University of Massachusetts Medical School
eScholarship@UMMS

GSBS Dissertations and Theses | Graduate School of Biomedical Sciences

2008-09-05

Checkpoint Regulation of S-Phase Transcription: A Dissertation

Chaitali Dutta
University of Massachusetts Medical School

Let us know how access to this document benefits you.
Follow this and additional works at: https://escholarship.umassmed.edu/gsbs_diss

 Part of the Amino Acids, Peptides, and Proteins Commons, Cells Commons, Fungi Commons, and the Genetic Phenomena Commons

Repository Citation
Dutta C. (2008). Checkpoint Regulation of S-Phase Transcription: A Dissertation. GSBS Dissertations and Theses. https://doi.org/10.13028/1y61-wk85. Retrieved from https://escholarship.umassmed.edu/gsbs_diss/391

This material is brought to you by eScholarship@UMMS. It has been accepted for inclusion in GSBS Dissertations and Theses by an authorized administrator of eScholarship@UMMS. For more information, please contact Lisa.Palmer@umassmed.edu.

Graduate School of Biomedical Sciences

GSBS Dissertations

University of Massachusetts Medical School

Year 2008

Checkpoint Regulation of S-phase Transcription

Chaitali Dutta

University of Massachusetts Medical School

CHECKPOINT REGULATION OF S-PHASE TRANSCRIPTION

A Dissertation Presented

By

Chaitali Dutta

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences,

Worcester

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

In

BIOMEDICAL SCIENCES

SEPTEMBER 5, 2008

CHECKPOINT REGULATION OF S-PHASE TRANSCRIPTION

A Dissertation Presented

By

Chaitali Dutta

Approved as to style and content by:

Nicholas Rhind, PhD., Thesis Advisor

Timothy Kowalik, PhD., Member of Committee

Janet Stein, PhD., Member of Committee

Job Dekker, PhD., Member of Committee

Peter Pryciak, PhD., Chair of Committee

Charles Hoffman, PhD., External Examiner

Anthony Carruthers, PhD

Dean of the Graduate School of Biomedical Sciences
University of Massachusetts Medical School

Interdisciplinary Graduate Program

September 5, 2008

Copyright notice

Portions of this dissertation are represented in the following publication:

Chaitali Dutta, Prasanta K Patel, Adam Rosebrock, Anna Oliva, Janet Leatherwood and Nicholas Rhind (2008) The DNA replication checkpoint directly regulates MBF-dependent G1/S transcription. Molecular and Cellular Biology, October 2008, Vol.28, No.19, p.5977-5985

Table of Contents

List of Tables and Figures

Acknowledgments

I would like to thank my mentor, Nick Rhind, for allowing me to stay in his lab for quiet a few years. During those years, he not only taught me how to address a question and answer it, he also taught me to ask the next question to take the project forward. He gave me the freedom I enjoyed to explore the multiple avenues and possibilities of a project. He trusted my abilities to drive a project and gave me the independence to work on a given question. I was fortunate enough to have an opportunity to work with a scientist like Nick, who is not only a great scientist but is also fun to work with. He always welcomed any questions and was very open for discussion. He is very considerate and always willing to help with students' personal problems or issues, and he freely offers his help when needed. I think because of him I was able to manage to be a graduate student and take care of my family.

I thank all the Rhind lab members: Prasant Patel, Mary Porter-Goff, Nicholas Willis, Naveen Kommajosyula, Daniel Keifenheim, Jillian Holmberg, Shankar Das and Kai-Di Fan. They were not only my friends; they provided scientific discussions, suggestions, and helpful tips for experiments. They helped me not only in the lab, but outside of the lab as well. They provided help countless number of times and always with a happy spirit. I would especially like to thank Mary, who was my backup person to pick up my daughter Puja, gave me rides when I was not allowed to drive, and babysat Puja when I needed.

I would like to thank my committee members Timothy Kowalik, Peter Pryciak, Job Dekker, Janet Stein and Charles Hoffman. They were very encouraging and

enthusiastic about my project. They provided extremely helpful discussion and helped me to successfully finish my projects. I appreciate the time and energy they offered to help me go forward with my project.

I would like to thank people in our department especially the members of the Munson lab, the Rando lab for providing fun environment, equipments, and reagents.

Finally I thank my parents for providing me strength, encouragement and support. I am grateful to my husband and my daughter for giving me their love, encouragement and support. I am extremely thankful to my daughter Puja who is so mature for her age and provided me a reason to love and live.

Abstract

The DNA replication checkpoint transcriptionally up-regulates genes that allow cells to adapt to and survive replication stress. Our results show that, in the fission yeast *Schizosaccharomyces pombe*, the replication checkpoint regulates the entire G1/S transcriptional program by directly regulating MBF (aka DSC1), the G1/S transcription factor. Instead of initiating a checkpoint-specific transcriptional program, the replication checkpoint targets MBF to maintain the normal G1/S transcriptional program during replication stress. We propose a mechanism for this regulation, based on *in vitro* phosphorylation of the Cdc10 subunit of MBF by the Cds1 replication-checkpoint kinase. Substitution of two potential phosphorylation sites with phospho-mimetic amino acids suffice to promote the checkpoint transcriptional program, suggesting that Cds1 phosphorylation directly regulates MBF-dependent transcription. The conservation of MBF between fission and budding yeast, and recent results implicating MBF as a target of the budding yeast replication checkpoint, suggest that checkpoint regulation of the MBF transcription factor may be a conserved strategy for coping with replication stress. Furthermore, the structural and regulatory similarity between MBF and E2F, the metazoan G1/S transcription factor, suggests that this checkpoint mechanism may be broadly conserved among eukaryotes.

