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G1/S Cell Cycle Regulated Transcription and Genome Stability

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Author's declaration of originality

I, Catia Maria Pereira Fraga Caetano, confirm that the work presented in this thesis is my own. Where information was driven from other sources, I confirm that this has been indicated in the thesis.

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I would like to thank all the members of my lab, past and present, for their help and scientific insights, in particular Rob de Bruin my PhD supervisor. I would also like to thank my PhD group year, my mum and my sister.

Abstract

Initiation of a new round of cell proliferation in eukaryotic cells is associated with coordinated transcriptional activation of genes during the G1-to-S transition. In the fission yeast *Schizosaccharomyces pombe*, genes involved in DNA replication are tightly regulated by the transcription factor MBF during G1/S. Recently, the MBF-associated corepressors Nrm1p and Yox1p were shown to have a non-redundant role in a negative feedback loop mechanism to repress MBF-dependent transcription outside of G1. Activation of the DNA replication checkpoint results in persistent expression of MBF-dependent genes. Here we show that in response to DNA replication stress both Yox1p and Nrm1p dissociate from MBF at promoters, leading to de-repression of MBF targets. Inactivation of Yox1p is an essential part of the checkpoint response. Cds1p checkpoint protein kinase-dependent phosphorylation of Yox1p promotes its dissociation from the MBF transcription factor. We establish that phosphorylation of Yox1p at Ser114 and Thr115 is required for maximal checkpoint-dependent activation of the G1/S cell-cycle transcriptional programme. G1/S transcription includes many genes required for replication and recovery of stalled replication forks. Although persistent expression of the G1/S transcriptional programme is an important part of the DNA replication checkpoint response we show that constitutive expression of the same group of genes outside of G1 and S is detrimental for cells undergoing normal cell cycle progression. Cells abrogated for *nrm1*+ and *yox1*+ experience increased genomic instability. We demonstrate that a delicate balance exists between levels of DNA replication initiation factors and CDK activity in these cells. Disruption of this balance induces further genome instability, a hallmark of cancer development.

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List of Abbreviations

APC – Anaphase promoting complex

ATM – Ataxia telangiectasia mutated

ATP – Adenine tri-phosphate

ATR – ATM- and Rad3-related

CAK(s) – CDK-activating kinase (s)

CDK(s) – Cyclin dependent kinase(s)

CDKI(s) – Cyclin dependent kinase Inhibitor(s)

ChIP – Chromatin immunoprecipitation

DNA – Deoxyribonucleic acid

DSBs – Double strand breaks

FACS – Fluorescent-activated cell sorting

Fkh2 – Forkhead 2 transcription factor

G1 – Gap phase 1

G2 – Gap phase 2

HR – Homologous recombination

HU - Hydroxyurea

M – Mitosis

MBF – *MluI* cell cycle box transcription factor

Mbx1 – MADS-box transcription factor

MCB – *MluI* cell cycle box

MRN complex - Mre11–Rad50–Nbs1 complex

ORIs – Origins of replication

ORC – Origin recognition complex

PCR – Polymerase chain reaction

RNA – Ribonucleic acid

RNR – Ribonucleotide reductase

RT-qPCR – Reverse transcriptase quantitative PCR

S – DNA synthesis

S. cerevisiae – *Saccharomyces cerevisiae*

S. pombe – *Schizosaccharomyces pombe*

SDS-PAGE – Sodium dodecyl sulfate – polyacrylamide gel electrophoresis

Sep1 – Forkhead transcription factor Sep1

ssDNA – Single stranded DNA

SSBs – Single strand breaks

YES – Yeast extract

EMM – minimal medium

WCE – Whole cell extract

Nomenclature

All strain names are written in italic.

Genes and RNA transcripts are written in small italic letters followed by a plus symbol: e.g. *nrm1+*.

Proteins start with a capital letter and end with a small letter p: e.g. Nrm1p.

Deletion mutants are indicated by the symbol Δ followed by the name of the gene deleted in italic: e.g. *Δnrm1*

1. Introduction

1.1 – The cell cycle of the model organism *S. pombe*

1.1.1 – Temporal and spatial organisation of the cell cycle of *S. pombe*

In order to divide cells must go through the mitotic cell cycle during which they replicate their DNA and separate their replicated chromosomes into two separate cells. The cell division cycle is an extremely important process that is linked to a wide range of physiologic (e.g. cell growth, embryonic development, tissue hyperplasia) and pathologic processes (e.g. cancer, Alzheimer's disease, some cardiovascular diseases) (Zhivotovsky and Orrenius, 2010). As observed for most eukaryotic cells, the mitotic or cell-division cycle of the fission yeast *Schizosaccharomyces pombe* is divided into two major periods known as interphase and mitotic phase (Figure 1.1A). Interphase, the stage whereby cells grow, replicate their DNA and prepare to undergo mitosis is further divided into three phases, gap phase 1 (G1), DNA synthesis (S) and gap-phase 2 (G2). The mitotic phase encompasses a process known as mitosis (M), the mechanism through which cells segregate their duplicated chromosomes into two daughter cells (Morgan, 2007). Cells resulting from mitosis are identical to each other and to their parental cell as they share a common genetic background. Mitosis can be further differentiated into five subphases: prophase, prometaphase, metaphase, anaphase and telophase. Once cells divide their nucleus and remaining cytoplasmic components into two identical cells, a mechanism termed cytokinesis separates the newly formed cells, which are then ready to enter a new cycle (Tyson and Novak, 2008).

Vegetative cycle - Unlike mammalian and other fungi cells such as *Saccharomyces cerevisiae* cells, which spend most of their time in G1 phase, wild-type *S. pombe* cells exist mainly as G2 cells and they can take up to 3h to complete a full cell cycle (Gómez and Forsburg 2004). As a result of the short G1 period in fission yeast cytokinesis is not completed until around the end of S phase. A direct consequence of this is that *S. pombe* haploid cells in S phase are binucleate cells with 2C-4C DNA content. Once a binucleate S phase cell divides by medial fission each replicating nucleus is packaged into a separate cell surrounded by a distinct cell wall and at the end of S phase

cells emerge as 2C DNA content G2 phase cells, ready to grow by polarised tip elongation, before entering another round of mitosis (Figure 1.1A). Given their extremely short G1 and S phases, fission yeast cells exert a high degree of control over their cell cycle, nutrition and cell growth at the G2/M transition (Forsburg and Nurse, 1991) (Egel, 2004).

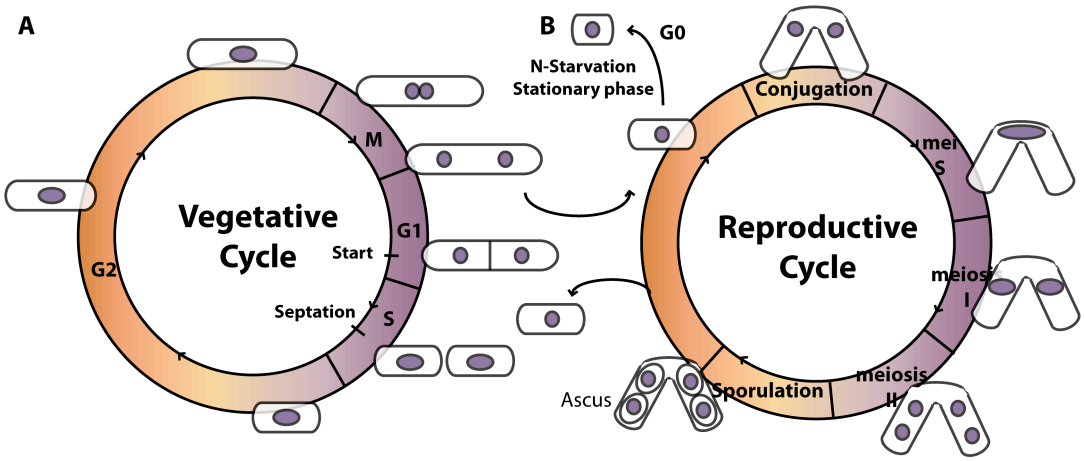

Figure 1.1 – The life cycle of *S. pombe*

(A) Wild type vegetative *S. pombe* cells undergoing exponential growth spend 70% of their cell cycle in G2 and 10% in each of the remaining phases. Note the equivalent to the mammalian restriction point, START, and septation, immediately before or during S phase, respectively. The nuclear envelope of *S. pombe* does not disassemble during mitosis. Instead, the microtubules necessary to form the mitotic spindle invade the intranuclear space. (B) In nitrogen-poor environments, haploid *S. pombe* cells can either enter stationary phase (small G0 phase cells) or pair up with a partner of opposite mating type and produce a resistant tetrad ascus. Each ascus segregates its spores in a 2:2 ratio fashion. Thus, fission yeast is easy to manipulate using Mendelian and classic genetics. In glucose deprived environments cells arrest in G2 [1].

Reproductive cycle - The life cycle of this unicellular eukaryote alternates between vegetative asexual division and sexual reproduction stages, depending upon nutrient availability. In carbon- and nitrogen-rich environments *S. pombe* cells are fast replicating haploid rod shape cells that divide by asexual division. When nutrients are in short supply, in particular that of nitrogen sources, and partners of the opposite mating type (termed

[1] Adapted from Gómez and Forsburg, 2004.

minus M or plus P) are present, two cells pair up and fuse with each other through a process known as conjugation (Figure 1.1B). The organism enters its sexual reproductive cycle. Conjugation is followed by nuclear fusion (karyogamy) and transient formation of a diploid zygote that following a round of meiosis develops into an elongated tetrad ascus composed of four haploid nuclei, the ascospores. When nutrients are replenished each dormant ascospore has the ability to germinate and enter the vegetative cycle at G1 phase (Egel, 2004).

1.1.2 –Regulation of cell cycle progression

In the recent past, much effort has been made to understand the molecular and biochemical mechanisms underlying the cyclic nature of the cell cycle. The extraordinary coordination of events that occur throughout the cell cycle is at present explained via the "clock + checkpoint" theory. The first part of this model, the clock, is understood as the temporal "cyclic sequence of states" that occur during the cell cycle and the second part, the checkpoints, as the processes necessary to ensure that the cell cycle progresses faultlessly (Tyson and Novak, 2008) (see section 1.2 and 1.5).

The master regulators governing progression through the different stages of the cell cycle, the Cyclin Dependent Kinases (CDKs), were first discovered through the pioneering work of three independent scientists who have won the Nobel Prize in Physiology or Medicine: Paul Nurse (in *S. pombe*), Tim Hunt (in sea urchin and clam eggs) and Leland H. Hartwell (in *S. cerevisiae*)[2]. CDKs are part of a complex regulatory network that is highly conserved across species and consists of many biochemical molecules including CDKs, cyclins, CDK inhibitors (CDKIs), CDK-activating kinases and phosphatases (CAKs) (Schafer, 1998). It is the periodic accumulation, inhibition and proteolytic destruction of these proteins that confers the cell cycle with its cyclic quality.

Mammalian cells present various CDK protein kinases and cyclins, however,

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[2] "The Nobel Prize in Physiology or Medicine 2001". Nobelprize.org. 3 Jul 2012 http://www.nobelprize.org/nobel_prizes/medicine/laureates/2001/

in *S. pombe* only one CDK, Cdk1 (also known as Cdc2), capable of driving both G1/S and G2/M transitions, and four cyclins, Puc1, Cig1, Cig2 and Cdc13, have been identified. Cdk1 is the master regulator of the fission yeast cell cycle (Nurse *et al*., 1976).

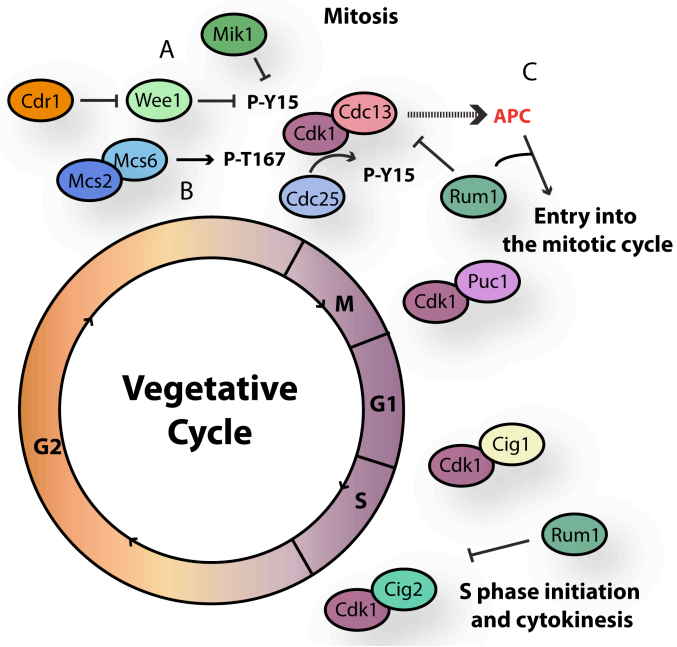

Figure 1.2 – Key regulators of the mitotic cycle of *S. pombe*

Progression through the vegetative cell cycle of *S. pombe* is secured by a unique Cdk protein, Cdk1. Cdk1 binds to cyclins Puc1, Cig1, Cig2 and Cdc13 to promote, passage through G1, G1/S, S and M phases, respectively. Mitosis is a highly regulated event. In order to ensure the correct timing of M, the Cdk1-Cdc13 complex is the target of an intricate regulatory phosphorylation system. Inactivation of Cdk1-Cdc13 by Y15 phosphorylation via the CDKI Wee1 during G1 and G2 phases, respectively, plays a role of extreme importance in preventing early entry into mitosis. Note that Rum1 can also inhibit Cdk1-Cig2 function outside of S and M phases. (B) In order to achieve its activity peak and promote chromosome segregation, Cdk1-Cdc13 must suffer both T167 phosphorylation and Y15 dephosphorylation. Whilst phophorylation at residue T167 is achieved via the CAK Mcs6-Mcs2, Y15 dephosphorylation is undertaken by the phosphatase Cdc25. (C) Start of a new cycle and entry into G1 requires inactivity of the Cdk1-Cdc13 complex which is attained via Rum1-dependent inhibition of Cdk1-Cdc13 and via APC-dependent destruction of cyclin Cdc13.

Succinctly, the mitotic cell cycle of *S. pombe* is thought to be regulated as follows (Figure 1.2) Cdk1 binds to cyclin Puc1 to promote mitotic cell division

and entry into G1 (Forsburg and Nurse, 1994). Subsequent accumulation and binding of Cdk1 to Cig1 during G1/S and then to Cig2 in S, promotes DNA replication initiation and cytokinesis (Connolly and Beach, 1994) (Mondesert *et al*., 1996). Then Cdk1 binding to the G2/M cyclin Cdc13 inhibits re-initiation of DNA synthesis and drives passage into M (Fisher and Nurse, 1996). Finally, degradation of Cdc13 via APC-ubiquitin-mediated proteolysis and re-accumulation of Puc1 throughout M leads to segregation of the sister chromatids, promotes entry into G1 and the beginning of a new cycle (Martín-Castellanos *et al*., 2000). The activity of the various Cdk1-cyclin complexes is regulated extensively by phosphorylation. Phosphorylation at T167 via the CAKs Mcs6-Mcs2 and Cak1 is crucial for Cdk1 kinase activity, whereas Y15 phosphorylation by the CDKIs Wee1 and Mik1 right before M inhibits its function. Furthermore, entry into mitosis requires dephosphorylation of Cdk1 at Y15 by the phosphatase Cdc25 (Figure 1.2). Some of these factors, including CDKIs and cyclins are further regulated by E3-mediated ubiquitination and proteasome-dependent proteolysis via either or both the APC and SCF. Finally cell cycle regulated transcription is required for the timely accumulation of many of the proteins involved, including but not limited to the cyclins Cig1 and Cig2, the CDKI Mik1 and components of the SCF (Bähler, 2005).

The widely accepted idea is that progression through the cell cycle is driven by the formation of complexes of Cdk1 with the different and phase specific cyclins described above. Interestingly, a recent study shows that *S. pombe* cells engineered to transcribe Cdk1-Cdc13 as a fused, monomolecular molecule, are able to drive their cell cycle almost identical to their wt counterparts, in the absence of all the remaining interphase cyclins. This suggests that oscillation of a single CDK-mitotic cyclin module is sufficient to activate many of the events that characterise the mitotic cell cycle, depending upon its activity and abundance status (Coudreuse and Nurse, 2010). In line with this hypothesis a different study undertaken in mouse embryos shows that all interphase Cdks (and possibly all interphase cyclins) are likely to play a redundant role in regulating the major cell cycle events, as ablation of these molecules does not affect organogenesis and development until midgestation (Santamaría *et al*., 2007).

1.2 – Periodic gene expression as a driving force of cell cycle progression

Cell-cycle dependent gene expression (also known as cell-cycle dependent waves of transcription) plays an important role in driving the eukaryotic cell division cycle. These waves of transcription, activated at different time during the cell cycle, encode proteins required for tasks in subsequent phases of the cell cycle. Hundreds of genes - 400 to 900, depending on the study - that are expressed as a function of cell cycle progression have been identified in fission yeast using genome-wide technology (Rustici *et al*., 2004) (Peng *et al*., 2005) (Oliva *et al*., 2005) (Marguerat *et al*., 2006). At least 800 genes displaying similar oscillatory behaviour have been reported for *S. cerevisiae* (Spellman *et al*., 1998). In fission yeast, as in most eukaryotes, periodically transcribed genes can be clustered into three main waves, each regulated by one or more specific transcription factors: G1/S (DNA replication), regulated by the MBF transcription factor complex; G2/M (entry into mitosis) regulated by the transcription factors Sep1p, Fkh2p and Mbx1; and M/G1 (exit from mitosis) regulated by the Ace2p transcription factor (Bähler, 2005). This project focuses on the G1/S wave of transcription in *S. pombe*, which regulation is undertaken by the transcription factor complex MBF.

1.3 – The G1/S transcriptional programme in *S. pombe*

Once yeast cells commit to the mitotic cell cycle, passing a point in G1 called START, DNA synthesis initiation becomes inevitable, irrespective of treatment with growth-stimulatory signals (Simanis *et al*., 1987). Passage through START and entry into mitotic S phase in *S. pombe,* is largely dependent on the expression of a group of genes tightly regulated by the *MluI* Cell Cycle Box (MCB) Binding Factor (MBF).

1.3.1 – The "anatomy" of the MBF transcription factor complex

The core components of the fission yeast MBF (Figure 1.3A) include two homologous DNA-binding, zing-finger proteins termed Res1 (aka Sct1) (Tanaka *et al*., 1992) (Caligiuri and Beach, 1993) and Res2 (aka Sct2) (Miyamoto *et al*., 1994) and the product encoded by the START gene *cdc10*+ (Aves *et al*., 1985) (Zhu *et al*., 1997). In budding yeast G1/S transition requires the activity of at least two heteromeric transcription factors, the MBF and the SBF (Horak *et al*., 2002). MBF and SBF are each composed of a Swi6 molecule (homologous to Cdc10) and one of two DNA-binding partners, Mbp1 and Swi4 (homologous to Res2 and Res1, respectively). Although there is no sequence or structural homology between the yeasts' MBF and SBF and the E2F family of transcription factors that regulate G1/S transcription in mammals, recent data suggests that the mechanism of regulation is conserved (Cooper, 2006). As a result, studying G1/S transcriptional regulation in yeast may contribute to the understanding of G1/S transcription regulation in higher eukaryotes, including humans (de Bruin and Wittenberg, 2009).

It is well established that conditional abrogation of Cdc10 prevents cells from entering S phase (Nurse *et al*., 1976) (Tanaka *et al*., 1992). Until recently, it was thought that MBF-dependent gene transactivation would take place via a partially redundant subunit switch mechanism in which Cdc10 would cluster independently with either Res1 (Cdc10-Res1 complex) or Res2 (Cdc10-Res2 complex) in order to regulate, primarily, entry into mitosis or meiosis, respectively (Tanaka *et al*., 1992) (Miyamoto *et al*., 1994). New data obtained

from electrophoretic mobility shift assays (EMSA) and time-course co-immunoprecipitation (Co-IP) experiments led to the development of an alternative model. In this model, Res1 and Res2 are hypothesised to form a heterodimer complex and bind constitutively to Cdc10 via their carboxy-terminal regions and to MCB-containing promoters via their amino-terminal ends (Zhu *et al*., 1997) (Baum *et al*., 1997) (Whitehall *et al*., 1999). Thus, Cdc10, Res1 and Res2 are thought to regulate G1/S transcription as a single integrated complex that switches between an active (during G1) and an inactive (outside of G1) state, rather than functioning as two independent transcription factor complexes. Four lines of evidence support this model: 1) the steady-state transcript and protein levels of Cdc10, Res1 and Res2 remain constant throughout cell cycle progression, (Figure 1.3B) (Simanis and Nurse, 1989) (Whitehall *et al*., 1999); 2) Res1, Res2 and Cdc10 form a high DNA-binding affinity complex, as detected by EMSA (Zhu *et al*., 1997); 3) MCB binding by the MBF is detectable in cell extracts deriving from all cell cycle phases, although it is only active during G1/S (Reymond *et al*., 1993) (Ayté *et al*., 1995); 4) the MBF remains constitutively associated to MCB-containing promoters as demonstrated by chromatin-immunoprecipitation (ChIP) analyses (Wuarin *et al*., 2002).

MBF regulates the expression of over 40 putative target genes, encoding proteins that are predominantly involved in DNA synthesis, DNA repair and cell-cycle control (Rustici *et al*., 2004). The promoter region of this cluster of genes is characterised by the presence of one or more cis-acting MCB1 (ACGCGT) or MCB2 (CGCGACGCGT) elements (Figure 1.3A) that serve as the platform for MBF binding (Lowndes *et al*., 1992). Some very well established MBF targets are *cdc22+* (Lowndes *et al*., 1992), *cdc18+* (Kelly *et al*., 1993) and *cdt1+* (Hofmann and Beach, 1994), which encode the large subunit of the ribonucleotide-diphosphate reductase (RNR - a key enzyme in the anabolism of deoxyribonucleotides) and two independent replication origin licensing factors, respectively (Table 1.1 for a description of the main MBF-dependent transcripts).

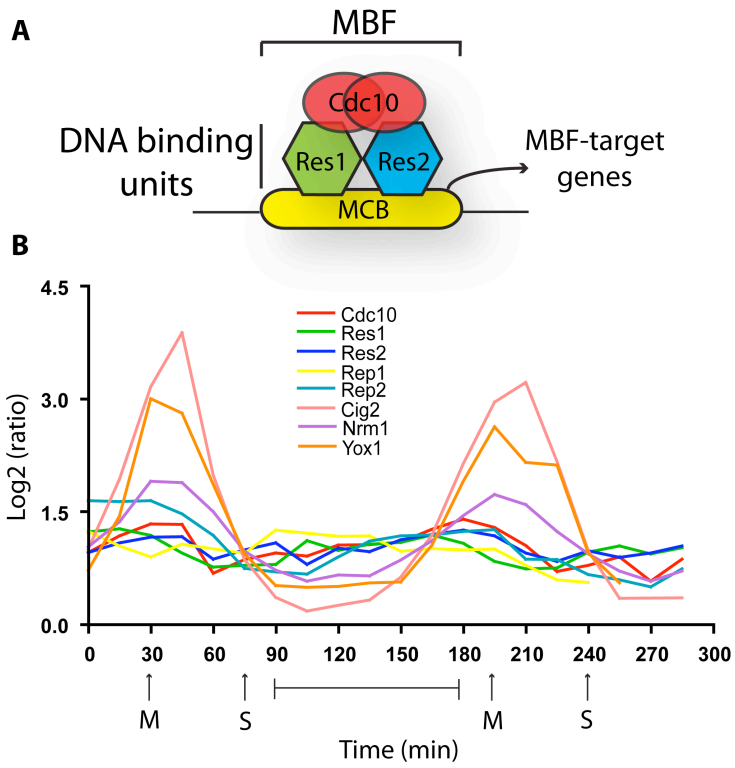

Figure 1.3 – The anatomy of the MBF transcription factor

(A) Main components of the MBF transcription factor. (B) Expression levels of the main components of the MBF and its regulator proteins throughout the cell cycle. Adapted from Rustici *et al* (2004).

The initial characterization of *res1+* and *res2+* null mutants revealed a downregulation and an upregulation of mRNA levels of *cdc18+*, *cdc22+*, *cdt1+* and *cig2+*, respectively (Zhu *et al*., 1997) (Baum *et al*., 1997). Whilst ectopic expression of Res1 enhances expression of *cdc18+*, *cdc22+*, *cdt1+* and *cig2+* transcripts, with loss of periodicity, ectopic expression of Res2 induces a slight repression of the same MBF-target transcripts (Ayté *et al*., 1995) (Baum *et al*., 1997) (Ayté *et al*., 2001). Taken together, these results suggest that Res1 may behave as a positive regulatory subunit of the MBF and Res2 as a repressor. Recent evidence has challenged this view, suggesting that Res2 may also play a positive and Res1 a negative regulatory role in MBF activity for certain targets (Whitehall *et al*., 1999) (Dutta *et al*., 2008).

Table 1-1 - Genes regulated by the transcription factor MBF

MBF Gene	Role and degradation
Cdc18	Replication licensing factor – Mcm2-7 loader; degraded via the SCF in a CDK-Cig2 dependent manner (Lopez-Girona *et al*., 1998) (Baum *et al*., 1998).
Cdt1	Replication licensing factor – Mcm2-7 loader; degraded via the SCF in a Cdt2- and Ddb1-dependent manner (Ralph *et al*., 2006).
Cdc22	Ribonucleoside reductase large subunit
Suc22	Ribonucleoside reductase small subunit
Nrm1	MBF co-repressor degraded via the APC (unpublished data)
Yox1	MBF co-repressor
Cig2	G1/S-specific B-type S-phase cyclin; degraded via the SCF in a Pop1- and Pop2-dependent manner (Yamano *et al*., 2004)
Mrc1	Mediator of checkpoint protein 1
Ams2	Cell cycle regulated GATA-type transcription factor
Mug40/polk	DinB translesion DNA repair polymerase
Psm3	Mitotic cohesin complex subunit
Rhp51	RecA family recombinase
Mik1	Mitotic inhibitor kinase Mik1
Rad21	Mitotic cohesin complex, non-SMC subunit
Ste9	CDK inhibitor
Ssb1	DNA replication factor A subunit
Pfh1	5 prime to 3 prime DNA helicase, involved in DNA recombination and repair
Pol1	DNA polymerase alpha catalytic subunit
Pof3	F-box protein
Cdt2	WD repeat protein – involved in the degradation of Cdt1

1.3.2 – Regulation of MBF transcription

The mechanism by which MBF-dependent transcription is confined to G1 has only recently been established in both *S. cerevisiae* and *S. pombe*. In fission yeast, MBF activity is modulated by at least four co-factors: the co-activators Rep1 and Rep2 and the co-repressors, Nrm1 and Yox1. The role of Rep1 and Rep2 in regulating periodic expression of MBF targets is still poorly understood. Rep1 is necessary for activation of premeiotic DNA synthesis and highly induced following nitrogen starvation (Sugiyama *et al*., 1994). Rep2, seems to be required for maximal transcriptional activity during the mitotic cell cycle, possibly by mediating inactivation of the repressor Res2, as suggested by biochemical and genetic evidence (Nakashima *et al*., 1995) (Baum *et al*., 1997) (Tahara *et al*., 1998). However, recent studies have uncovered that MBF-mediated mitotic transcription is restricted to the G1 phase of the cell cycle, mainly, by an auto-regulating negative feedback loop involving the products of the MBF targets *nrm1+* and *yox1+*. Nrm1 and Yox1 regulate normal cell cycle MBF activity via a double non-redundant transcriptional repression mechanism (Aligianni *et al*., 2009). Deletion of *nrm1+*, *yox1+* or both results in constitutive elevated levels of MBF-target transcripts (de Bruin *et al*., 2006) (Aligianni *et al*., 2009). The current view on the mechanism governing MBF-dependent cell cycle transcription is as follows (Figure 1.4): 1) activation of MBF-dependent transcription results in the accumulation of Nrm1 and Yox1 molecules synthesised *de novo* in late G1, resulting in binding and subsequent repression of MBF-dependent transcription as cells progress into S-phase 2) Nrm1 and Yox1 remain bound to MBF throughout S-, G2- and M-phases to repress MBF activity; 3) as cells enter G1, Nrm1 and Yox1 are degraded, relieving transcriptional repression of MBF transcripts and allowing DNA synthesis to occur (de Bruin *et al*., 2006) (de Bruin *et al*., 2008) (Aligianni *et al*., 2009). Together these events limit MBF activity to a very short time interval so that activation of genes necessary for DNA replication occurs once and only once per cell cycle. The mechanisms by which Nrm1 and Yox1 are targeted for destruction during entry into interphase or how they induce repression of MCB-containing genes remain elusive. Beside its role in normal cell cycle progression Nrm1 has also been implicated in the DNA replication checkpoint transcriptional

response. In response to DNA replication stress Nrm1 is inactivated via phosphorylation by the DNA replication checkpoint kinase Cds1 (de Bruin *et al*., 2008). This results in de-repression of G1/S transcription during the checkpoint response. Part of the work described here shows that Yox1 is also involved in maintaining the MBF transcription programme in response to replication stress.

