G1/S transcriptional regulation in *Saccharomyces cerevisiae* integrates cell cycle progression and genome stability

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Declaration

I, Michael Ross Harris, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Saccharomyces cerevisiae provides an ideal model to study the regulation of cell cycle commitment due to the high conservation of signalling pathways and regulatory modules through to higher eukaryotes. My work investigates the interplay of cell cycle progression and arrest via the action of transcription factor regulation. Cell cycle commitment is controlled by the cyclin-dependent activation of transcription factor complexes, MBF and SBF. Here I describe the dynamics of SBF and MBF using new polyclonal anti-sera against the three key components Mbp1, Swi4 and Swi6, and their interaction with the inhibitor of SBF, Whi5, and the MBF co-repressor Nrm1. I identify epigenetic modifications that occur on histone proteins at promoters of SBF and MBF genes during the cell cycle. The histone deacetylase Rpd3 has also been investigated as to the role it plays in regulating G1/S transcription. Finally, I have identified a new class of G1/S genes, named switch genes, which are regulated independently by G1/S transcription factors during different phases of the cell cycle. Switch genes are regulated by SBF during G1 and MBF upon entry into S phase, and are enriched for dosage sensitive and replication induced G1/S genes. Switching from SBF-to-MBF allows genes to be activated in response to replication stress, via inactivation of Nrm1. In addition, through switching a potential defect in one of the transcriptional factor complexes will not result in overexpression of these genes. Detailed analysis of the prototypical switch gene *TOS4* shows that it is regulated by SBF and MBF, accumulates in response to hydroxyurea, and delays cell cycle progression when over-expressed. The role Tos4 plays in the cell cycle and in response to checkpoint activation remains unclear, however, data suggests a role in modulating HDAC activity. The roles other switch genes play in response to checkpoint activation are yet to be investigated.

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Abbreviations

APC	Anaphase promoting complex
ARS	Autonomously replicating sequences
ATP	Adenosine triphosphate
bp	Base pair
Cdc	Cell division cycle
CDK	Cyclin-dependent kinase
ChIP	Chromatin immunoprecipitation
Clb	Cyclin B
Cln	Cyclin
Clr	Cryptic loci regulator
CPT	Camptothecin
CRISPR	Clustered, regularly interspaced, short palindromic repeats
CRT1	Constitutive RNR transcription 1
DBD	DNA binding domain
DDR	DNA damage response
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
Elp	Elongator protein
Esa1	Essential SAS family acetyltransferase
Esp1	Extra spindle pole bodies
FACS	Flouresence activated cell sorting
FHA	Forkhead associated
Gal	Galactose
Gcn5	General control nonderepressible
GFP	Green fluorescent proteinn
Grr	Glucose repression-resistant
GTB	G1/S Transcription factor Binding motif
H3K27Ac	Histone H3 Lysine (K) 27 acetylation

H3K9ac	Histone H3 Lysine (K) 9 acetylation
Hda	Histone deacetylase
Hap	Heme activator protein
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HDM	Histone demethylase
HDR	Homology directed repair
HIS-tag	Histidine tag
HMT	Histone methyltransferase
Hos	Hda one similar
HP	Heterochromatin promoter
HRP	Horseradish peroxidase
Hst	Homolog of SIR Two
HU	Hydroxyurea
HUG1	Hydroxyurea and UV and Gamma radiation induced
IgG	Immunoglobulin G
IP	Immunoprecipitation
KanMX	Kanamycin resistance gene
Kan^r	Kanamycin resistance gene
LC-MS	Liquid chromatography-mass spectrometry
Lcd	Lethal, checkpoint-defective
Leu	Leucine
MBF	MCB binding factor
Mbp1	MluI-box binding protein 1
MBP1	MluI-box binding protein
MCB	Mlu1 cell cycle box
MCD1	Mitotic Chromosome Determinant
Mih	Mitotic inducer homolog
mM	Millimolar
MMS	Methyl methanesulfate
Mre	Meiotic Recombination
mRNA	Messenger RNA

Myc	Myelocytomatosis
NaOH	Sodium (Na) hydroxide (OH)
NHEJ	Non-homologous end joining
Nrm1	Negative regulator of MBF1
NuA4	Nucleosomal acetyltransferases of H4
OD600	Optical density at 600 nm wavelength
ORF	Open reading frame
PBS-T	Phosphate buffered saline with Tween
PCR	Polymerase chain reaction
Pds	Precocious dissociation of sisters
pRb	Retinoblastome protein
QPCR	Quantitative PCR
Rad	Radiation sensitive
RNA	Ribonucleic acid
RNR	Ribonucleotide reductase
Rpd	Reduced potassium dependency
rpm	Revolutions per minute
RT	Reverse transcriptase
RT- qPCR	Reverse transcriptase - quantitative PCR
Rtt	Regulator of Ty1 transposition
SAC	Spindle assembly checkpoint
SAGA	Spt-Ada-Gcn5 Acetyltransferase
Sas	Something about silencing
SBF	SCB binding factor
SCB	Swi4/6 cell cycle box
SCF	Skp, Cullin, F-box containing complex
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-Polyacrilamide gel electrophoresis
SIR	Silent information regulator
Sml	Suppressor of Mec1 Lethality
ssDNA	Single stranded DNA
Swe	Saccharomyces Wee
Swi	Switching deficient

Taf	TATA binding protein-associated factor
Tos	Target of SBF
Tris	Tris(hydroxymethyl)aminomethane
Trp	Tryptophan
Ura	Uracil
WCE	Whole cell extract
Whi5	Whiskey 5
Xrs	X-ray sensitive
YEP	Yeast extract peptone
YES	Yeast extract with supplements
Yox	Yeast homeobox
YPD	Yeast extract, peptone and dextrose

Chapter 1. Introduction

1.1 The Cell Cycle

The cell cycle ensures the complete replication of cellular material for division into two identical daughter cells, a process that is imperative in organismal development and homeostasis (Morgan, 2007). Two key features of the cell cycle are uni-directionality and quality control mechanisms that ensure cells retain their genetic integrity by never under or over replicating the DNA. Misregulation of when to, and when not to enter the cell cycle program is integral to the progression of many diseases, particularly cancers (Massagué, 2004). Research on model organisms *Saccharomyces cerevisiae* (budding yeast) and *Schizosaccharomyces pombe* (fission yeast) have greatly contributed to our understanding of the cell cycle control mechanisms in eukaryotic cells. Here I outline the mechanisms of cell cycle control in the model organism *S. cerevisiae*, budding yeast. I also highlight the conserved mechanisms and regulatory processes in *S. pombe* and *Homo sapiens*, human cells.

1.1.1 The cell cycle of the model organism *Saccharomyces cerevisiae*

The cell cycle of eukaryotes contains four phases: Gap phase 1, S phase, Gap phase 2 and Mitosis. Gap phases 1 and 2 are responsible for growth of the cell, while S phase and M phase handle manipulation of the genetic material, DNA replication and sister chromosome division, respectively (Tyson and Novak, 2008). Cellular division of budding yeast is asymmetric and occurs via the mother cell developing a bud that grows in size as the cell cycle progresses. The mitotic spindle orientates one pole into the bud, before the separation of half the sister chromosomes into the bud during mitosis. Once mitosis is complete the mother and daughter cell separate by cytokinesis (Morgan, 2007). In fission yeast, the separation of sister chromosomes to opposite poles of the mother cell occurs before further growth. This is followed by symmetrical division, thereby creating two identical daughter cells (Coudreuse and Nurse, 2010; Moseley et al., 2009). The

majority of human cells undergo symmetrical cell division creating two identical daughter cells. Here I outline the function of each phase in budding yeast and how it differs in fission yeast and human cells.

Gap phase 1 (G1) in budding yeast is the longest phase of the cell cycle and regulates commitment to cell division by ensuring all external and environmental conditions and requirements are met (Morgan, 2007). In yeasts, this checkpoint is known as START whilst in human cells it is called the R point or restriction point. In budding yeast most cellular growth occurs during G1 and as in other species a growth threshold is reached before cells enter S phase. The mechanism of cellular growth signalling to regulate cell cycle entry in budding yeast is unclear (Lloyd, 2013; Tyers et al., 1993). G1 in budding yeast is similar to the G1 in human cells because cellular growth and nutrient sensing occur before commitment to cell division (Section 1.2.1). In fission yeast, G1 is very short and is indistinguishable as most cellular growth occurs during G2 (Morgan, 2007).

S phase (synthesis) is when cells replicate the genome. The mechanisms and the proteins involved in replicating DNA are highly conserved from yeast to man, although the length of time taken to complete S phase is not proportional to the size of the genome (Bell and Dutta, 2002; Gilbert, 2001; Robinson and Bell, 2005). Eukaryotes utilise multiple origins of DNA replication that fire in a temporal pattern allowing different sections of the genome to be simultaneously replicated (Bell and Dutta, 2002). Origins of replication are sites within the genome where the DNA replication machinery is recruited and begins to unwind the DNA helix. DNA polymerases are recruited and when activated, or 'fired', proceed to synthsise DNA using the template strands in a bi-directional manner. At each DNA polymerase there is a template strand of DNA and the newly synthesised DNA, creating a Y-shaped structure called a replication fork. Origins of replication ensure that regions of the genome are ony replicated once per cell cycle. The machinery used to initiate replication is removed following S phase and unable to reform until the next cell cycle. The initiator proteins are recruited to origins at the end of mitosis or early in G1 and the recruitment of DNA polymerases and firing occurs upon progression through G1 (Aparicio et al., 1997; Dahmann et al., 1995; Diffley et al., 1994; Liang et al., 1995; Morgan, 2007; Tanaka et al., 1997). In budding yeast, origins of

replication or autonomously replicating sequences (ARS) have been well characterised. They contain a short 11 bp sequence 'A' element and other 'B' elements that are responsible for the maintenance of plasmids during yeast cell division (Bell, 2002; Deshpande and Newlon, 1992; Stinchcomb et al., 1979; Theis and Newlon, 1994; Van Houten and Newlon, 1990). The order of ARS firing in budding yeast has been well characterised (Raghuraman et al., 2001). However, in fission yeast and human cells although the identification of sequences associated with origin of replication have been identified, the order of firing is not well-established.

Gap phase 2 (G2) is less distinct in budding yeast compared to most eukaryotic cells. In eukaryotic cells, G2 is when additional cellular growth occurs to provide enough material to support two daughter cells and prepare the cell for mitosis (Morgan, 2007). In budding yeast, a growth checkpoint is utilised during G1 and assembly of the mitotic spindle and bud emergence is initiated during S phase (Morgan, 2007). These two factors contribute to accelerated entry into mitosis making G2 very short. In fission yeast, G2 is the longest phase of the cell cycle as a cell size checkpoint (Section 1.3) is in place before cells can enter M phase. The checkpoint is regulated by the M phase inhibitor Pom1, which is located at the poles of the cell. As cells progress through G2 the poles grow apart and the concentration of Pom1 in the cellular mid-zone decreases. Pom1 inhibits two Wee1 kinase inhibitors, Cdr1 and Cdr2. Wee1 is a cell cycle regulator important for relating cell size to cell division through inhibition of entry into M phase. When the concentration of Pom1 decreases, Cdr1 and Cdr2 are able to inhibit Wee1, driving entry into M phase (Martin and Berthelot-Grosjean, 2009; Moseley et al., 2009; Sawin, 2009).

M phase, consisting mitosis and cytokinises, is the final phase of the cell cycle and ensures the accurate separation of duplicated sister chromosomes to both daughter cells. The mechansims and stages of segregating sister chromosomes during mitosis are highly conserved in eukaryotes (Morgan, 2007; Tyson and Novak, 2008). Separation of sister chromosomes to opposite ends of the mitotic spindle (nuclear division) is followed by cytokinesis (cell division), the physical separation of daughter cells. There are four stages of mitosis: prophase,

metaphase, anaphase and telophase. Prophase is initiated as soon as cells enter mitosis and activates the condensing of DNA into compact chromatin structures and disassembly of the nuclear membrane. During metaphase, sister chromatids are attached to either pole of the mitotic spindle by microtubules and aligned at the equidistantly to each pole at the metaphase plate. It is only when all sister chromatids are attached to either pole and aligned at the metaphae plate that progression to anaphase is permitted. The metaphase-to-anaphase transition is a checkpoint of the cell cycle (discussed in Section 1.3). Anaphase is the separation and segregation of sister chromosomes to either pole of the mitotic spindle into each daughter cell. Finally, telophase is when the spindle machinery is disassembled, the nuclear membrane reforms around each set of chromosomes creating new nuclei and chromosomes begin to decondense. In budding yeast and human cells, cytokinesis is coupled to the end of M phase. However, the cytokinesis of fission yeast differs in that completion of cell division is uncoupled from the end of M phase. Following nuclear division in fission yeast, cells re-enter the cell cycle and S phase. Once the two nuclei in the cell have completed S phase and replicated their DNA, cytokinesis symmetrically separates the daughter cells, which then undergo the next G2 and M phase (Morgan, 2007).

The conservation of time for cellular growth (G1 and/or G2) and the phases of the cell cycle are conserved in budding and fission yeast and human cells, although the anatomy and distribution of the phases varies (Fig.1.1A).

1.1.2 Cyclins and Cyclin-dependent kinases

Uni-directional progression through the cell cycle is driven by the accumulation and subsequent degradation of proteins, typified by the cell cycle regulators themselves: cyclins. Identified by Tim Hunt in 1983 using Sea Urchin embryos (Evans et al., 1983), cyclins are periodically expressed and degraded during the cell cycle. Upon accumulation, cyclins interact with the kinases they regulate: cyclin-dependent kinases, CDKs. CDKs were first identified by Paul Nurse and Leland Hartwell in fission and budding yeast, respectively (Hartwell et al., 1970; Nurse et al., 1976). The interaction of specific cyclins with CDKs activates the

kinase. Active CDK, with the help of several other protein kinases, phosphorylate multiple substrates to activate and inhibit multiple cellular processes that drive progression of the cell cycle (Amon et al., 1993; Mendenhall and Hodge, 1998). For example, the cyclin-CDK complex active at the start of S phase is responsible for initiating events that lead to DNA replication. The oscillation of CDK activity during the cell cycle is determined by transcriptional induction and subsequent accumulation of cyclins interacting with CDK, and then degradation of the cyclin by proteolysis. Degradation of cell cycle regulators is a key feature that drives the uni-directionality of the cell cycle (Morgan, 2007). Here I outline the circuitry of the cyclins and CDK network in budding yeast.

In budding yeast, there is one primary CDK, Cdc28 (cell division cycle) that drives the cell cycle (Dirick et al., 1995; Mendenhall and Hodge, 1998). Upon interacting with cell cycle specific cyclins, Cdc28 phosphorylates multiple downstream targets (Mendenhall and Hodge, 1998). In budding yeast, there are other classes of cyclins and CDKs (Cdc28, Pho85, Kin28, Ssn3, and Ctk1) that are involved in cellular regulation. Such proteins are involved in regulating other aspects of the cell during phases of the cell cycle. For example, the Pho85 CDK is known to play a role in cell morphology during the cell cycle when it interacts with the Pcl1 or Pcl2 cyclins (Mendenhall and Hodge, 1998). Here I focus on the regulation of cell cycle progression driven by Cdc28 (Cdk1 in other species) and its interaction with cell cycle specific cyclins.

Nine cell cycle cyclins form complexes with Cdc28: three G1 cyclins (Cln1, Cln2 and Cln3) and six B-type cyclins (Clb1-6) (Andrews and Mason, 1993; Mendenhall and Hodge, 1998; Morgan, 2007). During G1, Cln3 accumulates and interacts with Cdc28, forming an active Cln3-Cdc28 complex that phosphorylates target substrates (Cross, 1988; de Bruin et al., 2004; F R Cross, 1993; Nash et al., 1988). However, the only known target of Cln3-Cdc28 is the transcriptional inhibitor Whi5, although research is ongoing into identifying other targets of Cdc28 during G1 (Costanzo et al., 2004; de Bruin et al., 2004; Holt et al., 2009). Inhibition of Whi5 leads to expression of two other G1 cyclins *CLN1* and *CLN2,* amongst 200 other genes, at a cell cycle event known as START, late in G1 (Section 1.2.2). Expression of *CLN1* and *CLN2* creates a positive feedback loop, leading to further

accumulation of active Cln-Cdc28 complexes (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Dirick et al., 1995; Iyer et al., 2001; Marini and Reed, 1992; Stuart and Wittenberg, 1995; Tyers et al., 1993). Upon passage past START, the B-type cyclins are then expressed in successive waves from START through to mitosis, and regulate the remaining cell cycle events (Mendenhall and Hodge, 1998). The expression of *CLB5* and *CLB6* late in G1 form the Clb-Cdc28 complex responsible for the initiation of DNA replication (Schwob and Nasmyth, 1993). Clb3 and Clb4 are involved in forming the mitotic spindle apparatus (Amon et al., 1993; Richardson et al., 1992), and Clb1 and Clb2 are important regulatory molecules during mitosis. Clb2 plays a more prominent role than Clb1, as *clb2*Δ cells have severe mitotic defects (Fitch et al., 1992; Richardson et al., 1992; Surana et al., 1991). Figure 1.1B outlines the cell cycle control mediated in budding yeast and the order of cyclin accumulation.

The role of cyclins and CDKs are conserved across eukaryotes, although as organisms increase in complexity, the wiring of the networks controlling cyclin expression and regulation become more complex (Table 1.1).

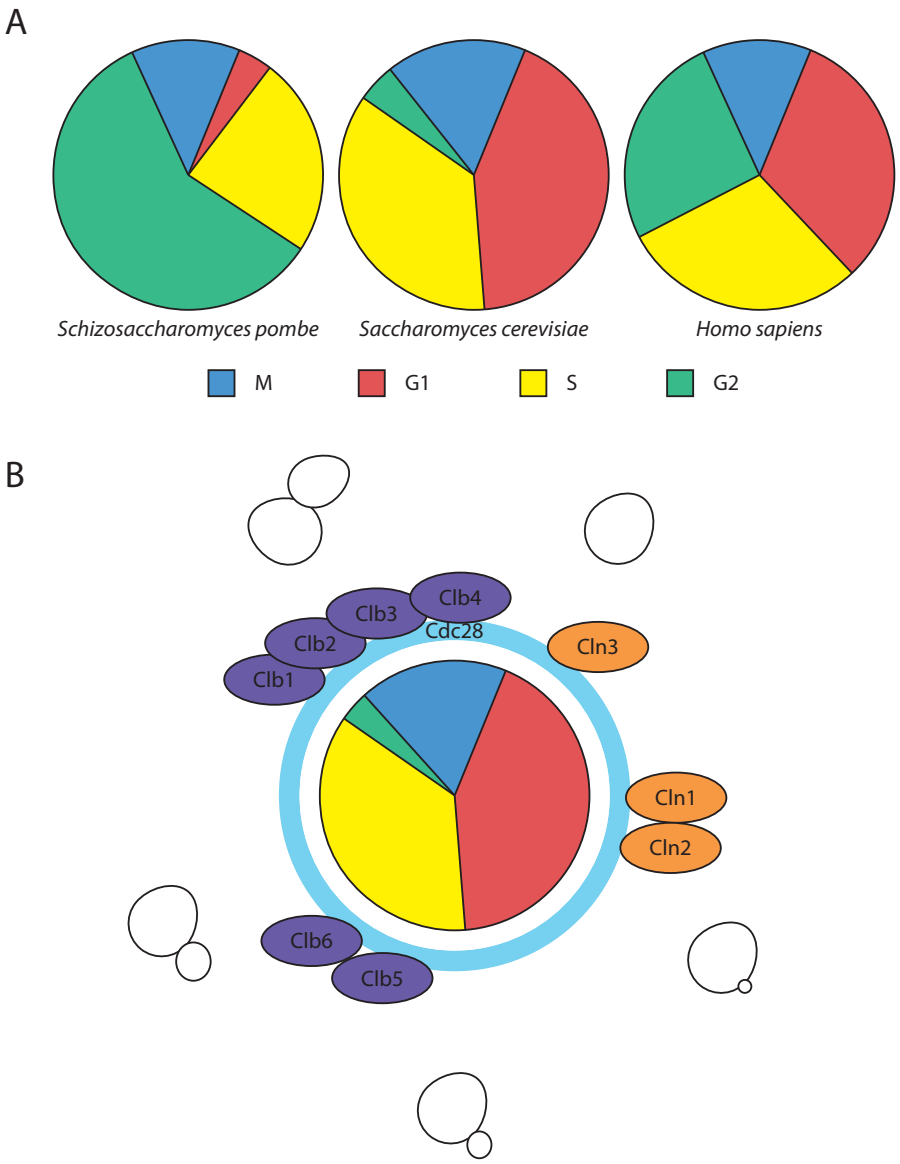

Figure 1.1 The distribution of the cell cycle in eukaryotes and its regulation in budding yeast

A. All eukaryotic cell cycles contain four phases: G1 (red), S phase (yellow), G2 (green) and M phase (blue). The length of the phases changes in accordance with evolutionary pressure and in regard to growth checkpoint dependence. **B.** The cell cycle of budding yeast is driven by the interaction of the cyclin-dependent kinase, Cdc28 (blue circle) and its interaction with the G1 specific cyclins (orange ovals) and the Clb family of cyclins (purple ovals) at different points during the cell cycle. The morphology of budding yeast during the cell cycle is represented by the schematics around the periphery, showing bud emergence late in G1.

	Saccharomyces cerevisiae		*Schizosaccharomyces pombe*		*Homo sapiens*	
Phase	*Cyclin*	*CDK*	*Cyclin*	*CDK*	*Cyclin*	*CDK*
G1	Cln3 Cln1 Cln2	Cdc28	Puc1	Cdc2	Cyclin D Cyclin E	Cdk4/6
S	Clb5 Clb6		Cig1		Cyclin E Cyclin A	Cdk2
G2	Clb3 Clb4		Cig2		Cyclin E Cyclin A	Cdk1/2
M	Clb1 Clb2		Cdc13		Cyclin B	Cdk1

Table 1.1 The conservation of cyclins and CDKs in eukaryotes

Listed are the functional orthologues between budding and fission yeast and humans, of cyclin and cyclin dependent kinases, CDKs. The functional orthologues of cyclins and CDKs share significant sequence homology between yeast and the higher eukaryotic cell cycle regulators.

1.1.3 Degradation of cell cycle regulators is key to the irreversibility of the cell cycle

A key feature of all cyclin-CDK complexes in budding yeast is the destruction of cyclins when they are no longer required to make the cell cycle program irreversible. For example, the proteolysis of cyclins during the cell cycle adds specificity to Cdc28, such that it progresses from regulating commitment to the cell cycle at START, to the initiation of DNA replication in S phase. There are two main complexes that regulate cyclin proteolysis, the SCF complex (Skp, Cullin, F-box containing) and the APC complex (Anaphase promoting complex) (Morgan, 2007).

Whilst the intrinsic stability of the cyclins is also a factor in how they regulate Cdc28, the addition of a ubiquitin moiety to the cyclin by the SCF or APC, signals them for destruction by the proteosome. The SCF complex is responsible for detruction of G1 and S cell cycle regulators such as Cln1 and Cln2. The F-box containing subunit of the SCF, Grr1, targets the SCF to ubiquitinate Cln1 and Cln2, whilst the Cdc4 subunit targets the SCF to Sic1, removing inhibition of the Clb-Cdc28 complex upon entry into S phase (Deshaies, 1999; Orlicky et al., 2003; Pickart, 2001). The APC is the larger of the two ubiquitin ligase complexes and is well characterised to play a role during mitosis. It is a key mediator of the mitotic spindle checkpoint, which is discussed in greater detail in Section 1.3.

1.2 Cell cycle regulated transcription

The periodic expression of proteins during the cell cycle in most eukaryotes has been characterised as a series of three key waves of transcription. These waves occur in a temporal pattern during the cell cycle at G1/S, G2/M and M/G1 boundaries (Bähler, 2005; Morgan, 2007). These waves of transcripts encode for proteins required for the subsequent phase of the cell cycle, and also lead to the proteolysis of inhibitors of progression, and cell cycle regulators of the previous phase (Section 1.1.3). The G1/S cyclins in budding yeast represent an example of how cell cycle regulated transcription controls unidirectional cell cycle progression. The transcription of B-type cyclins, activated by the previous Cln-Cdc28 complexes, creates positive feed-forward that drives the subsequent wave of transcription during G2/M of the cell cycle. In budding yeast, upon entry into S phase, the Clb-CDK complex brings about the proteolysis of the Clns used during G1 (Deshaies, 1999; Orlicky et al., 2003; Pickart, 2001).

The G1/S wave of transcription precedes DNA replication and encodes for many genes required for DNA synthesis and histones, which are responsible for the efficient packing of DNA into the nucleus (Section 1.4.1). The G2/M wave of transcription encodes for proteins required for mitosis and the M/G1 wave encodes for proteins required for exiting mitosis and includes *CLN3* for re-entry into the cell cycle (Bähler, 2005; Wittenberg and Reed, 2005). The expression of cyclins and

transcription factors involved in cell cycle regulation are known to play a key role in forming a continuous network throughout cell division (Wittenberg and Reed, 2005).

Regulating waves of expression involves the activation of transcription followed by repression, leading to genes switching between inactive and active states. Restricting the temporal expression of sets of genes is required so they do not impinge on the preceding or proceeding phase of the cell cycle. Different transcription factor complexes and co-regulators control expression of each of the cell cycle transcription waves. Understanding how these factors contribute to gene expression, and repression, has far-reaching implications into the understanding of diseases caused by genome instability and loss of cell cycle control, namely cancers (Sidorova and Breeden, 2003a). The high level of functional homology between yeasts and human cells in the regulation of cell cycle transcription means understanding the signalling pathways and regulatory mechanisms in one species can greatly expand the understanding in others.

To maintain uni-directionality of the cell cycle, in conjunction with repression of cell cycle transcription waves, proteins expressed during the previous phase of the cell cycle also need to be inhibited or degraded as highlighted in Section 1.1.3. The degradation of G1 cyclins Cln1 and Cln2 by the SCF, following phosphorylation by the Clb-CDK complexes during S phase, is an example of the removal of G1 regulators. The G1 regulators require removal such that they no longer lead to expression of G1/S genes, such as the histones, that when over expressed lead to a delay in cell cycle progression (Sopko et al., 2006).

Here I outline what is known about G1/S cell cycle regulated transcription in budding yeast and highlight similarities, where applicable, to fission yeast and human cells.

1.2.1 Periodic gene expression in *S. cerevisiae*

In budding yeast, 800 genes show periodic expression during the cell cycle, in three waves (Spellman et al., 1998). The G1/S wave of transcription serves a

crucial purpose, as upon activation of G1/S transcription, cells are committed to completing the cell cycle and no longer respond to external/environmental signals. In budding and fission yeast this commitment point is referred to as START and in mammalian systems the restriction point, or R point (Bloom and Cross, 2007; Breeden, 1996; Charvin et al., 2010; Dirick et al., 1995; Johnson and Skotheim, 2013; Koch et al., 1996). START transcription is characterised by the removal of transcriptional inhibitors and activation of transcription factors that regulate the expression of G1/S genes. Many studies have shown that the expression of this wave of transcription utilises a positive feedback loop to ensure robust activation and commitment into the cell cycle and S phase. Cln3-CDK activation leads to the expression of the other G1 cyclins *CLN1* and *CLN2*, leading to further formation of additional Cln-CDK complexes. The amplification of Cln-CDK signalling ensures cells cannot return to G1 until completing the cell cycle (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991; Skotheim et al., 2008). Recent evidence using a higher resolution of gene expression activation has also shown that the *CLN1* and *CLN2* genes are amongst the earliest genes to be expressed within the G1/S wave (Eser et al., 2011).

The G2/M wave contains around 120 genes transcribed by a Mcm1-Fkh1-Ndd1 transcription factor complex and includes the *CLB2* gene (Futcher, 2000; 2002; Wittenberg and Reed, 2005). The M/G1 wave comprises 110 genes expressed late in M phase by the Mcm1, Swi5 and Ace2 transcription factors (McInerny et al., 1997; Morgan, 2008; Wittenberg and Reed, 2005).

Due to the importance of the G1/S wave of transcription in regulating entry into the cell cycle program, here I focus on what is known about the regulation of G1/S transcription in budding yeast.

1.2.2 The G1/S transcriptional program in *S. cerevisiae*

There are two transcription factor complexes that regulate G1/S transcription in budding yeast, SBF and MBF (Section 1.2.3 and 1.2.4, respectively) and their mechanism of action is shown in Figure 1.2. Together, they are responsible for the

temporal expression of more than 200 genes that drive the G1 to S transition, and provide the required machinery for DNA replication (Breeden, 1996; Iyer et al., 2001; Koch et al., 1993). Based on chromatin immunoprecipitation arrays (ChIP-chip), genetic data and expression analysis, G1/S genes have been classified as regulated by either SBF or MBF or both (Iyer et al., 2001; Wittenberg and Reed, 2005). Some gene promoters contain binding sites for both SBF and MBF and there is redundancy in the binding of each transcription factor, although how SBF and MBF regulate G1/S gene expression is distinct (Iyer et al., 2001; Lee et al., 2002; Partridge et al., 1997; Simon et al., 2001; Wittenberg and Reed, 2005) (Figure 1.2). The G1 Cln-CDK complexes regulate the activation of SBF and MBF-dependent transcription (Dirick et al., 1995; Stuart and Wittenberg, 1995; Tyers et al., 1993). The active Cln3-Cdc28 complex phosphorylates the SBF inhibitor Whi5, initiating activation of G1/S transcription (Costanzo et al., 2004; de Bruin et al., 2004; Dirick et al., 1995; Koch et al., 1993).

Budding yeast cell size is indicative of when cells enter the cell cycle, and therefore activation of G1/S transcription. Many studies have focused on the regulation of Cln3 and its activation of G1/S transcription (Cross, 1988; Dirick et al., 1995; Nash et al., 1988; Stuart and Wittenberg, 1995; Tyers et al., 1993). They have identified various factors that regulate the Cln3 cyclin activity during G1 that includes transcription during late M phase (MacKay et al., 2001; McInerny et al., 1997), translational regulation (Gallego et al., 1997; Polymenis and Schmidt, 1997) and protein stability (Jorgensen et al., 2004; Tyers et al., 1992). The system of G1/S transcription activation in human cells is regulated in a similar manner (Cooper, 2006; Costanzo et al., 2004; de Bruin et al., 2004; Dyson, 1998; Nevins, 2001; Schaefer and Breeden, 2004). In human cells, the first identified tumour-suppressor gene *RB1* (Retinoblastoma, pRb) is the functional homolog of budding yeast Whi5 (Murphree and Benedict, 1984) (Section 1.2.3). A key regulator of cell cycle commitment, pRb is a transcriptional inhibitor that binds and inhibits the activator E2F1-3 transcription factors (Helin et al., 1993; Iaquinta and Lees, 2007; Schaefer and Breeden, 2004; van den Heuvel and Dyson, 2008). The E2F family of transcription factors regulate the expression of G1/S genes (Bertoli et al., 2013a; 2013b; Cooper, 2006; Stevens and La Thangue, 2003). Upon accumulation of the G1 cyclinD-CDK4/6 complexes and integration of external stimuli, pRb is

phosphorylated by the active CDK complex and removed from the E2F transcription factors. The removal of pRb and its associated repressive factors leads to activation of E2F-dependent G1/S transcription (Blais and Dynlacht, 2007; Stevens and La Thangue, 2003). Cells containing mutant pRb are unable to regulate entry into the cell cycle by repression of G1/S transcription and progress into S phase before all external conditions are met during G1.

The high conservation of function and regulation of G1/S transcriptional regulators across all species make studying G1/S regulation in yeast models advantageous. The analogous functions of proteins involved in regulating the cell cycle and transcription, combined with the importance of cell cycle commitment in diseases such as cancer, make it a focal point of much research (Cooper, 2006; Wang et al., 2009; Wittenberg and Reed, 2005).

1.2.3 SBF is an activator of G1/S transcription

SBF (SCB Binding Factor) is a heterodimeric transcription factor responsible for activating G1/S transcription (Andrews and Herskowitz, 1989a; 1989b; Breeden and Nasmyth, 1987; Nasmyth and Dirick, 1991; Sidorova and Breeden, 1993). The Swi4 subunit is a helix-loop-helix DNA binding protein that binds the SBF target sequence, the SCB (Swi4 cell cycle box; CRCGAAA) (Amon et al., 1993; Andrews and Herskowitz, 1989b; Bean et al., 2005; Primig et al., 1992; Sidorova and Breeden, 1993). The regulatory Swi6 subunit is responsible for the timely activation of G1/S transcription and is present in both SBF and MBF (Section 1.2.5).

Genetic analysis of *SWI4* identifies that it is an activator of transcription (Nasmyth and Dirick, 1991). SBF target genes are expressed late in G1 following activation by a Cln-CDK-dependent mechanism (Dirick et al., 1995; Stuart and Wittenberg, 1995; Tyers et al., 1992). DNA binding studies have shown that deletion of Swi4 prevents Swi6 binding, via the association between their C-termini, to target promoters, therefore removing SBF function entirely (Andrews and Moore, 1992; Primig et al., 1992; Sidorova and Breeden, 1993). Cells with mutant *SWI4* are no longer able to induce SBF-dependent transcription during the cell cycle (Breeden

and Mikesell, 1991; Nasmyth and Dirick, 1991; Partridge et al., 1997). However, *swi4*Δ cells remain viable, indicating that *SWI4* is not an essential gene (Breeden and Nasmyth, 1987). *swi4*Δ cells display a larger cell size phenotype because they spend a prolonged time in G1 due to a delay in activating G1/S transcription (Nasmyth and Dirick, 1991; Ogas et al., 1991).

The genes that are regulated by SBF are enriched for genes that encode proteins involved in cell morphogenesis, spindle pole body duplication and other growth-related functions, and include *CLN1/2*, *PCL1/2, GIN4, FKS1/2* (Bähler, 2005; Iyer et al., 2001; Wittenberg and Reed, 2005). SBF genes include non-essential genes responsible for controlling cell cycle progression such as the G1 cyclins *CLN1* and *CLN2* (Iyer et al., 2001). The regulation of non-essential cell cycle progression genes by a transcriptional activator is thought to be an effect of evolution (de Bruin and Wittenberg, 2009; Wittenberg and Reed, 2005).

The activation and repression of SBF target genes is restricted to G1/S by the utilisation of a transcriptional inhibitor during G1 and Clb-Cdc28-dependent inactivation during S phase (Amon et al., 1993; Costanzo et al., 2004; de Bruin et al., 2004; Koch et al., 1996; Siegmund and Nasmyth, 1996). Whi5, analogous to pRb in human cells, is bound to the SBF complex early in G1 and inhibits SBF-dependent transcription. Like pRb in human cells, studies have shown the involvement of histone deacetylase (HDAC) activity in Whi5 mediated transcriptional repression (Blais and Dynlacht, 2007; Frolov, 2004; Huang et al., 2009; Takahata et al., 2009) (Section 1.4.4). During G1, Cln3-Cdc28 phosphorylates Whi5, which leads to removal from SBF at promoters and nuclear export (Charvin et al., 2010; Costanzo et al., 2004; de Bruin et al., 2004; Skotheim et al., 2008; Taberner et al., 2009). The removal of Whi5 allows SBF to activate transcription. Deletion of Whi5 leads to cells entering the cell cycle at a smaller cell size indicative of earlier G1/S activation and a shorter G1 (de Bruin et al., 2004). Following G1/S transcription and entry into S phase, the Clb-Cdc28 complex phosphorylates the Swi4 DNA binding subunit leading to its dissociation from target promoters and loss of transcription (Fig. 1.2A) (Amon et al., 1993; Koch et al., 1996; Siegmund and Nasmyth, 1996).

1.2.4 MBF is a repressor of G1/S transcription

MBF (MCB Binding Factor) is a heterodimeric transcription factor responsible for the repression of G1/S genes (Dirick et al., 1992; Koch et al., 1993; Lowndes et al., 1991). The DNA binding subunit Mbp1 contains an N-termini helix-loop-helix domain that binds the MCB sequence (MluI Cell-Cycle Box; ACGCGN) in target promoters (Iyer et al., 2001; Koch et al., 1993). Similar to SBF, MBF also contains the regulatory subunit Swi6 (Section 1.2.5).

MBP1 is a non-essential gene and studies show that deletion of *MBP1* leads to elevated basal levels of MBF-targets throughout the cell cycle (Koch et al., 1993), This indicates that MBF is a negative regulator of transcription that is required for the repression of genes outside of G1/S. Cells with mutant *MBP1* have no cell size phenotype and cells enter the cell cycle at wild type cell size as determined by activation of SBF by Cln3-Cdc28 (de Bruin et al., 2006; Dirick et al., 1995; Horak et al., 2002; Koch et al., 1993; Stuart and Wittenberg, 1995; Tyers et al., 1993).

The genes regulated by MBF are involved in the control or execution of DNA synthesis, DNA repair and cell wall synthesis, including *POL2, CDC2, RNR1, CLB5/6* (Bähler, 2005; Iyer et al., 2001; Lowndes et al., 1991; Verma et al., 1992; Wittenberg and Reed, 2005). The regulation of essential genes by a transcriptional repressor has been suggested to be an evolutionary effect (de Bruin and Wittenberg, 2009; Wittenberg and Reed, 2005).

The mechanism of MBF regulating G1/S transcription is less clear in comparison to SBF. How MBF represses expression of transcripts in early G1 is currently unknown, although the repression is known to be MBF-dependent (Koch et al., 1993). The activation of expression of MBF bound transcripts is thought to be via Cln3-Cdc28-dependent phosphorylation of the Swi6 subunit (or associated regulator) in a manner similar to Whi5 and SBF (Ashe et al., 2008; de Bruin et al., 2008b; Dirick et al., 1995; Geymonat et al., 2004; Sidorova et al., 1995; Ubersax et al., 2003). Although, the role of Swi6 phosphorylation by CDK in regulating both SBF and MBF activation is unclear and studies have shown the regulation of G1/S transcripts in the absence on Cln3 (Cross and Tinkelenberg, 1991; Dirick and

Nasmyth, 1991; Marini and Reed, 1992). Recently, the mechanism of repressing MBF-dependent transcription during S phase has been characterised. One target of MBF-dependent transcription is the gene *NRM1* (de Bruin et al., 2006). Nrm1, negative regulator of MBF, upon accumulation during S phase binds MBF, via the regulatory Swi6 subunit, and co-represses MBF-dependent transcription (Travesa et al., 2013). This negative-feedback loop is required for the repression of MBF-dependent transcription, as in *nrm1*Δ cells, MBF-dependent transcripts are no longer repressed following entry into S phase (de Bruin et al., 2006). The mechanism of Nrm1 and MBF co-repression is currently unknown.