Our result shows that both the replication checkpoint and the S-phase DNA damage checkpoint are involved in activating MBF regulated S-phase gene transcription and that this coordinated transcriptional response is beneficial for survival during replication stress. I demonstrate that the beneficial role of the transcriptional response

during checkpoint activation is mediated by three major MBF transcripts: *cdc22, mrc1* and *mik1*. Mrc1 dependent stabilization of stalled fork is important during S phase arrest. However, cells ability to prevent mitosis (Mik1 dependent) along with stable fork (Mrc1 dependent) both are crucial for survival. Our data also suggest that the level of Cdc22 is a determining factor for replication checkpoint activation and when over-expressed can alleviate the effects not only in HU but also in MMS.

Chapter I

Introduction

Cell cycle and Checkpoints

During the DNA synthesis (S) phase of each cell division cycle, the entire genome is faithfully replicated. High fidelity duplication and error-free transmission of the genetic material to subsequent generations are critical for survival. As cells experience a continuous threat of adverse genetic changes from a plethora of DNA lesions, preserving the integrity of the genome is demanding. These lesions can be caused by environmental or endogenous genotoxic insults such as ionizing radiation (IR) or ultraviolet radiation (UV), various chemicals, drugs and reactive cellular metabolites. Consequently, continuous surveillance of the genetic material and prompt action to repair any DNA damage, or to eliminate hazardous, genetically unstable cells, are required. To cope with this challenging task, eukaryotes have evolved an elaborate network of molecular mechanisms to detect unreplicated or aberrant DNA structures, to spread the alert signal, and to respond through the coordinated activities of diverse DNA repair and cell cycle checkpoint pathways (Bartek et al., 2004; Elledge, 1996).

The term checkpoint was coined to describe a mechanism that actively delays a later cell-cycle event in response to DNA damage, thereby providing more time for repair of the damage (Hartwell and Weinert, 1989). Recent evidence showed that checkpoint pathways control not only cell cycle progression but also processes such as the transcription of DNA damage response genes, the activation of DNA repair pathways, recruitment of proteins to sites of damage, chromatin remodeling, death and senescence

(Shiloh, 2003; Abraham, 2001; Bartek and Lukas, 2001). Checkpoint failure can lead to an elevated mutation rate, chromosome instability, and the development of cancer (Falck et al., 2002).

Different Kinds of Checkpoints

There are three well-studied eukaryotic DNA damage checkpoints (Humphrey, 2000; Baber-Furnari et al., 2000; Melo and Toczyski, 2002). First, DNA damage sustained early in the cell cycle (G1) delays the start of S-phase (the restriction point in mammals) and thus protects against replication of damaged templates. G1 cells contain only one copy of the genome, and thus the options for accurately repairing specific forms of damage are limited. This limitation might explain why, in several mammalian cells, the G1 checkpoint is closely linked to programmed cell death or apoptosis (Elledge, 1996).

A second checkpoint called the S-phase DNA damage checkpoint reduces the rate of DNA synthesis if damage is present during replication without blocking or preventing replication completely. Indeed, cells that are irradiated at the beginning of S phase complete a slow S phase and arrest in G2 because damage is still present. The S-phase DNA damage checkpoint is related to, but distinct from the replication checkpoint that is initiated when the progression of replication forks becomes stalled in response to stresses such as dNTP depletion or chemical inhibition of polymerases. Both the S-phase DNA damage checkpoint and the replication checkpoint prevent mitosis. However, the down-stream targets for these checkpoints and their regulatory circuits could be overlapping but not identical (Elledge, 1996).

The third called G2-M DNA damage checkpoint delays entry into mitosis. The G2-M checkpoint is important because it provides time to repair damaged chromatids prior to segregation in mitosis. As G2 cells contain two full complements of genetic materials, repair of any kind of damage could be achieved without loss or change of the genetic information (Baber-Furnari et al., 2000).

Checkpoint Signaling Pathways in Human and in Yeast

Most of the DNA damage checkpoint proteins are well conserved and the overall organization of the pathways has been maintained through evolution (Table 1.1). Thus, a great deal of insight has been gained by combining information from yeast, *Xenopus*, murine and human studies (Melo and Toczyski, 2002). There are three major components of this pathway. 1) Sensors such as the phosphoinositide-3-kinase-related kinases (PIKKs), which directly or indirectly in collaboration with other factors, sense DNA damage or stalled replication structures and initiate the checkpoint-signaling pathway (Abraham, 2001). 2) Mediators and adaptors are thought to promote protein-protein interactions between checkpoint PIKKs and their down-stream targets. 3) Effector kinases are the downstream targets of PIKKs regulating cell cycle specific function of checkpoints (Figure 1.1)

