS (Gietz and Sugino, 1988)

pYCplac22 - CEN4 *TRP1* pUC19 MCS (Gietz and Sugino, 1988)

pYEplac112 - 2μ *TRP1* pUC19 MCS (Gietz and Sugino, 1988)

pYEplac195 - 2μ *URA3* pUC19 MCS (Gietz and Sugino, 1988)

pYES2 - 2μ *URA3* GAL promoter (Invitrogen)

pVT100U - 2μ *URA3* ADH promoter (Vernet *et.al.*, 1987)

2.2.6 Primers

2.2.6.1 Oligos for Band-shift probes

$SWI5_{wt}$: Top strand 5'-

tcgagtactttaacctgtttaggaaaaaggtaaacaataacaatacctaccattagcga-3'

Bottom Strand 5'- tcgatcgctaatggtaggtattgttattgtttacctttttcctaaacaggttaaagtac-3'

SWI5$_{A296}$: Top Strand 5'-

tcgagtactttaacctgtttaggaaaaaggtaaaaaataacaatacctaccattagcga-3'

Bottom Strand 5'- tcgatcgctaatggtaggtattgttatttttacctttttcctaaacaggttaaagtac-3'

CLB2$_{wt}$: Top Strand 5'-ctcgataaatatagcgaccgaatcaggaaaaggtcaacaacgaagttcgcgatatgg-3'

Bottom Strand 5'-ccatatcgcgaacttcgttgttgacctttcctgattcggtcgctatattta-3'

CLB2$_{mut}$: Top Strand 5'-

ctcgataaatatagcgaccgaatcaccaaaaggtcaacaacgaagttcgcgatatgg-3'

Bottom Strand 5'-ccatatcgcgaacttcgttgttgacctttggtgattcggtcgctatattta-3'

2.2.6.2 Cloning Primers

FKH2-F: 5'- cac aca gaa ttc cat g gcc agc agc aat ttt aac g-3'

FKH2-R: 5'- cac aca ctc gag tca gcg gcc gct tgg gtt gtt gat aat act gat ctt tgc-3'

FKH2-1DBD: 5'- cac aca ctc gag tca gcg gcc gct ggg taa act gtt gaa ttt tgc c-3'

FKH2-2DBD: 5'- c aca cag aat tcc atg gat ttt act tcg gac cta tcc cat gac-3'

FKH1-F: 5'- cac aca gaa ttc tca tga gtg tta cca gta ggg aac aaa aat tta gtg g-3'

FKH1-R: 5'- cac aca ctc gag tca gcg gcc gct tgg act cag aga gga att gtt cac gtt tgc g-3'

FKH1-8F 5'- atg tct gtt acc agt agg-3'

FKH1-8R 5'- tca act cag aga gag gaa ttg-3'

FKH2-13R: 5'- cct ttg gat ggg aca tgt-3'

FKH1-LEU2: 5'- ccg gct cta atg aag att

UF: 5'- gta aaa cga cgg cca gt-3'

2.2.7 Primary Antibodies

-12CA5 monoclonal antibody raised against HA epitope

-9E-10 monoclonal antibody raised against Myc epitope from Gerad Evan, ICRF, London

-GAPDH monoclonal antibody from Trevor Lithgow, University of Melbourne

2.2.8 Secondary Antibodies

Rabbit anti-mouse immunoglobin-HRP – obtained from DAKO

2.2.9 Yeast Strains and Media

Yeast strains used were isogenic with W303-1a (*MAT**a** ho, ura3-52, trp1-1, ade 2-1, lys 2-801, leu2-3, his 3-11, 15 can1-100 [psi+]*) unless specified differently.

YEPD Broth: 1% yeast extract, 2% bactopeptone, 0.5% adenine, 2% glucose

SD –Ura: 0.8% yeast nitrogen base, 1% casamino acids, 0.5% adenine, 0.5% leucine, 0.5% tryptophan, 2% carbon source.

SD –Trp: 0.8% yeast nitrogen base, 1% casamino acids, 0.5% adenine, 0.5% leucine, 0.5% tryptophan 2% carbon source.

SD –Leu: 0.8% yeast nitrogen base, 0.5% Adenine, 10% 10x Amino acid dropout (synthetic dropout –Leu), 2% carbon source

SD –His:‘8% yeast nitrogen base, 0.5% Adenine, 10% 10x Amino acid dropout (synthetic dropout –His), 2% carbon source

Solid Media: Agar plates were prepared by supplementing the above media with 1.5% bacto-agar.

5FOA Solid Media: 2.33g yeast nitrogen base, 332.5mg 5 FOA, 66.5mg uracil, 66.5mg leucine, 66.5mg adenine, 66.5mg histidine, 66.5mg tyrosine, 66.5mg tryptophan, 66.5mg glucose, 2% Agar. up to 500ml with H_2O

2.2.10 Bacterial Strains and Media

-DH5α (F$^-$ϕ80d*lac*Z ΔM15 Δ(*lac*ZYA-argF)U169 *deo*R *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+)*pho*A *sup*E44λ$^-$ *thi*-1 *gyr*A96 *rel*A1) (GIBCO).

-Calcium chloride competent cells were prepared as described by Ausubel *et. al.* (1992).

-L Amp plates: 1% sodium chloride, 1% trytone, 0.5% yeast extract, 1.5% bacto-agar, 100μg/ml ampicillin.

2.2.11 Miscellaneous Materials

Acid washed glass beads (500 micron)	Sigma
Broad Range Rainbow Markers	Amersham
Nitrocellulose	Schleicher and Schuell
Nylon Membranes	Amersham
Quickspin Columns	Roche Diagnostics

Whattman Paper	Whattman
X-ray Film	AGFA

2.3 Yeast Manipulations

2.3.1 Yeast Transformations

A single yeast colony was used to inoculate fresh medium and grown to OD_{660} 0.6 to 0.8. 1mL of cells was spun in a microcentrifuge at 20,800 rcf for 15 seconds. The supernatant was discarded and cells resuspended in the residual media. 50µg carrier DNA (salmon sperm) and 5µg of transforming DNA were added to the cells. 1ml of plate solution (2.2.4.) was added, cells were mixed gently and incubated overnight at room temperature. 200µL of cells were taken from the bottom of the tube and plated on selective media and incubated at 30°C for 2-3 days.

2.3.2 Yeast Genomic Integrations

10µg of linearised plasmid containing the 3' coding region of the yeast gene of interest was transformed along with a co-transforming plasmid into yeast (2.3.1). Cells were first plated onto media selecting for the co-transforming plasmid then replicated onto media selecting for an integration event. Integrations were screened using PCR.

2.3.3 Yeast Genomic DNA Extraction

5mL overnight cultures were harvested at 2,060 rcf for 5 minutes and the supernatant discarded. Cells were resuspended in 500µL of 1M sorbitol, 0.1M EDTA pH 7.5 and

transferred to 1.5mL eppendorf tubes. 50µg of Lyticase was added and cells incubated at 37°C for 1 hour. Cells were then spun at 3.800 rcf for 1 minute and the supernatant discarded. Cells were resuspended in 500µL 50mM TRIS pH 7.4, 20mM EDTA. Sphaeroplasts were lysed by addition of 50µL of 10% SDS and incubation at 65°C for 30 minutes. 200µL of 5M potassium acetate was added and tubes incubated on ice for 1 hour. Tubes were then spun at 20,800 rcf for 5 minutes. The supernatant was transferred to a new tube and nucleic acid precipitated by addition of 1 volume of isopropanol. Tubes were spun for 10 seconds at 20,800 rcf, the supernatant was removed and the pellets allowed to air dry. DNA was resuspended in 300µL of MQ water and 15µg of RNase A was added and incubated for 30 minutes a 37°C. DNA was precipitated with 0.1x volume of 3M sodium acetate pH 5.2 and 200µL of isopropanol. DNA was recovered by centrifugation for 10 seconds at 20,800 rcf. After washing with 70% ethanol, pellets were allowed to air dry before being resuspended in 50µl of MQ water.

2.3.4 Synchronisations

50mL overnight cultures grown from a single colony were used to inoculate fresh media to OD_{660} 0.1. Culture was then grown at 30°C to OD_{660} 0.4 and an asynchronous cell sample was collected by centrifugation. Cells were arrested in G1 using α-factor to a final concentration of 0.6µg/mL for bar⁻ strains and 6µg/mL for BAR strains, and allowed to arrest for 2 hours. Arrest at G1 was verified by microscopy. Cells were then released into fresh media by harvesting by centrifugation, washing twice in an equal

volume of water and then inoculating fresh media. Samples were collected every 15 minutes by centrifugation and the cell pellet split into two for Northern analysis.

2.3.5 Static Blocks

Overnight cultures, grown from a single colony, were diluted to an OD_{660} of 0.1. Cultures were then grown to OD_{660} 0.4 where hydroxy-urea was added to a final concentration of 0.1M to arrest cells at S phase. Nocodazole was added to a final concentration of .01µg/mL to arrest cells at mitosis. Cells were arrested in G1 using α-factor at a final concentration of 0.5µg/mL to arrest cells at G1. Cells were allowed to arrest for 3 hours at 30°C and checked for arrest by microscopy before harvesting by centrifugation. $1x10^7$ cells were collected to validate arrests through FACS.

2.4 Bacterial Manipulations

2.4.1 Bacterial Transformations

1µg of DNA was added to 45µL of $CaCl_2$ competent *E. coli* cells. Cells were incubated on ice for 20min., 42°C for 2 minutes, on ice for 2 min. then resuspended in 1ml of LB and incubated at 37°C for 20 minutes. The cells were plated onto L+AMP plates and incubated overnight at 37°C.

2.4.2 Mini Plasmid Preparation from Bacteria

A single bacterial colony was used to inoculate a 2mL LB + AMP (0.1mg/ml) culture which was grown overnight at 37°C. Cells were harvested from 1mL of culture by

centrifugation at 20,800 rcf for 15 seconds. The supernatant was discarded and cells resuspended in 100µL of solution 1 (2.2.4.). 200µL of solution 2 (2.2.4.) was added and mixed gently, followed by the addition of 150µL of solution 3 (2.2.4.). 400µL of 5M LiCl was added and after centrifugation at 20,800 rcf for 10 minutes the supernatant was collected. Nucleic acids were precipitated with 800µL of 95% ethanol and incubation on ice for 15 minutes. DNA was collected by centrifugation at 20,800 rcf for 10 min.. The supernatant was discarded and the pellet was washed with 70% ethanol and resuspended in 30µL of water. Plasmid preparations using this method were digested in the presence of RNase. DNA was also recovered using the Qiagen miniprep kit.

2.4.3 Maxi Plasmid Preparation from Bacteria

A single bacterial colony was used to inoculate a 2mL LB + AMP (0.1mg/ml) culture which was grown overnight at 37°C. 200µL of this culture was used to inoculate a 200mL LB + AMP (0.1mg/ml) culture and grown overnight at 37°C. Cultures were harvested by centrifugation at 2,060 rcf for 10 min.. The supernatant was discarded and cells resuspended in 5mL of solution 1 (2.2.4.) to which 10ml of solution 2 (2.2.4.) was added, mixed gently and left on bench for 10 minutes. 7.5mL of solution 3 (2.2.4.) was added followed by gentle mixing and incubation on ice for 15 minutes. The supernatant was recovered after centrifugation for 15 minutes at 2,060 rcf. Nucleic acids were precipitated with 1 volume of cold isopropanol and incubation on ice for 15 minutes and collected via centrifugation at 2,060 rcf for 10 min.. The pellet was resuspended in

1.5ml of water and high molecular weight RNA's were precipitated by addition of 2mL of cold 5M LiCl and incubation on ice for 5 minutes and collected via centrifugation for 5 min. at 2,060 rcf. The nucleic acids were precipitated with 2 volumes of cold 95% ethanol and harvested by centrifugation at 2,060 rcf for 10 min. and the pellet was allowed to air dry. After resuspension in 800µL of H2O the RNA was degraded by addition of 36µg of RNase and incubated for 15 min. at 37°C. DNA was precipitated by addition of 0.5 volumes of 20% PEG8000, 2.5M sodium chloride solution and incubation on ice for 5 minutes. DNA was collected by centrifugation at 20,800 rcf for 5 min.. The DNA pellet was resuspended in 600µL of water and extracted twice with 600µL of Phenol/Chloroform. Clean DNA was precipitated with 0.1 volumes of 3M sodium Acetate pH 5.2 and 2.5 volumes of cold 95% ethanol and harvested by centrifugation at 20,800 rcf. DNA pellets were washed with 70% ethanol and allowed to air dry on bench followed by resuspension in water.

2.5 Protein Detection Methods

2.5.1 Preparation of Whole Cell Protein Extracts

Cells were harvested by centrifugation at 2,060 rcf for 5 minutes. Media was aspirated and cells washed by resuspending in 1mL of cold water and centrifugation for 15 seconds at 20,800 rcf. Cells were resuspended in 500µL of cold protein extraction buffer (2.2.4) and transferred to tubes containing an equal volume of chilled acid washed 500-micron glass beads. Cells were lysed in a Biospec bead beater at 4°C using

4x 15 second bursts at maximum speed. Cellular debris was spun out at 20,800 rcf at 4°C. The supernatant was transferred to new tubes and protein was precipitated by slowly adding 0.8 volumes of saturated ammonium sulphate and tumbling at 4°C for 30 minutes. Protein was harvested by centrifugation at 20,800 rcf for 10 minutes. The pellet was retrieved and carefully washed with 1mL of cold protein resuspension buffer (2.2.4.) and resuspended in 50µL of resuspension buffer. Protein was snap frozen on solid carbon dioxide and stored at –80°C.

2.5.2 Determination of protein concentration

Protein concentrations of cell extracts were determined as described by Bradford (1976). Absorbance at 595nm was measured using a Pharmacia LKB Ultrospec III UV Spectrophotometer. Protein concentrations were calculated using a standard curve generated from known concentrations of BSA. Zero protein concentration was defined as the absorbence at 595nm of Bradford Reagent only.

2.5.3 SDS-PAGE Analysis

SDS-PAGE analysis was performed as described by Laemmli (1970). Separating gels used were between 8-12% acrylamide depending on the size of the protein of interest. Gels were composed of 40% Bis-acrylamide solution (29:1), 375mM Tris pH 8.8, 0.1% SDS, 0.2% APS and 0.1% TEMED. Separating gels were allowed to polymerise for 30 minutes to 1 hour under a layer of water. The water was then removed and a stacking gel comprising 4% Bis-acrylamide (29:1), 125mM Tris pH 6.8, 0.1% SDS, 0.2% APS and 0.1% TEMED was poured and allowed to polymerise with a gel comb to create

wells. Mini-gels (10cm x 7cm x 0.7mm) were loaded with 30µg of protein per well and run at 20mA per gel in SDS running buffer (2.2.4.).

2.5.4 Western Analysis

Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane using Biorad wet transfer apparatus at 250mA for 1 hour in Wet Transfer Buffer (2.2.4.). The membranes were then blocked with 5% skim milk powder in 0.1% Triton-X in PBS at 4°C overnight. Membranes were incubated with primary Ab with 1% skim milk powder in 0.1% Triton-X in PBS in sealed plastic bags for 2 hours while rotating. The membrane was then washed 4 x 10 min. with 0.1% Triton-X in PBS. Membranes were then incubated with HRP-conjugated secondary Ab with 1% skim milk powder in 0.1% Triton-X in PBS for 1 hour while rotating. The membrane was then washed 4 x 10 min. with 0.1% Triton-X in PBS and then washed with chemiluminescent substrate (Pierce) for 1 minute. The membrane was exposed to X-ray film and the film developed using an AGFA Curix 60 developer.

2.6 Determination of Cell Cycle Status

2.6.1 Preparation of cells for FACS analysis

$1x10^7$ cells were harvested by centrifugation for 30 seconds at 20,800 rcf and resuspended in 3mL of water. Cells were sonicated briefly for 3-5 seconds followed by the addition of 7mL of cold 95% ethanol while vortexing and incubated at 4°C overnight. Cells were harvested by centrifugation at 2,060 rcf for 5 min. and

resuspended in 5mL of 50mM sodium citrate pH7. Cells were pelleted again by spinning for 5 minutes at 2,060 rcf. The supernatant was discarded and cells were resuspended in 1mL of 50 mM sodium citrate pH 7 followed by sonication for 3-5 seconds. RNase A was added to a final concentration of 0.25mg/mL and incubated for 1 hour at 50°C. 50µL of 20mg/mL proteinase K was added followed by incubation at 50°C for 1 hour. At room temperature, 1mL of 50mM sodium citrate pH 7 containing 16mg/mL of propidium iodide was added and cells were analysed on a flow cytometer.

2.7 RNA Detection Methods

2.7.1 Isolation of Cellular RNA

Cells were harvested by centrifugation for 5 minutes at 2,060 rcf. Pelleted cells were washed with 1mL of water and collected by centrifugation for 30 seconds at 20,800 rcf. Cells were resuspended in 250µL of LETS (2.2.4.) and transferred to cold screw cap tubes containing 300µL of phenol and 300µL of acid washed glass beads. Cells were lysed in a Biospec bead beater at 4°C using 3x 15 second bursts at maximum speed. Tubes were then spun for 1 minute at 20,800 rcf and an extra 250µL of LETS was added and tubes vortexed for 15 seconds. Tubes were then spun for 5 minutes at 20,800 rcf. The supernatant was collected and extracted twice with an equal volume of phenol/chloroform. RNA was precipitated with 0.1x volumes of 5M lithium chloride and 2.5 volumes of cold ethanol and placed at -80°C for 1 hour. After centrifuging at 20,800 rcf for 10 min. the pellet RNA was dried at 65°C. The pellet was then

resuspended in 250µL water and RNA was re-precipitated by the addition of 0.1x volumes of 3M sodium acetate pH 5.2 and 2.5x volumes of cold ethanol. The RNA was collected by centrifugation at 20,800 rcf for 10min., dried and resuspended in 50µL of water after drying at 65°C.

2.7.2 Northern Blot Analysis

RNA isolated from whole cell extracts was run on a 2.2M formaldehyde gel (1.5g agarose, 108mL water, 15mL 10xMOPS (2.2.4.), 27mL 40% formaldehyde) using 1xMOPS as running buffer. 20µg of RNA was added to 20µL of loading dye (2.2.4) and final volume made up to 30µL with RNase free water. Samples were incubated at 65°C for 10 minutes before loading on to gel. Gel was run at 70V for 2 hours. RNA was visualised under UV light and the gel was washed for 10 minutes in RNase free water. RNA was capillary transferred to a pre-wet Amersham Hybond-N nylon membrane overnight using 20x SSC (2.2.4.) as the transfer buffer. After air drying the membrane was auto UV crosslinked using a Stratagene UV Crosslinker.

The membrane was pre-hybridised For 2 hours at 42°C using Ambion ULTRAhyb solution. Probes were generated using an Amersham Megaprime Labelling Kit using 1µg of probe DNA. The probe was purified using Sephadex G-50 Quick Spin columns by spinning for 5 min. at 330 rcf. The probe was transferred to a screw cap tube and denatured for 3 min. at 100°C before being added directly to the membrane/pre-hybridisation mixture. The membrane was incubated overnight at 42°C in a Hybaid

oven. The probe solution was removed and the membrane washed twice at 42°C in a Hybaid oven with 2xSSC, 0.1% SDS for 15 minutes followed by 2 washes with 0.2xSSC, 0.1% SDS for 15 minutes. The membrane was then placed in a sealed plastic bag and exposed to X-ray film at –80°C. Signals were also detected using a Molecular Dynamics PhosphorImager machine.

Membranes were stripped by incubation in 0.01% SDS at 65°C for 2 hours and stored between 2 pieces of Whattman Paper at room temperature.

2.8 In vitro Protein Translations

2.8.1 In vitro Coupled Transcription/Translation

All *in vitro* protein translations were carried out using a Promega TnT Rabbit Reticulocyte Lysate Kit according the manufacturers directions but using 2µg of DNA. Sample was stored at –80°C.

Translated protein was quantified by running 2µL of reaction mix on a 12% SDS PAGE gel. The gel was then fixed (50% MeOH, 10% Acetic acid), amplified and dried on a gel dryer followed by exposure to X-ray film at –80°C. Appropriate bands were cut from gel and counted in a LKB 1214 Rackbeta Liquid Scintillation Counter. The specific activity of each band was used to quantify the amount of translated protein according to the presence of Methionines in the protein.

2.9 Electrophoretic Mobility Shift Assays

2.9.1 Generation of Radiolabelled DNA Probes

100 pmoles each of two complimentary 50mer DNA oligonucleotides were added to 5µL of 10x annealing buffer (2.2.4.) and the final volume made up to 50µL with water. Oligonucleotides were annealed in a Perkin Elmer 9600 PCR machine using the following cycle: 3 minutes at 80°C, 60 minutes ramp to 25°C, 14 minutes 4°C. Or probe was retrieved from plasmid DNA via digestion.

Probe was radiolabelled in the following reaction: 5µL annealed probe, 5µL [γ-P^{32}] dATP (10mCi/mL), 5µ L [γ-P^{32}] dCTP (10mCi/mL), 1.3µL 0.5mM dGTP, 1.3µL 0.5mM dTTP, 0.5µL 1M DTT, 1µL Klenow and 2µL 10x annealing buffer. The reaction mixture was incubated for 1 hour at 25°C followed by the addition of 2µL of 0.5M EDTA to stop the reaction. 5µL of 10x load buffer was added and the mix was loaded onto a pre-run native 12% 19:1 acrylamide gel in 1x TBE and run for 2 hours at 170V. The gel was then wrapped in saran wrap and exposed to X-ray film. The appropriate band was cut from the gel and incubated at 37°C overnight in 500µL of Maxam-Gilbert buffer (2.2.4.). The liquid was then retrieved and DNA was precipitated with 2µL of glycogen and 3x volumes of cold ethanol. The tube was then spun for 30 minutes at 20,800 rcf. The pellet was retrieved and air-dried before being resuspended in 50µL of 1x binding buffer (2.2.4). 1µL was counted in a LKB 1214

Rackbeta Liquid Scintillation Counter and the probe activity was adjusted to 100,000 counts per minute. Probes were stored at 4^0C.