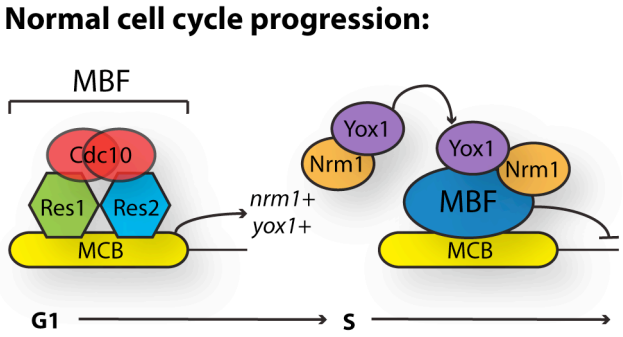

Figure 1.4 – The regulation of the MBF transcription factor

Inactivation of MBF-dependent transcription during late S phase of the normal cell cycle is dependent on binding of the co-repressors Yox1 and Nrm1 to the core components of the MBF transcription factor. MBF-dependent transcription is turned OFF during the G1 to S transition by a double non-redundant negative feedback mechanism involving Nrm1 and Yox1.

1.4 – DNA replication in *S. pombe*

Genomic instability is a threat to cell survival and a driving force of human diseases, including cancer. To prevent genomic instability cells must duplicate their chromosomes once and only once per cell cycle. This requires a high degree of coordination and accuracy (Maric and Prioleau, 2010). DNA replication is in general a highly conserved mechanism. DNA molecules are long polymers of adenine, thymine, guanine and cytosine deoxyribonucleotides, arranged as an anti-parallel double helix, with phosphate groups facing outwards and bases inwards (Watson and Crick, 1953). During DNA replication, the double-stranded DNA template unwinds at the sites of replication initiation, with the help of the Mcm2-7 (minichromosome maintenance) helicase. DNA replication undertaken by DNA replication polymerases initiates bidirectionally from the replication fork, in the 5 prime - 3 prime direction, a process known as elongation. Sites of replication initiation are known as origins of replication (ORI) and the complex of proteins taking part in this process together with the DNA to which they are bound to as the replication fork or the replisome. Whilst replication of the leading strand occurs in a linear and continuous fashion, due to the energetic requirements of 5 prime - 3 prime synthesis, synthesis of the lagging strand occurs via the formation of short discontinuous sections called Okazaki fragments (Ge and Blow 2009). Fork speed fluctuates along the chromosomes depending on genome primary structure and chromatin architecture.

For the purpose of this report we will focus our attention on the early events of replication, the mechanisms of replication initiation. This involves origin licensing, the transcriptional programme necessary for DNA replication initiation, the role of the cell cycle in regulating replication initiation events and the biological consequences of rereplication and endoreplication.

1.4.1 – DNA replication origins in *S. pombe*

DNA replication initiates at regions known as origins of replication (Miyabe *et al*., 2009). Contrary to prokaryotic cells, which present well-defined single sites of replication, eukaryote cells have hundreds if not thousands of ORIs

that fire asynchronously during S phase (Robinson and Bell, 2005). This increase in the number of origins verified in eukaryotes is directly correlated to genomic size, as it helps minimise the time necessary for DNA replication of larger genomes.

Although they lack a general recognisable sequence motif, *S. pombe* origins are generally 500-1000bp-long AT-rich regions, located in intergenic stretches of the DNA. Based on this feature, 384 potential origins have been identified in fission yeast, using genome-wide bioinformatics analysis (Segurado *et al*., 2003). According to a different genome-wide study, *S. pombe* may contain a total of 503 strong and weak putative ORIs interspersed every 14kb across the genome and divided into early and late origins, depending on the time at which they fire throughout the cell cycle (Heichinger *et al*., 2006).

Extensive research shows that DNA replication initiation in fission yeast and other eukaryotes such as *Xenopus laevis* and *Drosophila melanogaster*, is a stochastic event (Nishitani and Lygerou, 2002) (Robinson and Bell, 2005). At least half of the intergenic stretches have the potential of functioning as an ORI, depending on AT average content and length, thus suggesting a rather promiscuous DNA binding consensus system. This model diverges from the classical replicon model suggested for prokaryotes and budding yeast (Dai *et al*., 2005) (Robinson and Bell, 2005). In line with this data, the fission yeast origin of replication complex subunit protein Orc4, which binds ORIs, contains nine AT hooks, suggesting a high affinity towards AT-rich lengthy regions (Chuang and Kelly, 1999). In this scenario, the greater the AT content and the length of a region the greater its binding affinity. However, another study, undertaken by Cotobal *et al*, suggests that stretches as short as 30bp can determine an active ORI (Cotobal *et al*., 2010).

If the stochastic model view is correct then a problem arises, the “random completion problem”. According to this theory, replication of large genomic regions with few or no origins may be prone to be missed out by chance, resulting in gaps that would take longer than usual to replicate, through passive replication arriving from distantly located origins (Sclafani and Holzen, 2007). Two solutions have been suggested to resolve this issue: 1)

more origins are prepared to fire than those that actually do, a fail safe phenomenon known as origin redundancy and 2) there is a mechanism in place that ensures a regular and evenly distribution of origins across the genome, a model known as “the fixed spacing model” (Jun *et al*., 2004) (Maric and Prioleau, 2010). Origins of replication can be classified into early and late firing origins, depending on the time at which they fire during S phase. Whereas in budding yeast this temporal initiation programme is hereditary (Mantiero *et al*., 2011), in fission yeast origins that have fired during a specific cell cycle do not necessarily fire at the same time in subsequent cell cycles. They may not even fire at all until much later (Kim and Huberman, 2001) (Legouras *et al*., 2006). Hence, ORIs in *S. pombe* are stochastic both in space and in time. Furthermore, there seems to be a strong correlation between chromatin state/epigenetics and replication timing, as transcriptionally active euchromatic regions seem to be replicated earlier than inactive heterochromatic stretches of DNA (Sclafani and Holzen, 2007) (Maric and Prioleau, 2010).

1.4.2 – Molecular mechanism of DNA replication initiation in *S. pombe*

Replication initiation is a highly regulated multistep event divided into three main stages (Figure 1.5). Following S phase, origins are in a post-replicative state as they are unable to fire and commence replication. It is only after return into interphase, during G1, when ubiquitin ligase-dependent degradation of cyclins takes place and CDK activity is low and the G1/S transcriptional programme is turned ON, that origins become competent and get into the pre-replicative state. The origin licensing step involves binding of the Mcm2-7 helicase via the licensing factors and MBF-dependent genes Cdt1 and Cdc18 (Cdc6 in budding yeast and mammalian cells). This process requires low levels of CDK activity, since CDK-dependent phosphorylation inhibits this reaction. The activation step involves pre-replicative complexes to be activated by the helicase activator Cdc45 to form the pre-initiation complexes. Finally the initiation and licensing inhibition stage, when replication initiation and simultaneous inhibition reactions take place, prevents ORIs from being relicensed. The process of replication initiation and

the factors that bind to ORIs are well conserved across species (Legouras *et al.*, 2006).

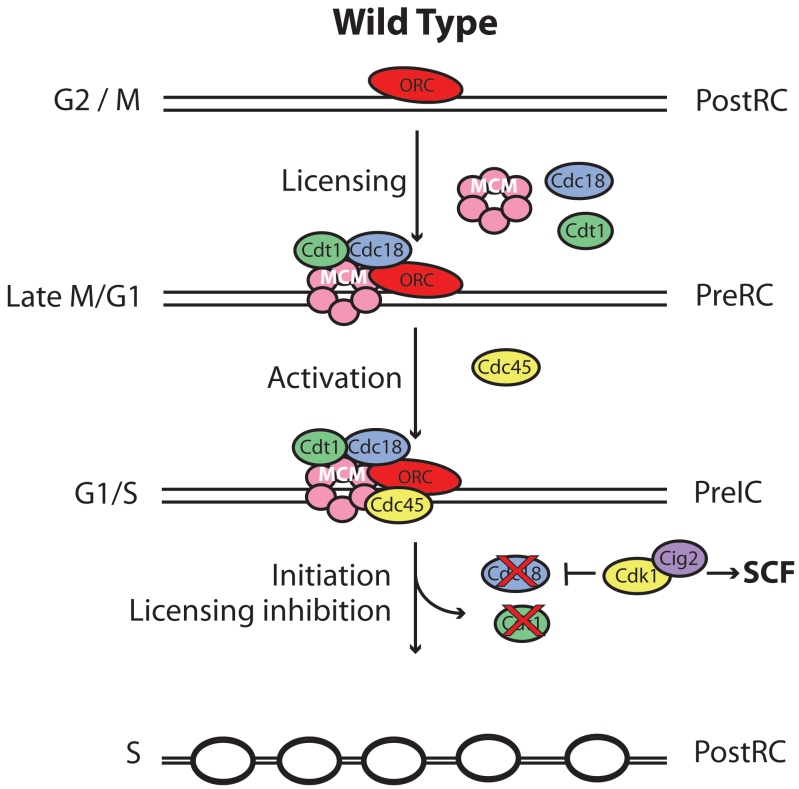

Figure 1.5 – DNA Replication initiation in *S. pombe*

DNA replication initiation is divided into three main stages: 1) licensing, 2) activation and 3) initiation and licensing inhibition. Licensing of origins requires the licensing factors Cdc18 and Cdt1 to enable the binding of the helicase Mcm2-7 to sites of ORC, in late M phase and when CDK activity is low. This step is followed by the loading of Cdc45 onto replisomes during activation. Following the activation step, S-phase-specific CDK and DDK activities activate Cdc45 helicase molecules and replication initiates in a bidirectional manner. Once origins have fired the licensing factors Cdc18 and Cdt1 are sent for proteolysis in order to prevent re-firing of already fired ORIs and hence prevent rereplication. CDK activity is required for proteolysis of Cdc18, which occurs via the SCF[3].

In fission yeast, ORIs are constitutively bound throughout the cell cycle by the origin recognition complex (ORC), a heteromeric assembly composed of six subunits (Orc1 to Orc6). ORCs preferentially bind to AT-rich stretches of DNA, possibly via multiple AT hook domains located in the N-terminus of

[3] Adapted from Legouras et al, 2006.

Orc4 (Lygerou and Nurse, 1999). ORCs provide the platform for binding of the remaining replication fork proteins onto chromatin. Licensing of post-replicative origins, during G1, involves the loading of the minichromosome maintenance complex (Mcm2-7), a doughnut-like or toroidal macromolecule composed of six AAA^+ domain-containing lobes surrounding a central cavity (Adachi *et al*., 1997). Biochemical and structural data obtained in yeast, bacteria and mammalian cells indicate that this complex may act as the DNA replicative helicase, which unwinds DNA during DNA synthesis (You *et al*., 1999) (Bell and Dutta, 2002) (Boos *et al*., 2012), thus, playing a role of extreme importance in both the initiation and elongation steps of DNA synthesis. Loading of the Mcm2-7 complex onto chromatin is dependent upon the AAA^+ ATPase activities of the ORC and the licensing factors Cdc18 and Cdt1 (Mehanna and Diffley, 2012). Following binding of inactive Mcm2-7 onto ORCs during G1, origins transition from a post-replicative state into a pre-replicative state, they are ready to be activated (Legouras *et al*., 2006).

In the second step of DNA replication initiation pre-RC complexes are transformed into transient pre-initiation complexes (pre-IC) via binding of the helicase activator Cdc45/Sld4. Recruitment of Cdc45 initiates Mcm2-7 helicase activity leading to origins firing in a bidirectional manner and cells initiating DNA synthesis. Cdc45 function is regulated by several factors, the S phase CDK, the Cdc7-Dbf4 kinase (DDK, Hsk1, Dfp1 in fission yeast), the GINS complex, Cut5/Rad4, Drc1/Sld2 and Sld3. Together these proteins form the CMG (Cdc45-Mcm2-7-GINS) complex. As verified for Mcm2-7, Cdc45 activity is crucial for DNA synthesis initiation and progression of active replication forks (Legouras *et al*., 2006) (Boos *et al*., 2012). Studies undertaken in *X. laevis* and in *S. cerevisiae* indicate that Cdc45 also has a pivotal role in loading the replication polymerases α and ε onto origins of replication (Mimura and Takisawa, 1998) (Aparicio *et al*., 1999).

In order to prevent rereplication, origins must fire once and only once per cell cycle. Origins switch automatically into their post-replicative state once they have fired or have been passively replicated via a passing replication fork. They are said to be in an “OFF” state as they can no longer sustain replication initiation. This step is known as licensing inhibition. Licensing

inhibition can be achieved in many ways. In fission yeast, this process is achieved mainly by directing Cdc18 degradation via the SCF, a CDK-Cig2-dependent reaction (Jallepalli *et al*., 1997) (Lopez-Girona *et al*., 1998). Degradation of Cdc18 thwarts further loading of Mcm2-7 onto chromatin, hence preventing re-firing of post-RCs. In fact degradation of Cdc18 is so important to prevent rereplication in *S. pombe* that overexpressing *cdc18*+ alone induces massive rereplication, a phenotype that is exacerbated by Cdt1 co-overexpression (overexpression of Cdt1 alone does not induce rereplication) (Lopez-Girona *et al*., 1998). Mammalian cells presenting Cdt1 overexpression seem to be particularly prone to rereplication. In mammalian systems Cdt1 hyper-accumulation can be achieved either by depleting Geminin, an inhibitor of Cdt1 or by ectopic expression of Cdt1. Geminin is not conserved in yeast.

1.4.3 – The role of CDK and DDK activities in replication initiation

Cdk activity plays a dual role in replication initiation. Together with DDK (Cdc7/Dbf4), S-phase CDK initiate origin firing by driving the stable association of Cdc45 with Mcm2-7 and the remaining components of the pre-RC machinery. There is strong biochemical evidence suggesting that the various components of the Mcm2-7 are directly phosphorylated by DDK during S phase (Weinreich and Stillman, 1999). Interactions between CDK1 and ORC and Cdc18 have also been reported via co-immunoprecipitation studies, suggesting a role for CDK in replication licensing. Furthermore, Sld3, which is necessary for the recruitment of Cut5, GINS, Drc1 and Cdc45 is phosphorylated in a CDK-dependent manner, in fission yeast (Fukuura *et al*., 2011). CDK plays a role of importance in temporally separating the first step of replication initiation, origin licensing, from the second step of the same process, activation of the helicase activator Cdc45. This temporal separation is crucial in preventing re-firing of already fired replication origins, something that would either cause lethality or survival of cells presenting an aberrant DNA copy number (Tanaka and Araki, 2010). CDK activity is low at the beginning of G1 and it rises throughout G1 until it reaches its first activity threshold at the beginning of S phase. This process allows for proper

activation of DNA replication origins and subsequent DNA synthesis (Tanaka and Araki, 2010).

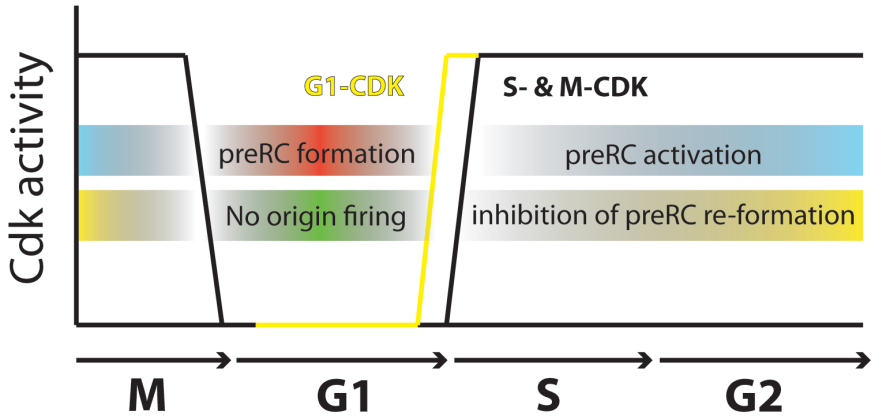

Figure 1.6 – CDK activity regulates DNA replication initiation

CDK activity fluctuates throughout the cell cycle with its highest and lowest peaks of activity at the beginning and end of M phase, respectively. Formation of preRCs takes place during G1 when CDK activity is low. However activation of preRCs requires high CDK activity, something that occurs at the beginning of S phase. CDK activity is also involved in the inactivation and proteolysis of various factors involved in origin firing. Thus, CDK activity plays a dual role in DNA replication initiation (see text for details)[4].

If on the one hand CDK activity contributes for the activation of preRCs by means of allowing loading of Cdc45, on the other and following activation of the pre-RCs, this protein kinase is implicated in inhibiting re-firing of origins that have already been activated. This is achieved by downregulating the activities of ORC, Cdc6, Cdt1 and Mcm2-7. In budding yeast Orc2 and Orc6 are know to be directly phosphorylated by Cdk1 (Nguyen *et al*., 2001) (Tanaka, 2010). In addition Cdc6 levels are reduced by Cdk1 via proteolysis and inactivation of G1/S transcription (Drury and Diffley, 2009) (Lopez-Girona *et al*., 1998). The second threshold of CDK activity at the end of G2 induces mitosis. Following mitosis, mitotic cyclins are degraded via the APC, thus bringing the levels of CDK activity down. Given that CDK activity is high solely from late M and G1 to beginning of M phase, pre-RCs can only be mounted onto chromatin during late M and G1. This prevents pre-RC assembly throughout S, G2 and early M phases (Figure 1.6), thus limiting

[4] Adapted from Tanaka *et al*, 2007.

firing of origins to once and only once per cell cycle (Tanaka and Araki, 2010).

1.4.4 – DNA rereplication and its biological consequences

In order to prevent rereplication cells must prevent replication origins from re-firing. Over-replication within the same cell cycle results in polyploidy, aneuploidy, double strand breaks and mitotic catastrophe, all hallmarks of cancer development (Hook *et al*., 2007) (Truong and Wu, 2011). A full reduplication of the DNA leads to duplication of the entire genome a phenomenon called endoreduplication. Rereplication comes about by re-firing of individual previously fired ORIs and not because of an additional round of replication, a phenomenon known as “the bubble within the bubble”. Together these two events are known as over-replication (Legouras *et al*., 2006). Rereplication results in activation of the ATM and ATR (Rad3 in fission yeast) kinases, markers of DNA structure checkpoint activation (see section 1.5) in both mammalian and yeast cells (Takeda and Dutta, 2005) (Fersht *et al*., 2007) (Hook *et al*., 2007). Elevated levels of Cdc6 and Cdt1 have been observed in tumours and in various transformed cell lines, suggesting that degradation of these licensing factors may act as “an important barrier to tumour formation” (Hook *et al*., 2007).

There are many mechanisms through which cells can acquire rereplicated DNA. In fission yeast overexpression of the licensing factor Cdc18 and depletion of the M-phase cyclin Cdc13 are known to induce rereplication, preferentially at early firing origins, as determined by FACS analysis (Lopez-Girona *et al*., 1998) (Snaith and Forsburg, 1999) (Hook *et al*., 2007). In mammalian cells and in *D. melanogaster*, overexpression of Cdt1 but not Cdc6 (the vertebrate ortholog of *S. pombe* Cdc18) induces rereplication. This can be achieved either by ectopic expression of Cdt1 (in $p53^{-/-}$ cells) or by depletion of geminin (in $p53^{+/+}$ cells), a substrate of the APC that binds and prevents Cdt1 from loading Mcm2-7 helicase molecules onto already-fired origins of replication during S and G2 phases (Mihaylov *et al*., 2002) (Melixetian *et al*., 2004). In budding yeast the general idea is that rereplication requires the co-overexpression of Cdc6, ORC and Mcm2-7

(Nguyen *et al*., 2001) (Hook *et al*., 2007). However, novel sensitive methods for detection of rereplication such as comparative genomic hybridisation analysis (CGH) have shown that deregulation of Cdc6 and ORC is sufficient to induce rereplication events that are not detectable via traditional FACS analysis methods (Green *et al*., 2006). In yeast rereplication is avoided mainly by CDK-dependent degradation of the licensing factors Cdc18/Cdc6 and Cdt1, and by CDK-dependent phosphorylation of Mcm2-7 and ORC. In mammalian cells, rereplication is kept in check primarily by geminin-mediated inhibition and Cdt2-dependent degradation of Cdt1 (Hook *et al*., 2007). In fission yeast Cdt1 is also degraded via the E3 ubiquitin ligase SCF in a Cdt2-Ddb1-depedent manner (Ralph *et al*., 2006).

1.5 – DNA structure checkpoint responses

Replicating cells are particular vulnerable to genotoxic stress and DNA damage. To safeguard their DNA against these unfavourable insults and preserve genomic stability, eukaryotic cells have evolved the DNA structure checkpoint signalling pathways (also known as the DNA-damage response, DDR) (Warmerdam and Kanaar, 2010). Although this response is not essential for regulating the cell cycle as such, its role is of extreme importance in ensuring genomic stability (Nyberg *et al*., 2002). Activation of the checkpoint results in alteration of the duration of the different cell cycle stages, a process commonly known as cell cycle checkpoint arrest.

Much of what is known about the DNA structure checkpoints, their control, activation, molecular mode of function and downstream targets has been acquired from work performed in the yeasts *S. pombe* and *S. cerevisiae* (Carr, 2002). As observed for all higher eukaryotes, the fission yeast DNA structure checkpoints can be divided into the DNA replication checkpoint and the DNA damage checkpoint. The fundamental difference between the two checkpoints is that the DNA replication checkpoint is essential to prevent DNA damage in response to replication stress during S-phase, whereas the DNA damage checkpoint is required to detect and resolve DNA damage before entry into mitosis. The DNA replication checkpoint prevents the accumulation of DNA damage as a result from replication stress by stabilizing stalled replication forks, preventing late origins from firing and enabling replication to resume once the stress has been resolved. For the purposes of this study we will mainly focus our attention on the DNA replication and the DNA damage checkpoints (Caspari and Carr, 1999).

Defects in the various components of the checkpoints are known to be involved in the development of genome instability, a hallmark of cancer aetiology. Several diseases have been discovered relating mutations in checkpoint proteins such as the cancer-prone and hereditary syndrome ataxia telangiectasia. Furthermore, given the importance of checkpoint activation in the survival of transformed or cancerous cells, checkpoint-

targeted chemotherapy has been of late, a very attractive therapeutic strategy (Chen *et al*., 2012).

1.5.1 – The checkpoint signalling pathway

In order to be able to mount an efficient response against DNA-damage or replication stress, cells must be able to, firstly, detect the damage or replication block through its sensor molecules, secondly, transform the sensed abnormality into a biochemical signal by transducer proteins and thirdly, relay the generated signal to downstream effectors capable of activating the machinery necessary to delay cell cycle progression and repair the insult encountered (Skladanowski *et al*., 2009). Such a response involves a complex signal transduction network, comprising many molecules and a series of post-translational modifications, such as phosphorylation and ubiquitination. An important part of the checkpoint response involves the activation of cell cycle arrest by means of temporarily reducing CDK (maintained by CDKIs) and Cdc25 activities (Caspari and Carr, 1999) (Zeng and Piwnica-Worms, 1999). Cell cycle arrest ensures that passage of damaged DNA to cells at subsequent cell cycle phases is avoided. Please note that we will use mainly *S. pombe* checkpoint-related nomenclature, making reference to other organisms whenever appropriate.

1.5.2 – Common elements of the DNA replication and the DNA damage checkpoints

The discovery of radiation-sensitive mutants led to the identification of various checkpoint-related genes in *S. pombe,* the so-called checkpoint *rad* family of genes (Carr, 2002). Checkpoint proteins fall into one of four major categories depending on their role in the checkpoint response and on genetic and biochemical findings: sensors (sense directly or indirectly the presence of genotoxic stress and initiate the checkpoint signalling), transducers (relay and amplify the signal of damage), effectors (lower downstream targets of the transducers, usually regulated via phosphorylation) and stabilisers or mediators (promote mediation between the transducers and the effectors) (Table 1.2) (Nyberg *et al*., 2002). Whilst the DNA replication checkpoint is generally activated by stalled replication forks, the DNA damage checkpoint

comes into play when the DNA is affected by a myriad of DNA lesions. These include but are not limited to single and double strand breaks (SSB and DSB), chemical modifications of the DNA structure caused by alkylation and oxidation, mismatched bases, products of ultraviolet (photoadducts and crosslinks) and X-ray radiation, amongst others (Boddy and Russell, 2001).

Table 1-2 Main checkpoint protein orthologs in *S. pombe*, *S. cerevisiae* and mammals[5]

Group	Type	*S. pombe*	*S. cerevisiae*	Mammals
Sensors	RFC1-like	Rad17	Rad24	Rad17
	PCNA-like	Rad9 Rad1 Hus1	Ddc1 Rad17 Mec3	Rad9 Rad1 Hus1
	DSB recognition/repair	Rad32 Rad50	Mre11 Rad50 Xrs2	Mre11 Rad50 Nsb1
	Clamp loader	Rfc2-5	Rfc2-5	Rfc2-5
Transducers (also understood as sensors)	PI3-kinases (PIKK)	Rad3 Tel1	Mec1 Tel1	ATR ATM
	PIKK binding partners	Rad26	Ddc2/Lcd1	ATRIP
Effectors	Kinase	Chk1 Cds1	Chk1 Rad53	Chk1 Chk2
Stabilisers/mediators		Mrc1 Crb2/Rhp9	Mrc1 Rad9	BRCA1

As we shall see in the following sections, there are several checkpoint proteins common to both the DNA replication and the DNA damage checkpoint signalling pathways. Examples are the ATR-ATRIP (Rad3-Rad26) complex and the RFC- (Rad17, Rfc2, Rfc2, Rfc4, Rfc5), and the PCNA-like (Rad1, Rad9, Hus1) complexes (Furuya and Carr, 2003). Whilst the activation of the DNA replication checkpoint is S-phase specific, the DNA damage checkpoint is activated mainly at G2. Initiation of the DNA replication and the DNA damage checkpoints leads to activation of the downstream effector protein kinases Cds1 and Chk1, respectively (Rhind and Russell, 2000). It remains largely elusive how, in the light of the existing complex and diverse plethora of DNA lesions, a specific lesion activates a particular downstream checkpoint response. The current model hypothesises that most

[5] Adapted from Nyberg *et al*, 2002.

DNA lesions, capable of changing the conformal structure of the DNA helix, are converted into either SSBs or DSBs, two relatively common lesions that activate the ATR (Rad3) and the ATM (Tel1) checkpoint pathways (Nyberg *et al*., 2002). Stalled replication forks are known to promote larger than usual stretches of ssDNA in *Xenopus laevis* egg extracts, a signal capable of triggering checkpoint responses (Van *et al*., 2010). It is important to notice that these checkpoints are not static. Instead, they are dynamic and highly integrated, as they may cross-talk with each other through a series of complex signal transduction pathways depending on the type of lesion being repaired. However, for reasons of clarity and simplicity they will be presented here as separate pathways.

1.5.3 – DNA replication checkpoint

1.5.3.1 – Molecular Mechanism of DNA replication checkpoint activation

The DNA replication checkpoint is activated by DNA replication stress resulting from stalled replication forks, which can be induced by the depletion of deoxyribonucleotide pools (induced for example by drugs such as hydroxyurea; HU), DNA replication polymerase inhibition (induced by treatment with aphidicolin) and when replication forks collide with unresolved lesions that impede normal fork progression along the DNA template (Xu *et al*., 2006).

In fission yeast, the DNA replication checkpoint cascade depends mainly on the Rad3/Cds1 pathway (Figure 1.7; *S. cerevisiae* Mec1/Rad53 and mammalian ATR/Chk1). The replisome continues to unwind DNA downstream of a stalled replication fork exposing ssDNA structures (Van *et al*., 2010), resulting in the loading and activation of the transducer and sensor phosphatidylinositol 3-kinase-related protein kinase Rad3 in complex with Rad26 (the regulatory unit of the complex) to sites of stalled replication forks (Bentley *et al*., 1996) (Chapman *et al*., 1999). In contrast to their wild type counterparts *Δrad3* cells are not able to arrest their cell cycle following DNA damage or replication stress, something that makes them lose viability in

response to genotoxic stress. This loss of viability illustrates the importance of the role of Rad3 in the establishment of the DNA replication and DNA damage checkpoints (al-Khodairy and Carr, 1992). Rad3 has the ability to autophosphorylate in a Rad26-dependent manner (Chapman *et al*., 1999). This autophosphorylation reaction is necessary for further increase of Rad3 activity and it is important for the activation of the DNA replication and DNA damage downstream checkpoint responses (Wolkow and Enoch, 2002).