In mammals there are two related pathways that respond to different types of DNA damage during replication. The first, described by Painter and Young, is defective in cells from patients with ataxia telangiectasia (AT), a hereditary disease in which ATM (PIKK) is mutated (Painter and Young, 1980). ATM is required to prevent firing of new origins in the presence of double strand breaks. A failure in this signaling pathway results in radiation resistant DNA synthesis (RDS). Even in normal cells, the presence of

damage does not completely stop replication. A second damage control pathway is regulated by the ATR, the ATM related protein (Figure 1.2). In addition to a role in responding to DNA damage, agents that directly interfere with replication fork progression such as HU, UV and MMS (a DNA alkylating agent) activate the ATR pathway. Directly downstream of ATM/ATR are two checkpoint effector kinases Chk1 and Chk2 (which are also known as Rad53 and Cds1 in budding yeast and fission yeast respectively) that are both activated by phosphorylation by upstream kinases (ATM/ATR). In human cells the Rad53/Cds1 structural homologue Chk2 is activated by ionizing radiation at any stage of the cell cycle, but it responds poorly to replication stress (Matsuoka et al., 1998). Chk2 phosphorylates key effectors of the mammalian checkpoint pathways, p53 and Cdc25 phosphatase (Blasina et al., 1999; Chehab et al., 2000; Hirao et al., 2000).

In fission yeast *Schizosaccharomyces pombe*, the central checkpoint kinase Rad3 (the homolog of the budding yeast Mec1 and metazoan ATR) responds to all kinds of DNA damage including replication block by HU (Figure 1.2). Rad3 phosphorylates the S-phase checkpoint effector kinase Cds1 (the homolog of the budding yeast Rad53and Dun1 and Chk2 in mammals), which regulates the downstream targets of the checkpoint (Boddy and Russell, 2001). Unlike Rad53 in budding yeast, Cds1 is not activated and does not mediate responses outside S-phase because Mrc1, the adaptor for Cds1 activation, is expressed only during S phase. Chk1 is the effector of the G2 DNA damage-induced checkpoint and it does not respond to incompletely replicated DNA. In fact, Cds1 actively inhibits Chk1 during activation of replication checkpoint and S-phase

DNA damage checkpoint (Brondello et al., 1999). Neither Cds1 nor Chk1 are essential genes in fission yeast, and even in the absence of both genes the organism is viable.

In budding yeast Rad53 is an effector of both the DNA damage and replication checkpoints (Figure 1.2). Consistent with these dual roles, Rad53 is phosphorylated and activated both by agents that induce double-strand breaks and by agents that cause replication stress. Unlike other organisms the effector kinase is essential in budding yeast because it is required to regulate ribonucleotide reductase (RNR) level through phosphorylation of SML1 (inhibitor of ribonucleotide reductase)(Osborn et al., 2002). Rad53 is required to resume DNA replication following replication blockage by HU or mutant polymerase or DNA damage. The second checkpoint kinase, Chk1, functions in response to DNA damage.

Significance of The Checkpoints

All three of these checkpoints provide time in which repair can occur. The G1 checkpoint prevents cells with damaged DNA from entering S-phase and hence prevents duplication errors. It is increasingly clear that the S-phase DNA damage checkpoint also preserves genetic integrity, but exactly how slowing of S phase does this is less clear. The G2 checkpoint provides opportunities for repair to prevent genetic instability as the cells progress from one cell cycle state into the next.

Protein function	*S. cereviviae*	*S. pombe*	Mammals
ATM/ATR-kinases	Mec1	Rad3	ATR
	Tel1	Tel1	ATM
ATR-interacting proteins	Ddc2	Rad26	ATRIP
RFC-like proteins	Rad24	Rad17	Rad17
	Rfc2-5	Rfc2-5	Rfc2-5
Mediators	Rad9	Crb2	BRCA1
	Mrc1	Mrc1	Claspin
Replication fork stabilizers	Tof1	Swi1	Timeless
DSB recognition processing	Mre11	Rad32	Mre11
	Rad50	Rad50	Rad50
	Xrs2	Nbs1	Nbs1
Effector Kinases	Rad53	Cds1	Chk2
	Chk1	Chk1	Chk1

Table 1.1 Proteins Involve in the S-Phase Checkpoint in Yeast and Mammals.

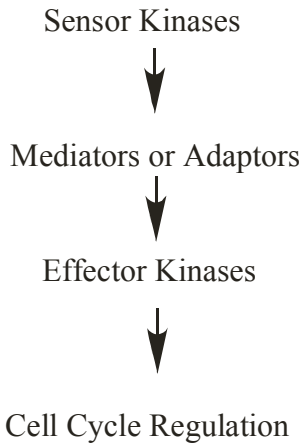

Sensor Kinases
Mediators or Adaptors
Effector Kinases
Cell Cycle Regulation

Figure 1.1

Figure 1.1 General Schematic of Checkpoint Regulation.

Sensors are proteins kinases, which either directly or in collaboration with other factors sense DNA damage or stalled replication forks and initiate signaling cascades. Mediators or adaptors are thought to promote protein-protein interactions between checkpoint kinases and down-stream effctor kinases. Activation of effector kinases has cell cycle specific multiple targets.