2.9.2 Mobility Shift Assays

Binding reactions were carried out as follows: 4μL 5x binding buffer, 1μL poly dIdC:dIdC, 1-5μL of *in vitro* translated protein (2.8.1.) and water to 20μL. The reaction mixture was allowed to sit on bench for 5 minutes before adding 1μL of radiolabelled DNA probe (100,000 counts). The reaction was then incubated at room temperature for 20 minutes followed by addition of 2μL of load dye. Samples were loaded on a pre-run native 4% 19:1 acrylamide gel consisting of 5mL 40% acrylamide (19:1), 1.25mL 20x TBE, 42.5mL water, 100μL TEMED and run at 170V for 2 hours in 0.5x TBE. The gel was then transferred to Whattman Paper and dried on a gel dryer, followed by exposure to X-ray film.

2.10 Enzymatic Assays

2.10.1 Blue/White Assay

N-Hybond nitrocellulose membranes were placed onto selective media yeast plates on which yeast was streaked in patches. After incubation overnight filters were submerged in liquid nitrogen for 5 seconds and allowed to air dry. Whattman paper was saturated with 1.8ml Z buffer + 25μL if X-gal solution (2.2.4) on which the filter was placed. This was incubated until the colour developed. Another piece of Whattman paper was

saturated with 1.8ml of 0.25M sodium carbonate and the filter was added to stop the reaction. Filters were allowed to dry.

Chapter 3:

Fkh2p is a Component of SFF

3.0 Fkh2p is a Component of SFF

3.1 Introduction.

Recently, R Kumar in our lab was able to purify a biochemical activity that followed SFF activity *in vitro*. Yeast extracts were put through two rounds of DNA affinity chromatography using concatenated $SWI5_{UAS}$ oligonucleotide duplexes that were biotinylated and coupled to streptavidin beads. The corresponding activity was examined via MALDI analysis and identified as Fkh2p (Kumar *et.al.,* 2000). Fkh2p is a member of a family of transcription factors that are characterised by their DNA binding domain (DBD), a winged-helix motif (see 1.3.3.2.3). In the budding yeast there are four forkhead proteins, one of which (Fkh1p) shows an 82% similarity to Fkh2p (see figure 1.7). For Fkh2p to be a component of SFF it must exhibit the biochemical and genetic characteristics of SFF. SFF was identified as a co-factor of Mcm1p in "CLB2 cluster" regulation. Examination of the UAS's of the "CLB2 cluster" has revealed the presence of Mcm1p/SFF consensus binding sites in 26 of the 33 genes (Spellman *et.al.,* 1998). Two of these UAS's (*SWI5* and *CLB2*) have been studied extensively and have shown SFF binding is dependent on Mcm1p (Lydall *et.al.*, 1991, Althoefer *et.al.*, 1995 and Maher *et.al.*, 1995). A single base substitution in the SFF consensus DNA binding site in the *SWI5* UAS eradicates SFF binding *in vitro* and inhibits reporter activation *in vivo* (Lydall *et.al.*, 1991). In this chapter I report the biochemical characterisation of Fkh2p as SFF in its ability to form ternary complexes with Mcm1p on the cell-cycle regulated

SWI5$_{UAS}$ and *CLB2*$_{UAS}$ and identify the Fkh2p and Mcm1p domains necessary for ternary complex formation.

3.2 Results

3.2.1 In vitro Binding Activity of Fkh2p on the SWI5$_{UAS}$ *and CLB2*$_{UAS}$

3.2.1.1 In vitro Binding on the SWI5$_{UAS}$

To examine the possibility that Fkh2p has SFF-like binding activity, *in vitro* band-shifts were used to test the ability of Fkh2p to form Mcm1p-dependent ternary complexes on the *SWI5*$_{UAS}$. *FKH2* was first PCR amplified from yeast genomic DNA using primers *FKH2*-F and *FKH2*-R. This PCR product was then cloned as a *Nco*I/*Xho*I fragment into T7pLINK (T_7*FKH2*) downstream of a T_7 promoter allowing *in vitro* translation. A 6-MYC epitope tag was inserted in frame as a *Not*I fragment at the C-terminus of *FKH2* for identification using the 9E10 mono-clonal antibody (Ab). Fkh2p and Fkh2p$_{6myc}$ were translated in rabbit reticulocyte lysate (RRL) and their ability to bind the *SWI5*$_{UAS}$ was tested using band-shifts (figure 3.1). Two probes were used in the band-shift assays. The first was a 55bp fragment from the *SWI5*$_{UAS}$ that contained the Mcm1p and SFF binding sites called *SWI5*$_{wt}$ and the second was a mutant probe (*SWI5*$_{A296}$) containing a single base substitution, an insertion of an adenine at position 296, abolishing SFF binding (Lydall *et.al.,* 1991). In the presence of recombinant Mcm1p$_{1-112}$ (rMcm1$_{1-112}$), Fkh2p and Fkh2p$_{6myc}$ were able to form ternary complexes on the *SWI5*$_{wt}$ probe (figure 3.1). Addition of α-myc Ab shifted the Mcm1p$_{1-112}$-Fkh2p$_{6myc}$

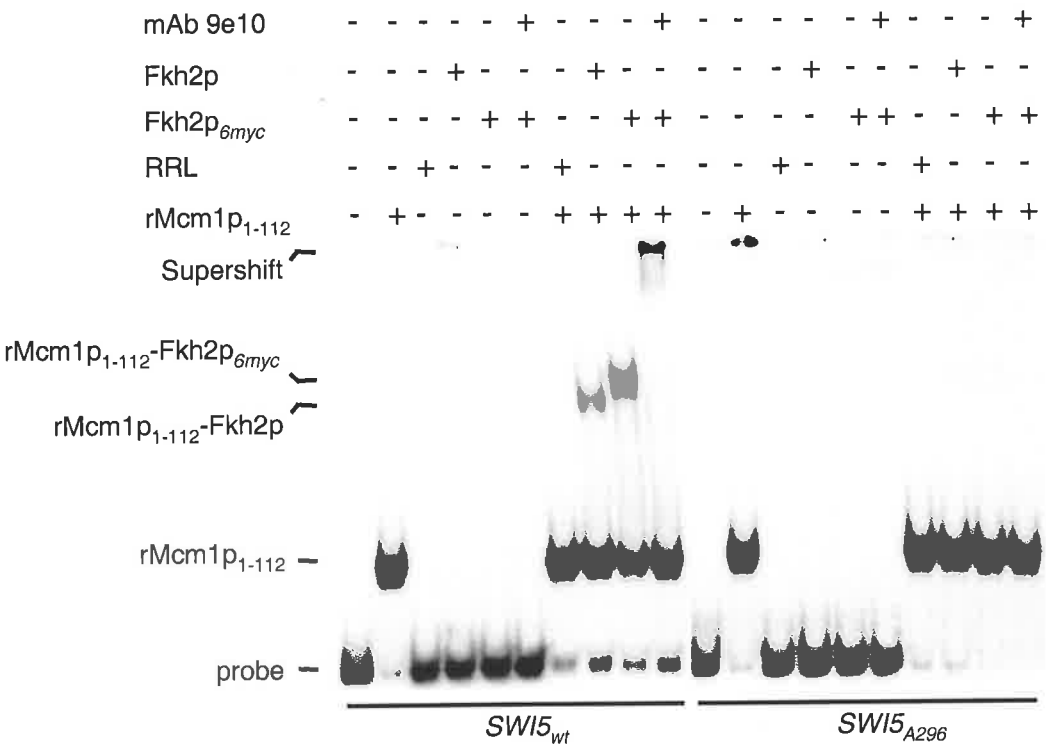


Figure 3.1 Ternary complex formation by Fkh2p on the *SWI5* promoter *in vitro*. $SWI5_{wt}$ and $SWI5_{A296}$ probes were used in band-shift assays with or without recombinant $Mcm1p_{1\text{-}112}$ ($rMcm1p_{1\text{-}112}$) and either Fkh2p or $Fkh2p_{6myc}$ or unprogrammed lysate (RRL). Free probe and the position of complexes containing $rMcm1_{1\text{-}112}$, Fkh2p and $Fkh2_{6myc}$ are indicated. The addition of α-myc Ab antibody and the resultant supershifted complex are indicated.

complex indicating $Fkh2p_{6myc}$ was indeed a part of the ternary complex (figure 3.1). Autonomous binding of Fkh2p and $Fkh2p_{6myc}$ could only be seen after long exposures (data not shown). On the $SWI5_{A296}$ probe however, neither Fkh2p or $Fkh2p_{6myc}$ could form complexes in the presence or absence of $rMcm1p_{1-112}$. The ternary complexes Fkh2p formed with $rMcm1p_{1-112}$ behave like those previously characterised for SFF from cell extracts (Lydall *et.al.*, 1991, and figure 3.3 a).

To further examine the ability of Fkh2p to form ternary complexes *in vitro*, a yeast strain containing a 6-MYC epitope tagged Fkh2p was constructed. A *FKH2* DNA fragment (encoding αα323-αα862) from the T_7 *FKH2* vector mentioned previously was cloned into the yeast vector pYIplac204 (*TRP1*) as a *Hind*III/*Xho*I fragment (figure 3.2 a). In addition a 6-MYC epitope tag was inserted 3' of the STOP codon as a *Not*I fragment (figure 3.2 a). This construct was linearised using *Nco*I (contained within *FKH2*) and transformed into a *W303* yeast strain expressing a truncated $Mcm1p_{198}$, S226 (*W303: MAT**a** mcm1::LEU2 ura3::ADH-mcm1*$_{1-98}$) for integration at the *FKH2* locus. Colonies that were selected on –trp media were tested using PCR (primers *FKH2*-F and UF) and Western analysis to identify integrants expressing $Fkh2p_{6myc}$ (figure 3.2 b and c). A successful integration formed a functional $Fkh2p_{6myc}$ and a non-functional truncated Fkh2p.

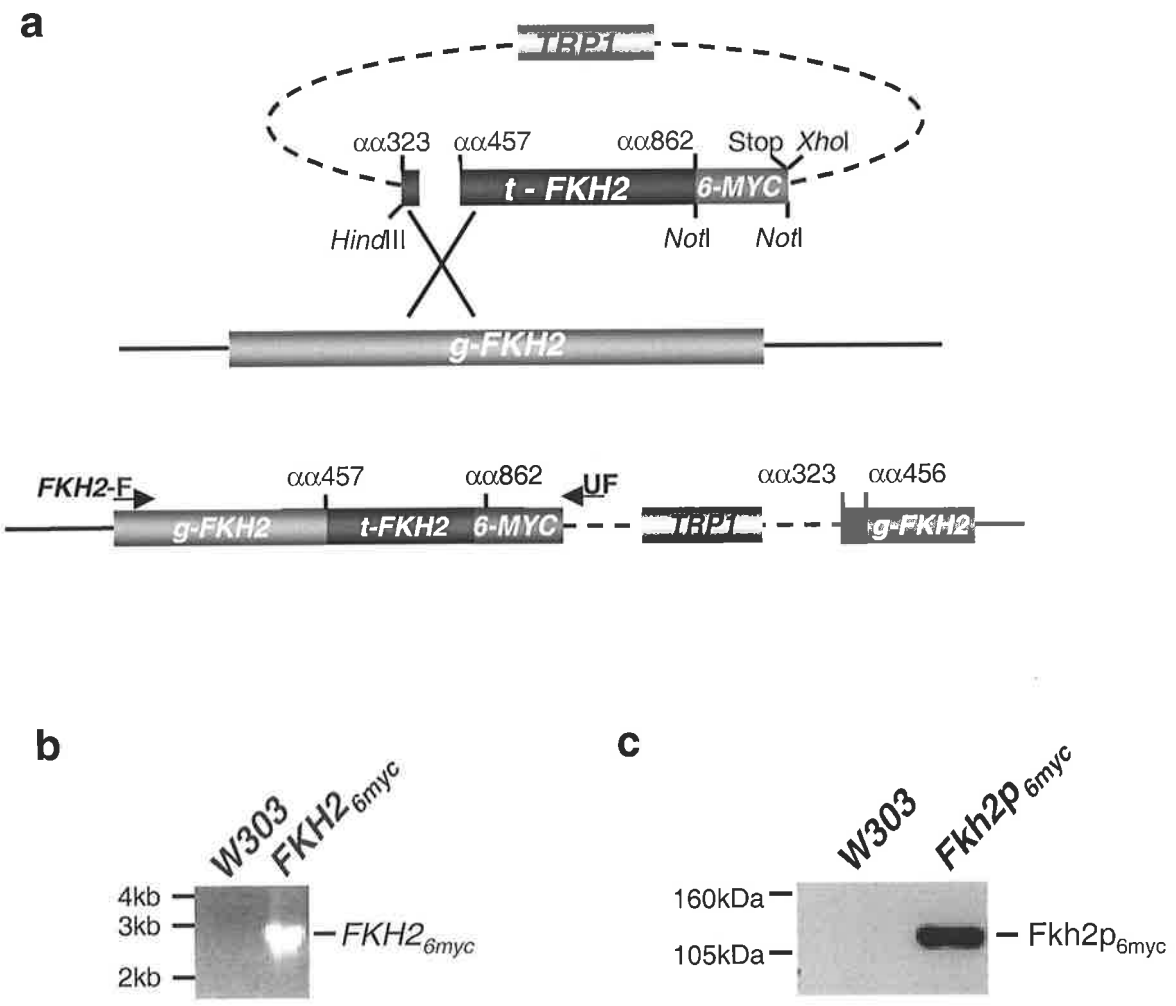


Figure 3.2 Construction of an epitope tagged Fkh2p yeast strain. (**a**) A *FKH2* fragment (bases 968-2589 (t-*FKH2*)) was cloned as a *Hind*III/*Xho*I fragment (*Xho*I was engineered by PCR) into pYIplac204. A 6-MYC epitope tag was inserted at the 3' end of t-*FKH2* as a *Not*I fragment (*Not*I was engineered by PCR). The plasmid was then linearised by *Nco*I and integrated into the *FKH2* locus (g-*FKH2*) in a *W303* yeast strain, S226 (W303: *MATa mcm1::LEU2 ura3::ADH1-mcm1*$_{1\text{-}98}$) through homologous recombination. The resultant clone produced a *TRP1* positive strain that has a truncated non -functional Fkh2p and a functional epitope tagged, full length Fkh2p. (**b**) The integration was confirmed by PCR using primers *FKH2*-F and UF. Genomic DNA was isolated *S226* and the *FKH2*$_{6myc}$ strain and used as the template in the PCR. The PCR product of a positive clone was 2889bp as indicated. (**c**) Cell lysate was isolated from *S226* and *FKH2*$_{6myc}$ yeast strains and tested using Western analysis. α-myc Ab was used to elucidate the presence of Fkh2p$_{6myc}$ which is a 106kDa protein.

Cell lysates from S226 and $FKH2_{6myc}$ (*W303: MATa mcm1::LEU2 ura3::ADH-mcm1*$_{1-98}$ *fkh2::FKH2*$_{6myc}$*-TRP1*) were collected and used in band-shift assays with $SWI5_{wt}$ and $SWI5_{A296}$ probes (figure 3.3 a and b). On the $SWI5_{wt}$ probe the previously characterised Mcm1p$_{1-98}$-SFF ternary complexes were formed (figure 3.3 a and b). The addition of the α-myc Ab supershifted the Mcm1$_{1-98}$-SFF complex in the strain containing Fkh2p$_{6myc}$ but not Fkh2p (figure 3.3 a and b) indicating the shift was specific for the presence of the 6-MYC epitope fused to Fkh2p. Neither of the *FKH2* and $FKH2_{6myc}$ strains formed ternary complexes on the $SWI5_{A296}$ as expected (figure 3.3 a and b), although there was an uncharacterised band present (marked with *) that does not contain Fkh2p$_{6myc}$ (figure 3.3 b). These binding activities are consistent with those expected for Mcm1p$_{1-98}$-SFF.

Further characterisation of Mcm1p-Fkh2p complexes in cell lysates were conducted using a galactose inducible $FKH2_{6myc}$ construct. *FKH2* was PCR amplified from yeast genomic DNA using primers *FKH2*-F and *FKH2*-R, containing engineered *Eco*RI, *Not*I and *Xho*I sites, and inserted into pYES2 vector as an *Eco*RI/*Xho*I fragment downstream of a *GAL1* promoter. A 6-MYC epitope tag was inserted at the C-terminus of *FKH2* as a *Not*I fragment 5' of the STOP codon. The pYES2 vector or the G.$FKH2_{6myc}$ were transformed into a *W303* yeast strain, S130 (*W303: MATa MCM1 ura3-52*), grown on raffinose containing medium until mid log phase, then supplemented with 2% glucose or 2% galactose and induced for 2 hours. The cell lysates from these cells were harvested and used in Western analysis and band-shifts using the $SWI5_{wt}$ probe (figure

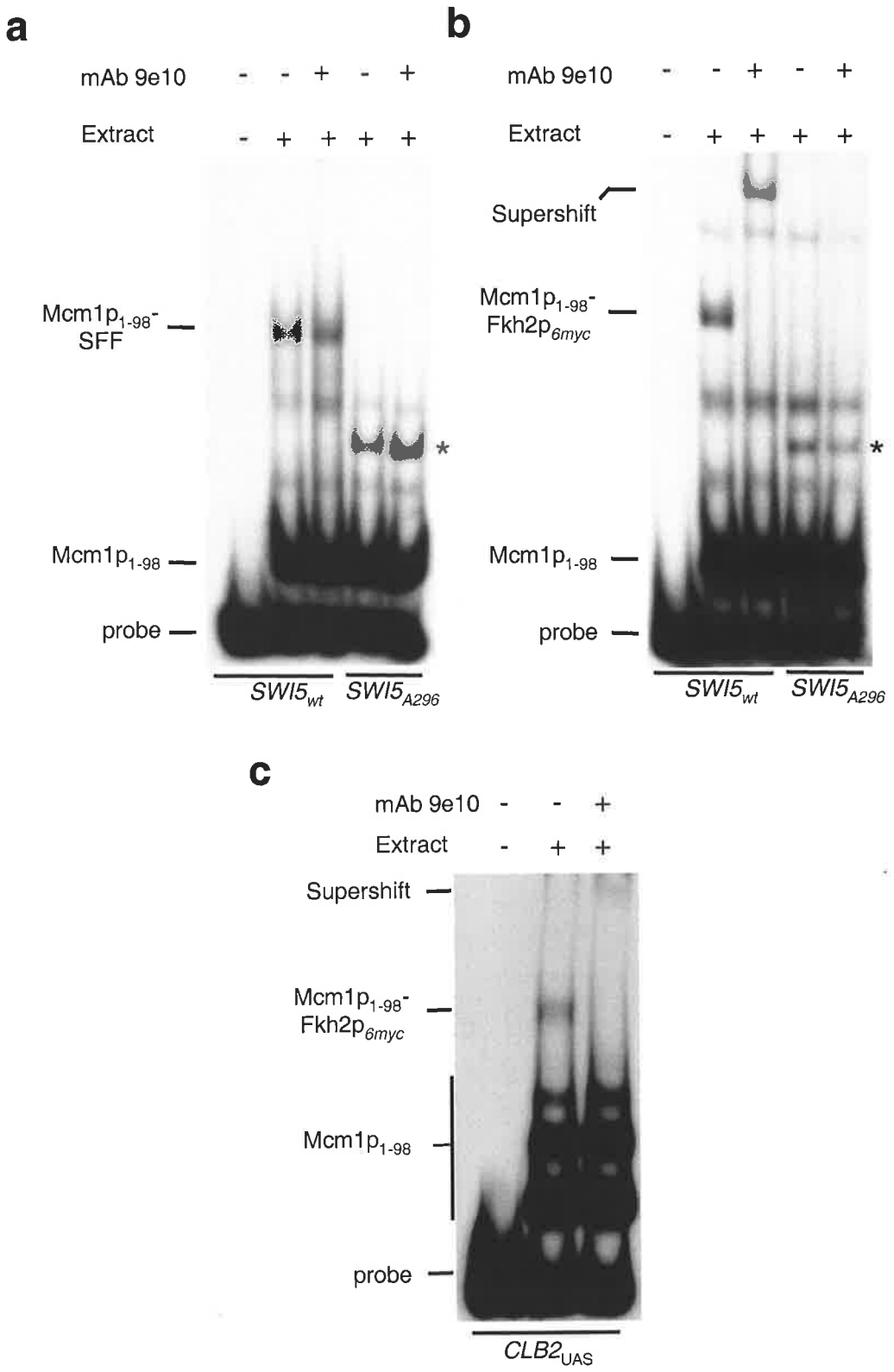


Figure 3.3 Ternary complex formation by Fkh2p. (**a**) Cell extracts were prepared from S226 and used in a band-shift assay with the $SWI5_{wt}$ or $SWI5_{A296}$ probe. The free probe, $Mcm1p_{1\text{-}98}$ and $Mcm1p_{1\text{-}98}$-SFF complexes and the addition of α-myc Ab are indicated. An uncharacterised complex forms on the $SWI5_{A296}$ mutant probe (indicated with *). (**b**) Same as **a** but using the $FKH2_{6myc}$ yeast strain. Complexes including supershifted complexes are as indicated. (**c**) Same as **b** but using the $CLB2_{UAS}$ probe.

3.4 a and b). The induction of $Fkh2p_{6myc}$ (figure 3.4 a) resulted in the formation of an additional complex on the $SWI5_{wt}$ probe indicative of Mcm1p-SFF ternnary complexes which was shifted with the addition of the α-myc Ab (figure 3.4 b). This further shows Fkh2p can form ternary complexes with Mcm1p *in vitro*.

3.2.1.2 *In vitro Binding on the $CLB2_{UAS}$*

The *in vitro* assembly of $Mcm1p_{1-112}$-Fkh2p complexes on the $CLB2_{UAS}$ was tested through band-shift assays using two probes. The first was a 55bp fragment containing wild type (wt) Mcm1p binding sites and a wt SFF site ($CLB2_{wt}$) (Maher *et.al.*, 1995). The second probe was a 240bp fragment of the *CLB2* UAS that contained three Mcm1p binding sites ($CLB2_{UAS}$) and has been shown to be essential for optimal UAS activity (Loy *et.al.*, 1999). *in vitro* translated Fkh2p and $Fkh2p_{6myc}$ were added to these probes with or without $rMcm1p_{1-112}$. Ternary complexes readily formed on the $CLB2_{wt}$ and $CLB2_{UAS}$ probes in the presence of $rMcm1p_{1-112}$, Fkh2p or $Fkh2p_{6myc}$ (figure 3.5 a and b) but only low level autonomous Fkh2p binding was observed after a long exposure as was the case on the $SWI5_{wt}$ (data not shown). On the $CLB2_{UAS}$ probe multiple $rMcm1p_{1-112}$ and $rMcm1p_{1-112}$-Fkh2p complexes were observed (Figure 3.5 b), probably due to the three Mcm1p-SFF binding sites found in this 240bp fragment of the *CLB2* UAS. The addition of α-myc Ab shifted the $rMcm1p_{1-112}$-$Fkh2p_{6myc}$ complexes on the $CLB2_{wt}$ and $CLB2_{UAS}$ probes (figure 3.5 a and b respectively). The observation of Fkh2p binding in a Mcm1p dependent manner on the *CLB2* UAS implicates Fkh2p as a component of SFF.