In addition to loading of the Rad3-Rad26 complex to sites of halted DNA replication, an independent reaction takes place, the loading of the PNCA-like heterotrimeric Rad9-Rad1-Hus1 clamp (also known as the 9-1-1 clamp) via the Rfc2-5-Rad17-like loading complex (Figure 1.7A) (Bermudez *et al*., 2003). The 9-1-1 and the Rfc2-5-Rad17 complexes are known to be structurally related to the replication processivity factor PCNA and the Rcf1-5 complexes operating during normal DNA replication, respectively. Binding of 9-1-1 clamp and Rfc2-5-Rad17-like complexes to sites of stalled replication forks is necessary for full activation of Rad3 and Cds1. Apart from its role in the activation of the DNA replication and DNA damage checkpoints, the 9-1-1 complex seems to be important for DNA repair such as in the mechanism of mutagenic translesion synthesis and in the repair of DSBs (Parrilla-Castellar *et al*., 2004).

Cds1 is activated via a two-stage mechanism and a complex phosphorylation network requiring one basal (T11) and three parallel phosphorylation steps mediated by Rad3, Mrc1 and Rad9 (Xu *et al*., 2006) (Yue *et al*., 2011). Mrc1 (mediator of replication checkpoint 1) is a component of the replisome during normal replication progression that travels along the replication fork together with the replication machinery.

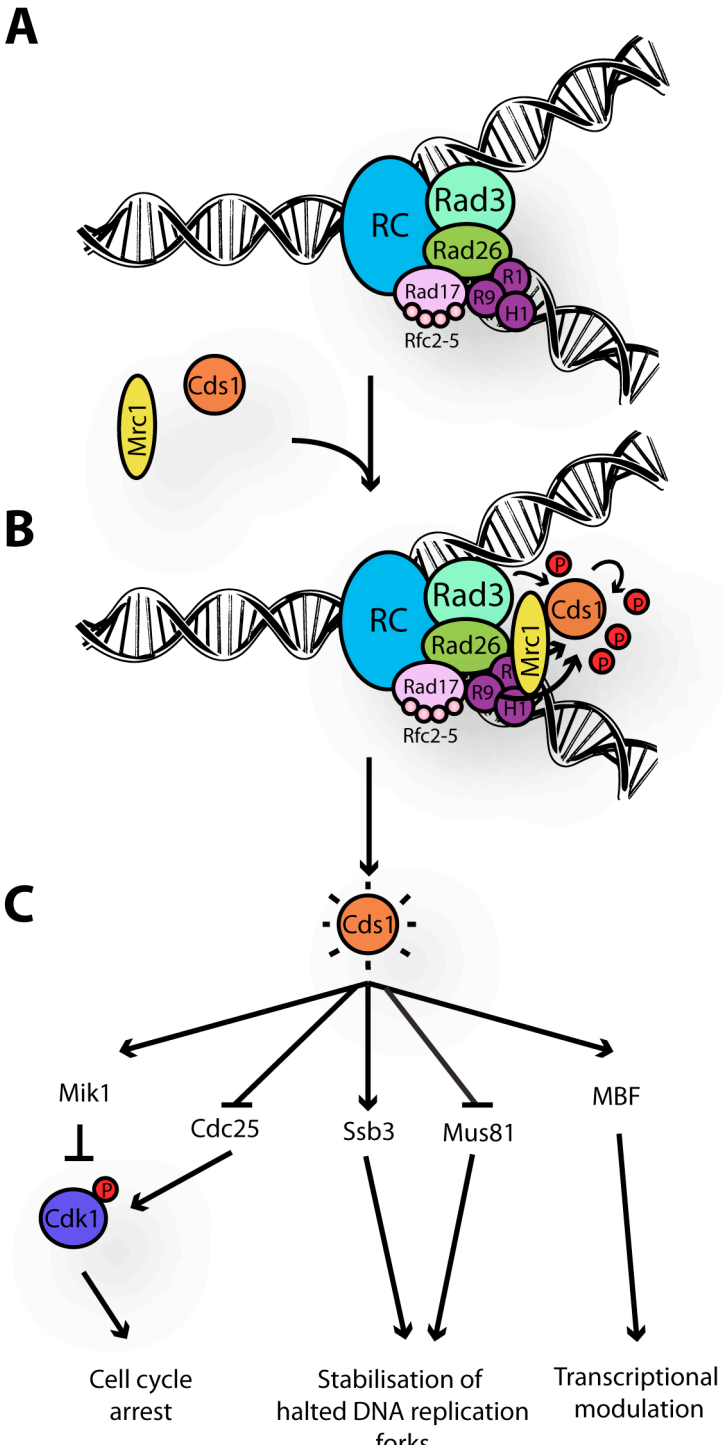

Figure 1.7 – The fission yeast DNA replication checkpoint

The molecular mechanism of the DNA replication checkpoint of *S. pombe* and corresponding responses to replication stress (see text for details).

In response to replication stress Mrc1 accumulates as a hyperphosphorylated molecule. SDS-PAGE experiments in a series of mutants have shown that Mrc1 phosphorylation at TQ repeats T645 and T653 is Rad3-dependent and that this occurs independently of Cds1

presence (Xu *et al*., 2006). The same study shows that phosphorylation of Mrc1 at TQ and SQ clusters is necessary for phosphorylation of Cds1 at residue T11 and that Mrc1 binds to inactive Cds1 via its FHA domain, an association that seems to facilitate phosphorylation of Cds1 by Rad3. Furthermore, *in vitro* studies have shown that activated Cds1 is capable of phosphorylating a kinase-dead form of Cds1, suggesting that full activation of Cds1 may include an autophosphorylation step (Boddy *et al*., 1998). Taken together these data suggest that Cds1 is activated via a two-stage biochemical mechanism, the priming and the autophosphorylation stages (Figure 1.7B). In the first step, the priming stage, Cds1 is recruited to sites of halted replication via Mrc1 and then phosphorylated by Rad3. Once bound to the chromatin and phosphorylated by Rad3, Cds1 enters the second stage of Cds1 activation, the autoactivation stage. At this stage phospho-Cds1 experiences a Rad3- and Mrc1-independent dimerisation and autophosphorylation mechanism, rendering it fully active (Lindsay *et al*., 1998) (Xu *et al*., 2006). A more recent study suggests that activation of Cds1 is also dependent upon phosphorylation of Rad9 via Rad3 and that Cds1 is only fully activated by a basal phosphorylation performed by Rad3 and three parallel phosphorylations performed by Mrc1 and Rad9 (Yue *et al*., 2011).

1.5.3.2 –DNA replication checkpoint responses

The activation of Cds1 promotes many checkpoint-related responses. These include cell cycle arrest, stabilisation of DNA replication forks and transcriptional induction (Branzei and Foiani, 2009).

One of the main features of the replication checkpoint is its ability to prevent entry into M phase of cells that are unable to fully synthesise their DNA. As we saw in section 1.1.2 the onset of M during cell cycle progression is inhibited by inactivation of Cdk1 by the CDKIs Wee1 and Mik1. In the fission yeast and other eukaryote cells, activation of the DNA replication checkpoint also promotes accumulation of Mik1 and its respective orthologs. Increase in Mik1 levels results in inhibitory phosphorylation of Cdk1 at Y15 and arrest of the cell cycle throughout S phase (Rhind and Russell, 1998) (Rhind and Russell, 2001). In addition, inactivation of Cdk1 is maintained via a

secondary mechanism that involves phosphorylation and nuclear extrusion of inactive Cdc25 molecules (Figure 1.7C) (Zeng and Piwnica-Worms, 1999).

A second response mediated by activation of Cds1 is the stabilisation of paused replication forks. In order to maintain the stability of a paused replication fork, association of the replisome with checkpoint proteins is essential (Branzei and Foiani, 2009). In mammalian cells RPA (replication protein A) is recruited to ssDNA at sites of stalled replication forks in order to prevent reannealing of the DNA strand (Sabatinos and Forsburg, 2009). In fission yeast, although the ortholog RPA, Ssb3 is not essential for cell viability, its deletion results in formation of a high number of Rad22 foci (Cavero *et al*., 2010). Rad22 is a protein that binds DSBs and has a function in both the repair of DSBs via homologous recombination and in mating type switching, and it is often used as a marker of genomic instability (Ostermann *et al*., 1993). Rad22 foci number will be used in this report as a marker for genomic instability. An essential pathway for maintaining stability of stalled replication forks corresponds to the Cds1-mediated inactivation of Mus81, a Holliday junction resolvase capable of cleaving stalled replication forks (Kai *et al*., 2005). Replication fork halting is not an everlasting event. Once cells resolve the block causing DNA replication stalling, they are ready to resume replication.

A third response corresponds to the DNA replication checkpoint transcriptional response, which includes the maintenance of the MBF-dependent G1/S transcriptional programme (de Bruin and Wittenberg, 2009). The MBF transcriptional programme includes many genes that promote repair of cellular lesions, including stabilization of stalled replication forks and induction of DNA repair functions. Persistent expression of MBF-dependent genes occurs in cells arrested in S phase with incompletely replicated DNA (de Bruin *et al*., 2008). DNA replication stress-induced activation of MBF-dependent transcription involves the initial activation of Rad3p, which phosphorylates and activates Cds1, which in turn, phosphorylates Nrm1, Cdc10, and Ste9, to keep MBF-dependent transcription active. Whereas phosphorylation of Nrm1 and/or Cdc10 inhibits the binding of the corepressor Nrm1p to MBF at promoters, phosphorylation of Ste9 is thought to inhibit the

transcriptional co-activator Rep2 being targeted for destruction by the Ste9/APC ubiquitin ligase complex (Dutta *et al*., 2008) (de Bruin *et al*., 2008) (Chu *et al*., 2009). Here we show that in response to DNA replication stress, and as observed for Nrm1, Yox1 is released from MBF promoters, correlating with induction of MBF-dependent transcription. We show that phosphorylation of Yox1 at Ser114 and Thr115 by the DNA replication checkpoint protein kinase Cds1 is sufficient to keep MBF-dependent transcription active. Furthermore we establish that activation of MBF-dependent transcription is critical for cell survival in response to replicative stress.

1.5.4 – DNA damage checkpoint

1.5.4.1 – Molecular Mechanism of DNA damage checkpoint activation

Activation of the DNA damage checkpoint pathway results in phosphorylation and activation of the DNA damage effector, serine/threonine protein kinase Chk1. Cells deleted for *chk1+* are compromised in their ability to respond towards DNA damage, hence extremely sensitive to DNA damage inducing drugs such as methylmethane sulfonate (MMS) and camptothecin (CPT) (Capasso *et al*., 2002) (Kuntz and O'Connell, 2009). The DNA damage checkpoint is highly conserved across eukaryotes, suggesting an early development in ancient eukaryote life (Tel1/Chk1 and Rad3/Chk1 in *S. pombe*, Mec1/Chk1 in *S. cerevisiae* and the ATM/Chk1 pathway in mammals) (Kuntz and O'Connell, 2009).

As verified for the DNA replication checkpoint, the first step in the activation of the DNA damage checkpoint cascade corresponds to the conversion of primary DNA lesions into ssDNA. These ssDNA stretches are recognised and bound independently by the Rad3/Rad26 complex and by the 9-1-1 clamp, which again, as established for the DNA replication checkpoint, is loaded with the help of the clamp loader Rfc2-5/Rad17 (Parrilla-Castellar *et al*., 2004) (Figure 1.8A). The ATM ortholog, Tel1 plays a role of significance in the processing of DSBs. Once these proteins are in place, two BRCT-domain mediators, Cut5 and Crb2 recruit Chk1 to the checkpoint complex, which is rendered active by Crb2-mediated phosphorylation. As discovered

for Mrc1, Crb2 contains a pair of conserved TQ/SQ phosphorylation clusters that promote the interaction between Crb2 and Chk1 (Saka *et al*., 1997) (Qu *et al*., 2012). The interaction between Crb2 and Chk1 brings Chk1 into close proximity with Rad3, resulting in Rad3-dependent phosphorylation of Chk1 at S317, a residue located in the C-terminal regulatory fraction of the protein (Lopez-Girona *et al*., 2001) (Figure 1.8B). Studies undertaken in *X. laevis* suggest that this induces a conformational change of Chk1, which unfolds, leaving the kinase domain of the protein free to participate in the phosphorylation of downstream targets (Katsuragi and Sagata, 2004). This suggests that Chk1 is regulated via an autoinhibition mechanism, whereby its conformation state regulates its activity in an "ON or OFF" state. Activated Chk1 binds the 14-3-3 proteins Rad24 and Rad25. This interaction is thought to be involved in the accumulation of active Chk1 within the nucleus (Dunaway *et al*., 2005). Inactivation of Chk1 is essential for cell recovery and re-commencement of the cell cycle once DNA lesions have been repaired. This is achieved by dephosphorylation of Chk1 by the phosphatases PP1 and Dis2 (den Elzen and O'Connell, 2004).

1.5.4.2 – DNA damage checkpoint responses

Activation of Chk1 leads to the establishment of many of the DNA damage repair pathways, depending on the lesion being repaired: 1) the homologous recombination (HR) and the non-homologous end joining repair pathways (NHEJ), which repair DSBs; 2) the base excision repair (BER) that excises and repairs damaged bases; 3) the nucleotide excision repair (NER), which is involved in repairing ssDNA stretches of approximately 24 nucleotides long; and 4) the direct reversal DNA repair proteins, alkyltransferases and dioxygenases, which repair alkylated and oxidised bases, respectively. These pathways are highly integrated (Figure 1.8C) (Helleday *et al*., 2008).

As observed for the DNA replication checkpoint, activation of Chk1 results in cell cycle arrest by means of Cdk1 and Cdc25 inactivation. Inhibitory phosphorylation of Cdk1 at Y15 is achieved by Chk1-dependent accumulation of the CDKIs Mik1 and Wee1. Chk1 directly mediates

stabilisation of Wee1 and cellular localisation of Cdc25, the latter being exported out of the nucleus (Raleigh and O'Connell, 2000).

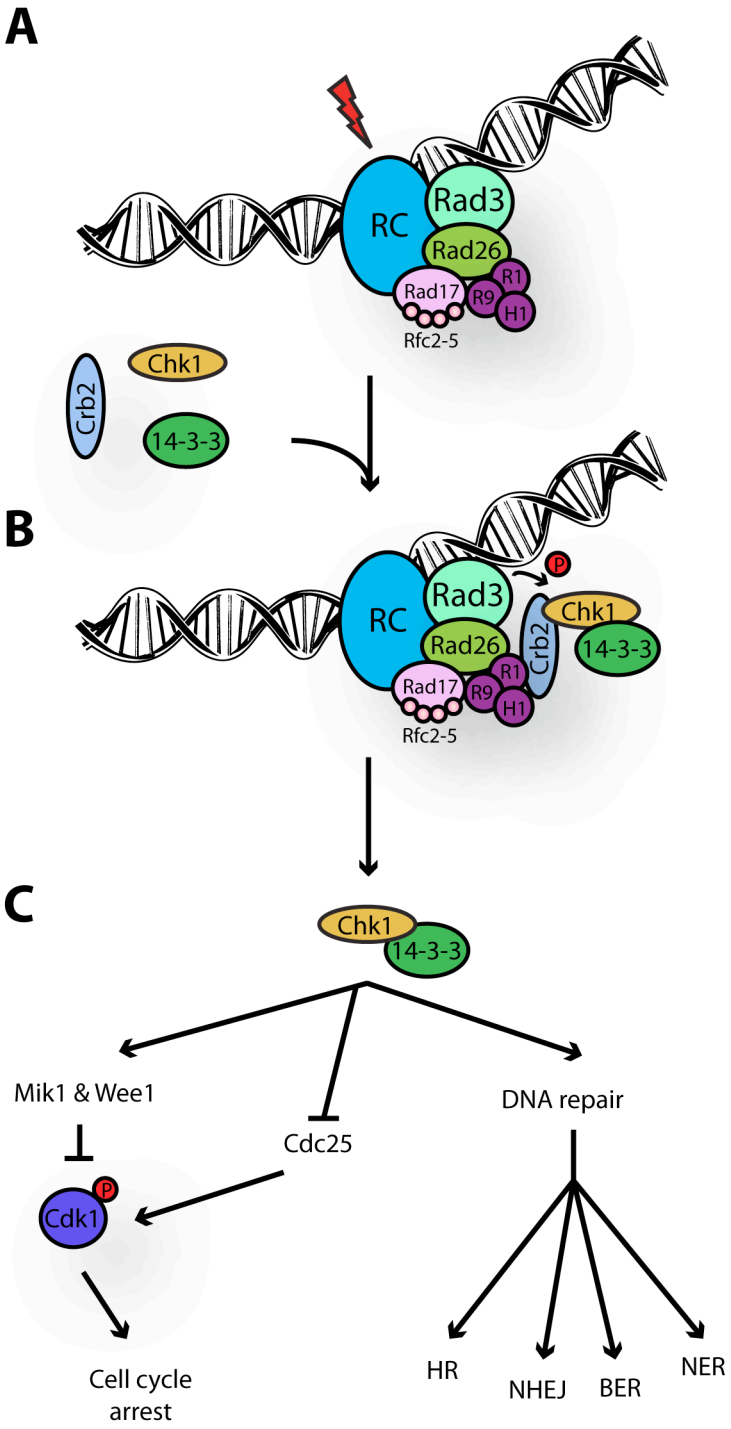

Figure 1.8 – The fission yeast DNA damage checkpoint

The molecular mechanism of the DNA damage checkpoint in *S. pombe* and corresponding responses to DNA damage (see text for details).

1.6 – Objectives

1.6.1 – Objective 1

Activation of Cds1 leads, amongst other downstream effects to persistent expression of MBF-dependent transcripts (de Bruin and Wittenberg, 2009). This is achieved, in part, by alleviating Nrm1-mediated MBF transcriptional repression (de Bruin *et al*, 2008). The current model postulates that replication stress-induced activation of Rad3 leads to dimerisation and activation of the DNA replication checkpoint effector kinase Cds1, which in turn phosphorylates and inactivates Cdc10p, Nrm1p, and Ste9p (Dutta *et al*., 2008) (de Bruin *et al*., 2008) (Chu *et al*., 2009). Given the involvement of Yox1 in repressing MBF transcription during normal cell cycle progression we ask if there is a role for Yox1 in the DNA replication checkpoint response. In this work we show that together with inactivation of Nrm1p, Yox1p phosphorylation constitutes a second mechanism through which the DNA replication checkpoint acts to maintain MBF transcription in response to DNA replication arrest.

1.6.2 – Objective 2

G1/S transcription levels are often upregulated in human cancers. In human cells the G1/S transcriptional network encompasses hundreds of transcripts. In fission yeast over 40 genes have been identified. These include but are not limited to the DNA replication initiation factors, responsible for loading of the MCM helicase Cdc18 and Cdt1 and the S-phase cyclin Cig2. Cdc18 and Cdt1 overexpression has been shown to induce rereplication in *S. pombe* and mammalian cells, respectively. Rereplication is highly detrimental for cells. In this report we use the fission yeast model to explore the biological effects of keeping G1/S cell-cycle regulated transcription constitutively expressed throughout all stages of the cell cycle. We show that cells that are unable to turn the G1/S transcriptional programme OFF accumulate genome instability. Furthermore, we demonstrate that this is the result of a fine and delicate balance between the levels of licensing factors and CDK activity, which results in sporadic re-firing of DNA replication origin.

2. Methods

2.1 – Yeast specific methods

2.1.1 – Yeast strains and media

The *nrm1Δ* and *yox1Δ* mutants and the *yox1*-3xHA C-terminal 3xHA-tagged strains are as described previously (de Bruin *et al*., 2008) (Aligianni *et al*., 2009). All strains were grown in either YES rich (YE+supplements) or EMM (Edinburgh minimal medium) media at 30°C or at 25°C (see Table 2.1 for strains list).

Table 2-1 Strains used in this report

Strain Number	Source	Mating Type	Strain	Notes
RBP4	Russell lab	h^-	*cdc25-22*	Temperature sensitive
RBP7	Russel lab	h^+	*Wt leu1-32 ura4-D18*	
RBP9	de Bruin lab	h^+	*Δnrm1::hyg*	
RBP10	Rhind lab	h^{90}	*Δres2::ura4*	
RBP11	Russell lab	h^-	*Wt leu1-32 ura4-D18*	
RBP31	de Bruin lab	h^-	*Δnrm1::hyg*	
RBP35	Boddy lab	h^-	*Δcds1::ura4*	
RBP66	de Bruin lab		*Δcds1::ura4 Δnrm1::hyg*	
RBP127	de Bruin lab	h^-	*Δres1::ura4*	
RBP190	Russell lab	h^-	*rad22-YFP::kan*	
RBP354	Russell lab	h^+	*rad22-YFP::kan*	
RBP361	de Bruin lab	h^+	*Δnrm1::hyg rad22-YFP::kan*	Cross of RBP9 with RBP190
RBP364	de Bruin lab		*Δres1::ura4 rad22-YFP::kan*	Cross of RBP127 with RBP354
RBP367	de Bruin lab		*Δres2::ura4 rad22-YFP::kan*	Cross of RBP10 with RBP190
RBP386	Bähler lab	h^-	*yox1-3HA::kan*	
RBP387	Bähler lab	h^+	*Δyox1::kan*	
RBP388	de Bruin lab		*nrm1-myc::kan yox1-3HA::kan*	

RBP403	de Bruin lab	h^+	*cdc25-22*	
RBP405	de Bruin lab	h^+	*Δnrm1::hyg Δyox1::kan*	Cross of RBP31 with RBP387
RBP406	de Bruin lab		*Δyox1::kan Δcds1::ura4*	Cross of RBP35 with RBP387
RBP412	de Bruin lab	h^+	*Δyox1::kan rad22-YFP::kan*	Cross of RBP190 with RBP387
RBP425	de Bruin lab		*Δcds1::ura4 yox1-3HA::kan*	Cross of RBP35 with RBP386
RBP450	de Bruin lab	h^-	*P41nmt1-cig2::kan*	Nmt1 tagging using the Bahler plasmids
RBP451	de Bruin lab	h^+	*nrm1Δ::hyg Δyox1::kan rad22-YFP::kan*	Cross of RBP361 with RBP387
RBP452	de Bruin lab	h^-	*yox1^{2A}-3HA::kan*	Site directed mutagenesis
RBP496	de Bruin lab		*Δnrm1::hyg cdc25-22*	Cross of RBP4 with RBP9
RBP514	Russel lab	h^-	*Δcig2::ura4*	
RBP519	de Bruin lab		*Δcig2::ura4 rad22-YFP::kan*	Cross of RBP514 with RBP354
RBP520	de bruin lab		*Δnrm1::hyg cig2Δ::ura4*	Cross of RBP9 with RBP514
RBP529	de Bruin lab		*P41nmt1-cig2::kan Δnrm1::hyg rad22-YFP::kan*	Cross of RBP9 with RBP190 and RBP450
RBP533	de Bruin lab		*P41nmt1-cig2::kan rad22-YFP::kan*	Cross of RBP190 with RBP450

2.1.2 – Mating/crosses

Cells were grown from frozen onto selective YES or EMM agar plates and allowed to grow for two days at 30°C. Loops of h- and h+ cells were mixed in 100μl of ddH_2O and 10μl plated onto ME plates and left at room temperature for 2 or more days. Mating efficiency was checked using a light microscope. Cells were picked into 400μl H_2O in an Eppendorf tube, 4μl of glusulase (lytic enzyme that degrades the cell wall of *S. pombe*) added and cells incubated for at least 4h at 30°C or at room temperature for temperature-sensitive mutants. The generated endospores were then washed in water, resuspended at 1/100 and 1/1000 dilutions in water and plated onto selective plates. Colonies were re-streaked on selective plates and their genotype assessed via PCR. HA-tagged strains were identified by western blot analysis.

2.1.3 –Spot assays

Cells were grown in YES to saturation in 10ml. Cultures were then 5-fold serially diluted and spotted on drug-free and HU- or MMS-containing YES plates using a purpose-built, replica-pin apparatus. Plates were incubated for at least four days at 30°C and pictures taken using an Epson Expression 1680 Pro scanner.

2.1.4 –Transformation –Lithium acetate method

50ml of *S. pombe* cultures were grown to OD_{595}=0.8-1. Cell pellets were washed with ddH_2O and resuspended in 1ml of 0.1M LiAc (pH 7.5), 1MTris-HCl (pH 8.0) by tube inversion. 100μl of the mixture were aliquoted for each transformation, to which 1μg of DNA and 1μg of salmon sperm carrier ssDNA were added. The mixture was allowed to sit at room temperature for 5min following which, 280μl 40% PEG, 0.1M LiAc (pH7 .5) 10mM Tris-HCl (pH 8.0) were added. The mixture was mixed carefully by inverting the tube several times and placed in a water bath set at 30°C, without shaking, for 1h. After 1h 43μl of DMSO were added and the cells heat-shocked at 42°C for no more than 5min. Cells were spun down and washed once with ddH_2O, resuspended in water and spread onto YES/EMM-agar plates. The plates were incubated at 30°C for two days and cells replica-plated onto selective agar plates. Three to four days later the obtained colonies were re-streaked onto fresh selective plates, grown overnight and checked for transformation efficiency using either PCR or western blot analysis.

2.1.5 –Spore microdissection

Cells were mated as described in section 2.1.2. A loop of crossed cells was streak on fresh YES/EMM plates and individual asci picked and placed in line with the help of a microdissection microscope (Singer Instruments). Plates were then placed at 30°C and following cell wall disruption individual spores were picked and placed along a grid. The picked single cells were allowed to grow at 30°C or at room temperature (temperature-sensitive mutants), the plates photographed using an Epson Expression 1680 Pro scanner and individual colonies re-streaked onto selective plates. Genetic background was confirmed using PCR analysis.

2.2 – Molecular biology

2.2.1 – PCR – genotyping

The genotype of transformed or crossed strains was verified by PCR using the Bähler method (Bähler *et al*., 1998). Primers were designed to check gene deletion, Pnmt1 N-terminal tagging and C-terminal tagging with for example HA. DNA was extracted as described in section 2.3.1 and PCR performed using the GoTaq Flexi DNA polymerase (Promega) and a BioRad iCycler PCR machine. PCR reactions were run on 0.8 or 1.2% agarose gels depending on size of the PCR product and gel photographed using a GeneFlash UV transilluminator machine.

2.2.2 – RT-PCR

Total RNA was prepared using the RNeasy Plus Kit (Qiagen) as indicated in the manufacturer's manual. Briefly, cells were resuspended in 400μl and disrupted using glass beads and a Fast-prep machine. The obtained samples were then centrifuged at 13000 rpm for 1min and the supernatant applied to a DNA column. 350μl of 70% ethanol were added to the obtained samples and the mixture applied to an RNA column. Columns were washed with RW1 and RPE buffers and eluted in 100μl RNase-free water. Transcript levels were determined by RT qPCR using the iScript One-Step RT-PCR kit with SYBR Green Supermix (Bio-Rad). RT-PCR reactions were run on a Chromo-4 Real-Time PCR Detector (Bio-Rad) and obtained data analysed using MJ Opticon Analysis Software 3.0. Finally data was normalised against actin and investigated using the C(t) method.