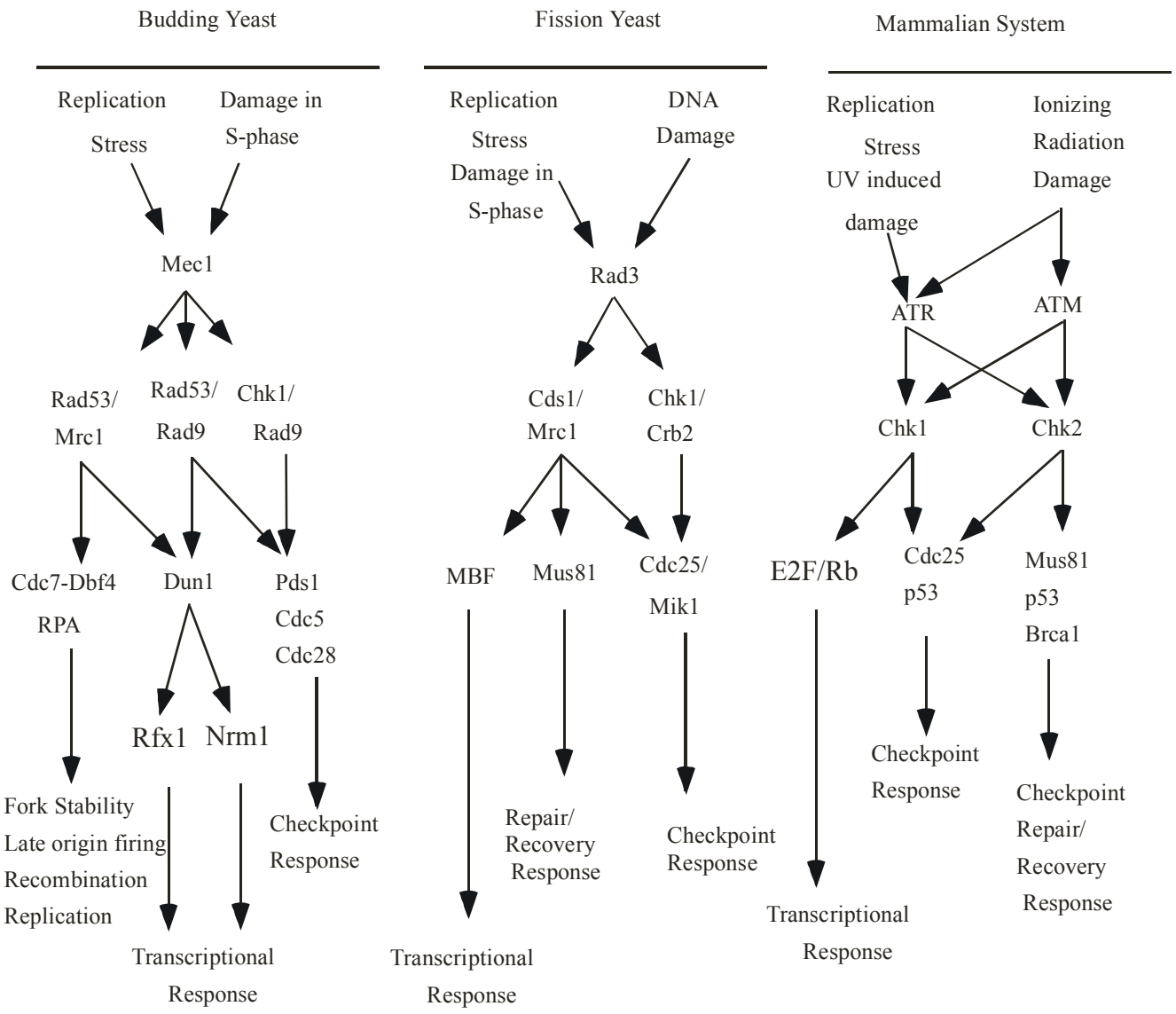

Budding Yeast
Fission Yeast
Mammalian System
Replication Stress
Damage in S-phase
Mec1
Rad53/ Mrc1
Rad53/ Rad9
Chk1/ Rad9
Cdc7-Dbf4
RPA
Dun1
Pds1
Cdc5
Cdc28
Rfx1
Nrm1
Fork Stability
Late origin firing
Recombination
Replication
Checkpoint Response
Transcriptional Response
Replication Stress
Damage in S-phase
DNA Damage
Rad3
Cds1/ Mrc1
Chk1/ Crb2
MBF
Mus81
Cdc25/ Mik1
Repair/ Recovery Response
Checkpoint Response
Transcriptional Response
Replication Stress
UV induced damage
Ionizing Radiation Damage
ATR
ATM
Chk1
Chk2
E2F/Rb
Cdc25
p53
Mus81
p53
Brca1
Checkpoint Response
Checkpoint Repair/ Recovery Response
Transcriptional Response

Figure 1.2

Figure 1.2 Schematic Representations of the DNA Damage and Replication Checkpoint in Yeast and Mammal.

Arrows represent either biochemical or genetic evidence for a connection.