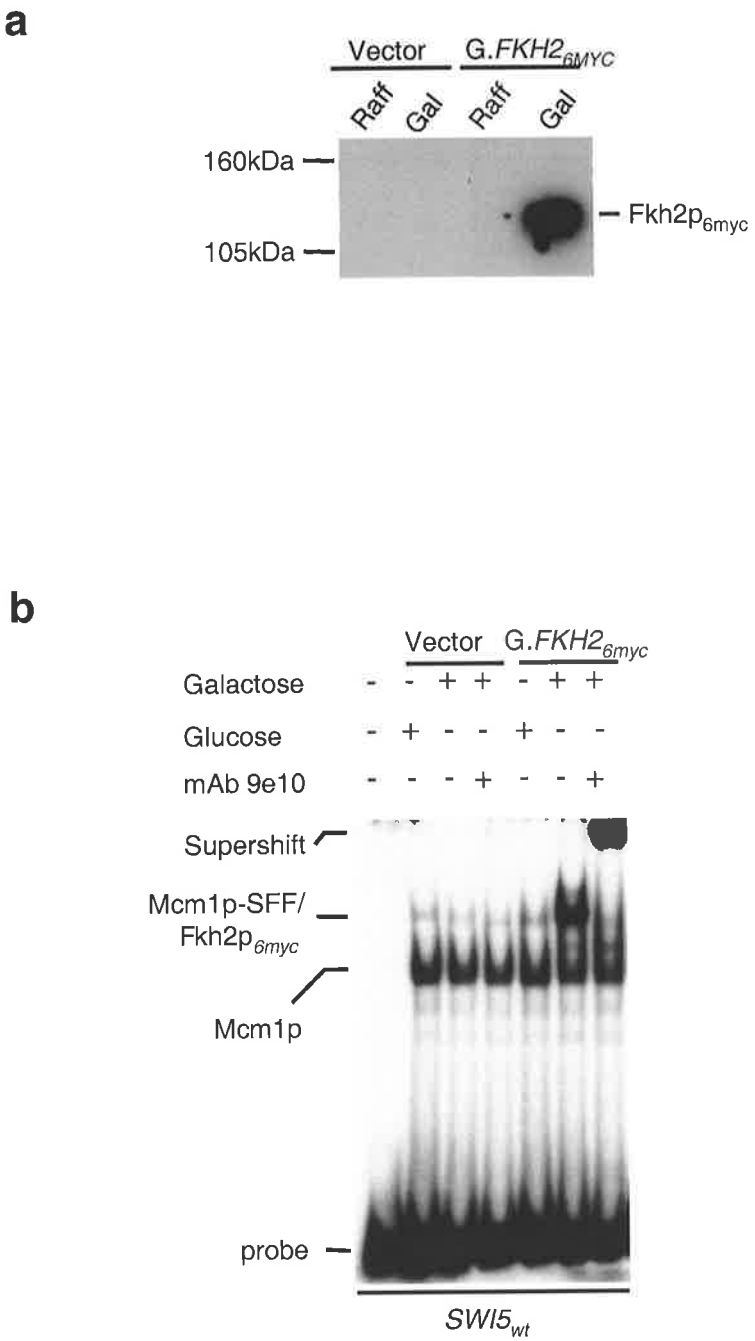


Figure 3.4 Overexpressed Fkh2p forms ternary complexes with Mcm1p. (**a**) *W303* cells (S130) were transformed with the pYES2 (vector) or the corresponding galactose-inducible $FKH2_{6myc}$ vector (G.$FKH2_{6myc}$). Asynchronous log-phase cells were grown in Raffinose-containing medium and then supplemented with either 2% Glucose or 2% Galactose. After 2hrs of induction, cells were harvested and lysates were checked for the presence of $Fkh2_{6myc}$ as indicated. (**b**) The lysates from **a** were used in band-shift assays using the $SWI5_{wt}$ probe. Complexes are as indicated.

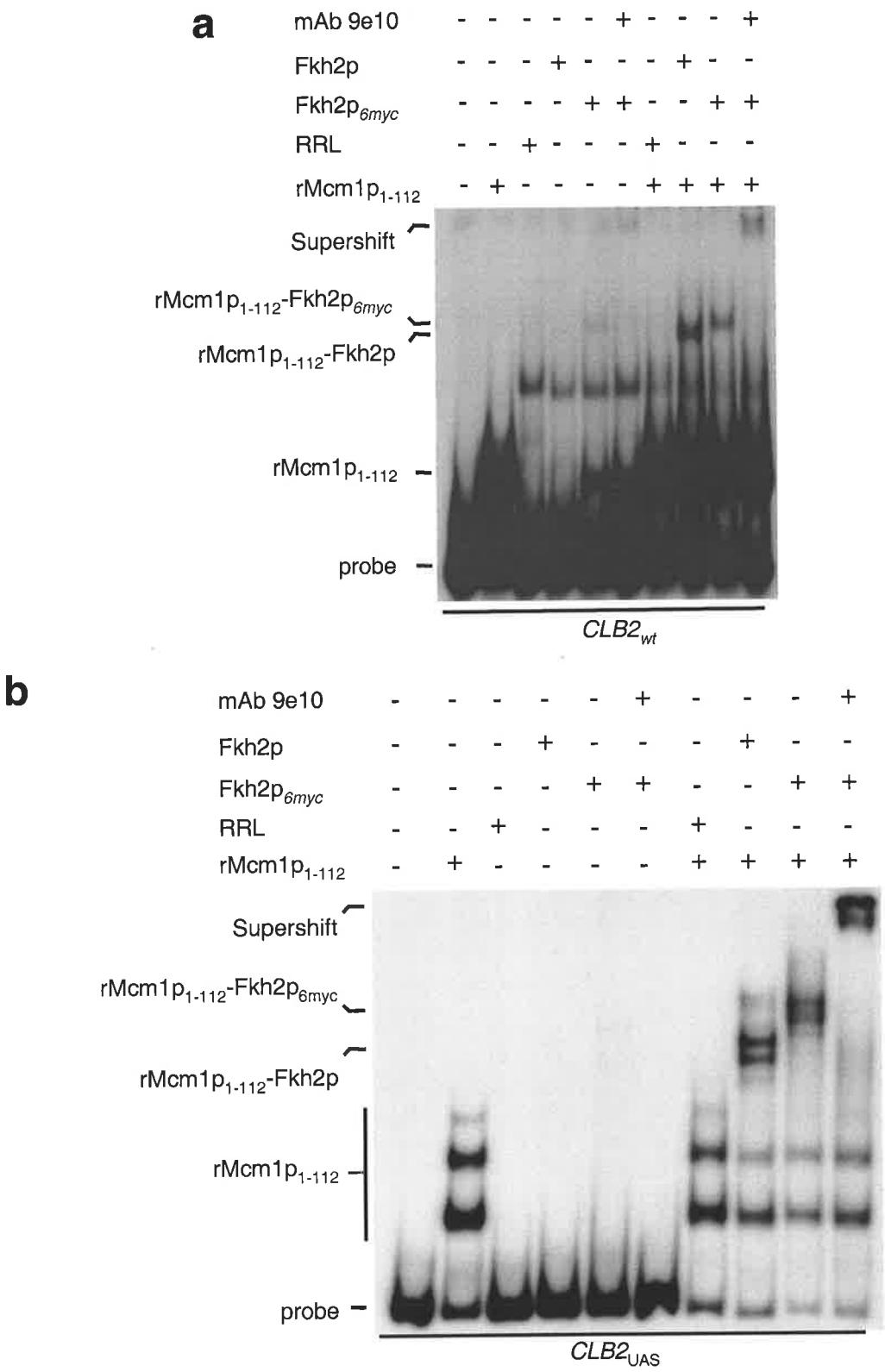


Figure 3.5 Ternary complex formation by Fkh2p on the *CLB2* promoter *in vitro*. (**a**) $CLB2_{wt}$ probe was used in band-shift assays with or without recombinant $Mcm1p_{1\text{-}112}$ ($rMcm1p_{1\text{-}112}$) and either Fkh2p or $Fkh2p_{6myc}$ or unprogrammed lysate (RRL). Free probe and the position of complexes containing $rMcm1_{1\text{-}112}$, Fkh2p and $Fkh2_{6myc}$ are indicated. The addition of α-myc Ab antibody and the resultant supershifted complexes are indicated. (**b**) Same as **a** but using $CLB2_{UAS}$ probe.

Cell lysates from the $Fkh2p_{6myc}$ yeast strain used in the *SWI5* experiments (figures 3.3 and 3.4 a and b) were added to the $CLB2_{UAS}$ probe in band-shift assays. Multiple $Mcm1p_{1-98}$ complexes were observed indicative of the multiple Mcm1p binding sites (figure 3.4 c). A $Mcm1p_{1-98}$-SFF ternary complex was also observed which was shifted with the addition of α-myc Ab (fig 3.4 C) indicating $Fkh2p_{6myc}$ as a component of this ternary complex.

3.2.2 Binding of Fkh1p to the $SWI5_{UAS}$ in vitro

FKH1 was cloned into the T_7pLINK vector (used previously for *FKH2*) as a *Bsp*MI/*Xho*I fragment generated by PCR from yeast genomic DNA using primers *FKH1*-F and *FKH1*-R. *FKH1* was translated in RRL and its ability to bind to the *SWI5* UAS in band-shift assays was tested. Fkh1p was unable to form complexes in the presence or absence of $rMcm1p_{1-112}$ on the $SWI5_{wt}$ or $SWI5_{A296}$ probe used in figure 3.1 (figure 3.6), indicating that Fkh1p does not share the properties of Fkh2p in binding the *SWI5* UAS *in vitro*, therefore it is unlikely to be SFF.

3.2.3 Mapping the Mcm1p Dependent DNA Binding Domain of Fkh2p.

Several Fkh2p truncation mutants were made based around the Fkh2p DBD (figure 3.7 a). T_7FKH2_{1-457} was amplified from T_7FKH2 using primers *FKH2*-1DBD and *FKH2*-F and inserted into T_7pLINK as a *Nco*I/*Xho*I fragment. $T_7FKH2_{236-862}$ was also amplified using primers *FKH2*-2DBD and *FKH2*-R and cloned into T_7pLINK as *Nco*I/*Nco*I (αα326-αα457), *Nco*I/*Xho*I (αα458-αα862) fragments. $T_7FKH2_{326-457}$ was constructed

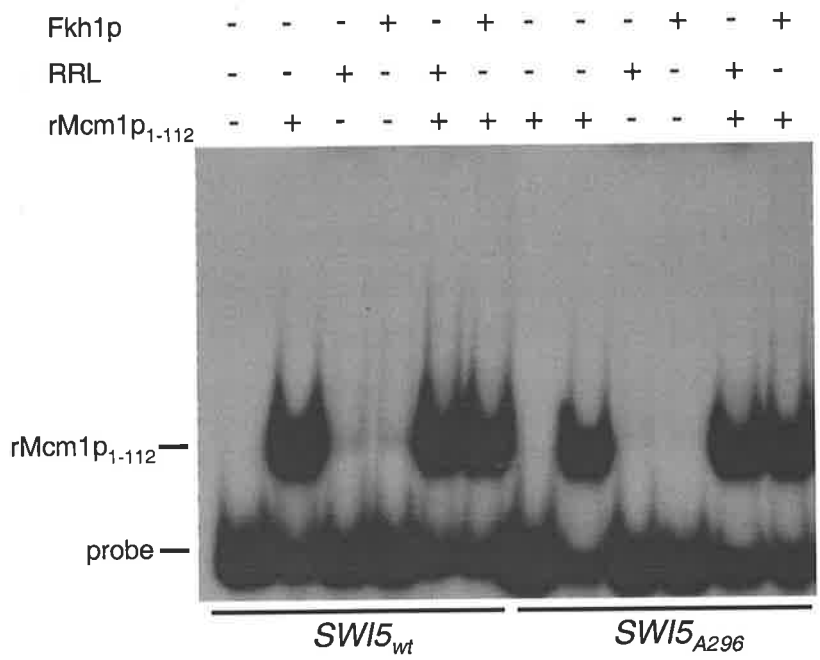


Figure 3.6 Lack of ternary complex formation by Fkh1p on the *SWI5* promoter *in vitro*. $SWI5_{wt}$ and $SWI5_{A296}$ probes were used in band-shift assays with or without recombinant $Mcm1p_{1-112}$ ($rMcm1p_{1-112}$) and either Fkh2p or unprogrammed lysate (RRL). Free probe and the position of complexes containing $rMcm1_{1-112}$, are indicated.

as a *Nco*I/*Xho*I (αα326-αα457) fragment generated by PCR using primers *FKH2*-2DBD and *FKH2*-1DBD. $T_7FKH2_{326\text{-}554}$ was constructed by removing a *Mun*I/*Not*I fragment from $FKH2_{236\text{-}862}$. $T_7FKH2_{\Delta DBD}$ was constructed by removing the DBD of T_7FKH2 generating D G I D K P. Each of the constructs were translated in RRL and used in band-shift assays with the $SWI5_{wt}$ probe with or without $rMcm1p_{1\text{-}112}$ (figure 3.7 b). $Fkh2p_{\Delta DBD}$ and $Fkh2p_{326\text{-}457}$ were unable to form ternary complexes in the presence or absence of $rMcm1_{1\text{-}112}$ (figure 3.7 b) demonstrating that the DBD is essential but not sufficient for complex formation. The other mutants formed $rMcm1p_{1\text{-}112}$ ternary complexes as efficiently as Fkh2p (figure 3.7 b) indicating that neither the amino or carboxy-termini are important for Mcm1p dependent DNA binding while a small extension of the DBD is sufficient for ternary complex formation.

3.2.4 The Protein Interaction Domain of Mcm1p is Necessary for Fkh2p Recruitment.

To determine if Fkh2p recruitment to DNA is due to the Mcm1p DNA binding or the protein interaction domain, fusion constructs between Mcm1p and Serum Response Factor (SRF) were utilised (Nurrish and Treisman, 1995 and Wynne and Treisman, 1992). These constructs (figure 3.8 a) were translated in RRL and used in band-shift assays with *in vitro* translated Fkh2p and ActL-SFF and $SWI5_{wt}$ probes. The ActL-SFF probe contains an SFF binding site and an optimal SRF DNA binding element which Mcm1p does not bind. SRF is also unable to bind the $SWI5_{wt}$ probe. Autonomous Mcm1p and $Mcm1p_{1\text{-}112}$ binding to the ActL-SFF probe was not observed, however,

ternary complexes were formed in the presence of Fkh2p implicating Fkh2p in stabilising Mcm1p DNA binding through protein-protein interaction (figure 3.8 b). MMSSp (figure 3.8 a), which contains the Mcm1p DBD and the SRF protein interaction domain, could not form complexes in the presence or absence of Fkh2p on the ActL-SFF probe (figure 3.8 b). This implicates the ternary complexes that were observed with Mcm1p and $Mcm1p_{1\text{-}112}$ and Fkh2p on this probe are due to protein-protein interactions and not DNA binding. Furthermore, Mcm1p protein interaction domain, when fused to the SRF DBD (SSMMp (figure 3.8 a)), was able to recruit Fkh2p into a ternary complex whereas SRF could not (figure 3.8 b). These experiments implicate the protein-protein interaction domain of Mcm1p as the key component for Fkh2p recruitment.

In addition to the above experiments, $Mcm1p_{1\text{-}112}$ was able to form complexes on the $SWI5_{wt}$ probe with and without Fkh2p as previously shown in figure3.1 (figure 3.8 c). MMSSp was able to bind the $SWI5_{wt}$ probe but only formed weak ternary complexes in the presence of Fkh2p probably due to the absence of the optimal Mcm1p protein-protein interaction domain (figure 3.8 c). SRF could not bind to the $SWI5_{wt}$ probe, but when fused to the Mcm1p protein interaction domain (SSMMp) it could form ternary complexes with Fkh2p (figure 3.8 c). This complex was stronger than that observed with MMSSp implicating the Mcm1p interaction domain as the dominant domain for Fkh2p recruitment.

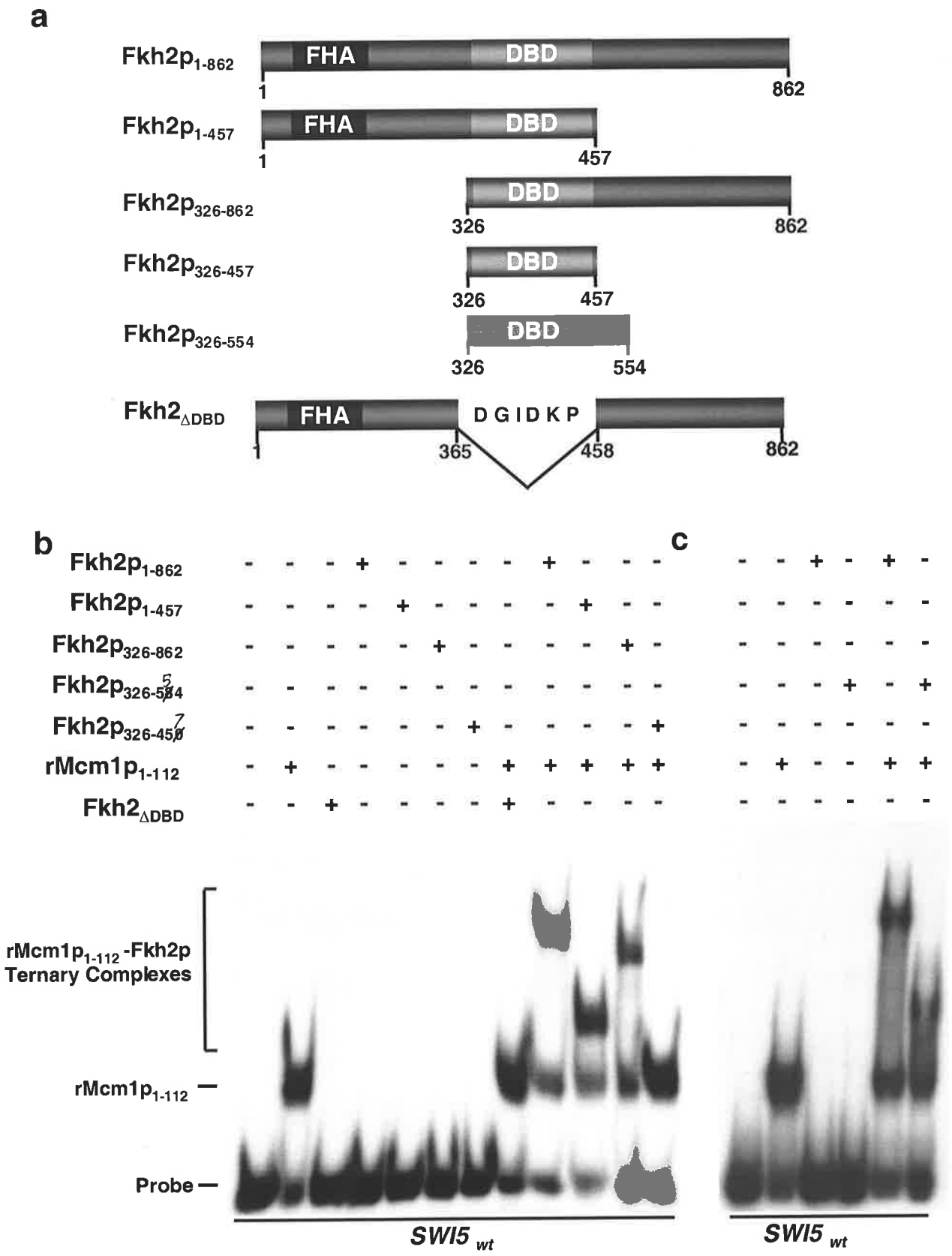


Figure 3.7 Mapping the Mcm1p dependent Fkh2p DBD. (**a**) Schematic representation of the truncation mutants constructed to map the essential components of Fkh2p for Mcm1p dependent ternary complex formation. (**b**) Band-shift assays were used to test the ability of *in vitro* translated Fkh2p mutants from **a** to form complexes on the $SWI5_{wt}$ probe with or without $rMcm1p_{1\text{-}112}$. Complexes are as indicated. (**c**) $Fkh2_{\Delta DBD}$ and $Fkh2p_{1\text{-}862}$ were used in the same experiment as **b** and complexes are as indicated.