Table 2-2 Primers used for performing RT-PCR analysis

Name	Target	Purpose	Sequence
Spcdc18 RTRT Fw	Spcdc18	RTRT	GTAGGCATGCAATTGAACTTGCGG
Spcdc18 RTRT Rv	Spcdc18	RTRT	TCATAGCAGATGTCGCTCGGACAA
Spcdc22 RTRT Fw	Spcdc22	RTRT	TGCAACGTGTTGAACGTAACGAGC
Spcdc22 RTRT Rv	Spcdc22	RTRT	AGGTAATGAACGACGACCACGGTT

Spact1 RTRT Fw	Spact1	RTRT	CGCCGAACGTGAAATTGTTCGTGA
Spact1 RTRT Rv	Spact1	RTRT	TCAAGGGAGGAAGATTGAGCAGCA
Spcig2 RTRT Fw	Spcig2	RTRT	AGGCATTACTGCTCTTCTCATCGC
Spcig2 RTRT Rv	Spcig2	RTRT	ACGTTCAGCGACACAGACATCTTC
Spste9 RTRT Fw	Spste9	RTRT	TTGTGGTGAGGGATCTCAAGCGAA
Spste9 RTRT Rv	Spste9	RTRT	TTCAACAGAGCCATTGTGTGTGCC
Spsuc22 RTRT Fw	Spsuc22	RTRT	TCCACTGTACTCGCCTATTTCGCT
Spsuc22 RTRT Rv	Spsuc22	RTRT	AACACAACGAGCTTCAGGGATCTG
Sppfh1RTRTFw	Sppfh1	RTRT	CCTCCTTGAACAAATTGGGCGGTT
Sppfh1RTRTRv	Sppfh1	RTRT	TGACGGAAGGTTATCAGACTGGCT
Sppof3RTRTFw	Sppof3	RTRT	GGCCGGTACTAGATTTGTTTCGCA
Sppof3RTRTRv	Sppof3	RTRT	TCTCTCCAGTGTTTGCAGACTTGC
Spams2RTRTFw	Spams2	RTRT	GGCACGTCAAATTCCGACACAGTT
Spams2RTRTRv	Spams2	RTRT	TCCAACCTCGAAGGAGTTCTGCAA
Spcdt2RTRTFw	Spcdt2	RTRT	TGAGACTGGAGCTCTTGAGCTGTT
Spcdt2RTRTRv	Spcdt2	RTRT	TAGCATTGTTGTGAGCAAGCCAGC
Sprhp51RTRTFw	Sprph51	RTRT	TCTGCCATACACTTGCGGTAACCT
Sprhp51RTRTRv	Sprph51	RTRT	GCCAACAAACGAACAGGACGGAAA
SpC644.05cRTRTFW	SpC644.05c	RTRT	GATTTGTACGCGGCTGCTGAATGT
SpC644.05cRTRTRV	SpC644.05c	RTRT	GCTAATCCGCTTCTTGGAGCAACA
Spcdt1RTRTFw	Spcdt1	RTRT	ACCGTATGGCCAGAGTCATTTGCT
Spcdt1RTRTRv	Spdct1	RTRT	AATTCAATGGAGCGGGAGAAGGCT
Sppol1RTRTFw	Sppol1	RTRT	ACCGATTGAAGGAACAAACCGTGC
Sppol1RTRTRv	Sppol1	RTRT	AGACTCATACCGCTGAAACGCAGA
Spmr1cRTRTFw	Spmrc	RTRT	TTGCAACGCAACCAACTTGAGGAG
Spmrc1RTRTRv	Spmrc	RTRT	ATCATTTCTAGCGCGTTCAAGCGG

Spmik1RTRTFw	Spmik1	RTRT	AAACCACCTCAATCTCCGCTGTCA
Spmik1RTRTRv	Spmik1	RTRT	ACGTTTCATCCGAGCTGTCAGAGT
Spssb1RTRTFw	Spssb1	RTRT	AGGTTCAAGCCGAACATCTGGGAA
Spssb1RTRTRv	Spssb1	RTRT	AGGGCAAGCAGGATATGACACGTT
Sppsm3RTRTFw	Sppms3	RTRT	TGGACATTGATACGCCATCCCAGA
Sppsm3RTRTRv	Sppms3	RTRT	AGTGACTTCTGACCGCCACTCAAT
mug40RTRTFw	Spmug40	RTRT	GCGCGGATTAGTCACAAACTCGAT
mug40RTRTRv	Spmug40	RTRT	TGACGCAACAATTGCAAAGCTGG
rad21RTRTFw	Sprad21	RTRT	AGCTTGGTTCGCCAATGGGATTTC
rad21RTRTRv	Sprad21	RTRT	CCAATCGTTCGCTTCCCAGCAAAT

2.2.3 –ChIp analysis

ChIP analysis was carried out as described in Aligianni *et al*. In summary, 45ml of exponentially growing cells were treated with formaldehyde (37% v/v) for 30min, to 1% final concentration for DNA-protein crosslinking. Crosslinking reaction was then stopped by adding glycine (2.5M) to a final concentration 125mM. Pelleted cells were washed 3 times with cold TBS, resuspended in 500μl lysis buffer complemented with protease and phosphatase inhibitors and disrupted as described before. Resulting chromatin fractions were subsequently resuspended in fresh lysis buffer, sonicated in a Bioruptor (Diagenode) for a total time of 30 min (30 sec ON, 5 min OFF) and immunoprecipitated with anti-HA antibody (12CA5, Roche) overnight, plus 50μl 50% PAS for four more hours. Protein-DNA-bead complexes were washed 2 times in 1ml lysis buffer (no inhibitors), 2 times in 1ml lysis buffer containing NaCl (360mM), 2 times in 1ml wash buffer and 1 time in 1ml TE buffer, for 15 min in each individual solution.

Table 2-3 Primers used for performing ChIP analysis

Name	Target	Purpose	Sequence
Spcdc22 RTChIP Fw	Spcdc22	RT ChIP	ACTTAAAGTTCGGATGACGCGACG
Spcdc22 RTChIP Rv	Spcdc22	RT ChIP	GTTTGTAAGGTGGTAAATACCGGG
Spcdt2 RTChIP Fw	Spcdt2	RT ChIP	CTGGGTAACGTTTGGTGCATGTGA
Spcdt2 RTChIP Rv	Spcdt2	RT ChIP	TGAGGTCGTGTGTTCCAGTT
Spcdc18 RTChIP Fw	Spcdc18	RT ChIP	GGCATTTCATATCTTTGAGGATGAGTCGT
Spcdc18 RTChIP Rv	Spcdc18	RT ChIP	ATGTCGCGTTCAACTCTACGTGTC

Washed complexes were incubated in 100μl of elution buffer for 30 min at 65°C and resulting supernatants and previously prepared WCEs further incubated at 65°C overnight to reverse crosslinking. Finally, samples were purified and quantified using the Qiaquick PCR Purification (Qiagen) as described in the manufacturer's protocol. The iQ SYBR Green supermix (Bio-Rad) kit was used and RT-PCR reactions ran on a Chromo-4 Real-Time PCR Detector (Bio-Rad). Data was analysed using MJ Opticon Analysis Software 3.0 and values normalised against WCE.

2.2.4 – Promoter switch of *cig2+* to the *P41nmt1*-inducible promoter

Cig2 was placed under the control of the nmt1 promoter at the endogenous locus using the Bähler method (Bähler *et al.*, 1998). Succinctly, the plasmid pFA6a-KANMx6-P41nmt1 was amplified using TTTTGGTTACAAACAACTA GATATATTTCTATACGTTGATAAAAGGGTAATTTATCAATCCATATTTCAT GAATTCGAGCTCGTTTAAAC and TGTTTTCATCTTGATAACTATGCTTAT TGATTTTAGAACCAACAGGCTTTGAAATTGAATAGAGAGCCATGATTTAA CAAAGCGACTATA primers; the generated PCR product was then transformed into Wt cells using the lithium acetate transformation protocol described in section 2.1.4 and positive clones detected by ability to grow in kanamycin-YES plates. P41nmt1-tagging of *cig2*+ was confirmed by PCR for insertion of the *P41nmt1::kan* cassette and by RT-PCR for detecting reduced expression levels of *cig2*+, following addition of thiamine (vitamin B1).

2.2.5 – Mutagenesis of *Yox1*^{2A} mutant

DNA corresponding to the Yox12p-3HA stretch (Aligianni *et al*., 2009) was amplified using the GoTaq Flexi DNA polymerase (Promega) and CACGGATCTTAAGGGTGACTTC and ATTCGCAACTTCTTTGCTCTTC primers. Following PCR purification (Qiagen) the obtained PCR product was cloned into the TOPOpCR2.1 plasmid according to the manufacturer's protocol.

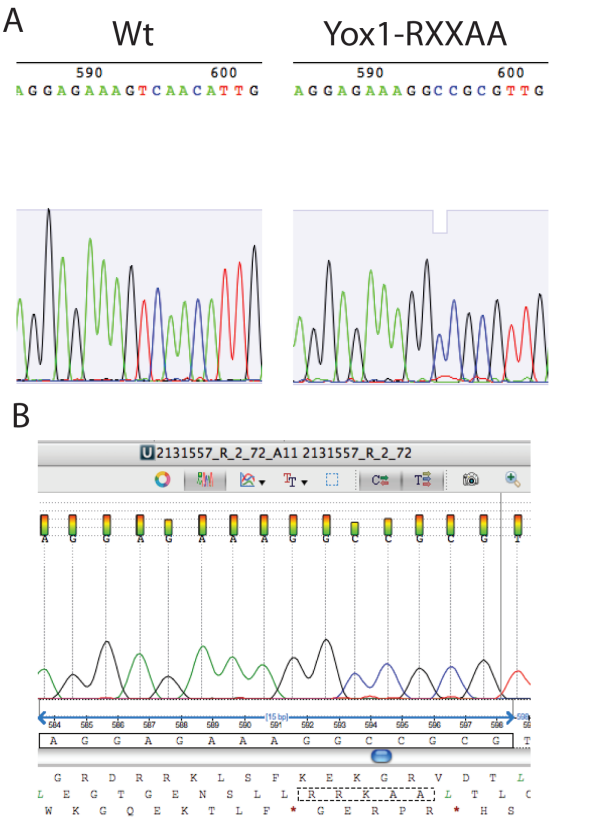

Figure 2.1 – *Yox1*^{2A} mutant

(A) Mutagenesis of the TOPO plasmid and (B) mutagenesis of *S. pombe*.

The *yox1*^{2A}-3H*A* mutants carrying amino acid substitutions S114A and T115A were then generated by PCR using the Quick-Change XL site-directed mutagenesis strategy (Stratagene) and primers CTCTCTTTTAAGGAGAAAGGCCGCGTTGACACTCTGCGAAACTTCTAC and GTAGAAGTTTCGCAGAGTGTCAACGCGGCCTTTCTCCTTAAAAGAG AG. *Yox1*^{2A}*-3HA* was integrated at the endogenous locus via homologous recombination by means of the lithium acetate method and incorporated mutations confirmed by DNA sequencing (Figure 2.1).

2.3 – Biochemistry

2.3.1 – DNA extraction

For each clone 10ml cultures were grown overnight in YES or EMM medium. Cells were pelleted and washed in 1ml ddH_2O and 200µl TE/SDS buffer (10mM TrisCl pH7.5, 1mM EDTA, 3% SDS) were added to the pellet followed by a small amount of glass beads. Cells were vortexed for 30sec and 500µl of TE added to the mixture. Cells were vortexed again for 30sec, followed by addition of 500µl Phenol. Once again cells were vortexed for 3min and centrifuged for 10min at full speed. 500µl of supernatant (aqueous phase) were pipetted into a fresh Eppendorf tube followed by addition of 350µl isopropanol. The mixture was vortexed for 10min at 13000rpm, the supernatant aspirated off and the pellet air-dried for 10min. Finally the pellet, containing DNA was dissolved in 30-50µl ddH_2O and used for genotyping or for cloning of Yox1p-3HA into the TOPO vector.

2.3.2 – Protein extraction

Samples were prepared using the TCA protein extraction method. Briefly, cell pellets were resuspended in 100µl 20% TCA in a 2ml Eppendorf, glass beads added to mixture and cells disrupted on a vortex for 3 minutes. 200µl 5% TCA were added to the disrupted cells, everything transferred into a fresh 1.5ml Eppendorf and cells centrifuged for 10 minutes at 3000rpm. Pellets were resuspended in 100µl Laemmli sample buffer and 50µl 1M Tris-HCl and boiled for 3 minutes in a heat block. Samples were centrifuged for another 10 minutes at 3000rpm and the supernatants transferred to fresh Eppendorfs.

2.3.3 – Western Blot analysis

Samples were prepared as described in section 2.3.2. Samples were ran in either 10% precasted Bis-Tris NU-PAGE gels from Invitrogen using MOPS or 8% and 10% costume-made Tris-Glycine gels using the Laemmli system. Proteins were transferred onto PVDF or nitrocellulose membranes by means of the semi-dry transfer system, the membranes blocked in 10% milk and primary and secondary antibodies diluted in 2% milk as described in table

2.4. Membranes were developed using the Luminata Crescendo, Western HRP substrates system from Millipore.

Table 2-4 List of antibodies

Name	Host	Purpose	Dilution	Company and Catalogue number
Anti-HA (12CA5) (1ry)	Mouse	Yeast/WB/ChiP	1/1000	Roche, 11583816001
Anti-HA (3F10) (1ry)	Rat	Yeast/WB	1/500	Roche, 11867423001
Anti-Myc (1ry)	Mouse	Yeast/WB	1/1000	Santa Cruz Biotechnology, sc-40
Anti-Cig2 (1ry)	Mouse	Yeast/WB	1/2000	Abcam, ab10881
Anti-Tubulin (1ry)	Mouse	Yeast/WB/ Immunofluorescence	1/5000 and 1/100	Abcam
Anti-E2F6 (1ry)	Rabbit	Mammalian/WB	1/1000	Santa Cruz Biotechnology, sc-22823
Anti-Cyclin E (1ry)	Mouse	Mammalian/WB	1/1000	Santa Cruz Biotechnology, sc-247
Anti-Cyclin A(1ry)	Mouse	Mammalian/WB	1/1000	Santa Cruz Biotechnology, sc-53230
Anti-Cdt1 (1ry)	Mouse	Mammalian/WB	1/1000	Millipore, #04-1524
Anti-Cdc6 (1ry)	Mouse	Mammalian/WB	1/1000	Santa Cruz Biotechnology, sc-9964
Anti-GAPDH (1ry)	Mouse	Mammalian/WB	1/1000	GeneTex, GTX627408
Anti-mouse-HRP (2ry)	Goat	WB	1/1000	Fisher Scientific, AFPA1-74421
Anti-rat-HRP (2ry)	Goat	WB	1/1000	GE Healthcare Life Sciences, NA935
Anti-rabbit-HRP (2ry)	Goat	WB	1/3000	Sigma, A9169
Anti-mouse-FITC (2ry)	Goat	Immunofluorescence	1/200	Sigma, F2653

2.3.4 – Co-immunoprecipitation

For each IP, 50ml of exponentially growing cells were mechanically disrupted (FastPrep) in lysis buffer containing protease inhibitors (Complete Mini, Roche) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail 1, Sigma-Aldrich) and glass beads (BioSpec) by 4x30s cycles with 4 minutes cool down periods. Subsequently Nrm1p-13myc, Nrm1p-3HA and Yox1p-3HA

were immunoprecipitated with either anti-HA (12CA5, Roche) or anti-myc (9E10, Santa Cruz Biotechnology) antibodies, by incubating lysates for 2h at 4°C with 50μl of 50% protein A Sepharose beads. SDS sample buffer was added to protein purified on beads and resolved by 10% SDS-PAGE. Nrm1p-13myc was detected using the previously described antibody, (12CA5, Roche) and Nrm1p-13HA and Yox1p-3HA a high affinity anti-HA (3F10, Roche) antibody.

2.3.5 – Phosphatase treatment

Lysates deriving from 50ml of exponentially growing cells were enriched for Yox1p-3HA as indicated above. Bead bound protein was washed 3x in IP buffer containing protease but not phosphatase inhibitors, resuspended in 900μl of washing buffer, divided into 3 and treated with either IP buffer alone, and IP buffer plus active λ-protein-phosphatase (1200 units final concentration, Sigma). Samples were then allowed to incubate at room temperature for 30 min, disrupted in SDS sample buffer and resolved in 10% SDS-PAGE as described previously.

2.3.6 – Flow cytometry

Samples were prepared as described in Sabatinos & Forsburg (Sabatinos and Forsburg, 2009). Briefly, cells were fixed in 1ml 70% ethanol; 300μl of this mixture washed in 3ml 50mM Na citrate, pellets resuspended in 0.5ml 50mM Na citrate containing 0.1mg/ml RNase A (Sigma) and left at room temperature for 2h. A volume of 0.5ml 50mM Na citrate containing 8μg/ml propidium iodide (Sigma) was subsequently added to a final concentration of 4μg/ml and samples sonicated for 30 sec., output control 4 using a Brason Sonifier 450 Sonicator prior to flow cytometry analysis. 20,000 single events were analysed for FSC, SSC and DNA content using the BD LSR II Flow Cytometer and the Flow_Jo v.9.2 Software.

2.4 – Cell Biology

2.4.1 – Rad22 foci quantification

For each strain, 25ml cultures were grow for a total time of 4.5h in EMM at 25°C and fixed by resupension in ice-cold 70% ethanol. Fixed cells were washed in sterile deonised water, mounted on glass slides using vectashield mounting medium, and captured using a Zeiss Axioskop Fluorescence Microscope set with a 100x objective, phase contrast 3, attached to a Hamamatsu Orca-ER Digital Camera connected to OpenLab software. Cells containing Rad22 foci were quantified using Image J Software 1.46j and classified according to number, 1 foci or 2 or more foci.

2.4.2 – Time-lapse microscopy

Cells were inoculated in 10 ml YES medium and grown overnight at 30°C. The resulting culture was diluted to OD_{595}=0.1 in 10ml of EMM medium and grown until OD_{595}=0.8. Following growth at 30°C, 1µl of cells were pipetted onto a bed of 2% EMM-rich electrophoresis agarose, prepared as follows: 1) 200mg of agarose were added to 10ml of EMM medium and heated in a microwave oven until the agarose was melted and mixed completely with the EMM medium; 2) the resulting mixture was aliquoted into individual Eppendorfs and stored at room temperature until needed; 3) immediately before mounting the cells, the previously prepared EMM-agarose was melted in a water bath at 95°C, 50µl pipetted onto a glass slide and the volume compressed into a fine layer (~200µm thick) using another glass slide; 4) following solidification, 1µl of freshly resuspended cells were mounted onto the EMM-agarose, a coverslip placed on the top of the cells and sealed with a 1:1:1 VALAP mixture (vaseline, lanolin and paraffin). The slide was then placed in an Axiovert 135 inverted fluorescence microscope. The temperature of the microscope chamber was set at 30°C and cells allowed to grow for 12h. Images were captured using the Volocity 5.5.1 software for 26ms, every 3 min with a 20x objective (aperture 0.5), with the focus set for autofocus.

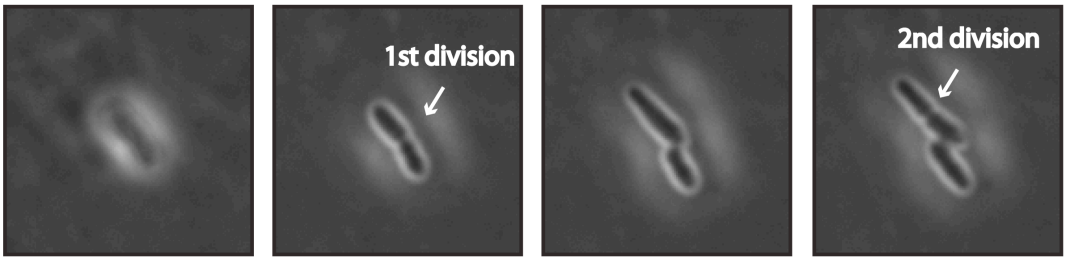

Figure 2.2 – Time of division quantification

Freshly grown cells were followed throughout 12 hours and their time of division from the first observed division until the second observed division was measured.

2.4.3 – Cell size quantification

Using the data obtained from the previously described time-lapse experiments, the length of 100 cells was measured from cell-tip to cell-tip, immediately before fission using the Volocity v.5.5.1 software.

2.4.4 – Time of cell division

Using the data obtained from the time-lapse experiments described in section 2.4.2, the time of division was acquired by measuring the time of 100 fully observed divisions (from the time of a firstly observed fission to the time of a subsequent fission) using the Volocity v.5.5.1 software.

2.4.5 – Cell staining – Hoechst 334 and Calcofluor

Exponentially growing cells were fixed with 1 ml 70% ice-cold ethanol and stored at -20°C until use. Cells were re-hydrated in 1ml PBS, spun down for 1min at 3000rpm and the supernatant discarded. The pellet was resuspended in 100μl 0.02125mg/ml calcofluor and incubated at room temperature for 5min. Cells were then washed with 1ml PBS, three times. The resulting pellet was resuspended in 5μl 2μg/ml Hoechst (prepared in ddH_2O) and mounted onto a microscope slide using Vectashield as the mounting medium. Images were captured using a Zeiss Axioskop Fluorescence Microscope set with a 100x objective, phase contrast 3, attached to a Hamamatsu Orca-ER Digital Camera connected to OpenLab software.

2.4.6 – Immunofluorescence

Exponentially growing cells were fixed with 1ml ice-cold methanol and samples stored at -20°C until needed. Cells were washed three times in 1ml PEM (100mM PIPES, 1mM EGTA, Mg_2SO_4, pH6.9) and digested with 50units/reaction Zymolyase at 37°C for 20 minutes. Digested cells were then washed three times in PEMS (PEM, 1.2M Sorbitol), permeabilised for 30sec. in 1ml 1% Triton X-100 in PEMS and washed once in PEM. Cells were then blocked by incubating for 50 minutes in PEMBAL (PEM, 0.1M L-lysine, 1% BSA, 0.1% Sodium Azide) on a rotary inverter at room temperature and resulting pellets resuspended in 30μl 1:100 mouse anti-tubulin (Abcam) antibody. Cells were incubated on a rotary inverter for 18 hours, following which they were washed three times with 1ml PEM. Finally cells were resuspended in 100μl 1:200 anti-mouse-FITC secondary antibody in PEMBAL, wrapped in aluminium foil, incubated on a rotary inverter overnight, washed three times in PEM, resuspended in 50μl 2μg/m Hoechst and mounted onto a microscope slide using Vectashield mounting medium. Images were captured using a Zeiss Axioskop Fluorescence Microscope set with a 100x objective, phase contrast 3, attached to a Hamamatsu Orca-ER Digital Camera connected to OpenLab software.

2.5 – Cell culture

2.5.1 – Tissue culture – media and growth conditions

hTERT immortalised retinal pigmentum epithelial (RPE) cells were grown in Dulbecco's Modified Eagle Medium (DMEM)/F12+Glutamax (Gibco,#10565-018), with 10% fetal calf serum (FCS), supplemented with penicillin and streptomycin (Invitrogen) antibiotics at 37°C in T75 flasks (Nunc). Cell dissociation was achieved using 0.05% Trypsin-EDTA (1X) (Gibco).

2.5.2 – SiRNA

Transfection of cells with siRNA was performed using the Lipofectamine 2000 (Invitrogen) system according to the manufacturer's protocol. Succinctly, cells were plated in 3cm tissue-culture Petri dishes. 250μl Optimem were then mixed with 5μl siRNA (20μM, either siControl, siE2F6a, siE2F6b or siE2F7) and in a separate reaction 250μl Optimem mixed with 5μl of Lipofectamine 2000. Five minutes after, these two reactions were mixed and allowed to form liposomes at room temperature for 20 minutes. Cells were then washed two times with PBS and siRNA+Lipofectamin2000 reactions added to cells together with 1.5ml Optimem. Cells were incubated with the referred mixture for up to 6 hours, following which fresh DMEM/F12+Glutamax containing antibiotics was added. Cells were collected and analysed 48 hours after.

2.5.3 – Western Blot analysis

Protein was extracted using RIPA buffer supplemented with protease (1/1000) and phosphatase inhibitors (1/1000). Succinctly, Petri dishes were washed twice with PBS, 200μl of RIPA buffer added to the Petri dishes, cells scraped using a plastic scraper and pipetted into an Eppendorf tube. Tubes were centrifuged at 14000rpm for 5min at 4°C and supernatants transferred into fresh Eppendorf tubes. Protein concentration was measured using the Bradford method as described in the manufacture's protocol. Samples were run using the NUPAGE gels system from Invitrogen as described in section

2.3.3. Membranes were blotted with primary and secondary antibodies as described in table 2.4.

3. The role of Yox1 in the DNA replication checkpoint response

3.1 – Objective 1 – The role of Yox1 in the DNA replication checkpoint response

In response to replication stress the DNA replication checkpoint is required to prevent entry into mitosis by blocking Cdc2 activity, stabilize stalled replication forks, aid replication fork processivity, and facilitate reinitiation of DNA replication once DNA replication stress has been resolved (Murakami *et al*., 2002) (Osborn *et al*., 2002) (Branzei and Foiani, 2008). A widely used method for inducing the DNA replication checkpoint response consists of the application of the ribonucleotide reductase inhibitor hydroxyurea (HU), a drug known to prevent expansion of the dNTP pool used by cells to replicate their DNA (Koç *et al*., 2004) (Alvino *et al*., 2007). Several studies have shown that HU-dependent activation of Rad3p leads to activation of Cds1p, which in turn phosphorylates Cdc10p, Nrm1p, and Ste9p, to keep MBF-dependent G1/S transcription on (Dutta *et al*., 2008) (de Bruin *et al*., 2008) (Chu *et al*., 2009). Persistent expression of MBF-dependent genes, via inactivation of Nrm1p function, is critical for cell survival from DNA replication stress (de Bruin and Wittenberg, 2009). Recently Yox1p was identified as an additional MBF co-repressor to Nrm1p, required to repress MBF transcription outside of G1 (Aligianni *et al*., 2009). The level of MBF transcription observed in HU-treated cells is comparable to that of both Δ*yox1* and Δ*nrm1* single mutants (Caetano *et al*., 2011). Based on these observations we hypothesised that just as established for Nrm1p (de Bruin *et al*., 2008), Yox1p could represent an additional target of the DNA replication checkpoint to keep MBF transcription active.

3.1.1 – Yox1p and Nrm1p dissociate from MBF promoters in response to DNA replication stress.

In response to HU treatment Nrm1 is unable to bind and repress MBF-dependent targets. To check if binding of Yox1p to MBF target promoters is affected in response to replication stress we carried out Chromatin Immuno-Precipitation (ChIP) in Yox1p-3HA-tagged cells with or without HU. Enrichment of *the cdc22+*, *cdc18+* and *cdt2+* promoters by Yox1p-3HA

pulldown was measured by means of quantitative PCR (qPCR) analysis. As depicted in Figure 3.1 Yox1p is released from *cdc22+*, *cdc18+* and *cdt2+* promoters following treatment with HU, suggesting that Yox1p is evicted from MBF promoters in response to DNA replication stress. Nrm1-3HA-tagged cells and actin were used as positive and negative controls, respectively.

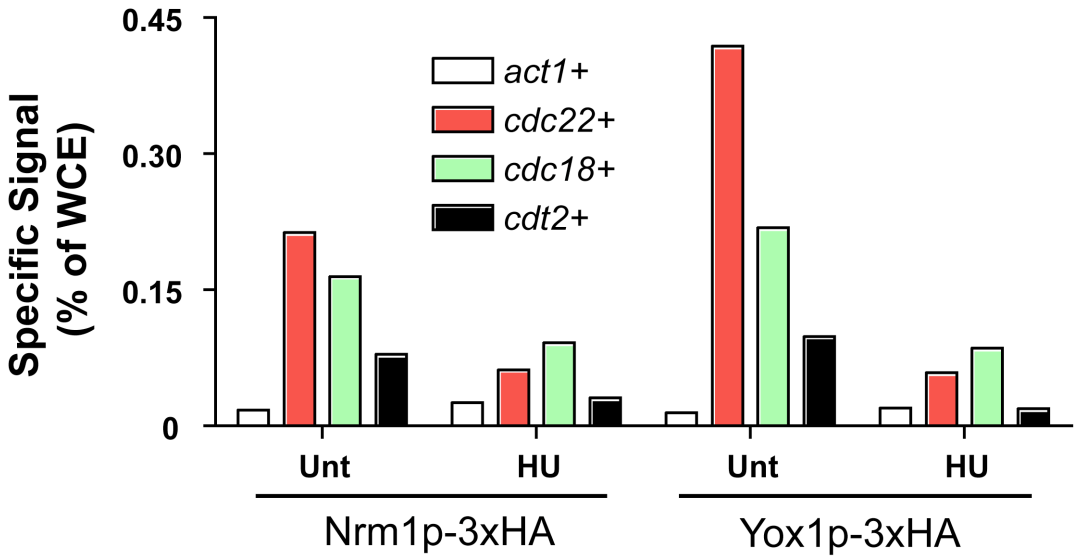

Figure 3.1 – Yox1p is unable to bind MBF promoters in response to DNA replication stress

Exponentially growing cultures were allowed to grow for 4h in the absence or presence of HU. Promoter fragments obtained from Nrm1p-3HA and Yox1p-3HA ChIPs were quantified using qPCR for MBF-dependent genes *cdc22+*, *cdc18+* and *cdt2+* and for *act1+*. Bar graphs represent percentage of whole cell extract (WCE) signal. Data representative of multiple independent experiments.

Inactivation and release of Nrm1p from MBF-dependent promoters results in increased levels of MBF transcription. In order to see if loss of Yox1p from MBF-dependent promoters has the same effect on MBF transcription as verified for Nrm1p, we monitored the expression levels of *cdc22+*, *cdc18+* and *cdt2+* in the untreated and HU-treated Yox1-3HA tagged cells by Reverse Transcriptase (RT) qPCR. As expected, the transcription levels of the referred transcripts are upregulated following addition of HU (Figure 3.2). Treatment with HU resulted in an average 5.2-, 5.9- and 6.1-fold increase in the levels of *cdc22+*, *cdc18+* and *cdt2+* transcripts in Yox1-3HA cells, respectively.