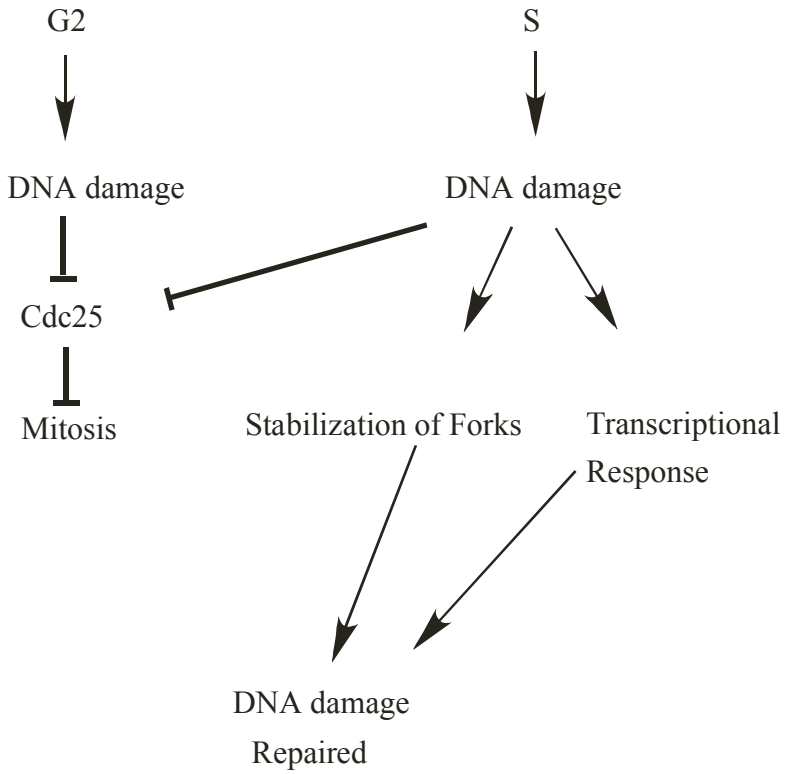

G2
S
DNA damage
DNA damage
Cdc25
Mitosis
Stabilization of Forks
Transcriptional
Response
DNA damage
Repaired

Figure 1.3

Figure 1.3 Functions of G2 DNA Damage Checkpoint and the Replication Checkpoint.

The main function of G2 DNA damage checkpoint is to provide time to repair by preventing mitosis. However, replication checkpoint/S-phase DNA damage checkpoint performs additional functions. Both stabilization of forks and transcriptional response ensure that cells can cope with the damages until the sister chromatids are available to repair in G2.

The Mechanism of The G2 DNA Damage Checkpoint

The function of the G2 DNA damage checkpoint activation is to regulate the kinase Cdc2 (Figure 1.3). The kinase needs to be dephosphorylated in order to become active and drive the cells into mitosis (Rhind et al., 1997). Mik1, along with Wee1, phosphorylate Cdc2 at tyrosine 15 (Gould and Nurse, 1989; Featherstone and Russell, 1991; Lee et al., 1994; Parker et al., 1992; Russell and Nurse, 1987).

The inhibitory phosphate on tyrosine 15 of Cdc2 is removed by Cdc25 phosphatase for mitotic entry (Millar et al., 1991; Russell and Nurse, 1986). The checkpoint controls both Cdc25 and Mik1 through the Chk1 dependent pathway (Furnari et al., 1997; Rhind and Russell, 1998). Phosphorylation of Cdc25 promotes its nuclear export; presumable sequestering it away from Cdc2 and also directly inhibits Cdc25 (Dalal et al., 1999; Lopez-Girona et al., 1999; Yang et al., 1999; Zeng and Piwnica-Worms, 1999; McGowan, 2002)

The Mechanism of The Replication and S-phase DNA Damage Checkpoints

The replication checkpoint also regulates Cdc2 via Cdc25 and Mik1. However, in addition to inhibiting Cdc2, this checkpoint is involved in performing multiple functions that are necessary to complete replication. Providing time for repairing damage is not enough when cells are arrested in S phase with partially replicated DNA. Therefore, the additional roles of the checkpoint needed to maintain cell viability primarily rely on two known important steps. 1) Stabilization of stalled forks is important for survival. In fission yeast, the S-phase checkpoint kinase Cds1 is known to regulate three proteins that

are implicated in maintaining replication fork stability: Mus81-Eme1, a heterodimeric structure-specific endonuclease complex; Rqh1, a RecQ-family helicase involved in suppressing inappropriate recombination during replication; and Rad60, a protein required for recombinational repair during replication (Kai M 2005; Taylor ER 2008; Miyabe I 2006; Boddy MN 2000). 2) The replication checkpoint is also known to regulate transcription. The transcriptional response is different in budding yeast, fission yeast and metazoans. An extensive study of transcriptional response has been done in budding yeast and the target of the checkpoint has been identified. However when I began my work very little was known about how the transcriptional response is regulated in fission yeast and the target (s) for checkpoint dependent transcription was unknown (Figure 1.3).

Regulation of Normal S-phase Transcription

In *S. cerevisiae*, periodic transcription of the S-phase genes is mediated by two transcriptional complexes, Swi4-Swi6 and Swi6-Mbp1, which act through conserved promoter elements known as SCB (SWI4/6-dependent cell cycle box) and MCB (Mlu1 cell cycle box) sites respectively (Figure 1.4A)(Breeden and Nasmyth, 1987; Andrews and Herskowitz, 1989; Lowndes et al., 1991; Taba et al., 1991; Dirick et al., 1992; Koch et al., 1993). A DNA binding complex containing Swi6 and Mbp1, termed MBF (MCB binding factor) or DSC1 (DNA synthesis control) that recognizes MCB elements, is thought to be involved in transcriptional activation (Dirick et al., 1992; Koch et al., 1993). Similarly DNA binding activity containing Swi4 and Swi6 complex known as SBF recognizes SCB elements is also involved in G1 specific transcription. Although neither Swi4 nor Mbp1 is essential, inactivation of both Swi4 and Mbp1 is lethal (Bean et