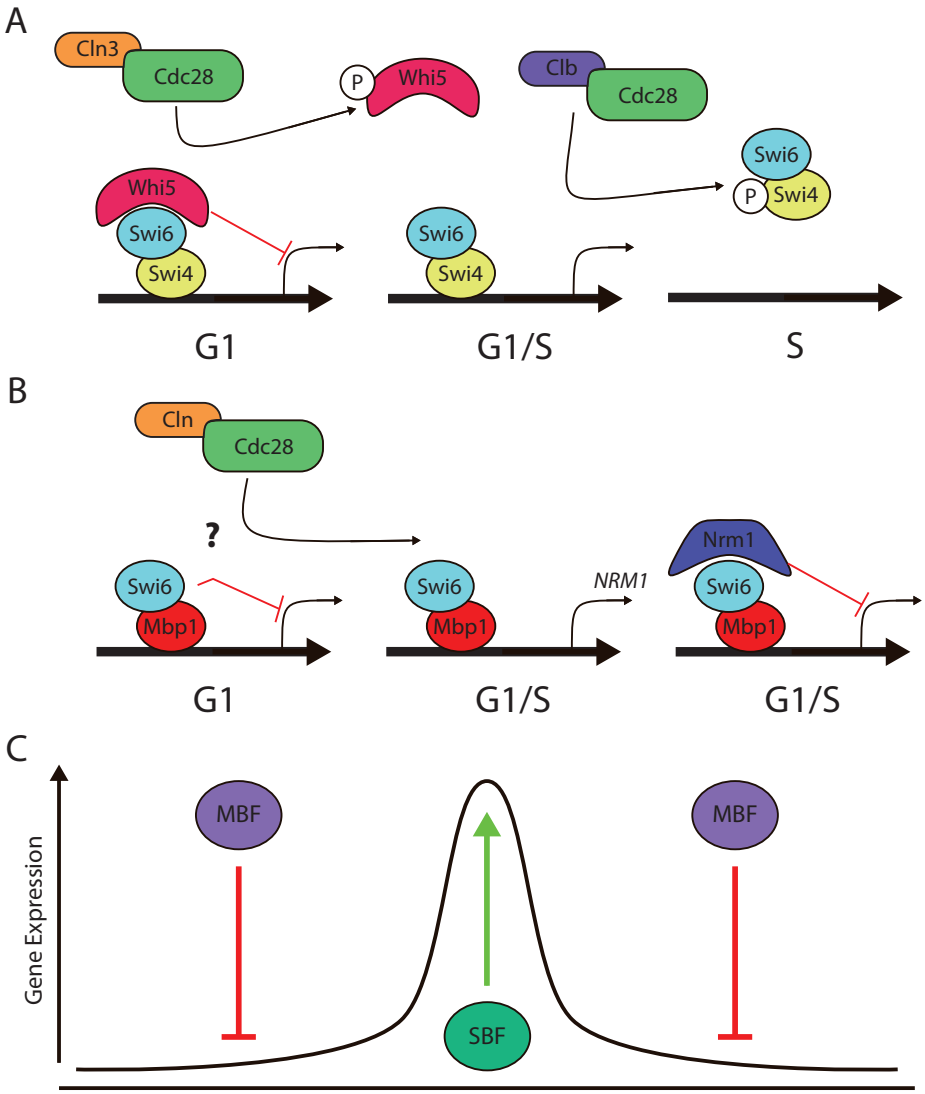

Figure 1.2 The regulation of SBF and MBF in *S. cerevisiae*

A. In early G1, SBF transcription is inhibited by Whi5 binding to the SBF complex at target promoters. Cln3–Cdc28 relieves transcriptional inhibition by phosphorylating Whi5, which induces its nuclear export and activates G1/S transcription. During S phase, Clb-Cdc28 activity phosphorylates Swi4 resulting in dissociation from target promoters. **B.** In early G1, the MBF complex represses transcription by an unknown mechanism. Cln-Cdc28 activity activates G1/S transcription by an unknown mechanism. During G1/S transcription, *NRM1* is expressed and binds to MBF during S phase creating a negative feedback loop to repress MBF-dependent transcription. **C.** The functions of SBF and MBF create different transcription profiles where MBF represses transcripts outside of G1/S and SBF is required for activation of G1/S transcripts.

1.2.5 Swi6 is a modulator of G1/S transcription

Both SBF and MBF contain the regulatory Swi6 subunit (Costanzo et al., 2003; Dirick et al., 1992; Koch et al., 1993; Lowndes et al., 1992; Moll et al., 1992; Nasmyth and Dirick, 1991; Primig et al., 1992). As well as being a direct target of Cdc28 phosphorylation (Dirick et al., 1995; Geymonat et al., 2004; Sidorova et al., 1995; Ubersax et al., 2003), Swi6 also provides a platform for recruitment of many other regulators of G1/S transcription, such as Stb1, Whi5 and Nrm1 (Travesa et al., 2013). Double *SWI4* and *MBP1* mutation is lethal, whereas a single *SWI6* mutant, although sickly, is not inviable, indicating that Mbp1 and Swi4 have some Swi6-independent function (Koch et al., 1993). A double mutant of *SWI4* and *SWI6* is inviable, indicating that Mbp1 alone is not sufficient for cell viability (Breeden and Nasmyth, 1987). *swi6*Δ mutant cells phenotypically show altered cell cycle dynamics, with cells taking longer to complete G1 and therefore displaying a larger cell size phenotype indicative of a delay in the activation of G1/S transcription (Wijnen et al., 2002). G1/S gene expression analysis in Swi6 mutant cells shows characteristics of both Mbp1 and Swi4 mutants. That is, unregulated increased basal level of MBF target transcripts and lower levels of SBF transcripts during the cell cycle (Moll et al., 1992; Nasmyth and Dirick, 1991).

1.2.6 Evolution of G1/S transcription factors

SBF and MBF are the G1/S transcription factors in budding yeast. In fission yeast, MBF is present (spMBF), but SBF is not found nor SCBs found in the promoters of G1/S genes (Baum et al., 1997; Bertoli et al., 2013b; Cooper, 2006). The anatomy of spMBF in fission yeast differs to that in budding yeast. Two DNA binding subunits, spRes1 and spRes2, and two Swi6 related regulatory spCdc10 subunits comprise spMBF (Ayté et al., 1997; Baum et al., 1997; Bähler, 2005). Analysis of each of the individual subunits identifies that the Mbp1 related spRes2 subunit is required for repression of G1/S targets and the Swi4 related spRes1 subunit is responsible for activation of expression (Baum et al., 1997). Alongside the conservation of function from fission yeast spMBF to budding yeast MBF, the MBF

co-repressor Nrm1 is also conserved in the same capacity. Thereby, creating a negative feedback loop to repress G1/S transcripts upon entry into S phase (Aligianni et al., 2009; Andrews and Mason, 1993; Bähler, 2005; de Bruin et al., 2006).

In human cells, the regulators of G1/S transcription are far more complex and are composed of the E2F family of transcription factors. There are eight members of the E2F family of transcription factors. E2F1-3 are activators of G1/S transcription and E2F4-8 are considered repressors of G1/S transcription (Bertoli et al., 2013b; Stevens and La Thangue, 2003; Takahashi et al., 2000). The activator E2F1-3 transcription factors are bound by the transcriptional inhibitor pRb to repress transcription during G1. pRb phosphorylation by the cyclinD-CDK4/6 complex leads to removal from the E2F1-3 transcription factors and activation of G1/S transcription, analogous to the regulation of Whi5 in budding yeast (Costanzo et al., 2004; de Bruin et al., 2004; Helin et al., 1993). Further analogy to the regulation of G1/S transcription in budding yeast to human cells is the presence of a negative feedback loop to turn G1/S transcription off. Activation of G1/S transcription leads to the accumulation of E2F6 which then binds promoters leading to repression of G1/S transcripts upon entry into S phase (Bertoli et al., 2013a; Dimova and Dyson, 2005; Giangrande et al., 2004; Westendorp et al., 2012).

There is no recognisable sequence or structural homology between the G1/S transcription factors in yeasts and human cells. However, the functional and regulatory mechanisms involved are highly conserved (Bertoli et al., 2013b; Hateboer et al., 1998). The conservation of G1/S transcription factors and their regulators in budding and fission yeast and human cells is shown in Table 1.2.

Regulator Type	*Saccharomyces cerevisiae*	*Schizosaccharomyces pombe*	*Homo sapiens*
Activator	SBF (Swi4-Swi6)	spMBF (Cdc10-Res1-Res2)	E2F1, E2F2, E2F3
Repressor	MBF (Mbp1-Swi6)		E2F4, E2F5, E2F6, E2F7, E2F8
Inhibitor	Whi5		pRb
Co-repressor	Nrm1	Nrm1, Yox1	p107, p130

Table 1.2 The conservation of G1/S transcriptional regulators in eukaryotes

Listed are the functional orthologues between budding and fission yeast and humans of G1/S phase transcriptional regulators. There is little identified sequence homology between yeast and the higher eukaryotic G1/S transcriptional regulators.

1.3 Cell cycle checkpoints

The uni-directionality of the cell cycle is a fundamental feature that ensures genomic material is replicated only once per cycle. By ensuring a cell cannot re-enter G1, start S phase again, and re-replicate, the cell cycle program ensures the integrity of the genome sequence. However, during a cells lifetime its DNA can encounter many forms of damage caused by internal and external sources, such as UV light or errors during DNA replication or nuclear division. To prevent the damaged DNA being inherited by daughter cells the cell cycle contains quality control mechanisms called checkpoints. The checkpoints enable progression of the cell cycle program to be arrested in response to genomic insult, and for the damaged DNA to be repaired before continuing. The ability of the program to be arrested at checkpoints until certain conditions are satisfied is a fundamental feature of the eukaryotic cell cycle (Alberts et al., 2002; Hartwell and Weinert, 1989; Morgan, 2007; Weinert and Hartwell, 1988).

Initial discovery of cell cycle checkpoints established that they arrest cell cycle progression in response to internal conditions not being satisfied, such as incomplete DNA replication (Hartwell and Weinert, 1989; Weinert and Hartwell, 1988). Additional studies have shown that cell cycle checkpoints affect more than cell cycle progression. The checkpoint response also includes the activation of pathways that repair damaged DNA, that express genes required to repair DNA, and the recruitment of proteins to sites of DNA damage (Foiani et al., 2000; Lowndes and Murguia, 2000; Nyberg et al., 2002; Rhind and Russell, 2000; Zhou and Elledge, 2000).

The first checkpoint in the cell cycle is late in G1. In budding yeast and human cells this checkpoint monitors the growth and environment of the cell. Should all conditions be permissible to cell division then G1/S transcription is activated (Section 1.2.1).

The next cell cycle checkpoint is the G2/M checkpoint. The G2/M checkpoint ensures the genome is free from damage and completely replicated, and the cell contains sufficient material for two daughter cells before entering mitosis (Alberts et al., 2002; Raleigh and O'Connell, 2000). Should conditions not suit progression into mitosis the G2/M checkpoint inhibits the M-phase cyclin-CDK complex. This inhibition is mediated by the Wee1 kinase that phosphorylates the CDK on the conserved tyrosine 15 (Y15) residue, preventing it from activating proteins involved in carrying out prophase. When G2 is complete and the genome free from damage, the Cdc25 phosphatase removes the Y15 CDK phosphorylation leading to activation of the M-phase cyclin-CDK (Alberts et al., 2002; Morgan, 2007; O'Connell et al., 1997; Rhind et al., 1997). Activation of the M-phase cyclin-CDK leads to robust entry into mitosis by activating two positive feedback loops. Firstly, active CDK phosphorylates Cdc25 leading to further Cdc25 activation. Secondly, the active CDK phosphorylates and inhibits the Wee1 kinase, preventing further inhibition of the CDK. The G2/M checkpoint has been characterised in fission yeast and is conserved in human cells, but is largely cryptic in budding yeast (Amon et al., 1992; Morgan, 2007). In budding yeast should DNA damage be present or replication incomplete the next checkpoint, the spindle assembly checkpoint, arrests cell cycle progression.

The spindle assembly checkpoint (SAC) or the metaphase-to-anaphase checkpoint is the terminal cell cycle checkpoint. The SAC ensures that each pair of sister chromatids are properly attached to each pole and aligned at the metaphase plate before permitting chromosome separation at anaphase. The checkpoint inhibits the APC complex from targeting substrates for degradation, by targeting the APC co-activator subunit Cdc20. Once chromosomes are properly aligned, the M-phase cyclin-CDK complex phosphorylates the APC leading to Cdc20 recruitment and APC activation. The APC targets the M-phase cyclins for degradation leading to low CDK activity. The APC also activates the separation of sister chromosomes by targeting securin for degradation. Securin degradation leads to activation of separase that cleaves the cohesin complex, which tethers sister chromosomes to one another. In budding yeast, the active APC ubiquitinates Pds1 marking it for destruction by the proteasome. Pds1, the homolog of Securin in human cells (Cohen-Fix et al., 1996; Yamamoto et al., 1996), binds and inhibits the Esp1

protease, which is specific for the cohesin complex, which keeps sister chromatids together following S phase (Alexandru et al., 2001; Ciosk et al., 1998; Gruber et al., 2003).

Should the structure of DNA be altered through for example errors in replication or UV light damage, there are two quality control checkpoints. The DNA structure checkpoints function prior to M phase and ensure the integrity of the DNA is maintained throughout cell division. Here I outline the functions of the DNA replication checkpoint and the DNA damage checkpoint in budding yeast and highlight the differences in the signalling pathways compared to fission yeast and human cells.

1.3.1 DNA structure checkpoints

Eukaryotic cells have developed mechanisms that can detect aberrations in the physical structure of the genome (Elledge, 1996; Nyberg et al., 2002; Weinert, 1998a; Zhou and Elledge, 2000). If not repaired the structural aberrations could be potentially catastrophic (Hartwell and Kastan, 1994). The DNA replication checkpoint and DNA damage checkpoint in budding yeast function through overlapping signalling pathways making it difficult to distinguish between each checkpoint pathway. However, studies performed in fission yeast and human cells have enabled distinction of the functions of each checkpoint and the role they play in cell division.

The DNA replication checkpoint is activated in response to the replication machinery stalling or slowing down, such as when it encounters a shortage of dNTPs (Branzei and Foiani, 2005; 2006). Therefore, the DNA replication checkpoint is only activated during S phase. The replication complex is highly efficient at synthesising new DNA when all conditions are suitable. Should conditions not be optimal for replication and the replication machinery progression stalls or slows down, the DNA replication checkpoint is activated. The checkpoint prevents DNA damage occurring by stabilising replication forks and preventing breaks in the DNA from occurring (Lopes et al., 2001; Tercero and Diffley, 2001).

The DNA replication checkpoint also elicits other responses including inhibition of later origin firing, delaying cell cycle progression by CDK inhibition, and preparing re-initiation of replication forks when replication stress is resolved (Alberts et al., 2002; Morgan, 2007; Santocanale and Diffley, 1998; Santocanale et al., 1999; Shirahige et al., 1998; Sidorova and Breeden, 2003b; Tercero and Diffley, 2001; Tercero et al., 2003; Weinberger et al., 1999).

The DNA damage checkpoint monitors DNA for structural aberrations including double stranded breaks (DSBs), and functions throughout interphase. Activation of the DNA damage checkpoint promotes cell cycle arrest and repair of the damaged DNA (Wahl and Carr, 2001). Depending on when the DNA damage is detected in the cell cycle, for example DSBs, two pathways of repair can be instigated by the DNA damage checkpoint (Hoeijmakers, 2001; Morgan, 2007; Sancar et al., 2004). Non-homologous end joining (NHEJ) or homology directed repair (HDR) repair pathways differ in their use of template DNA in the resynthesis of a new sequence of DNA. NHEJ occurs during G1 when the absence of a replicated sister chromatid results in no template sequence being used for resynthesising new DNA. The two exposed ends of a double stranded break are resynthesised back together during G1 at the risk of losing a few bases of sequence (Morgan, 2007). In contrast, the HDR repair pathway occurs following DNA replication and uses the replicated sister chromatid as a template from which to repair DNA. The ends of the damaged DNA strands are resected by an exonuclease, generating ssDNA ends. The ssDNA generated then invades the double stranded sister chromatid DNA and uses it as a template for resynthesising new DNA. The HDR pathway is thus able to replicate the complete sequence without loss of information (Morgan, 2007).

1.3.1.1 DNA replication checkpoint Vs. DNA damage checkpoint

There is little distinction between the DNA replication checkpoint and the DNA damage checkpoint in budding yeast. This is due to a number of reasons.

Firstly, the stimulus that activates each checkpoint is different in budding and fission yeast and human cells, but in budding yeast the two checkpoints initiate the

same response. The DNA replication checkpoint is activated in response to slowing or stalled replication forks, whereas the DNA damage checkpoint is activated in response to damaged DNA structures. In fission yeast and human cells, the signalling cascades activated by replication stress or DNA damage are distinct. Both checkpoints in fission yeast and human cells have an activating sensor kinase, which activates an effector kinase, and each pathway displays some redundancy with the other (Table 1.3). However, in budding yeast the sensor kinase and effector kinase of the DNA damage response, Tel1 and Chk1 respectively, are neither essential nor responsible for the same cell cycle regulatory processes as their homologs from fission yeast and human cells (Bartek and Lukas, 2003; Bertoli et al., 2013b; Jackson and Bartek, 2009; Morgan, 2007; Sørensen and Syljuåsen, 2012; Zegerman and Diffley, 2009). In budding yeast both checkpoints lead to activation of the checkpoint effector kinase Rad53.

Secondly, the two responses differ in when they function during a cell cycle. The DNA replication checkpoint is activated during S phase, whereas the DNA damage response functions throughout interphase (Bertoli et al., 2013b; Morgan, 2007). It is difficult to distinguish the two checkpoints in budding yeast due to a short and indistinct G2 phase. In fission yeast and human cells, the presence of distinct G2 phases have allowed for studies to be performed when S phase has been completed. Such studies have led to the clear distinction of the pathway and the response of the DNA damage checkpoint, independent of the DNA replication checkpoint pathway being activated by replication fork stalling (Bartek and Lukas, 2003; Bartek et al., 2004; Carr, 2002; O'Connell et al., 1997; Rhind et al., 1997; Sørensen and Syljuåsen, 2012). In fission yeast, activation of the DNA damage checkpoint during G2, by UV irradiation causing DSBs, leads to an increase in the inhibitory Y15 phosphorylation of Cdc2. Cdc2 is the main regulator of cell cycle progression in fission yeast (Table 1.1). The kinase responsible for the inhibition of Y15 phosphorylation of Cdc2 is Wee1 (Nurse and Thuriaux, 1980). Therefore, DNA damage detected during G2 leads to a delay in entry into mitosis (O'Connell et al., 1997; Rhind et al., 1997).

Thirdly, evolutionary pressure has led to the re-wiring of the signalling cascades in budding yeast to suit differences in cell cycle progression. In human cells, the DNA

replication checkpoint functions by a signalling pathway (outlined in Table 1.3) dependent on ATR, leading to activation of CHK1. However, the DNA damage response throughout interphase depends on the ATM sensor kinase, which leads to activation of CHK2. Similarly in fission yeast the terminal effector kinase in response to replication checkpoint activation is spCds1, while in response to DNA damage spChk1 is activated. Studies have shown in budding yeast that activation of both the pathways, using various treatments, leads to activation of the checkpoint effector kinase Rad53 (Bartek et al., 2001; Carr, 2003; Foiani et al., 2000; Lopes et al., 2001; Muzi-Falconi et al., 2003; Pellicioli and Foiani, 2005; Vialard et al., 1998).

Ultimately, when the DNA replication checkpoint cannot prevent replication stress induced DNA damage, the DNA damage response is activated. In human cells this leads to stabilisation of p53 to promote either cell senescence, to allow more time to repair the genome, or induce apoptosis to prevent the DNA damage being inherited and the accumulation of genomic instability (Carvajal and Manfredi, 2013; Harris and Levine, 2005; Vousden and Lu, 2002; Wahl and Carr, 2001). In budding yeast, the two checkpoints are activated together in most circumstances although certain treatments are thought to induce specific pathways. For example, the treatment with hydroxyurea (HU) and methylmethansulpahte (MMS) induces the DNA replication checkpoint, whereas doxorubicin and UV radiation induces the DNA damage checkpoint. The work presented in this thesis focuses on the use of hydroxyurea to induce the DNA replication checkpoint.

	Saccharomyces cerevisiae		*Schizosaccharomyces pombe*		*Homo sapiens*	
DNA Structure Checkpoint	***DDR***	***DRC***	***DDR***	***DRC***	***DDR***	***DRC***
Sensor and/or Transducer	Tel1	Mec1*	Tel1	Rad3	ATM	ATR*
Effector	Chk1	Rad53*	Chk1	Cds1	CHK2	CHK1*

Table 1.3 The conserved DNA structure checkpoint regulators in eukaryotes

Listed are the functional orthologues between budding and fission yeast and humans of the DNA structure checkpoint protein kinases. The checkpoint protein kinases share significant sequence homology between yeast and the higher eukaryotic protein kinases. *proteins are essential. DDR, DNA damage response; DRC, DNA replication checkpoint; Tel1, telomere length regulation 1; Mec1, mitosis entry checkpoint 1; CHK, checkpoint kinase; ATM, ataxia-telangiectasia mutated; ATR, ataxia-telangiectasia and Rad3-related protein; Rad, Radiation sensitive.

1.3.2 DNA replication checkpoint in budding yeast

Reasons for the replication fork progress slowing and/or stalling include a shortage of dNTPs, hard to replicate repetitive sequences, encountering damaged DNA templates or when the progressing replication fork encounters sequences being actively transcribed (Bertoli et al., 2013a; Morgan, 2007; Sidorova and Breeden, 2003a; Smolka et al., 2012). When replication forks stall they activate a signalling cascade via the Mec1 kinase that leads to activation of the effector kinase Rad53 and the downstream effector kinase Dun1 (Allen et al., 1994; Ma et al., 2006; Pellicioli and Foiani, 2005; Sanchez et al., 1996; Siede et al., 1996).

In response to the replication complex stalling sections of single stranded DNA (ssDNA) are exposed that signal replication stress and DNA damage (Alcasabas et al., 2001; Sidorova and Breeden, 2003a; 2003b; Zou and Elledge, 2003). Stalled

replication forks need stabilising to prevent the fork from collapsing and generating damaged DNA and also for re-starting forks when the checkpoint is resolved (Branzei and Foiani, 2006). The stabilisation of replication forks leads to the recruitment of the a phosphatidylinositol-3-kinase (PI3K), Mec1 (D'Amours and Jackson, 2002; Huang et al., 1998; Weinert, 1998b; Zhou and Elledge, 2000; 1993). The recruitment of Mec1, the regulatory subunit Lcd1 and the replication checkpoint mediator Rad9, lead to the phosphorylation and activation of Rad53 (Alcasabas et al., 2001; Ma et al., 2006; Sweeney et al., 2005). Active Rad53, also a PI3K-like kinase, mediates pathways responsible for eliciting the effects of the DNA replication checkpoint and DNA damage checkpoint (Allen et al., 1994). Rad53 kinase activates responses to replication stress by activation, inhibition, stabilisation and degradation of key proteins that include further kinases, cell cycle inhibitors and transcription factors (reviewed in Weinert (1998)) (Elledge, 1996; Sidorova and Breeden, 1997; 2003b; Weinert, 1998a; Weinreich and Stillman, 1999).

1.3.3 The transcriptional response to DNA replication checkpoint activation

Studies in budding yeast suggest that there might be a limited role for the transcriptional induction of genes in response to genotoxic stress, as when translation of new proteins is inhibited, cells are able to survive genomic insults and complete S phase (Tercero et al., 2003). However, other studies suggest that it is the degree of genotoxic stress, either acute or chronic, that determines the need for a transcriptional response, as cell survival increases when G1/S transcription is turned on (de Bruin et al., 2008a; 2006). Many studies have monitored gene expression changes that occur in response to various cellular stresses, including DNA structure checkpoint activation and environmental stresses (Fu et al., 2008; Gasch et al., 2001; 2000; Jaehnig et al., 2013; Putnam et al., 2009; Workman et al., 2006). These studies suggest that the induction of transcription in response to various stresses induces overlapping classes of genes.

In budding yeast, Rad53 is activated in response to DNA replication stress and DNA damage. Activated Rad53, in response to various stresses, induces an

overlapping, but distinct response. Here I focus on the Rad53-dependent response to DNA replication stress and its effect on transcriptional regulation.

The most established role of Rad53 is activation of the DNA damage response (DDR) genes that are involved in mediating the repair of DNA. In budding yeast, one of the established targets of the DDR pathway is the *RNR3* gene. Rnr3 is a subunit of the ribonucleotide reductase (RNR) complex, responsible for the synthesis of dinucleotide triphosphates (dNTPs) for incorporation into newly synthesised DNA (Elledge et al., 1993). *RNR3* expression is under the control of the transcriptional repressor Crt1. Active Rad53 phosphorylates the Dun1 kinase (Bashkirov et al., 2003; Chen et al., 2007) that targets and inactivates Crt1, leading to expression of DDR genes (Huang et al., 1998) (Fig 1.3). *CRT1* expression is a target of Crt1 repression, creating a negative auto-regulatory feedback loop so that when the checkpoint stress is resolved, expression is quickly repressed. Another key target of the Dun1 kinase is the RNR inhibitor Sml1, which is phosphorylated and targeted for degradation (Zhao et al., 1998; Zhao and Rothstein, 2002).

Activation of cell cycle regulated genes in response to DNA replication checkpoint activation has been identified in fission yeast. Studies show that activation of the replication checkpoint with the RNR inhibitor HU, led to inhibition of the spMBF co-repressors spNrm1 and spYox1, and expression of MBF target genes outside of G1/S (Fig 1.3) (de Bruin et al., 2008a). The conservation of the transcriptional co-repressor Nrm1 in budding yeast has suggested that this is a conserved regulatory mechanism of the DNA replication checkpoint (de Bruin and Wittenberg, 2009).

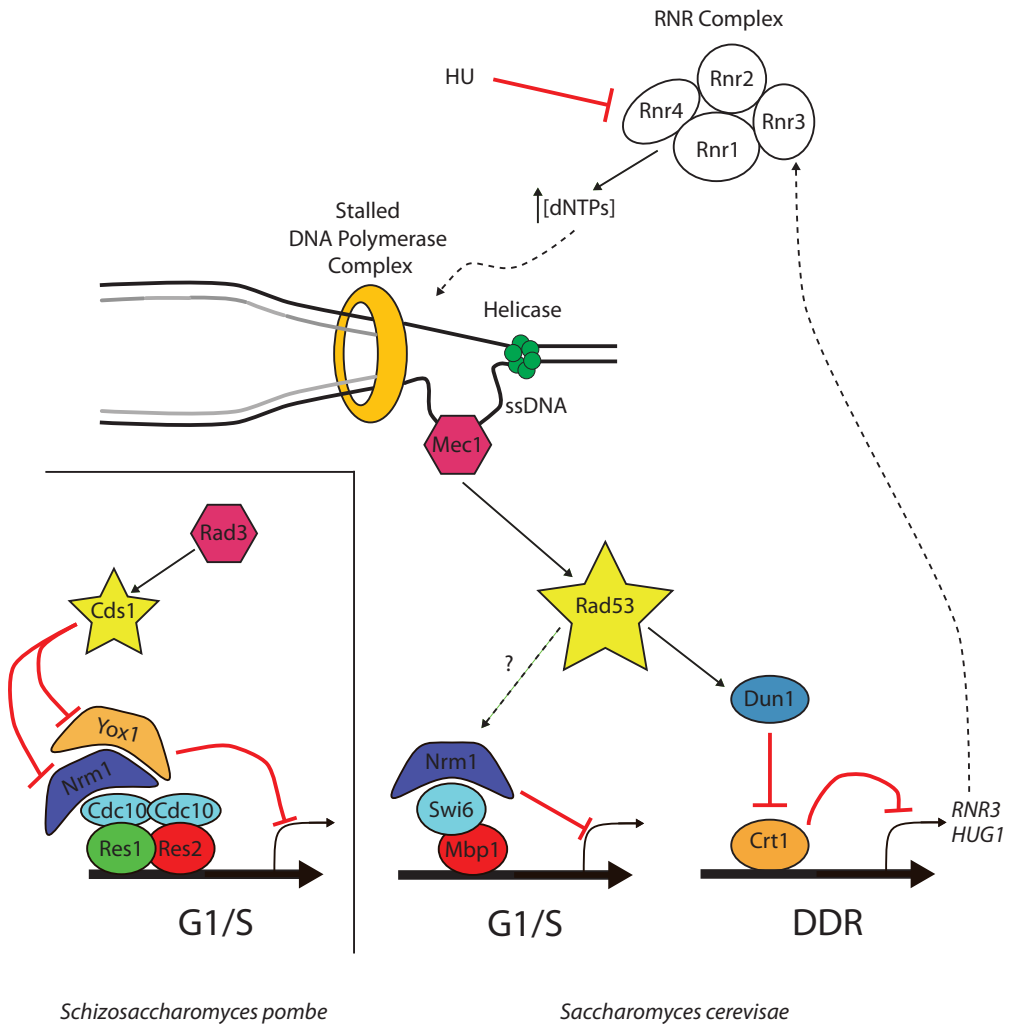

Figure 1.3 The transcriptional response of the DNA Replication Checkpoint in *S. cerevisiae*

In response to hydroxyurea, HU, the RNR complex is inhibited and the pool of dNTPs decreases leading to replication fork stalling. The single stranded DNA, ssDNA exposed by the uncoupling of the DNA helicase recruits a complex leading to activation of the Mec1 protein kinase. Mec1 activates the effector kinase Rad53, which leads to activation of Dun1 and inhibition of the transcriptional repressor Crt1, leading to expression of the DNA damage response genes, DDR. In fission yeast, the ortholog of Rad53, spCds1, activates MBF-dependent transcription via inhibition of the co-repressors spNrm1 and spYox1 (boxed area). It is thought activation of G1/S genes in response to replication stress would be conserved in budding yeast. dNTPs; deoxynucleotide triphosphates.

1.4 Histone modifications and gene expression

Histones are amongst the most highly conserved class of proteins. They play an essential role in the efficient packaging of DNA into chromatin (the collective name for the collection of DNA, histone proteins and non-histone proteins associated with DNA) in the eukaryotic cell nucleus. Histones are small (102-130 amino acids in length), highly abundant proteins, rich in arginine and lysine residues, that associate with high affinity to DNA (Alberts et al., 2002). Histones comprise roughly the same mass as DNA in chromatin. It was initially thought that the function of histones was to aid in the compaction of DNA within the nucleus. However, studies have begun to show that these highly diverse and conserved proteins are able to regulate the structure of chromatin and DNA-dependent processes such as transcription, DNA replication and DNA repair (Alberts et al., 2002; Khorasanizadeh, 2004; Morgan, 2007).

1.4.1 Histones and the Nucleosome

There are four core histones involved in the packaging of DNA: H2A, H2B, H3 and H4 (Kornberg, 1977; Luger et al., 1997). The structure of the core histones has been characterised as the histone fold. Each of the core histones contains three **α**-helices connected by two loops, giving them a U-shape. The N- and C-terminal tail of each core histone is flexible and unstructured (Khorasanizadeh, 2004). Interactions through the central **α**-helices of the histone fold core domains lead to the formation of H3-H4 and H2A-H2B heterodimers (two interlocking U-shapes). Two copies of each heterodimer create a histone octamer (H3-H4 tetramer and two H2A-H2B heterodimers), called the nucleosome core, around which 147 bp of DNA wrap (Khorasanizadeh, 2004; Luger et al., 1997). Together the histone octamer and the associated DNA are referred to as the nucleosome. It is a repeating structure along eukaryotic chromosomes with ~50 bp of DNA between nucleosomes, often referred to by its 'beads-on-a-string' appearance in electron microscopy. This first-order of DNA packaging reduces the length of DNA by a approximately a third (Alberts et al., 2002).

The concentration of lysine and arginine residues within the histone octamer creates a highly positive charge. The deposition of nucleosomes along DNA is non-sequence specific and is mediated by an electrostatic interaction between the histone core and the negative phosphodiester backbone of DNA (Luger et al., 1997). A key feature of the nucleosome is the protrusion of the N-terminal tails of the histones, 10-30 amino acids in length, beyond the surrounding DNA. This enables post-translational covalent modifications of the histone lysine and arginine residues to occur and regulate the electrostatic interaction of DNA with the histone core particle (Section 1.4.2).

The second order of folding chromatin comes from the recruitment of the non-core histone H1. H1 is a linker histone that secures the entry and exit of the DNA wrapped around a nucleosome (Bednar et al., 1998; Brown et al., 2006). H1 can also tether adjacent nucleosomes to one another by interaction with another H1 molecule at another nucleosome, as can the adjacent H4 N-terminal tails between nucleosomes. Nucleosome to nucleosome interaction begins to distort the 'string' of DNA and create a 30 nm coil. There are two proposed models for the 30 nm coil referred to as the zig-zag model and the solenoid model (Khorasanizadeh, 2004; Woodcock and Horowitz, 1995). This 30 nm wide coil is flexible enough to expand and contract and, excluding during M phase, is the most common form of chromatin in the nucleus (Alberts et al., 2002; Cui and Bustamante, 2000; Katritch et al., 2000). During mitosis, chromosomes need to de tightly condensed for efficient nuclear division. Scaffold proteins are recruited to the 30 nm fiber and result in further coiling of the fiber to make a super-helical structure 100 nm wide. Additional to this structure, further compaction is achieved via the recruitment of more scaffold proteins that form the highly condensed mitotic chromosomes that are visible under the microscope.

1.4.1.1 Regulating the nucleosome

Chromatin in the nucleus needs to be a dynamic structure that is readily accessible for DNA-dependent processes whilst highly compacted for efficient organisation of DNA. Through numerous mechanisms, both the core and non-core histones allow

for the regulation of the chromatin to provide this dynamicity by altering the DNA-histone and histone-histone interactions within the nucleosomes. The presence of histones is inherently repressive to transcription indicating that DNA-based processes that require specific DNA sequences need to overcome the inhibition and sequence sequestration bought about by the packaging of chromatin (Becker and Hörz, 2002; Grunstein, 1990; Kayne et al., 1988). The focus of my thesis is on the covalent chemical modification of histones and is discussed in further detail in Section 1.4.2. However, other mechanisms used to regulate the state of chromatin include the use of variant histones and ATP-dependent remodelling of the nucleosomes and ejection of the histone octamer core form nucleosomes.

Firstly, variant histones are alternative forms of the core histones that can be incorporated into the nucleosome in place of the canonical histone. Histone variants are understood to play a key role in determining various structures of the chromosomes such as the centromere, by incorporation of histone CenH3, the structure responsible for binding to the mitotic spindle. The incorporation of CenH3 provides the nucleosome with an alternate N-terminal tail region. This is responsible for the recruitment of non-histone proteins to the DNA that assemble the necessary machinery for kinetochore attachment (Ahmad and Henikoff, 2002; Blower et al., 2002; Kamakaka and Biggins, 2005). Histone variants are also known to highlight sites of actively transcribed genes (incorporation of H2A.Z) and also sites of DNA damage (incorporation of H2A.X) (Billon and Cote, 2012; Jackson and Gorovsky, 2000; Meneghini et al., 2003; Paull et al., 2000; Raisner et al., 2005; Rogakou et al., 1999; Suto et al., 2000).

Secondly, chromatin-remodelling complexes use the energy generated from the hydrolysis of ATP to actively manipulate the interaction between DNA and the histone octamer core. There are three mechanisms the family of chromatin remodelling complexes use to alleviate the histone-DNA interaction: sliding, looping and displacement of core histones (Becker and Hörz, 2002; Fan et al., 2003; Fazzio and Tsukiyama, 2003; Kassabov et al., 2003; Khorasanizadeh, 2004; Mizuguchi et al., 2004). Sliding of nucleosomes relocates the position of the H1 linker histone such that an alternative sequence is wrapped around the octamer core and sequences previously wrapped are then exposed as part of the linker

DNA are more accessible (Aoyagi et al., 2002; Narlikar et al., 2002). Looping is a proposed model that suggests chromatin remodelling complexes temporarily expose DNA sequences wrapped around the octamer core by 'pulling' the DNA strand away from the core. The DNA sequence is then accessible to binding by sequence specific factors, that when bound can lead to nucleosome mobilisation, or if the factor is only bound transiently, the DNA sequence will return to the octamer core surface (Fan et al., 2003; Kassabov et al., 2003; Narlikar et al., 2002). The displacement of core histones uses ATP-dependent remodelling complexes to eject core histone proteins from the octamer and replace them with histone variants. This mechanism is used in the incorporation H2A.Z at actively transcribed regions in budding yeast (Billon and Cote, 2012).

The combined action of regulating the chromatin through different mechanisms can lead to a drastic shift between the formation of the chromatin. For example, the action of incorporation of the CenH3 histone variant leads to the stabilisation of DNA associated factors that provide the scaffold around the centromere (Ahmad and Henikoff, 2002; Blower et al., 2002; Kamakaka and Biggins, 2005). As such, the genes, if any, that are located in such an area are repressed by the highly compacted chromatin. Such a formation of chromatin is referred to as heterochromatin. The structure of chromatin can also be open and more accessible during DNA-dependent processes, such as transcription and replication, where many regulatory factors are required. The density of the nucleosomes in such areas/regions is generally low and the structure/state of chromatin is referred to as euchromatin. The two states are associated with a measure of how transcriptionally active genes are within, although not all euchromatin is transcriptionally active.

Throughout the cell cycle the open euchromatin and closed heterochromatin can fluctuate through a continuum of states. During M phase, all chromatin is heterochromatin and tightly packaged for nuclear division (Alberts et al., 2002). However, during interphase most of the DNA is in a euchromatic state apart from two forms of heterochromatin. 1) Chromosomal structures such as the telomere and centromere that contain highly repetitive sequences are regarded as transcriptionally silent and comprise constitutive heterochromatin. 2) Centric heterochromatin at the centromere is surrounded by pericentric heterochromatin

and the region is generally transcriptionally silent in all chromosomes, across all species (Meneghini et al., 2003; Oberdoerffer and Sinclair, 2007). Areas where genes are being actively repressed, such as by histone deacetylation (Section 1.4.2), are referred to as facultative or adaptive heterochromatin as the formation of chromatin is actively regulated. Facultative heterochromatin is therefore more likely to change state to euchromatin upon activation of transcription and replication than constitutive heterochromatin (Craig, 2005; Oberdoerffer and Sinclair, 2007).

The work of my thesis focuses on the transcriptional regulation of G1/S cell cycle regulated genes in budding yeast. One mechanism that transcriptional regulators use to promote or repress transcription is through the reversible covalent modification of the histone N-terminal tails. Here I outline what is known about the effect of chemical modifications of histones on transcription and the enzymes that carry out such modification in budding yeast.

1.4.2 Histone modifications in budding yeast

The N-terminal tails of core histones that protrude out from the nucleosome are rich in positive arginine, serine and lysine residues thought to be important for the electrostatic attraction to DNA. Such residues, like the histones themselves, are highly conserved throughout eukaryotic evolution (Alberts et al., 2002; Ekwall, 2005; Santos-Rosa and Caldas, 2005). The chemical modification of the amino acid side chain can change the biochemistry of the histone-DNA interaction and can also act as a binding site for the recruitment of chromatin regulators (Morgan, 2007). Transcription factors utilise enzymes that catalyse post-translational modifications of the core histone N-terminal tails in order to switch the chromatin between a heterochromatic (repressive) and euchromatic (permissive) state. Modifications of histones are a key feature in regulating transcription, DNA replication and DNA repair. Here I focus on the role of histone modifications in transcription.