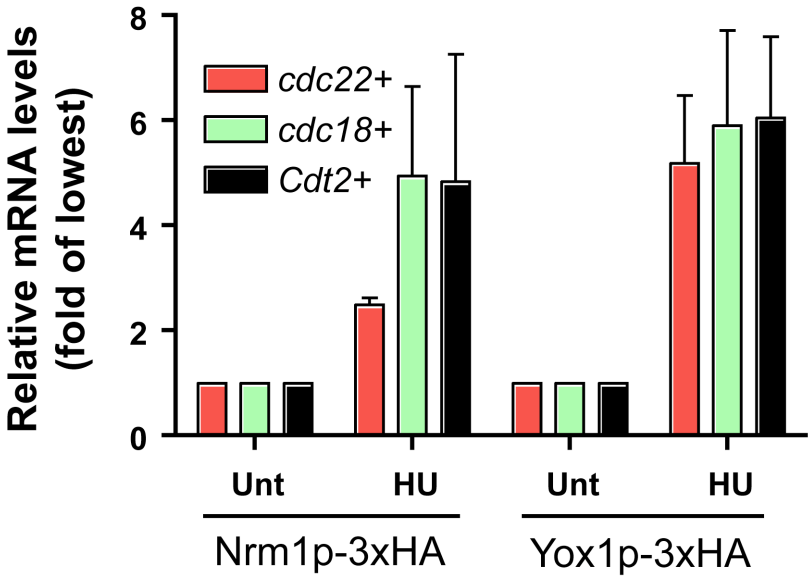

Figure 3.2 – Yox1p is unable to repress MBF transcripts in response to DNA replication stress

RT qPCR analysis of RNA levels in untreated and HU-treated Yox1-3HA-tagged cells. Fold induction over levels detected in untreated cells is shown. Values are normalised against *act1+*. Error bars represent the SE of three independent biological repeats.

Yox1p-HA was shown to interact with Nrm1p-Myc by co-immunoprecipitation (co-IP) experiments of affinity-purified Nrm1p complexes, during normal cell cycle progression (Caetano *et al.*, 2011) (Figure 5.1). To investigate the interaction between Nrm1p and Yox1p in response to checkpoint activation we carried out additional co-IPs and we can conclude that in the presence of HU the indicated proteins no longer interact (Figure 3.3). Together these data suggest that Nrm1p and Yox1p dissociate not only from MBF promoters but also from each other following HU-induced DNA replication stress.

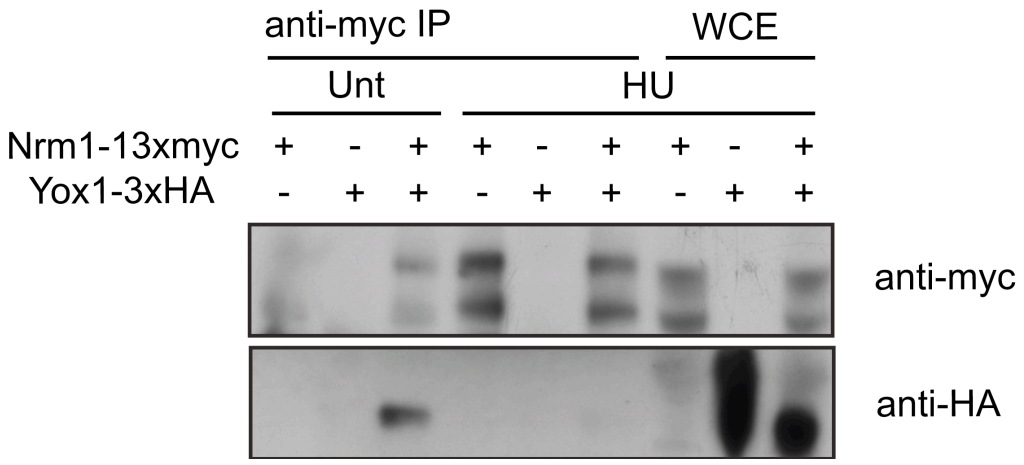

Figure 3.3 – Yox1p-3HA dissociates from Nrm1p-13myc following treatment with HU

Western blot analysis of Nrm1p and Yox1p from untreated (Unt) and HU-treated (HU) cells. Membranes were blotted for anti-myc (Nrm1p) and anti-HA (Yox1p) as described in section 2.3.3. Immunoprecipitation experiments were undertaken as indicated in section 2.3.4.

3.1.2 – Yox1p phosphorylation in response to HU-induced replication stress is Cds1p-dependent.

It has been shown that Nrm1p is phosphorylated in a checkpoint dependent manner following HU treatment (de Bruin *et al*., 2008). Furthermore, phosphorylation of the C-terminal region of Cdc10p has also been implicated in the mechanism by which the checkpoint activates MBF dependent transcription (Dutta *et al*., 2008). To establish whether Yox1p is phosphorylated in response to HU treatment we monitored Yox1p migration pattern via SDS-PAGE analysis from untreated and HU-treated Yox1-3HA cell lysates. As shown for Nrm1p (de Bruin *et al*., 2008) treatment with HU results in accumulation of a series of higher molecular weight species of Yox1p-3HA that migrate slower in the SDS-polyacrylamide matrix compared to Yox1p-3HA from untreated cells (Figure 3.4A and 3.4B). To test if the slower migrating species of Yox1p-3HA present in HU-treated samples are the result of phosphorylation, immunoprecipitated Yox1p-3HA was treated with λ-phosphatase. Phosphatase treatment collapses the slower migrating species (Figure 3.4A and 3.4B) indicating that Yox1p, like Nrm1p, is

phosphorylated in response to checkpoint activation. Given the involvement of Cds1p in the phosphorylation and inactivation of Nrm1p (de Bruin *et al*., 2008), we sought to determine whether phosphorylation of Yox1p in response to HU is also Cds1-dependent. Analysis of Yox1p-3HA mobility in *Δcds1* cells after HU treatment reveals that the phospho-shift is impaired in the absence of Cds1p, as no species of higher molecular weight were detected in the obtained anti-HA blots (Figure 3.4B). Thus, Yox1p phosphorylation in response to HU treatment is Cds1-dependent.

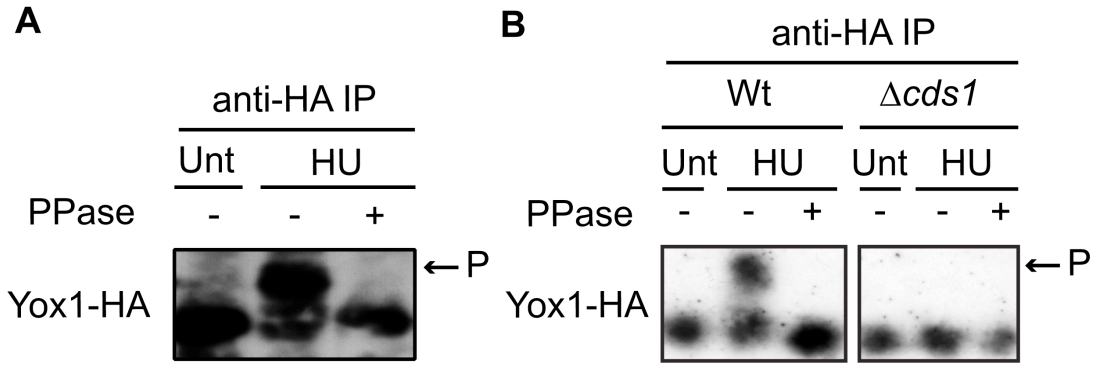

Figure 3.4 – Yox1p HU-induced phosphorylation is Cds1p dependent

(A) Yox1-3HA deriving from untreated and HU-treated cells detected by high-affinity anti-HA antibody. Part of Yox1-3HA from HU treated cells was treated with phosphatase (PPase) as described in the methods and materials section. (B) Yox1p-3HA in HA-enriched lysates from untreated and HU-treated wild type and *Δcds1* cells. Arrow and letter P indicate, slower migrating, phosphorylated Yox1p-3HA.

3.1.3 – Inactivation of Yox1p is an essential part of the checkpoint response.

Cds1+ null mutant cells are extremely sensitive to the deleterious effects caused by HU and MMS (Murakami and Okayama, 1995) (Lindsay *et al*., 1998). This is attributable, in part, to their inability to maintain the MBF transcriptional programme (de Bruin *et al*., 2008). Constitutive activation of MBF-dependent transcription, as observed in *Δnrm1* cells, suppresses the sensitivity of *Δcds1* cells to chronic exposure but does not seem to have a role in the acute response to genotoxic stress (Dutta *et al*., 2008). Based on

these observations, we hypothesised that deletion of *yox1+*, would suppress sensitivity of Δ*cds1* cells to HU and MMS, by restoring sustained MBF transcription. To test this hypothesis we compared the sensitivities of wild type, *Δcds1*, *Δnrm1*, *Δyox1*, *Δcds1Δnrm1* and *Δcds1Δyox1* cells to chronic exposure to HU and MMS, through the application of survival assays. The obtained results revealed that, like Δ*nrm1*, deletion of *yox1+* suppresses the sensitivity of *Δcds1* cells to both HU and MMS (Figure 3.5). In this context, failure to inactivate Yox1p due to absence of Cds1p is rescued by abrogation of Yox1p itself, demonstrating that inactivation of Yox1p is a vital step in the checkpoint response.

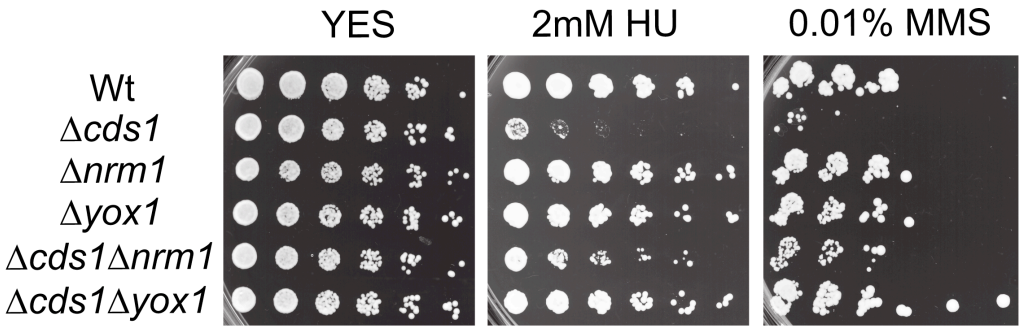

Figure 3.5 – Inactivation of Yox1p following genotoxic stress is essential for cell survival

Spot assays. Cultures were allowed to grow to stationary phase. Five-fold serially dilution of wild type, *cds1Δ*, *Δnrm1*, *Δyox1*, *Δcds1Δnrm1* and *Δcds1Δyox1* cells were then spotted onto YES or YES plus indicated concentrations of HU or MMS. Cells were allowed to grow for 3-5 days and pictures taken using a high definition scanner.

3.1.4 – Phosphorylation of Yox1p at S114, T115 sites plays an important role in checkpoint regulation of MBF transcription.

Since Nrm1p has been shown to be a direct target of Cds1p *in vitro* (de Bruin *et al*., 2008) we hypothesized that phosphorylation of Yox1p by Cds1 might also be involved in its dissociation from MBF. In an effort to establish the requirement of Yox1p phosphorylation for its release from the transcription complex, following treatment with HU, we looked for putative Cds1p-recognition motifs in the Yox1p amino-acid sequence (O'Neill *et al*., 2002) (Seo *et al*., 2003) (Smolka *et al*., 2007). We identified one such consensus

sequence at amino-acids 111-115 of Yox1p (Figure 3.6A; RRKST). Conversion of the Ser114 and Thr115 sites to alanine residues at the endogenous locus (*yox1*^{2A} mutant strain, section 2.2.5), results in a dramatic effect on the mobility shift of the HA-tagged mutant protein in response to HU *in vivo* (Figure 3.6B).

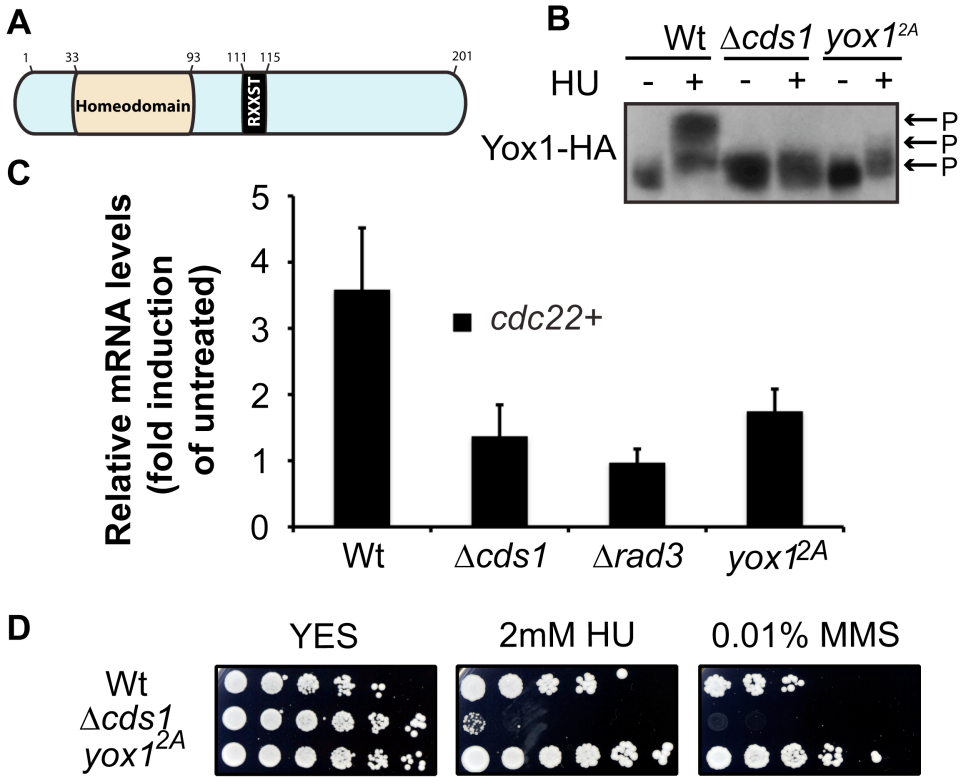

Figure 3.6 – HU challenge induces phosphorylation of Yox1p at its RXXST consensus

(A) Cartoon diagram displaying the molecular arrangement of the homeodomain and the putative RXXS/T motif in Yox1p. Not to scale. (B) SDS-PAGE electrophoresis of HA-tagged Yox1 in untreated and HU-treated wild type, *Δcds1*, and *yox1*^{2A} cells as described before. (C) RT-qPCR analysis of *cdc22+* transcript levels corresponding to fold induction over untreated wild type for wild type, *Δcds1*, *Δrad3* and *yox1*^{2A} cells. Bars represent the average value, and error bars represent their SE, obtained by qPCR of triplicate biological samples. (D) Five-fold serially dilution volumes of wild type, *yox1*^{2A} and *cds1Δ*, cells were spotted onto YES or YES plus indicated concentrations of HU or MMS.

This indicates that the RRKST site is one of the main sites at which Yox1p is phosphorylated following activation of the DNA replication checkpoint.

Consistent with a possible role in phosphorylation-dependent inactivation of Yox1p by Cds1p, we observe significant repression of the MBF target *cdc22*+ in response to HU treatment in the *yox1*^{2A} mutant (Figure 3.6C). The level of expression is significantly lower than that observed in wild-type cells but somewhat higher than that observed in the Δ*cds1* and Δ*rad3* checkpoint mutants. However, inability to fully induce MBF-dependent transcription in response to checkpoint activation in the *yox1*^{2A} mutant does not result in an increase in HU sensitivity (Figure 3.4D). We have also monitored the levels of Rad22 foci, a marker of genome instability, in *yox1*^{2A}, however no increase in genome instability was observed (data not shown).

4. G1/S cell-cycle transcription and genome stability

4.1 – Objective 2 – G1/S-cell cycle regulated transcription and genome stability in *S. pombe*

The primary regulation of cell proliferation in mammalian cells is imposed during the G1-S transition of the cell cycle. Proliferation requires the activation of the G1/S transcriptional programme, which is regulated by the MBF transcription factor and the E2F family of transcription factors in fission yeast and in mammalian cells, respectively (Reymond *et al*., 1993) (Cam and Dynlacht, 2003). The E2F family of transcription factors is composed of three activators (E2F1-3) and five repressors (E2F4-8). In addition it requires three co-regulators, the pocket proteins pRb, p107 and p130, to confine transcription to G1-S. The high frequency of genetic alterations found in tumour cells that affect E2F-dependent transcriptional repression suggests that misregulation of this pathway, and hence constitutive G1/S transcription, may be necessary for the development of cancer (Chen *et al*., 2009).

Whereas derepression of G1/S transcription has clearly been shown to contribute to uncontrolled cell division in cancer cells it has also been suggested to directly contribute to the accumulation of genome instability (Chen *et al*., 2009). To study the consequences of derepressing G1/S transcription on genome stability independent of the loss of control over the G1-to-S transition in mammalian cells is hard to do. However, fission yeast has an exceedingly short G1 interval in unperturbed cycling cells growing in nutrient-rich media. Furthermore G1/S transcription is regulated by a unique transcription factor (MBF), which repression can be easily alleviated via inactivation of either or both *nrm1+* and *yox1+* co-repressors. Hence, *S. pombe* represents the perfect model organism to study the effect of derepression of G1/S transcription on genome stability.

In this section we will show that inactivation of the G1/S transcriptional co-repressors Nrm1 and Yox1 results in de-repression of MBF-dependent transcription outside of the G1 phase of the cell cycle. Our work establishes that this induces genomic instability probably due to sporadic re-firing of DNA replication origins and thereby rereplication. We suggest that this is the result of a delicate balance between high levels of the MBF-dependent genes

encoding a DNA replication licensing factor (Cdc18) and cyclin (Cig2), hence between replication initiation factors and CDK activity.

4.1.1 – Deletion of *nrm1+* and *yox1+* leads to constitutive de-repression of MBF transcripts

ChIP and co-immunoprecipitation studies have shown that is it possible that Yox1p binds to MBF promoters through Nrm1p to repress expression of MBF transcripts outside of G1/S phases (Figure 5.1) (Caetano *et al*., 2011) (Caetano *et al*., 2011). To determine the contribution of Nrm1p and Yox1p to the repression of MBF-dependent transcription we analysed the expression levels of 19 MBF-dependent transcripts in wild type, *Δnrm1*, *Δyox1* and *Δnrm1Δyox1* cells (Figure 4.1).

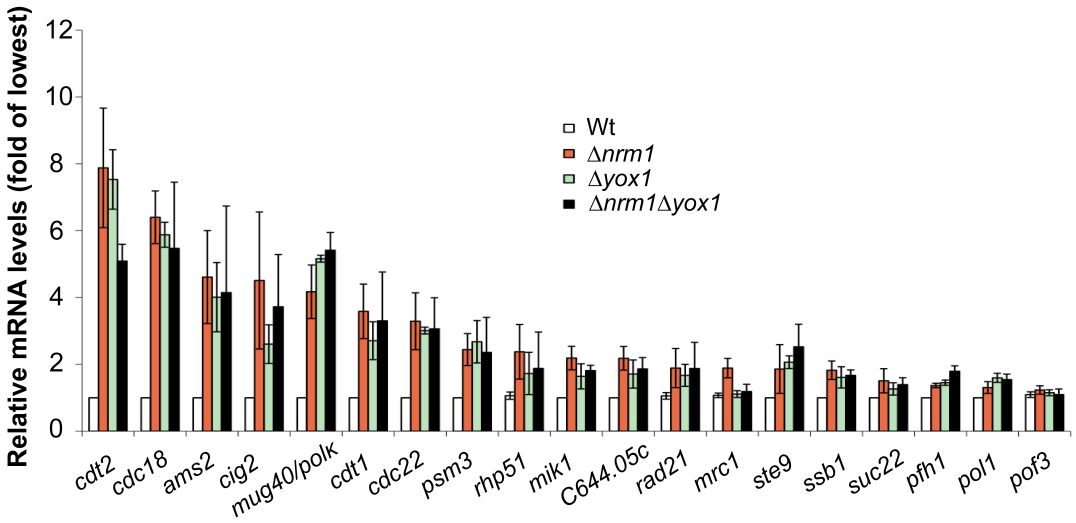

Figure 4.1 – Nrm1p and Yox1p require each other to repress MBF transcription

Relative mRNA levels obtained by RT-qPCR for 19 MBF-dependent transcripts in wild type, Δ*nrm1*, Δ*yox1* and Δ*nrm1*Δ*yox1* cells. Transcript levels are shown as fold-induction of the lowest transcript levels detected for each gene. Bars represent the average value, and error bars represent their SE, obtained by RT-qPCR of triplicate biological samples.

Consistent with data obtained previously from RT-qPCR experiments and microarray expression profiling, deletion of either *nrm1+* or *yox1+* promotes an overall upregulation of the MBF transcriptional programme (de Bruin *et al*., 2008) (Aligianni *et al*., 2009). The fold-induction generated by abrogation

of *nrm1+* and *yox1+* varies widely across the studied transcripts, with a maximum of 7.9-fold and 7.5-fold for transcript *cdt2+* and a minimum of 1.1-fold and 1.1-fold for transcript *pof3+* in *Δnrm1* and *Δyox1* cells, respectively.

The expression signature of the studied transcripts in the single mutants is similar, if not identical, to that observed for cells co-deleted for both *nrm1+* and *yox1+*, with the exception of transcript *cdt2+*, which seems to be less upregulated in the double mutant than in each individual mutant strain. These data indicate that Nrm1p and Yox1p have a mutual dependency for proper promoter binding and that this is likely to be the cause for their non-redundant role in transcriptional repression of MBF.

4.1.2 – Constitutive expression of G1/S transcription induces abnormal progression through the cell cycle and activates the DNA damage checkpoint

In fission yeast cell elongation is normally used as a diagnostic marker for cells with cell division defects, cells that do not progress normally through the cell cycle, a phenotype known as the cd*c*- (cell division cycle) phenotype, and often associated with genome instability (Nurse *et al*., 1976). Microphotographs of Δ*nrm1*, Δ*yox1,* Δ*nrm1*Δ*yox1* cells (Figure 4.2A) show that constitutive expression of MBF-dependent transcripts generates a cell elongation phenotype in these cells. To establish this possibility we measured the cell length of 100 cells from cell tip to cell tip of the different strains immediately before cell division using time-lapse images (Figure 4.2B). This revealed that (n=100; mean+/-SE) Δ*nrm1* (18.5+/-0.36μm), Δ*yox1* (18.1+/-0.26μm) and Δ*nrm1*Δ*yox1* (18.9+/-0.6μm) cells are about 1.31-, 1.28- and 1.34-times more elongated on average than their wild type (14.2+/-0.15μm) counterpart, respectively. This phenotype was particularly accentuated in the Δ*nrm1*Δ*yox1* double mutants, with a maximum length of 52.8μm for these cells compared to a maximum length of 17.5μm for wild type cells.

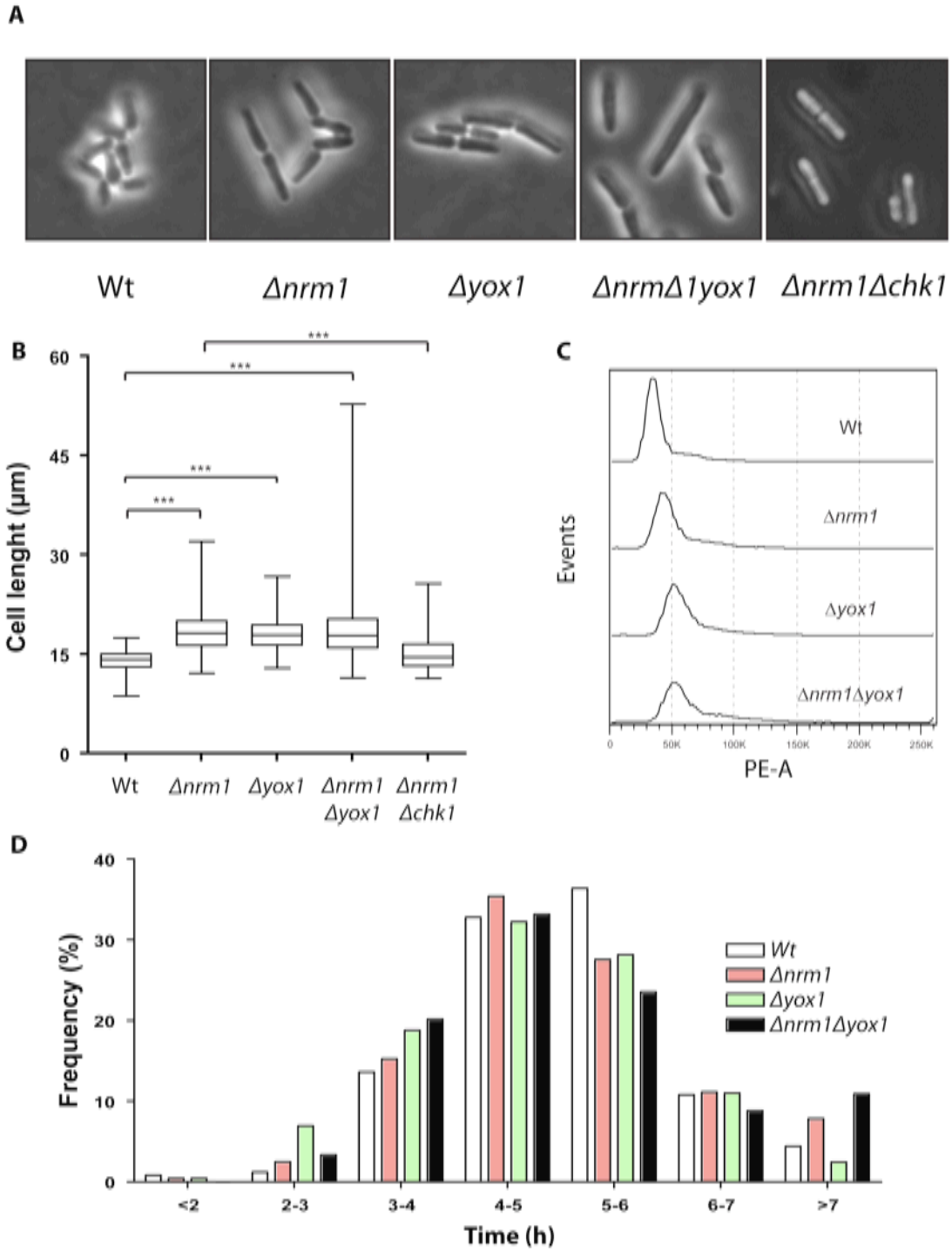

Figure 4.2 – Constitutive expression of the MBF transcriptional programme induces cell cycle delay

(A) Microphotographs showing wild type, Δ*nrm1*, Δ*yox1,* Δ*nrm1*Δ*yox1* and Δ*nrm1*Δ*chk1* cells. (B) Whisker plot displaying the maximum, minimum and quartile lengths of 100 cells immediately before septation was observed. Statistical treatment: unpaired, 2-tail t-student-test, * $p<0.05$, ** $p<0.005$, *** $p<0.001$. (C) Flow cytometry DNA profiles of the same cells (see methods and text for further details). PE-A=DNA content (D) Frequency histogram displaying the profile of cell division for the same strains. Data was binned according to time of division group.

Furthermore, FACS analysis of asynchronous populations (Figure 4.2C) shows that Δ*nrm1*, Δ*yox1* and Δ*nrm1*Δ*yox1* cells display a typical cdc-phenotype, as their main peaks are wider, lower and more to the right than that of wild type cells. Taken together these data suggest that Δ*nrm1*, Δ*yox1* and Δ*nrm1*Δ*yox1* cells may be delayed in S and/or G2 phases, due to activation of the DNA replication and/or DNA damage checkpoints.

In order to assess if the elongated phenotype observed in cells with constitutive G1/S expression is the result of DNA damage checkpoint activation we have monitored the cell length of 100 cells deleted for both *nrm1*+ and the DNA damage checkpoint effector kinase *chk1*+. If the elongated phenotype was checkpoint-dependent, inactivation of *chk1*+ should suppress cell elongation. Indeed Δ*nrm1*Δ*chk1* cells are on average 15.14μm long, about 3.3μm less elongated than *nrm1*+ single mutants and only slightly longer than wild type cells. This suggests that Δ*nrm1* cells may develop DNA damage, which is likely the result of constitutive G1/S transcription expression (Figures 4.2A and 4.2B). To confirm if Δ*nrm1*, Δ*yox1* and Δ*nrm1*Δ*yox1* cells progress abnormally through the cell cycle we have examined their cell division profiles using the same time-lapse images as before. Whilst the majority of wild type cells take between five to six hours to divide, the majority of Δ*nrm1*, Δ*yox1* and Δ*nrm1*Δ*yox1* cells take either longer or shorter periods of time to complete the same task (Figure 4.2D). This allows us to conclude that although the average time of division does not change significantly between strains, their time distribution does, with Δ*nrm1*, Δ*yox1* and Δ*nrm1*Δ*yox1* cell subpopulations progressing either slower or faster through the cell cycle than wild type cells. In addition, and not included in the frequency histogram we have observed a much higher frequency of cells that were unable to divide in the Δ*nrm1*Δ*yox1* strain compared to results obtained for wild type and single mutants.