al., 2005). Binding of SBF and MBF to G1-specific promoters is insufficient for activation of G1 specific transcription (Harrington and Andrews, 1996; Koch et al., 1993); they additionally require the activity of Cln3/Cdc28 (Dirick et al., 1995; Koch et al., 1996; Siegmund and Nasmyth, 1996). Although both SBF and MBF are regulated by Cln3/Cdc28, the mechanism by which these factors are regulated is distinct (Amon et al., 1993; de Bruin et al., 2004; Koch et al., 1996; Siegmund and Nasmyth, 1996). Whi5 acts as a suppressor of SBF in G1; subsequent inactivation of Whi5 via phosphorylation by Cln3/Cdc28 is responsible for activation of G1 specific transcription (Costanzo et al., 2004; de Bruin et al., 2004). Transcription from SCB elements is then switched off in G2 by the CDK activity of Cdc28-Clb complexes (Koch et al., 1996). Recently Nrm1 has been identified as a repressor of MBF to repress transcription when cells exit from G1 phase (de Bruin et al., 2006).

Analogous systems function in metazoans. E2F, which is a G1 dependent transcription factor, forms a heterodimer with members of the related DP family (DP1 and DP2) (Nevins 1998). E2F-DP preferentially recognizes the nucleotide sequence TTTCCCGC. There are multiple members in the E2F family, the activity of which is regulated by Rb (retinoblastoma). Cdk4/6-cyclin D and Cdk2-cyclin E are known to phosphorylate Rb and sequentially render it inactive, leading to activation of E2F dependent S phase progression and S phase dependent gene expression (Figure1.4C) (Martin et al., 1995; Slansky and Farnham, 1996). Cdk2-cyclin A inactivates the complex in G2 (Xu et al., 1994; Krek et al., 1994).

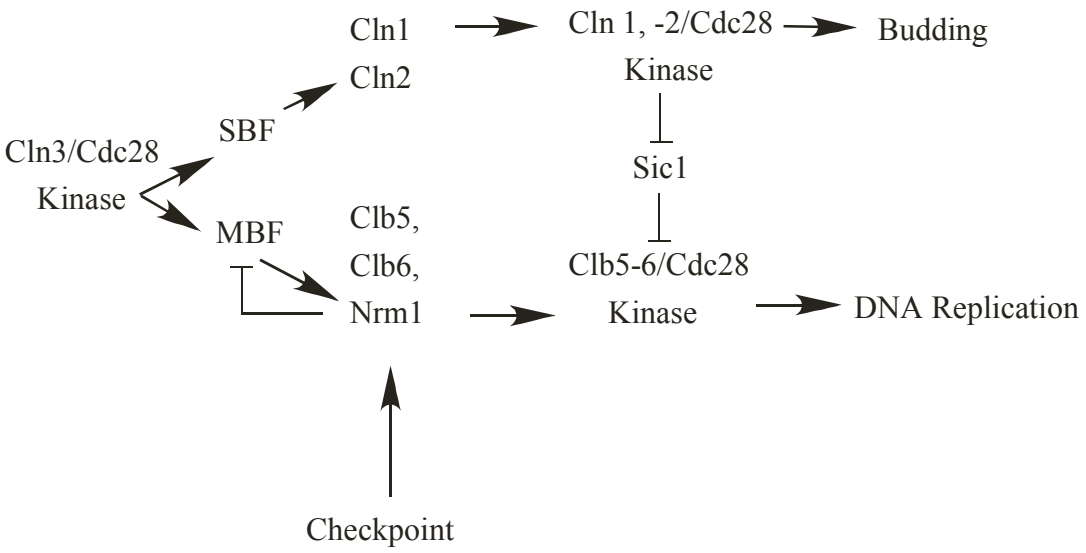

Cln1
Cln2
Cln 1, -2/Cdc28
Kinase
Budding
SBF
Cln3/Cdc28
Kinase
Sic1
MBF
Clb5,
Clb6,
Nrm1
Clb5-6/Cdc28
Kinase
DNA Replication
Checkpoint

Figure 1.4A

Figure 1.4 A Schematic of S-phase Transcription Pathway in Budding Yeast.

In budding yeast Cln3-Cdc28 transiently induces expression of two complexes. SBF (Swi4/Swi6) drives the transcription of genes required for budding. MBF (Swi6/Mbp1) complex on the other hand drives the transcription of genes required for DNA replication. Nrm1 is negative regulators of the checkpoint dependent transcription of MBF.