Post-translational modifications of histones include phosphorylation, SUMOylation, ubiquitination, methylation and acetylation (Kouzarides, 2007; Millar and Grunstein,

2006). Many of the studies on histone modifications have been carried out in budding yeast and have focused on the role of acetylation and methylation in regulating chromatin. Each modification is associated with a general effect on histone-DNA biochemistry. For example, acetylation of lysine residues is thought to neutralise the positive charge on the histone tail decreasing the attraction between the histone octamer and the DNA, therefore promoting a euchromatic state (Tse et al., 1998; Zheng and Hayes, 2003). Further, genome wide ChIP-on-chip studies have identified correlations between specific modifications and gene expression patterns (Kurdistani et al., 2004; Liu et al., 2005; Millar et al., 2006; Pokholok et al., 2005; Rao et al., 2005; Xu et al., 2005). However, such studies provide only a snapshot of the modification state of histones, which is thought to be a highly regulated and dynamic process (Kouzarides, 2007).

The modification of a histone tail can provide a binding site for regulators of chromatin structure and function through the use of specialised domains. For example, the establishment of constitutive heterochromatin utilises the recruitment of heterochromatin promoter 1, HP1, to methylated histones via its chromodomain. Further, HP1 can recruit a histone methyltransferase, HMT, to methylate nearby histone tails and perpetuate the spread of heterochromatin (Cheutin et al., 2003; Maison and Almouzni, 2004; Rea et al., 2000).

There is no rule for a specific histone modification, either acetylation or methylation, for eliciting a specific effect on transcription (Berger, 2007), but the effect seems to be largely context-dependent. For example, methylation has been associated with both gene expression and repression. It is the specificity of the methylated lysine residue and the position of the nucleosome relative to the ORF that has been associated with the transcriptional outcome. This led to the hypothesis of a ‘histone code’, whereby a combination of modifications at a nucleosome within a promoter would predict an effect for the corresponding gene’s expression (Jenuwein and Allis, 2001; Suganuma and Workman, 2008). This model accounts for the observation that both methylation and acetylation neutralise a lysine positive charge, but each modification is associated with a specific outcome due to the other marks that must be associated with the observed histone modification (Berger, 2007; Gardner et al., 2011).

1.4.3 Histone modifying enzymes in budding yeast

As highlighted in Section 1.2, the initiation of cell cycle entry is governed by the regulated expression of G1/S genes at START. In human cells, unregulated transcription of G1/S genes leads to early cell cycle entry and unregulated cell division is one of the hallmarks of cancer (Hanahan and Weinberg, 2000), highlighting the need to repress G1/S transcription during the G1 until the R point. Additionally, the inhibition of G1/S transcription upon entry into S phase is a key factor in preventing the overexpression of DNA replication factors that can lead to genome instability. Therefore, my work has focused on the transcriptional repression mediated by transcription factors, and the HAT antagonising role of histone deacetylase enzymes, HDACs, in budding yeast.

To catalyse the modification of histones, transcription factors recruit enzymatic subunits to promoter regions where they act on nearby nucleosomes. When a transcriptional activator mediates acetylation of nucleosomes in the promoter it can lead to an increase in the accessibility of the associated DNA sequences, such as the TATA box. RNA polymerase is then able to bind the promoter region and following activation lead to transcription of the downstream gene. In contrast, negative regulators of transcription are thought to recruit enzymes that inhibit the recruitment of the transcriptional machinery to the promoter region. Here I outline the role of histone modifying enzymes in budding yeast. I focus on the role that acetylation plays in budding yeast promoters, as this is the most studied and associated promoter modification with gene expression (Millar and Grunstein, 2006; Saunders et al., 2006).

There are nine histone acetyltransferases, HATs, in budding yeast: Hat1, Gcn5, Taf1, Esa1, Sas2, Sas3, Elp3, Hap2 and Rtt109. The HAT enzymes catalyse the addition of acetyl groups to the lysine amino acid side chains in target substrates using acetyl Co-A as a donor (Berndsen and Denu, 2008; Roth et al., 2001). Although initially identified as acetyltransferases of histones, in recent years it has become clear that lysine residues in many non-histone proteins are also targets for

acetylation and regulation (Allis et al., 2007; Gu and Roeder, 1997; Yang, 2004; Yuan and Marmorstein, 2012). The most characterised HATs in budding yeast are Gcn5 and Esa1 that are part of multi-subunit chromatin-modifying complexes, the SAGA and NuA4 complexes, respectively (Allard et al., 1999; Grant et al., 1997; Jenuwein and Allis, 2001; Lee and Workman, 2007). Target residues of Gcn5 in histones tails are H3 (K9, K14, K18, K23, K27), while deletion of Esa1 is associated with a decrease of H4 (K5, K8, K12, K16) acetylation (Kouzarides, 2007; Millar and Grunstein, 2006). Overall, the SAGA and NuA4 complexes have been identified to be associated with gene activation and cell cycle regulation (Allard et al., 1999; Clarke et al., 1999; Kuo and Allis, 1998; Kuo et al., 1998; Marmorstein and Roth, 2001).

Histone deacetylases, HDACs, are highly conserved throughout evolution and are found in organisms that lack histones, further supporting the notion that acetylation is not a histone-specific modification (Frye, 2000; Hubbert et al., 2002). The budding yeast genome contains 10 HDACs, split into 3 phylogenetic classes that are responsible for removing the acetylation marks on lysine residues (catalysed by HATs). Class I HDACs include Rpd3, Hos1 and Hos2, Class II HDACs include Hda1 and Hos3 and class III HDACs, also known as the Sirtuins, include Hst1, Hst2, Hst3, Hst4 and Sir2 (Denu, 2003; Ekwall, 2005; Grozinger and Schreiber, 2002; Yang and Seto, 2008). The classes also differ in the catalysis/mechanism/reaction of deactylation. The class I and II HDACs utilise a H_2O molecule to hydolyse the acetyl group from target lysines to generate acetate. The Class III HDACs are nicotinamide adenine dinucleotide (NAD+)-dependent HDACs. For example, Sir2 catalyses deacetylation to the hydrolysis of the coenzyme NAD+, generating nicotinamide, the unique metabolite *O*-Acetyl-ADP-ribose and lysine (Denu, 2003). The high conservation of HDACs means the classes are based on their sequence and functional conservation from within different systems, for example class I HDACs in human cells are based on their ubiquitous expression, and class I HDACs in yeasts are classified as such due to their sequence and structural homology to class I HDACs in human cells. Additionally, the high functional conservation between species for HDACs makes the orthologous relationships between yeasts rather straightforward, whereas the human HDACs appear to have gained additional functions (Ekwall, 2005; Gu and

Roeder, 1997; Hubbert et al., 2002). The budding yeast HDACs and their homologs from fission and human cells are shown in Table 1.4. A schematic of the action of histone acetylation on chromatin structure is shown in Figure 1.4.

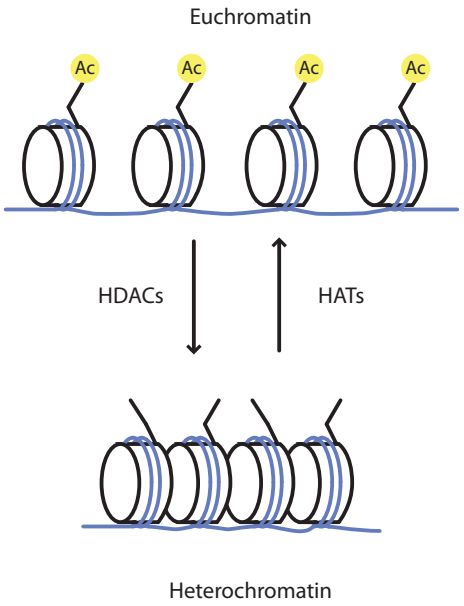

Figure 1.4 A schematic of the action of histone acetylation on chromatin structure

The acetylation of histone N-terminal tails decreases the electrostatic attraction between the positive nucleosome core and the negative phosphate backbone of DNA. This leads to exposure of DNA sequences for binding by DNA-sequence specific proteins. The deacetylation of histones by HDACs promotes facultative heterochromatin formation and preventing binding to specific sequences.

The function of budding yeast HDACs has been extensively studied using genome-wide genetic screens looking into the gene expression and acetylation profiles of strains lacking the HDACs (Bernstein et al., 2000; De Nadal et al., 2004; Kurdistani

et al., 2004; 2002; Robert et al., 2004; Robyr et al., 2002; Sabet et al., 2004; Wang et al., 2002). From these analyses correlations between individual HDACs and their effect on gene expression and individual modification of histones have been identified. The class I HDAC Rpd3 has been associated with gene repression and is involved in the deacetylation of H4 (K5, K8, K12) and H3 (K9, K14, K18, K23, K27) amongst others. Researchers have also identified genes with lower level expression in *rpd3*Δ cells, suggesting a role of Rpd3 and other class I HDACs in regulating gene activation (Sharma et al., 2007; Wang et al., 2002). In further juxtaposition to the Gcn5 HAT, Rpd3 has also been identified in numerous chromatin modifying complexes (Shevchenko et al., 2008). One such complex, the Rpd3S complex, plays a role in preventing spurious transcription by association with the transcription machinery and deacetylating histones in regions already transcribed (Carrozza et al., 2005).

The different classes of HDACs have also been shown to play a role in regulating different sets of genes. Hda1 associates with and represses genes regulating carbohydrate transport and carbon metabolism, Sir2 with amino acid biosynthesis genes and Rpd3 with cell cycle regulated genes (Bernstein et al., 2000). The identification of Rpd3 with cell cycle regulatory genes has also been identified in studies directly on Rpd3 (Huang et al., 2009; Takahata et al., 2009)

The class III HDACs have also been shown to play an extensive role in heterochromatin formation, further associating the action of HDACs with repression of gene expression. Sir2 is a specific deacetylase of H4 K16 residues through the formation of a silencing complex that spreads heterochromatin through the sequential deacetylation of histones (Hoppe et al., 2002; Imai et al., 2000).

In addition to roles in transcription, Rpd3, Hst3 and Hst4 HDACs have been identified to play a role in regulating the chromatin at origins of replication and in response to DNA damage signals (Aparicio et al., 2004; Maas et al., 2006; Mantiero et al., 2011). In *rpd3*Δ cells the association of DNA polymerase with late firing origins of replication leads to earlier firing, thus suggesting that Rpd3 plays a role in delaying S phase progression (Aparicio et al., 2004; Mantiero et al., 2011). In response to DNA damage signals, the Hst3 and Hst4 HDACs are downregulated

leading to persistence of H3K56 acetylation during the arrest, thought to lead to maintained expression of acetylated genes (Maas et al., 2006).

HDAC Class	*Saccharomyces cerevisiae*	*Schizosaccharomyces pombe*	*Homo sapiens*
I	Rpd3 Hos1 Hos2	Clr6 Hos2	HDAC1 HDAC2 HDAC3 HDAC8
II	Hda1 Hos3	Clr3	HDAC4 HDAC5 HDAC6 HDAC7 HDAC9 HDAC10
III	Hst1 Hst2 Hst3 Hst4 Sir2	Hst2 Hst4 Sir2	SIRT1 SIRT2 SIRT3 SIRT4 SIRT5 SIRT6 SIRT7

Table 1.4 The conserved HDACs enzymes in eukaryotes

Listed are the functional orthologues between budding and fission yeast and humans of the histone deacetylase enzymes. The HDACs share significant sequence homology between yeast and the higher eukaryotic protein kinases.

1.4.4 HDACs and Cancer

Deregulation of HDACs has been shown to be a key driving force in the progression of cancers (Ropero and Esteller, 2007). A decrease in the acetylation of H4K16 has been associated with the early stages of tumorigenesis (Fraga et al., 2005) and aberrant regulation of HDACs have been shown to cause repression of

E-cadherin (Peinado et al., 2004) a key feature in the development of cancer migration and invasion (Christofori and Semb, 1999; Hajra and Fearon, 2002). Due to the role of histone modifications in regulating gene expression and increased activity in many cancers (Ropero and Esteller, 2007), it has been suggested that histone modifying enzymes play a role in the progression of cancers (Nevins, 2001)Weinberg:1995tn, Sidorova:2003tq, (Pandolfi, 2001; Ropero and Esteller, 2007).

The G1/S repressor pRb in human cells represses E2F-dependent gene expression during G1, by the recruitment of HDAC1 that deacetylates nearby histones repressing G1/S genes (Brehm et al., 1998; Harbour and Dean, 2000; Stevens and La Thangue, 2003). Mutations of pRb or E2F1-3 that prevent inhibition by pRb, lead to uncontrolled commitment to cell division and progression of cancers. The removal of pRb by cyclin-CDK-dependent phosphorylation relieves the deacetylation by HDAC1, promoting the recruitment of HATs and the acetylation of histones, leading to expression of cell cycle entry genes (Blais and Dynlacht, 2007; Frolov, 2004; Nevins, 2001; Trouche et al., 1996). In normal cells that aberrantly express E2F transcripts, apoptosis is induced via stabilisation of p53 and apoptosis (Kowalik et al., 1998; Nevins, 2001; Wu and Levine, 1994), and in conjunction with deregulated HDAC activity in cancers, this led to the development of using HDAC inhibitors in cancer therapy.

HDACi's, have been used in clinical trials and are effective drugs at inducing transformed cell death (Xu et al., 2007). The method of action for these drugs is unclear due to the large number of targets of HDACs in human cells (Richon and O'Brien, 2002). However, gene expression analysis identifies that p21 expression, a CDK inhibitor, is up regulated in response to HDACi treatment, due to an increase in histone acetylation in the promoter region (Gui et al., 2004; Sambucetti et al., 1999). Understanding the roles that different classes and individual HDACs play in regulating gene expression, in particular cell cycle transcription, would aid in understanding the mechanism of HDACi and development of new treatments.

1.5 Aims of this thesis

Currently, studies in budding yeast have shown that the class I HDACs Rpd3 and Hos3 are recruited to SBF via Whi5 at promoters, analogous to how pRb recruits HDAC1 in human cells (Blais and Dynlacht, 2007; Huang et al., 2009; Takahata et al., 2009). The specific lysine residues on either histones or other proteins targeted by Rpd3 and Hos3, however, has not been established at the promoters of G1/S genes. How transcriptional repression is mediated by MBF on its target genes is also currently unclear.

Regulating the correct entry into the cell cycle is crucial for development and tissue homeostasis. G1/S transcription is de-regulated in many if not all cancers. Therefore, understanding the mechanisms of G1/S transcriptional regulation presents many potential targets of therapeutic intervention. My thesis aims to expand what is currently known about G1/S transcription in budding yeast. I set out to identify new regulatory mechanisms involved in the regulation of G1/S transcription and the role that histone modifications may play in gene expression.

Furthermore, the derepression of MBF-dependent transcription in response to the DNA replication checkpoint leads to an increase in the number of new transcripts. I set out to investigate the mechanism of Nrm1 inhibition in response to replication stress and identify the downstream functions of the large transcriptional induction.

The aim of my thesis is to identify new features of G1/S transcription in budding yeast, which due to the high functional conservation in eukaryotes, can be transferred and identified in both fission yeast and human cells.

Chapter 2. Materials & Methods

2.1 Yeast Methods

2.1.1 Budding yeast strains and media

Yeast strains used in this thesis are listed in Table 2.1. Strains used in this work were generated by standard genetic methods and derived from S288C (*MATa, ura3-52, trp1-63, his3-200*), MBS164-YPH499, congenic to S288C (*MATa, ura3-52, leu2Δ1, trp1-63, his3-200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, arg4Δ, sml1::TRP bar1::HIS3*)(Smolka et al., 2007) or 15DaubΔ (*MATa, ade1, leu2-3, 112 his2, trp1-1, ura3Δns, bar1Δ*)(de Bruin et al., 2006; Richardson et al., 1989).

All liquid cultures were grown in YPD, rich media (10 g/L yeast extract, 20 g/L Bacto-peptone, 20 g/L dextrose (CCM0210), 0.15 g/L adenine sulphate (DOC0230), uracil 0.05 g/L (DOC0214)) at 30°C unless otherwise stated. Strains were grown at 30°C on YPD agar plates containing 20 g/L agar (AGA03) or selective dropout plates (6.7 g/L yeast nitrogen base without amino acids (CYN0410), 0.15 g/L adenine sulphate (DOC0230), 20 g/L dextrose, 20 g/L agar (AGA03) containing appropriate drop-out amino acid powder -Ura (DCS0289), -Trp (DCS0141), -Ura-Trp (DCS0691), -Leu (DCS0091). G418 (Sigma, G1279-1G) was added (200 µg/ml) for *KanMX* (Wach et al., 1994) and *KAN^r* selection (Sherman, 2002). All reagents are from FORMEDIUM unless otherwise stated.

Strain	Genotype	Source
RBY1	*MATa, ade1, leu2-3, 112 his2, trp1-1, ura3Δns, bar1Δ*	(de Bruin et al., 2004)
RBY206	*RBY1 with SWI4-6xmyc::KAN^r*	(de Bruin et al., 2006)
RBY91	*RBY1 with SWI6-6xmyc::URA3*	(de Bruin et al., 2004)
RBY467	*RBY1 with swi6::TRP1, WHI5-TAP:: KAN^r, SWI4-6xMyc::URA3*	(de Bruin et al., 2006)
RBY124	*RBY1 with mbp1::LEU2*	(de Bruin et al., 2006)

RBY125	*RBY1 with swi4::KAN^r*	(de Bruin et al., 2006)
RBY312	*RBY1 with MBP1-13xmyc::TRP1*	(Bastos de Oliveira et al., 2012)
RBY205	*RBY1 with MBP1-13xmyc::URA3*	(de Bruin et al., 2006)
RBY207	*RBY1 with NRM1-13xmyc::URA3*	(de Bruin et al., 2006)
RBY46	*RBY1 with WHI5-13xmyc::KAN^r*	(de Bruin et al., 2006)
RBY110	*RBY1 with nrm1::TRP1*	(de Bruin et al., 2006)
RBY466	*RBY1 with NRM1-13xmyc::URA3, MBP1-TAP::KAN^r, swi6::TRP1*	(de Bruin et al., 2006)
RBY209	*RBY1 with MBP1-13xmyc::URA3, swi4::Kan^r*	(de Bruin et al., 2006)
RBY144	*RBY1 with ura3::YIplacGAL-sic1∆p::URA3*	(de Bruin et al., 2006)
MBS164	*MATa, ura3-52, leu2∆1, trp1-63, his3-200, lys2∆Bgl, hom3-10, ade2∆1, ade8, arg4∆, sml1::TRP bar1::HIS3*	(Smolka et al., 2007)
MBS430	*MBS164 with TOS4 (R122A;N161A)-3xHa::KanMX*	(Bastos de Oliveira et al., 2012)
MBS595	*MBS626 with tos4::URA3*	(Bastos de Oliveira et al., 2012)
MBS626	*MATa, ura3-52, trp1-63, his3-200, bar1::HIS3*	(Bastos de Oliveira et al., 2012)
MBS636	*MBS164 with TOS4-3xHa::KanMX*	(Bastos de Oliveira et al., 2012)
MBS659	*MBS626 with TOS4-3xHa::KanMX*	(Bastos de Oliveira et al., 2012)
MBS660	*MBS626 with TOS4 (R122A;N161A)-3xHa::KanMX*	(Bastos de Oliveira et al., 2012)
MBS759	*MBS626 with tos4::TOS4-3xHa::KanMX, RPD3-3xFlag::TRP1*	(Bastos de Oliveira et al., 2012)
MBS760	*MBS626 with tos4::TOS4 (R122A;N161A)-3xHa::KanMX, RPD3-3xFlag::TRP1*	(Bastos de Oliveira et al., 2012)
MBS815	*MBS626 with dun1::URA3*	(Bastos de Oliveira et al., 2012)
MBS824	*MBS626 with TOS4 (R122A;N161A)-3xHa::KanMX, dun1::URA3*	(Bastos de Oliveira et al., 2012)
MBS832	*MBS626 with tos4::URA3, dun1::KanMX*	(Bastos de Oliveira et al., 2012)
MBS870	*ura3-52, trp1-63, his3-200, rpd3::TRP1*	(Bastos de Oliveira et al., 2012)
MBS874	*MBS870 with dun1::KanMX, tos4::HIS3*	(Bastos de Oliveira et al., 2012)
MBS886	*ura3-52, trp1-63, his3-200, dun1::KanMX, rpd3::TRP1*	(Bastos de Oliveira et al., 2012)

MBS1134	*ura3-52, trp1-63, his3-200, hst1::TRP1*	(Bastos de Oliveira et al., 2012)
MBS1136	*MBS1134 with dun1::HIS*	(Bastos de Oliveira et al., 2012)
MBS1137	*ura3-52, trp1-63, his3-200, tos4::URA, hst1::TRP1*	(Bastos de Oliveira et al., 2012)
MBS1138	*MBS1137 with dun1::HIS*	(Bastos de Oliveira et al., 2012)
MBS1479	*MBS626 with tos4::TOS4-3xHa::KanMX, HST1-3xFlag::TRP1*	(Bastos de Oliveira et al., 2012)
MBS1480	*MBS626 with tos4::TOS4 (R122A;N161A)-3xHa::KanMX, Hst1-3xFlag::TRP1*	(Bastos de Oliveira et al., 2012)

Table 2.1 List of budding yeast strains used in this thesis

2.1.2 Fission yeast strains and media

Strains used in this study are *wild type* RBP7 (*h+ leu1-32 ura4-D18*) and *nrm1*Δ RBP8 (*h+ leu1-32 ura4-D18 nrm1Δ::kan*^{r}) (de Bruin et al., 2006). All strains were grown in YES media (5 g/L yeast extract, 30 g/L dextrose, 0.05 g/L adenine, histidine, leucine, lysine and uracil (PCM0310)) or YES plates containing 20 g/L agar (PCM0410) at 30°C. G418 (Sigma, G1279-1G) was added (2 µg/ml) for *KAN*^{r} selection. All reagents are from FORMEDIUM unless otherwise stated.

2.1.3 Spot Assays

Strains were incubated overnight at 30ºC to saturation and then diluted to the same OD_{600} (0.5 – 1.0). Four-fold serial dilutions were then made before being spotted on drug-free or 12 mM hydroxyurea (HU) (Sigma, H8627-100G) YPD agar plates using a purpose-built, replica-pin apparatus. Agar plates were incubated for four days at 30°C and pictures taken using an Epson Expression 1680 Pro scanner.

2.1.4 Synchronisation

Mating pheromone arrest synchrony experiments were carried out as described (Stuart and Wittenberg, 1995). Overnight stationary cultures were diluted to OD_{600}

= 0.3 and incubated for 2 hours at 30ºC. Log phase cultures (OD_{600} = ~0.5) were incubated with 50 µM alpha-factor (Genescript, RP01002-10mg) for a further 90 minutes before cultures were centrifuged (3000 rpm, 3 minutes), washed with room temperature YPD (3000 rpm, 3 minutes) and resuspended in 30ºC YPD. Time point 0' was taken at the end of the 90 minute incubation. For experiments where hydroxyurea (HU) treatment was used, 100 mM HU was added 20 minutes after releasing cells from arrest. A budding index was taken with all time-course experiments at each time point to establish the percentage of budded cells, as a measure of synchrony.

In experiments involving strains carrying *GAL-sic1∆p*, an overnight culture was grown in YEP (10 g/L yeast extract, 20 g/L peptone (FORMEDIUM, CCM0410) with 20 g/L raffinose (RAF02) and 2 g/L dextrose. The overnight culture was used to inoculate a 20 g/L raffinose YEP culture. This culture was grown for two hours before alpha-factor (50 µM) was added. Two hours after mating pheromone addition, 20 g/L galactose (GAL03) was added and two hours later cells were washed and released from the G1 arrest in 2% galactose medium.

2.2 Molecular Biology

2.2.1 Quantitative PCR

2.2.1.1 Reverse Transcriptase qPCR-RT-qPCR

Relative mRNA transcript levels (see Section 2.3.1 for RNA purification) were determined by RT-qPCR using the One-Step Mesa green qRT-PCR and RNase Inhibitor Mastermix Plus for SYBR Assay (Eurogentech, RT-SYRT-032XNR). RT-qPCR reactions were run on a Chromo-4 Real-Time PCR Detector or CFX Connect Thermal Cycler Real-Time System (Bio-Rad) and obtained data analysed using MJ Opticon Analysis Software 3.0 or CFX Manager software, respectively. Finally data was normalised against actin mRNA levels (*ACT1*) and investigated using the $\Delta\Delta C_t$ method (Schmittgen and Livak, 2008).

Purified RNA samples were normalised to 20 ng/µl and 5.9 µl used per 20 µl qPCR reaction. 2 µl of forward and reverse primers (5 pmol) were used, 10 µl of mastermix and 0.1 µl of RT enzyme mix. Each sample was loaded in triplicate per qPCR plate.

Primers for analysis of mRNA transcript levels were designed against the open reading frame using the PrimerQuest design tool from IDT DNA. A list of primers used in the thesis is shown in Table 7.1 in the appendix.

2.2.1.2 ChIP - qPCR

Analysis of ChIP DNA samples (see Section 2.2.2 for ChIP DNA purification) was performed using Mesa Green or Blue Mastermix Plus for SYBR Assay (Eurogentech). qPCR reactions were run on a Chromo-4 Real-Time PCR Detector or CFX Connect Thermal Cycler Real-Time System (Bio-Rad) and obtained data analysed using MJ Opticon Analysis Software 3.0 or CFX Manager software, respectively. Enrichment of ChIP samples was determined by comparing to the signal from 1% WCE samples using the $\Delta\Delta C_t$ method (Nolan et al., 2006; Schmittgen and Livak, 2008). These enrichment values were then normalised to either background levels at a non-target promoter (such as *ACT1*), and Histone H3 occupancy in data from Chapter 5, and then either to wild type enrichment levels, time point 0' or maximum ChIP enrichment (% of maximum ChIP) values, as indicated in figure legends.

5.9 µl of purified ChIP DNA samples (see 2.2.2 for ChIP DNA purification) was used in each 20 µl qPCR reaction as described in 2.2.1.1 without the addition of the reverse transcriptase enzyme mix. Each ChIP DNA sample and whole cell extract were analysed in triplicate.

Primers for analysis of ChIP DNA enrichment levels were designed against 500bp 5' to the open reading frame using the PrimerQuest design tool from IDT DNA. A list of primers used in the study is shown in Table 7.1 in the appendix.

2.2.2 Chromatin Immunoprecipitation - ChIP

ChIP experiments were carried out as in (Aligianni et al., 2009; Flick et al., 2003). In all ChIP experiments 43.75 ml of log phase culture was incubated with 1.25 ml formaldehyde (1%) for 20 minutes at room temperature. For time course experiments 43.75 ml of log phase culture was used per time point. The cross-linking reaction was quenched with 2.3 ml of 2.5 M glycine for five minutes at room temperature. Samples were centrifuged and washed 3 times in ice-cold TBS (3000 rpm, 3 minutes) and lysed in 500 µl of ChIP lysis buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% Na Deoxycholate, 1 mM EDTA and protease inhibitors (Roche, 04693124001)). 500 µl of 0.5 mm diameter glass beads (Biospec. 11079105) were added and pellets either processed in a fast-prep (4 times 30 seconds with 3 minutes rest at 4ºC) or vortexed for 20 minutes at 4ºC. The bottom of the tubes containing lysates where then pierced with a hot 25G needle (BD Microlance 3, 300600) and placed into a new tube and centrifuged briefly (6 seconds) to collect the lysate. The lysates were cleared (10 minutes, 14000 rpm, 4ºC) and the chromatin pellet resuspended in 500 µl fresh lysis buffer. Chromatin samples were sonicated to generate 500-1500 bp fragments of DNA. 5 µl of whole cell extract (WCE, 1%) was kept and remaining lysate incubated with specific antibody overnight at 4ºC. Antibodies used in this study are shown in Table 2.2. 50 µl of 50% Protein A-Sepharose beads (Sigma, P3391) in ChIP lysis buffer were added and samples incubated at 4ºC for 3-4 hours to immunoprecipitate complexes. Beads were washed (2000 rpm, 1 minute) in cold ChIP lysis buffer twice, ChIP lysis buffer (500 mM NaCl) twice, ChIP wash buffer (10 mM Tris-HCl pH 8, 250 mM LiCl, 0.75% NP-40, 0.75% Na Deoxycholate, 1mM EDTA) twice and Tris EDTA pH 8.0 once with a 15 minute 4ºC incubation between washes. 100 µl ChIP elution buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS) was added to the beads before being incubated at 65ºC for 30 minutes. Samples were cleared (13000 rpm, 30 seconds) and 90 µl of supernatant removed to a new 1.5 ml tube. 95 µl of ChIP elution buffer was added to WCE samples and all samples incubated overnight at 65ºC to reverse cross-linking. The following day DNA was purified using the Qiagen PCR purification kit according to manufacturers protocol and analysed by qPCR (see 2.2.1.1).

2.2.3 Fast ChIP

A faster protocol was optimised to facilitate faster processing of ChIP samples and was based on the referenced protocols (Kuo and Allis, 1999; Nelson et al., 2006). The protocol for fixing, lysing and immunoprecipitating chromatin remains the same as the ChIP protocol in 2.2.2.

Following incubation with Protein A-Sepharose beads, samples were washed (3000 rpm, 30 seconds) eight times with 1 ml of cold Fast ChIP IP buffer (50 mM Tris-Cl ph 7.5, 1% Triton X-100, 150 mM NaCl, 5mM EDTA, 0.5% NP-40). 100 µl of 10% Chelex (Bio-Rad) in ultra pure milliQ H_2O (Millipore) added to beads and whole cell extract (WCE) samples, vortexed and boiled for 10 minutes. 70 µl were removed and 120 µl of ultra pure milliQ was added to wash beads. 120 µl of supernatant removed and collated with the previous 70 µl. Samples were analysed by qPCR (see 2.2.1).

This protocol takes approximately 24 hours compared to 3 days for the ChIP protocol in 2.2.2, largely due to the utilisation of chelex in facilitating the elution of the reverse cross-linked immunoprecipitated DNA. The cost per IP was also reduced due to omitting the use of PCR purification columns.

2.2.4 Co-immunoprecipitation

25 ml of log phase growing yeast cultures at the same OD_{600} (0.5 - 1) were pelleted (3000 rpm, 3 minutes), washed with 45 ml water (3000 rpm, 3 minutes) and moved to 1.5 ml tubes in 1 ml of water and centrifuged (13000 rpm, 30 seconds). Pellets were then resuspended in 500 µl co-IP lysis buffer (50 mM Tris-HCl pH 7.5, 1% Triton X-100, 250 mM NaCl) containing protease inhibitors (Roche, 04693124001) and phosphatase inhibitors (Sigma, P2850). 100 µl of 0.5 mm diameter glass beads (Biospec. 11079105) were added to pellets and vortexed for 20 minutes at 4ºC. Lysates were cleared (14000 rpm, 15 minutes, 4ºC) and 40 µl WCE aliquoted and prepared for western analysis with 40 µl of 2% SDS/DTT loading buffer (50 mM Tris pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100 mM DTT). 400 µl of remaining lysate was immunoprecipitated with specific antibody (Table

2.2) for two hours at 4ºC. 50 µl of 50% cold Protein A-Sepharose beads in co-IP lysis buffer were added and IPs incubated for a further hour. Beads were washed (2000 rpm, 1 minute) three times in 1 ml co-IP lysis buffer with 15 minute incubations at 4ºC between washes. IP samples were prepared for SDS-PAGE analysis by adding 50 µl 2% SDS/DTT sample buffer to beads before being boiled and vortexed for one minute, three times, before being cleared (12000 rpm, 5 minutes) and resolved by SDS-PAGE (Section 2.3.3).

Company (cat. no.)	Raised in	Antibody/Raised against	ChIP (µl/IP)	WB	Co-IP (µl/IP)
N/A	Rabbit	NL-11 (swi4)	3	1/2000	3
N/A	Rabbit	NL-20 (Mbp1)	3	1/1000	3
N/A	Rabbit	NL-02 (Swi6)	3	1/2000	3
Santa Cruz Biotechnology (Sc-40)	Mouse	Anti-myc	3	1/1250	3
Sigma (P7962)	Mouse	Anti-PSTAIRE		1/1000	
Abcam (ab1791)	Rabbit	H3	1.5		
Millipore (07-352)	Rabbit	H3K9Ac	1.5		
Millipore (07-353)	Rabbit	H3K14Ac	1.5		
Millipore (07-360)	Rabbit	H3K27Ac	1.5		
Millipore (07-327)	Rabbit	H4K5Ac	1.5		
Thermo Scientific (31460)	Goat	Anti-Rabbit IgG		1/1250	
Thermo Scientific (31430)	Goat	Anti-Mouse IgG		1/1250	

Table 2.2 List of antibodies used in this study and the concentration used in different protocols

2.3 Biochemistry

2.3.1 RNA extraction

For RNA extraction 25 ml of log phase growing yeast cultures were used and RNA purified using the Qiagen RNEasy Plus mini kit (Qiagen). 600 µl of RLT buffer with 1% 2-mercaptoethanol was added to frozen pellets. 500 µl of 0.5 mm diameter glass beads (Biospec. 11079105) were added and pellets either processed in a fast-prep (4 times 30 seconds with 3 minutes rest at 4ºC) or vortexed for 20 minutes at 4ºC. Bottom of tubes containing lysates where then pierced with a hot

25G needle (BD Microlance 3, 300600) and placed into a new tube and centrifuged briefly (6 seconds) to collect lysate. Lysates were cleared (14000 rpm, 2 minutes). Protocol was followed according to manufacturers guidelines. RNA was eluted in two aliquots of 30 µl RNAse-free water and RNA quantity determined by using a LabTech ND-1000 NanoDrop spectrophotometer normalised to blank RNAse-free water and associated software. All samples were then diluted to 20 ng/µl with RNAse-free water before being analysed by RT-qPCR (see 2.2.1.1).

2.3.2 Protein extraction

For whole cell lysates 25ml of log-phase growing cells were used at the same OD_{600} (0.5 - 1) in all samples. Pellets were washed with 45 ml water (3000 rpm, 3 minutes) and moved to 1.5 ml tubes in 1 ml of water and centrifuged (13000 rpm, 30 seconds). Pellets were then resuspended in 100 µl 2% SDS/DTT sample buffer and 100 µl of 0.5 mm diameter glass beads (Biospec. 11079105) were added. Pellets were then vortexed and boiled for one minute three times. Samples were cleared (14000 rpm, 5 minutes) before being resolved by SDS-PAGE (see 2.3.3).

Alternatively, a post-alkaline extraction procedure was used where the washed cell pellets were resuspended in 100 µl water and 100 µl of 200 mM NaOH was added. Samples were incubated for 5 minutes at room temperature and pelleted (13000 rpm, 30 seconds). Pellets were resuspended in 50 µl of 2% SDS/DTT sample buffer and boiled for 3 minutes. Samples were cleared (14000 rpm, 5 minutes) before loading onto polyacrylamide gels.

2.3.3 Sodium dodecyl sulphate – polyacrylamide gel electrophoresis - SDS-PAGE

Protein lysates and co-IP samples were resolved using 10% Bio-Rad Mini PROTEAN TGX Precast gels with Tris-Glycine buffer (25 mM trizma base, 192 mM glycine, 0.1% SDS) according to manufacturers instructions and transferred onto nitrocellulose membranes (Roche) by semi-dry transfer using Bio-Rad Transblot SD Semi-dry transfer cell with semi-dry transfer buffer (48 mM trizma base, 39 mM

glycine, 0.037% SDS, 20% ethanol). Membranes were blocked for 20 minutes in 10% milk in PBS-T (0.2% Tween) at 65ºC. Membranes were incubated with primary antibody (Table 2.2) in 2% milk in PBS-T for one hour at room temperature, washed 3 times with PBS-T for 10 minutes and then incubated with secondary antibody (Table 2.2) for 30 minutes at room temperature or 4ºC overnight. Membranes were washed five times with PBS-T before being developed using the Luminata Crescendo, Western HRP substrates system (Millipore, 00112798).

Mbp1, Swi4 and Swi6 were detected using the described antisera (Chapter 3) and myc-tagged proteins detected using anti-myc antibody (9E10, Santa Cruz Biotechnology) and HA-tagged proteins using anti-HA (12CA5, Roche).

To minimise interference from immunoprecipitated rabbit immunoglobulin heavy and light chains, co-IPs using anti-rabbit antibodies were probed using the TrueBlot anti-rabbit IgG IP beads and secondary antibody according to manufacturers instructions (eBioscience, 88-1688-31). TrueBlotH enables detection of protein bands that would otherwise be obscured by the presence of reduced and denatured heavy and light chain immunoglobulins.

2.4 Statistical analysis

The data from ChIP experiments containing error bars are representative of the standard error and mean from biological triplicates. Where no error bars are present, data representative of multiple independent experiments is shown. However, in Figure 3.5 error bars are derived from experimental triplicates.

Data for gene expression analysis where no error bars are shown during time course experiments is representative of multiple independent experiments. Where error bars are shown these are representative of the standard error of biological or experimental triplicate experiments and this is indicated in the figure legends.

To test for enrichment for classes of genes within groups of genes (Fig. 4.13), the χ^2 test for differences was used with an expected outcome of 30% dosage-sensitive genes.

Error bars shown in data using fission yeast represent standard error calculated from biological replicates.

Chapter 3. Binding specificity of the G1/S transcriptional regulators in budding yeast

3.1 Introduction

Entry into the cell cycle in all cells comes as a consequence of a series of conditions being satisfied and the signalling of approval being conveyed to G1/S transcription factor complexes. The incorrect signalling or regulation of G1/S transcription is thought to be a driving force in many, if not all, cancers. Current studies into G1/S transcriptional regulation in both yeasts and human cells has identified many mechanisms involved in regulating cell cycle entry (Section 1.2) (Amon et al., 1993; Ashe et al., 2008; Breeden, 2003; Costanzo et al., 2003; 2004; Cross et al., 1994; de Bruin et al., 2008a; 2004; 2006; Li et al., 2008; Siegmund and Nasmyth, 1996; Wijnen et al., 2002). Approaches for treating cancers by targeting G1/S transcriptional regulation are led by the hypothesis that multiple growth factor receptors and signal transduction pathways eventually feed into G1/S transcription factor complexes and initiate cell cycle entry. Specifically, targeting the final transducers of cellular growth pathways, such as the E2F1-3 transcription factors in human cells, is thought to bypass redundancies that occur in many of the upstream signalling proteins. However, studies carried out in mice in which the three G1/S activating transcription factors, E2F1, E2F2 and E2F3 were mutated, suggests there is redundancy between the transcription factors and their isoforms. Thus, attempting to target them specifically for cancer treatments may not be beneficial (Cloud et al., 2002). Due to the high conservation and ease of studying G1/S transcription in yeasts, I set out to identify features of G1/S transcriptional regulation to add further mechanistic insight into the eukaryotic system. In order to achieve this, I sought to develop new tools for further research into features of G1/S transcription in budding yeast.