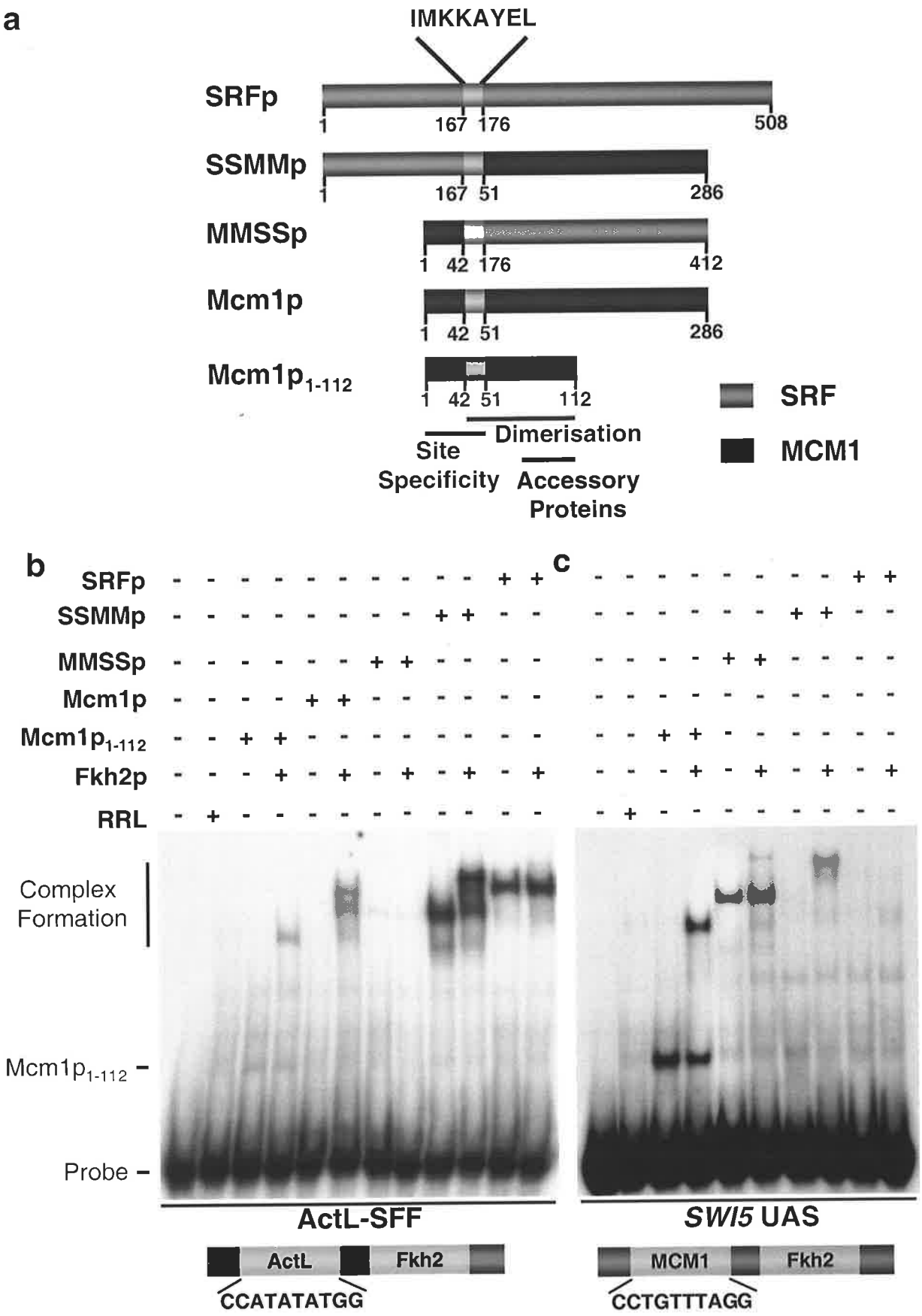


Figure 3.8 Domains of Mcm1p required for Fkh2p ternary complex formation. (**a**) Schematic representation of Mcm1-SRF fusion proteins. (**b**) Band-shift assays were used to test the ability of the *in vitro* translated fusion proteins from **a** to form ternary complexes with Fkh2p on the ActL-SFF probe. Complexes are as indicated. (**c**) Same as **b** but using the $SWI5_{wt}$ probe.

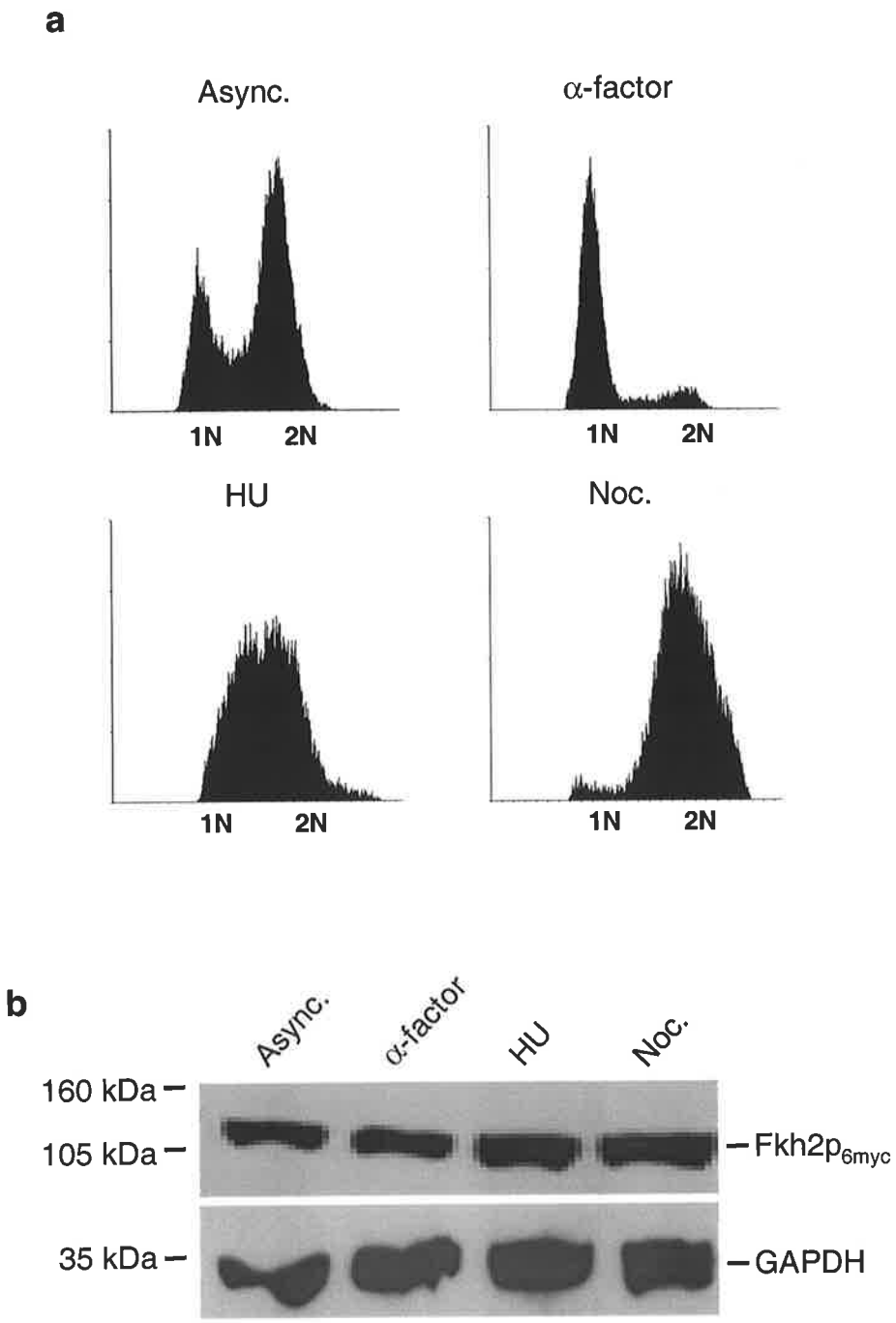


Figure 3.9: Fkh2p is constitutive throughout the cell cycle. (**a**) Cells containing $FKH2_{6myc}$ were arrested in G1, S-phase and mitosis and the blocks were confirmed using FACS analysis. (**b**) Cell lysates from **a** were analysed for the presence of $Fkh2p_{6myc}$ through the addition of α-myc Ab. GAPDH was used as a loading control.

Through the use of these SRF/Mcm1p fusion experiments we have identified the Mcm1p protein-protein interaction domain as being vital for the recruitment of Fkh2p to the promoter rather than the DNA binding domain of Mcm1p.

3.2.5 Fkh2p is present throughout the cell cycle

To examine the abundance of Fkh2p in throughout the cell cycle the $FKH2_{6myc}$ strain from 3.2.1.1 (see figure 3.2) was blocked in G1 using α-factor, S-phase using hydroxyurea (HU) and mitosis with nocodazole (Noc). The $FKH2_{6myc}$ strain was grown to an OD_{660} 0.4 on YEP media supplemented with 2% glucose at which time the blocking agents were added. The cells were allowed to block for two hours, blocks were checked visually and the cells were harvested. $1x10^7$ cells were taken, fixed, DNA stained using propidium iodide and the blocks checked using FACS analysis (figure 3.9 a). Lysates were made from the remaining cells and run on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane and probed for the presence of $Fkh2p_{6myc}$ using α-myc Ab and GAPDH as a loading control (figure 3.9 b). Fkh2p was found in all phases of the cell cycle (figure 3.9 b).

3.3 Discussion

Here it has been demonstrated that the previously unidentified Mcm1p dependent DNA binding activity of SFF is Fkh2p. Fkh2p, a member of the forkhead family of transcription factors, binds to the *CLB2* and *SWI5* UAS's ("CLB2 cluster" genes) in a

Mcm1p dependent manner forming ternary complexes indistinguishable from that of SFF (figure 3.3). The consensus site that is present in the *SWI5* and *CLB2* promoters is identical to that reported for other members of the Forkhead family in other species (Kaufman and Knochel, 1996). This consensus sequence, along with the Mcm1p consensus, can be found in 26 of the 35 "CLB2 cluster" genes identified by microarray analysis (Spellman *et.al.*, 1998), implicating Fkh2p as a regulator of "CLB2 cluster" activation. The formation of Mcm1p/Fkh2p complexes *in vitro* does not exclude the possibility that other, as yet, unidentified factors may be involved in complex formation. Supporting these observations, work by other groups published at the same time have confirmed Fkh2p as a Mcm1p dependent binder of "CLB2 cluster" promoters (Pic *et.al.*, 2000 and Koranda *et.al.*, 2000). Further to this we, and others, have shown the *in vivo* recruitment of Fkh2p and Fkh1p to the "CLB2 cluster" promoters (Kumar *et.al.*, 2000, Koranda *et.al.,* 2000 and Zhu *et.al.*, 2000). However, we have shown that Fkh1p does not form ternary complexes with Mcm1p *in vitro* even though it has the same consensus sequence as Fkh2p. Recently however, Fkh1p and Fkh2p have been shown to bind autonomously to these UAS's *in vitro* when present at very high levels and the binding of Fkh2p is enhanced 100 fold in the presence of Mcm1p (Hollenhorst *et.al.*, 2001). This indicates Fkh2p is the dominant Mcm1p-dependent binder of the "CLB2 cluster" UAS's, even though Fkh1p has a 72% indentity with Fkh2p over the DBD and shares the same DNA consensus site (Zhu *et.al.*, 2000).

Mcm1p could be playing two roles in recruiting Fkh2p to the promoter. Firstly, the recruitment of Fkh2p via protein-protein interactions is supported by the ability of SFF to stabilise binding of Mcm1p to mutant *SWI5* binding sites (Lydall *et.al.*, 1991), however, DNA bending could play the major role in Fkh2p recruitment. This is supported by FREAC (human forkhead protein) whose DNA binding is thought to be influenced by DNA bending (Pierrou *et.al.*, 1994). In this chapter, it has been demonstrated that protein-protein interactions are the more probable role of Fkh2p recruitment by Mcm1p (figure 3.8). The protein-protein interaction domain of Mcm1p was able to recruit Fkh2p and stabilise ternary complex formation irrespective of the DNA binding domain. Furthermore, the DNA binding domain of Fkh2p is essential and with a small extension was sufficient to impart Mcm1p ternary complex formation.

Althoefer *et.al.* (1995) have previously shown the Mcm1p and SFF DNA binding sites are occupied continuously throughout the cell cycle. Here we have shown that the Fkh2p protein is present in all phases of the cell cycle leading to the possibility of Fkh2p constitutively occupying the SFF site. In support of this, a recent study has revealed Fkh2p binding the UAS's of the "CLB2 cluster" continuously throughout the cell cycle *in vivo* (Koranda *et.al.*, 2000). However, "CLB2 cluster" gene expression is still cell cycle regulated even when Fkh2p is present throughout the cell cycle indicating there is another activator and that Fkh2p could play a repressive role in its absence.

From these studies, it is evident that Fkh2p is part of the Mcm1p-SFF activity. The recruitment of Fkh2p to the "CLB2 cluster" is via the forkhead DNA binding consensus sequence recognised by its DBD and protein-protein interactions with its partner protein, Mcm1p.

Chapter 4:

Forkhead Proteins are Involved in "CLB2 Cluster" Regulation

4.0 Forkhead Proteins Involved in "CLB2 Cluster" Regulation

4.1 Introduction

At the G2/M transition there are 33 genes that share similar transcriptional profiles known as the "CLB2 cluster" (Spellman *et.al.,* 1998). It has been shown that Mcm1p binds as a homodimer and recruits SFF to form ternary complexes on the *SWI5* and *CLB2* promoters *in vitro* (Lydall *et.al.*, 1991, Althoefer *et.al.*, 1995 and Maher *et.al.*, 1995) and activate UAS's *in vivo* (Lydall *et.al.*, 1991). The binding site for Mcm1p homodimers and SFF can be easily identified in a majority of the "CLB2 cluster" gene proximal promoters implicating the Mcm1p-SFF complex as the regulator of the "CLB2 cluster of transcription (Spellman *et.al.*, 1998). In chapter 3, we identified Fkh2p as a protein that shares the biochemical binding characteristics of SFF, however, to further characterise Fkh2p, its ability to regulate the "CLB2 cluster" must be examined. To this end, deletion studies of *FKH2* were performed and recorded in this chapter. An important fact to be considered in this study was the high degree of similarity Fkh2p shared with Fkh1p and a possible functional redundancy that could exist between these two proteins. Based on their similarity deletion studies were conducted on *FKH1* singly and in conjunction with *FKH2*.

4.2 Fkh1p and Fkh2p are Required for Cell Cycle Regulation of the "CLB2 Cluster"

4.2.1 Construction of FKH deletion strains

To elucidate the role of Fkh1p and Fkh2p in the transcription of the "CLB2 cluster" at the G2-M transition, strains containing single or double deletions of the *FKH* genes were made. Bases 608 to 1267 of the coding region of *FKH1,* which contained the DBD, was replaced by a *LEU2* gene in T_7*FKH1* (figure 4.1 a and b). Using primers *FKH1*-8F and *FKH1*-8R the *Δfkh1::LEU2* construct was amplified and transformed into a *W303* yeast strain containing a $CLB2_{UAS}$-*UbiYLacZ* reporter construct, S287 *(W303: MATa bar1 leu2-3 ura3-1* $CLB2_{UAS}$-*UbiYLacZ)* replacing the endogenous *FKH1* via double homologous recombination (figure 4.1 b, c and d). Using primers *FKH1-LEU2* and *FKH1*-8R, PCR of genomic DNA from *LEU2* positive colonies was used to confirm the deletion of *FKH1* (*Δfkh1)* (figure 4.1 d and 4.3 b).

A deletion construct of *FKH2* was also made by cloning a *Xba*I/*Xho*I fragment from T_7*FKH2* into pBS (figure 4.2 a and b) then replacing bases 968 to 1315 of *FKH2*, which contained most of the DBD, with a *URA3* gene (*Δfkh2::URA3)* (figure 4.2 b). The *Δfkh2::URA3* was released from pBS as a *Xba*I/*Kpn*I fragment and transformed into S287 *(W303: MATa bar1 leu2-3 ura3-1*$CLB2_{UAS}$-*UbiYLacZ*) replacing the endogenous *FKH2* (figure 4.2 c and d). Again the presence of *Δfkh2::URA3* was confirmed via PCR

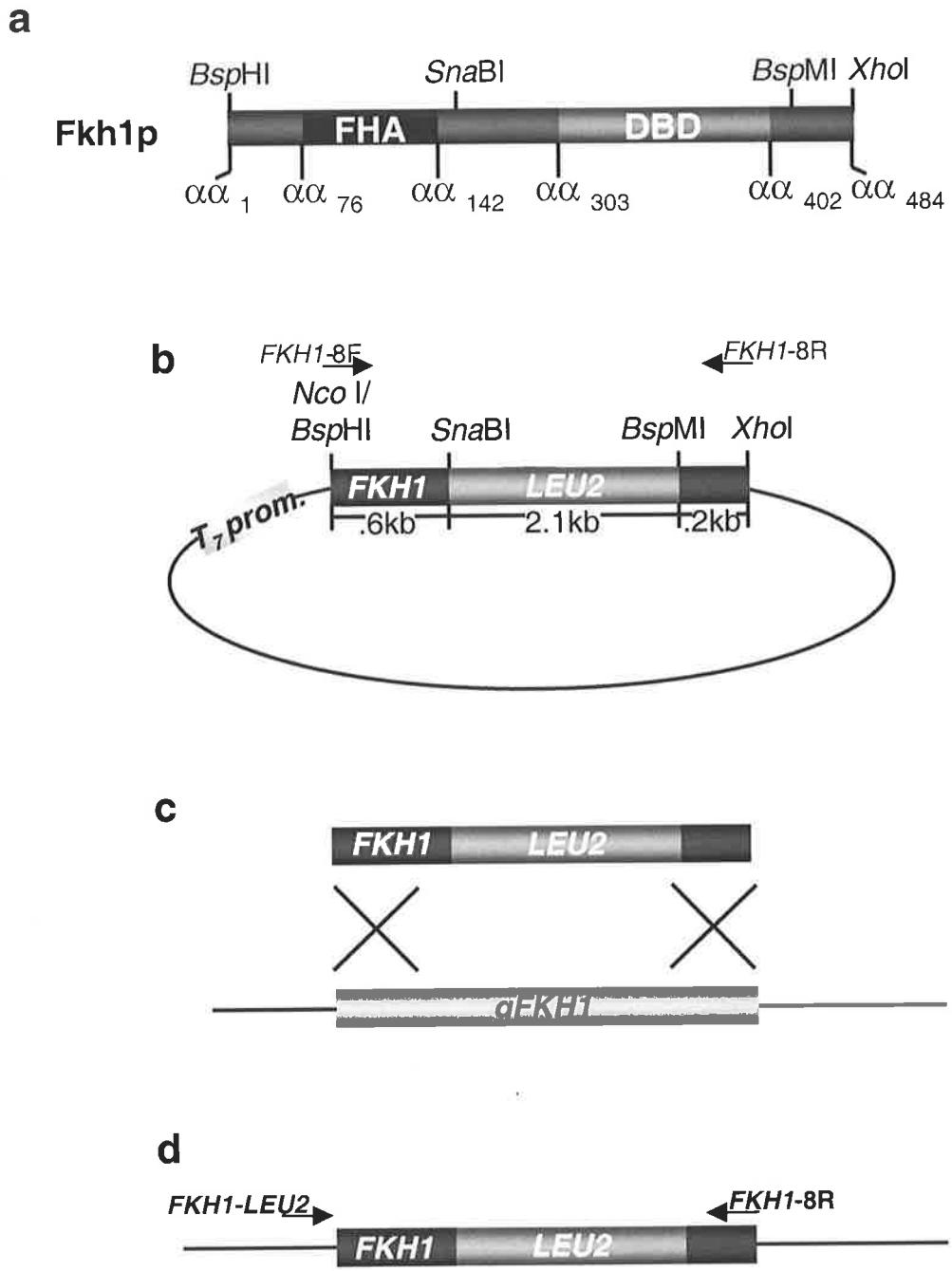


Figure 4.1: Schematic representation of the *FKH1* deletion strategy. **a** Schematic of *FKH1* indicating the important enzyme restriction sites and domains of Fkh1p. **b** Schematic of the *LEU2* insertion into T_7*FKH1* including the primers *FKH1*-8F and 8R used to amplify the *Δfkh1::LEU2* construct for transformation. **c** Representation of the *fkh1::LEU2* PCR fragment undergoing a double homologous recombination at the *FKH1* locus (*gFKH2*). **d** The resultant deletion at the genomic *FKH1* locus. The primers used to check for *Δfkh1* are as indicated.

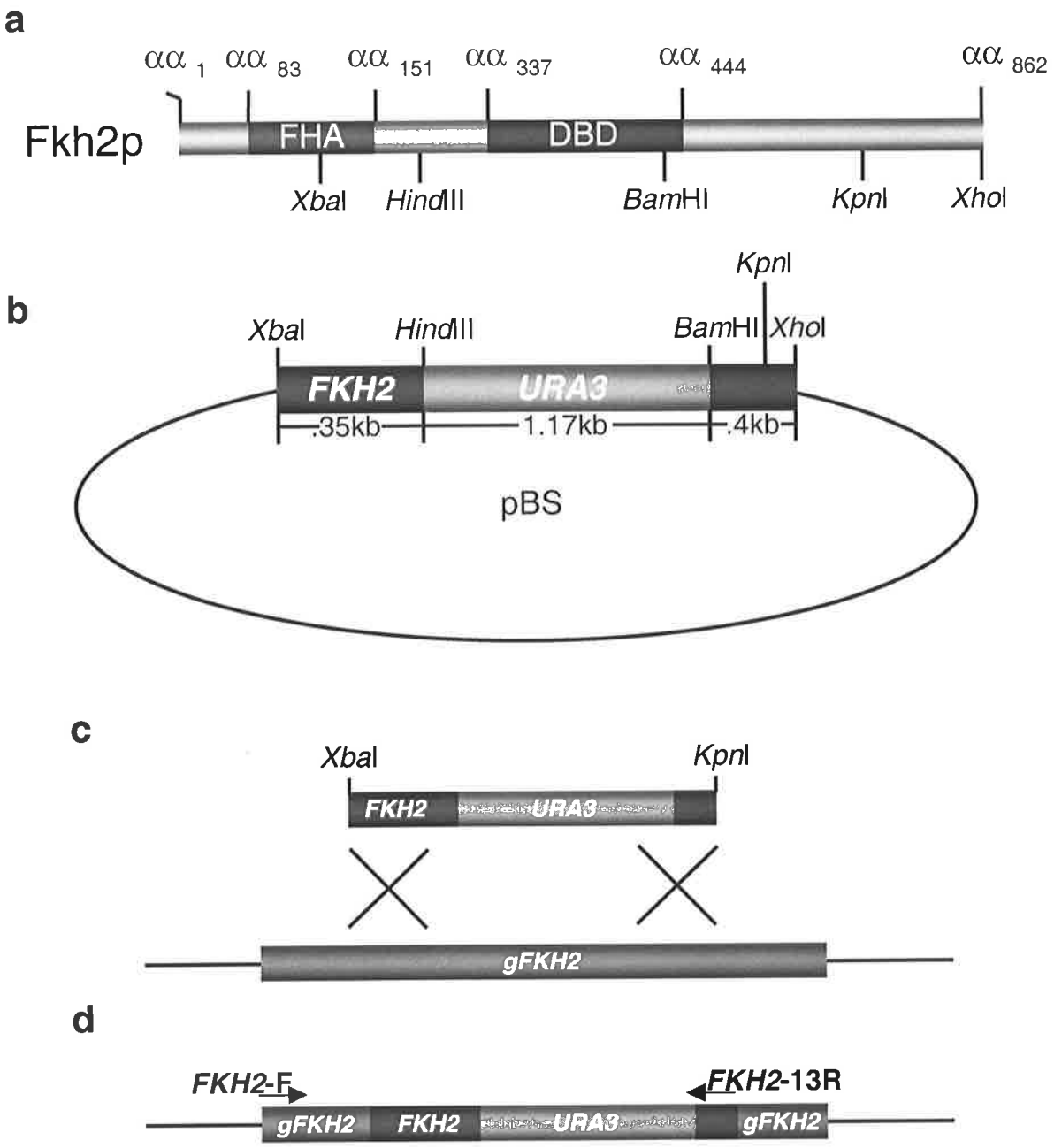


Figure 4.2: Schematic representation of the *FKH2* deletion strategy. **a** Schematic of *FKH2* indicating the important enzyme restriction sites and domains of Fkh2p. **b** Schematic of the *URA3* insertion into the truncated *FKH2* in pBS. **c** Representation of the *fkh2::URA3* fragment released from pBS which undergoes a double homologous recombination with the genomic *FKH2* (*gFKH2*). The restriction enzymes used to release the fragment are as indicated. **d** The resultant deletion at the genomic *FKH2* locus. The primers used to check for *Δfkh2* are as indicated

using primers *FKH2*-F and *FKH2*-13R (figure 4.2 d and 4.3 a). To construct the double delete strain (*Δfkh1, Δfkh2*), *fkh1::LEU2* was transformed into *FKH1 Δfkh2 (W303: MATa bar1 leu2-3 fkh2::URA3 CLB2*$_{UAS}$*-UbiYLacZ*) and confirmed by PCR (figure 4.1 d and 4.3 c).