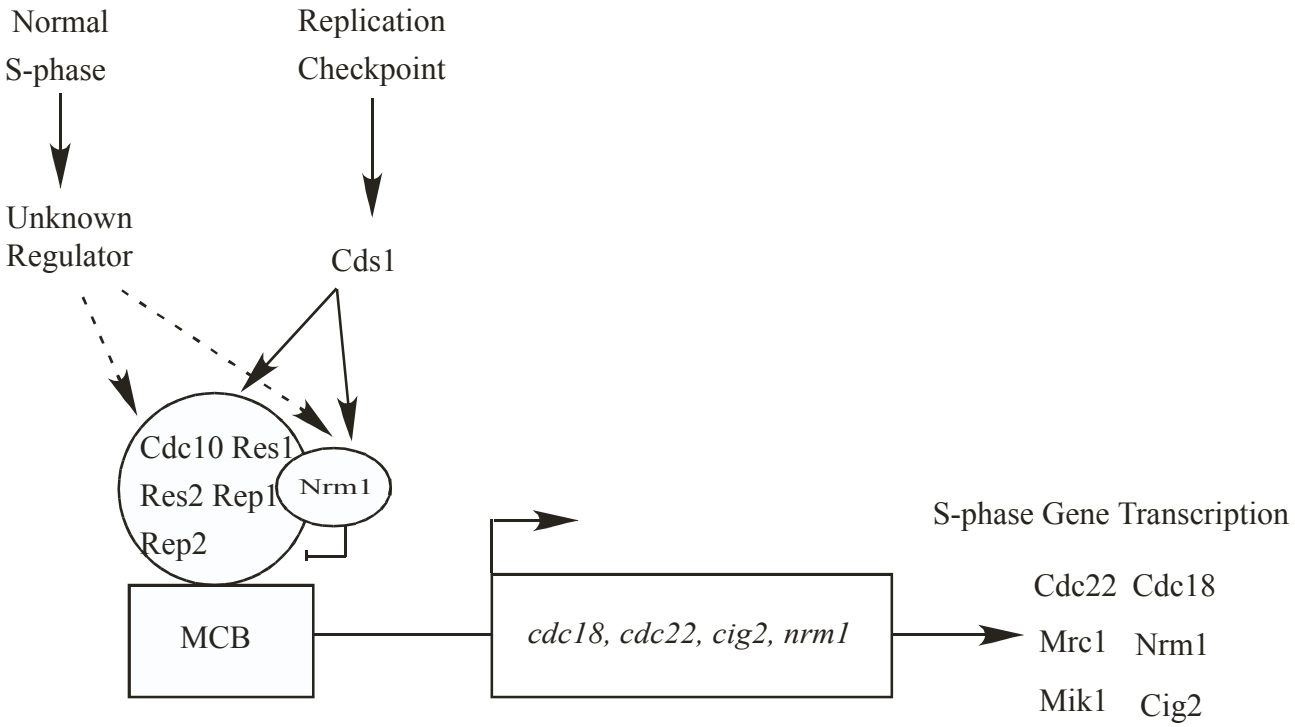

Normal
S-phase
Replication
Checkpoint
Unknown
Regulator
Cds1
Cdc10 Res1
Res2 Rep1
Rep2
Nrm1
MCB
cdc18, cdc22, cig2, nrm1
S-phase Gene Transcription
Cdc22 Cdc18
Mrc1 Nrm1
Mik1 Cig2

Figure 1.4 B

Figure 1.4 B Schematic of S-phase Transcription Pathway in Fission Yeast.

In fission yeast the MBF complex drives S-phase specific genes required for replication. Cdc10 and Nrm1 both have been suggested as targets for checkpoint dependent transcriptional regulation.

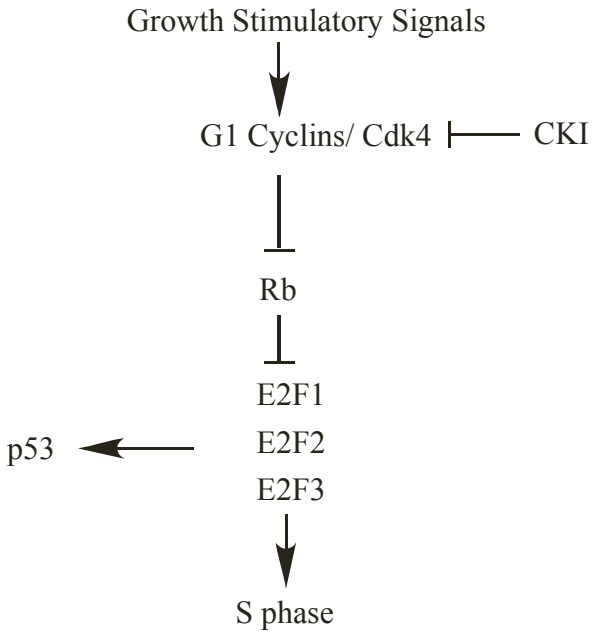

Growth Stimulatory Signals
G1 Cyclins/ Cdk4
CKI
Rb
E2F1
E2F2
E2F3
p53
S phase

Figure 1.4C

Figure 1.4 C Schematic of S-phase Transcription Pathway in Mammals.

The Rb/E2F signaling pathway. Rb is the target of cell cycle dependent kinases as well as checkpoint kinase. Rb phosphorylation leads to the activation of E2F dependent S-phase transcription. Deregulation of this pathway induces p53-mediated apoptosis.