Here I describe the development and characterisation of new polyclonal antisera against all three components of the two transcription factors SBF and MBF: Swi4, Swi6 and Mbp1. In 1999, David Lee, a PhD student in the laboratory of Professor

Noel Lowndes, immunised rabbits using purified peptides from Swi4, Swi6 and Mbp1 and analysed the resulting serum for their specificity against the individual proteins. Their aim was to identify novel components of SBF and MBF transcription factor complexes. Specific antibodies would provide a reagent to allow the study of SBF and MBF complexes in single cultures without the need to generate multiple strains. However, for over 10 years the antisera were left uncharacterised and their potential for characterising Swi4, Swi6 and Mbp1 function left unexplored.

Due to the ease of manipulating the budding yeast genome through homologous recombination (Longtine et al., 1998), C- or N-terminal epitope tagging of endogenous proteins has been used to study many features of yeast proteins, for example the addition of the influenza virus hemagglutinin (HA) epitope (Field et al., 1988; Tyers et al., 1993) or the Myc epitope (Evan et al., 1985; Munro and Pelham, 1987). Consequently, commercially available antibodies can be utilised to study protein localisation, via immunoflouresence, and the biochemical detection and isolation of proteins and protein complexes can be carried out without the need to generate specific antibodies against a gene of interest. However, the generation of specific antibodies, although more costly, is seen to be more beneficial due to the possibility that tagging a protein may alter the folding and structure and therefore function of the wild type protein.

The generation of specific antibodies is widely used in studies using human cells due to the difficulty of manipulating the genome. Although new techniques are emerging such as CRISPR (Gasiunas et al., 2012; Ran et al., 2013), that enable genome editing to be carried out in human cells in a manner analogous to yeast cells. The generation of specific antibodies against proteins can yield either polyclonal or monoclonal antibodies, each with their own advantages and disadvantages. Polyclonal antibodies are quicker to generate and consist of multiple antibodies with differing specificity against epitopes within the antigen. Due to the multiple antibodies generated polyclonal antibodies can often produce non-specific background signal in many applications. However, polyclonal antibodies are more tolerant of minor changes in protein structure and therefore are able to identify denatured proteins due to the detection of multiple epitopes. Monoclonal antibodies take longer to generate but are specific to one epitope within the antigen

and therefore detect proteins with high specificity and little background signal in many applications. Depending on the application, the high specificity of monoclonal antibodies can be a hindrance when studying proteins with sequence homology across species, as they are less forgiving to subtle changes in target epitopes.

3.2 Results

3.2.1 Specific antibodies to Swi4, Swi6 and Mbp1

In order to study protein-protein and protein-DNA interactions of the G1/S cell cycle transcription factors of budding yeast, David Lee and his colleagues generated antibodies against Swi6, Swi4 and Mbp1. Polyclonal antisera were raised in rabbits against the C-terminal regions of Mbp1 and Swi4 and against the whole Swi6 protein. C-terminal fragments of Swi4 (residues 683-1092) and Mbp1 (residues 632-833) were cloned in frame with the His-tag of the vector pET21C and then purified from transformed *Escherichia coli* lysates using nickel-affinity chromatography (Fig. 3.1A). The addition of the His-tag enables purification of the C-terminal fragments as the histidine residues within the tag have a high affinity for nickel metal ions (Hochuli et al., 1988). The resulting purified peptides were used to immunise rabbits and the resulting polyclonal sera were tested on yeast whole cell extracts and labelled NL11, NL20, NL02 (Fig. 3.1A).

To test the quality and specificity of the antisera, I carried out western blot analysis using asynchronous whole cell extracts from a wild type strain, single deletion strains for all three components and three strains containing myc-tagged variants of Swi4, Swi6 and Mbp1. My data shows that the antisera, NL02, NL11 and NL20 recognise Swi6, Swi4 and Mbp1, respectively (Fig. 3.1B). Swi6 has an apparent molecular weight of 100 kDa, Swi4 of 150 kDa and Mbp1 of 120 kDa compared to their predicted weights of 90.5 kDa, 123.8 kDa and 94 kDa, respectively (Fig. 3.1B). The antisera not only recognise the wild type proteins but also the C-terminal myc-tagged versions. No specific bands are identified in the deletion strains using the anti-Mbp1 antiserum, whereas the anti-Swi6 and anti-Swi4 antisera identify some

non-specific bands in all strains. These data show that the generated antisera each identify specific components of the SBF and MBF transcription factors.

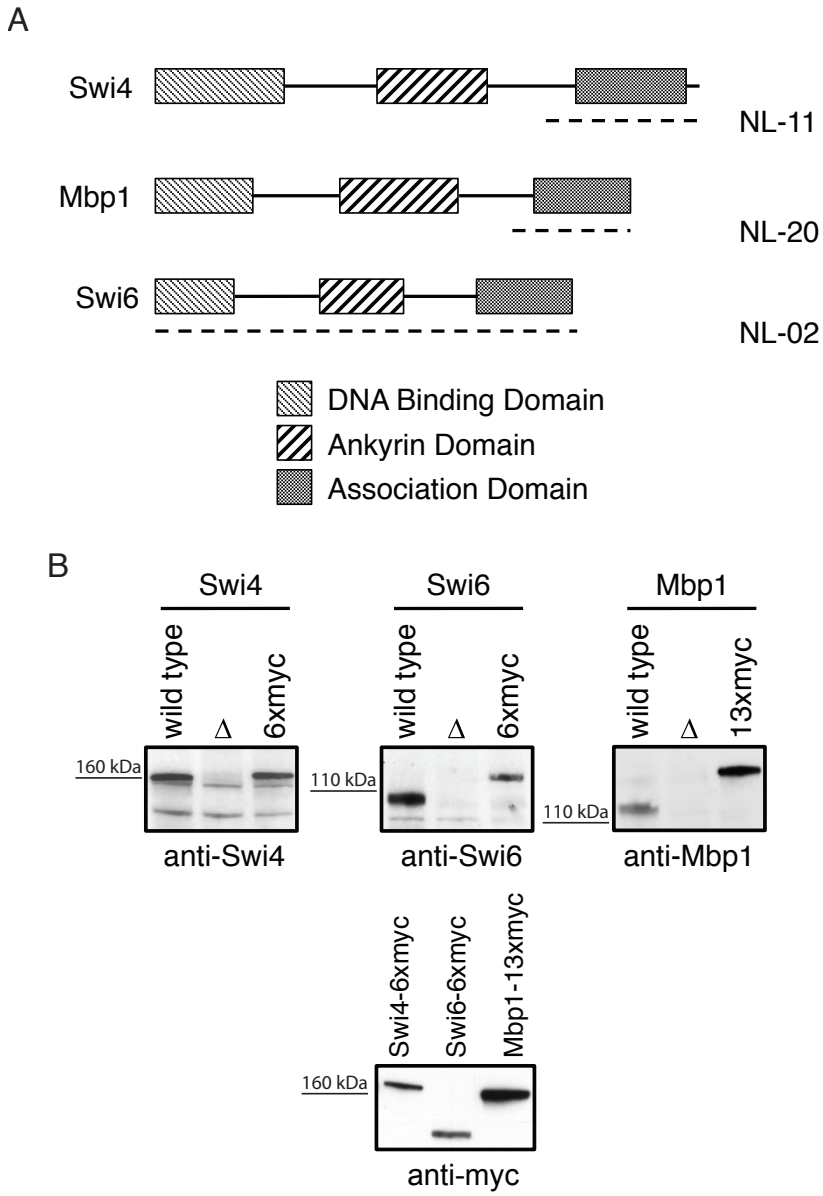

Figure 3.1 Polyclonal antisera generated against budding yeast G1/S transcription factor components

A. Functional domains within the three G1/S transcription factor components are represented by the boxed regions. Peptides (dashed lines) from the C-terminal regions of Swi4 (NL11) and Mbp1 (NL20) and full length Swi6 (NL02) were used to immunise rabbits and the resultant polyclonal antisera tested. **B.** Whole cell lysates of asynchronous wild type (wild type), Swi4, Swi6 or Mbp1 deleted (Δ) and Swi4, Swi6, or Mbp1 myc-tagged (6xmyc or 13xmyc) cultures were resolved. Antisera to detect Swi4, Swi6, Mbp1 or myc tagged versions of these components were used as indicated.

3.2.2 MBF not SBF interacts with Nrm1

MBF (Mbp1 and Swi6) has been shown to specifically interact with the co-repressor Nrm1 during S phase to repress MBF dependent transcription (de Bruin et al., 2006). SBF (Swi4 and Swi6) specifically interacts with the transcriptional inhibitor Whi5 to repress transcription during G1 to prevent early entry to the cell cycle (de Bruin et al., 2004) (Section 1.2.3). To clarify these previously characterised protein-protein interactions of MBF with Nrm1 and SBF with Whi5 in single cultures, I performed co-immunoprecipitations with each of the antisera in single asynchronous cultures using either myc-tagged Nrm1 or Whi5 strains.

My data shows that myc-tagged Nrm1 co-immunoprecipitates with Swi6 and Mbp1 specific antisera but not Swi4 antiserum (Fig. 3.2A, top panel). The increased presence of Nrm1 with Swi6 as opposed to Mbp1 is indicative that Nrm1 binds and is recruited to MBF by interacting with Swi6 as shown in other studies (de Bruin et al., 2006; Travesa et al., 2013). The data also shows that Swi6 immunoprecipitates Nrm1, Mbp1 and Swi4, both SBF and MBF complexes and Nrm1 (Fig. 3.2A, lane 2). The Mbp1 antiserum only pulls-down Swi6 and Nrm1 and not Swi4 whilst the Swi4 antiserum only pulls-down Swi6 (Fig. 3.2A). Overall, this data supports the previously identified specificity of the interactions of key components of SBF and MBF, specifically with Nrm1, using specific antibodies in a single culture.

3.2.3 Whi5 pulls down SBF, not MBF, components

The two initial papers that identified and characterised the role of Whi5 in START transcription both identified the binding of Whi5 to SBF, however, they disagreed on what role Whi5 may play at the promoters of MBF target genes (Costanzo et al., 2004; de Bruin et al., 2004). To clarify the binding specificity of the transcriptional inhibitor Whi5 to G1/S promoters, I performed a co-immunoprecipitation in an asynchronous Whi5 myc-tagged strain as in the previous experiment. As Whi5 binds SBF during G1, to identify additional interacting components, the SBF-Whi5 complex was enriched by immunoprecipitating Whi5-myc, before probing for interacting partners using the specific antisera. My data shows that only Swi6 and

Swi4 immunoprecipitate with Whi5, and not Mbp1 (Fig. 3.2B). This data supports a role for Whi5 in SBF-dependent transcription inhibition and not MBF regulated transcription.

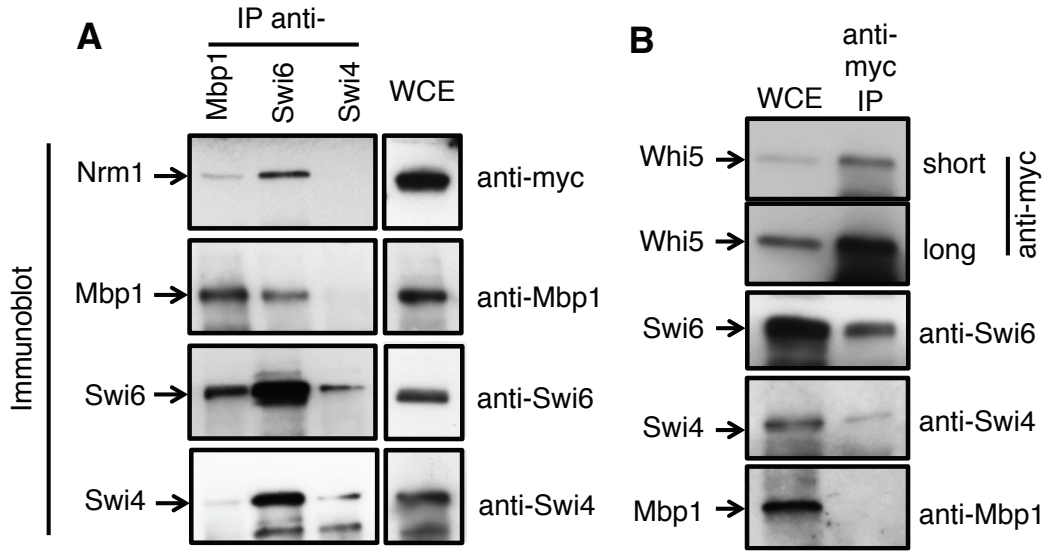

Figure 3.2 Nrm1 is a component of MBF and Whi5 of SBF

A. Lysate of an asynchronous culture of Nrm1-myc cells was immunoprecipitated using polyclonal antisera against Swi4, Swi6 and Mbp1 (IP anti-Mbp1, Swi6 or Swi4) and analysed for Nrm1, Mbp1, Swi6, or Swi4 by immunoblotting with anti-myc, anti-Mbp1, anti-Swi6 and anti-Swi4, respectively, as indicated. Whole cell extract (WCE) was immunoblotted with the same antibodies and provided as a control. **B.** Lysate of an asynchronous culture of Whi5-myc cells was immunoprecipitated using anti-myc antibody (anti-myc IP) and analysed for Whi5, Swi6, Swi4, and Mbp1 by immunoblotting with anti-myc, anti-Swi6, anti-Swi4, and anti-Mbp1, respectively, and as indicated. Long and short exposure of the anti-myc blot is provided. Whole cell extract (WCE) was immunoblotted with the same antibodies and provided as a control.

3.2.4 MBF preferentially binds the *RNR1* promoter and SBF the *CLN2* promoter.

In order to assess if the specific antisera can be used to study the protein-DNA interactions of the components of SBF and MBF, I performed a ChIP analysis in asynchronous wild type and myc-tagged strains. Thus, I was able to test whether the antisera could pull-down promoter regions of previously characterised SBF and MBF target genes (Iyer et al., 2001), as compared to the myc-tagged control strains. My data shows that the Swi4 antiserum preferentially immunoprecipitates the SBF target promoter *CLN2* over the MBF-specific promoter *RNR1* (Fig. 3.3A). Conversely, the Mbp1 antiserum ChIPs are specifically enriched for the *RNR1* promoter and significantly less for the *CLN2* promoter (Fig. 3.3A). As anticipated, anti-Swi6 serum is able to pull-down promoters of both SBF and MBF target genes *CLN2* and *RNR1*, respectively. The results using the specific antisera are comparable to that of ChIP analysis performed using the commercially available anti-myc antibody against Swi4, Swi6 and Mbp1 myc-tagged strains. My data therefore indicate that the antisera generated are suitable for ChIP analysis.

The ChIP data shows that both the specific antisera and anti-myc pull-downs show binding of Mbp1 to the SBF-regulated *CLN2* promoter and Swi4 to the MBF-regulated *RNR1* promoter, above *ACT1* background levels (Fig. 3.3A). In order to establish if this is representative of true Swi4 or Mbp1 binding levels or non-specific enrichment, I compared ChIP analysis results between wild type and *swi4*Δ, *swi6*Δ and *mbp1*Δ strains using the specific antisera. My data confirm that there is low-level binding of Swi4 to the *RNR1* promoter and of Mbp1 to the *CLN2* promoter (Fig. 3.3B). The data also show enrichment of Swi6 at the *RNR1* promoter over the *ACT1* promoter in *swi6*Δ cells, which could be an effect of non-specific recognition by the polyclonal antiserum, although the data shows significant enrichment in cells with wild type Swi6.

It had been reported that *CLN2* expression is dependent on Swi4 and *RNR1* expression on Mbp1 (Elledge and Davis, 1990; Koch et al., 1993; Verma et al.,

1992). In order to clarify this and investigate the role of low-level cross-binding identified in my previous experiment, I analysed the mRNA transcript of *CLN2* and *RNR1* by qPCR in synchronised wild type, *swi4*Δ and *mbp1*Δ strains. The data show that induction of *CLN2* is dependent upon Swi4 and that the low-level binding of Mbp1 is not sufficient to promote expression late in G1 (Fig. 3.4). The data also show that repression of *RNR1* outside of G1/S is dependent upon Mbp1 during S phase, however, the repression during early G1 could be due to the low-level binding of Swi4 and recruitment of the SBF inhibitor Whi5, in the absence of Mbp1, although this remains to be studied.

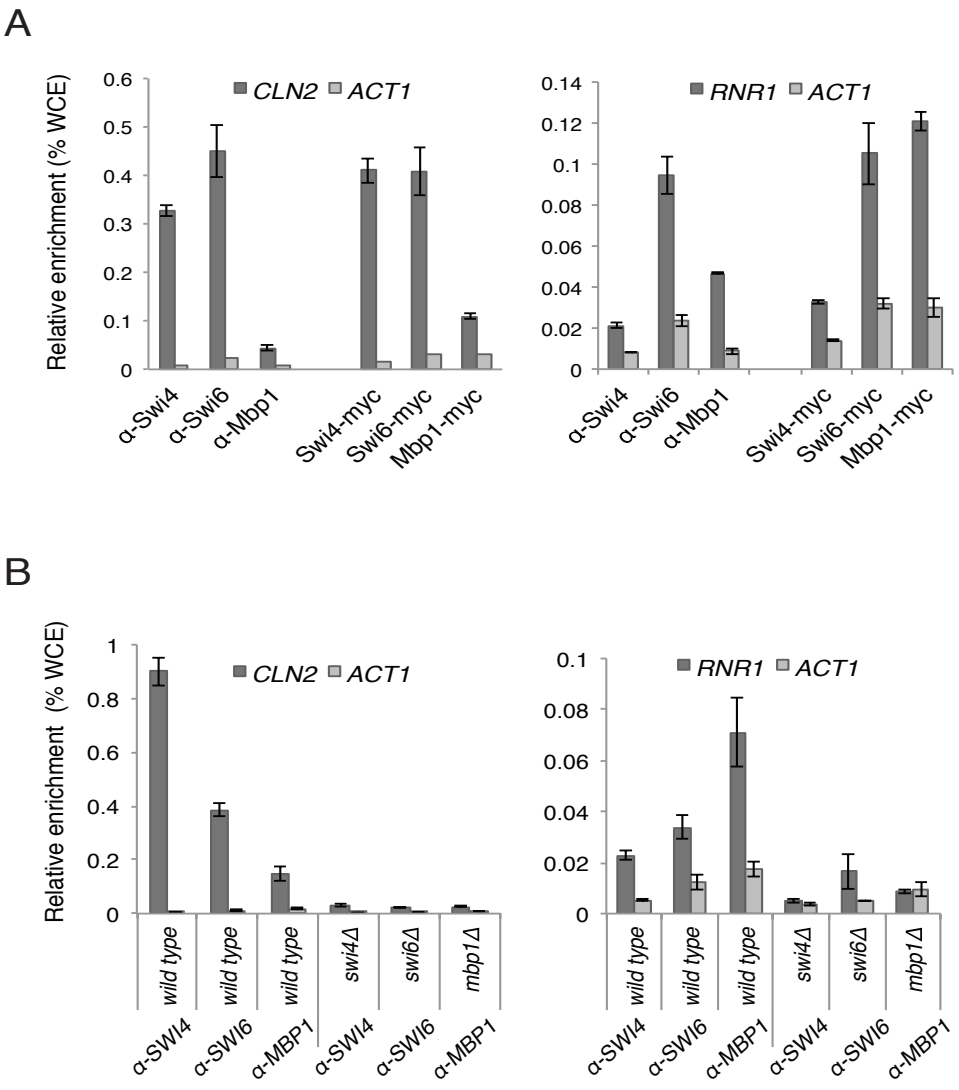

Figure 3.3 Mbp1 and Swi4 bind the *RNR1* and *CLN2* promoters, respectively

A. and B. ChIP analysis for Swi6, Swi4 or Mbp1 at the promoters of *CLN2* (SBF target) and *RNR1* (MBF target). Analysis was performed in asynchronous cells and enrichment levels were assessed by qPCR and normalised to WCE signals (percentage of WCE). *ACT1* signal corresponds to non-specific background binding. Error bars represent standard error calculated from biological triplicates. **A** ChIP analysis was carried out in wild type and myc-tagged Swi4, Swi6 and Mbp1 cell lysates using anti-Swi4, anti-Swi6, anti-Mbp1 or anti-myc antibodies, as indicated. **B.** ChIP analysis was carried out on wild type and *swi4*Δ, *swi6*Δ and *mbp1*Δ cell lysates using anti-Swi4, anti-Swi6, or anti-Mbp1 antibodies as indicated.

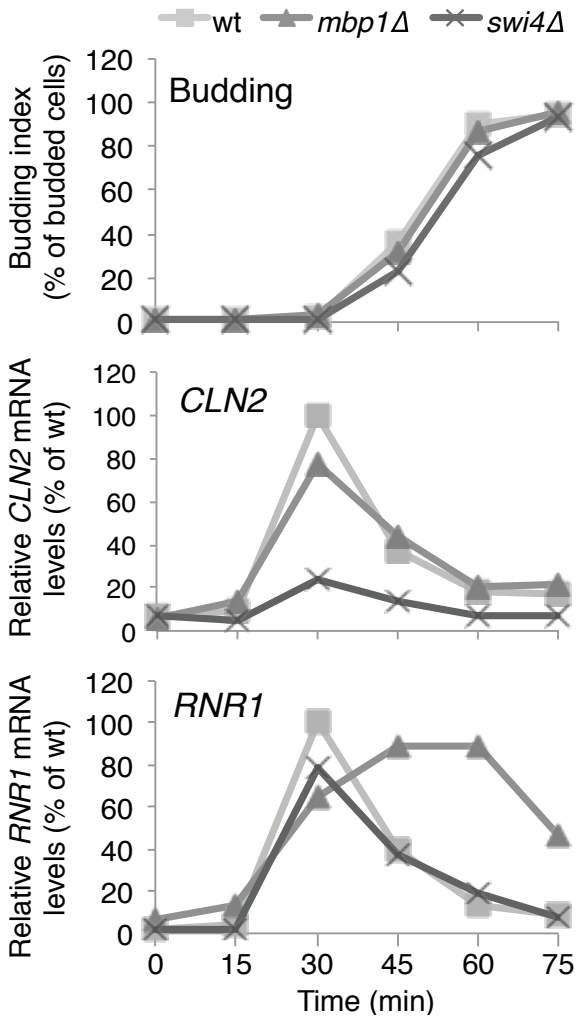

Figure 3.4 Mbp1 binding to the *CLN2* promoter does not regulate transcription

Cultures of indicated strains were synchronised by alpha factor arrest and release. Budding index (% budded cells, upper panel) is provided as an indicator of cell cycle progression. Relative mRNA levels of *CLN2* (SBF target, middle panel) and *RNR1* (MBF target, lower panel) were analysed by qPCR during the cell cycle in wild type (dark grey), *mbp1*Δ (medium grey) and *swi4*Δ (light grey) cells. Expression levels are plotted as percentage of maximum value detected in wild type experiment (100%). Data is representative of multiple experiments.

3.2.5 Swi4 binding to SBF target promoters is enhanced during G1

My work has established that the Mbp1, Swi4 and Swi6 specific antisera are suitable for ChIP analysis. Studies have shown the dissociation of the transcriptional inhibitor Whi5 from SBF promoters prior to activation of G1/S transcription (Costanzo et al., 2004; de Bruin et al., 2004). Therefore, using the specific antisera I sought to re-examine the dynamics of SBF during the cell cycle and its interaction with Whi5 to identify new features of SBF-dependent transcription.

Expression of *SWI4* has been shown to be activated by the M/G1 cell cycle transcription factor Mcm1, and also MBF, and peaks early in G1. This regulation is thought to be important for timely activation of G1/S transcription (MacKay et al., 2001; McInerny et al., 1997), although the effect of expression of *SWI4* on Swi4 binding to SBF promoters has never been assessed by ChIP.

To assess this I monitored binding of Whi5 and Swi4 via ChIP, and expression of *SWI4* and the SBF target *CLN2* in Whi5 myc-tagged synchronised cells. My data show that both Whi5 and Swi4 are bound to the *CLN2* promoter early in G1 (Fig. 3.5A, upper panel). Whi5 dissociates from the promoter at 30 minutes coinciding with transcriptional activation. Swi4 remains bound to the promoter until transcriptional inactivation and bud emergence at 40 minutes, due to the Clb-CDK activity removing SBF from promoters (Koch et al., 1996). My data indicate enhanced binding of Swi4 to the *CLN2* promoter after dissociation of Whi5 at 30 minutes, suggesting a stronger interaction between Swi4 and the *CLN2* promoter, or enhanced antigen recognition, in the absence of Whi5. Alternatively, as the enhanced recruitment precedes activation of G1/S, the enhanced binding might be a direct consequence of the transcriptional activation of *SWI4* (Fig. 3.5A, upper panel) and Swi4 protein accumulation (Fig. 3.5B).

Expression of *SWI4* remains periodic when the Mcm1 binding site, the ECB (Early Cycle Box), is mutated (MacKay et al., 2001; McInerny et al., 1997). The mutation results in a delay in peak transcript levels of *SWI4* such that it coincides with G1/S

transcription, thought to be dependent on MBF, due the MCB site in the *SWI4* promoter. To test if MBF can drive expression of *SWI4* I observed expression levels in synchronised wild type and *mbp1*Δ strains. My data show that *SWI4* expression depends on Mbp1 in the first cell cycle after release from alpha factor arrest, whereas, peak expression levels are unaffected in the subsequent cell cycle, driven by Mcm1 and peaking during M/G1, just before budded cells return into G1 (Fig. 3.5C). Overall, my data show that following alpha factor arrest and release, *SWI4* peak expression depends on Mbp1 during G1/S, suggesting MBF plays a role in accumulation of Swi4.

Taken together, these data suggest there is a positive feedback loop controlling activation of G1/S transcription. This results in extended accumulation of Swi4 and further recruitment of active SBF to target promoters, thus driving further G1/S transcription and commitment to the cell cycle.

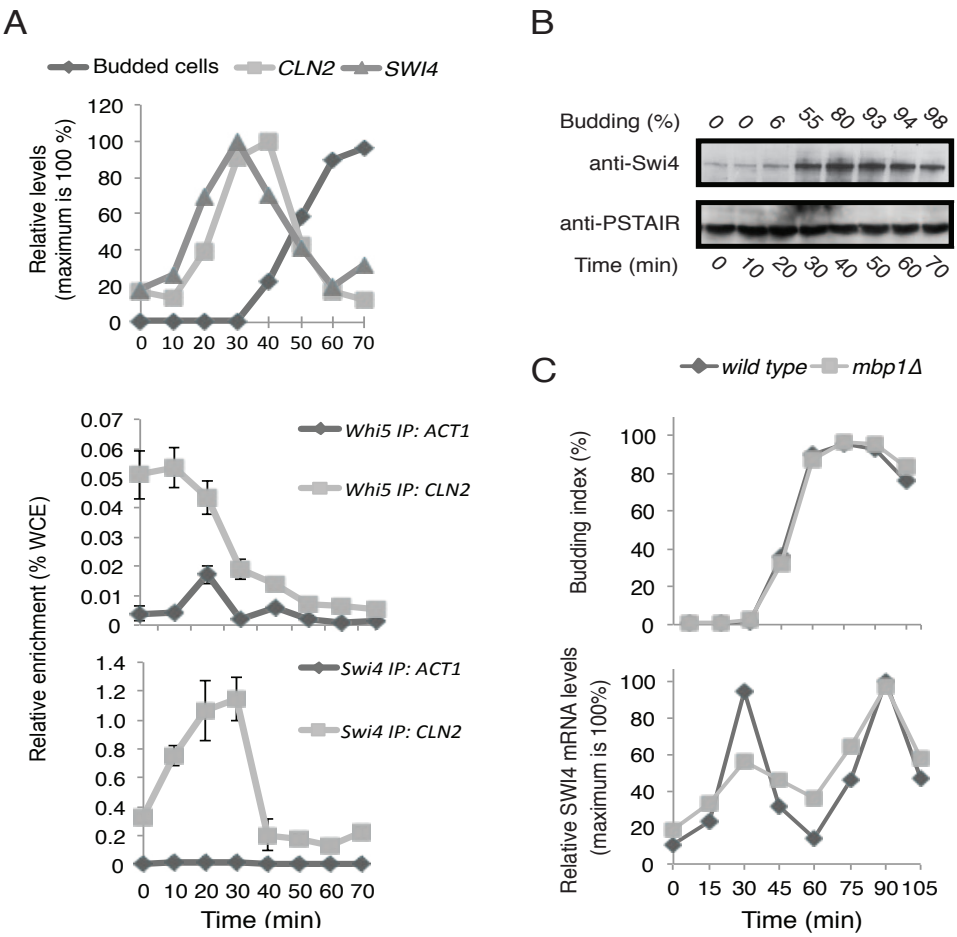

Figure 3.5 Dynamics of SBF target gene regulation during the cell cycle

A. Cells were synchronised by alpha factor arrest and release. Relative mRNA levels of *CLN2* (SBF target) and *SWI4* (G1/S target) gene expression in synchronised Whi5-myc cells were analysed by qPCR. Expression levels are plotted as percentage of highest value detected (100%). Budding index (% budded cells, dark grey line, upper panel) is provided as an indicator of cell cycle progression. ChIP analysis for Whi5-myc (light grey line, middle panel) and Swi4 (light grey line, lower panel) binding to the *CLN2* promoter during the cell cycle was achieved via anti-myc and anti-Swi4 pull downs. Enrichment levels of pulled down DNA was assessed by qPCR and signals were normalised to WCE signals (percentage of WCE). *ACT1* signal (dark grey line: Whi5 IP, middle panel; and Swi4 IP, lower panel) represents non- specific background binding. Error bars represent standard error calculated from experimental triplicates and representative data for multiple independent experiments is shown. **B.** Whole cell lysates of synchronised Whi5-myc cells were resolved by SDS-PAGE. Anti-Swi4 antiserum was used to detect Swi4 and anti-PSTAIR to detect Cdc28 as a loading control. **C.** Cultures of wild type or *mbp1*Δ cells were synchronised by alpha factor arrest and release. Budding index (% budded cells, upper panel) is provided as an indicator of cell cycle progression. Relative mRNA levels of *SWI4* were analysed by qPCR during the cell cycle in wild type (dark grey) and *mbp1*Δ (light grey). Expression levels are plotted as percentage of highest value detected in wild type experiment (100%).

3.3 Summary

This chapter highlights the use of new specific antibodies against the three budding yeast G1/S transcription factor components that make up SBF and MBF: Swi4, Swi6 and Mbp1. These antibodies were used to clarify and expand upon previous observations made using tagged versions of these proteins. Using the specific antibodies, I investigated the interactions between Swi4, Swi6 and Mbp1 and also their interactions with the MBF and SBF co-regulators Nrm1 and Whi5, respectively, in single strains. My data show that wild type versions of the proteins display the same protein-protein and protein-DNA dynamics as myc-tagged versions. I have shown that some Swi4 can be found at the promoter of the MBF target gene *RNR1*, and that Mbp1 can be located at the promoter of the SBF target gene *CLN2*.

Finally, I determined the binding dynamics of Swi4 during the cell cycle to the promoters of the SBF target gene *CLN2*, alongside the recruitment of Whi5, and the transcriptional activation of *CLN2* in a single culture. The data of which suggests that SBF might be further recruited following initial inactivation of Whi5 and that expression of *SWI4*, driven by MBF, results in a positive feedback signal to drive G1/S transcription and cell cycle commitment.

Overall, these data support the use of a range of new highly specific antibodies against Swi4, Swi6 and Mbp1 in further G1/S transcriptional research in budding yeast.

A manuscript resulting from this work has been published (Harris et al., 2013).

Chapter 4. Linking the DNA replication checkpoint to MBF cell cycle transcription reveals a distinct class of G1/S genes

4.1 Introduction

Activation of the DNA replication checkpoint impacts on cell cycle progression by eliciting many responses, including the initiation of transcription. However, how this effect is mediated in budding yeast is not entirely clear. Data from fission yeast research has identified a mechanism whereby the spMBF-dependent G1/S transcripts are de-repressed in response to activation of the DNA replication checkpoint (Caetano et al., 2011; de Bruin et al., 2008a). The de-repression of spMBF target genes is mediated by the checkpoint effector kinase, spCds1, which phosphorylates and inhibits the spMBF co-repressors spNrm1 and spYox1 (Caetano et al., 2011; de Bruin et al., 2008a). Due to the high conservation of functional homologs in budding yeast (Rad53, Nrm1 and MBF), I sought to investigate the effect of DNA replication checkpoint signalling on G1/S cell cycle regulated genes, as well as further downstream effects.

In order to investigate the proteomic changes that occur in response to DNA replication checkpoint activation, Dr. Francisco Bastos de Oliveira, a collaborator in the lab of Dr. Marcus Smolka at Cornell University, New York, performed a quantitative proteomic SILAC (Stable Isotope Labelling by Amino acids in Cell culture) screen. A relatively new technique, SILAC utilises ‘heavy’ and ‘light’ amino acids to differentially label the proteome in two different conditions and identify a peptide ratio between the conditions via mass-spectrometry analysis (Fig. 4.1A) (Mann, 2006; Ong et al., 2002)..

4.2 Results

4.2.1 Expression of G1/S genes is induced during replication stress

Treating cells with hydroxyurea (HU), a specific inhibitor of the ribonucleotide reductase (RNR) complex, leads to the depletion of the deoxynucleoside triphosphate (dNTPs) pool (Koc, 2003; Slater, 1973). A lack of dNTPs leads to replication fork stalling and arrest during S phase. Arrested replication forks become stabilised to prevent the occurrence of double stranded breaks, DSBs. Detection of the stalled replication forks and associated ssDNA, leads to activation of Mec1 and initiation of the DNA replication checkpoint (Section 1.3.2).

Dr. Bastos de Oliveira utilised activation of the checkpoint via HU treatment in a SILAC screen to establish which proteins accumulate in response to genotoxic stress when comparing treated and untreated samples. Wild type cells were incubated in 'light' media containing normal amino acids or in 'heavy' media containing lysine (^{13}C6, ^{15}N2) and arginine (^{13}C6, ^{15}N4). Cultures were then arrested using alpha-factor and released into the same 'light' or 'heavy' media containing 100 mM HU. Samples were taken at 20 minutes and 120 minutes following release, corresponding to conditions prior to, and following, activation of Rad53 at 25 minutes. The average ratio of peptides at 120/20 minutes was then analysed by liquid chromatography-mass spectrometry, LC-MS/MS. A full list of the proteomic changes can be found in (Bastos de Oliveira et al., 2012) along with more information on how the screen was performed and analysed.

The results of the screen showed accumulation of well-established replication checkpoint induced Crt1 targets, Hug1 and Rnr3 (Fig. 4.1A). In addition, numerous targets of G1/S transcription were highly accumulated in response to replication stress including Mcd1 (Mitotic Chromosome Determinant 1, a putative MBF-only target), Tos4 (Target of SBF 4, a putative SBF-only target), Ndd1 (Nuclear Division Defective 1, a SBF-only target) and the established MBF target Rnr1 (Fig. 4.1A).

The increase of both Tos4 and Ndd1 was not anticipated due to their expression being regulated by SBF, where it was anticipated that only MBF targets would be induced (de Bruin and Wittenberg, 2009). However, the increase of Tos4 abundance in response to HU treatment could be validated by western blot (Fig. 4.1B).

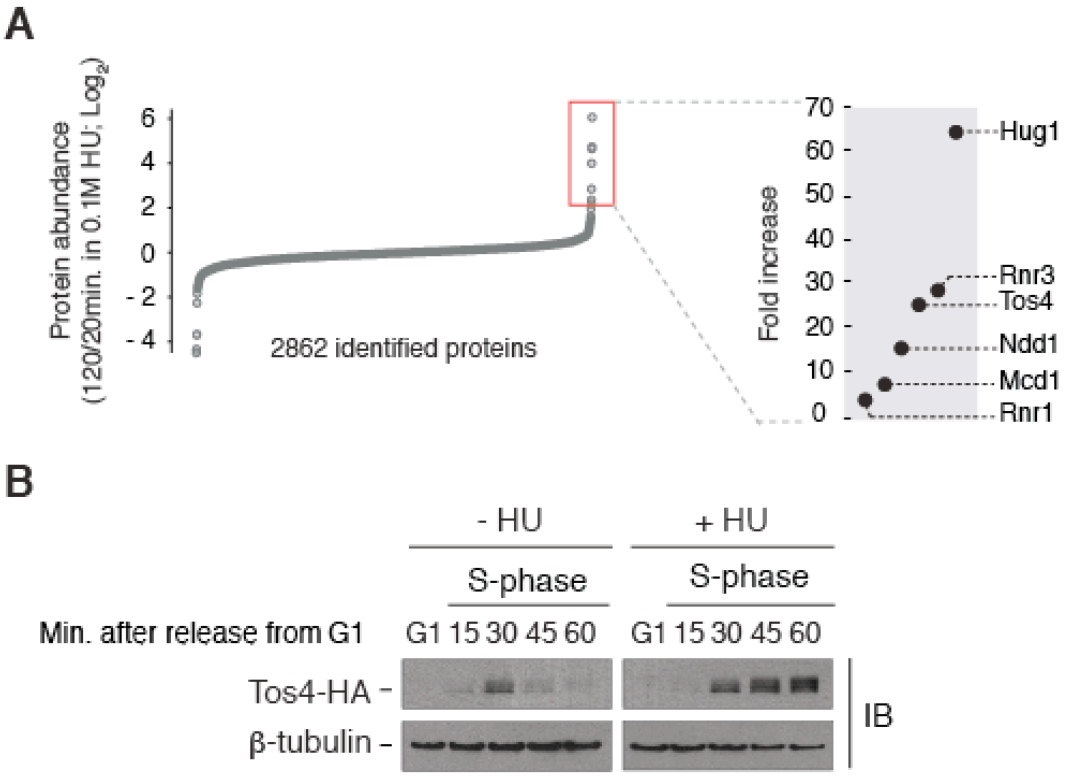

Figure 4.1 Replication stress induced accumulation of proteins

A. Proteomic analysis of changes in protein abundance following replication stress as identified by Dr. Bastos de Oliveira. Cells were arrested in G1 and released for 20 or 120 min in media containing 100 mM HU. Rad53 is activated around 25 minutes after release from G1 arrest so samples were taken before and after activation of the DNA replication checkpoint. Shown results are the average ratio (120/20 min) for each of the 2862 proteins identified and quantified in the experiment. **B.** Tos4 proteins levels in cells synchronised by alpha-factor arrest and release, in the presence or absence of 100 mM HU. Tos4 levels were detected by western blot using anti-HA antibody. Anti β-tubulin was used as a loading control.

The impact of the DNA replication checkpoint on G1/S transcription targets has not been studied in budding yeast. In fission yeast it has been shown that the checkpoint effector spCds1 inhibits spMBF co-repressors spNrm1 and spYox1 (Caetano et al., 2011; de Bruin et al., 2008a). Therefore, I sought to determine if the accumulation of G1/S proteins in response to HU treatment was due to transcriptional induction, as opposed to protein stabilisation or inhibition of proteolysis. This was achieved by monitoring the mRNA levels of accumulated targets using qPCR in untreated and HU-treated synchronised wild type cells (Fig. 4.2).