4.2.2 Characterisation of the FKH deletion strains

S287 (*FKH1 FKH2*), *Δfkh1 FKH2, FKH1 Δfkh2* and *Δfkh1 Δfkh2* yeast strains were grown on YEP plates supplemented with 2% glucose. Visual examination of the colonies that formed showed wild type smooth colonies for S287, *Δfkh1 FKH2* and *FKH1 Δfkh2* strains whereas *Δfkh1 Δfkh2* produced small, rough colonies (figure 4.3 d). In addition, *Δfkh1 Δfkh2* grew slower than the other strains hence giving giving rise to the smaller colonies (figure 4.3 e). To further investigate this phenotype, the four strains were grown in liquid culture (YEP supplemented with 2% glucose) to and OD_{660} 0.8 and prepared for microscopy. The first observation was the slower growth rates of *Δfkh1 Δfkh2* when compared to S287. The doubling rate of S287 was 90 minutes whereas the *Δfkh1 Δfkh2* doubled every 150 minutes. *Δfkh1 FKH2* and *FKH1 Δfkh2* cells doubled every 120 minutes (S287 is 90 minutes) but looked similar to wild type with a few examples of larger cells (figure 4.4). However, when the *Δfkh1 Δfkh2* cells were examined, there was a greater phenotypic change observed (figure 4.4). The most obvious defect was in the inability for mother and daughter cells to undergo cytokinesis at the end of M-phase forming chain-like projections (figure 4.4). There were also

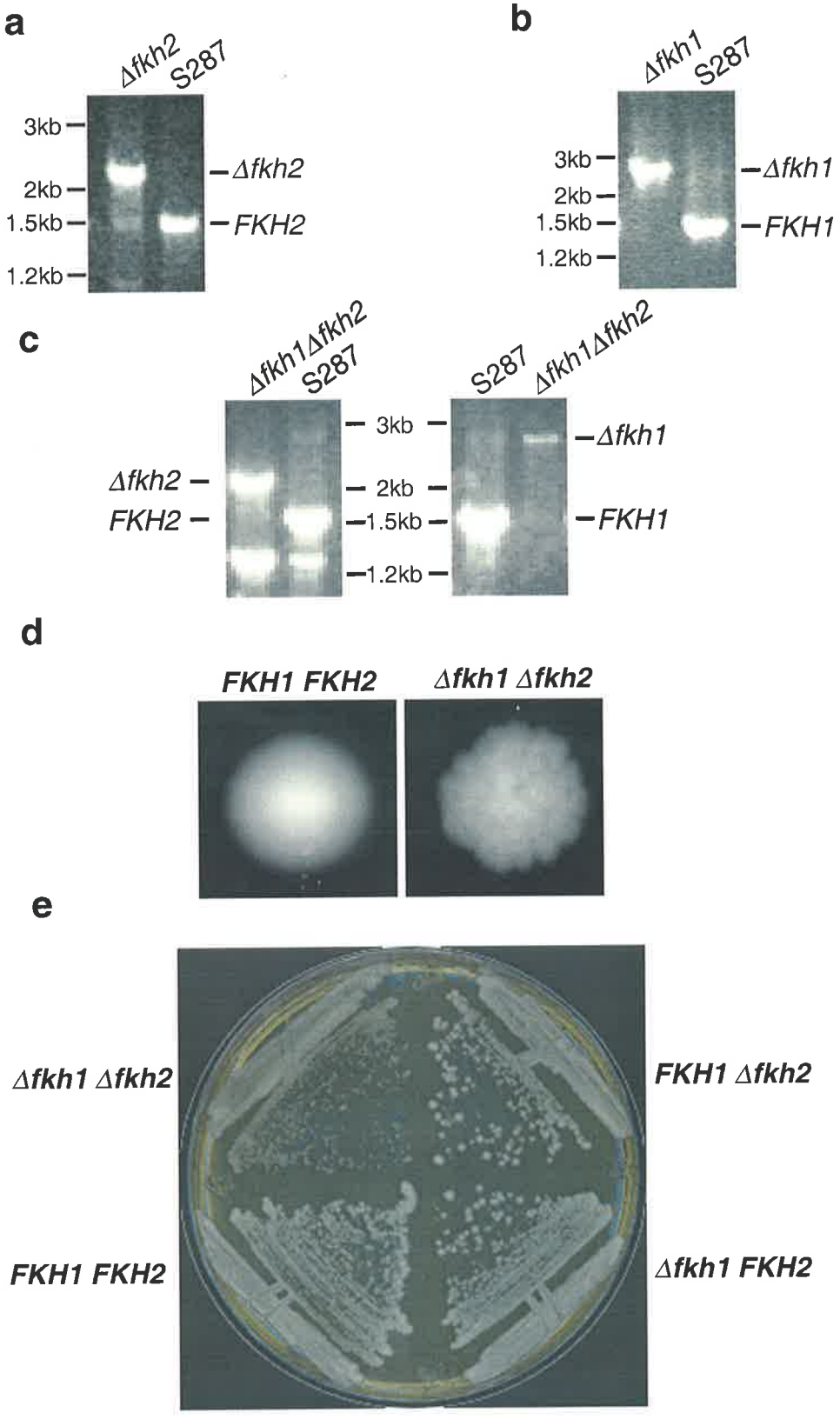


Figure 4.3: Confirmation of delete strains, colony morphology and growth rate. **a** PCR analysis of *Δfkh2* strain and parental S287 using primers *FKH2*-F and *FKH2*-13R. Resultant products are as indicated. **b** PCR analysis of *Δfkh1* strain and parental S287 using primers *FKH1-LEU2* and *FKH1*-8R. Resultant products are as indicated. **c** PCR analysis of the double *FKH* delete strain using the primers from a and b. **d** Colony morphology of *FKH1 FKH2* (S287) and *Δfkh1 Δfkh2* on YEPD media. **e** Growth comparisons of *FKH1 FKH2, Δfkh1 FKH2, FKH1 Δfkh2* and *Δfkh1 Δfkh2* on YEPD media.

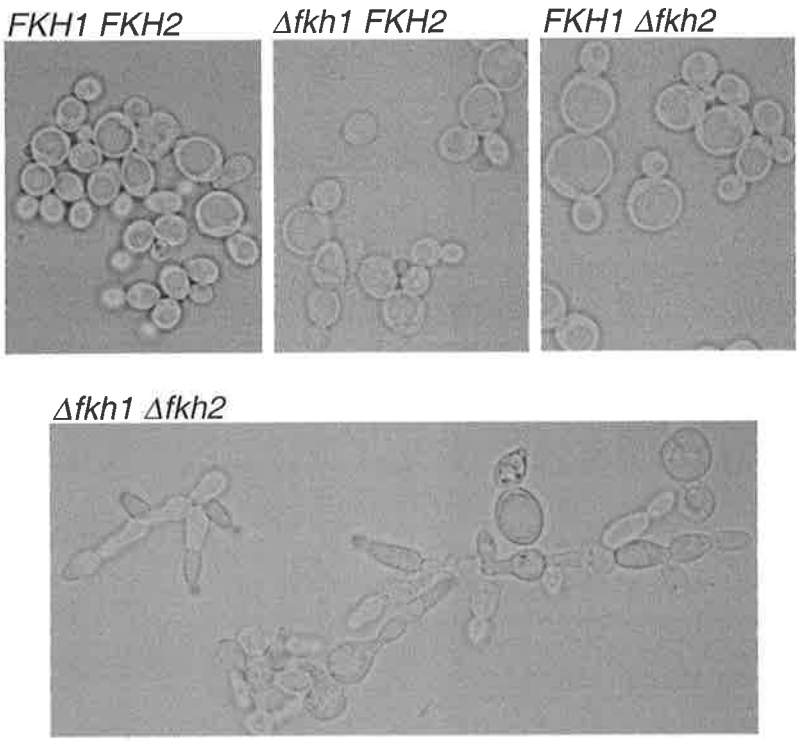


Figure 4.4: Phenotype of Δ*fkh* mutants. The four Δ*fkh* mutants were grown to an OD_{660} 0.8 rich media and photographed on a microscope slide using a 60x objective.

budding defects where there was a loss of axial bud growth and extended buds (figure 4.4).

4.2.3 "CLB2 cluster" regulation

To test the effects on the "CLB2 cluster" by deleting the *FKH*'s, RNA from synchronised cells was examined for "CLB2 cluster" gene expression. S287, *Δfkh1 Δfkh2, Δfkh1 FKH2* and *FKH1 Δfkh2* yeast strains were grown in YEP supplemented with 2% glucose to an OD_{660} 0.4 to which 0.6μg/ml of α-factor was added and allowed to progress through another cell division. The arrest of cells in G1 was checked visually by microscopy where at least 95% percent of cells were shmoos. The cells were released from the block, samples were taken every 15 minutes, RNA was extracted, run on 1% formaldehyde-agarose gels, blotted onto nitrocellulose filters and probed with ^{32}P-labelled *CLB2*, *SWI5* and *CDC5* ("CLB2 cluster" genes), *CLN2* (G1 transcript), LacZ (reporter under the control of the $CLB2_{UAS}$) and *PRT1/H2A* (*PRT1* as a loading control) probes (figure 4.5 a). In the different *FKH* backgrounds, *CLN2* and *H2A* were regulated normally and *PRT1* levels did not vary significantly. Each of the "CLB2 cluster" representatives and LacZ were cell cycle regulated in G2/M in S287, *Δfkh1 FKH2* and *FKH1 Δfkh2* with little difference in expression periodicity and levels (figure 4.5 a). However, *Δfkh1 Δfkh2* showed a great reduction in expression of "CLB2 cluster" genes along with a loss of periodic regulation (figure 4.5 a). When quantified and compared to S287, *CLB2* and LacZ expression was shown to be flat (non-periodic) over the cell cycle whereas the *CLN2* expression was periodic but delayed due to the slower

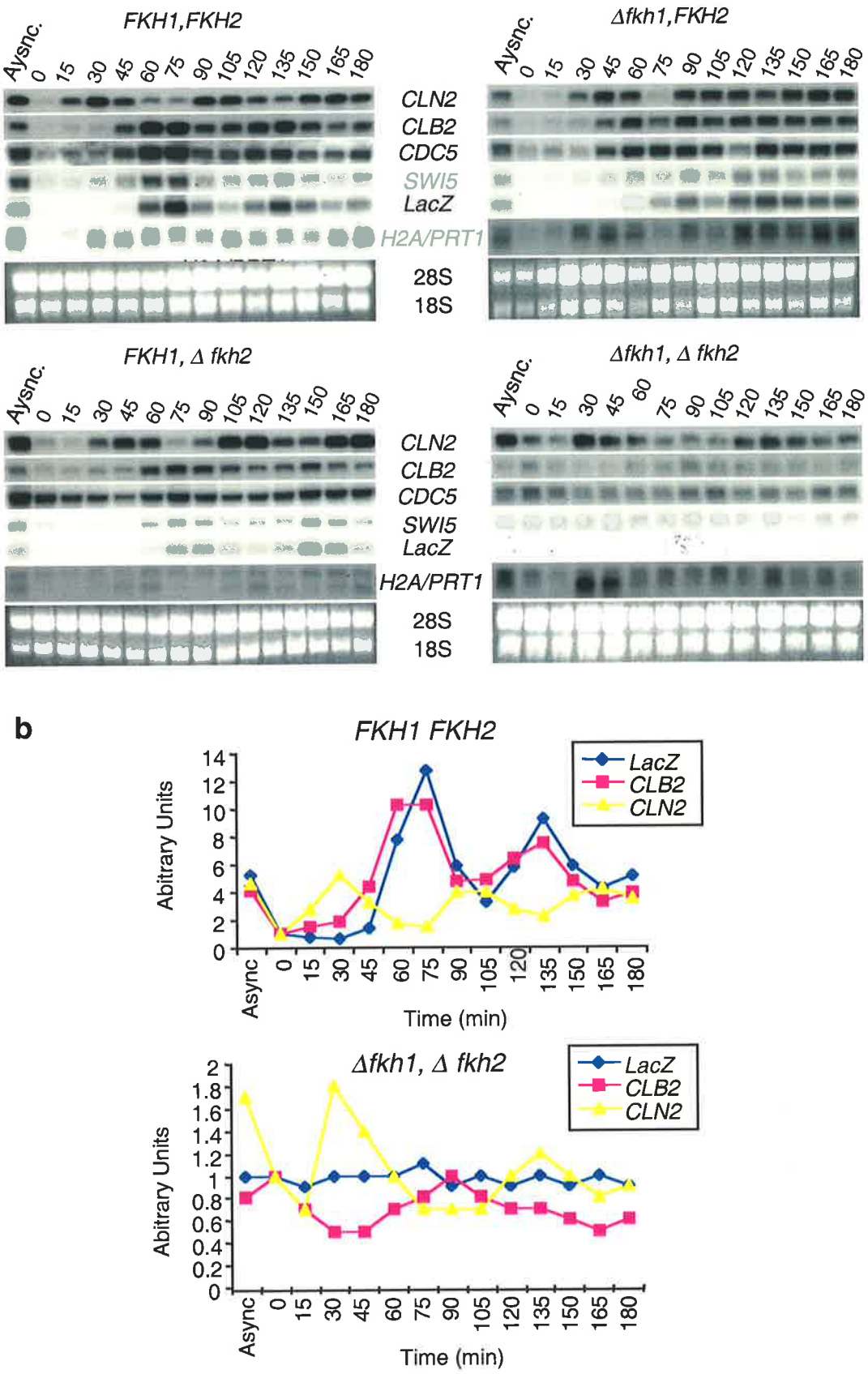


Figure 4.5: *FKH1* and *FKH2* are required for cell cycle regulation during G2-M. **a** Cell from different forkhead genetic backgrounds and carrying a $CLB2_{UAS}$-LacZ reporter were synchronised in G1 and released with samples taken every 15 minutes. The mRNA was *used* to examine the transcriptional periodicity of the indicated genes. **b** LacZ, *CLB2* and CLN2 were quantified by phosphoimaging analysis *and plotted* for over two consecutive cell cycles.

cell cycle time (figure 4.5 b). This data clearly shows the "CLB2 cluster" requirement for *FKH1* and *FKH2*.

4.3 Discussion

There seems to be an overlapping role between Fkh1p and Fkh2p in the regulation of the "CLB2 cluster". It has been shown here that the deletion of either of the *FKH's* has very little effect on the cell morphology, periodic and level of "CLB2 cluster" expression. However, when both *FKH's* are deleted together, there is a great morphological change accompanied by significant lowering of these transcript levels and a loss of periodicity. Other groups that published at the same time as this work found similar effects (Pic *et.al.*, 2000, Zhu *et.al.*, 2000 and Hollenhurst *et.al.*, 2000). Furthermore, $SWI5_{UAS}$ and $CLB2_{UAS}$ elements that are sufficient for cell cycle regulation of reporter activity, are affected similarly in the double *FKH* delete background (Kumar *et.al.*, 2000). In addition, Fkh2p, when fused to a transactivation domain, can inappropriately activate "CLB2 cluster" expression (Kumar *et.al.*, 2000) implicating the importance of *FKH1* and *FKH2* in the periodic and absolute expression of the "CLB2 cluster" expression. However, unlike *MCM1*, the *FKH's* are not essential for cell survival. This could possibly be due to Mcm1p ability to recruit other transcriptional activators to the "CLB2 cluster" which give the low, non-periodic transcription seen here in the absence of the *FKH's*. Possible activators could be the other forkhead proteins that are found in yeast (*HCM1* and *FHL1*) (Zhu and Davis,1998). Alternatively, the Mcm1p-forkhead pathway is not the only Mcm1p-dependent pathway

operating at the G2-M transition implicating other pathways may be required for mitotic entry.

Chapter 5:

Functional Analysis of Fkh2p and Ndd1p

5.0 Functional Analysis of Fkh2p and Ndd1p

5.1 Introduction

It has been shown in the past two chapters and in the recent literature that Fkh2p is a component of SFF but there still may be other components of this transcription complex (Kumar *et.al.*, 2000, Pic *et.al.*, 2000, Koranda *et.al.*, 2000 and Zhu *et.al.*, 2000). Another protein, Ndd1p, was discovered as a high copy number suppressor of the *cdc28-1n*^{ts} mutant and has recently been shown to be recruited to "CLB2 cluster" UAS's in a Fkhp dependent manner (Loy *et.al.*, 1999 and Koranda *et.al.*, 2000). Ndd1p does not exhibit any DNA binding properties of its own but has been shown to have putative transactivating abilities through Gal4 DBD fusion experiments (Loy *et.al.*, 1999). Furthermore, overexpression of Ndd1p upregulated "CLB2 cluster" transcription and Ndd1p was shown to be cell cycle regulated over the G2/M transition (Loy *et.al.*, 1999). Deletion analysis showed Ndd1p to have an essential role in cell cycle progression which can be rescued through deleting *FKH2* (Loy *et.al.*, 1999 and Koranda *et.al.*, 2000). Along with this, Fkh2p has been shown to be present throughout the cell cycle and bound to "CLB2 cluster" UAS's and only active in G2/M implicating Fkh2p as a possible repressor in the absence of Ndd1p (see 3.5 and Koranda *et.al.*, 2000). ***The above evidence implicates the essential recruitment of Ndd1p to the "CLB2 cluster" UAS's via Fkh2p could (i) de-repress Fkh2p allowing further recruitment of co-regulators or, (ii) activate "CLB2 cluster" gene expression directly.*** However, in the

budding yeast there is another forkhead protein, Fkh1p, that can functionally compensate for the absence of Fkh2p but does not share all the binding characteristics of SFF. Even though Ndd1p can be recruited to the "CLB2 cluster" UAS's through Fkh1p, it is not essential for Fkh1p's redundant function at the G2/M transition (Koranda *et.al.,* 2000). This indicates Fkh1p has its own activating properties either by itself or through the recruitment of other transcriptional activators independent of Ndd1p.

Common structural characteristics between Fkh1p and Fkh2p are the DBD and FHA domain and they differ by a C-terminal extension of Fkh2p that contains six putative cdk phosphorylation sites. The DBD is important for DNA binding whereas FHA domains have been shown to recruit pT phospho-proteins in other organisms (Hollenhorst et.al., 2000, Hofmann and Bucher, 1995, Durocher *et.al.*, 1999, Durocher *et.al.*, 2000 and Li *et.al.*, 2000). ***Ndd1p contains four putative cdk phosphorylation sites, three of which are pT cdk sites making Ndd1p a putative interactor with FHA domains, possibly with Fkh2p.*** Amon *et al.* (1993) reported that the "CLB2 cluster" gene expression is active in the presence of mitotic cdk activity but repressed when it is removed implicating a possible role for the putative phosphorylation sites in Fkh2p and Ndd1p in "CLB2 cluster" regulation. In this chapter functional studies on both Fkh2p and Ndd1p are performed and a possible link between Fkh2p and Ndd1p is examined.

5.2 Results

5.2.1 Mitotic-Cdk activity acts through the "CLB2 cluster" UAS's

To examine the effects of mitotic-Cdk activity on "CLB2 cluster" regulation two *ts* strains were utilised. The first, *clb2*^{ts}, contains deletions in *clb1,3* and *4*, a *ts* allele of *clb2* and a LacZ reporter downstream of a *CLB2*$_{UAS}$. The second strain is the *cdc28-1n*^{ts} that has a cdk defect in mitosis at the restrictive temperature. The two strains were grown at 24^{O}C to an OD_{660} 0.4 in YEP media supplemented with 2% glucose. The strains were arrested in G1 through addition of α-factor and then released in glucose containing YEP supplemented with nocodazole at 24^{O}C for two hours, shifted to 37^{O}C (restrictive temperature) for a further two hours and then back down to the permissive temperature. Cells were harvested at each stage, RNA extracted, run on a 1% formaldehyde-agarose gel, blotted to a nitrocellulose membrane and probed using "CLB2 cluster" members, *CLN2*, *LacZ* and *PRT1/H2A* ^{32}P-labelled probes (figure 5.1 a). The "CLB2 cluster" genes were regulated in a mitotic-cdk dependent manner and *CLN2* was inhibited in the presence mitotic-cdk as expected (figure 5.1 a). The observation of LacZ being regulated in a pattern similar to the "CLB2 cluster" indicates that the Cdk activity is acting directly through the UAS's of the "CLB2 cluster" (figure 5.1 a).