4.1.3 – MBF transcriptional repression outside of G1/S is essential for maintaining genome stability

Cells abrogated for the MBF repressor Res2p and the co-repressors Nrm1p and Yox1p show constitutive overexpression of MBF transcripts, although

expression induction is lower in Δ*res2* cells (Figure 4.1) (Zhu *et al*., 1997) (de Bruin *et al*., 2008) (Aligianni *et al*., 2009). Although persistent MBF transcription is required for survival of cells undergoing replicative stress (e.g. HU treatment) as seen in section 3 of this report, results described above (4.2.2) prompted us to hypothesise that expression of the MBF transcriptional programme outside of G1/S phases may be deleterious to cells undergoing normal mitotic division and induce genomic instability.

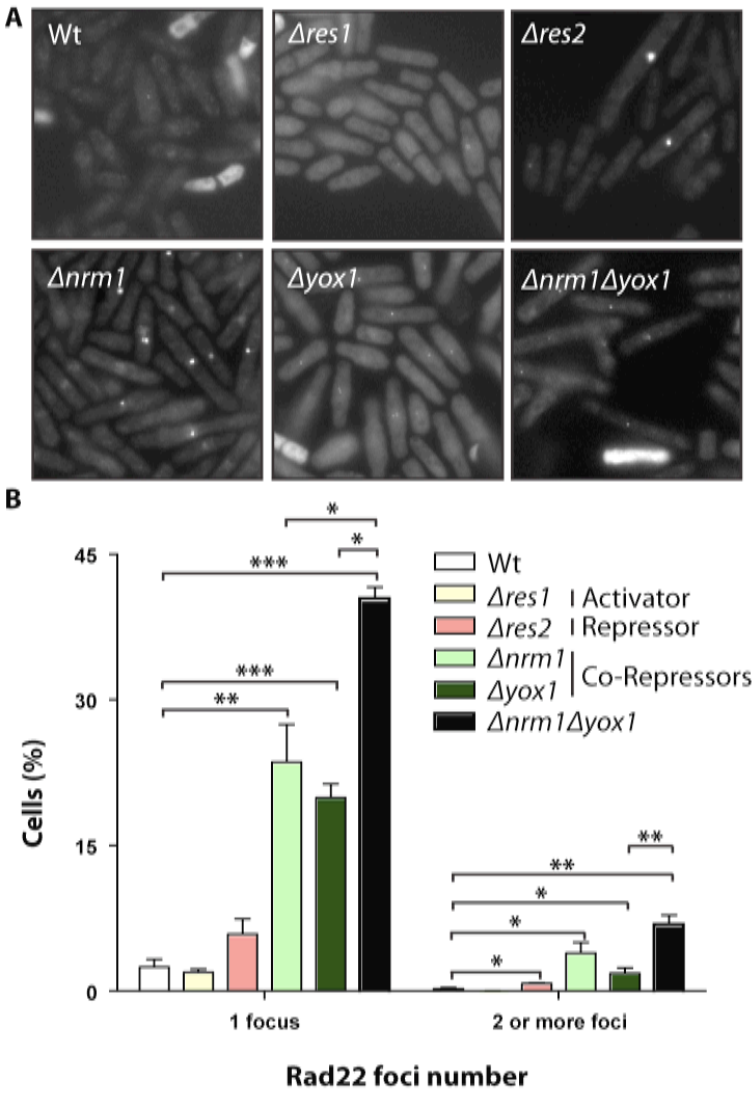

Figure 4.3 – Δ*nrm1*, Δ*yox1* and Δ*nrm1*Δ*yox1* cells present genome instability

(A) Fluorescent microphotographs of Rad22-YFP foci in wild type, Δ*res1*, Δ*res2*, Δ*nrm1* Δ*yox1* and Δ*nrm1*Δ*yox1* cells. (B) Bar graph displaying the quantification of Rad22 foci for the strains described in figure 4.3A. Error bars correspond to the SE of three independent biological experiments. Statistical treatment: unpaired, 2-tail t-student-test, * p<0.05, ** p<0.005, *** p<0.001.

To further investigate this we monitored formation of Rad22 foci, a marker for single stranded DNA, DSBs, collapsed DNA replication forks and persistent recombination intermediates in wild type and mutant cells (Kim *et al*., 2000). Abrogation of the MBF repressors Nrm1p, Yox1p and Res2p (Δ*res2* mutants are also more elongated than wild type cells), but not the activator Res1p results in accumulation of Rad22 foci (Figure 4.3A). Circa 23.6% and 20% of Δ*nrm1* and Δ*yox1* cells present formation of single Rad22 foci, respectively, compared to only 2.5% of wild type cells (Figure 4.3B). The same is true for formation of two or more Rad22 foci. These data further suggest that derepression of G1/S transcription causes accumulation of DNA damage.

4.1.4 – E-MAP analysis reveals that derepression of MBF-dependent transcription causes replication stress

Next, to establish the cause and nature of the damage, we carried out genome wide epistasis map (E-MAP) analysis of the Δ*nrm1* deletion mutant in collaboration with the Krogan lab (Table 4.1). We anticipated that negative interactions identified by this analysis would reveal the proteins required to deal with the genotoxic stress caused by *nrm1*+ inactivation and G1/S transcriptional induction. Conversely, positive interactions might reveal proteins that cause the genomic instability in Δ*nrm1* cells, which we anticipated to be direct or indirect targets of MBF. Gene ontology enrichment analyses (using cytoscape) revealed that 8% (data not shown) of the genes obtained in the E-MAP dataset had functions in DNA repair and/or DNA replication checkpoint control (Table 4.1). See Ryan *et al* (2012) for methods.

Of the negative interactions many genes encode proteins involved in the DNA replication checkpoint control, indicating that the genotoxic stress caused by *nrm1*+ inactivation is likely to result from DNA replication stress. In addition a negative interaction was found when inactivating Whi5. Whereas Whi5 function in fission yeast is not well established, in budding yeast Whi5 was identified as an inhibitor of G1/S transcription (de Bruin *et al*., 2004) (Costanzo *et al*., 2004). This suggests that inactivation of Whi5 might further derepress G1/S transcription in Δ*nrm1* cells resulting in additional genotoxic stress. Based on the negative interaction found in the E-MAP analysis of Δ*nrm1* we conclude that the nature of the genotoxic stress caused by

derepression of G1/S transcription is likely to be replication stress, which is induced by the upregulation of one or more MBF targets.

Table 4-1 Δ*nrm1* epistasis interaction screen

Interaction	Gene	Effect	Function
Negative	Mms22	-16.4	Repair of resolving DNA replication intermediates and prevention of damage caused by blocked replication forks
	Eme1	-6.2	Holliday junction resolvase subunit
	Rad3	-5.9	ATR checkpoint kinase
	Mrc1	-5.9	Mediator replication checkpoint
	Nse5	-4.9	Replication fork protection
	Rad32	-4.6	Nuclease involved in homologous recombination
	Whi5	-4.2	G1/S transcriptional repressor
	Swi3	-4.2	Replication fork protection complex subunit
	Rad26	-3.8	Cds1 activation. ATR checkpoint kinase regulatory subunit (ATRIP)
	Nse6	-2.0	Replication fork protection
Positive	Rad24	2.1	DNA damage checkpoint (14-3-3)
	Rad25	2.1	DNA damage checkpoint (14-3-3)
	Res2	2.9	G1/S transcription. MBF subunit

Interestingly a positive interaction was found with Δ*res2*. As discussed earlier Res2p is thought of as the repressor subunit of MBF. However, whereas inactivation of *res2+* results in constitutive MBF-dependent transcription, transcript levels are around 60% of those observed in Δ*nrm1* cells (data not shown). Even though Nrm1p is unable to bind MBF in the absence of Res2p (de Bruin *et al.*, 2008) this is thought to be the result of the MBF transcription

factor complex being compromised in the absence of *res2*+. Importantly inactivation of both Res2p and Nrm1p results in transcript levels similar to that observed in Δ*res2* (data not shown). The positive interaction observed therefore suggests that the level of induction of MBF targets is a direct measurement of the genotoxic stress induced by the derepression. Disappointingly, none of the known MBF targets tested in the E-MAP analysis were found to have a positive interaction with *nrm1*+ inactivation. Based on those results we were unable to predict which MBF targets cause the replication stress. However, not all MBF targets were included in the E-MAP analysis. Most notably *cig2*+ and the essential genes *cdc18*+ and *cdt1*+ were missing. Interestingly all three are involved in regulating DNA replication, and our E-MAP analysis suggests that derepression of MBF targets causes replication stress.

4.1.5 – High levels of DNA replication licensing factors may cause genomic instability

Based on the previous results we hypothesise that accumulation of the licensing factors Cdc18p and Cdt1p (Figure 4.1) may be the cause of why these cells present more genome instability than their wild type counterparts, as their overexpression is known to generate rereplication and endoreduplication. We also predict, based on the DNA profiles of these cells (Figure 4.2) that de-repression of G1/S transcription induces sporadic rereplication and not full endoreduplication. Surprisingly, given the redundant role of Nrm1p and Yox1p in repressing MBF transcription, a much higher frequency of cells containing Rad22 foci was observed for the *nrm1*+ and *yox1*+ double mutant than for each of the individual mutant strains. Analysis of the G1/S transcriptional programme of Δ*nrm1*Δ*yox1* cells (Figure 4.1) shows that this strain presents lower levels of *cdt2*+ than Δ*nrm1* or Δ*yox1* single mutant strains. As observed for mammalian cells Cdt2p is involved in the degradation of Cdt1p in fission yeast. These results indicate that Δ*nrm1*Δ*yox1* may accumulate higher levels of Cdt1p than Δ*nrm1* and Δ*yox1* single mutant cells and that, given that *cdc18*+ is also upregulated in these cells, sporadic rereplication may be enhanced. Hence the additional genomic instability observed in the double mutant strains. It is reasonable to suggest

that Nrm1p and Yox1p may regulate the expression of *cdt2*+ differently compared to other G1/S genes.

4.1.6 – Derepression of G1/S transcription causes aberrant DNA

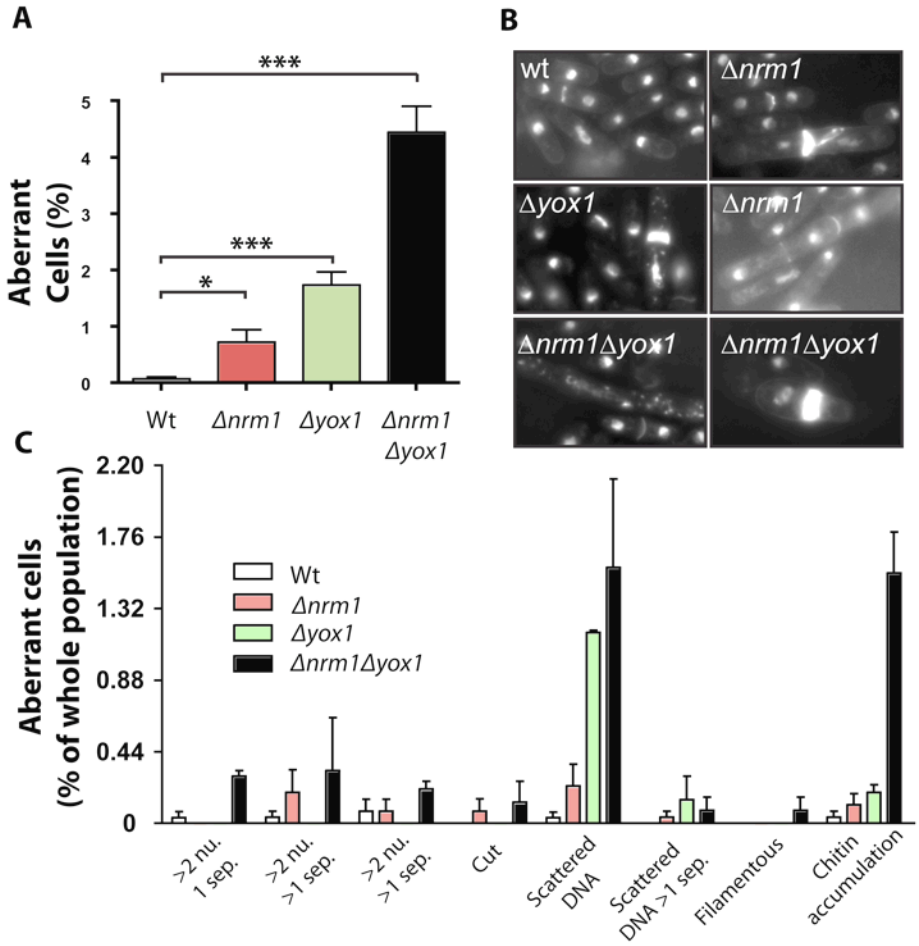

Figure 4.4 – *Δnrm1*, *Δyox1* and *Δnrm1Δyox1* cells show morphologic defects

(A) Quantification of wild type, *Δnrm1*, *Δyox1* and *Δnrm1Δyox1* cells presenting aberrant phenotype. Results are represented as percentage of whole cell population. Statistical treatment: unpaired, 2-tail t-test, * $p<0.05$, ** $p<0.005$, *** $p<0.001$. (B) Photomicrographs displaying examples of the aberrant phenotypes observed. (C) Quantification of aberrant cells per morphological defect group. Note the higher incidence of scattered DNA and chitin accumulation compared to the other aberrant phenotypes. Results are presented as percentage of whole cell population. Error bars correspond to the SD of three independent biological experiments looking at approximately 500 cells per experiment.

Another indicator of genomic instability is the detection of aberrant DNA. Expression of G1/S transcription outside of G1/S phases causes a

statistically significant increase in cells presenting aberrant cell morphology compared to wild type cells (Figures 4.4A, B and C). We have stained all strains for their nucleus (Hoechst 33342) and septum (calcofluor) and measured the number of cells presenting aberrant morphology. In average (n>250; mean+/-SD), about 0.7+/-0.38%, 1.7+/-0.4% and 4.4+/-0.8% of Δ*nrm1*, Δ*yox1* and Δ*nrm1*Δ*yox1* cells present aberrant phenotypes, respectively, compared to only 0.07+/-0.06% of wild type cells.

Several morphological defects have been detected: cells with more than 2 nuclei and 1 septum, trinucleated cells presenting more than 1 septum, cells presenting chromosome misegregation (scattered DNA), cells with cut phenotype, cells with accumulated chitin, cells donated with filamentous growth, amongst others (Figure 4.4C). We attribute the pleiotropic nature of the aberrant morphologies observed to the fact that these are the consequence of the replication stress caused by the induction of G1/S transcripts in these mutants (Figure 4.1). We hypothesise that Cdc18p and Cdt1p may play a predominant role in indirectly causing the genomic instability detected in the studied mutants.

4.1.7 – Genetic interaction of Nrm1p and Yox1p with Cig2p

A number of observations have prompted us to analyse the interaction between *Δnrm1* and *Δyox1* mutants with *Δcig2* cells. Firstly *cig2*+ was the only non-essential MBF target that was not represented in our E-MAP study. Secondly, Cig2p is involved in the degradation of Cdc18p through the ubiquitin ligase SCF at the beginning of S-phase (Lopez-Girona *et al*., 1998) (Kominami and Toda, 1997). Since we hypothesise that overexpression of Cdc18p in Δ*nrm1* cells might cause replication stress, Cig2p might be required for viability of Δ*nrm1* cells. Thirdly, Cig2p/Cdc2p has been reported to phosphorylate MBF on its DNA-binding subunit Res1p at residue S130 (Ayté *et al*., 2001). Inactivation of *cig2*+*,* or loss of S130 phosphorylation has been suggested to induce prolonged activation of G1/S transcription (Ayté *et al*., 2001). If Cig2p and Nrm1p or Yox1p have independent effects on MBF-regulated transcription, then the Δ*nrm1*Δ*cig2* and Δ*yox1*Δ*cig2* double mutants should be additive in terms of their effect on MBF transcription and, perhaps, in terms of cellular phenotype. In order to test this hypothesis we have

crossed Δ*nrm1* and Δ*yox1* cells with Δ*cig2* cells. As predicted, crosses of Δ*nrm1* and Δ*yox1* with Δ*cig2* give rise to lethal or highly compromised haploid segregants (Figure 4.5A).

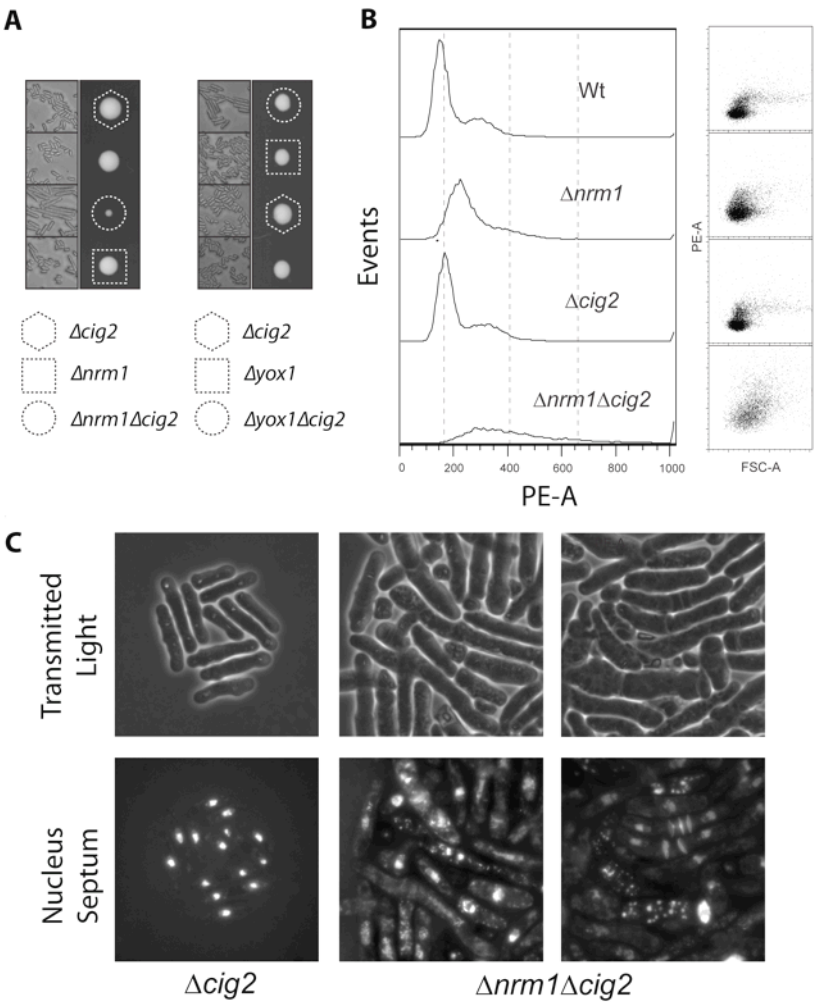

Figure 4.5 – Genetic interaction of Δ*nrm1* and Δ*yox1* mutants with Δ*cig2* cells

(A) Haploid Δ*nrm1* and Δ*yox1* mutants carrying an integrated Rad22-YFP construct were crossed with a Δ*cig2* mutant and haploid meiotic segregants dissected as described in the methods and materials section. Segregants were analysed for presence of wild type, each of the parental disruption and the double mutant. No healthy double mutants were observed (B) DNA FACS profile of the same cells. DNA was stained with propidium iodine (C) Photomicrographs displaying the aberrant morphology of the Δ*nrm1*Δ*cig2* double mutant. Cells were stained for nucleus and septum using the dyes Hoechst 33242 and calcofluor, respectively.

Examination of Δ*nrm1*Δ*cig2*-germinated spores reveals a dramatic elongated shape, characteristic of cell cycle arrest, and striking morphological defects widely spread across the cell population (Figure 4.5C). Examples include but

are not limited to missegregated and broken chromosomes, multiseptated cells and filamentous growth. Furthermore, these cells present enhanced DNA damage as measured by formation of Rad22 foci compared to Δ*nrm1* single mutants (Figure 4.6A and B). Furthermore, Δ*nrm1*Δ*cig2* cells present a statistically significant increase in the formation of two or more Rad22 foci compared to Δ*nrm1* cells (Figure 4.6A and B).

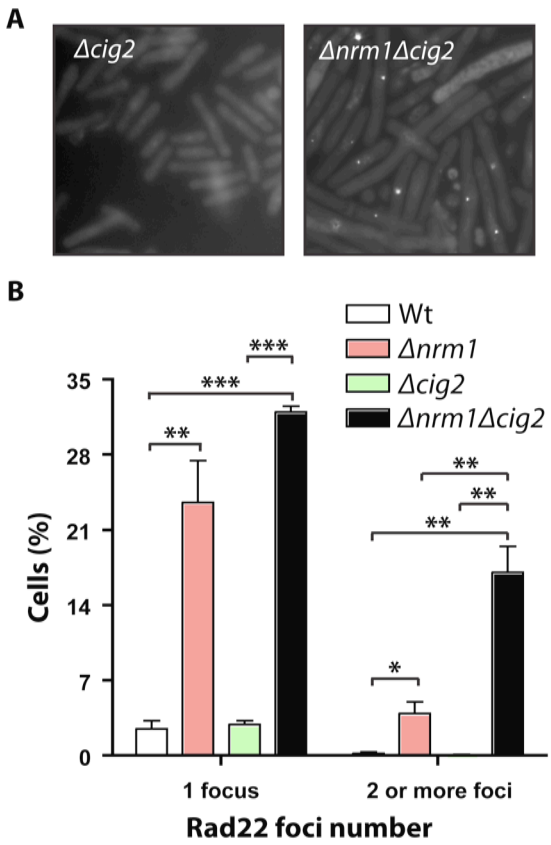

Figure 4.6 – Genome instability is enhanced in Δ*nrm1*Δ*cig2* mutants compared to Δ*nrm1* and Δ*cig2* single mutants

(A) Microphotographs displaying formation of Rad22 foci in Δ*cig2* and Δ*nrm1*Δ*cig2* cells. Cells were grown for 4h in EMM medium as described in the methods and materials section. (B) Quantification of Rad22 foci in Δ*cig2* and Δ*nrm1*Δ*cig2* mutants. Error bars correspond to the SE of three independent biological experiments. Statistical treatment: unpaired, 2-tail t-test, * $p<0.05$, ** $p<0.005$, *** $p<0.001$.

We have considered two hypotheses to explain the synthetic lethality or aberrant morphology observed between Δ*nrm1* and Δ*cig2*, and Δ*yox1* and Δ*cig2* interactions: 1) deletion of *cig2*+ in a Δ*nrm1* and a Δ*yox1* background

may induce further rereplication due to accumulation of Cdc18p protein levels (Lopez-Girona *et al*., 1998); 2) alternatively, given the role of Cig2p in negatively regulating the MBF activator Res1p, deletion of *cig2+* may lead to enhanced levels of MBF transcription and hence more genome instability (Ayté *et al*., 2001). Additionally both issues might contribute to the negative synergy.

To test the first hypothesis we analysed the DNA profile of Δ*nrm1*Δ*cig2* double mutant cells for increase in DNA content compared to single mutants alone via FACS analysis. As depicted in Figure 4.5B, the DNA content of these cells is much higher than that of wild type cells and Δ*nrm1* and Δ*cig2* single mutant cells, indicating that these cells present enhanced rereplication. If the second hypothesis is right then we expect Δ*cig2* single mutant cells to contribute to some extent to the presence of genome instability, as measured by means of formation of Rad22 foci and as observed for the repressor Res2p and the co-repressors Nrm1p and Yox1p. As shown in figures 4.6B this is not the case. *cig2+* mutant cells look rather normal compared to Δ*nrm1 and* Δ*nrm1*Δ*cig2* mutants (Figure 4.5C and Figure 4.6B). Furthermore, only circa 2.7% of Δ*cig2* cells present formation of single Rad22 foci, a value that is highly comparable to that of wild type cells (2.5%). These results prompted us to hypothesise that disruption of *cig2+* is not sufficient to fully alleviate MBF transcriptional repression. To test this hypothesis we measured the expression levels of *cdc18+* and *cdt1+* transcripts in wild type, Δ*nrm1*, Δ*cig2* and Δ*nrm1*Δ*cig2* asynchronous populations (Figure 4.7). In contrast to previous findings (Ayté *et al*., 2001), we have not observed an increase in the levels of MBF transcription in Δ*cig2* mutant cells compared to wild type counterparts (Figure 4.7). Furthermore, deletion of *cig2+* in a Δ*nrm1* background does not seem to further enhance MBF transcription, as levels of *cdc18+* and *cdt1+* transcripts in Δ*nrm1*Δ*cig2* double mutants are similar to those observed for Δ*nrm1* cells (Figure 4.7). Altogether these data suggest that the basis of the synthetic lethality and morphological defects observed for Δ*nrm1*Δ*cig2* and Δ*yox1*Δ*cig2* mutants is the result of enhanced accumulation of Cdc18p protein levels due to unavailability of Cig2p to target Cdc18p for proteolysis.

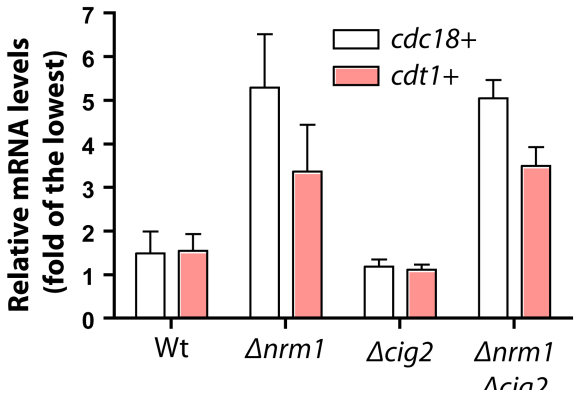

Figure 4.7 – Deletion of *cig2*+ does not promote alleviation of MBF transcriptional repression

RT-PCR relative levels for *Cdc18*+ and *Cdt1*+ transcripts in wild type, Δ*nrm1*, Δ*cig2* and Δ*nrm1*Δ*cig2* cells. Cells were grown for 4h in EMM medium. Results are represented as relative mRNA levels. Error bars correspond to the SE of three independent biological experiments.

4.1.8 – Cdc18p and Cig2p proteins accumulate in *Δnrm1* cells

Abrogation of *nrm1*+ leads to constitutive upregulation of G1/S transcripts, and the accumulation of genomic instability (Figure 4.1). Our results, discussed above, suggest that the genomic instability is caused by replication stress as a result of overexpression of the licensing factor Cdc18. Furthermore the extent of the replication stress is likely limited by the overexpression of Cig2p. In order to check if Cdc18p and Cig2p accumulate at the protein level in cells that are unable to repress MBF-dependent transcription, we have measured the protein levels of these genes in cell extracts deriving from wild type and *Δnrm1* cells. In agreement with the transcriptional data depicted in Figure 4.1, Cdc18p and Cig2p protein hyper-accumulate in *Δnrm1* cells (Figure 4.8). This reveals that overexpression of MBF transcripts at the mRNA level upon abrogation of *nrm1*+ correlates with the protein level, and that proteins encoded by MBF targets hyper-accumulate in the absence of Nrm1p-dependent repression (see also section 6.2). The same is expected to be true for abrogation of *yox1*+.

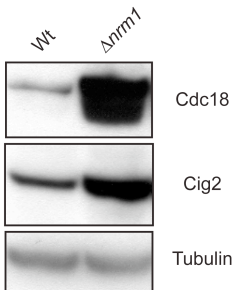

Figure 4.8 – Cdc18p and cig2p protein levels in *Δnrm1* cells.

Western blot analysis of the protein levels of Cdc18p and Cig2 in Wt and *Δnrm1* cells. Cdc18p was tagged with HA and detected with anti-HA antibodies. Cig2p was detected with a commercially available anti-Cig2 antibody as described in section 2.3.3.

4.1.9 – Genomic instability in *Δnrm1* cells is dosage dependent on Cig2 levels

Constitutive derepression of MBF by means of abrogation of the MBF co-repressors *nrm1+* and *yox1+* results in elevated transcript levels of both DNA replication initiation factors *cdc18+* and *cdt1+* and the S-phase cyclin *cig2+* (Figure 4.1 and 4.8). Δ*nrm1*Δ*cig2* double mutants present more genome instability than Δ*nrm1* single mutants, suggesting that a delicate balance of high levels of Cig2p and Cdc18p exists in Δ*nrm1* cells and that hyperaccumulation of Cdc18p may be counteracted by hyperaccumulation of Cig2p, which targets Cdc18p for proteasome-dependent protein degradation.

In order to determine the role of Cig2p in counteracting the effects of *cdc18+* overexpression observed in our Δ*nrm1* mutant, we have established a strain carrying an inducible *cig2+* construct. To construct this strain we have replaced the wild type promoter of *cig2+* by an inducible promoter, the *P41nmt1* promoter, at the endogenous locus and crossed the generated strain with Δ*nrm1* cells (Figure 4.9B). The *nmt1* (for *n*o *m*essage in *t*hiamine 1) promoter is regulated by thiamine, also known as vitamin B1. Addition of thiamine to exponentially growing cells results in full repression of transcripts regulated by the *nmt1* promoter within 3h (Maundrell, 1990), making it a very powerful tool in yeast genetics.