My data shows transcriptional induction of DDR genes, *RNR3* and *HUG1* in response to HU treatment and not in untreated cells (Fig. 4.2). The data also shows that G1/S target genes, *TOS4*, *RNR1* and *MCD1* expression peaks at 30 minutes and decreases as cells enter S phase by 60 minutes during an unperturbed cell cycle. However, in response to HU treatment, transcript levels are sustained during S phase (Fig. 4.2). Other studies have shown similar cytotoxic agents such as methyl methanesulfonate (MMS) and camptothecin (CPT) give similar results (Travesa et al., 2012). Transcript levels of *NDD1* were not increased in response to replication stress and displayed normal cell cycle dynamics, suggesting that the accumulation of Ndd1 in response to replication stress is under an alternate form of regulation, possibly by protein stabilisation, although this remains to be assessed (Fig. 4.2B).

Taken together these data show that transcript and protein levels of numerous G1/S targets accumulate in response to activation of the DNA replication checkpoint, in particular *TOS4*, *MCD1* and *RNR1*. The large fold accumulation of these transcripts in the SILAC proteomic screen supports the idea that G1/S transcription is impacted upon in response to replication stress, in conjunction with the induction of DDR genes. The data also show that the transcriptional regulation of G1/S targets is distinct from DDR genes whose transcription is only upregulated in response to replication stress.

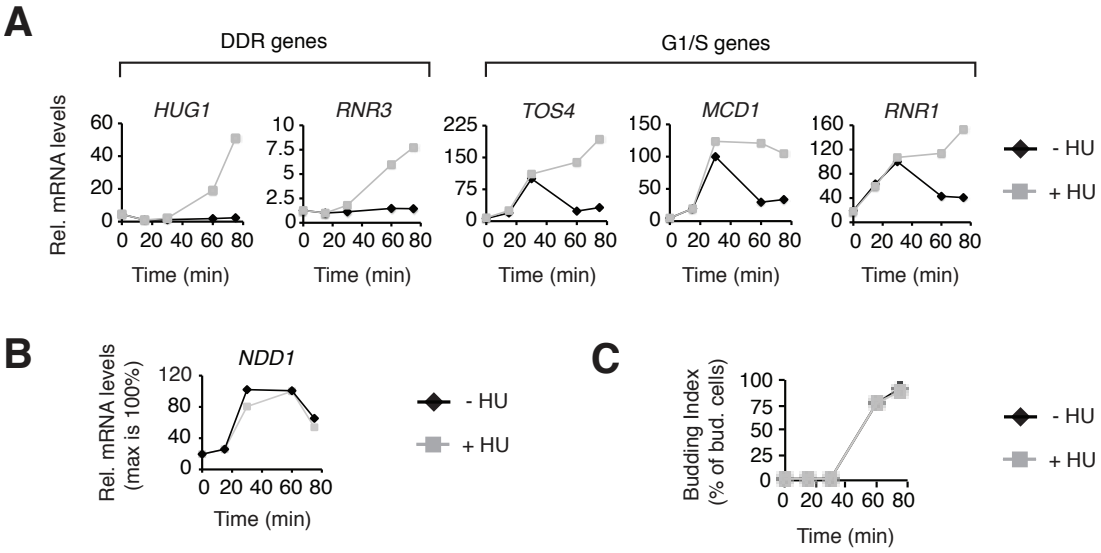

Figure 4.2 Replication stress-induced reprogramming of gene expression includes transcriptional up-regulation of G1/S genes

A. and B. Relative mRNA levels of indicated genes in cells synchronised by alpha-factor arrest and release in the presence or absence of HU. Transcript levels are represented as a fold over 0 min for the Crt1 targets and as percentage of maximum in untreated for the G1/S targets. For samples treated with HU, 100 mM HU was added 20 minutes after release from alpha-factor arrest. Levels of mRNA were quantified by RT-qPCR and normalised to *ACT1*. **C** Budding index (% budded cells) of wild type cells synchronised by alpha-factor arrest and release in the presence or absence of HU as a measure of synchrony. All data is representative of multiple experimental repeats.

4.2.2 The DNA replication checkpoint induces expression of G1/S genes via a Rad53-dependent, but Dun1-independent pathway

The regulation of G1/S targets in response to replication checkpoint activation led me to investigate the mechanism of gene induction in response to HU treatment as this was previously unknown. Induction of DDR genes by the DNA replication checkpoint is via Rad53 activating the Dun1 kinase, which in turn phosphorylates and inactivates the transcriptional repressor Crt1. This induction is dependent on Dun1 (Zhou and Elledge, 1993). To determine if the induction of the G1/S genes *TOS4*, *MCD1* and *RNR1* is also Dun1-dependent, I measured the transcript levels of Crt1 target genes, and G1/S genes during a normal cell cycle with and without HU treatment in wild type and *dun1*Δ strains.

As had been previously shown, induction of Crt1 targets *RNR3* and *HUG1*, in response to replication stress, is Dun1-dependent (Fig. 4.3) (Zhou and Elledge, 1993). My data show that in *dun1*Δ cells the G1/S transcripts *TOS4*, *MCD1* and *RNR1* remain cell cycle-regulated and HU-induced. This suggests that the induction of G1/S genes in response to DNA replication checkpoint activation is Dun1-independent (Fig. 4.3).

The upstream kinase responsible for activating Dun1 is Rad53, and it is known to mediate many effects of the DNA replication checkpoint (Allen et al., 1994). Therefore, I next sought to determine if the induction of the G1/S target genes *TOS4*, *MCD1* and *RNR1* was dependent on Rad53. I repeated the experiment above looking into the transcript levels of DDR and G1/S genes with and without treatment with HU in wild type and *rad53*Δ strains. Rad53 is an essential gene, so I performed the experiment in an *sml1*Δ*rad53*Δ background, which suppresses *rad53*Δ lethality (Zhao et al., 1998). Sml1 inhibits the RNR complex and is relieved via phosphorylation by Dun1 following activation by Rad53, therefore removal of Sml1 maintains an active RNR complex, which allows tolerance to *rad53*Δ lethality (Zhao et al., 1998; Zhao and Rothstein, 2002). My data show that Crt1 targets *RNR3* and *HUG1* induction is Rad53-dependent, as expected (Fig. 4.3). The data also show that the induction of *TOS4*, *MCD1* and *RNR1* is also dependent on

Rad53 (Fig. 4.3). Together these data suggest that the vast majority of the transcriptional induction carried out in response to DNA replication checkpoint activation, is dependent on Rad53 and that the induction of G1/S targets is Dun1-independent.

4.2.3 Targets of MBF, but not SBF, are up-regulated in response to replication stress

Data from fission yeast shows the induction of spMBF target genes in response to DNA replication checkpoint via the inhibition of co-repressors spNrm1 and spYox1 (Caetano et al., 2011; de Bruin et al., 2008a). The conservation of Nrm1 and MBF in budding yeast suggested that only MBF targets would be induced in response to replication stress and not SBF target genes (de Bruin and Wittenberg, 2009). Therefore, I sought to determine if the induction of the G1/S genes in response to replication stress is a general feature of G1/S cell cycle genes or specific to SBF or MBF targets.

I monitored mRNA levels of SBF target genes *SVS1* and *CLN2* and the MBF target gene *CDC21,* with and without treatment with HU, during the cell cycle. The data show that in response to replication stress SBF targets *SVS1* and *CLN2* are inactivated in a timely manner at 60 minutes (Fig. 4.4A). The MBF target *CDC21* is de-repressed similarly to what is seen with *TOS4*, *MCD1* and *RNR1* (Fig. 4.4A and Fig. 4.2A). Further analysis of the induction of *CDC21*, as performed in the previous experiment, showed that induction is dependent upon Rad53 and independent of Dun1 (Fig. 4.4B).

Together, this data shows that MBF-dependent, but not SBF-dependent cell cycle transcripts are de-repressed in response to replication stress in a Rad53-dependent manner. Interestingly, *TOS4* is annotated as a target of SBF (Iyer et al., 2001), but displays a pattern of behaviour similar to that of an MBF-dependent transcript in response to replication stress, suggesting it might be regulated by MBF during S phase.

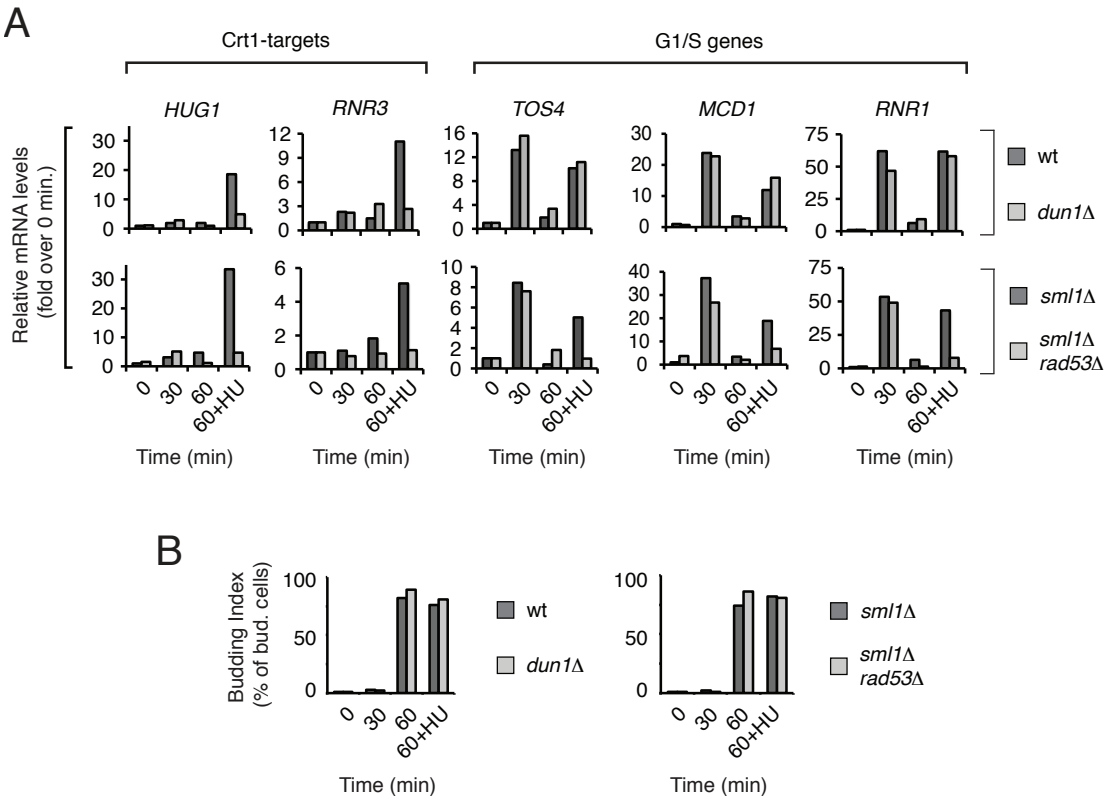

Figure 4.3 Replication stress-induced reprogramming of gene expression of G1/S genes is Rad53-dependent

A. Relative mRNA levels of indicated genes in wild type, *dun1*Δ, *sml1*Δ or *sml1*Δ*rad53*Δ cells. Gene expression levels at indicated time points (minutes), are presented as fold induction of levels detected at time 0 following alpha-factor arrest and release. For samples treated with HU, 100 mM HU was added 20 min after release from alpha-factor arrest. Levels of mRNA were quantified by RT–qPCR and normalised to *ACT1* levels. **B.** Budding index (% budded cells) of cells synchronised by alpha-factor arrest and release as a measure of synchrony. All data is representative of multiple experimental repeats.

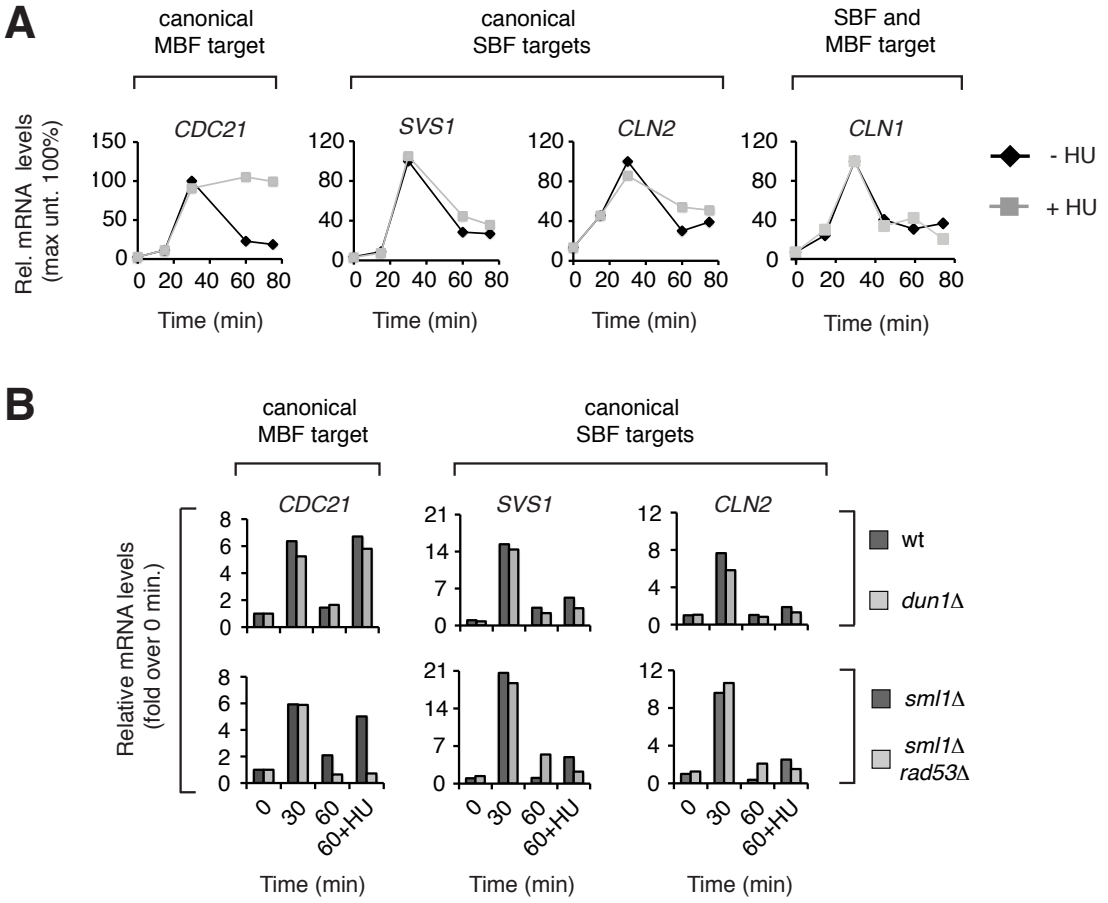

Figure 4.4 Expression of canonical MBF and SBF targets during replication stress

A. Relative mRNA levels of the canonical SBF targets *SVS1* and *CLN2*, the canonical MBF target *CDC21*, and of the SBF and MBF dual regulated gene *CLN1* in wild type cells synchronised by alpha-factor arrest and release, in the presence or absence of 100 mM HU. Transcript levels are represented as a percentage of highest level (100%) observed in untreated samples. **B.** Relative mRNA levels of *CDC21*, *SVS1* and *CLN2* in wild type, *dun1*Δ, *sml1*Δ or *sml1*Δ*rad53*Δ cells. Gene expression levels at indicated time points are presented as fold induction of levels detected at time 0 following alpha-factor arrest and release. For samples treated with HU, 100 mM HU was added 20 min after release from alpha-factor arrest. Levels of mRNA were quantified by RT–qPCR and normalised to *ACT1 levels*. Data are representative of multiple repeated experiments. Budding index (% budded cells) for these two experiments **A.** and **B.** are shown in Figures 4.2 and 4.3, respectively.

4.2.4 SBF is replaced by MBF at the promoter of *TOS4* during the G1/S transition

My previous data suggest that *TOS4*, a G1/S gene annotated as a target of SBF-dependent transcription, was regulated in a manner similar to that of MBF-dependent genes in response to replication stress. In order to determine if *TOS4* expression is regulated by SBF or MBF during the cell cycle I performed a ChIP analysis by immunoprecipitating the myc-tagged DNA binding components Swi4 and Mbp1, SBF and MBF, respectively. Their binding to the promoter region of *TOS4* was determined by qPCR, at different time points in the cell cycle following arrest with alpha-factor. My analysis shows that Swi4 (SBF) was predominantly bound to the *TOS4* promoter during G1, but upon entry into S phase (60 minutes), Swi4 leaves the *TOS4* promoter and the binding of Mbp1 (MBF) increases (Fig. 4.5A). This data suggests that SBF and MBF regulate *TOS4* expression in a mutually exclusive manner at different stages of the cell cycle. This SBF-to-MBF switch is a feature that differentiates *TOS4* regulation from other G1/S genes such as *SVS1* (SBF-only), *CDC21* (MBF-only) and *CLN1* (SBF and MBF) (Fig. 4.5A).

The switching of transcription factors at the G1/S promoters during the cell cycle had not previously been observed in budding yeast. However, in human cells, the E2F family of G1/S transcription factors has been shown to switch at the promoters of G1/S genes during the cell cycle, although until recently the reason for switching was unclear (Bertoli et al., 2013a; Dimova and Dyson, 2005; Moon and Dyson, 2008).

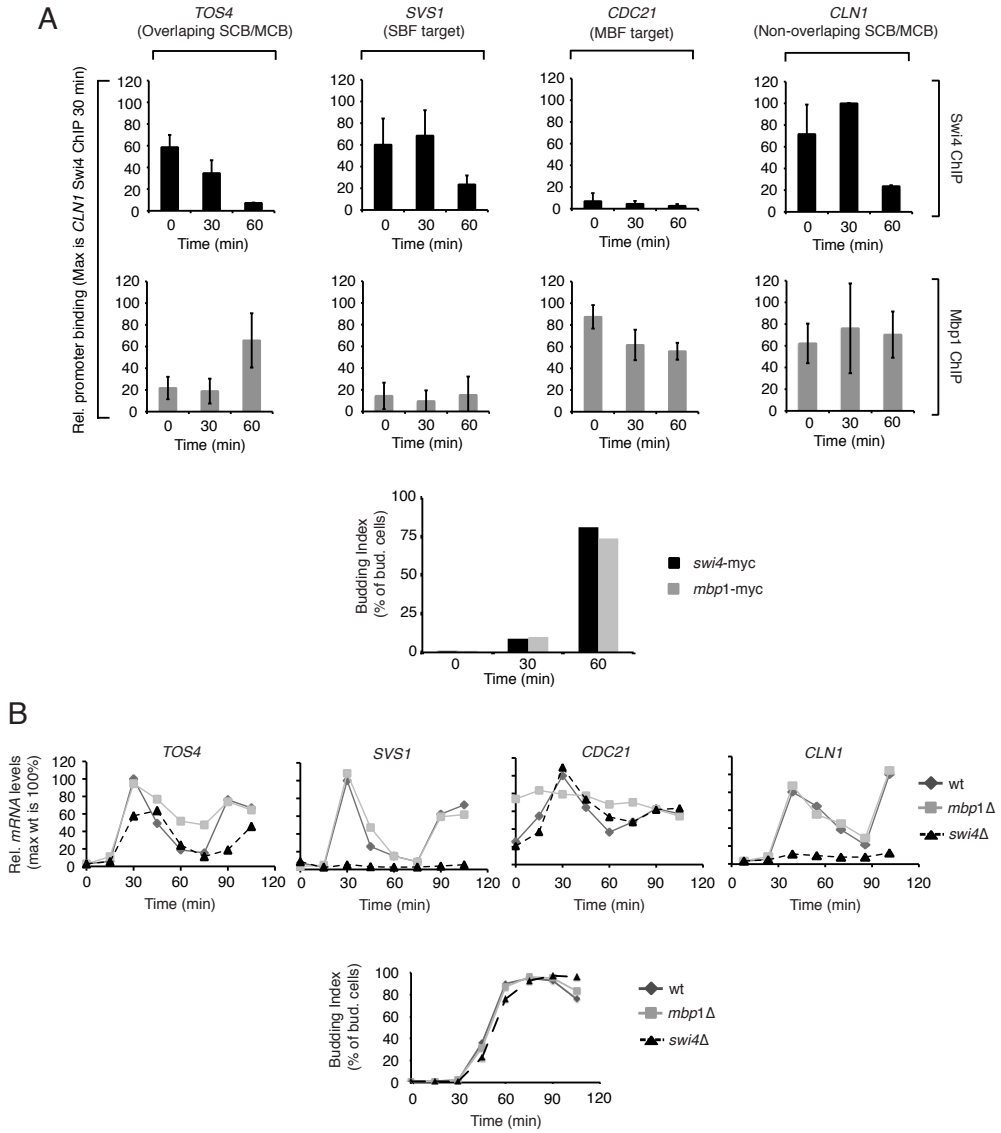

Figure 4.5 An SBF-to-MBF switch at the promoter of the G1/S gene *TOS4*

A. ChIP analysis for Swi4–myc or Mbp1–myc at the *TOS4, SVS1, CDC21* and *CLN1* promoters during the G1 and S phase of the cell cycle. Analysis was performed in G1 synchronised cells released from alpha-factor arrest and release, samples were taken at the indicated intervals. Enrichment levels were assessed by qPCR and are presented as a percentage of highest obtained level in the separate Swi4 ChIPs and Mbp1 ChIPs after normalization to whole cell extract (WCE) levels. Average value and error bars are derived from biological replicates. **B.** Relative mRNA levels of indicated genes during the cell cycle in wild type, *swi4*Δ and *mbp1*Δ cells. Cells were synchronised in G1 by alpha-factor, released from the arrest and collected at the indicated times after release. Levels of mRNA were quantified by RT–qPCR and normalised to *ACT1*. Transcript levels are represented as a percentage of highest level (100%) observed in wild type cells.

4.2.5 *TOS4* transcription is activated by SBF during G1 and repressed by MBF during S phase

Previous studies have shown that SBF functions as an activator during G1/S transcription and MBF functions as a repressor outside of G1/S (Amon et al., 1993; Bean et al., 2005; de Bruin et al., 2006). In order to determine the roles that SBF and MBF play in regulating *TOS4* expression during the cell cycle, I measured *TOS4* expression during the cell cycle using wild type cells, and strains lacking the DNA binding components of either MBF (*mbp1*Δ) or SBF (*swi4*Δ). The transcription profile of the established SBF-only target *SVS1*, the MBF-only target *CDC21* and the SBF and MBF dual-regulated target *CLN1* were used as controls (Bean et al., 2005; de Bruin et al., 2006; Eser et al., 2011). My data shows that the loss of MBF or SBF has no effect on the periodic expression of *SVS1* and *CDC21*, respectively. As expected, the loss of the activator *SWI4* reduced the transcriptional induction of *SVS1*, completely removing its periodicity (Fig. 4.5B). Conversely, loss of the repressor *MBP1* abolished periodicity of *CDC21* whilst displaying complete loss of repression outside of G1/S throughout the cell cycle (Fig. 4.5B). Expression of *TOS4* in the two DNA binding mutants shows characteristics of both SBF and MBF loss seen in the expression profiles of *SVS1* and *CDC21*. That is, a loss of optimal induction late in G1 upon loss of *SWI4* and lack of repression during S phase upon loss of *MBP1*. This confirms that *TOS4* expression is under regulation of SBF during G1 and under the control of MBF during S phase, as indicated by our ChIP data (Fig. 4.5A). The dual-regulated *CLN1* gene expression profile shows that although bound by both SBF and MBF (Fig. 4.5A), the regulation of expression is like that of an SBF target, that is reduced transcriptional induction and removal of periodicity upon loss of *SWI4* (Fig. 4.5B).

Together these data shows that while SBF is important for the transcriptional activation of *TOS4* during G1, MBF is important for the repression of *TOS4* outside of G1/S in S phase. However, clarification of these results using other synchrony methods, such as elutriation, should be used to give my findings more confidence.

4.2.6 During G1, Swi4 competes with Mbp1 for binding to the *TOS4* promoter

My previous data show that *TOS4* expression is regulated by SBF during G1 and by MBF outside of G1/S. The target promoters of SBF and MBF have been traditionally defined by the presence of consensus sequence binding sites (Iyer et al., 2001). The DNA regulatory sequence representative of Swi4 binding is an SCB (Swi4 Cell-cycle Box: CRCGAAA) site, whereas the MBF binding site is a MCB (*Mlu*I Cell-cycle Box: ACGCGN) site. The promoter of *TOS4* contains sequences representative of both an SCB and MCB site (Fig. 4.6A). Distinct from dual-regulated genes, such as *CLN1* that contains separate SCB and MCB elements in the *TOS4* promoter (Eser et al., 2011), the two elements overlap with one another (Fig. 4.6A). These overlapping binding sites could be the basis of a mechanism of competition between SBF and MBF for the promoter of *TOS4*.

To investigate the theory of competition between SBF and MBF I tested to see what effect removing one of the transcription factors would have on the binding of the other to the *TOS4* promoter. I performed ChIP analysis immunoprecipitating myc-tagged Swi4 and Mbp1 in wild type, *mbp1*Δ and *swi4*Δ asynchronous cultures, respectively. My data shows that Swi4 binds the *TOS4* promoter and inactivation of *MBP1* has no effect on Swi4 binding (Fig. 4.6B). In contrast, in the absence of *SWI4* there is a significant increase in the binding of Mbp1 to the *TOS4* promoter (Fig. 4.6B). Swi4 is predominantly bound during G1 during the cell cycle as it is removed from promoters during S phase by Clb-Cdc28 activity, therefore, I reasoned that the increase in Mbp1 binding to the promoter of *TOS4* in the absence of *SWI4* is due to increased binding during G1. This was confirmed when performing a similar ChIP analysis as above, but in a synchronous culture to monitor binding during G1 specifically (Fig. 4.6C).

Taken together, my data shows that Swi4 competes with Mbp1, during G1, for binding to the *TOS4* promoter, suggesting that there exists a cell cycle-dependent switch from SBF to MBF. I hypothesise that the overlapping MCB/SCB binding sites present in the promoter region of *TOS4* provide the foundation for such competition.

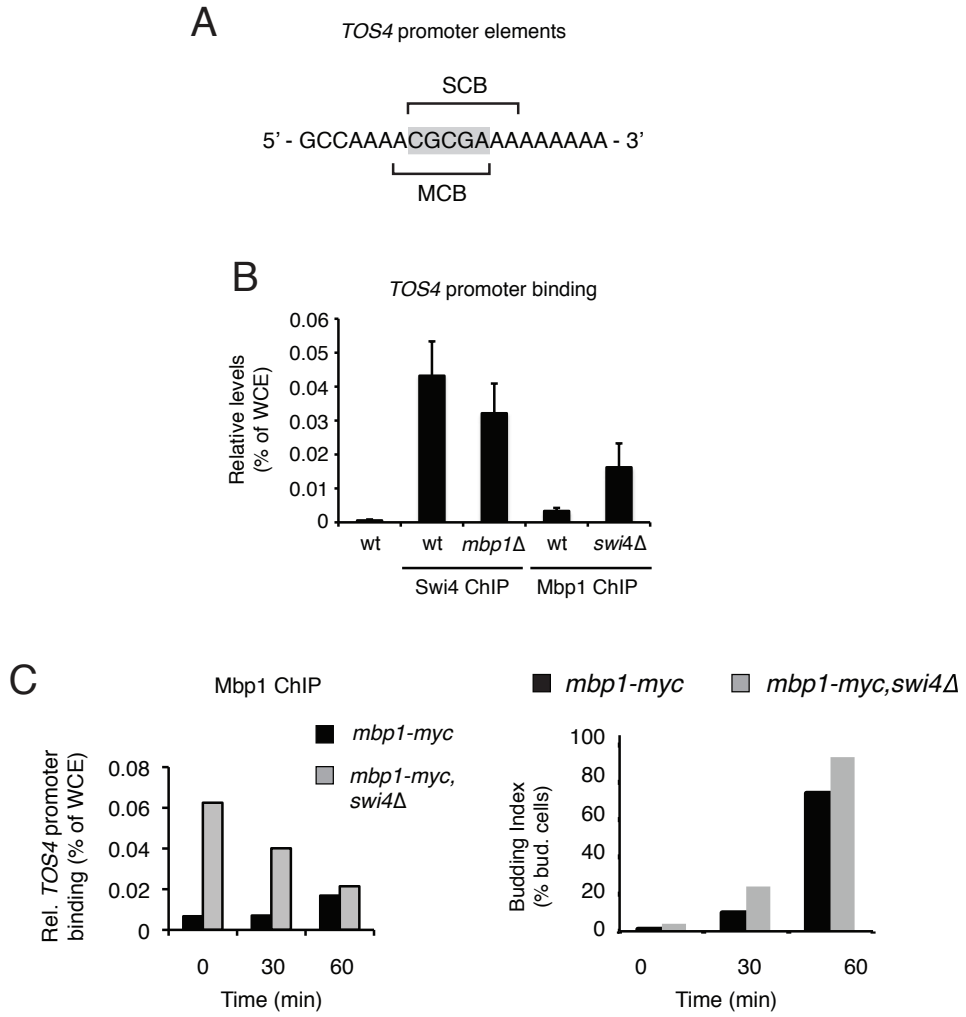

Figure 4.6 SCB/MCB overlapping motifs at G1/S promoters provides the rationale for SBF and MBF competition

A. Schematic representation of overlapping SBF (SCB) and MBF (MCB) DNA-binding motifs identified in the *TOS4* promoter element (-232 to -214 from the ATG start codon). Grey shade represents the SCB/MCB overlapping region. **B.** ChIP analysis for Mbp1–myc and Swi4–myc at the *TOS4* promoter in wild type, *swi4*Δ and *mbp1*Δ cells. Analysis was performed in asynchronous cells and enrichment levels were assessed by qPCR and are shown as a percentage of WCE signal. Untagged strains were included as negative control. Average value and error bars are derived from biological triplicates. **C.** ChIP analysis for Mbp1–myc at *TOS4* promoter in wild type and *swi4*Δ alpha-factor arrested and released synchronised cells. Budding index (% budded cells) of cells synchronised by alpha-factor arrest and release is shown as a measure of synchrony. All data is representative of multiple experimental repeats.

4.2.7 During S phase, the replacement of SBF by MBF on the *TOS4* promoter is likely an active process

My previous data shows that upon entry into S phase, MBF is the dominant transcription factor bound to the *TOS4* promoter. Upon entry into S phase, the active Clb-Cdc28 complex directly phosphorylates Swi4, dissociating its binding to target promoters and leading to the repression of SBF targets (Siegmund and Nasmyth, 1996). Therefore, I reasoned that either the removal of SBF by Clb-Cdc28 activity is the mechanism that allows MBF to bind the *TOS4* promoter during S phase or MBF actively replaces SBF at promoters upon entry into S phase. To test this I carried out a cell cycle experiment utilising a strain that contains a hyper-stable form of the Clb-Cdc28 inhibitor, Sic1, under the control of a galactose inducible promoter. Overexpression of *sic1∆p* inhibits Clb-Cdc28 activity into S phase and prevents the removal of SBF from target promoters maintaining their expression, whilst MBF-dependent transcription is repressed in a timely manner via Nrm1 repression (de Bruin et al., 2006). Therefore, if MBF binding is a consequence of SBF leaving promoters then in the absence of Clb-Cdc28 activity, the *TOS4* promoter should remain bound by SBF and expression maintained. Conversely, if MBF actively replaces SBF at promoters, then *TOS4* expression would still be repressed following G1/S transcription in the absence of Clb-Cdc28 activity.

My data shows that overexpression of *sic1*∆p has no effect on the timing and repression of the MBF-only target gene *RNR1*, as expected (Fig. 4.7). However, the SBF-only target *CLN2* and the SBF and MBF dual-regulated (predominantly SBF, as has been shown Fig. 4.5) gene *CLN1* show increased expression during S phase (100 minutes) when *sic1*∆p is overexpressed (Fig. 4.7). *TOS4* expression behaves like that of an MBF-only target and is repressed in a timely manner throughout S phase in the absence of Clb-Cdc28 activity. Therefore, this data suggests that MBF actively replaces SBF at the *TOS4* promoter upon entry into S phase by an as yet unknown mechanism.

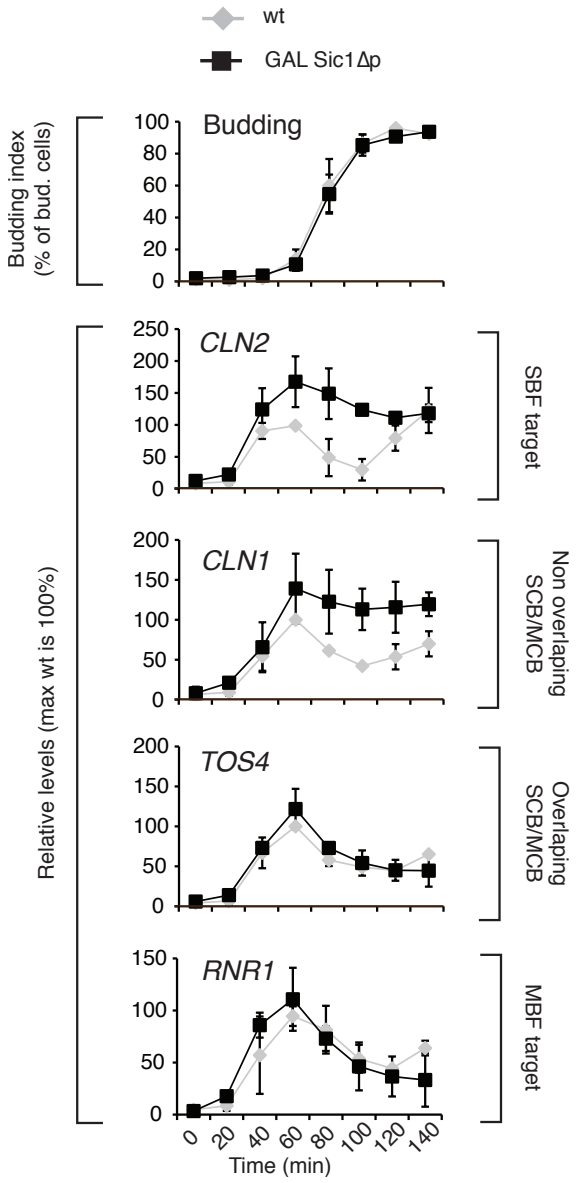

Figure 4.7 The replacement of SBF by MBF is an active process

Relative mRNA levels of indicated genes during the cell cycle in wild type cells and cells expressing *sic1Δp* from the inducible *GAL1* promoter. Cells were synchronised in G1 by alpha-factor arrest and release. Samples were collected at the indicated times after release. Levels of mRNA were quantified by RT–qPCR and normalised to *ACT1*. Transcript levels are represented as a percentage of highest level (100%) observed in wild type cells. Budding index (% budded cells) was measured as an indicator of synchrony. Average value and error bars are derived from biological triplicates.

4.2.8 Overexpression of Tos4 halts progression of the cell cycle

My data (Section 4.2.6 and 4.2.6) show that expression of *TOS4* is under an elaborate form of regulation involving both G1/S transcription factors SBF and MBF, in order to restrict *TOS4* expression to G1/S**.** This mechanism of *TOS4* transcriptional regulation, having not previously been seen in budding yeast, provided me with a target to test the functional relevance of transcription factor switching at the promoters of G1/S genes, as has been seen with E2F targets in human cells (Bertoli et al., 2013a; Dimova and Dyson, 2005; Moon and Dyson, 2008).

To test why it is important to control *TOS4* expression, Dr. Bastos de Oliveira performed a cell cycle arrest and release experiment using wild type cells and cells overexpressing *TOS4* from a galactose inducible promoter from a pYES2/NT-C plasmid, and monitored cell cycle progression using FACS (Fluorescence-activated cell sorting). The data show that when *TOS4* is overexpressed prior to G1/S activation, the progression through the cell cycle is delayed with fewer cells able to exit G1 (Fig. 4.8). This result revealed that *TOS4* is a dosage-sensitive gene where overexpression delays cell cycle progression. The number of cells able to complete S phase could be a consequence of the plasmid used in this experiment, therefore the experiment should be repeated using an integrated GAL promoter to endogenous *TOS4*, to validate this result.

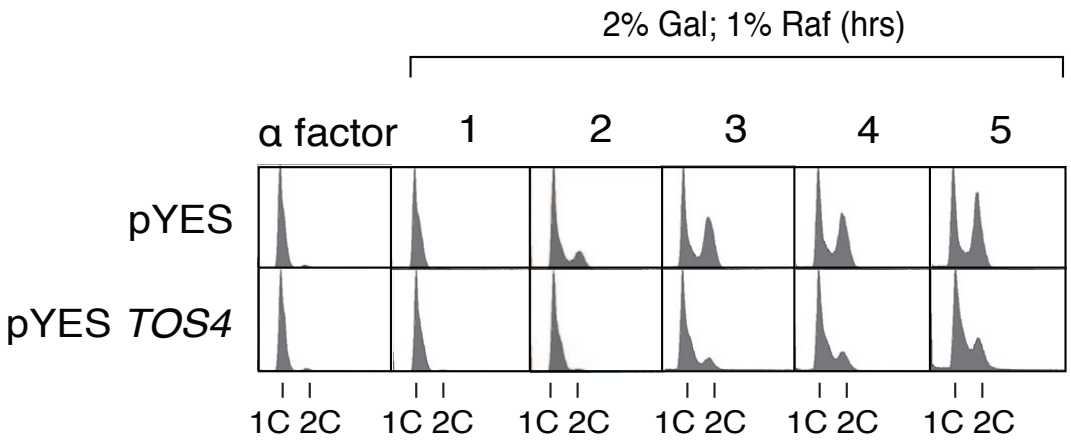

Figure 4.8 *TOS4* overexpression delays cell cycle progression

FACS analysis of cells overexpressing *TOS4* as carried out by Dr. Bastos de Oliveira. Cells were synchronised in G1 by alpha-factor arrest and released in 2% galactose 1% raffinose for the indicated intervals. No budding index was taken to measure synchronicity of the experiment.

4.2.9 Rad53 mediates induction of *TOS4* and other MBF targets via inactivation of the co-repressor Nrm1

My data shows that the replacement of SBF by MBF at the *TOS4* promoter upon entry into S phase leads to the repression of *TOS4* expression. The previous data also shows that upon activation of the DNA replication checkpoint *TOS4* expression is de-repressed in a Rad53-dependent manner (Fig. 4.3). Previous studies have shown that the transcriptional repression of MBF targets as cells enter into S phase depends on a negative feedback loop involving the MBF transcriptional co-repressor Nrm1. Expression of *NRM1*, during G1/S transcription, results in Nrm1 protein accumulation and binding to MBF, leading to repression of transcription (de Bruin et al., 2006). Together, my data suggests that Nrm1 could be a target of the DNA replication checkpoint as a mechanism of de-repressing MBF targets, including *TOS4*, in response to replication stress.