To examine the possibility of the mitotic-cdk acting directly through Fkh2p, *FKH2* was deleted in both *ts* strains using the construct from chapter 4 (see figure 4.2). The same

experiment was performed as above using these *Δfkh2 ts* strains and the regulation of the "CLB2 cluster" genes was examined. There seemed to be no change in "CLB2 cluster" regulation in the absence of Fkh2p possibly due to the functional redundancy that exists between Fkh1p and Fkh2p. In addition to the "CLB2 cluster" genes, a ^{32}P-labelled *NDD1* probe was used which revealed an up-regulation of *NDD1* in the absence of Cdk activity implicating a negative feedback loop involving mitotic-cdk may exist in mitosis to down-regulate *NDD1*. To see if Ndd1p followed the same pattern, an ADH driven $_{3HA}$*NDD1* plasmid (made by B Shi) was transformed into *cdc28-1n^{ts}* and subjected to a temperature shift. This strain was grown to an OD_{660} 0.4 in –ura media supplemented with 2% glucose, arrested in mitosis using nocodazole at the permissive temperature, shifted to 37^{O}C (restrictive temperature) and then back to 24^{O}C. Cell lysates were prepared from cells harvested at these temperatures and run on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane and probed with α-HA Ab and GAPDH Ab (as a loading control) (figure 5.1c). Ndd1p followed the same pattern as its RNA, which is possibly the result of elevated transcripts (figure 5.1c).

5.2.2 Fkh2p putative cdk phosphorylation sites do not affect "CLB2 cluster" regulation

To examine the possible role of the six putative cdk phosphorylation sites of Fkh2p in "CLB2 cluster" gene regulation, point mutations of each of the cdk sites (*fkh2$_{6*cdk}$*) and a C-terminus truncation of Fkh2p (*fkh2$_{\Delta C}$*) (which contained the six sites) were made (plasmids constructed by B Shi). The plasmids contained the truncated form of *FKH2*

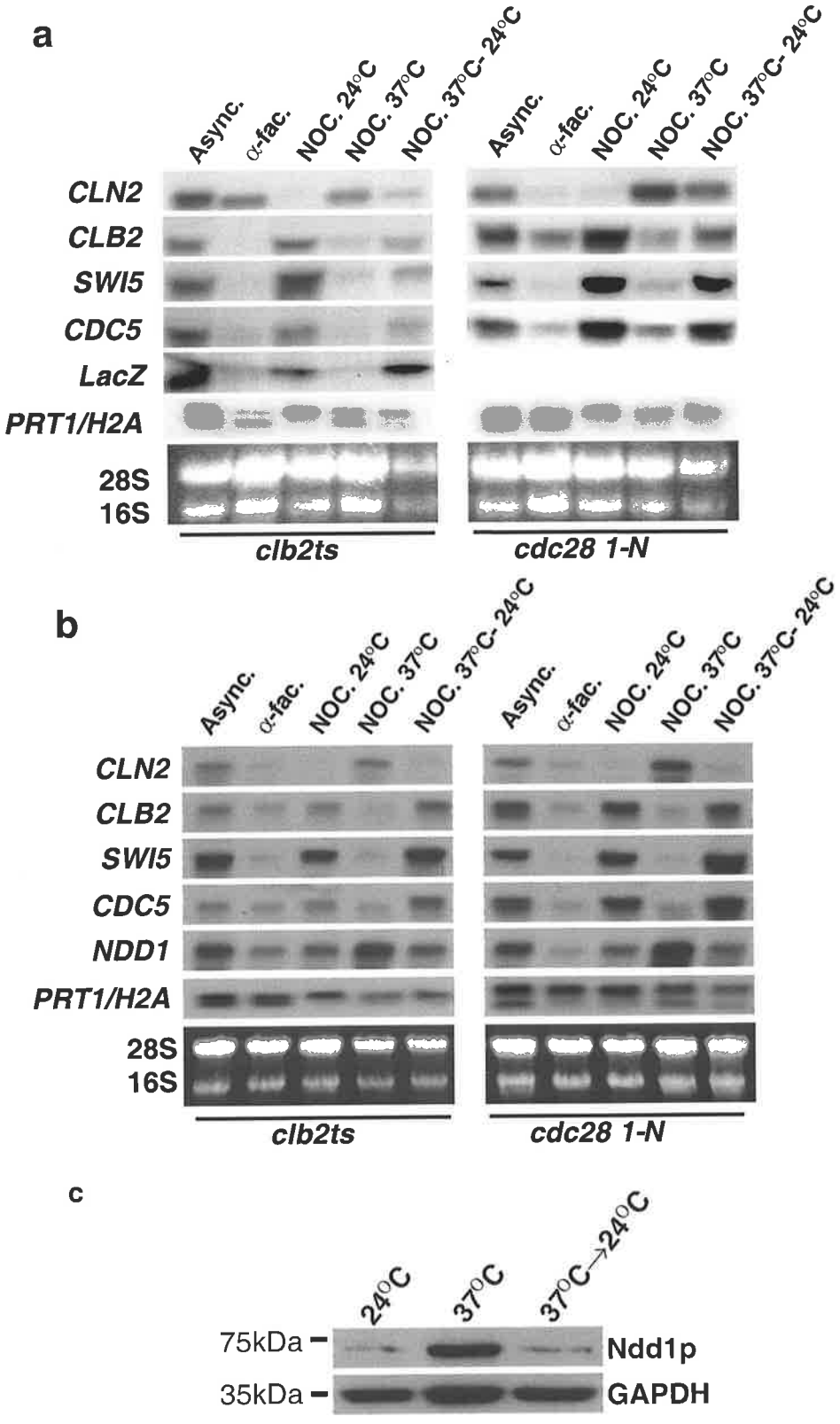


Figure 5.1: "CLB2 cluster" gene expression is directly regulated by mitotic-cdk activity. (**a**) *cdc28-1n*^{ts} and *clb2*^{ts} (containing a $CLB2_{UAS}$ UbiYLacZ reporter) yeast strains were blocked in G1 and M-phase and subjected to the temperature shifts as indicated. mRNA was used to examine the transcriptional profile of the indicated genes. (**b**) Same as **a** but in a *Δfkh2* background. (**c**) *cdc28-1n*^{ts} containing an ADH-$_{3HA}$*NDD1* plasmid was arrested in M-phase and under went a temperature shift as indicated. Cell lysates were probed for $_{3HA}$Ndd1p using α-HA Ab as indicated. GAPDH was used as a loading control.

with 6MYC epitope tag at the C-terminus used previously for homologous recombination (see figure 3.2). These constructs, along with int. $FKH2_{6myc}$ (figure 3.2), were linearised using the restriction enzyme *Nco*I and transformed in the yeast strain *Δfkh1 FKH2* (*W303: MATa bar1 fkh1::LEU2*). The resultant strains, $FKH2_{6myc}$, $fkh2_{6*\ 6myc}$ and $fkh2_{\Delta C\ 6myc}$, were confirmed via PCR, using primers UF and *FKH2*-F, and Western analysis using α-myc Ab (data not shown).

$FKH2_{6myc}$, $fkh2_{6*\ 6myc}$ and $fkh2_{\Delta C\ 6myc}$ were grown in YEP supplemented with 2% glucose to an OD_{660} 0.4 where they were arrested in G1 through the addition of α-factor. After two hours the cells were released into glucose containing YEP media and samples were taken every 15 minutes. RNA was extracted from the cells, run on a 1% formaldehyde-agarose gel, blotted to a nitrocellulose membrane and probed with ^{32}P-labelled *CLN2, CLB2, SWI5, CDC5, LACZ* and *PRT1/H2A* probes (figure 5.2 a). The resultant RNA profiles of $fkh2_{\Delta C}$ and $fkh2_{6*cdk}$ strains varied little, if any, to the parental $FKH2_{6myc}$ strain indicating no obvious role for the putative cdk phosphorylation sites of Fkh2p (figure 5.2 a). The resultant quantitation revealed no difference between the mutants and the parental strain (figure 5.2 b).

5.2.3 Ndd1p putative phosphorylation sites affect "CLB2 cluster regulation

To investigate the possible role of the putative cdk phosphorylation sites of Ndd1p, point mutants of the three published cdk sites, pT180, pS254 and pT319, were made (constructed by B Shi). The point mutants at the third position ($ndd1_{3*}$) and the three

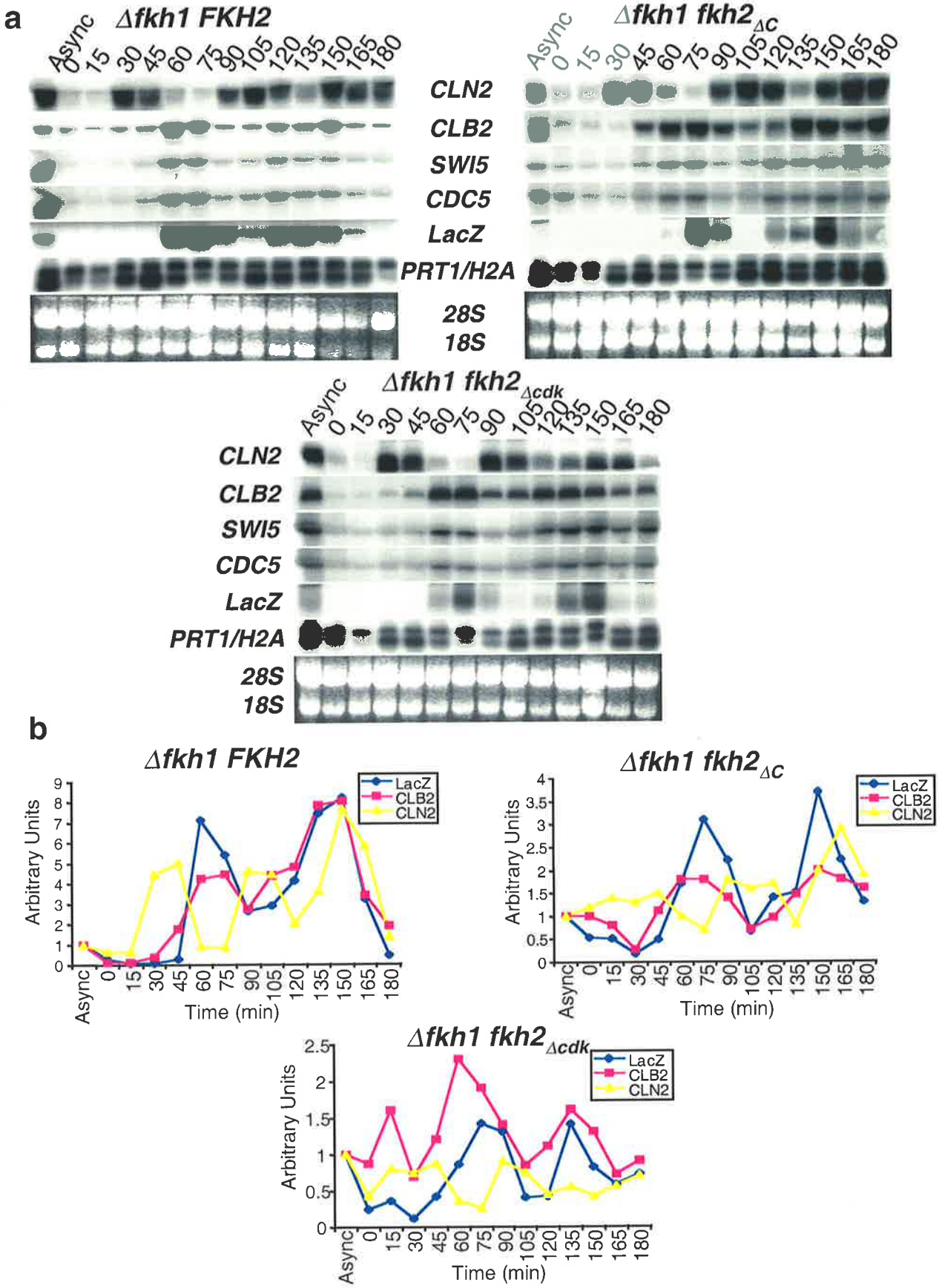


Figure 5.2: The putative cdk phosphorylation sites do not influence "CLB2 cluster" gene expression. (**a**) Cells from different forkhead backgrounds and carrying a $CLB2_{UAS}$-UbiYLacZ reporter were synchronised in G1 and released with samples taken every 15 minutes. The mRNA was used to examine the transcriptional periodicity of the indicated genes. (**b**) LacZ, *CLB2* and *CLN2* were quantified by phosphoimaging analysis and plotted

point mutants (*ndd1*$_{123*}$) were moved into an ADH driven pYCplac112 plasmid (*TRP1* marker) containing a 3HA epitope tag (constructed by B Shi). The mutants, along with $_{3HA}$*NDD1*, were transformed into the *Δndd1* strain from Loy *et.al.* (1999) maintained by a wt *NDD1* plasmid on a *URA3* marker. The strains were struck onto –ura -trp and 5 FOA –trp plates to eject the *NDD1::URA3* covering plasmid (figure 5.3 b and c). The three introduced plasmids were able to sustain growth of the *Δndd1* strain but at different efficiencies. The *NDD1*$_{wt}$ sustained normal growth with a doubling time of 1.5 hours in liquid culture, whereas the *ndd1*$_{3*}$ and *ndd1*$_{123*}$ grew slower and doubled every 2.5 and 3.5 hours respectively. To make sure the plasmids were expressing, the cells were grown on –trp media supplemented with 2% glucose after FOA curing, harvested, lysates made, run on a 10% SDS-PAGE gel, transferred to a nitrocellulose membrane and probed with α-HA Ab to check for $_{3HA}$Ndd1p, $_{3HA}$Ndd1p$_{3*}$ and $_{3HA}$Ndd1p$_{123*}$ expression using α-HA Ab (figure 5.3 a).

To examine the effects of the ndd1 point mutants on cell morphology, the cells were grown to mid-log phase and examined by microscopy (figure 5.4 a). The effect on morphology was gradual when the mutants were compared with the wt NDD1 plasmid. The most severe phenotype was observed for the triple cdk-phospho mutant where there was an inability for mother and daughter cells to undergo cytokinesis at the end of M-phase, forming chain-like projections, budding defects with a loss of axial bud growth and extended buds were observed (figure 5.4 a). This phenotype is indicative of a *CLB2*

expression defect and was observed previously in the *Δfkh1 Δfkh2* double delete strain. To examine what effects this had on "CLB2 cluster" expression, the cells from the 5 FOA plate were grown in YEP media supplemented with 2% glucose and arrested in mitosis using nocodazole. RNA was extracted from harvested cells, run on a 1% formaldehyde-agarose gel, blotted to a nitrocellulose membrane and probed with *CLN2, CLB2, SWI5, PRT1/H2A* ^{32}P-labelled probes (figure 5.4 b). The resultant northern revealed a 1.5-2 fold reduction in "CLB2 cluster" expression and loss of periodicity (figure 5.4 b and c). This reduction in *CLB2* expression accounts for the cell morphology observed. Closer examination of the Ndd1p sequence revealed a fourth putative pT^{193} phosphorylation site previously not published which may cause a greater effect of cell viability. Due to time constraints, investigations into the fourth site are being performed by other members of the lab.

5.2.4 A possible role for Ndd1p through de-repressing Fkh2p

The lethality of *Δndd1* can be rescued by deleting *FKH2* implicating the essential nature of Ndd1p is through Fkh2p (Koranda *et.al.,* 2000). To determine if the periodicity and expression levels of the "CLB2 cluster" are affected in a *Δfkh2Δndd1* a cell synchrony was performed. The yeast strain used, *Δfkh2Δndd1*, was a *W303* background containing a Lac Z reporter downstream of a $CLB2_{UAS}$ (*W303: MATa fkh2::LEU2 ndd1::HIS3* $CLB2_{UAS}$*-UbiYLacZ*) (constructed by B Shi). The *Δfkh2Δndd1* strain was grown on YEP media supplemented with 2% glucose to an OD_{660} 0.4 then arrested in G1 through the addition of α-factor. After arrest the cells were released and samples were taken

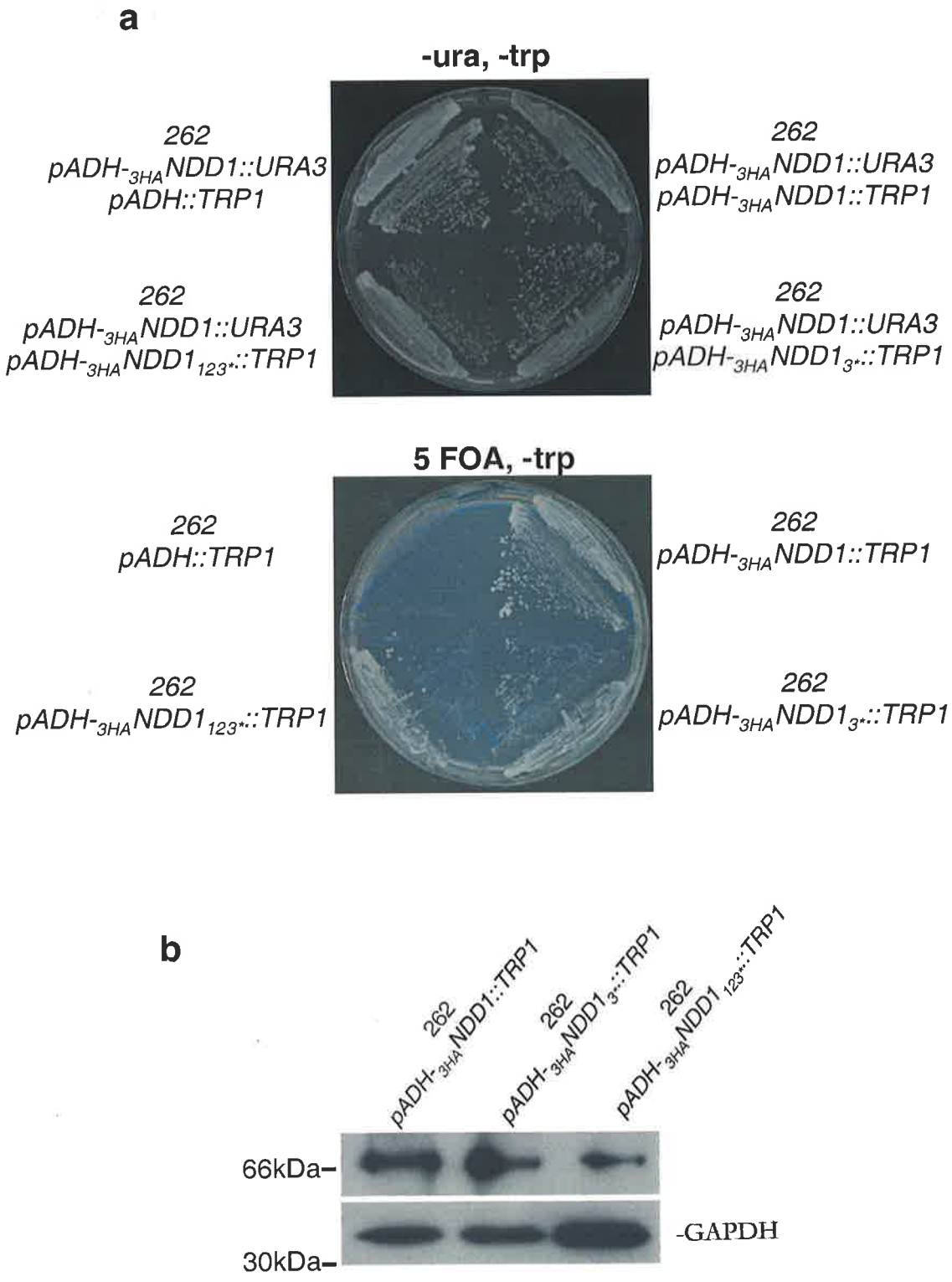


Figure 5.3: Ndd1p phosphorylation sites are important for cell growth. (**a**) The strains indicated were struck onto onto -ura -trp and 5FOA -trp plates and incubated at 30^{O}C. (**b**) Cell lysates were prepared from the strains indicated and probed for the presence of the $_{3HA}$Ndd1p derivatives after FOA curing.

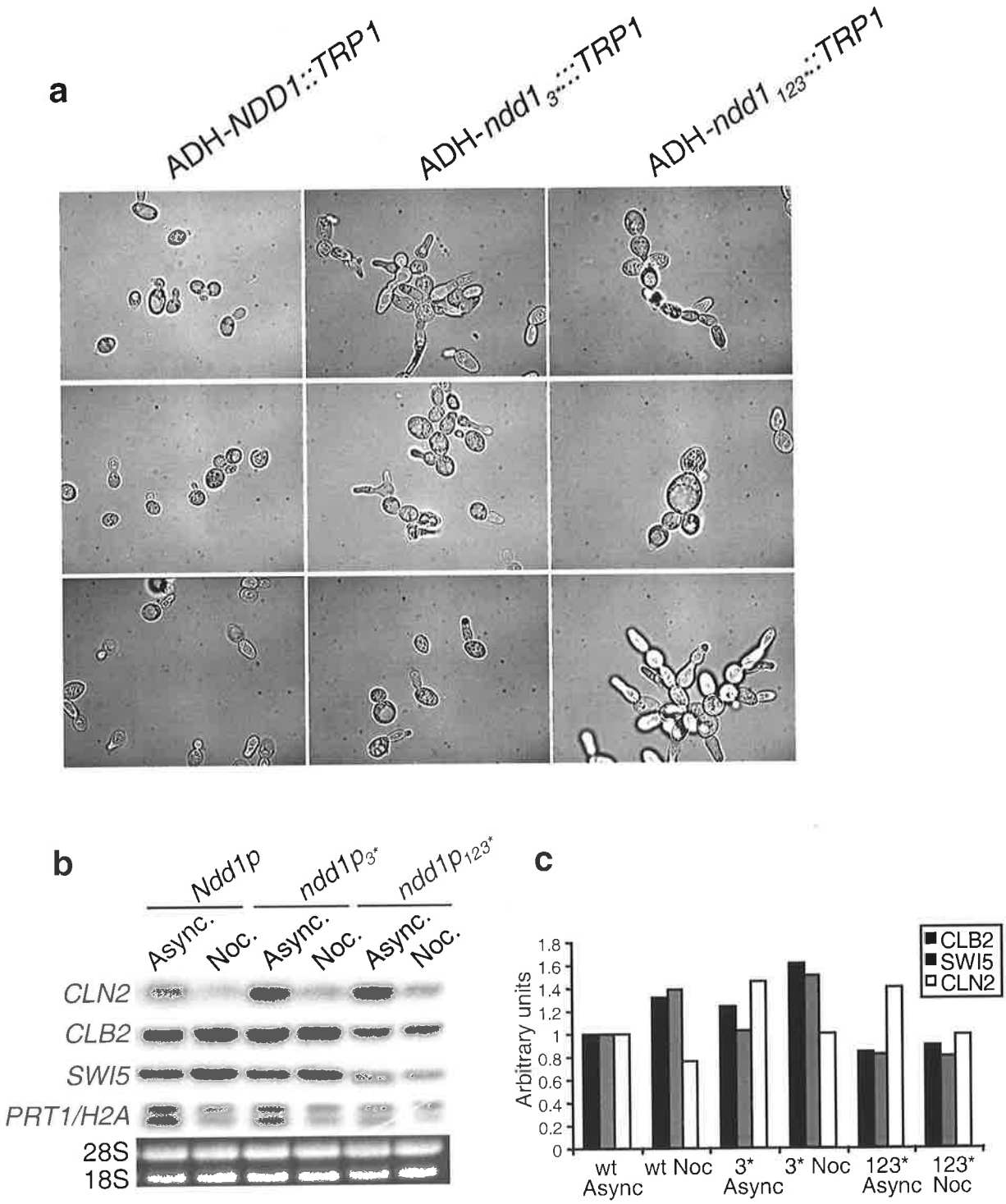


Figure 5.4: Ndd1p phosphorylation sites important for normal cell cycle progression. (**a**) Cells from the different genetic backgrounds indicated were viewed through microscopy. (**b**) The yeast strains from **a** were blocked in M-phase using nocodazole (Noc) and mRNA was used to examine the indicated genes. (**c**) Quantitation of *CLN2*, *CLB2* and *SWI5* by phosphoimaging was graphed as indicated.

every 15 minutes, RNA extracted, run on a 1% formaldehyde-agarose gel, blotted onto a nitrocellulose membrane and probed using *CLN2, CLB2, SWI5, CDC5, PRT1/H2A* ^{32}P-labelled probes (figure 5.5 a and b). The "CLB2 cluster" genes were regulated at wt levels and with normal periodicity (figure 5.5 a and b). This is probably due to the functional redundancy Fkh2p shares with Fkh1p.