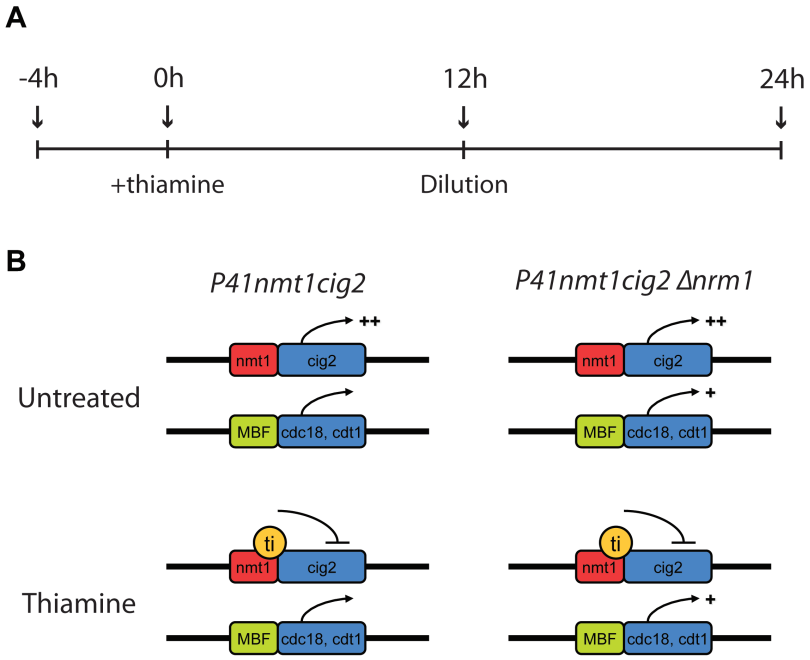

Figure 4.9 –The interaction of *Δnrm1* with *P41nmt1cig2* - experimental design

(A) Cells were allowed to grow in thiamine-free EMM medium for 4h before point 0h was taken. Thiamine was added, to repress transcription from the *P41nmt1* promoter, at a final concentration of 15μM and cultures allowed to grow for 24h. Cultures were diluted to OD_{595}=0.025 at 12h to ensure that cultures were at exponential growth throughout the entirety of the experiment. (B) Cartoon diagram displaying the expected levels of *cig2*+ and MBF transcripts in untreated and thiamine-treated *P41nmt1cig2* and *P41nmt1cig2* Δ*nrm1* cells.

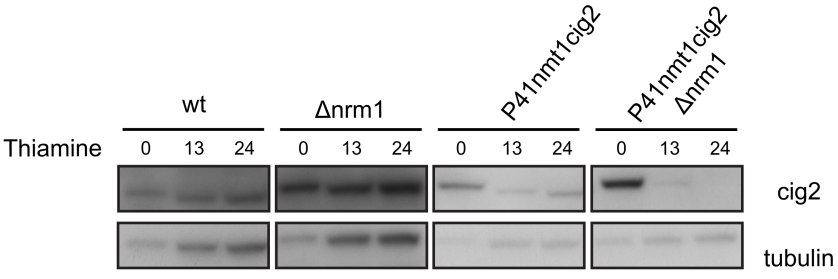

Figure 4.10 – Cig2p levels in the *P41nmt1cig2* inducible system

(A) Wild type, *Δnrm1*, *P41nmt1cig2* and *P41nmt1cig2* Δ*nrm1* cells were allowed to grow for 24h in the presence of thiamine and the levels of Cig2p measured by means of SDS-PAGE analysis at 0h, 13h and 24h time points. Tubulin is shown as the loading control.

To test if our inducible system was working we have monitored the protein levels of Cig2p in wild type, Δ*nrm1*, *P41nmt1cig2* and *P41nmt1cig2* Δ*nrm1* cells by western blot analysis, according to the experimental conditions described in Figure 4.9A. As expected the levels of Cig2p were downregulated, 13h and 24h after addition of thiamine, both in *P41nmt1cig2* and *P41nmt1cig2* Δ*nrm1* cells but not in wild type and Δ*nrm1* cells (Figure 4.10). Furthermore, Cig2p levels are slightly increased in *P41nmt1* cells compared to Δ*nrm1* mutants.

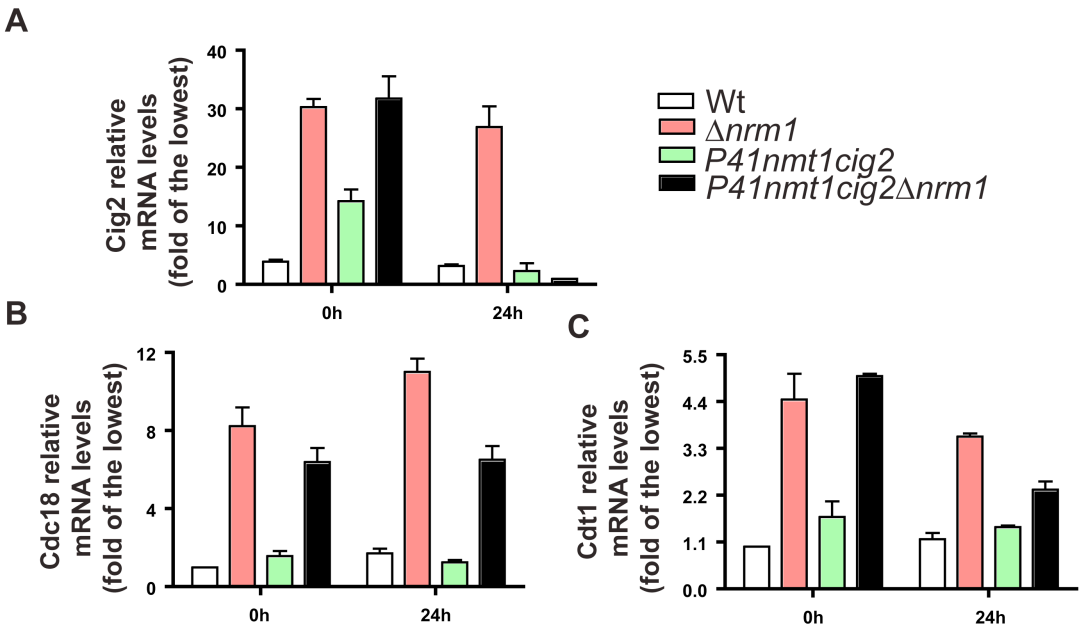

Figure 4.11 – *cig2+*, *cdc18+* and *cdt1+* mRNA levels in the *P41nmt1cig2* inducible system

Experimental conditions were as explained in Figure 4.9. Bar graphs representing the relative mRNA levels for transcripts *cig2+* (A), *cdc18+* (B) and *cdt1+* (C) of wild type, *Δnrm1*, *P41nmt1cig2* and *P41nmt1cig2 Δnrm1* cells 0h and 24h after addition of thiamine. Transcript levels are shown as fold-induction of the lowest transcript measured. Bars represent the average value, and error bars represent their SE, obtained by RT-qPCR of triplicate biological samples.

Next we examined the RNA levels of *cdc18+*, *cdt1+* and *cig2+* in the same cells. These studies revealed that *cdc18+* and *cdt1+* transcripts were unaffected by the repression or induction of *P41nmt1cig2* (Figure 4.11B and C). Repression of *cig2+* in a Δ*nrm1* background (*P41nmt1cig2* Δ*nrm1* cells) does not result in further increase in the levels of *cdc18+,* similar to what we

observed in Δ*cig2*Δ*nrm1* double mutants (Figure 4.11B). Overall, addition of thiamine represses *cig2*+ levels in both *P41nmt1cig2* and *P41nmt1cig2* Δ*nrm1* cells, proving that the system is working as expected (Figure 4.11A).

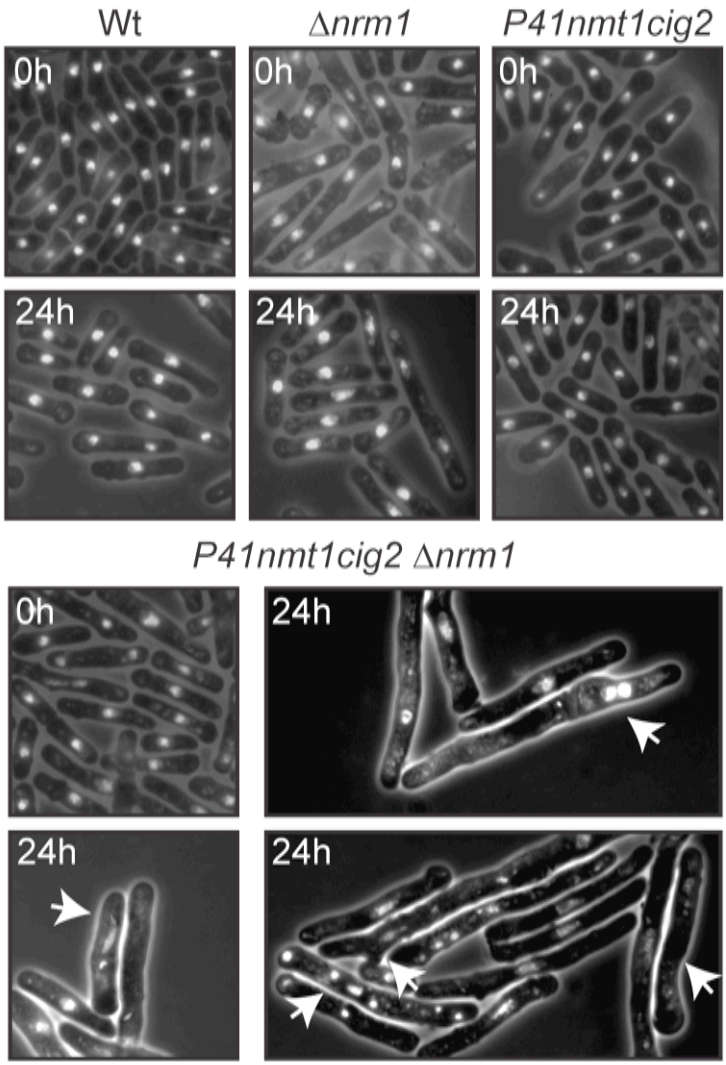

Figure 4.12 – Addition of thiamine induces aberrant phenotypes in *P41nmt1cig2 Δnrm1* cells

Microphotographs of wild type, *Δnrm1*, *P41nmt1cig2* and *P41nmt1cig2 Δnrm1* cells stained for their nucleus (Hoechst 33242) and septum (Calcofluor) before and after treatment with thiamine. White arrows indicate cells with aberrant phenotypes. Examples include missegregated chromosomes, multinucleated cells and filamentous growth.

In order to measure the effects of downregulating *cig2*+ on the cell morphology of *P41nmt1cig2* Δ*nrm1* mutants we treated cells with thiamine and co-stained cells for their septum and nucleus using calcofluor and

Hoechst 33242, respectively. Observation of thiamine-treated *P41nmt1cig2* Δ*nrm1* cells under the microscope revealed a phenotype that highly resembles that of Δ*nrm1*Δ*cig2* cells, suggesting that high levels of *cig2*+ are essential for the survival of cells deleted for *nrm1*+. These cells are wider and highly elongated compared to wild type, Δ*nrm1* and *P41nmt1cig2* cells. Furthermore, they also present filamentous growth and missegregated chromosomes, phenotypes that are symptomatic of genome instability (Figure 4.12). In addition we have immunostained thiamine-treated *P41nmt1-cig2* Δ*nrm1* cells for their microtubules using anti-tubulin antibodies (Figure 4.13). We have noticed that the microtubules of these cells are highly elongated and that chromatin is stretched along the mitotic spindle in the majority of these cells, suggesting that cytokinesis may also be compromised.

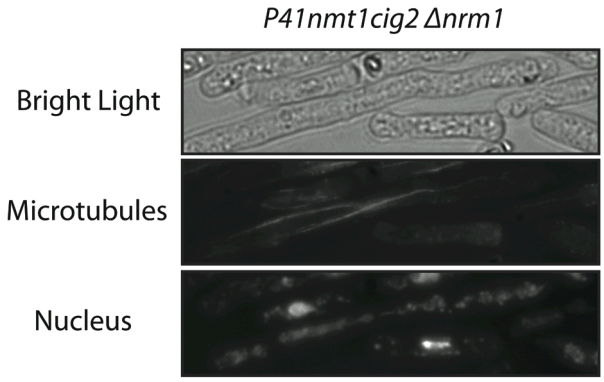

Figure 4.13 – Chromatin stretches across the mitotic spindle in *P41nmt1cig2 Δnrm1* cells following treatment with thiamine.

Microphotographs of thiamine-treated *P41nmt1cig2* Δ*nrm1* cells stained with Hoechst for nucleus and immunostained with anti-tubulin antibodies for microtubules as described in sections 2.2.4.5 and 2.4.6, respectively. Note the highly elongated phenotype of the microtubules and the chromatin stretched along the microtubule spindle.

To determine if repressing *cig2*+ induces further genomic instability in our Δ*nrm1* single mutants as verified for Δ*nrm1*Δ*cig2* cells we have treated *P41nmt1cig2* Δ*nrm1* cells with thiamine as described above and measured the levels of Rad22 foci formation in these cells. As expected addition of thiamine to *P41nmt1cig2* Δ*nrm1* cells induces an increase in genome

instability compared to Δ*nrm1* and control *P41nmt1cig2* cells (Figure 4.14B). Although these cells continue to divide, their cell cycle is highly disturbed, as they progress extremely slowly through the cell cycle. Conversely, *cig2*+ overexpression in the *P41nmt1cig2* Δ*nrm1* cells, observed before thiamine addition, partially rescues the genomic instability phenotype observed in Δ*nrm1* cells, as these present slight less formation of Rad22 foci than Δ*nrm1* cells (Figure 4.14). Although this event is not statistically significant this suggests that further overexpression of Cig2p in these cells may more effectively counteract the rereplication induced by overexpression of Cdc18p. Further experiments would need to be performed to confirm this hypothesis.

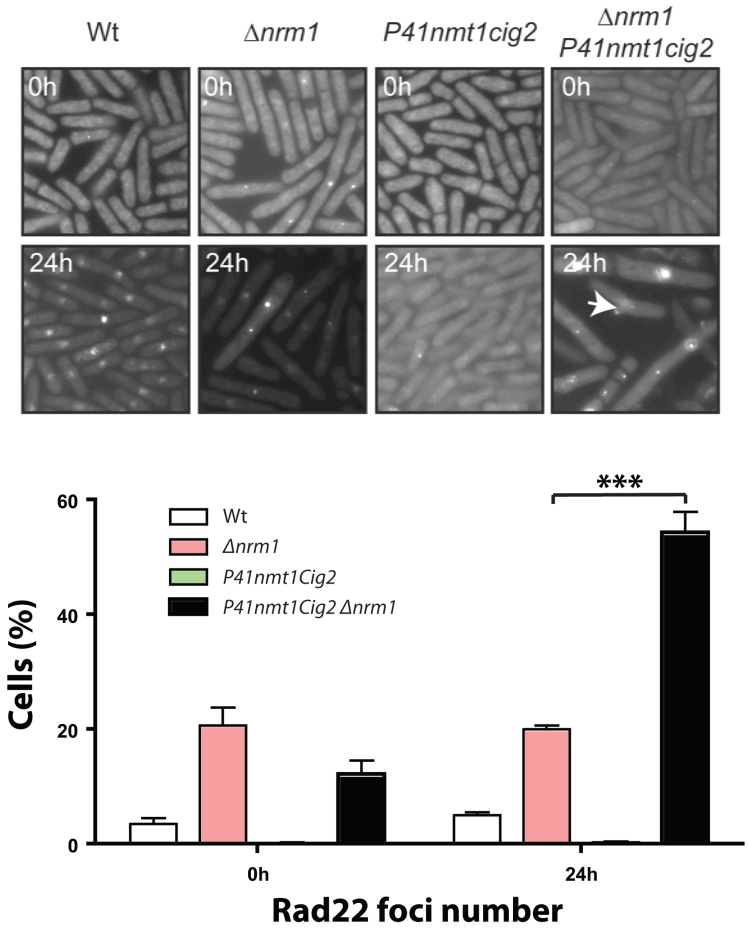

Figure 4.14 – *cig2*+ repression in *P41nmt1cig2 Δnrm1* cells results in increased genome instability

(A) Microphotographs of wild type, *nrm1*, *P41nmt1cig2* and *P41nmt1cig2* Δ*nrm1* cells expressing Rad22-YFP, 0h and 24h after treatment with thiamine. (B) Quantification of A. Bars represent the average value, and error bars represent their SE, obtained by RT-qPCR of triplicate biological samples.

If the derepression of MBF transcription observed in Δ*nrm1* cells results in a delicate balance between high levels of Cdc18p, that induces re-firing of replication origins, and Cig2p counteracts this by targeting Cdc18p for destruction, downregulation of Cig2p in Δ*nrm1* cells should induce high levels of rereplication. To test this we analysed the DNA profile of *P41nmt1cig2* Δ*nrm1* cells following addition of thiamine to *repress cig2+* levels. True to our assumption downregulation of Cig2 in Δ*nrm1* cells causes the accumulation of >4C DNA content (Figure 4.15A). In addition the analysis of the FCS vs. PE dotplot of these cells reveals the existence of a cohort of cells (6.11% of the cells) with reduced DNA content. We assume that these correspond to cells that underwent chromosomal mis-segregation and display a so-called cut phenotype (Figure 4.15B).

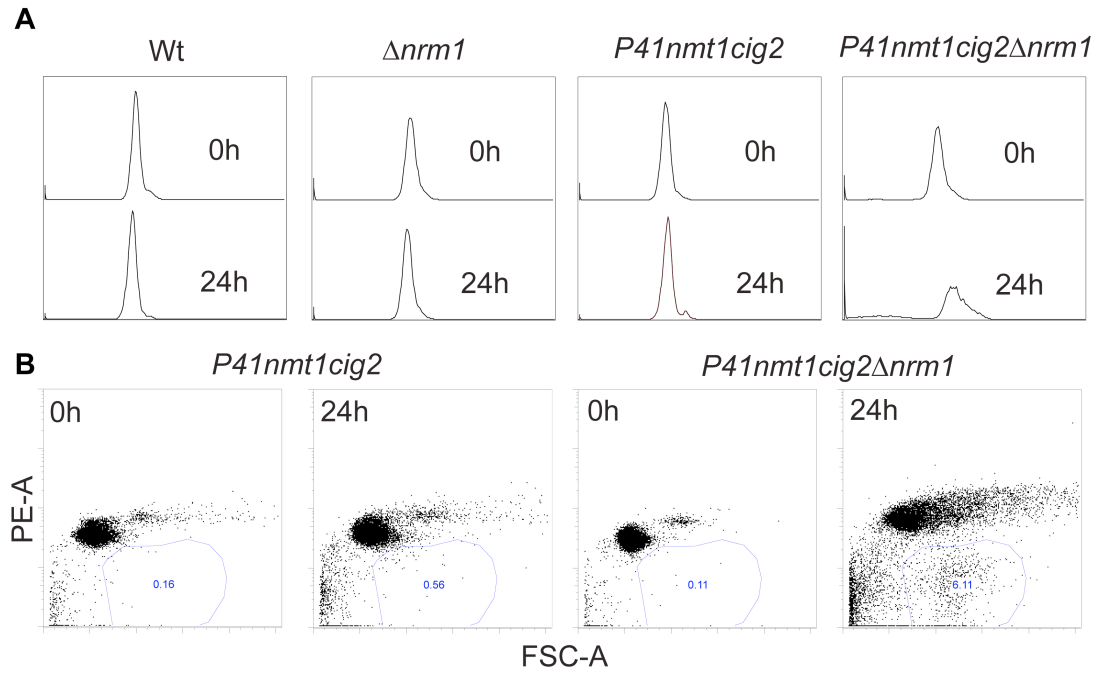

Figure 4.15 – *cig2+* repression in *P41nmt1cig2 Δnrm1* cells results in enhanced rereplication and cells presenting cut phenotype

(A) DNA FACS profile of wild type, Δ*nrm1*, *P41nmt1cig2* and *P41nmt1cig2* Δ*nrm1* before and after addition of thiamine. Cells were treated with thiamine for 24h. (B) FSC-A vs PE-A dotplot of the same cells. Cells were gated for cut-phenotype cells.

Cells depleted for Cdc13 have been shown to re-replicate their DNA without intervening mitoses (Lopez-Girona *et al*., 1998). One could argue that lower levels of Cdc13p could be the cause of why Δ*nrm1* and thiamine-treated *P41nmt1cig2* Δ*nrm1* cells undergo rereplication. In order to check if Δ*nrm1* and thiamine-treated *P41nmt1cig2* Δ*nrm1* cells present normal levels of Cdc13p, we have monitored the protein levels of Cdc13p in these strains by western blot analysis. We observed that the levels of Cdc13p are not downregulated in either Δ*nrm1* or thiamine-treated *P41nmt1cig2* Δ*nrm1* cells (Figure 4.16). Altogether these results indicate that the rereplication phenotype observed in these mutants is directly related to downregulation of Cig2p levels and not with indirect downregulation of Cdc13p.

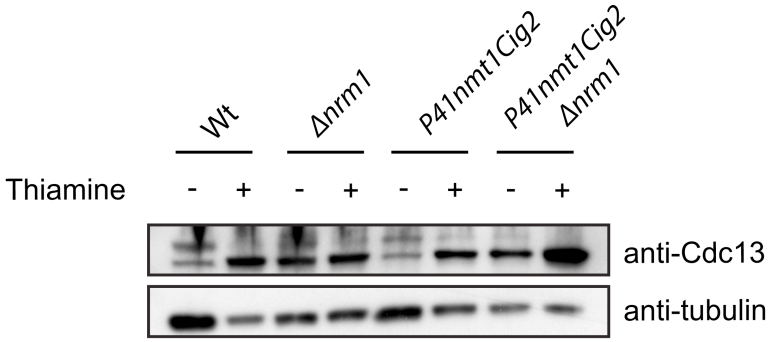

Figure 4.16 – Rereplication observed in *Δnrm1* and thiamine-treated *P41nmt1cig2 Δnrm1* cells is not due to decrease in Cdc13 levels

Cells were grown in the absence and presence of thiamine, protein extracted and samples analysed using SDS-PAGE analysis. Membranes were probed for Cdc13p using an anti-Cdc13 antibody kindly provided by Hiro Yamano. Tubulin is used as loading control.

4.1.10 – Downregulation of E2F6 induces an increase in the levels of Cdt1 and Cyclin A in immortalised RPE human cells

Our work in fission yeast has shown that derepression of G1/S transcription leads to genomic instability via the upregulation of licensing factors resulting in the induction of rereplication. Next we sought to establish if this is also true for mammalian cells. G1/S cell cycle dependent transcription in mammalian cells is regulated by the E2F family of transcription factors (Zhu *et al*., 2004).

E2F6 and E2F7 are repressors of the E2F transcriptional programme. Much like Nrm1 and Yox1 in fission yeast they are G1/S targets themselves and therefore involved in a negative feedback loop to turn off transcription during the G1-to-S transition (Di Stefano *et al*., 2003) (Lyons *et al*., 2006). Amongst a large number of genes the E2F transcription factors are involved in the controlling the expression of the licensing factors Cdc6 and Cdt1, and Cyclin E and Cyclin A (Schulze *et al*., 1995) (Yoshida and Inoue, 2004) homologs of the fission yeast Cdc18 and Cdt1, and Cig1 and Cig2, respectively. In mammalian cells overexpression of CDT1 is known to induce rereplication, whereas as discussed above in fission yeast overexpression of Cdc18p (CDC6 homolog) has the most pronounced effect. Cyclin A is involved in the degradation of CDT1, much like Cig2p is involved in targeting Cdc18p in fission yeast (Sugimoto *et al*., 2004).

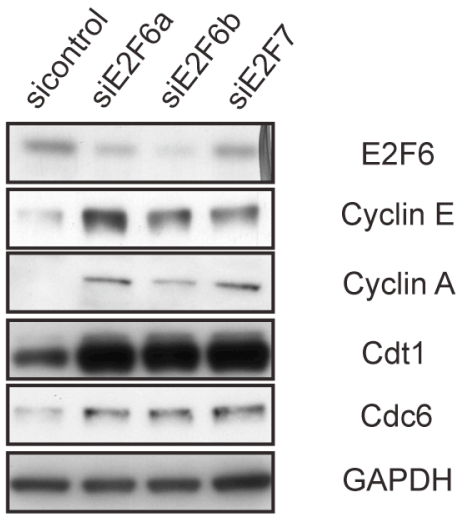

Figure 4.17 – Protein levels of several E2F-regulated genes in siE2F6- and siE2F7-treated cells

RPE cells were left untreated or treated with 2 types of siE2F6 and one type of siE2F7 and the proteins levels of E2F6, Cyclin E, Cyclin A, CDT1 and CDC6 detected by western blot analysis as explained in the methods section. GAPDH was used as loading control.

Based on this we speculated that, as shown for *S. pombe,* depletion of cyclin A in a background where the levels of CDT1 are constitutively upregulated by derepression of E2F-dependent transcription may lead to induction of rereplication and possibly genomic instability. To mimic our Δ*nrm1* yeast

model in RPE immortalised human cells we depleted cells for E2F6 and E2F7 by means of siRNA and measured the protein levels of CDT1 and Cyclin A. Preliminary data indicates that as expected, CDT1 and Cyclin A are upregulated in RPE cells following treatment with siRNA directed against E2F6 and E2F7 (Figure 4.17).

5. Miscellaneous

5.1 – Yox1p and Nrm1p interact with each other to repress MBF transcription

The MBF is composed of two DNA-binding proteins, Res1p and Res2p and the product of the START gene Cdc10p (Aligianni *et al*., 2009). Furthermore, it has been shown that transcriptional repression of MBF involves the coordinated action of two co-repressors, Nrm1p and Yox1p, which are bound to chromatin throughout G2 and M phases of the cell cycle and are released from MBF-dependent genes upon entry into G1 and treatment with HU. Recent studies suggest that Yox1p associates directly or indirectly with the Cdc10p and Res2p components of the MBF complex (Aligianni *et al*., 2009) but nothing is known about the interactions between Nrm1p and Yox1p. To determine if Yox1p physically interacts with Nrm1p we carried out myc-targeted immunoprecipitations in wild type strains carrying Yox1-3HA-tagged and Nmr1-13myc-tagged versions of these two proteins and blot membranes for HA and Myc. We establish that Yox1p and Nrm1p associate with each other in wild type cells as depicted in Figure 5.1. This interaction seems to be independent of inactivation of *res2*$^{+}$, as deletion of *res2*+ does not abolish the interaction verified between Nrm1p and Yox1p (Caetano *et al*., 2011). It has also been observed that Yox1p does not bind detectably to MBF promoters in the absence of *nrm1*+ (*nrm1*Δ cells), suggesting that Nrm1p may function as a scaffold for Yox1p binding to MBF promoters.

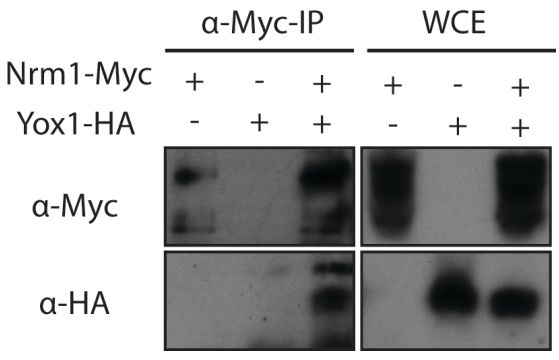

Figure 5.1 – Yox1p and Nrm1p co-immunoprecipitate

Wild type cells carrying Nrm1p-13myc and Yox1p-3HA were allowed to grow for 4 hours. Lysates were enriched for myc and membranes tagged for both myc and HA.

5.2 – Deleting *yox1+* induces overexpression of Nrm1p at the protein level

Deleting either *nrm1+* or *yox1+* results in upregulation of MBF transcripts. As we saw before this leads to accumulation of Cdc18p and Cig2p at protein levels. We have also looked at the protein levels of Nmr1p in *Δyox1* cells and as expected we see a hyperaccumulation of Nrm1p in these cells (Figure 5.2). Although *Δyox1* cells accumulate Nrm1p at higher levels than in wild type cells they are still unable to repress MBF transcription, suggesting that *nrm1+* requires *yox1+* for proper repression of G1/S transcripts and vice versa.

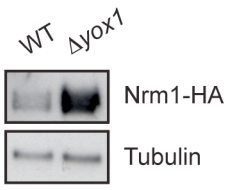

Figure 5.2 – Nrm1p accumulates in *Δyox1* mutant cells

Western blot analysis of the protein levels of Nrm1p in wild type and *Δyox1* cells. Nmr1p was tagged with HA and detected with anti-HA antibodies as described in section 2.3.3.