I therefore investigated the function that Nrm1 plays in response to DNA replication checkpoint activation induced by HU treatment. If Nrm1 were inactivated as part of the checkpoint response, then MBF transcript levels would resemble the same as in wild type or *nrm1*Δ cells treated with HU. In addition, HU treatment should not further induce expression of MBF transcripts in *nrm1*Δ cells, as they cannot be de-repressed in the first place. To investigate this I carried out gene expression analysis in synchronised wild type and *nrm1*Δ cells treated with HU to induce activation of the DNA replication checkpoint. My data show that both assumptions hold true; MBF targets, including *TOS4*, are de-repressed during S phase in *nrm1*Δ cells comparable to levels observed in HU-treated wild type cells (Fig. 4.9A). My data also shows that HU-treatment in *nrm1*Δ cells does not induce further expression of MBF-dependent transcripts during S phase. As anticipated the Crt1 target *HUG1* is still induced in response to HU treatment in both wild type and *nrm1*Δ cells (Fig. 4.9A). Together, this data suggests that in wild type cells, where Nrm1 is able to bind and repress MBF-dependent transcription during S phase, upon HU treatment, Nrm1, and thereby its repression, is removed from promoters. To test this I performed cell cycle ChIP analysis using Nrm1 myc-tagged strains looking at the binding of Nrm1 to target promoters in untreated and HU treated cells.

My data shows that in response to replication stress there is a loss of Nrm1 from the promoters of *TOS4*, *CDC21* and *RNR1* (Fig. 4.9B).

Taken together this data shows that upon activation of the DNA replication checkpoint, MBF–dependent transcription is de-repressed via the removal of the co-repressor Nrm1 from the promoters of MBF transcripts similar to what is seen in fission yeast (de Bruin et al., 2006). Further data supporting this has been published (Travesa et al., 2012) and shows that Nrm1 is a direct target of the Rad53 effector kinase and it is this phosphorylation that leads to the dissociation of Nrm1 from target promoters.

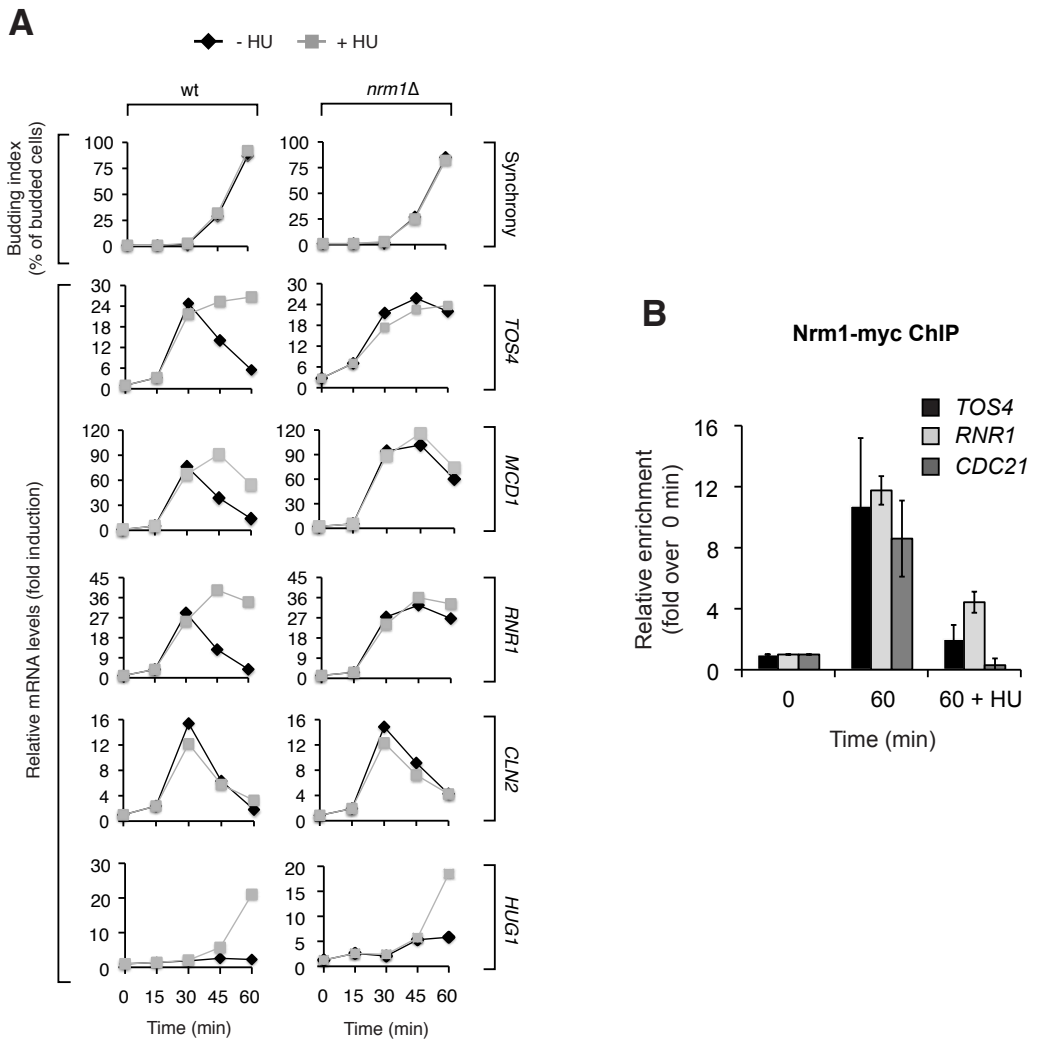

Figure 4.9 Nrm1 represses expression of MBF targets during unperturbed S phase but not during replication stress

A. Relative mRNA levels of indicated genes in wild type and *nrm1*Δ cells in the presence or absence of HU. For HU treatment, 100 mM HU was added 20min after release from alpha-factor arrest. Levels of mRNA were quantified by RT–qPCR, normalised to *ACT1*, and presented as fold induction of levels detected in time 0 in wild type cells. Budding index (% budded cells) was measured as an indicator of synchrony. **B.** ChIP analysis of Nrm1-myc binding to the *TOS4* promoter in wild type cells in the presence or absence of 100 mM HU. G1 synchronised cells were released from alpha-factor arrest and samples taken at indicated intervals. HU was added 20 minutes after release. Enrichment levels were assessed by qPCR, normalised to WCE and presented as fold increase over levels detected in time 0.

4.2.10 Tos4 is essential for cell survival during replication stress in the absence of the checkpoint protein kinase Dun1

The strong Rad53-dependent up-regulation of *TOS4* transcription in response to replications stress, combined with the cell cycle arresting effect of *TOS4* overexpression, suggest that Tos4 is an effector of the DNA replication checkpoint. In order to assert the role Tos4 plays in response to replication stress, I used a *tos4*Δ strain and tested it for sensitivity to HU and monitored colony growth. The data shows that *tos4*Δ cells are not sensitive to HU and are still viable (Fig. 4.10A).

The role of Rad53 in response to replication stress places it at the top of many branches of the checkpoint response, both activating the expression of DDR genes, *RNR3* and *HUG1*, and MBF-dependent G1/S transcripts *RNR1* and *TOS4*, as I have previously shown (Fig. 4.3). The products of such transcriptional induction are therefore likely to be required for the role they play in the replication stress response to promote cell survival. To assess the importance of *TOS4* in promoting cell survival, I monitored sensitivity of strains lacking the DDR gene induction of the DNA replication checkpoint. My data shows when the DDR branch of the checkpoint response is removed, via deletion of *DUN1*, the presence of Tos4 is essential for cells to tolerate replication stress (Fig. 4.10A). The deletion of *DUN1* alone shows slight sensitivity to HU treatment. This result suggests an important role for Tos4 in the DNA replication checkpoint response. It also supports my data that suggest expression of *TOS4* is Dun1-independent, and that the G1/S transcriptional de-repression in response to replication stress represents a branch of the checkpoint pathway that functions in parallel to the Dun1 mediated response.

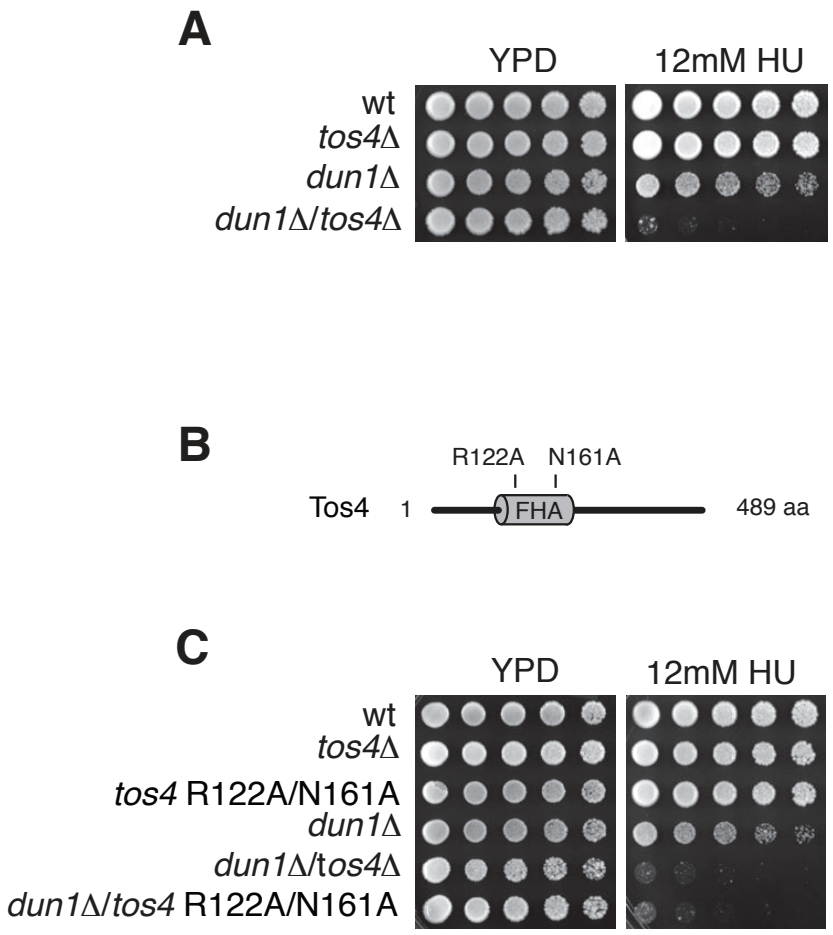

Figure 4.10 The FHA domain is important for Tos4 function in response to replication stress

A. HU sensitivity of indicated strains. Four-fold serial dilutions were spotted on plates and grown for 2–3 days at 30ºC. **B.** Schematic representation of Tos4 protein displaying its FHA domain and the position of residues that were mutated to disrupt FHA domain function. **C.** HU sensitivity of indicated strains. Four-fold serial dilutions were spotted on plates and grown for 2–3 days at 30ºC.

4.2.11 The FHA domain of Tos4 mediates an interaction with the Rpd3L and Set3 HDAC complexes to promote cellular tolerance to replication stress

Tos4 is an FHA (ForkHead-associated) domain-containing protein of unknown function. FHA domains are phosphoprotein interaction motifs originally identified in the ForkHead cell-cycle transcription factors, but are also found in checkpoint proteins, including Rad53 and Dun1 (Mohammad and Yaffe, 2009). I sought to determine if the FHA domain is important for the function of Tos4 in response to replication stress. I introduced two point mutations in the FHA domain of Tos4 at residues 122 and 161 (arginine and asparagine, respectively to alanine), which are predicted to perturb FHA domain interactions and tested the strains sensitivity to HU as in the previous experiment (Durocher et al., 1999; Durocher and Jackson, 2002) (Fig. 4.10). Dr. Bastos de Oliveira had observed that the protein abundance in response to HU treatment was unaffected by the mutations to the FHA domain (supplementary data in Bastos de Oliveira et al. (2012)). My data show that mutation of the FHA domain of Tos4 renders the cells sensitive to HU in the absence of Dun1 comparable to that of *tos4*Δ*dun1*Δ cells (Fig. 4.10C). This suggests that the FHA domain is essential for the role of Tos4 in response to replication stress.

Work by Dr. Bastos de Oliveira to identify proteins that interact with Tos4 in response to replication stress found peptides from two histone deacetylase complexes (HDACs), Rpd3L and Set3 that specifically interact with wild type Tos4 (Fig. 4.11A) (supplementary data in Bastos de Oliveira et al. (2012)). Follow-up co-immunoprecipitation experiments confirmed that wild type Tos4 interacts with components of the Rpd3L complex and Set3 complex of HDACs supporting previous observations (Shevchenko et al., 2008). Dr. Bastos de Oliveira also showed that the FHA mutant Tos4 does not interact with the HDAC components (supplementary data in Bastos de Oliveira et al. (2012)).

HDACs are well known to play a key role in modifying the biochemistry of histone N-terminal tails and are key regulators of gene expression (Section 1.4). In

particular, the Rpd3L and Set3 complexes have previously been shown to mediate transcriptional responses to various environmental stresses and in the regulation of the DDR response (Alejandro-Osorio et al., 2009; Sharma et al., 2007). The identification of Tos4 interacting with HDAC complexes, via its FHA domain, suggests that in response to replication stress Tos4 hyper-accumulates and can mediate HDAC activity, thereby modulating chromatin and gene expression. I reasoned that if Tos4 positively regulates HDAC activity during replication stress, then in the absence of Tos4 and Dun1, inactivation of the catalytic component of the HDACs, Rpd3 and Hst1, should have no effect or a negative effect on cell viability in response to replication stress. Alternatively, if Tos4 negatively regulates HDAC activity in response to replication stress, then inactivation of the catalytic component would suppress sensitivity to HU in the absence of Tos4 and Dun1. I tested this by using strains lacking the HDACs catalytic components, *RPD3* and *HST1*, in strains lacking *DUN1* and *TOS4* and tested their sensitivity to HU treatment. My data shows that deletion of *RPD3* and *HST1* suppresses the sensitivity of *tos4*Δ*dun1*Δ mutants to HU, suggesting that Tos4 interferes with HDAC activity in response to replication stress (Fig. 4.11B).

To investigate a potential role in chromatin regulation in response to DNA replication checkpoint activation, Dr. Bastos de Oliveira observed the localisation of Tos4 in response to HU treatment using a Tos4 GFP-tagged strain. The microscopy analysis shows that the hyper-accumulation of Tos4 in response to HU treatment occurs within the nucleus of cells, further supporting a role of Tos4 mediating HDAC activity in the nucleus, either by inhibition or removing HDACs from their target regions (Fig. 4.11C).

Taken together, these data suggest that Tos4 is a nuclear effector of the DNA replication checkpoint that couples Rad53 signalling to the regulation of HDAC function in response to replication stress.

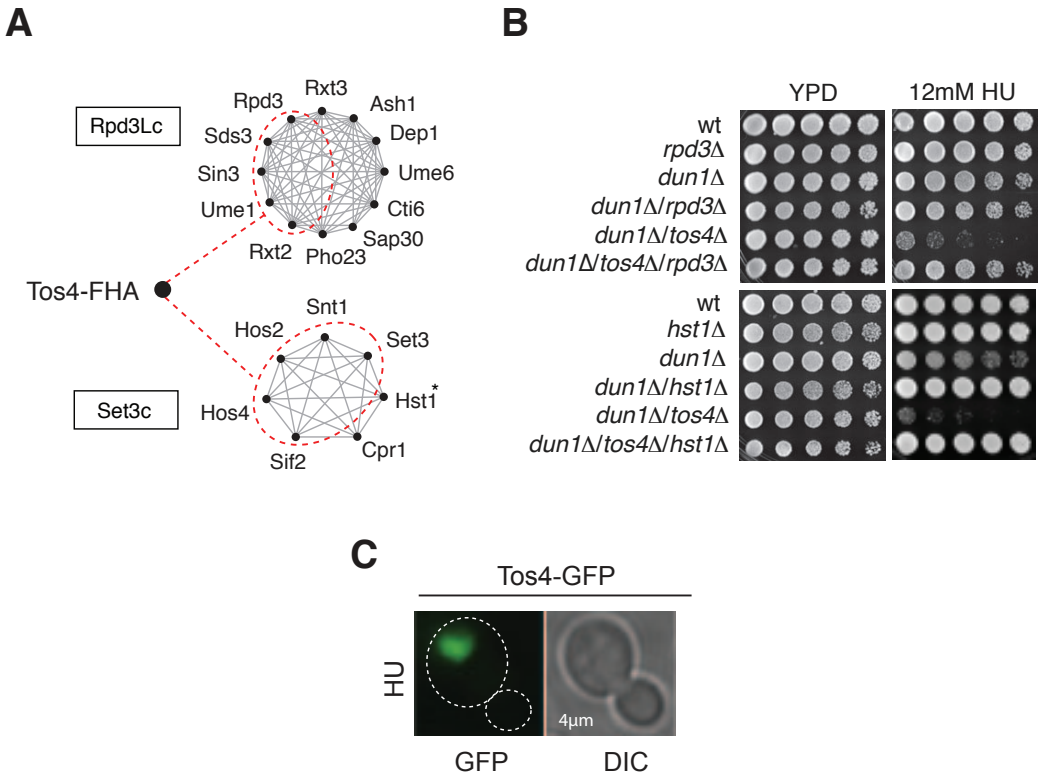

Figure 4.11 Tos4 plays an important role in the replication stress response via interaction with HDACs

A. The network of Tos4 FHA-interacting proteins during replication stress as identified by Dr. Bastos de Oliveira. According to available information in the Biogrid Database (http://thebiogrid.org/), the identified interacting proteins were grouped into two distinct complexes represented by the Rpd3 and Set3 HDACs. Grey lines represent previously known interactions between the different subunits that comprise both HDACs. Dotted line indicates the interactions identified in this work. (*) Peptides for Hst1 were not detected in the MS experiment, but the interaction was validated by co-IP (see Supplementary material (Bastos de Oliveira et al., 2012)). **B.** HU sensitivity of indicated strains. Four-fold serial dilutions were spotted on plates and grown for 2–3 days at 30ºC. **C** Cellular localization of Tos4-GFP during HU treatment. G1 synchronised cells were released from alpha-factor arrest in media-containing 100 mM HU for 60 minutes and subjected to fluorescence microscopy analysis. The fluorescence and corresponding DIC images are shown. **A. and C.** Data from Dr. Bastos de Oliveira.

4.2.12 Overlapping SCB/MCB motifs characterises a distinct group of G1/S genes

My data points to various novel characteristics of the regulation of *TOS4* and also features of the Tos4 protein's function in response to replication stress. It establishes switching from SBF to MBF as the dominant transcriptional regulator of *TOS4*, at an overlapping SCB/MCB binding site, making it what I term a 'switch gene'. The data suggest that the switch is required to tightly control *TOS4* expression to prevent cell cycle delay effects. My data also shows the de-repression of *TOS4* in response to HU treatment via the inhibition of Nrm1. It shows that this leads to hyper-accumulation of Tos4, which is important to mediate the DNA replication checkpoint response likely via mediating HDAC regulation. The suggestion that Tos4 is an effector of the DNA replication checkpoint point led me to question if there were other genes not previously known to play a role in the DNA replication checkpoint response that share the same characteristics.

Using these characteristics I manually screened the known targets of G1/S transcription (Iyer et al., 2001), looking for other genes containing overlapping SCB/MCB sites to characterise other 'switch genes'. Of the gene promoters I found, I then compared their response to HU treatment using both the SILAC screen results and data from other studies (Travesa et al., 2012). Analysis of the promoters of other G1/S genes strongly upregulated in response to replication stress revealed that the promoter of *MCD1*, the fifth most abundant protein in response to HU treatment (Fig. 4.1A), also contains overlapping SCB/MCB motifs (Fig. 4.12A). Mcd1 is an essential subunit of cohesin involved in preventing separation of sister chromosomes (Guacci et al., 1997; Michaelis et al., 1997). I assessed the binding of SBF and MBF during different stages of the cell cycle by performing ChIP analysis using myc-tagged Swi4 and Mbp1 strains and confirmed that the switching from one transcription factor to the other occurred at the *MCD1* promoter (Fig. 4.12B). As in the regulation of *TOS4* expression by both SBF and MBF (Fig. 4.5B), I observed that the transcriptional profile of *MCD1* expression in wild type, *mbp1*Δ and *swi4*Δ cells shows characteristics of being regulated by both SBF during G1 and then MBF during S phase (Fig. 4.12C). Further to this, I

observed that the switching from SBF to MBF is an active process, similar to that seen in the regulation of *TOS4* (Fig. 4.7 and 4.12D).

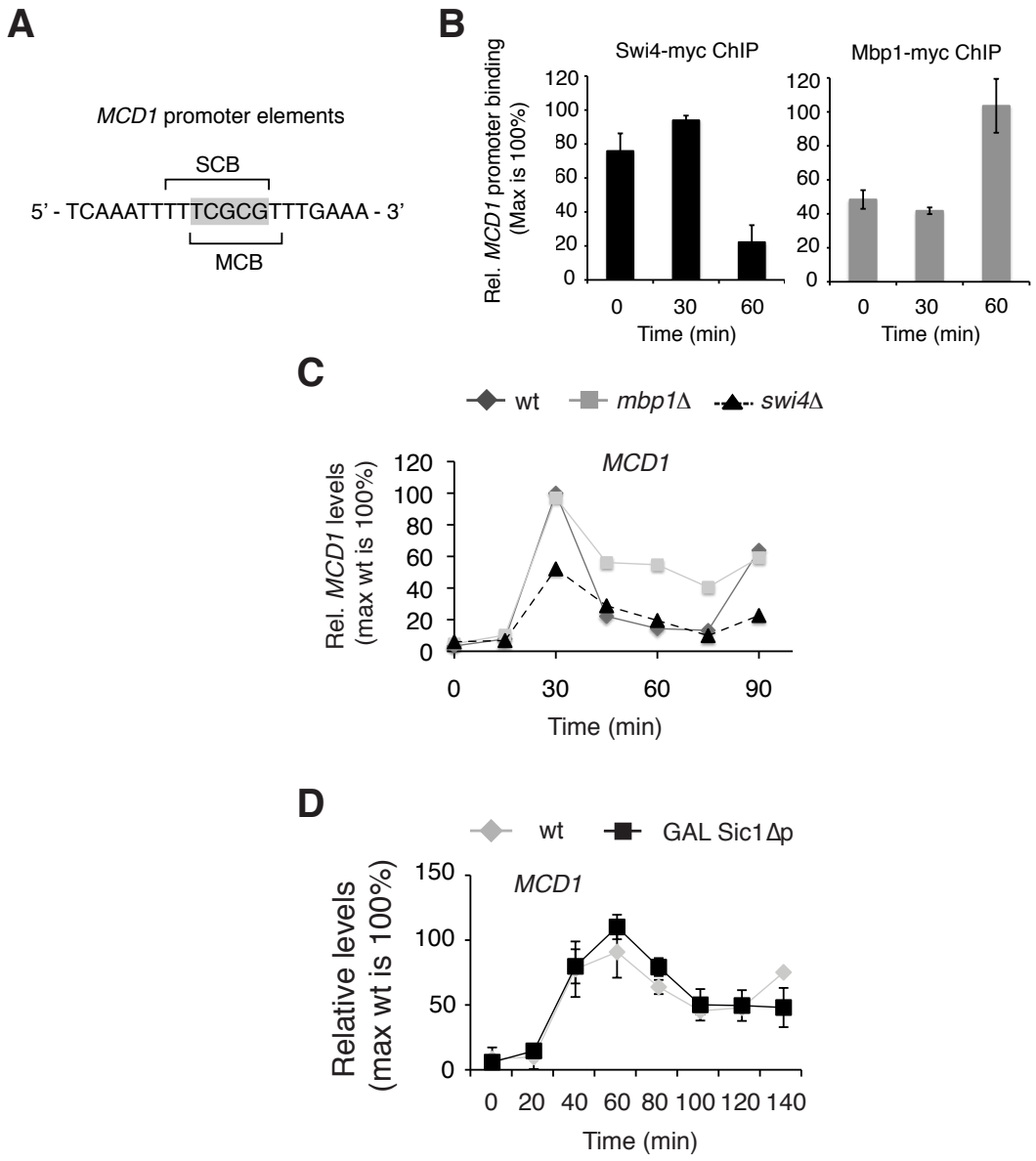

Figure 4.12 SBF-to-MBF switch at the promoter of *MCD1*

A. Schematic representation of overlapping SCB and MCB motifs identified in the *MCD1* promoter element (–336 to –329 from the ATG start codon). Grey shade represents SCB/MCB overlapping region. **B.** ChIP analysis for Swi4–myc or Mbp1–myc at the *MCD1* promoter during the G1 and S phase of the cell cycle. Experiment was performed as described in Figure 4.5. **C.** and **D.** Relative mRNA levels of *MCD1* during the cell cycle in wild type, *swi4*Δ and *mbp1*Δ cells (**C**) and wild type and cells expressing *sic1*Δ*p* from the inducible *GAL1* promoter (**D**) Experiments were performed as described in Figures 4.5B and 4.7, respectively, where budding indexes can be found.

I next wanted to investigate the possibility of other 'switch genes' and identified 44 genes containing overlapping SCB/MCB motifs that maintain the consensus binding sequences (SCB; CRCGAAA and MCB; ACGCGN) from three genome-wide screens (Harbison et al., 2004; Iyer et al., 2001; Simon et al., 2001) (Table 4.1).

Interestingly only the Iyer et al. (2001) data set contained the founding 'switch gene' *TOS4* so therefore my initial research focused on analysing the 'switch genes' identified from the Iyer et al. (2001) list. In the data set the majority of G1/S promoters were designated as SBF-only targets with very few as targets of both SBF and MBF and none as MBF-only targets. I selected six of my identified Iyer et al. (2001) 'switch genes' (*SWE1*, *TOS8*, *HCM1*, *PCL2*, *TOS3*, *SWI4*) and analysed the binding of SBF and MBF during the cell cycle using ChIP, pulling down myc-tagged versions of Mbp1 and Swi4. The data shows that all six genes display the switch from SBF-to-MBF during the G1 to S transition (Fig. 4.13A).

Another characteristic of *TOS4*, and possibly switch genes in general, is its toxicity when over-expressed. To investigate this I compared the list of G1/S genes (Iyer et al., 2001) and my list of 'switch genes', to two combined genome-wide overexpression screens (Sopko et al., 2006; Yoshikawa et al., 2011). My analysis revealed that amongst the G1/S genes identified by Iyer et al. (2001), 32% cause a delay in cell cycle progression when overexpressed. This is in line with the number of genes in the entire genome, 30%, which cause a delay when overexpressed. In line with the identified characteristics of *TOS4*, my list of identified 'switch genes' is significantly ($p = <0.01$) enriched for genes which cause a delay in cell cycle progression when overexpressed, 52% (Fig. 4.13B).

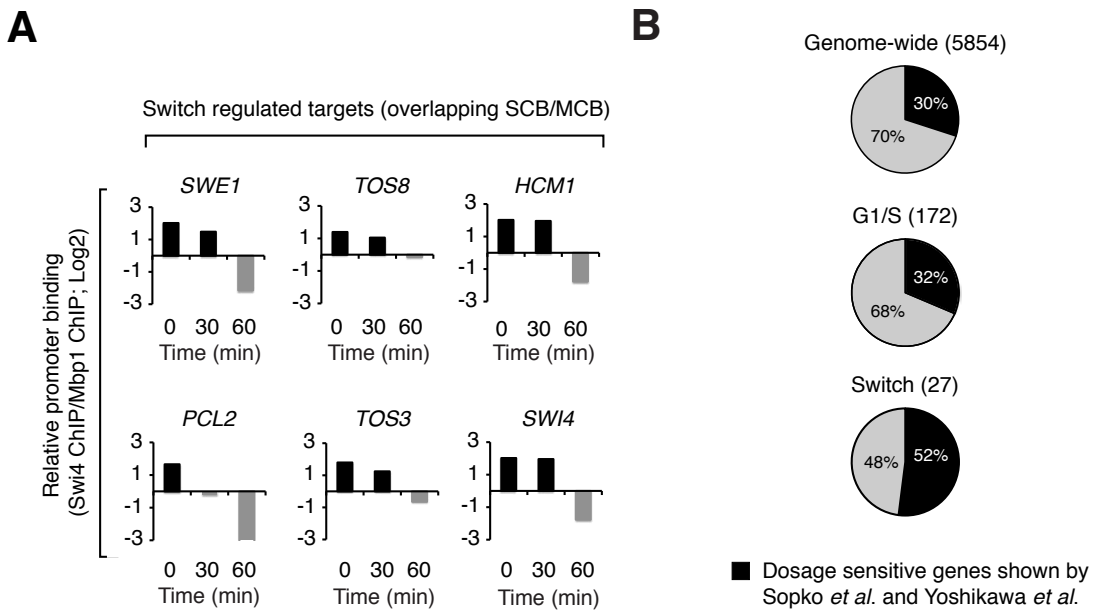

Figure 4.13 SBF-to-MBF switch at the promoter of other G1/S genes

A. ChIP analysis for Swi4–myc or Mbp1–myc at the promoter of the indicated genes. Analysis was performed in G1 synchronised cells released from alpha-factor arrest and samples were taken at the indicated intervals. G1: 0 min, G1/S: 30 min and S phase: 60 min after release. Enrichment levels were assessed by qPCR and are presented as the ratio between Swi4–myc/Mbp1–myc percentages after normalisation of each ChIP for its corresponding maximum value. **B.** Percentage (%) of dosage-sensitive genes as compared with the number of genes tested in the combined genome-wide overexpression studies of Sopko et al. (2006) and Yoshikawa et al. (2011) for the indicated gene categories. The number of genes tested for overexpression in each of the categories is indicated in parenthesis.

An additional characteristic of *TOS4* is that it is a cell cycle regulated gene that is activated in response to activation of the DNA replication checkpoint. I took my initial list of 'switch genes' from the Iyer et al. (2001) screen and assessed gene expression during the cell cycle and in response to HU treatment. I also compared this data to the screen performed by Travesa et al. (2012). My data shows that of the 27 genes analysed, 18 display cell cycle regulated transcription and are induced in response to HU treatment (Fig. 4.14).

All these data taken together suggests that the switching from SBF to MBF at overlapping binding sites during the cell cycle identifies a new class of G1/S genes. This new class is enriched for genes induced in response to replication stress and are dosage-sensitive when overexpressed.

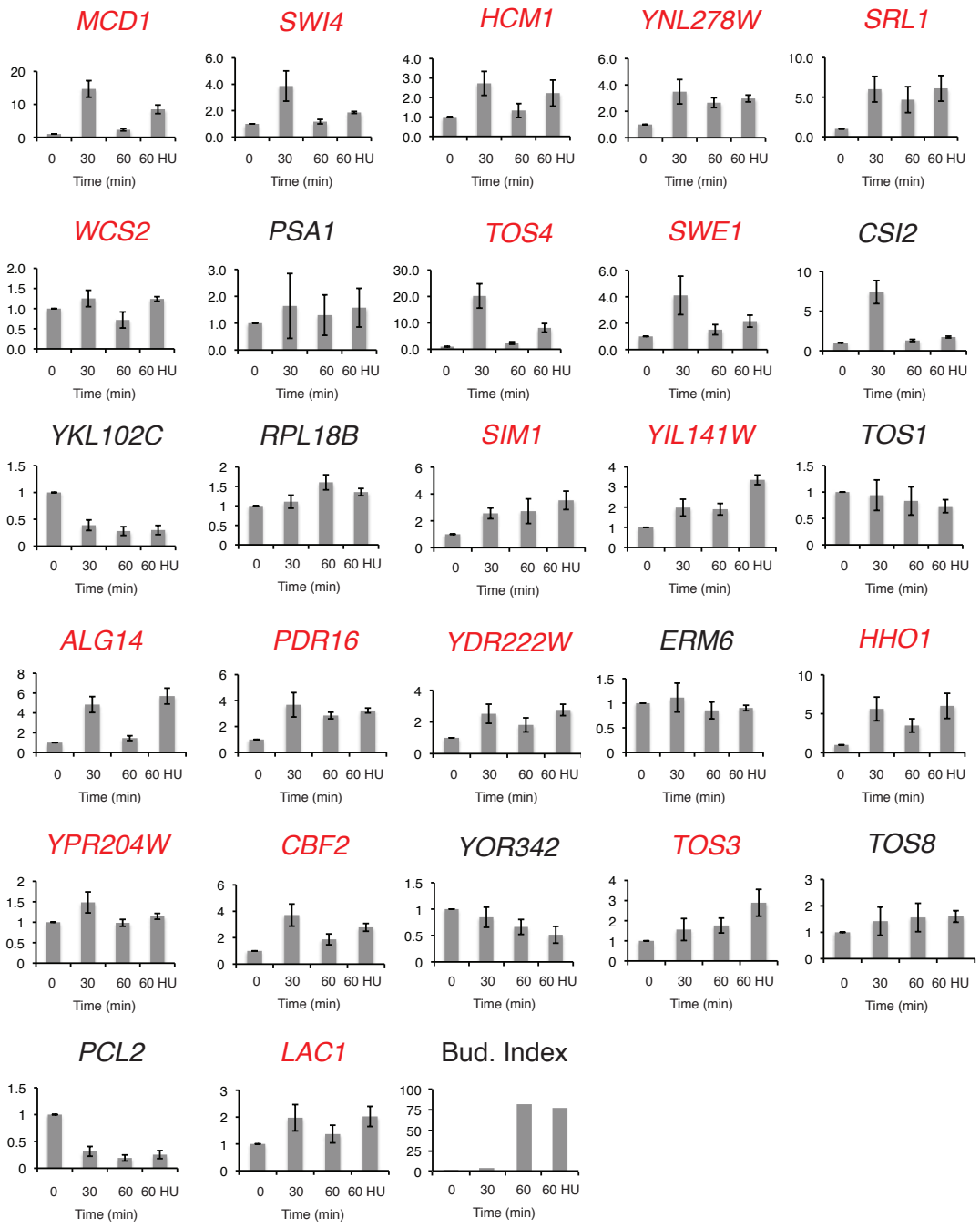

Figure 4.14 Replication stress induced transcription of switch genes

Relative mRNA levels of the 27 switch genes identified in the Iyer et al. (2001) data set in the presence or absence of 100 mM HU. Cells were synchronised with alpha-factor and released, HU was added 20 minutes after release and samples taken at indicated times. Genes upregulated upon HU treatment are in red. Levels of mRNA were quantified by RT-qPCR and normalised to *ACT1*.

#	*Gene*	Overexpression (Phenotype)	Iyer et al. 2001	Simon et al. 2001	Harbison et al. 2004	Up in HU?
1	*ALG14*	N/A	✓		✓	✓
2	*YBR071W*	Vegetative growth		✓	✓	-
3	*TOS1*	N/A	✓		✓	✗
4	*HCM1*	N/A	✓	✓	✓	✓
5	*MCD1*	Vegetative growth	✓	✓	✓	✓
6	*PSA1*	N/A	✓	✓	✓	✗
7	*PCL2*	N/A	✓	✓	✓	✗
8	*YDR222W*	Vegetative growth	✓			✓
9	*HTB1*	Vegetative growth		✓	✓	-
10	*HTA1*	Vegetative growth		✓	✓	-
11	*PLM2*	Vegetative growth		✓	✓	-
12	*MNN1*	N/A		✓	✓	-
13	*SWI4*	Vegetative growth; G2	✓	✓	✓	✓
14	*LSM4*	N/A			✓	-
15	*TOS8*	Vegetative growth	✓			✗
16	*TOS3*	N/A	✓		✓	✓
17	*CBF2*	Vegetative growth	✓			✓
18	*YGR151C*	N/A		✓	✓	-
19	*TOS10*	N/A		✓	✓	-
20	*SIM1*	N/A	✓	✓	✓	✓
21	*YIL141W*	N/A	✓			✓
22	*SWE1*	Vegetative growth	✓	✓	✓	✓
23	*ERM6*	Vegetative growth	✓		✓	✗
24	*LAC1*	N/A	✓	✓	✓	✓
25	*YKL102C*	N/A	✓			✗
26	*TOS4*	Vegetative growth; G1/S	✓			✓
27	*MID2*	Vegetative growth			✓	-

#	*Gene*	Overexpression (Phenotype)	Iyer et al. 2001	Simon et al. 2001	Harbison et al. 2004	Up in HU?
28	*YMR144W*	N/A		✓	✓	-
29	*PDR16*	Vegetative growth	✓	✓	✓	✓
30	*CAF120*	Vegetative growth	✓			✓
31	*WSC2*	Vegetative growth	✓			✓
32	*RPL18B*	N/A	✓		✓	✗
33	*CSI2*	Vegetative growth	✓			✗
34	*SRL1*	Vegetative growth	✓	✓	✓	✓
35	*YOR342C*	N/A	✓			✗
36	*NAN1*	N/A			✓	-
37	*HHO1*	Vegetative growth; G1	✓	✓	✓	✓
38	*YPR204W*	N/A	✓			✓
39	*CAP1*	N/A			✓	-
40	*ACM1*	Vegetative growth			✓	-
41	*CSH1*	Vegetative growth			✓	-
42	*GLS1*	Vegetative growth		✓		-
43	*TOS2*	N/A		✓	✓	-
44	*TOS6*	N/A		✓	✓	-

Table 4.1 List of G1/S switch genes with overlapping SCB/MCB motifs.

'Switch genes' were identified by observing the overlapping SCB/MCB motifs in the promoters of G1/S genes as identified by Iyer et al. (2001). Cell cycle and/or growth defects caused by gene overexpression were based on Sopko et al. (Sopko et al., 2006) and Yoshikawa et al. (Yoshikawa et al., 2011).The upregulation of 'switch genes' in response to HU treatment was confirmed either by myself or from a screen carried out by Travesa et al. (2012).

4.3 Summary

Here my work describes how the expression of G1/S cell cycle genes is maintained as part of the transcriptional response to DNA replication checkpoint activation and the mechanism of such induction. I reveal that replication stress specifically induces expression of MBF target genes and not SBF genes in a Rad53-dependent manner. The mechanism of de-repression is via inhibition of the MBF co-repressor Nrm1, a suggestion that was validated by another study (Travesa et al., 2012). I have also highlighted that this Nrm1 inhibition pathway functions in parallel to the Rad53-Dun1-dependent inhibition of the Crt1 repressor, which leads to the induction of DDR genes (Fig. 4.3 and 4.9A).

In addition my work establishes a novel mechanism of transcriptional regulation of G1/S genes via switching of the dominant regulatory transcription factor from SBF to MBF during the G1-to-S transition (Fig. 4.5). This is the first reported data of transcription factor switching at G1/S genes in budding yeast during the cell cycle. My data on the regulation of *TOS4,* the prototypical switch gene, suggests that the importance of switching from SBF to MBF is two-fold.

Firstly, by switching to MBF regulation in S phase, this leads to repression via the recruitment of Nrm1. The recruitment of Nrm1 to MBF creates a nexus of the DNA replication checkpoint to activate expression of genes required in response to replication stress.

Secondly, the switching from SBF in G1 to MBF in S phase allows for recruitment, by association, of the transcriptional inhibitors Whi5 and Nrm1, respectively, to 'switch gene' promoters. My data suggests that it is important to turn off expression of *TOS4* and other 'switch genes' outside of G1/S so it does not delay cell cycle progression when over-expressed. By recruiting both SBF and MBF to the promoter of 'switch genes', should normal SBF or MBF function be compromised, there is a 'fail-safe' mechanism that can repress expression of *TOS4* to prevent cell cycle progression defects (Fig. 6.2B, in Discussion).

Overall my data suggests that the reason for a SBF to MBF switch at promoters of G1/S genes is a transcriptional control mechanism, which allows checkpoint activation but prevents loss of periodicity and hyper-accumulation of dosage-sensitive proteins.