Ndd1p's essential role is through Fkh2p but what function does it have? Genetic analysis implicates Fkh2p as a represser of "CLB2 cluster" gene expression, which is activated by the production of Ndd1p in G2/M. Ndd1p could then be either an activator or a de-represser of Fkh2p function. What domain of Fkh2p is vital for Ndd1p function? Fkh2p contains three domains of interest; the DBD important for Mcm1p dependent binding of the DNA, the FHA domain implicated in pT phospho-protein-protein interactions and the C-terminus, which contains six putative cdk phosphorylation sites. To investigate the importance of the C-terminus, $FKH2_{6myc}$, $fkh2_{6*\ 6myc}$ and $fkh2_{\Delta C\ 6myc}$ (from 5.2.2) were integrated into the *FKH2* locus of a *Δndd1* yeast strain covered by a ADH-$_{3HA}$*NDD1* plasmid (262 (Loy *et.al.*, 1999)). These strains were confirmed by PCR, using primers *FKH2*-F and UF, and western analysis, using α-MYC Ab for the Fkhp and α-HA Ab for Ndd1p (figure 5.6 a-c). To check for viability, the strains were first struck on –ura media supplemented with 2% glucose to keep the *NDD1* plasmid and then struck onto 5 FOA plates to eject the *NDD1* covering plasmid (figure 5.6 d). Of the three strains, only $fkh2_{\Delta C\ 6myc}$ *Δndd1* grew without Ndd1p

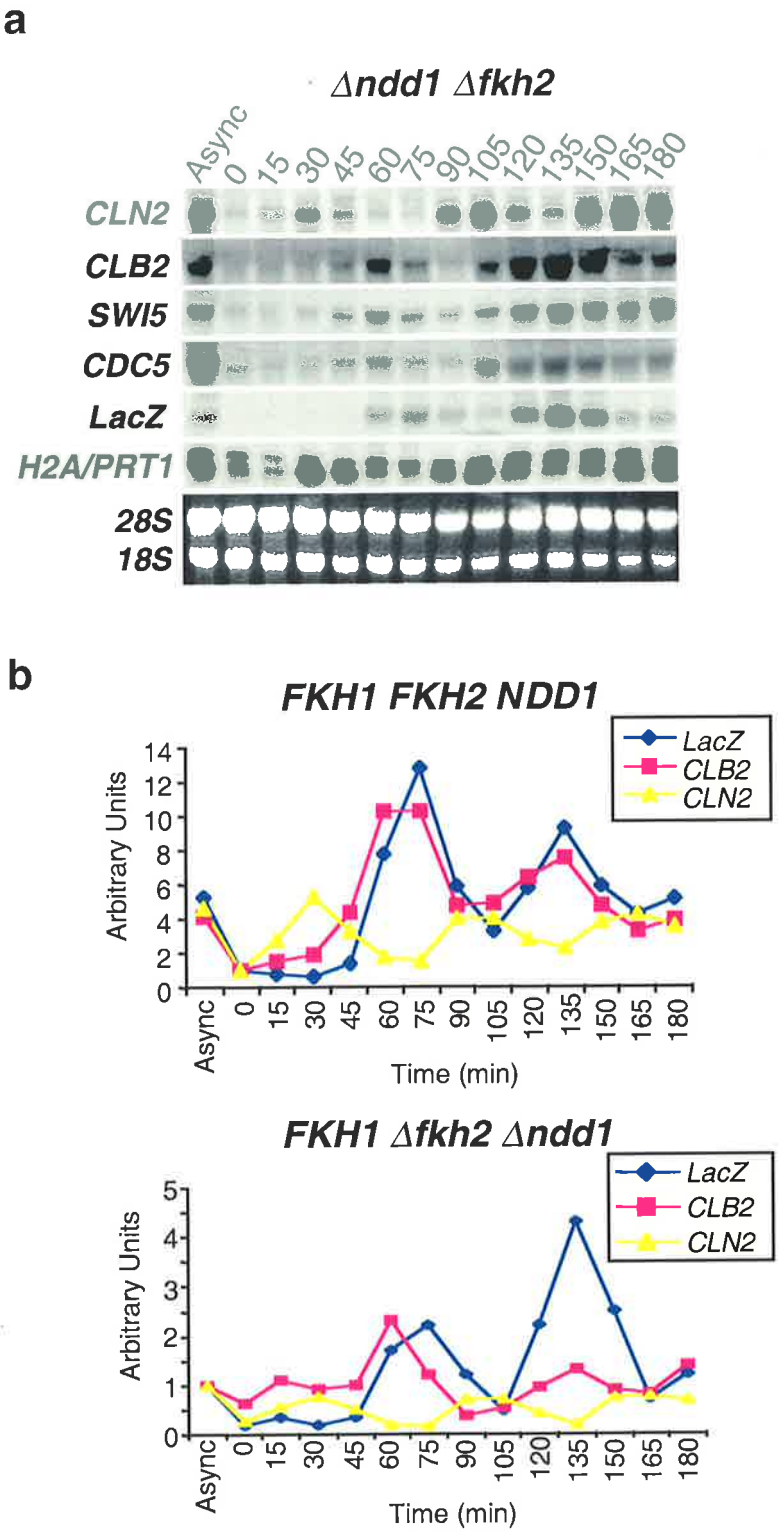


Figure 5.5: "CLB2 cluster" gene expression is still cell cycle regulated in the absence of *FKH2* and *NDD1*. (**a**) Cells from a *Δfkh2 Δndd1* genetic background were synchronised in G1 and released and samples harvested every 15 minutes for 2 cell cycles. mRNA was used to examine the transcription profile of the indicated genes. (**b**) *CLN2*, *CLB2* and LacZ were quantitated on a phosphoimager in respect to *PRT1* and plotted.

present indicating the C-terminus of Fkh2p is involved in the essential role of Ndd1p but not the putative cdk phosphorylation sites (figure 5.6 d).

To investigate the effects of *fkh2*$_{\Delta C}$ *Δndd1* on "CLB2 cluster" gene expression, *fkh2*$_{\Delta C\ 6myc}$ was integrated at the *FKH2* locus and *NDD1* was deleted in *Δfkh1 FKH2* (*W303: MATa bar1 fkh1::LEU2 CLB2*$_{UAS}$*-UbiYLacZ*). The resultant strain (*fkh2*$_{\Delta C\ 6myc}$ *Δndd1*) was synchronised in G1 and released with samples taken every 15 minutes as performed previously. RNA was made, run on a 1% formaldehyde-agarose gel, blotted to a nitrocellulose membrane and probed using *CLN2, CLB2, SWI5, CDC5, PRT1/H2A* ^{32}P-labelled probes (figure 5.7 a). The resultant profile showed little difference in periodicity and expression of the "CLB2 cluster" genes and was confirmed by quantitation (figure 5.7 a and b) indicating "CLB2 cluster" expression can be restored by removing the C-terminus of Fkh2p.

FHA domains have been shown to be important for pT phospho-protein-protein interactions and are contained in both Fkh1p and Fkh2p. To see if they have functional relevance in the role Fkh's play in "CLB2 cluster" gene expression, the FHA domains were deleted in both *FKH1* and *FKH2* and cloned into ADH-driven vectors containing a *HIS3* marker gene. These constructs were integrated singly into the *HIS3* locus of the *Δfkh1 Δfkh2* strain from chapter 4 (constructed by B Shi). *FKH2* was also constructed in this plasmid and integrated into the *Δfkh1 Δfkh2* strain to see if it could rescue the

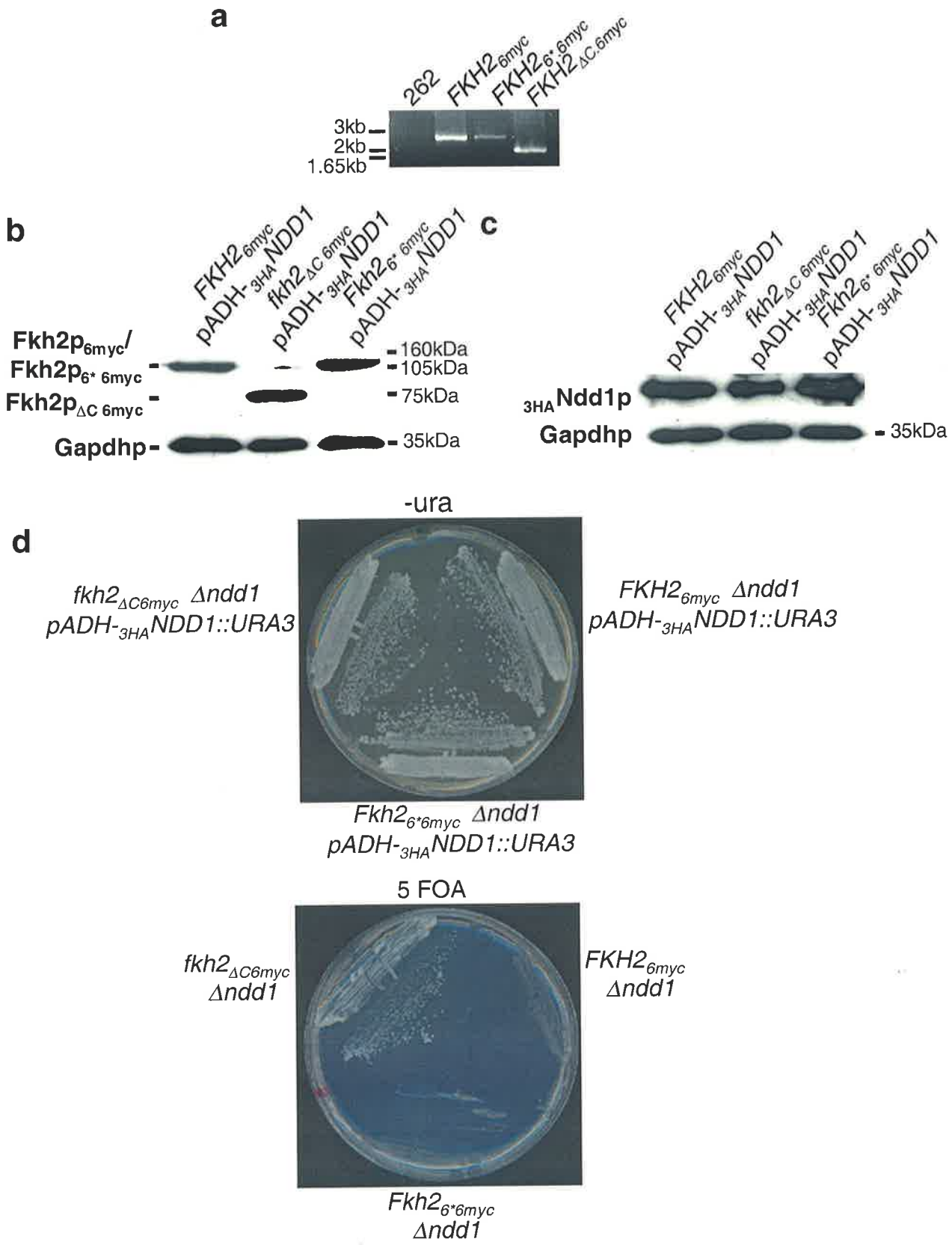


Figure 5.6: The lethality of *Δndd1* can be rescued by deleting the Fkh2p C-terminus. (**a**) PCR confirmation of the integration of the *FKH2* derivatives. (**b**) Western analysis using α-myc Ab to confirm the presence of $Fkh2p_{6myc}$ derivatives. (**c**) Western analysis using α-HA checking expression of $_{3HA}$Ndd1p from the covering plasmid. (**d**) Streaks of each of the confirmed strains of -ura and 5 FOA plates supplemented with 2% glucose.

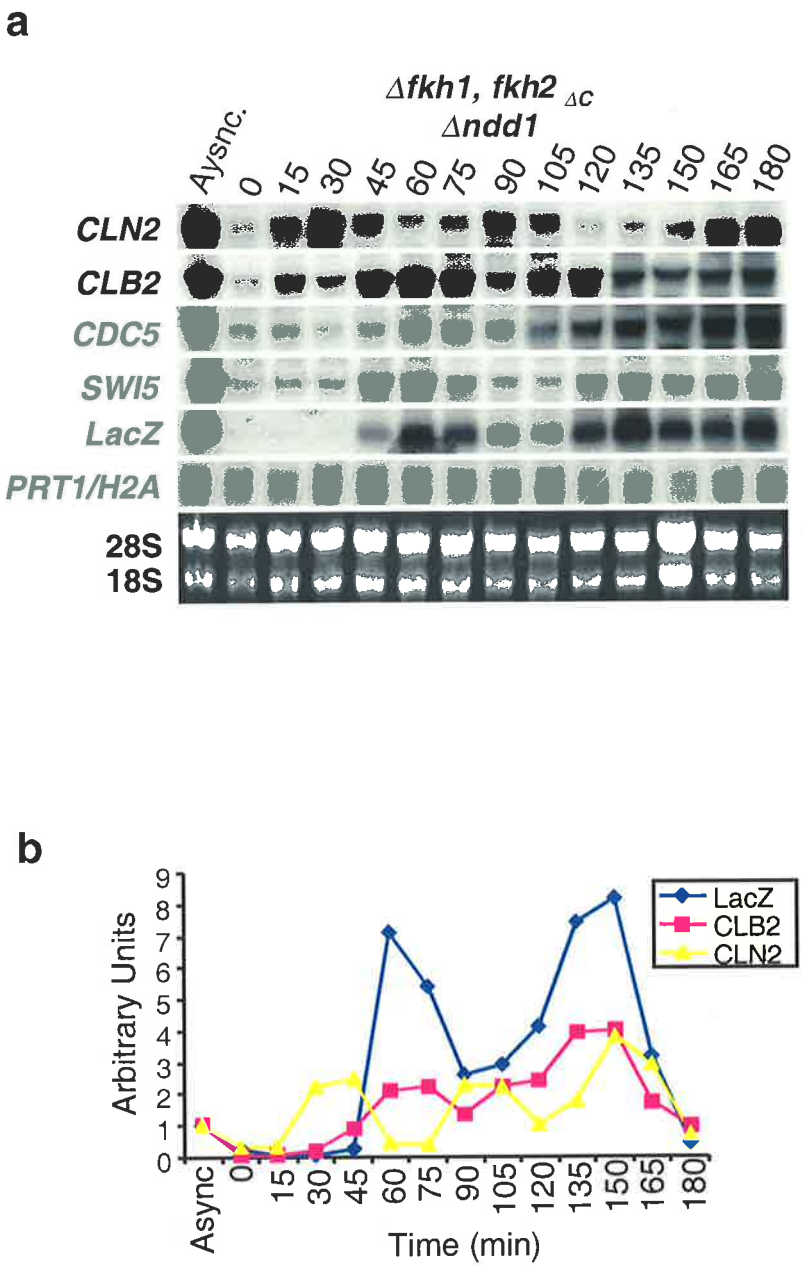


Figure 5.7: The essential nature of Ndd1p can be rescued through deleting the C-terminus of Fkh2p. (**a**) Cells from a *Δfkh1 fkh2*$_{\Delta C}$ *Δndd1* were synchronised in G1 and released and cells harvested every 15 minutes. mRNA was used to examine the transcription profile of the indicated genes. (**b**) *CLN2*, *CLB2* and LacZ were quantitated on a phosphoimager and plotted.

Δfkh1 Δfkh2 phenotype. The resultant *Δfkh1 FKH2* strain rescued the *Δfkh1 Δfkh2* phenotype, whereas the $fkh1_{\Delta FHA}$ *Δfkh2* and *Δfkh1* $fkh2_{\Delta FHA}$ strains did not (data not shown). To investigate the effects of these constructs on "CLB2 cluster" gene expression, synchrony experiments were performed as done previously (figure 5.8). The *Δfkh1 FKH2* strain rescued the "CLB2 cluster" periodicity and gene expression levels (figure 5.8). However, the $fkh1_{\Delta FHA}$ *Δfkh2* and *Δfkh1* $fkh2_{\Delta FHA}$ did not show any significant difference to *Δfkh1 Δfkh2* expression patterns (figure 5.8) indicating the FHA domain delete forkheads are non functional.

For further characterisation, the FHA domain of Fkh2p was fused to a GAL4 DBD downstream of an ADH promoter (constructed by C McLean). Furthermore, a R87A point mutation was constructed based on other similar point mutations disrupting protein-protein interactions (Durocher *et.al.,* 1999). These two plasmids were transformed into either *Δfkh2 NDD1* or *Δfkh2 Δndd1* yeast strains (*W303*: ***MATa*** *fkh2::LEU2* or 465 (Koranda *et.al.*, 2000) respectively) along with a GAL4-LacZ reporter (p55), a mutant GAL4-LacZ reporter (p53)or CYC-LacZ reporter (p52) plasmids (figure 5.9 a). The different strains (figure 5.9 a) were patched onto a nitrocellulose filter on –trp-ura plates and allowed to grow overnight and used in a blue white assay (see 2.10.1). *Δfkh2 NDD1* pFHA/p55, *Δfkh2 NDD1* p*TRP1*/p52 *and Δfkh2 Δndd1* p*TRP1*/p52 patches turned blue whereas those patches containing pFHA* remained white in both strains indicating the point mutants are unable to recruit co-

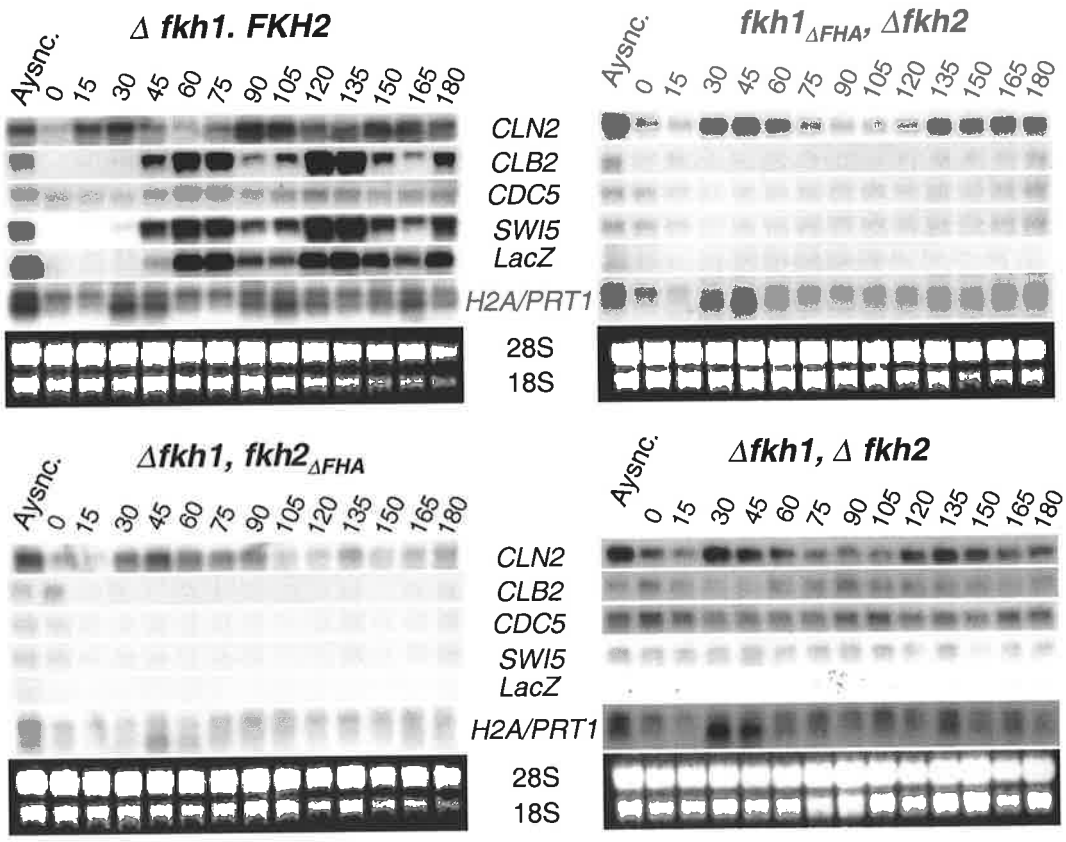


Figure 5.8: The FHA domain of Fkh1p and Fkh2p is important for "CLB2 cluster" regulation. Cells from different *FKH* FHA backgrounds were synchronised in G1 released with samples taken every 15 minutes. mRNA was used to examine the transcription profile of the indicated genes.

a

Strains	Transformed Plasmids				
Δfkh2 NDD1	pFHA/ p55	pFHA/ p53	pFHA*/ p55	pFHA*/ p53	p*TRP1*/ p52
465	pFHA/ p55	pFHA/ p53	pFHA*/ p55	pFHA*/ p53	p*TRP1*/ p52

b

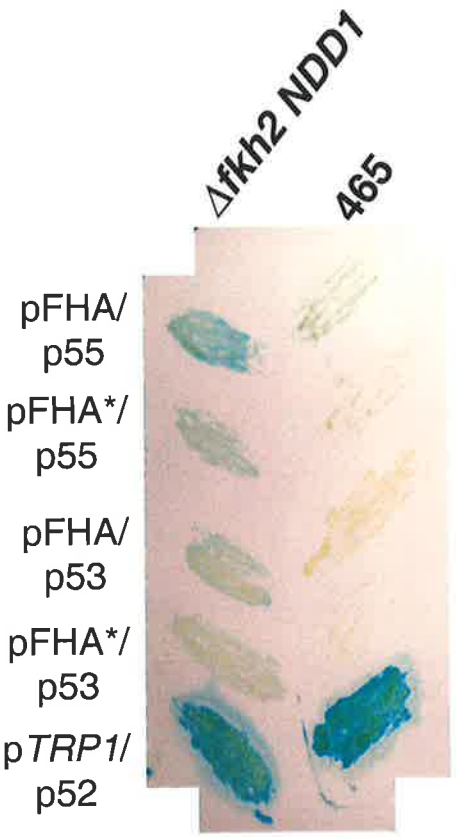


Figure 5.9: The Fkh2p FHA domain is important for transcriptional activation and optimal activation occurs in the presence of Ndd1p. (**a**) A table of the plasmids transformed into the indicated strains. (**b**) Blue/White assay performed on the strains from **a**. p52 was used as a positive control.

activators (figure 5.9 b). *Δfkh2 Δndd1* containing pFHA/p55 turned blue at a much slower rate than *Δfkh2 Δndd1* p*TRP1*/p52, *Δfkh2 NDD1* pFHA/p55 and *Δfkh2 NDD1* p*TRP1*/p52 indicating that in the absence of Ndd1p the Fkh2p FHA domain may recruit co-activators but with less efficiency (figure 5.9 b).