5.3 – Genetic interaction of *Δnrm1* with *cdc25-22*

Temperature sensitive *cdc25-22* cells are known to arrest at G2 phase of the cell cycle, when grown at non-permissive temperatures (37°C). Cdc25p dephosphorylates Cdc2p/Cdk1p at Tyr15, to render it active and allow cells to progress into mitosis. Wee1p largely counteracts this reaction by phosphorylating Cdc2p at the same residue, thus, preventing entry into mitosis. In an attempt to study the levels of MBF transcription in synchronous Δ*nrm1* cells via the Cdc25-22 block and release method we have crossed Δ*nrm1* cells with *cdc25-22* mutants and subjected the obtained asci to tetrad analysis. To our surprise, we have observed that Δ*nrm1cdc25-22* mutant cells are synthetic lethal and unable to grow at permissive temperatures (Figure 5.3).

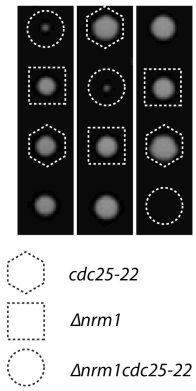

Figure 5.3 – Crossing *Δnrm1* with *cdc25-22* mutants gives rise to synthetic lethal mutants

Haploid Δ*nrm1* cells were crossed with *cdc25-22* cells and the generated spores dissected as described in section 2.1.5. Experiments were repeated at least three times and all crosses gave rise to synthetic lethal double mutants.

Nuclear staining of these cells revealed a highly elongated phenotype and scattered DNA, possibly due to chromosome missegregation (Figure 5.4). We hypothesise that the synthetic lethality observed for Δ*nrm1cdc25-22* is due to the inability of Δ*nrm1* cells to progress into mitosis and that prolonged G2 phase may result in the accumulation of further DNA damage in these cells. Alternatively it is possible that, given the role of Cdc25p in activating

Cdc2p, Cdc2p is no longer accessible to target Cdc18p for proteolysis together with Cig2p.

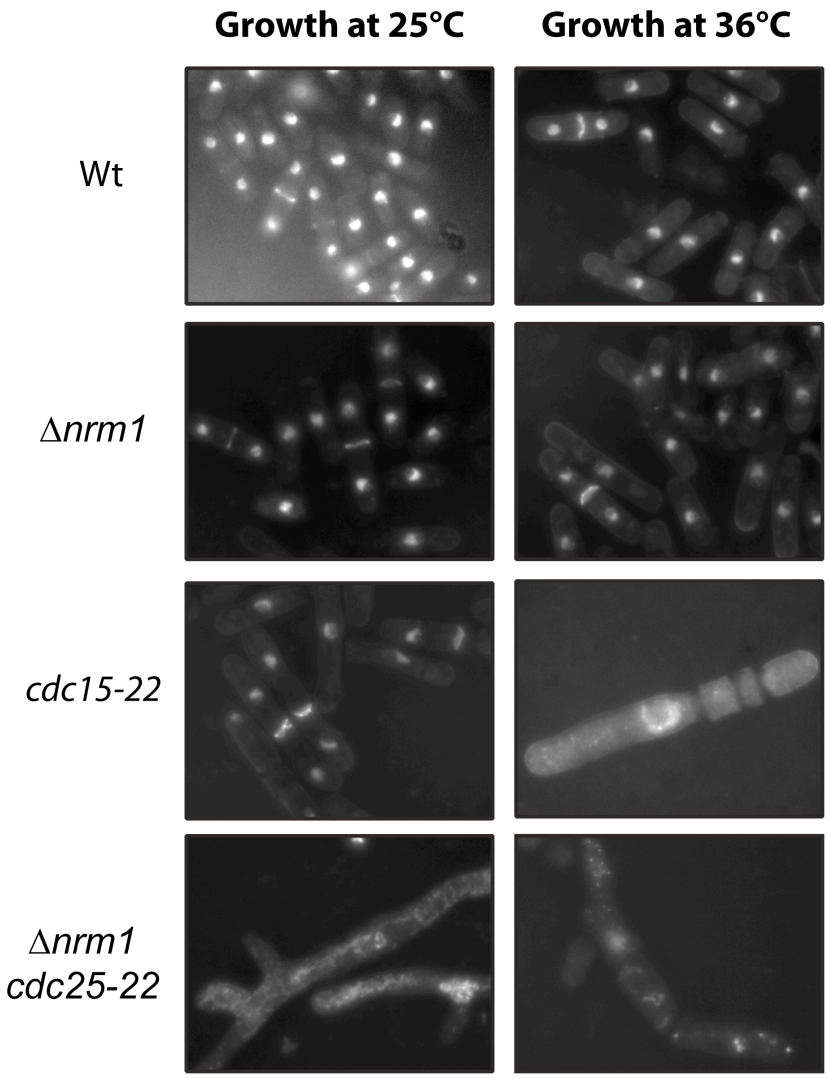

Figure 5.4 – *Δnrm1cdc25-22* double mutants are highly elongated and present scattered DNA

Fluorescent microphotographs of *Δnrm1cdc25-22* synthetic mutants showing scattered DNA at both permissive and non-permissive temperatures.

6. Discussion

6.1 – Objective 1 - Discussion

In order to properly replicate their DNA and avoid genomic instability, cells utilize the DNA replication and the DNA damage checkpoints. The DNA replication checkpoint is mediated via the Rad3p-Cds1p signal transduction pathway, which is activated in the presence of DNA replication stress (Lindsay *et al*., 1998) (Xu *et al*., 2006) (Xu and Kelly, 2009). An important feature of the DNA replication checkpoint response consists of the activation and maintenance of the regular cell-cycle dependent MBF transcriptional programme (Dutta *et al*., 2008) (Dutta and Rhind, 2009) (de Bruin *et al*., 2008) (Chu *et al*., 2007) (Chu *et al*., 2009) (Caetano *et al*., 2011). The inability to do so, as observed in cells lacking the checkpoint protein kinases Rad3p and Cds1p, results in extreme sensitivity to agents that promote loss of integrity of the replication fork, such as HU and MMS (Murakami and Okayama, 1995). In *S. pombe* and during normal cell cycle progression, MBF transcription is regulated by a double negative feedback loop involving the actions of the co-repressors Nrm1p and Yox1p (de Bruin *et al*., 2008) (Aligianni *et al*., 2009).

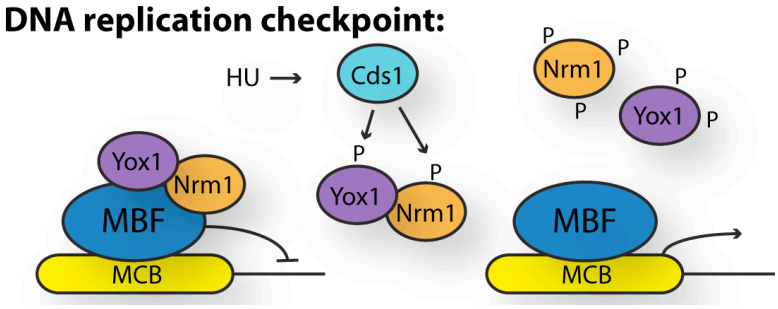

Figure 6.1 – Regulation of Yox1p in response to HU-dependent checkpoint activation

Activation of the DNA replication checkpoint and maintenance of the MBF transcriptional programme following HU challenge occurs via phosphorylation and inactivation of both Nrm1p and Yox1p by the DNA replication checkpoint effector kinase Cds1.

In this report we have shown that, as observed for Nrm1p, inactivation of Yox1p and its dissociation from MBF promoters is necessary for full de-

repression of MBF transcripts, following HU-induced activation of the DNA replication checkpoint. Treatment with HU promotes loss of Yox1p from MBF at promoters (Figure 3.1). This correlates with maintenance of high levels of expression of the MBF transcriptional programme, which amongst others includes enzymes involved in the production of dNTPs (*cdc22*+) and the licensing factors *cdc18*+ and *cdt1*+ (Figure 3.2) (Caetano *et al*., 2011). In line with this, inactivation of *yox1*+ makes DNA replication checkpoint Δ*cds1* mutants less sensitive to the deleterious effects of HU and MMS, indicating that de-repression of MBF-dependent transcripts is a crucial step for viability of cells undergoing replicative stress (Figure 3.5). Furthermore, we have shown that inactivation of Yox1p is Cds1p-dependent, and that phosphorylation at residues Ser114 and Thr115 plays an important role in the activation of checkpoint-dependent MBF transcription (Figure 3.6).

Although the *yox1*^{2A} mutation prevents Yox1p phosphorylation at the RRKST locus, which significantly reduces the induction of MBF-dependent transcripts following activation of the DNA replication checkpoint, it does not result in increased sensitivity to HU and MMS (Figure 3.6). In addition, mutating a combination of checkpoint-dependent phosphorylation sites in *nrm1*^{8A} and *cdc10*^{8A} result in a partial loss of induction of MBF transcription following HU treatment, but cells do not present a higher sensitivity to the same drug (data not shown). Furthermore, no difference in HU sensitivity, when compared to wt cells, is observed for the combination of the c*dc10*^{8A} mutant with the *yox1*^{2A} mutant (data not shown). Based on this we hypothesise that a triple *crm1*^{8A} *yox1*^{2A} *cdc10*^{8A} mutant may be required for complete abrogation of the checkpoint-dependent MBF transcriptional response. To answer this question a strain carrying the referred triple mutant should be produced and checked for sensitivity to HU. It could also be assessed whether reduced levels of MBF-dependent transcription following activation of the checkpoint occur in this triple-mutant strain as observed in our *yox1*^{2A} mutant. Furthermore, this strain could be tagged with Rad22p-YFP, a marker for DNA damage, treated with HU and quantified for Rad22 foci number to determine if these cells experience more genomic instability, when treated with HU, than their wild type counterparts.

Recently, two studies have been published with similar findings to the ones described here. These suggest, as described in this report, that Yox1p is a direct target of the DNA replication checkpoint effector kinase Cds1p, and that this reaction is necessary for derepression of the MBF transcriptional response following HU-induced DNA replication stress (Gómez-Escoda *et al*., 2011) (Purtill *et al*., 2011). In addition to the findings reported here, it is also demonstrated that Yox1p is a target of CDK activity. *In vitro* kinase assays have shown that Yox1p is phosphorylated by Cdc2p-Cdc13p at residue Ser6. Substitution of Ser6 into an alanine residue completely abolishes the Cdc2p-Cdc13p-mediated phosphorylation of Yox1p, however no *in vivo* effects of loss of Ser6 phosphorylation have been found (Gómez-Escoda *et al*., 2011). The biological importance of this reaction remains obscure.

Yox1p and Nrm1p are involved in a negative feedback loop to confine G1/S transcription to the G1-phase of the cell cycle. As we saw in section 4, genetic perturbation of either *nrm1*+ or *yox1*+ leads to increased MBF-dependent transcription indicating that both proteins are required, but are not sufficient, to repress MBF transcription outside of G1 phase. One can argue that this creates a less robust system to repress transcription, since mutations that affect either Yox1p or Nrm1p will result in loss of cell cycle regulated transcription. Based on the same argument the use of two non-redundant proteins creates a more robust regulatory system when MBF-dependent transcription needs to be de-repressed outside of normal G1 phase (e.g. following activation of the DNA replication checkpoint). So why use two non-redundant proteins to repress transcription during the cell cycle? Here we show that in response to DNA replication stress the DNA replication checkpoint de-represses MBF-dependent transcription by releasing both Yox1p and Nrm1p from MBF at promoters (Figure 7.1). So whereas confining MBF-dependent transcription to the G1 phase of the cell cycle is not essential in rapidly growing cells, as Δ*nrm1* and Δ*yox1* deletion mutants are viable, we show that de-repression of MBF-dependent transcription is essential in response to genotoxic stress. Overall the requirement for these

multiple, non-redundant negative feedback loops is striking and may reflect the importance of keeping transcription ON in response to genotoxic stress over turning transcription OFF outside of G1. In addition, by targeting an autoinhibitory negative feedback loop the checkpoint ensures that once the replication stress is dealt with MBF transcription is switched OFF immediately. Whilst cells deal with DNA replication stress, Cds1p keeps MBF-dependent transcription active via phosphorylation of Nrm1p and Yox1p, which, being MBF targets themselves start to hyperaccumulate. Once replication stress terminates, Cds1p is made inactive and accumulated Nrm1p and Yox1p are free to bind to promptly turn OFF G1/S transcription.

Work from the de Bruin lab has shown that, although there is no sequence homology between the mammalian G1/S transcription factors, the E2F family, and the yeasts MBF and SBF, the mechanism by which cells activate DNA replication checkpoint-dependent G1/S transcription is conserved from yeast to humans. As observed for *S. pombe* and *S. cerevisiae*, in human cells, treatment with HU results in maintenance at high levels of expression of the G1/S transcriptional programme. This is mediated by CHK1-dependent phosphorylation, and inactivation of the E2F repressor E2F6, much like the Cds1p-dependent inactivation of Nrm1p and Yox1p in fission yeast. Like Nrm1p and Yox1p, E2F6 is a G1/S transcript, which is involved in a negative feedback loop to repress transcription when cells progress into S phase. In response to replication stress Chk1 directly phosphorylates E2F6 resulting in its dissociation from E2F promoters (unpublished data). The conservation of this regulation and the mechanism by which it is implemented demonstrates the importance of G1/S transcriptional induction for the survival of cells undergoing DNA replication stress and highlights the significance of this particular mechanism of regulation that allows induction during the checkpoint response and rapid repression once satisfied.

Following DNA replication stress the same transcriptional programme is maintained at high levels of expression (Chu *et al*., 2007) (Dutta *et al*., 2008) (Caetano *et al*., 2011). The reason why this happens remains unresolved but is likely to relate to the function of the transcripts regulated by MBF. Many MBF-dependent transcripts are known to have a role in the DNA checkpoint

response. These include, but are not limited to *mik1+*, a kinase that inhibits mitosis, *mrc1+*; the Cds1p mediator in the DNA replication checkpoint pathway; *ssb1+*, an RPA ssDNA-binding protein; *ctp1+*, a subunit of the MRN complex; *rhp51+,* a Rad51p recombinase; and the repair helicase *pfh1+* (Dutta *et al*., 2008). Although some MBF transcripts do not have an obvious function in the DNA replication checkpoint response, it may be that their activities are required for later events. For example *cdc22+*, which encodes the large subunit of ribonucleotide reductase involved in the synthesis of ribonucleotides and the licensing factors *cdc18+* and *cdt1+*, may be required for restarting of stalled DNA replication. Furthermore, Cdc18p has also been implicated in the generation and maintenance of intermediate replication structures necessary for DNA replication checkpoint activation (Liu *et al*., 2000) (Murakami *et al*., 2002).

6.2 – Objective 2 - Discussion

Activation of G1/S transcription initiates the G1-to-S transition and commits cells to a new cell cycle. In *S. pombe* the G1/S transcriptional wave is controlled by the transcription factor MBF. In mammalian cells G1/S cell-cycle-regulated transcription is under the control of a large family of transcription factors collectively known as the E2F transcription factors, which are regulated by the pocket proteins, the tumour suppressor pRB and p107 and p130 (Polager and Ginsberg, 2008). Uncontrolled cell growth is an invariable characteristic of human cancer. Constitutive activation of E2F-dependent transcription, found in every type of cancer, allows cancer cells to sustain proliferation in the absence of growth factors and renders them insensitive to growth-inhibitory signals. (Chen *et al*., 2009). However, it has been postulated that there are alternative tumour-promoting activities for the E2F family, which are independent of cell cycle regulation. Here we have used the model organism *S. pombe* to study the effects of keeping G1/S transcription ON throughout all stages of the cell cycle. Since *S. pombe* cells spend little to no time in G1 and have, virtually, no G1-to-S transition control during an unperturbed mitotic cell cycle we could study the effect of de-repressing G1/S transcription independent of its role in cell cycle regulation. Our work establishes that in addition to its effect on cell cycle progression de-repression of G1/S transcription also induces sporadic rereplication and thereby actively contributes to the accumulation of genomic instability, another hallmark of cancer.

In this report we confirm that fission yeast cells abrogated for *nrm1+* or *yox1+* show constitutive overexpression of MBF-dependent transcripts throughout the cell cycle (Figure 4.1) (de Bruin *et al*., 2006) (de Bruin *et al*., 2008) (Aligianni *et al*., 2009). We show that constitutive mRNA overexpression is reflected downstream in the protein level as confirmed by accumulation of Cdc18p and Cig2p in *nrm1+* deletion mutants and Nrm1p in *Δyox1* cells (Figure 4.8 and 5.2). *nrm1+* and *yox1+* deletion mutants are viable, however, they display a large variation in cell cycle length and are more elongated than their wt counterparts, a phenotype often linked with delay in S and/or G2

phases (de Bruin *et al*, 2008; Aligianni *et al*, 2009). We have shown that this cell cycle delay occurs due to activation of the DNA damage checkpoint as abrogation of the DNA damage checkpoint effector protein kinase *chk1+* in a ∆*nrm1* background partially rescues the elongation phenotype observed for the ∆*nrm1* cells, respectively. The same was observed for deletion of the upstream checkpoint protein kinase *rad3+* in a ∆*nrm1* background (data not shown).

Our results indicate that activation of MBF-dependent transcription, by deletion of the MBF co-repressors *nrm1+* or *yox1+* or the repressor *res2+*, promotes accumulation of Rad22 foci, a marker for single stranded DNA (ssDNA), indicative of DSBs, presence of collapsed or stalled DNA replication forks and persistent recombination intermediates (Kim *et al*., 2000). Based on these observations we speculate that de-repression of MBF-dependent transcripts outside of G1/S phases results in DNA damage accruement and genomic instability, both hallmarks of cancer aetiology. In agreement with this, ∆*nrm1*, ∆*yox1* and ∆*nrm1*∆*yox1* cell populations present higher levels of cells with aberrational morphologic phenotypes than their wild type counterparts. Several classes of aberrant cell morphology phenotypes have been observed including cells with scattered DNA, cut phenotype and chitin accumulation, the last being an indicator of compromised cytokinesis.

Cells prevent rereplication by allowing DNA replication origins to be licensed, via binding of licensing factors, when there is no CDK activity at the end of mitosis and beginning of G1 (Tanaka and Araki, 2010). In addition licensing factors accumulate solely during G1, limiting licensing activity to the end of mitosis. This further ensures that replication happens once and only once per cell cycle. However, overexpression of licensing factors has been shown to bypass this failsafe mechanism by licensing origins even in the presence of CDK activity and absence of mitosis, causing rereplication, demonstrating that accurate control of Cdc18p and Cdt1p activities is required at all times in the cell cycle, in order to prevent rereplication (Jallepalli *et al*., 1997). Inactivation of *nrm1+* and *yox1+* leads, amongst others, to overexpression of the replication initiation factors *cdc18+* and *cdt1+* and the S-phase cyclin

cig2+ both at the transcription and at the protein levels (Figure 4.1 and Figure 4.8). Whereas overexpression of Cdc18p alone has been shown to induce rereplication (Jallepalli *et al*., 1997) (Lopez-Girona *et al*., 1998), our work shows that simultaneous overexpression of the CDK cyclin Cig2p largely counteracts this. We show that the number of Rad22 foci is slightly lower than the number of Rad22 foci observed for ∆*nrm1* cells alone when *cig2*+ is further induced and significantly higher when *cig2*+ is repressed. Furthermore, deletion of *cig2*+ in a ∆*nrm1* background promotes formation of further genomic instability compared to genomic instability measured for ∆*nrm1* single mutants. Cig2p is known to target Cdc18p for proteolysis via the ubiquitin E3 ligase Cullin F-box containing complex (SCF) in a CDK-dependent manner (Lopez-Girona *et al*., 1998). Hence, since both licensing factors and cyclin Cig2p are G1/S transcripts, derepression of G1/S transcription results in a delicate balance of high levels of licensing factors and CDK activity, which cause only sporadic rereplication but a significant increase in genomic instability, as determined by formation of Rad22 foci and aberrant morphological defects in ∆*nrm1* and ∆*yox1* cells (Figure 7.2). Disruption of this delicate balance by means of downregulating CDK activity results in formation of further genomic instability, as ∆*nrm1*∆*cig2* and thiamine-treated *P41nmt1cig2* ∆*nrm1* cells are extremely sick and present very high numbers of Rad22 foci (Figure 4.7 and 4.14). To establish whether this is the result of hyper-accumulation of Cdc18p, levels of Cdc18p in ∆*nrm1*∆*cig2* and in thiamine-treated *P41nmt1cig2* ∆*nrm1* cells should be determined. We hypothesise that the protein levels of Cdc18p hyper-accumulate in these cells compared to ∆*nrm1* alone, due to a disruption in the ability to target Cdc18p for proteolysis via the Cig2-CDK pathway.

Our data suggests that the genomic instability observed in ∆*nrm1*, ∆*yox1* and ∆*nrm1*∆*yox1* cells is largely due to hyperaccumulation of Cdc18p (Jallepalli *et al*., 1997) (Lopez-Girona *et al*., 1998). However, given the relatively large number of genes regulated by MBF, it is possible that other MBF transcripts may also contribute to the generation of the genomic instability encountered. In order to explore the contribution of *cdc18*+ to the generation of genomic instability in our ∆*nrm1* cells, a strain bearing an inducible *nmt1-cdc18* promoter should be constructed, crossed with ∆*nrm1* cells and the number of

Rad22 foci quantified following repression of *cdc18*+ with thiamine. However, since *cdc18*+ is both an essential and a dosage sensitive gene (constitutive expression causes rereplication) it can't be overexpressed or repressed beyond certain levels, which might be tricky. Alternatively, the expression of *cdc18*+ could be placed under the control of an MBF-independent cell cycle regulated promoter in an ∆*nrm1* background. Candidate promoters could be those regulated by the M/G1 cell cycle transcription factor, Ace2, given that their expression pattern is quite close to that of the MBF transcriptional wave (Bähler, 2005). In an ∆*nrm1* mutant this would reconstitute the cyclical expression pattern of *cdc18*+ while the remaining MBF genes are maintained a high levels. If the number of Rad22 foci generated in these strains was to be close to wild type levels then we could conclude that accumulation of Cdc18p is in fact the main reason why *nrm1*+ mutant cells display elevated levels of genomic instability. If the number of Rad22 foci generated was similar to or only slightly lower than ∆*nrm1* cells but higher than wild type cells we would conclude that other genes contribute to Rad22 focus accumulation when constitutively expressed. These could include, but are not limited to, the RNR subunits *cdc22*+ and *suc22*+, which are required for dNTP synthesis; the translesion polymerase *polκ*+*;* and *ctp1*+, a protein involved in homologous recombination (HR). Elevated dNTP pools are known to induce genomic instability likely by allowing error prone replicative DNA polymerases to bypass certain DNA lesions (Sabouri *et al*., 2008). In the yeast *S. cerevisiae*, upregulation of the RNR subunit induces an increase in the copy number of mtDNA (Xu *et al*., 2008). In mice altered RNR expression promotes lung carcinogenesis and increases the metastic potential (Fan *et al*., 1996) (Xu *et al*., 2008). Hyperaccumulation of translesion polymerases such as *polκ*+ was reported to allow these error-prone polymerases to access undamaged DNA and increase untargeted mutagenesis (Rattray and Strathern, 2003). Finally increased *ctp1*+ levels may lead to over-induction of homologous recombination repair (Limbo *et al* 2007, Mol Cell).

The role of genomic instability in cancer development remains poorly understood. The most accepted view on cancer aetiology relies on the mutator hypothesis, whereby genomic instability (e.g. chromosomal instability and microsatellite instability), which is believed to be present in most precancerous lesions, drives formation of neoplasia by increasing the rate of spontaneous mutations (Negrini *et al*., 2010). According to this theory increase in the spontaneous mutation rate occurs early in cancer development and it leads to emergence of mutations in classical caretaker genes such as DNA repair genes and mitotic checkpoint genes (e.g. TP53 and ATM). Although this has been shown to be involved in the formation of hereditary cancers, wide genome analyses have failed to demonstrate the same to be true for the formation of sporadic human cancers, as mutations in caretaker genes seem to be infrequent amongst this class of tumours. This led to the formulation of an alternative model, the oncogene-induced DNA replication stress model (Halazonetis *et al*., 2008). According to the oncogene-induced DNA replication model activation of oncogenes leads to the establishment of DNA damage and DNA replication stress, which in turn drive formation of tumours. In this report we show that the G1/S transcriptional programme has an important role in response to replicative stress and that de-repression of G1/S transcription contributes to the formation of genome instability via the misregulation of DNA replication.

Understanding how unregulated G1/S transcription and DNA replication stress contribute to the generation of genomic instability is becoming increasingly important in cancer biology. In mammalian cells G1/S transcription is turned off in S phase by the E2F repressors E2F6 and E2F7, much like Nrm1 and Yox1 in fission yeast. Our preliminary data shows that knocking down either E2F6 or E2F7 in RPE cells induces the accumulation of licensing factors CDT1 and CDC6 (cdc18 in *S. pombe*) and cyclins E and A (Cig2 in *S. pombe*) at the protein level, much like that observed in fission yeast (Figure 4.17). In mammalian cells cyclin A is involved in the degradation of CDT1. Future work should focus on determining if co-downregulating E2F6/7 and cyclin A, therefore creating an unbalance between licensing factors and CDK activity, leads to formation of bulk genomic instability in RPE cells, as verified for fission yeast. Given the role of

Geminin in degrading CDT1, in mammalian cells, co-downregulation of E2F6 and geminin could also be tested for generation of further genomic instability. If the lethal synergy observed between Δ*nrm1* and Δ*cig2* cells in fission yeast is conserved in RPE cells, E2F6/7 and cyclin A could represent two potential therapeutic targets for the treatment of cancer.

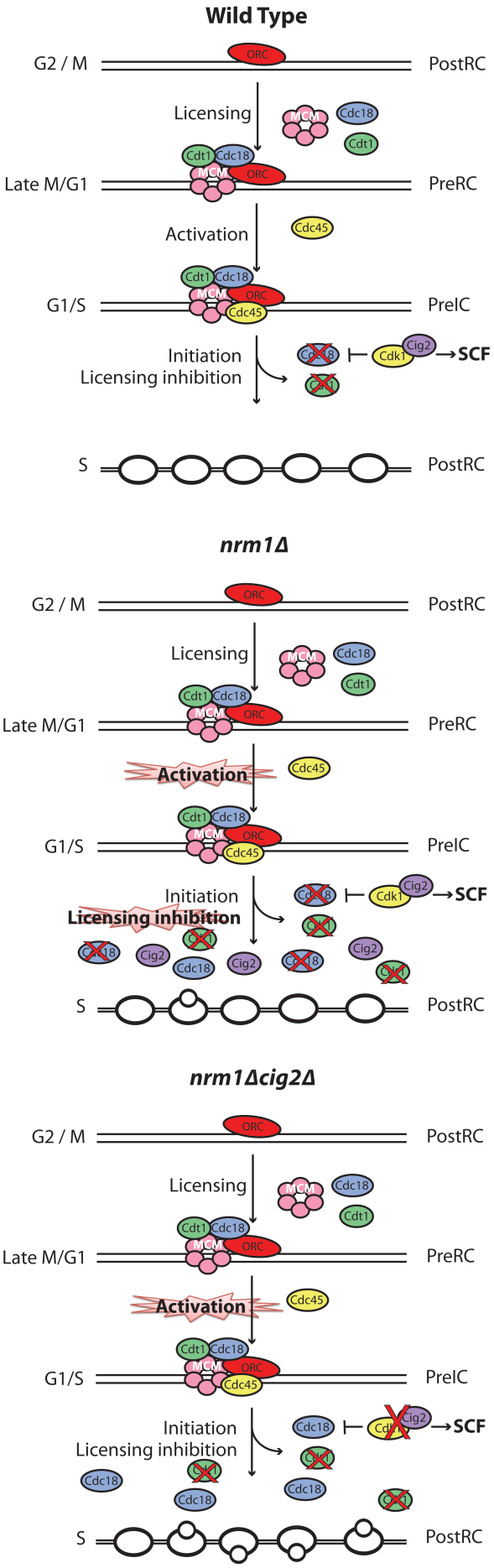
Wild Type
G2 / M
ORC
PostRC
Licensing
MCM
Cdc18
Cdt1
Late M/G1
PreRC
Activation
Cdc45
G1/S
PreIC
Initiation
Licensing inhibition
Cig2
Cdk1
SCF
S
nrm1Δ
Licensing inhibition
nrm1Δcig2Δ

Figure 6.2 – Model

(Wild type cells) Cdc18p and Cdt1p are involved in loading the MCM helicase onto origins of replication during a step known as origin licensing. In order to prevent re-rereplication, Cdc18p is sent for proteolysis via the Cig-CDK pathway, thus inhibiting re-licensing of already fired origins. (*Δnrm1* cells) Deletion of *nrm1+* promotes upregulation of both Cdc18p and Cig2p, resulting in a delicate balance between licensing factors and CDK activity, hence, between licensing activation and licensing inhibition. This results in accumulation of genomic instability and sporadic rereplication. (*Δnrm1Δcig2*) Disruption of this balance by abrogation of Cig2p-CDK activity results in enhanced licensing activation, hence, further accumulation of genomic instability and rereplication.

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7.1 – Journal articles and book chapters

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7.2 – Websites

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8. Appendices