A manuscript and a review resulting from this work were published in 2012 (Bastos de Oliveira et al., 2012; Smolka et al., 2012).

Chapter 5. Histone modifications at targets of G1/S transcriptional regulators during commitment to the cell cycle

5.1 Introduction

Regulation of the acetylation state of histones has been observed to play a key role in regulating G1/S transcription in human cells (Blais and Dynlacht, 2007). Recruitment of HDAC1 to the promoters of G1/S genes, thought to be mediated via the transcriptional repressor pRb, is responsible for promoting a heterochromatic state of chromatin. Cyclin-dependent kinase activity inhibits pRb, removing it from promoters of E2F regulated genes, permitting histones to be acetylated and promoting transcription and cell cycle entry (Frolov, 2004; Lehrmann et al., 2002; Suzuki-Takahashi et al., 1995; Takahashi et al., 2000; Trouche et al., 1996). However, little is known about how histone modifications are involved in turning off transcription during S phase.

Owing to the high conservation of functional homology of the G1/S transcriptional machinery in both human cells and budding yeast, I aimed to identify and characterise the role of acetylation and deacetylation at the promoters of G1/S genes in budding yeast. I sought to further identify the role that both SBF and MBF and their co-regulators, Whi5 and Nrm1, respectively, play in histone acetylation and the HDACs responsible for repression of G1/S genes in budding yeast during the cell cycle. It is thought that MBF, as a repressor of G1/S genes recruits HDACs to promote heterochromatin and that during S phase this is mediated via the recruitment of the co-repressor Nrm1. Alternatively, SBF as an activator of G1/S genes is inhibited during G1 by Whi5, which is thought to recruit a HDAC, either Rpd3 or Hos3 (Huang et al., 2009; Takahata et al., 2009), to repress transcription until Whi5 is inactivated by Cln-Cdc28 activity. Upon entry into S phase, SBF is removed from promoters leading to a loss of transcription, although how the

histone modifications are regulated in the absence of a transcription factor are unknown.

5.2 Results

5.2.1 Acetylation of G1/S promoter histones correlates with cell cycle regulated transcription

The role of modifying histones at the promoters of SBF and MBF genes had not previously been studied in budding yeast although the interaction of the transcription factors with various histone-modifying enzymes had previously been reported (Huang et al., 2009). I set out to characterise the histone modifications that take place at G1/S genes during the cell cycle in budding yeast. In order to characterise the role of histone modifications in G1/S regulation, I performed ChIP analysis against specific histone acetylation marks in wild type cells during the cell cycle. I analysed the association with SBF and MBF promoters and the mRNA transcript levels by qPCR. In order to analyse my data following ChIP I normalised to both the level of histone acetylation at a control promoter (*ACT1*) and to levels of unmodified histone 3 at the promoter, as a measure of nucleosome occupancy (as histones have been shown to be fewer at active genes). My data show that acetylation and deacetylation of histone 3 lysine 9 (H3K9Ac) and H3K27 correlates with SBF and MBF gene expression and repression, respectively (Fig. 5.1 and 5.2). Other histone marks H3K14Ac and H4K5Ac also show association with acetylation increasing alongside gene expression but to a lesser degree than H3K9 and H3K27.

Interestingly over the time-course of my experiment I capture two cell cycles. In the second, less synchronous cell cycle, from 75 minutes onwards, the peak expression of SBF-dependent genes is earlier than MBF-dependent genes (Fig. 5.1 and Fig. 5.2). However, the timing of acetylation marks of all histones increases at the promoters of both SBF and MBF-dependent genes in correlation with the expression of *SVS1* and *CLN2* (Fig. 5.1 and Fig. 5.2). Studies showing the

temporal expression of the G1/S wave of transcription have suggested that SBF targets are activated earlier although my data may not show this following release due to a lower resolution which is exaggerated as the cell become less synchronous during the second cell cycle (Eser et al., 2011). The same study by Eser et al. (2011) showed that the method of synchronisation affects the order of gene expression of G1/S genes. However, the sharp second peak in acetylation at 90 minutes is common to all samples and gene promoters and may indicate that a control sample was inaccurate, either histone H3 occupancy or acetylation at the *ACT1* promoter may account for this observation. The experiment should be repeated to confirm these observations and also be re-assessed using alternative synchronisation methods.

Overall these data show the that expression of SBF and MBF genes during the cell cycle correlates with an increase in the acetylation of histones at the promoter region followed by deacetylation in conjunction with transcriptional repression.

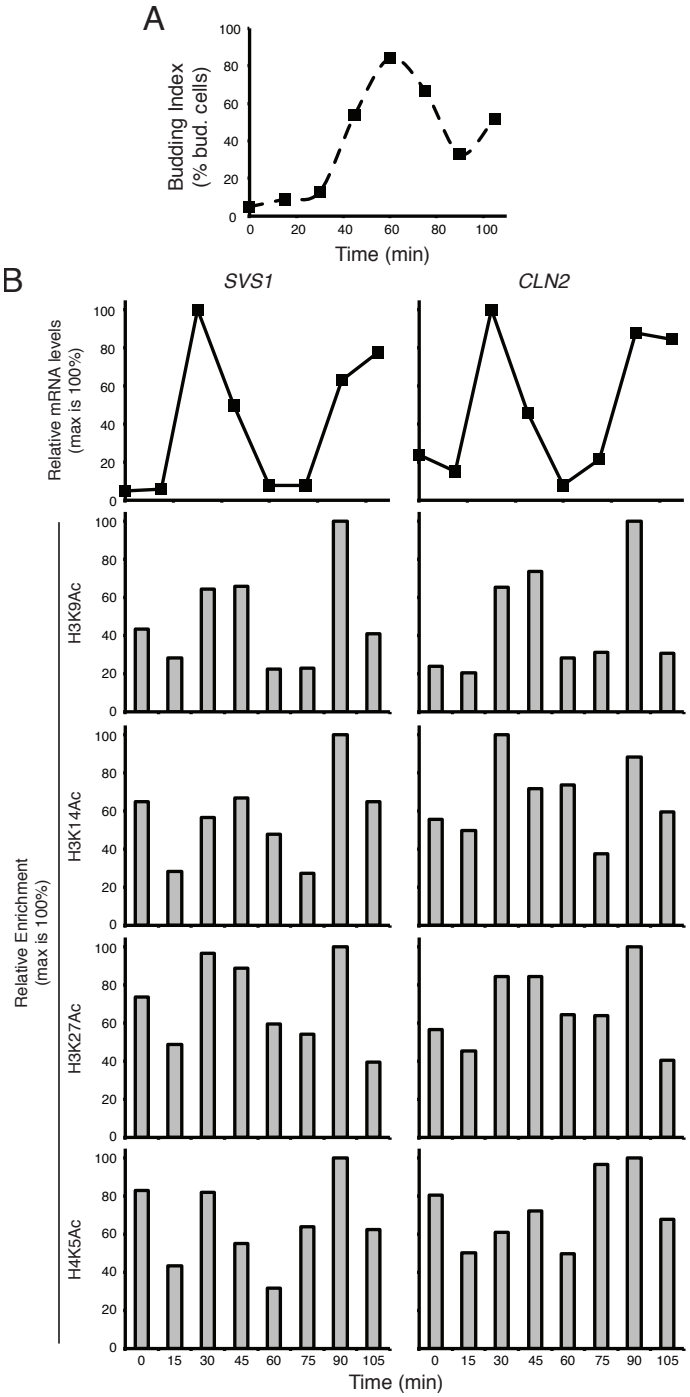

Figure 5.1 Acetylation of SBF promoters during the cell cycle

Wild type cells were synchronised in G1 using alpha-factor and released and samples taken every 15 minutes **A.** Budding index (% budded cells) was measured as an indicator of synchrony. **B.** Relative mRNA levels of SBF genes *SVS1* and *CLN2*. Levels of mRNA were quantified by RT–qPCR and normalised to *ACT1*. Transcript levels are represented as a percentage of highest level (100%) observed. **C.** ChIP analysis of histone modifications at the promoter of *SVS1* and *CLN2*. Enrichment levels were assessed by qPCR and are presented as fold enrichment over time 0 after normalisation to whole cell extract (WCE) levels, modifications at the *ACT1* promoter and H3 occupancy.

5.2.1.1 The deacetylation of H3K27ac occurs at a faster rate at MBF dependent promoters

My previous data identified the association between histone acetylation and gene expression at both SBF and MBF-regulated promoters. The differences in how SBF and MBF are regulated with Whi5 inhibiting SBF during G1 and Nrm1 co-repressing during S phase, I set out to identify if the two transcription factors target different histone lysine residues that they regulate during the cell cycle. In order to see the relative fold changes from one time point to another, and the apparent stronger signal in the second cell cycle, I focused on comparing changes that occur during the first cell cycle following alpha-factor release. My analysis identified that following maximal levels of H3K27 acetylation, MBF-dependent genes *CDC21* and *RNR1* have a faster rate of deacetylation compared with the SBF genes, *SVS1* and *CLN2* (Fig. 5.3). This suggests that the recruitment of Nrm1 following entry into S phase, via a negative feedback loop, actively recruits a HDAC to remove the acetylation marks from H3K27Ac. Conversely, H3K27 acetylation takes longer to be removed from the promoters of SBF genes in the absence of a repressor, or SBF as it has been removed by this stage (Section 5.3), at the promoter. This suggests that deacetylation of H3K27 at SBF genes is a passive process as a result of a loss of HATs that are possibly recruited via SBF at promoters.

5.2.1.2 The deacetylation of H3K9ac occurs at a faster rate at SBF dependent promoters

During S phase, MBF recruits the co-repressor Nrm1 to repress MBF-dependent transcription. Nrm1 has also been shown to play a role in the DNA replication checkpoint response and is inhibited to de-repress MBF targets to maintain expression (Section 5.3?). SBF targets are not induced in response to replication checkpoint activation. My data set show that there is an association between H3K9 acetylation and gene expression. My data looking into differences between SBF and MBF target deacetylation, show that the rate of H3K9 deacetylation is faster at the SBF promoters *SVS1* and *CLN2* compared to MBF promoters *CDC21* and *RNR1* (Fig. 5.3).

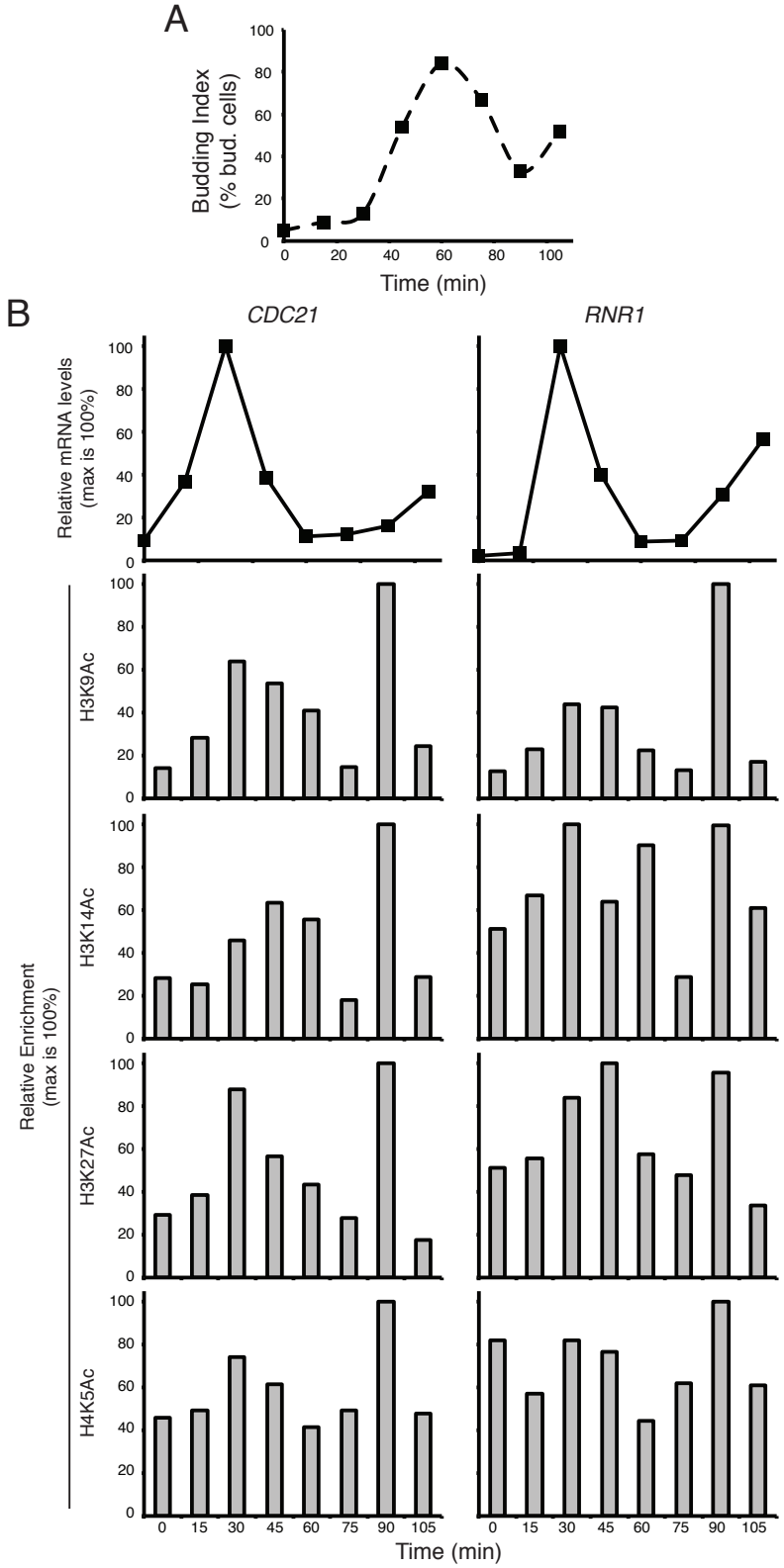

Figure 5.2 Acetylation of MBF promoters during the cell cycle

Experiment performed as in Figure 5.1**. A.** Relative mRNA levels of MBF genes *CDC21* and *RNR1*. **B.** ChIP analysis of histone modifications at the MBF promoters of *CDC21* and *RNR1*.

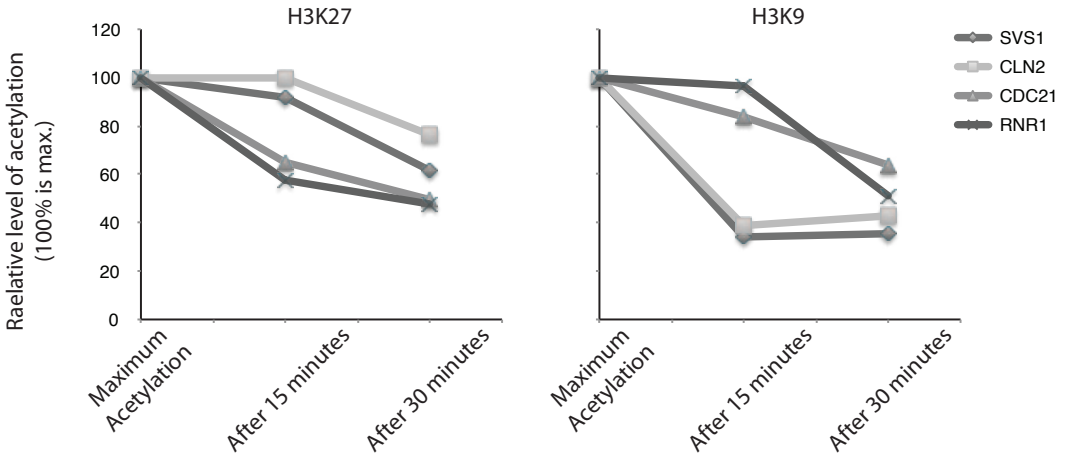

Figure 5.3 The dynamics of H3K27 and H3K9 deacetylation differ between SBF and MBF promoters

ChIP analysis of indicated histone acetylation marks at the promoters of SBF (*SVS1* and *CLN2*) and MBF (*CDC21* and *RNR1*) promoters following maximum acetylation. Experiment is the same as (Fig. 5.1 and Fig. 5.2) with data presented as percentage of maximum enrichment normalised to WCE, *ACT1* promoter acetylation and histone H3 occupancy.

5.2.2 Histone acetylation changes are a general feature of cell cycle regulated transcription.

My previous data suggest that specific histone acetylation changes occur at the promoters of G1/S genes. To identify if this is a specific feature of G1/S transcription or a common feature of cell cycle regulated transcription, I analysed histone changes at the promoters of genes in the G2/M and M/G1 wave of transcription, *CLB2* and *SIC1*, respectively. My data show that G2/M transcription peaks at 60 minutes and this correlates with maximal acetylation of H3K9, K14 and K27, but not H4K5 (Fig. 5.4). My data also show that there is rapid deacetylation following maximum levels and that this correlates with repression, similar to the regulation seen at G1/S promoters. In contrast, my data show that the peak expression of *SIC1* occurs at 75 minutes after release from alpha-factor arrest and the histone acetylation marks decrease as expression increases (Fig. 5.4). This is a distinct form of regulation of cell cycle regulated transcription. Interestingly, acetylation of the *SIC1* promoter occurs during the cell cycle prior to deacetylation and activation of transcription.

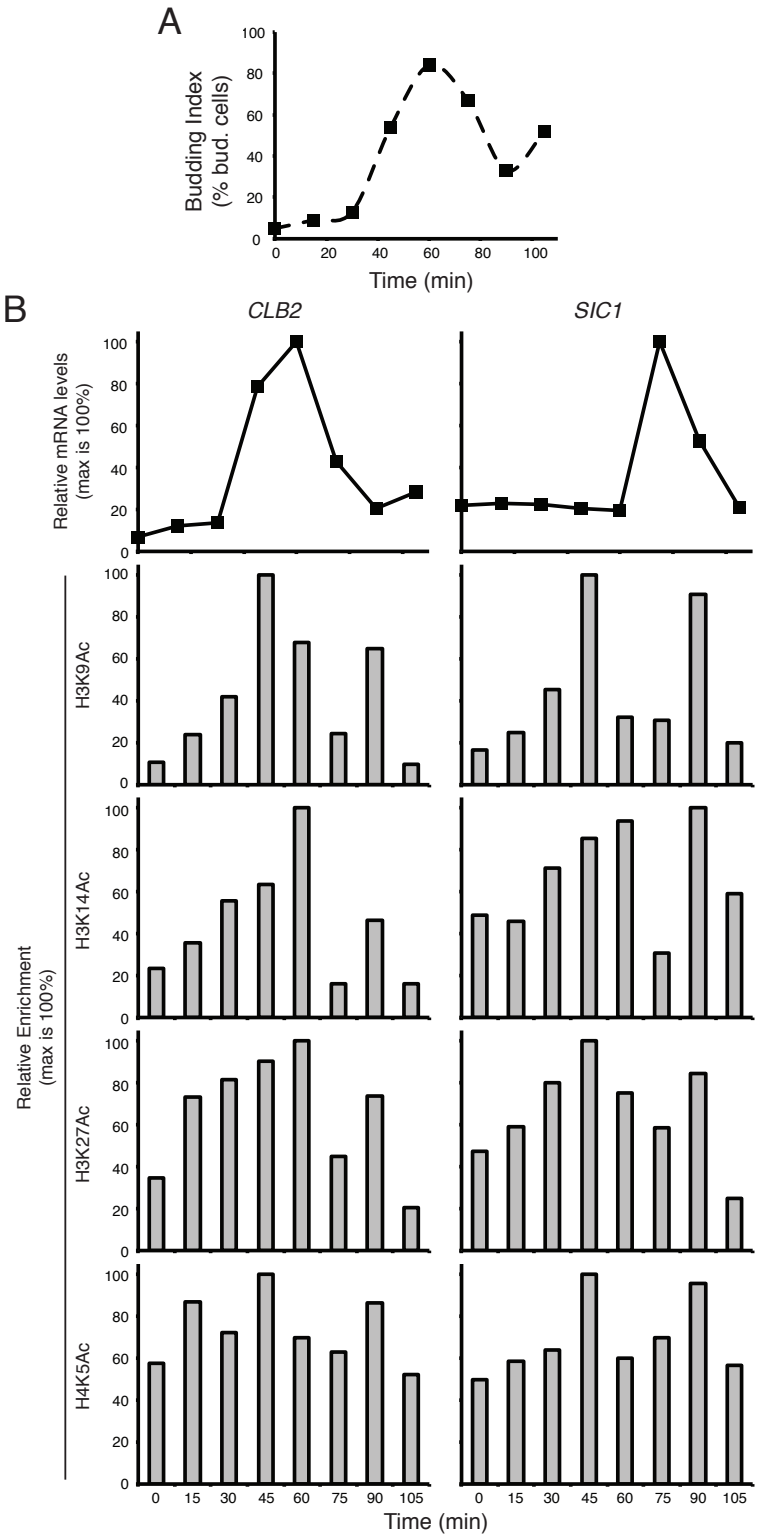

Figure 5.4 G2/M and M/G1 transcription waves have different histone acetylation dynamics.

Experiment performed as in Figure. 5.1. **A.** Relative mRNA levels of G2/M gene *CLB2* and M/G1 gene *SIC1*. **B.** ChIP analysis of histone modifications at the promoter of *CLB2* and *SIC1*.

5.2.3 MBF-dependent promoters are acetylated during S phase in an MBF-independent manner.

Different mechanisms lead to transcriptional repression of SBF and MBF-dependent expression during S phase (Fig. 1.3). This, in conjunction with my previous data, that identifies differences in the dynamics of histone deacetylation of the same residues H3K9 and H3K27, suggests that there are specific dynamics associated with each transcription factor. To test this hypothesis, I repeated the above experiment in *mbp1*Δ cells and monitored H3K9 acetylation changes in conjunction with transcript levels of G1/S transcripts, to determine MBF-dependent effects on histone dynamics. The data show that the expression of *SVS1*, which is SBF-dependent, is not affected during the cell cycle and the dynamics of H3K9 acetylation correlate with expression and repression as seen in wild type cells (Fig. 5.1 and Fig. 5.5). My data also show that the repression of the MBF-dependent target *CDC21* outside of G1/S is compromised in the absence of Mbp1 (Fig. 5.5). The data show that the acetylation changes that occur at nucleosomes in the promoter of *CDC21* are not regulated in the absence of MBF, however, upon entry into S phase there is a increase in the acetylation of H3K9, a mark my data suggests is associated with expression. Overall, these data show that MBF mediates specific effects on the acetylation and deacetylation of H3K9 in target promoters during G1 and S phase, respectively. My data also suggests H3K9 acetylation during S phase occurs in an MBF-independent manner at MBF promoters.

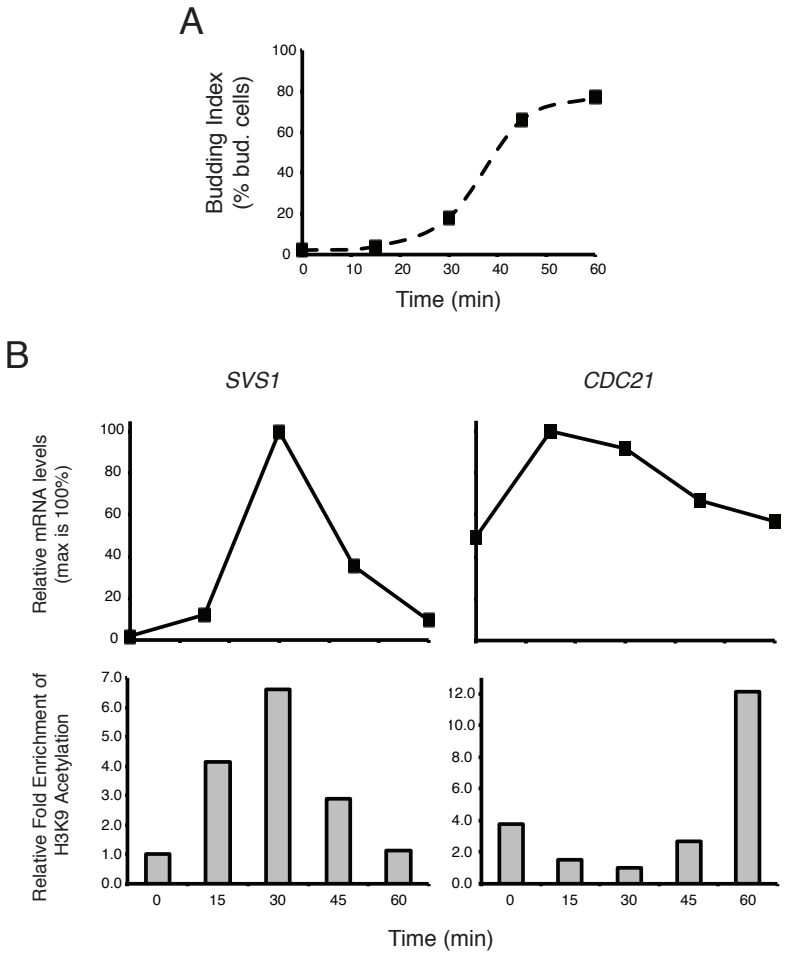

Figure 5.5 MBF-dependent acetylation of Histone 3 Lysine 9 in S phase

Wild type and *mbp1*Δ cells were synchronised in G1 using alpha-factor and release and samples were taken every 15 minutes. Relative mRNA levels of SBF gene *SVS1* and MBF gene *CDC21* were quantified by RT–qPCR and normalised to *ACT1*. Transcript levels are represented as a percentage of highest level (100%) observed (upper panel). ChIP analysis of H3K9 acetylation at the promoter of *SVS1* and *CDC21*. Enrichment levels were assessed by qPCR and are presented as fold enrichment over lowest value after normalisation to whole cell extract (WCE) levels, modifications at the *ACT1* promoter and H3 occupancy.

5.2.4 Acetylation and deacetylation of G1/S promoters is a conserved feature in fission yeast, dependent on spNrm1

The identification of histone modifications led to the hypothesis that specific modifications would lead to a specific outcome for the associated genes, known as the histone code (Jenuwein and Allis, 2001). My budding yeast experiments aimed to characterise the histone acetylation changes that occur at the promoters of G1/S genes in budding yeast and attribute modifications to a specific transcription factor. While G1/S transcriptional regulation in budding yeast is well defined and dependent on SBF, MBF and their respective repressive co factors Whi5 and Nrm1, it is not known which HDAC is responsible for mediating repression. Therefore, in order to focus my research on histone changes dependent on G1/S transcription factors I used fission yeast. Due to the high conservation of the functional homologs to MBF and Nrm1, the lack of a recognisable SBF, and the conservation of histone residues across eukaryotes, I hoped that the use of fission yeast would aid in narrowing down specific histone residues modified in an MBF or Nrm1-dependent manner in budding yeast.

Briefly, the cell cycle dynamics of fission yeast differ and they spend most of the cell cycle in G2 (Fig1.1A). The role of spNrm1 in fission yeast, to repress MBF-dependent transcription outside of G1/S, is therefore obvious in asynchronous cells (de Bruin et al., 2006). Therefore, to monitor histone acetylation changes dependent on spNrm1 in fission yeast, I performed ChIP analysis of histone modifications in asynchronous wild type and *spnrm1*Δ cells, as the majority of the cellular population would be in G2 (i.e. repressed outside of G1/S). I correlated the data with expression of the spMBF G1/S target genes *spcig2*, *spcdc18* and *spcdc22*. My data show that the spMBF-dependent transcripts are significantly upregulated in the absence of spNrm1 with a 8-11 fold induction (Fig. 1.6A). My ChIP data identifies that the acetylation of H3K14 and H4K5 within the promoter region of these G1/S genes is increased in the absence of spNrm1. My data show the acetylation of H3K9 in the promoters of *spcdc18* and *spcdc22* is increased in the absence of spNrm1 but that the acetylation state of H3K9 in the *spcig2* promoter is comparable to wild type levels (Fig. 1.6B).

Overall, these data identify that gene expression and the modification of histones at the promoters of G1/S genes in fission yeast is regulated by spNrm1 outside of G1/S. The data also show that spNrm1, or another factor, contributes to promoter specific effects on histone modifications.

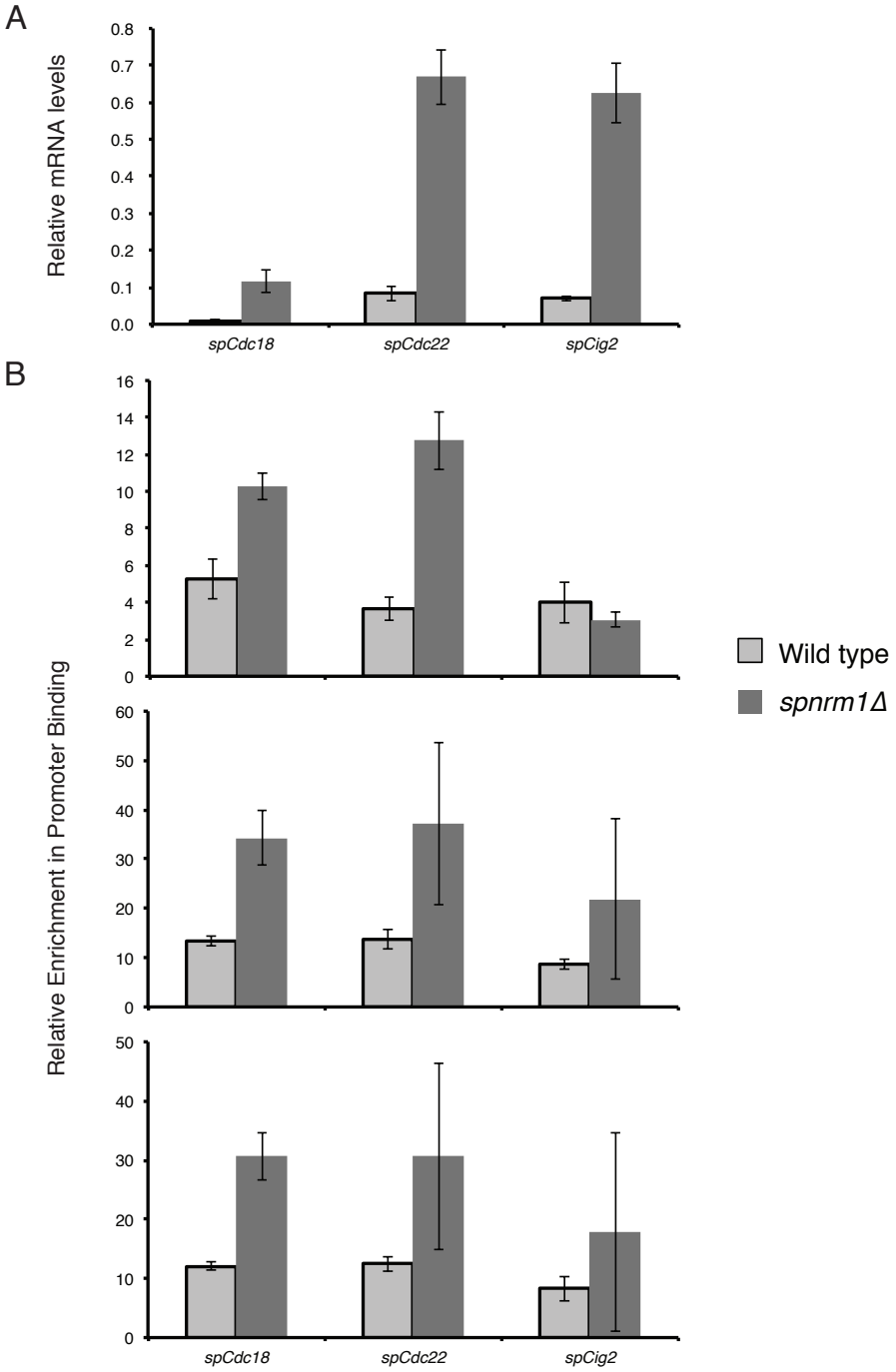

Figure 5.6 Histone deacetylation is dependent on spNrm1 in fission yeast

Asynchronous wild type and *spnrm1*Δ cells cultures were incubated.**A.** Absolute mRNA levels of spMBF genes *spcdc18, spcdc22 and spcig2* were quantified by RT–qPCR and normalised to *spact1*. **B.** ChIP analysis of histone modifications at the promoters of *spcdc18, spcdc22 and spcig2*. Enrichment levels were assessed by qPCR and are presented as relative enrichment over after normalisation to 1% whole cell extract (WCE) levels. Error bars represent standard error of biological triplicates.

5.3 Summary

In this chapter, I have identified that the histones in promoters of G1/S genes in budding and fission yeast are acetylated and deacetylated during the cell cycle.

Firstly, I characterised the histone acetylation changes that occur during the cell cycle in budding yeast at the promoters of SBF and MBF genes, and identified differences in the dynamics of specific lysine residues being deacetylated upon entry into S phase between SBF and MBF promoters. I have identified that the budding yeast MBF is required to regulate H3K9 acetylation at the *CDC21* promoter during the cell cycle and that H3K9 is acetylated during S phase in an MBF-independent manner.

Secondly, I identify that the spMBF co-repressor spNrm1 is required for the deacetylation of histones at G1/S in fission yeast in a promoter specific manner. The data also suggest that the acetylation of H3K9 is associated with increased gene expression in a promoter specific manner.

Chapter 6. Discussion

Cell cycle regulated transcription is critical for correct entry into the cell cycle program and also for regulating progression of the cell cycle in response to checkpoint activation. Therefore, cell cycle regulated transcription requires stringent temporal regulation. In this thesis, I have used *Saccharomyces cerevisiae* as a model organism to study the regulatory modules that govern cell cycle entry and how the DNA replication checkpoint impinges on cell cycle regulated transcription. I have characterised a set of specific antibodies useful for further applications in the study of G1/S transcription. I have identified a new class of G1/S genes with a unique regulatory process enriched for cell cycle effectors/DNA damage responders. Furthermore, I identify a correlation of histone modifications that coincides with G1/S gene expression. My findings further support the power of using *S. cerevisiae* as a model organism to study the control of the cell cycle in eukaryotes.

6.1 New antibodies for further study into G1/S regulation in *S. cerevisiae*

Understanding the cell cycle dynamics of Swi4, Swi6 and Mbp1 protein levels, protein-protein binding and protein-DNA interactions will be improved by use of the antisera characterised in Chapter 3. These antibodies have already been used in a study to investigate the role of a conserved region in the G1/S repressors Whi5 and Nrm1 (Travesa et al., 2013). This study shows that the G1/S Transcription factor Binding motif, GTB, is necessary and sufficient for binding of Whi5 to SBF and Nrm1 to MBF. The study uses the antibodies to identify SBF and MBF complexes showing that the identified GTB motif provides specificity to Whi5 and Nrm1 in binding to the Swi6 subunit in SBF and MBF, respectively.

6.1.1 Evolution of G1/S transcription factors

Fission yeast spMBF contains two DNA binding subunits spRes1 and spRes2. spRes2 is related to the budding yeast Mbp1 in its function to repress G1/S

transcription outside of G1/S (Ayté et al., 1997; Baum et al., 1997; Bähler, 2005). Budding yeast and fission yeast are distantly related and through evolution budding yeast has evolved SBF and the presence of SCBs in the promoters of G1/S genes. Yeasts more closely related to budding yeast such as *Kluyveromyces lactis* and *Candida albicans* contain both SBF and MBF homologs, although *C. albicans* does not contain SCB sequences in G1/S promoters (Côte et al., 2009; Hussein et al., 2011; Koch et al., 1993). *Neurospora crassa* and *Yarrowia lipolytica* yeasts contain both Swi6 and Mbp1 yet no homolog of Swi4 and are more closely related to fission yeast (Bean et al., 2001; Dujon et al., 2004; Wolfe, 2006). These five separate yeasts represent different branches of the phylogenetic tree. There is currently a study into the co-evolution of the DNA binding domains of the G1/S transcription factors and the DNA recognition motifs using the antibodies I characterised. By replacing the DNA binding domain of Mbp1 or Swi4, using other yeasts DNA binding domains, the new antibodies are being used to study G1/S promoter binding, protein complex formation and G1/S gene expression in chimera strains.

6.1.2 Dynamics of *SWI4* during the cell cycle

The binding dynamics of myc-tagged Swi6 and Mbp1 during the cell cycle have been previously determined using ChIP analysis followed by qPCR (de Bruin et al., 2006). Mbp1 is bound to its target promoters throughout the cell cycle, as is Swi6, although there is a decrease in their respective binding outside of G1. This is in contrast to the binding of Swi6 to SBF-dependent promoters during the cell cycle, as it is only found bound to promoters during G1. The removal of Swi6 from promoters is thought to occur along with Swi4 in a Clb-Cdc28-dependent manner upon entry into S phase (Amon et al., 1993; Koch et al., 1996; Siegmund and Nasmyth, 1996).

Here I investigated the dynamics of Swi4 binding during the cell cycle using a new specific antibody and found enhanced binding to the SBF-regulated *CLN2* promoter once G1/S transcription was activated. The activation of G1/S transcription is shown by the dissociation of Whi5 from SBF and accumulation of Swi4 protein (Fig. 3.5). As a target of both the M/G1 cell cycle transcription factor Mcm1 and MBF,

SWI4 expression peaks in early G1 and this regulation, in conjunction with Mcm1 driven expression of *CLN3*, is thought to be important for timely activation of G1/S transcription (MacKay et al., 2001; McInerny et al., 1997). Following G1/S transcription, Swi4 is phosphorylated and removed from promoters by Clb-Cdc28 activity, leading to repression of SBF transcripts (Amon et al., 1993; Koch et al., 1996; Siegmund and Nasmyth, 1996). The dynamics of Swi4 following the removal from promoters has not been studied although a recent screen has identified a KENbox within Swi4 that would target it for degradation by the APC during M phase (Jensen et al., 2006). This remains to be studied in detail, however, my data does suggest this could be the case at 70 minutes after alpha factor arrest and release (Fig. 3.5). The new antibodies generated would be able to study the protein levels of Swi4, Swi6 and Mbp1 during the cell cycle

6.1.3 Regulation of Swi4 highlights a fundamental feature of cell cycle entry

Despite periodic expression of *SWI4* largely depending on MBF, early activation at M/G1 involves Mcm1. My data shows that enhanced binding of active SBF to G1/S promoters could be a result of Swi4 accumulation (Fig. 3.5). Since *SWI4* is a target of MBF-regulated G1/S transcription, the Swi4 accumulation and binding to G1/S promoters could further induce G1/S transcription. This would represent a Swi4 positive-feedback loop (Fig. 6.1). Positive feedback loops are a conserved feature of the eukaryotic cell cycle and a similar positive feedback loop has been suggested to be important for G1/S transcriptional activation in mammalian cells (Cross et al., 2011). The mammalian G1/S transcriptional activators E2F1, E2F2 and E2F3a (an E2F3 isoform) accumulate in response to activation of G1/S transcription. These factors then bind to target promoters and drive further G1/S transcription.

In budding yeast, a positive-feedback involved in regulating cyclin expression, where Cln3-Cdc28 leads to transcription of other G1 cyclins *CLN1* and *CLN2* promoting further G1/S transcription, is well established (Skotheim et al., 2008). The potential role of Swi4 in a positive-feedback loop activating G1/S transcription requires additional research. However, it would represent a second positive-

feedback loop involved in robust activation of G1/S transcription that could be conserved from yeast to humans.