5.3 Discussion

Previously it has been reported that *CLB2*-cdk activity is necessary for "CLB2 cluster" gene expression through establishing a positive feedback loop (Amon *et.al.*, 1993). Here it has been demonstrated that this activity is directly through the "CLB2 cluster" UAS's and possibly though the recruitment of Ndd1p, a putative phospho-protein that, when a least three of its phospho-sites are mutated, causes a morphological change to the cell attributed to a defect in *CLB2* gene expression. Fkh2p also contains six putative cdk phosphorylation sites at its C-terminus, which do not affect cell cycle progression or "CLB2 cluster" gene expression when mutated or removed. However, the C-terminus does play a role in repressing "CLB2 cluster" gene expression because in its absence it can rescue the essential nature of Ndd1p. This is partly supported by the deletion of *FKH2* rescuing the lethality of *Δndd1* implying Ndd1p's essential role is through Fkh2p (Koranda *et.al.*, 2000). Furthermore, this evidence implies Fkh2p is a repressor of transcription in the absence of Ndd1p which is supported by its constitutive production and occupation of the SFF binding site (Althoefer *et.al.*, 1995, Pic *et.al.*, 2000 and Koranda *et.al.*, 2000).

The FHA domain is another important domain of Fkh1p and Fkh2p because its deletion fails to rescue a forkhead double deletion phenotype and transcriptional profile. It has been shown that these FHA domain deletes can be recruited to the "CLB2 cluster" UAS's *in vivo* implicating the FHA domain is important for transcriptional activation (S Dalton personal communication). Previous studies on FHA domains showed that point mutations at one of two key conserved residues abolishes protein-protein interaction with its pT phospho-binding partner (Durocher *et.al.*, 1999). Here we have shown that such a mutation of the Fkh2p FHA domain abolishes *in vivo* reporter activity implicating the FHA domain is important in recruitment of co-activators. Furthermore, in the absence of Ndd1p, the FHA domain's ability to activate is reduced indicating its preferred co-activator is Ndd1p. One possibility for the residual reporter activity in the absence of Ndd1p is the putative binding partners of Fkh1p. Fkh1p can functionally compensate for the absence of Fkh2p and Ndd1p to wild-type efficiency indicating it has its own co-activators, which are probable phospho-proteins interacting with Fkh1p through its FHA domain. Fkh1p shares a 59% identity with Fkh2p through the FHA domain supporting the theory of Fkh2p's recruitment of Fkh1p's co-regulators at a lower efficiency. Furthermore, this identity between the two forkheads explains the recruitment of Ndd1p to the "CLB2 cluster" UAS's through Fkh1p (Koranda *et.al.*, 2000).

Chapter 6:

Discussion

6.0 Discussion

6.1 Transcriptional regulation at the G2/M transition

Microarray analysis performed on the budding yeast, *Saccharomyces cerevisiae*, revealed several waves or "clusters" of transcriptional activity associated with cell cycle progression (Spellman *et.al.,* 1998). One of these "clusters", the "CLB2 cluster", is comprised of 35 genes that are important for the G2/M transition and mitotic progression (Spellman *et.al.,* 1998). Previous work on the UAS's of two "CLB2 cluster" members, *CLB2* and *SWI5*, revealed the binding of an Mcm1p homodimer and, until recently, an unidentified activity SFF (*SWI5* Factor) (Lydall *et.al.*, 1991, Maher *et.al.*, 1995 and Althoefer *et.al.*, 1995). Recently in our laboratory an activity that followed SFF binding *in vitro* was purified and identified as Fkh2p (Kumar *et.al.*, 2000). In this thesis the biochemical and genetic characterisation of Fkh2p has identified it to be a major component of SFF and close examination of the SFF and previously reported consensus forkhead binding sites reveal that they are almost identical (Kaufman and Knochel, 1996 and Zhu *et.al.*, 2000). Of the 35 members of the "CLB2 cluster" genes, 26 have easily identifiable binding sites for Mcm1p-SFF/Fkh2p in their proximal promoter regions (Spellman *et.al.*, 1998) implicating Mcm1p-Fkh2p as the key transcriptional regulator at the G2/M transition.

6.2 Fkh2p binding to the UAS's of the "CLB2 cluster"

In this study, Fkh2p has been shown to bind the *CLB2* and *SWI5* UAS's *in vitro* in an Mcm1p dependent manner (3.2.1.1 and 3.2.1.2). Other studies published at the same time as this work confirmed the ability of Fkh2p to bind the promoter regions of members of the "CLB2 cluster" *in vitro* and *in vivo* (Kumar *et.al.*, 2000, Pic *et.al.*, 2000 and Koranda *et.al.*, 2000 and Zhu *et.al.* 2000). Fkh1p, a yeast forkhead protein that shares an 82% similarity to Fkh2p, was also shown to be recruited to promoters of the "CLB2 cluster" *in vivo* but with less affinity than Fkh2p (Kumar *et.al.*, 2000, Koranda *et.al.*, 2000 and personal communication S. Dalton). Furthermore, a recent study has shown that high levels of Fkh2p and Fkh1p can bind DNA autonomously *in vitro,* with Fkh2p, not Fkh1p, binding enhanced a 100 fold in the presence of Mcm1p (Hollenhurst *et.al.*, 2001). However, *in vitro* band-shifts using cell lysates and *in vitro* translated Fkh1p failed to detect Fkh1p binding the "CLB2 cluster" UAS's (3.2.2, Pic *et.al.,* 2000 and Koranda *et.al.,* 2000). These observations implicate Fkh2p, and not Fkh1p, as SFF and hence the key regulator of "CLB2 cluster" gene expression.

The ability of Fkh2p to bind to DNA in a Mcm1p dependent manner is conferred through its DBD and its interaction with the protein-protein interaction domain of Mcm1p (3.2.3 and 3.2.4). Previous studies of Mcm1p have revealed its need to recruit co-activators and co-repressers to regulate gene expression and it seems to be true at the G2/M transition through the recruitment of SFF/Fkh2p (Lydall *et.al.*, 1991). Althoefer

et.al. (1995) showed the continual occupation of the Mcm1p and SFF binding sites throughout the cell cycle and it has now been shown that Fkh2p levels are constitutive throughout the cell cycle and it occupies "CLB2 cluster" promoters in G1 and mitosis (3.2.5, Koranda *et.al.*, 2000 and Pic *et.al.*, 2000). If Fkh2p is occupying the SFF binding site throughout the cell cycle then what is imparting periodic expression of the "CLB2 cluster"? This will be discussed later.

6.3 The transcriptional regulation of the "CLB2 cluster" by Forkhead proteins

There appears to be a level of redundancy at the G2/M transition in the regulation of the "CLB2 cluster" genes. The deletion of *FKH2* has little effect on the transcriptional profile of this cluster of genes but when combined with a *FKH1* deletion, a reduction and loss of periodic expression are observed (4.2.3, Hollenhorst *et.al.,* 2000, Kumar *et.al.*, 2000, Pic *et.al.*, 2000, Zhu *et.al.*, 2000 and Koranda *et.al.*, 2000). This is not the expected lethality seen with the deletion of *MCM1* which arrests at the G2/M transition with no "CLB2 cluster" activity (Althoefer *et.al.,* 1995), indicating there may be more redundancy with other factors, even the other yeast Forkheads (*HCM1* and *FHL1* (Zhu and Davis, 1998)). Another alternative is the presence of an Mcm1p-dependent pathway that acts independently of the forkhead proteins to regulate mitotic progression.

Furthermore, the deletion of both *FKH1* and *FKH2* changes the yeast cells morphology and has been attributed to a reduction in Clb2p production by rescuing the phenotype through the overexpression of *CLB2* (Hollenhorst *et.al.*, 2000). In addition to the transcriptional read out, *CLB2* and *SWI5* UAS driven reporter activity is switched off in the forkhead double delete (Kumar *et.al.*, 2000). This data shows the importance of *FKH1* and *FKH2* as regulators of the "CLB2 cluster" in both absolute transcriptional activity and establishment of periodicity. Taken with the DNA binding affinities of the two forkhead proteins, Fkh2p may be the main regulator of the "CLB2 cluster" but Fkh1p can compensate for its absence.

6.4 Are there other factors important for "CLB2 cluster" regulation?

Ndd1p was first isolated as a high copy suppressor of the *cdc28-1n*^{ts} mutation and was shown to be essential for mitotic progression (Loy *et.al.*, 1999). Although Ndd1p shows no DNA binding properties of its own it can be recruited to "CLB2 cluster" UAS's *in vivo* (Loy *et.al.*, 1999 and Koranda *et.al.*, 2000). Furthermore, the recruitment of Ndd1p is dependent of forkhead proteins indicating a role for Ndd1p through Fkh1p or Fkh2p (Koranda *et.al.*, 2000), however, genetic evidence implicates Fkh2p as the essential forkhead through which Ndd1p activates "CLB2 cluster" gene expression (Koranda *et.al.*, 2000).

Both Fkh1p and Fkh2p contain a FHA domain that has been shown to be important for pT phosphoprotein-protein interactions in other situations. Ndd1p contains three

putative cdk pT phospho-sites implicating it as a possible FHA binding partner. Indeed deletion of two of these sites in conjunction with a third pS phospho-site confers a morphological change to the cell in the absence of Ndd1p (5.2.1) which, like the double forkhead deletion, has been attributed to a *CLB2* defect (5.2.1). This implicates the pT sites of Ndd1p in an important role in "CLB2 cluster" regulation. In addition, the essential nature of Ndd1p can be rescued by deleting *FKH2* (Koranda *et.al.,* 2000) and by deleting the C-terminus of Fkh2p (5.2.1). This implies that the C-terminus of Fkh2p is important in "CLB2 cluster" gene repression and can only be de-repressed in the presence of Ndd1p. Furthermore, it has been shown that Ndd1p is still recruited to the "CLB2 cluster" UAS's in the absence of the Fkh2p C-terminus *in vivo* indicating that Ndd1p does not interact solely, if at all, through the C-terminus (S Dalton personal communication). It appears that Ndd1p may interact with the FHA domain of Fkh2p (5.2.4) indicating a dual role for Ndd1p in de-repressing Fkh2p by displacing its C-terminus, and activating the "CLB2 cluster" through an interaction with the FHA domain figure 6.1 b).

Another factor important for "CLB2 cluster" regulation is Clb2p-cdk activity. Previously Clb2p-cdk activity had been shown to regulate "CLB2 cluster" activity and here it has been shown to act directly through the "CLB2 cluster" UAS's (Amon *et.al.,* 1993 and 5.2.1). The cdk activity does not work through Fkh2p directly because loss of Fkh2p's putative cdk sites has no affect on "CLB2 cluster" regulation (5.2.1), however,

it does seem to play an important role indirectly through Ndd1p. As mentioned above, the mutation of the Ndd1p putative cdk phospho-sites produces a defect similar to a double forkhead deletion that has been attributed to low *CLB2* levels (5.2.1). Furthermore, recruitment of Ndd1p to the "CLB2 cluster" UAS's is disrupted in the absence of Clb2p-cdk activity (S. Dalton personal communications). This sets up a model at the G2/M transition where Cdk activity activates Ndd1p allowing it to bind the FHA domain of Fkh2p, displacing the C-terminus and activating the "CLB2 cluster" (figure 6.1 a). As a consequence a positive feedback loop is established through Clb2-cdk activity (figure 6.1 b). At the end of mitosis when Clb2p is degraded and the mitotic cdk is switched off, Ndd1p is released and degraded and the C-terminus of Fkh2p again represses "CLB2 cluster" gene expression.

6.6 Are the transcriptional mechanisms at the G2/M transition conserved in eukaryotes?

Through evolution, cell cycle transcriptional pathways are only loosely conserved. However, in fission yeast, sep1(+), a forkhead transcription factor, is required for cell separation and causes a hyphal phenotype when deleted (Ribar *et.al.*, 1999). Furthermore, it has been recently demonstrated that Forkhead transcription factors are important for mitotic progression in mammals (Alvarez *et.al.*, 2001). There are also other members of the MADS box family of transcription factors in higher eukaryotes

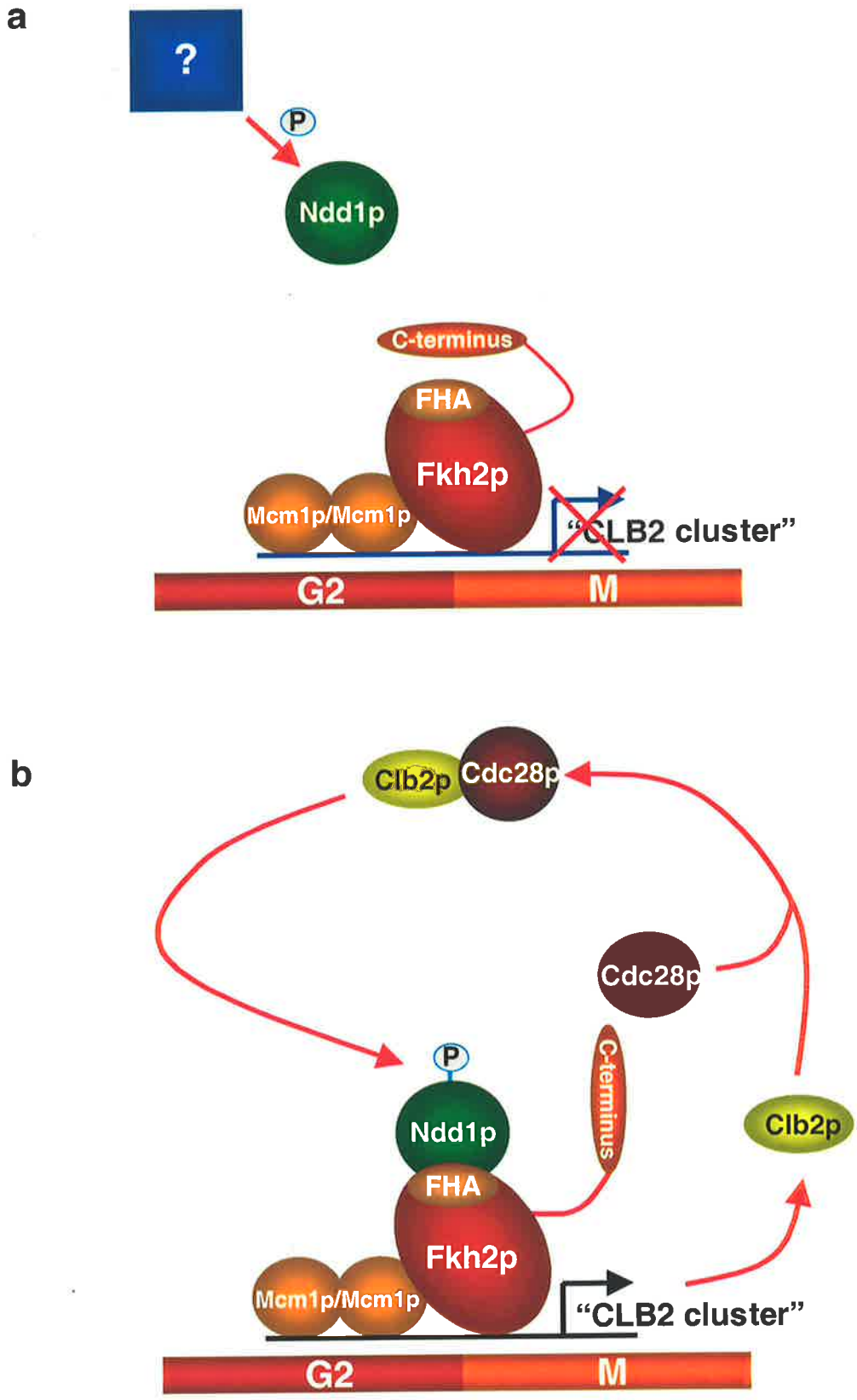


Figure 6.1: Model of transcriptional activation of the "CLB2 cluster". (**a**) The C-terminus of Fkh2p represses the "CLB2 cluster" in the absence of Ndd1p binding. Ndd1p is activated by phosphorylation by an unknown factor. (**b**) The phosphorylation of Ndd1p allows it to bind Fkh2p's FHA domain, displacing the C-terminus and activation the "CLB2 cluster. "CLB2 cluster" activation establishes a positive feedback loop through Ndd1p phosphorylation by Clb2p-cdk complexes.

which recruit co-regulatory proteins but whether these or any unidentified members can recruit forkhead transcription factors to regulate mitosis has not yet been determined.

6.7 Future directions

Future work in this area will concentrate on the interaction between Ndd1p and Mcm1p/Fkh2p. It is proposed that this interaction will be localised to the FHA domain of Fkh2p and be reliant on the cdk phosphorylation of Ndd1p at its pT-sites. Further work on characterising the C-terminus of Fkh2p as a repressing domain should be carried out as well as identifying roles for the putative cdk-phospho sites of Fkh2p. The role of Fkh1p, if any, should also be determined.

6.8 Summary

Regulation of Cdk activity through the binding of different cyclin partners is important for cell cycle progression in an ordered fashion. The cyclins are regulated, for the most part, at the level of transcription and hence are members of subsequent clusters of genes associated with cell cycle progression. At the G2/M transition a cluster of genes, which include the mitotic cyclins *CLB1,2*, share a similar transcriptional pattern and are regulated by transcription factor complex. The initial complex contains Fkh2p and Mcm1p, which occupy the UAS's of the "CLB2 cluster" throughout the cell cycle. This complex is proposed to be activated through the recruitment of Ndd1p, which is itself regulated by cdk activity. At the end of mitosis, the trancriptional machinery is

switched through the collapse of mitotic cdk activity and degradation of Ndd1p. This regulation of the "CLB2 cluster" helps ensure the fidelity of cell cycle progression.

Chapter 7:

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7.0 References

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All the strains used in this thesis are derived from W303 and seen in 2.2.9.

In 3.2.5 a static block experiment was used to determine the level of Fkh2p. From this experiment Fkh2p could be clearly seen in each phase of the cell cycle. An α-factor release would only confirm what was already shown.

Page 65: The presence of Fkh2p bound to promoters throughout the cell cycle and "CLB2 cluster" activity only present late G2/M, indicated the possible presence of a co-activator. Even though further experiments show the presence of other activators, this is not the only explanation for the constitutive presence of Fkh2p. Post-translational modifications of Fkh2p could affect its activity in a cell cycle dependent manner.

The defect seen in *Δfkh1Δfkh2* is attributed to a low Clb2p-Cdc28p kinase activity a consequence of low "CLB2 cluster" expression. This cluster contains genes that are or affect cell budding and cytokinesis. Using plasmolysis would determine if cytokinesis had occurred but this phenotype may be the result of both a defect in cytokinesis and cell separation.

In figure 3.4 the Mcm1p-Fkh2p complex is only shifted in the Gal-inducible strain because it is the Gal-inducible Fkh2p that contains the 6myc epitope tag. The abundance of the Mcm1p-Fkh2p complex after Gal induction shows a limitation in endogenous Fkh2p.

Figure 3.6 shows the absence of any Mcm1p dependent Fkh1p complexes. The *in vitro* translation product Fkh1p was comparable to those performed previously producing Fkh2p (data not shown). A similar result was observed when using $Fkh1p_{6myc}$ yeast strains where no complex was shifted with the addition of α-myc Ab (data not shown). Furthermore, ChIP analysis and further bandshift assays show Fkh2p has a greater affinity for these promoters in an Mcm1p dependent manner than Fkh1p (S. Dalton personal communications and Hollenhorst *et.al.*, 2002).

Figure 5.3 shows a slightly smaller colony for $ndd1_{123*}$ on –W-U. This is an experimental artefact and further analysis by cell spotting has shown $ndd1_{123*}$ has the same colony size on –W-U (data in publication).

Figure 5.8 shows the effects of deleting the FHA domain of Fkh1p and Fkh2p on "CLB2 cluster" gene expression. Western analysis did show a slightly lower expression of these *fkhp* mutants but further ChIP analysis revealed the presence of these mutants on target promoters (data not shown and S. Dalton personal communications).

Page 84: There are five (not two as previously sited) conserved residues in FHA domains that abolish pT binding (Li *et.al.*, 2000 and Pike *et.al.*, 2001).

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