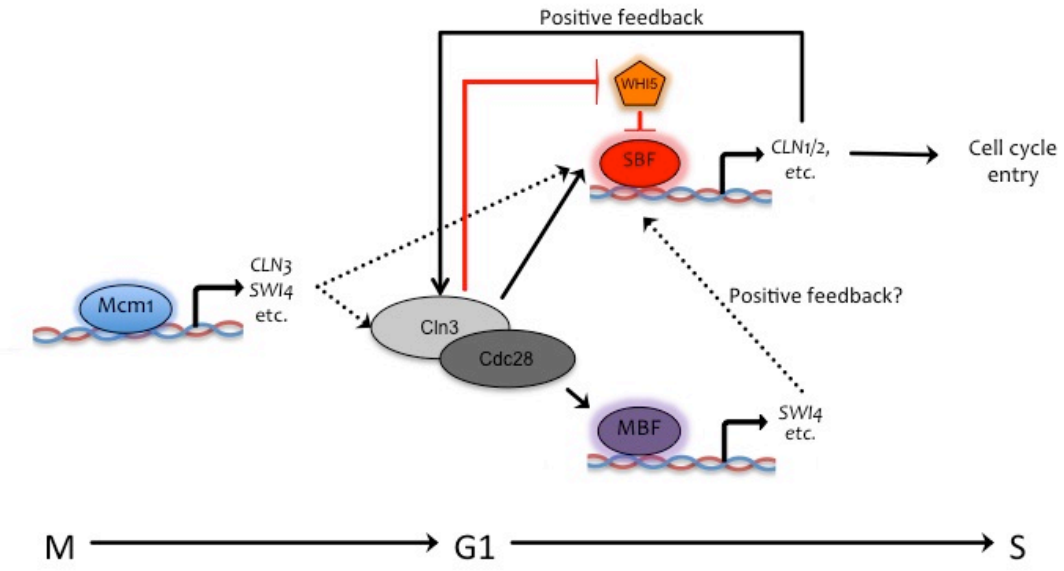

Figure 6.1 Model of SBF transcriptional regulation

Activation of Mcm1-dependent transcription at M/G1 results in the initial accumulation of Cln3 and Swi4. During G1, SBF (Swi4-Swi6) is bound to target promoters in complex with the transcriptional inhibitor Whi5, which represses transcription. Cln3-Cdc28-dependent phosphorylation removes Whi5 from SBF at promoters, activating transcription at START. Transcriptional activation results in SBF-dependent accumulation of Cln1 and Cln2 and further MBF-dependent accumulation of Swi4. Cln1 and Cln2 in complex with Cdc28 is involved in a positive feedback loop to further phosphorylate Whi5, which leads to robust activation of G1/S transcription. Accumulation of Swi4 during G1 coincides with enhanced detection of Swi4 at the *CLN2* promoter, possibly representing an additional positive feedback loop to ensure timely activation of G1/S transcription.

6.2 SBF-to-MBF 'Switch Genes': a new group of G1/S genes

The data presented in Chapter 4 shows the detailed analysis of the transcriptional regulation of the G1/S gene *TOS4*. The data reveals a switching from SBF to MBF transcription factor at the promoter as the cell cycle enters S phase. The overlapping SCB and MCB sites in the promoter of *TOS4* is thought to be the basis for SBF to MBF switching by only permitting binding of one transcription factor at a time. Presumably, there exists a higher affinity of SBF to the SCB element than for MBF to the MCB element during G1, prior to Swi4 being phosphorylated by Clb-Cdc28 during S phase, removing it from promoters (Amon et al., 1993; Koch et al., 1996; Siegmund and Nasmyth, 1996). However, my data shows that even in the absence of Clb-Cdc28 activity, MBF actively displaces SBF at the *TOS4* promoter upon entry into S phase, leading to timely inactivation of SBF-regulated transcription. The overlapping SCB/MCB site again provides the basis for this theory, as the promoter of the G1/S gene *CLN1*, which contains both SCB and MCB motifs that do not overlap, is still expressed in the absence of Clb-Cdc28 activity regulated by SBF (Partridge et al., 1997)(Fig. 4.7). Other features of *CLN1* regulation identify it as part of a SBF and MBF dual-regulated group of genes. My data shows that in *mbp1*Δ cells, *CLN1* expression is repressed during S phase as opposed to being upregulated like other MBF target genes. Also, in wild type cells in response to HU treatment *CLN1* expression is repressed, even with MBF being bound to the promoter throughout the cell cycle, there is no checkpoint-dependent de-repression of *CLN1* expression (Fig 4.4 and 4.5). This shows a clear difference to the 'switch genes' I uncover, such as *TOS4* and *MCD1*, establishing them as a novel group of G1/S genes. In total, when I analysed three screens of G1/S genes I discovered 44 'switch genes' (Harbison et al., 2004; Iyer et al., 2001; Simon et al., 2001) (Table 4.1).

6.2.1 The rationale for an SBF-to-MBF switch in the regulation of G1/S genes

Analysis of the 200+ G1/S promoters identified by a ChIP-chip screen by Iyer et al. (2001), looking for overlapping SCB/MCB motifs resulted in 27 genes being found.

This suggested that switching form SBF-to-MBF might be a common mode of regulation. G1/S genes are segregated based on their role in the cell cycle with MBF genes being largely essential genes involved in DNA synthesis and repair, and SBF genes being non-essential and involved in timing or efficiency of cell-cycle events (Iyer et al., 2001). This is thought to be an evolutionary effect to protect cells in response to transcription factor insult, so that if MBF is mutated, essential genes are expressed and cell cycle timing genes are expressed at a low level in response to SBF malfunction. Therefore, I sought to identify the rationale of switching from one transcription factor to another. My analysis of *TOS4* suggests that G1/S genes that need to be induced in response to the DNA replication checkpoint, but whose overexpression is detrimental to cell cycle progression, would benefit from switching from SBF to MBF. By switching to MBF during S phase, 'switch genes' are repressed via the recruitment of the co-repressor Nrm1. In response to DNA replication stress Nrm1 is inhibited via phosphorylation by Rad53, leading to de-repression of MBF-dependent transcription (Fig. 6.2A). By switching from SBF to MBF during the cell cycle, cells are able to protect themselves, which presumably represents an evolutionary advantage. Should cells experience loss of MBF function, basal levels of *TOS4* expression would normally be increased throughout the cell cycle, as with other genes in *mbp1*Δ cells. However, due to the binding of SBF to the promoter during G1 and the action of the transcriptional inhibitor Whi5, *TOS4* expression is repressed, preventing Tos4 hyper-accumulation and prevention of cell cycle progression (Fig. 6.2B). Supporting this, my data suggest that Tos4 has an important role in the DNA replication checkpoint response, possibly by arresting cell cycle progression, as this is the phenotype when *TOS4* is overexpressed. However, the role that Tos4 mediates in the DNA replication checkpoint remains to be established (Section 1.3.1).

Analysis of the cluster of genes with overlapping SCB/MCB motifs, when compared with genome-wide overexpression screens by Sopko et al. (2006) (Sopko et al., 2006; Yoshikawa et al., 2011), shows that the SCB/MCB overlapping group are enriched for genes that cause cell cycle delay or growth defects when overexpressed. The class of 'switch genes' are also enriched for genes that are transcriptionally induced in response to HU treatment via the Rad53 inhibition of Nrm1.

Together my data reveals that the SBF to MBF switch preferentially regulates dosage-sensitive genes that also play important roles in response to activation of the DNA replication checkpoint as seen with Tos4 and previously with Mcd1 and Swe1 (Covo et al., 2010; Enserink et al., 2006; Liu and Wang, 2006; Russell et al., 1989; Ström et al., 2004). This suggests that other 'switch genes', which do not have any known function, may include other dosage-sensitive genes and/or effectors of the DNA replication checkpoint, and would be an interesting avenue of future research.

6.2.2 Conservation of G1/S transcription factor switching

The data presented here suggest that G1/S transcription factor switching assures that malfunction of any one transcription factor does not result in a loss of the cell cycle programs integrity (Fig. 6.2B). In mammalian cells, switching of the E2F transcription factors at G1/S promoters during the cell cycle seems to be the standard (Bertoli et al., 2013a; Dimova and Dyson, 2005; Moon and Dyson, 2008), suggesting that loss of periodicity is detrimental. Supporting this is the high frequency of genetic alterations involved in E2F-dependent G1/S regulation found in human tumours. This suggests that the proper regulation of the E2F family plays a key role in the prevention of tumour development (Stevens and La Thangue, 2003).

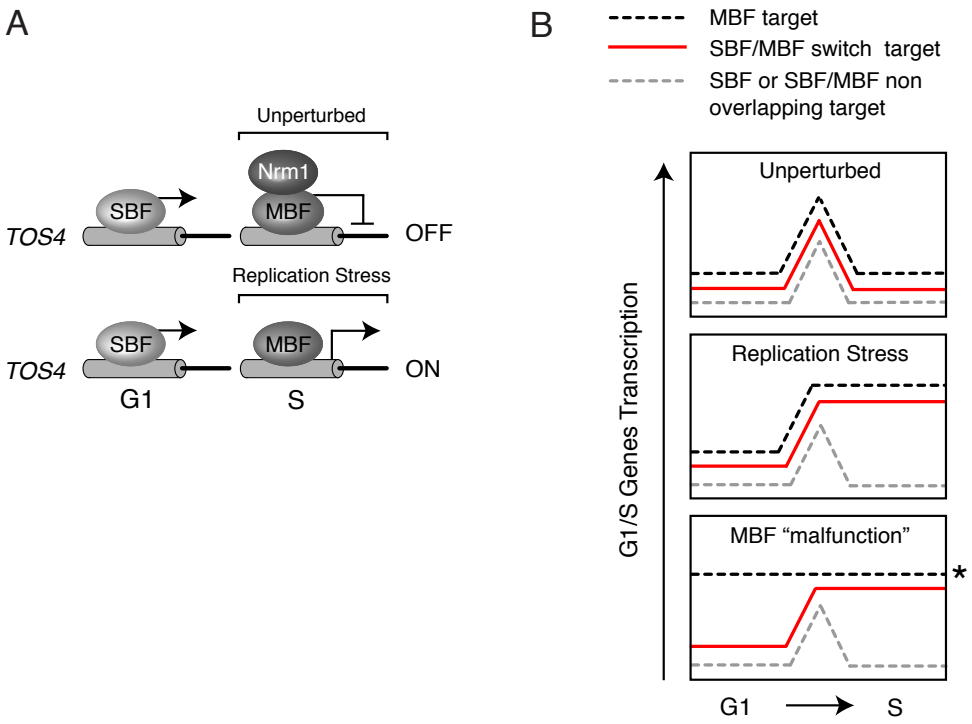

Figure 6.2 The rationale for an SBF to MBF switch at G1/S promoters

A. Regulation of *TOS4* as a prototypical example of an SBF-to-MBF switch gene. *TOS4* transcription is regulated via subunit switching and replication checkpoint signalling. During G1, SBF binds to *TOS4* promoter and activates transcription. Upon entry into S phase, SBF dissociates from the *TOS4* promoter in a Clb-Cdc28-dependent manner leaving the *TOS4* promoter available for binding by MBF and its co-repressor Nrm1. Nrm1 dissociates from MBF in response to replication checkpoint activation leading to de-repression of *TOS4* transcription. **B.** Proposed importance of SBF-to-MBF subunit switching. In an unperturbed cell cycle SBF, MBF and 'switch genes' display normal G1/S transcriptional dynamics. In response to replication stress MBF and 'switch genes' are de-repressed. Should cells experience MBF malfunction, SBF and the associated transcriptional inhibitor Whi5 binding during G1, represses the expression of dosage sensitive 'switch genes' that would cause a loss of periodicity if unregulated. (*) Indicate loss of periodicity in case of MBF 'malfunction'.

6.3 MBF as a link between G1/S transcription and the DNA replication checkpoint

My data in Chapter 4 shows that the specific accumulation of MBF-regulated targets upon replication stress involves Rad53-dependent regulation of the MBF co-repressor Nrm1. This is a feature also identified by another group using a genome-wide transcriptional approach (Travesa et al., 2012). The regulation of the DNA replication checkpoint targeting the co-repressor Nrm1 is a feature conserved in both budding yeast and fission yeast (de Bruin et al., 2008a).

In fission yeast, G1/S transcription is regulated by a single transcription factor complex (Table 1.2), that is also called spMBF based on its homology to subunits of budding yeast G1/S transcription factors and its binding to MCBs. In addition to spNrm1 in fission yeast, spYox1 is also required to repress G1/S transcription outside of G1 phase (Aligianni et al., 2009; Gómez-Escoda et al., 2011). In response to activation of the DNA replication checkpoint in fission yeast, the checkpoint effector protein kinase spCds1 is activated and it phosphorylates and inactivates the spMBF co-repressors spNrm1 and spYox1 (Caetano et al., 2011; de Bruin et al., 2008a; Gómez-Escoda et al., 2011; Purtill et al., 2011).

The data here shows that the mechanism that controls remodelling of the cell cycle transcription program, in response to activation of the DNA replication checkpoint, is conserved between the distantly related yeasts.

6.3.1 Rad53-MBF-Tos4 pathway; a novel branch of the DNA replication checkpoint

Initially, the identification of Tos4 suggested it was an SBF target and a putative transcription factor that repressed genes during the cell cycle and in the presence of alpha-factor (Horak et al., 2002). My data presented here suggest that Tos4 is a crucial effector of the DNA replication checkpoint.

Firstly, the expression of *TOS4* is strongly up-regulated in a Rad53-dependent manner. Second, Rad53 activation of *TOS4* expression leads to the rapid accumulation of Tos4 within the nucleus. Third, overexpression of Tos4 causes cell cycle delay, a feature that is common to cell cycle checkpoints. Fourth, Tos4 contains a FHA domain, commonly found in checkpoint proteins, important for its function during the checkpoint response (Mohammad and Yaffe, 2009) (Fig 4.10C).

Furthermore, the data shown here identifies that Tos4 plays a parallel role to Dun1 in the replication checkpoint. This is supported by the findings that transcription of *TOS4* is regulated in a Dun1-independent manner and that *tos4*Δ*dun1*Δ cells exhibit greater sensitivity to HU compared with the single deletes (Fig 4.3 and 4.10).

Together, my data establishes that the Rad53-MBF-Tos4 pathway represents a novel branch of the DNA replication checkpoint (Fig. 6.3). The exact role of Tos4 remains unclear. However, the ability of Tos4 to interact with Rpd3 and Set3 HDAC complexes, and the importance of this interaction in cell survival in response to replication stress, suggest that Tos4 couples the replication checkpoint to the regulation of HDAC activity.

One hypothesis is that the role of Rpd3 in regulating origin firing is diminished in response to DNA replication checkpoint activation, and this is mediated via the interaction with Tos4. This would support the role of Tos4 in arresting cell cycle progression during S phase as the data suggest when *TOS4* is overexpressed (Fig 4.8).

6.3.2 Conservation of the DNA replication checkpoint regulating transcriptional repressors

My data identifies a simple but elegant mechanism by which checkpoint activation can override the G1/S transcriptional program by directly targeting the transcriptional repressor Nrm1. Nrm1 is involved in a negative auto-regulatory feedback loop that represses MBF-dependent transcription following G1/S upon entry into S phase. This is similar to the inactivation of Crt1, involved in the

regulation of DDR genes, in response to checkpoint activation (Huang et al., 1998). The mechanism of targeting a transcriptional repressor that regulates its own expression and creating a negative-feedback loop means that upon replication stress being resolved, proteins such as Tos4 are rapidly repressed. In the case of dosage-sensitive genes that delay cell cycle progression, it is key to down-regulate expression rapidly. The mechanism of targeting a transcriptional repressor is a conserved feature of the cell cycle checkpoint response from bacteria to yeast and humans.

In human cells, the main regulator of the DNA damage-inducible genes in G1 is the transcription factor p53, which is a target of both ATM and CHK2 (Carr, 2000). p53 regulates the expression of its own negative regulator Mdm2, thus creating a negative-feedback loop. Upon resolving DNA damage, p53 is rapidly shut down by the Mdm2 inactivation of further p53-dependent transcription (Wu et al., 1993). However, in human cells the transcriptional response induced by the DNA replication checkpoint in S phase is regulated in a p53-independent manner through an as yet unknown mechanism. Recent data from our lab has shown that, as in yeast, G1/S transcription regulated by the E2F family of transcription factors is maintained at high levels in a Chk1-dependent manner in response to activation of the DNA replication checkpoint (Bertoli et al., 2013a). In bacteria, a similar mechanism is observed in response to DNA damage during the SOS response. Activation of the SOS response leads to inactivation of the transcriptional repressor LexA which regulates its own expression creating a negative-feedback loop (Butala et al., 2009).

The recent discovery that remodelling of the G1/S cell cycle transcriptional program by the DNA replication checkpoint is conserved from yeasts to humans, establishes it as the largest group of co-regulated genes amongst the DNA replication stress induced targets. This would indicate that the G1/S transcriptional targets comprise important mechanisms to prevent the accumulation of genomic instability and therefore it would be important to establish the mechanism of control in human cells and the importance of this regulation for genome integrity in eukaryotes.

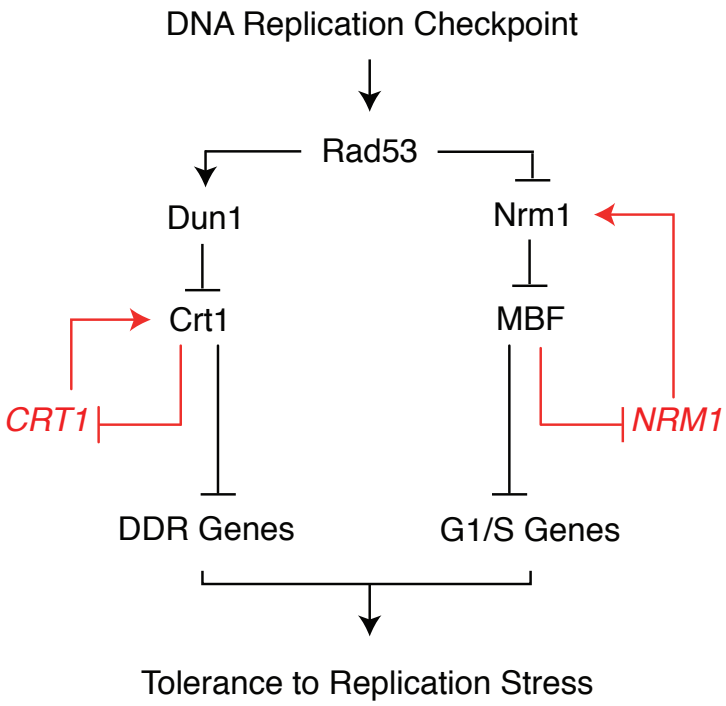

Figure 6.3 Parallel pathways for the replication checkpoint-dependent transcriptional regulation in budding yeast

Upon genotoxic stress, Rad53 de-represses transcription of DNA damage response (DDR) and G1/S cell cycle genes via Dun1-Crt1 and Nrm1-MBF pathways, respectively. Crt1 and Nrm1 negative auto-regulation is highlighted in red.

6.4 G1/S transcription factors modify histones to regulate gene expression.

The data presented in Chapter 5 characterises some of the histone modifications that take place in the promoters of G1/S genes in budding and fission yeast during the cell cycle. In particular my data show that certain histone acetylation marks have promoter specific dynamics and the dependency of transcription factors for mediating modification of certain histone lysine residues.

The data suggest that the transcriptional repressor MBF regulates the cell cycle acetylation of H3K9 to coordinate expression of G1/S genes late in G1. Also the data show that H3K9 acetylation increases in an MBF-independent manner during S phase. Alternatively, the increase in acetylation may be due to the absence of MBF, indicating that deacetylation and the recruitment of HDACs could be MBF-dependent. The data characterises that H3K9 acetylation is the most associated with expression of G1/S genes and that histones at MBF promoters are more acetylated at H3K9 during S phase than SBF promoters. This could be through recruitment of a HAT being during S phase, as suggested by my mbp1Δ data (Fig 5.5). The persistence of H3K9 acetylation at MBF promoters during S phase in the absence of gene expression could be a mechanism that maintains MBF target promoters in a state that is rapidly activated in response to the DNA replication checkpoint and inhibition of the MBF co-repressor Nrm1.

The repression of MBF genes during S phase requires the MBF co-repressor Nrm1. Nrm1 could utilise a HDAC to actively remove acetylation marks on H3K27 residues at a faster rate than at the promoters of SBF genes where the mark persists for longer before decreasing. H3K27 acetylation persisting at SBF promoters could be due to the removal of SBF from the promoter, via Clb-Cdc28 activity, so there is no complex present for HDACs to be recruited to actively deacetylate H3K27. This would suggest that either the loss of H3K27 acetylation is passive, or that nucleosome turnover could be responsible for replacing the acetylated nucleosome with a non-modified nucleosome.

The differences identified between the histone acetylation modifications at SBF and MBF regulated genes, if established with their link to specific histone modifying enzymes, would go some way to completing the mechanism of action of the G1/S transcriptional regulators in budding yeast. In addition, identifying the histone modifications that occur to regulate MBF target expression in response to activation of the DNA replication checkpoint, and if this differs to that of G1/S cell cycle transcription, would also add further insight into the role of integrating cell cycle regulated transcription to the maintenance of genome integrity.

This work leaves many questions unanswered with regards to the mechanism of transcriptional co-regulators in regulating G1/S transcription during the cell cycle and in response to genotoxic stress. However, with insights from other eukaryotes and with the development of new tools and techniques, understanding how the commitment to cell division is regulated can be further understood.

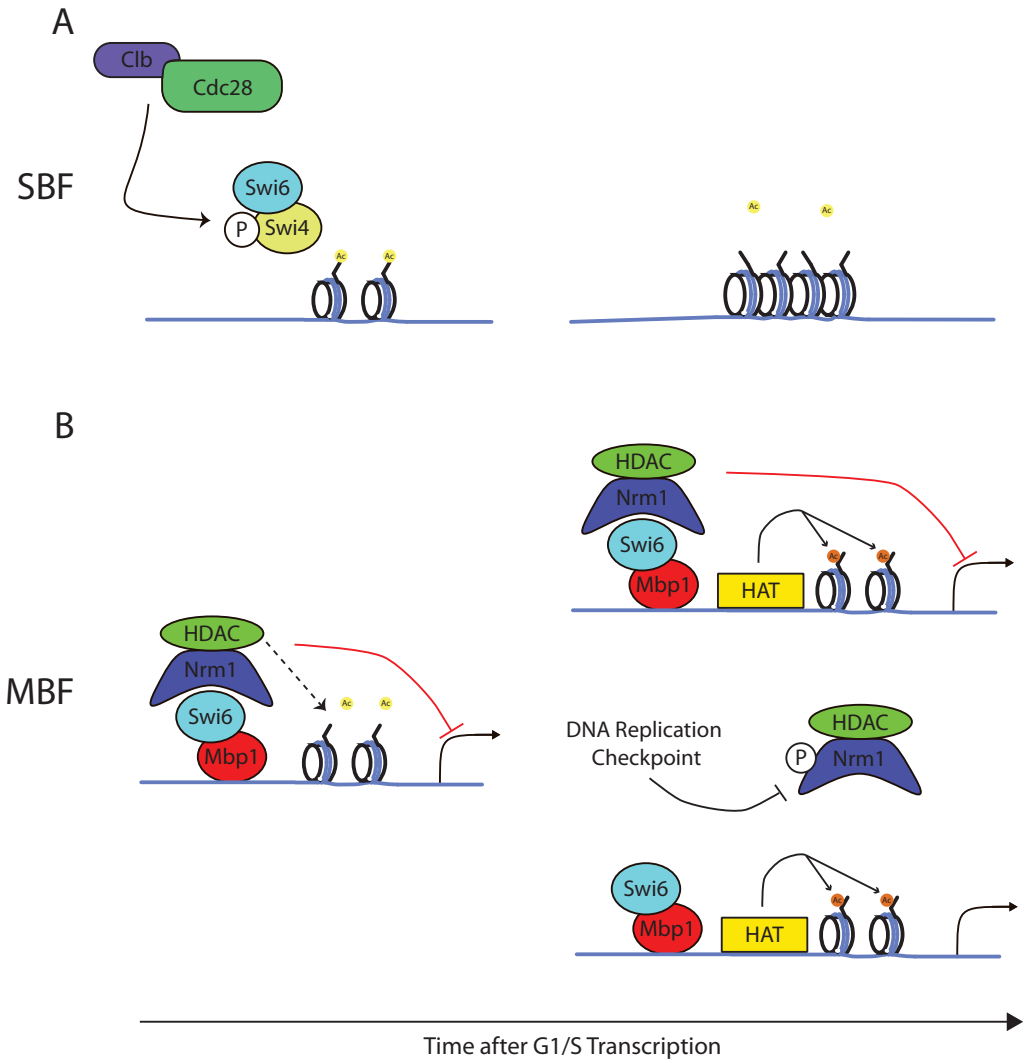

Figure 6.4 Differential deacetylation of SBF and MBF promoters during S phase

A. My data suggest that the loss of H3K27 acetylation from SBF promoters is a passive process compared to the loss of the mark from MBF promoters and the loss of H3K27 acetylation either by dissociation or nucleosome turnover, leads to gene repression. **B.** At the promoters of MBF genes H3K27 acetylation is rapidly removed indicating the action of an unknown HDAC is used by the co-repressor Nrm1 to repress transcription. Secondly, H3K9 becomes acetylated at MBF promoters in an MBF-independent unknown manner. Acetylation of H3K9 is thought to ensure rapid expression in response to replication stress, removing the repression by Nrm1.

6.5 Future experiments

6.5.1 Issues to address

Much of the data presented here was from experiments carried out in budding yeast synchronised by alpha-factor arrest and release. This synchronisation method was used due to the ease of handling the cultures, the length and efficiency of the arrest, omission of specialist equipment and as the cells do not experience a change in temperature. The background strain 15Daub used is optimal for monitoring the expression of G1/S transcription, however, it is not widely used in all budding yeast research. These two factors (synchronisation method and strain) therefore need to be addressed for some of the findings of my work (Futcher, 1999). By identifying the features I observed in my work through other methods and using alternative background strains would add weight to my findings being conserved through all strains of *S. cerevisiae* and other eukaryotes.

6.5.1.1 Cell synchrony experiments

The identified switching from SBF to MBF during the cell cycle, the positive feedback loop involving *SWI4* expression and the histone acetylation dynamics at G1/S promoters are all data that require confirmation by other synchronisation methods. It has been shown that the order of G1/S transcript activation in alpha-factor arrested cells is different to cells arrested at the anaphase to metaphase transition (Eser et al., 2011). Therefore, my data may appear different under alternative synchronisation methods. Other methods of synchronisation include elutriation, which isolates small G1 cells based on size and is considered a more 'natural' method of synchronising cells as no manipulation of the genome or treatment with hormones required. The study by Eser et al. (2011) observed that elutriation and alpha-factor synchronisation had the same profile of G1/S transcript activation, suggesting that alpha-factor arrest is physiologically accurate. The disadvantage of elutriation is the cost of the equipment required and the length of time needed in order to collect a sufficient number of cells for a time course

experiment. Numerous studies also arrest cells via the inhibition of *CDC20*, an APC co-activator, using a galactose-regulated promoter (Di Talia et al., 2009; Eser et al., 2011; Huang et al., 2009; Takahata et al., 2009; Wang et al., 2009). Repression of *CDC20* expression prevents the APC from marking target proteins for degradation, including those of the cohesin complex that holds sister chromatids together. In experiments where cell cycle feedback loops may play a role, arresting the cell cycle using alpha factor could possibly remove or disturb the feedback mechanisms that would otherwise alter my observations. The timing of the SBF to MBF switch at the promoters may also differ under a different synchronisation method and, for example, may precede G1/S transcriptional activation. This would represent a method of repressing MBF-dependent transcription activation by the competition for promoter binding by SBF.

6.5.1.2 TOS4 overexpression

The experiment performed by Dr. Bastos de Oliveira looking into the effect of *TOS4* overexpression requires further clarification. The design of the experiment needs to address the issues of plasmid copy number and the loss of the expression plasmid, leading to detection of wild type cells progressing through the cell cycle. By integrating a GAL inducible promoter at the endogenous loci of *TOS4*, both artefacts can be addressed. By repeating the experiment I would hope to clarify the effects of *TOS4* overexpression on cell cycle progression by FACS and monitoring synchrony of the cells during the experiment with a budding index.

6.5.2 Future directions

The observations reported in my thesis provide many avenues for investigating both G1/S transcriptional regulation and the downstream effects of the DNA replication checkpoint.

6.5.2.1 The function of Tos4

The identification of Tos4 as an essential effector of the DNA replication checkpoint, and the unknown function of Tos4, are the most intriguing. The mechanism of *TOS4* G1/S expression during an unperturbed cell cycle indicate that it may also play a role outside of the DNA replication checkpoint as it is not regulated in the same manner as a DDR gene. This function of Tos4 during a normal cell cycle may also be redundant with the Tos4 paralog Plm2 (Plasmid maintenance 2). The functions of the DNA replication checkpoint mediated through Rad53 include arresting the cell cycle, stabilising replication forks and inhibiting late origins of replication from firing. Tos4 interacts with HDAC complexes in response to genotoxic stress that include the HDAC Rpd3. The Rpd3 HDAC has been implicated in regulating the timing of ARS firing in budding yeast (Aparicio et al., 2004; Mantiero et al., 2011) and therefore the interaction with Tos4 in regulating this action as part of the DNA replication checkpoint remains to be investigated.

6.5.2.2 The function of 'switch genes' in response to the DNA replication checkpoint

The number of 'switch genes' identified from my work includes numerous genes of unknown function (Table 4.1). The enrichment of switch genes being involved in the DNA replication checkpoint response suggest that maybe other mediators of the checkpoint response are amongst the list of 'switch genes', and may include new unknown factors as well as add features to genes of known function. Investigating the function of each of the 'switch genes' provides an intriguing proposition.

6.5.2.3 The mechanism of SBF and MBF dependent repression

My data on the modification of histones during G1/S activation and repression is aimed at understanding the complete mechanism of SBF and MBF in regulating gene expression. In addition to my data in *mbp1*Δ cells, SBF-dependent effects on histone acetylation need to be investigated and the mechanism of Whi5 mediated repression. The high conservation of regulatory modules (SBF-Whi5 and E2Fs-

pRb) and the identified recruitment of HDAC1 by pRb strongly suggest that Whi5 will recruit a class I HDAC in order to repress transcription during G1. Rpd3 has been implicated in repressing SBF and MBF-dependent transcription during G1 (Huang et al., 2009; Takahata et al., 2009). Therefore, the Rpd3-dependent role in histone acetylation levels at G1/S promoters remains to be investigated to completely understand the mechanism of SBF, Whi5, HDAC and histone modifications in repressing transcription until START.

The MBF-dependent mechanisms of repression during G1 and S phase also remain unknown. The identification that Mbp1 is required for either acetylation at START, or deacetylation outside of G1/S, raises interesting questions. Is MBF responsible for the direct recruitment of HDACs during G1 and/or HATs at START? Does the co-repression mediated by Nrm1 involve the recruitment of HDACs? My data also suggest that there is MBF-independent histone acetylation during S phase that has not been previously reported. The acetylation of H3K9 and its correlation to gene activation indicate that the MBF-independent acetylation may be a precursor to DNA replication checkpoint activation. Further work into the mechanism of MBF and Nrm1-dependent repression may be aided by my observations.

Chapter 7. Appendix

Target Gene	Assay	Sequence of forward primer	Sequence of reverse primer
ScACT1	RT	TGATGGTGTTACTCACGTCGTTCC	GCAGTGGTGGAGAAAGAGTAACCA
	ChIP	TTCTCTGTCACCCGGCCTCTATTT	GAGAGA GAG GCGAGTTTGGTTTCA
ScCLN2	RT	TCCCAGGATAGTGATGCCACTGTA	GTACTGCCACGCGGATACATCAAT
	ChIP	GAAATTCGTCCCGCTGAACCTCAA	TAACAGGCTACGCCAAATGTGCTC
ScRNR1	RT	GCTCCATTCAAGGCTTACCAAACG	GAACGATCGGCTGCCATGTTAATG
	ChIP	ACGCGTAAACAGTGTCGGGTAAGT	ACGCGTCCTCTATTCAACACCCAA
ScSWI4	RT	TGACCCTGTGGTCAACTCCATCTT	AGTTATCTTCGTTCCCGGCGAAGT
	ChIP	TTACTCTGTGTACGGTGGCGGAAA	GTACGCAGTCACAATGCGGTTGAA
ScHUG1	RT	GACCAAGGCCTTAACCCAAAGCAA	CGGCAATGATGTTGGCAGAAGGAA
ScRNR3	RT	CCATTTGGCATGTGGGATTGGGAA	AATTTGGGAAGTTGAGGCGGTTGG
ScTOS4	RT	GTTGGCAGAAACGTCACCCAAGTT	ATCACATTGCGAACTATTGCGCCC
	ChIP	TGATGAGAATGAACAGCGGCAGTC	GTGTCGTGGCAGATGTATATGGAC
ScMCD1	RT	TGAGCAAGGAAGAAGGCTAGGTGA	TCTATATTGCCTGCGGGTGCTTCT
	ChIP	GATGATGATTTCATTCCCGGCCTC	GCGTCCCTCCTCGAGTTATTTGTT
ScCDC21	RT	CGGAGATCTTTCCTTGTTGGCAGT	GGGAAAGAAGCTTGTTCGCATGATCC
	ChIP	TCTCGTCTGTAAAGGACGGGATTG	AGGTAGCTGTATAATGACGGG
ScSVS1	RT	AGTTACAGCTGCTGCAGTTACCGA	TGGGTACCGTTGTTAGCAGAACCT
	ChIP	TGGGAAAGCATTCTCCGCTAGAAG	TGAATATCCAATGCGGCGGGAA
ScCLN1	RT	AGCTCGTATTCCACGCCTTTCTGA	TCATGGGCTCATAAACGTCCCAGT
	ChIP	ATTGAGCTGAATGGTGCCAGGT	CACGCTGCCTTTACTCCATTCTCA
ScSWE1	RT	ATAGTAACAACGCTGGCACCTCCA	AAGACGATTCTTGGTAGCGGCAGT
	ChIP	CAACACAGTTGAACATTGGCGTGC	GCGCAAGATGATGTGCAGGATAGT
ScTOS8	RT	AACTTCTTCGCAGCCCGAGTATGA	CATTTACCGGATGAGGTTGTGCCA
	ChIP	GAGGCAGCTGCGCGAAATAAGAAA	AGTCGGAACCGAACTGGGAATGTT
ScHCM1	RT	TGCGCTCATGTCGAAACCACAATC	ATCGGAATGCCTCATCTTGGGTGT
	ChIP	AAACGACATGCCGCGCATAAAGAC	TTGCTGCGAGCTTAGACGACTTGT
ScPCL2	RT	TGTCTCTCGCCATTCTTGAAGCCT	AGACATATTGGACGATGAGCGCGA
	ChIP	AGATCATGAGACGCGAAGCCCTAT	AGCTGGAATGCCGTGACAGATGTA
ScTOS3	RT	AACCTTAGGACTGGTGCTGACAGA	AGCGGTGGATGATGTGAAAGAGGA
	ChIP	TTCGGTTTGCACAACGTCCTTCAG	AAGGCACACCCGCTCTTCGTATAA
ScNDD1	RT	TGGACTCTCCGTCCACCAATTTCA	TTGTTGACCTGGGTTCTCTGTGGT
ScWSC2	RT	TTGTGGTACAGTTGCCTTGTTGGC	ACGGTGGAATAACAGGGTTAGCGT
ScPSA1	RT	CCAAAGCCACTGGTTGAATTCGGT	TGTCAGTAACACCAGCGTTGGCTA
ScYNL278W	RT	ATGAATACGCACATGGGCTCTCCT	ATGGCTGCTGAGGCCTAGTATTGT
ScSRL1	RT	TGCTGCTAACCCTTCATGACTCCA	TTGGGACGCAACCATTGGAAGTTG
ScYKL102C	RT	ACAGCTGCAGCCAGTTGTTCATTC	ACCAACAAGCGCAAGAGATAAGGG
ScCSI2	RT	ACCTTCCAGCGTTTATATCCCGGT	TTCAATGCAATCCATGCACCCGTC
ScRPL18B	RT	CTTTGAAGCAAGAAGGTGCTGCCA	AAAGCAGCAACAGTGGTCTTTGGG

ScSIM1	RT	TCTGGTGCCATCGTGTCTGCTTTA	AAACGTATTTGTACGCAACGGCCC
ScYIL141W	RT	ACTCGAGCCCTGTCTCAAGAATGT	TCTAGTGATGGTGGCTGTTGCAGT
ScTOS1	RT	TTCTGGTAGCTTGGCTCCCTTCAA	TGGAGACAACAGCTTCACCTTCCT
ScYOR342C	RT	GTCCGCTCAAAGTTTGCCCATGAA	AGTGTTGTGCCAATGGAAGGAACG
ScALG14	RT	ATTGAATGGGCCTGGAACATGCTG	TGTCCCTCAATTCTTGCCACTGGA
ScPDR16	RT	AAATAACTGCTGACTTGGTGGCCG	ACCAAGTGCTGTACCTGTCTGTGA
ScYDR222W	RT	TACAGATAAGGAAAGCGGCTGGGT	TGAGCTAACGACCTGACCATGCTT
ScERM6	RT	GGCCAGACCAATCATGAAGAAGCA	ACTCACGCAACCCGCTATATTCCT
ScHHO1	RT	CTTCTGTGAGTGCAACCGCATCAA	TCTTGGCGGTAACAGTAGGCGATT
ScCBF2	RT	ATGTCAACTACTGCATCGCCGTCT	GCATTGGTTGGCGTACTTAGCGTT
ScYPR204W	RT	ACGGCATTCCTGTCGATGCTGATA	ATGTGGTAACAACCACACCTCCGA
ScLAC1	RT	CAAGACCAAGACGCAAGTCTTCCA	GGCATGACGGTAACTTATCTCTCGGA
Spact1	RT	CGCCGAACGTGAAATTGTTCGTGA	TCAAGGGAGGAAGATTGAGCAGCA
Spcdc22	RT	TGCAACGTGTTGAACGTAACGAGC	AGGTAATGAACGACGACCACGGTT
	ChIP	ACTTAAAGTTCGGATGACGCGACG	GTTTGTAAGGTGGTAAATACCGGG
Spcig2	RT	AGGCATTACTGCTCTTCTCATCGC	ACGTTCAGCGACACAGACATCTTC
	ChIP	GGACGATTTCTTTCCCTTTCTTCC	GGGAAATTGAGCGATCGAGAAACAG
Spcdc18	RT	GTAGGCATGCAATTGAACTTGCGG	TCATAGCAGATGTCGCTCGGACAA
	ChIP	GGCATTTCATATCTTTGAGGATGAGTCG	ATGTCGCGTTCAACTCTACGTGTC

Table 7.1 List of primers used in this thesis

RT; Primers used for gene expression analysis by qPCR. ChIP; primers used for ChIP association analysis by qPCR.

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