The *Schizosaccharomyces pombe* G1/S transition is coordinated by the DSC1 (DNA synthesis control) or MBF (MCB binding factor) transcription factor complex (Figure 1.4B). MBF contains the *cdc10, res1, res2, rep1* and *rep2* gene products and recognizes the MCB element (Lowndes et al., 1992; Miyamoto et al., 1994; Slansky and Farnham, 1996; Zhu et al., 1997; Caligiuri and Beach, 1993; Tanaka et al., 1992). MCB is the common repeated DNA sequence in the promoters of genes, such as *cdc22, cdc18, cig2* and *cdt1* all of which are expressed only at the G1-S boundary during the mitotic cell cycle and whose products are required either directly or indirectly for DNA synthesis (Gordon and Fantes, 1986; Kelly et al., 1993; Obara-Ishihara and Okayama, 1994; White et al., 2001). Furthermore, isolated MCBs confer cell cycle-regulated transcription on a heterologous gene (Lowndes et al., 1991). The periodicity of transcription is controlled independent of CDK activity (Baum et al., 1997). Several studies have proposed the possibility that two independent complex Cdc10-Res1 and Cdc10-Res2 are active during mitotic and meiotic cycle respectively (Tanaka et al., 1992; Miyamoto et al., 1994). However, later studies have provided evidence that both Res1 and Res2 are associated with Cdc10 throughout the cell cycle (Whitehall et al., 1999). Recently, Nrm1 has been identified as a negative regulator of MBF in fission yeast and to be a direct target of the replication checkpoint (de Bruin RA 2008)

Checkpoint Dependent Transcriptional Regulation

The mechanism of checkpoint dependent transcription regulation has been best studied in budding yeast. The effector kinase Dun1 downstream of Mec1 and Rad53, is activated in response to DNA damage and replication arrest (Zhou and Elledge, 1993;

Allen et al., 1994) and is required for the transcriptional response. *RFX1*, also known as *CRT1* gene whose product has been identified as a repressor of RNR. Rfx1 binds specifically to sequences in the promoters of the RNR genes, and Rfx1 inhibition leads to high levels of RNR expression. Phosphorylation of Rfx1/Crt1 by Dun1 is necessary for de-repression of RNR synthesis (Zhou and Elledge, 1993). De-repression of *RFX1* suppresses the lethality of *mec1* and *rad53* null alleles and is essential for cell viability during replication stress (Huang et al., 1998). However, this signaling pathway is not conserved among eukaryotes.

In metazoans, Rb/E2F is known to be involved in the G1/S transition (Figure 1.4C). E2F1 is a member of the E2F family of transcription factors that modulates expression of many genes involved in the transition from G1 to S phase of the cell cycle (Nevins, 1998). Many S-phase genes are known to be upregulated in HU induced cells (Ishida et al., 2001). In addition deregulation of E2F1 expression has been shown to lead to S-phase entry (Qin et al., 1994; Kowalik et al., 1995). Chk2 kinase had been shown to phosphorylate E2F1 in response to UV and etoposide induced DNA damage (Stevens et al., 2003). These studies imply that in metazoans, checkpoints might be targeting S-phase transcriptional machinery and driving the expression of all the S-phase genes. However, there is no study directly addressing checkpoint driven S-phase transcription by E2F1/Rb.

Unlike budding yeast, in fission yeast there is no specific DNA damage induced transcriptional pathway; instead it had been shown that in response to the replication stress many S-phase genes are up-regulated in an MBF dependent manner (Baum et al., 1997; Whitehall et al., 1999; Kelly et al., 1993). These genes are usually periodically expressed at the G1-S transition called START in yeast, (Restriction Point in higher

eukaryotes); which suggests that the checkpoint targets the MBF, the S-phase transcription factor, to mediate the transcriptional response (Figure 1.4B). Recent genomic analysis of budding yeast replication stress response and recent work on budding yeast Nrm1 suggest that MBF is a conserved checkpoint target in yeast (de Bruin et al., 2006; Gasch et al., 2001). Therefore, the checkpoint regulation of MBF may be conserved in budding yeast, but largely obscured by the more dramatic Rfx1-dependent response.

Specific Aims of My Research

The goal of my thesis research has been to determine if MBF is a checkpoint target in fission yeast. It was known that HU arrested S phase cells modulate S-phase dependent *cdc18* (replication initiation factor) transcription. In addition, the transcription of *cdc18* was dependent on Cdc10, a member of MBF complex (Baum et al 1997). From these results, we hypothesized that MBF is a checkpoint target.

In the second chapter, I focused on the hypothesis that MBF is a checkpoint target and tested three predictions. 1) Normal MBF function is necessary for any checkpoint regulation, 2) all S-phase genes should be affected and 3) one or multiple members of the MBF complex should be the targets of the checkpoint dependent S-phase kinase Cds1. I provide experimental evidence that the checkpoint specifically targets Cdc10; a member of the MBF complex, to modulate its function and that Cdc10 is an *in vitro* target of the replication checkpoint kinase Cds1.

In my third chapter, I studied the relative importance of the transcriptional response in survival of cells in stress compared to prevention of mitosis and stabilization

of forks. I show that the checkpoint dependent S-phase transcription is important specifically during S phase arrest. There are three major MBF transcripts that play important roles in survival: *mrc1* maintains stalled forks, *mik1* prevents mitosis, and *cdc22* provides additional nucleotides.

My data suggest that during S phase arrest, fork stabilization is the most crucial step for survival and if cells are able to reach G2, their chance of survival increases dramatically. The checkpoint dependent transcriptional response on the other hand provides additional functions in both scenarios (S-phase arrest in HU and slowed S phase progression in MMS) to ensure the cells will complete replication and be able to repair the damage when the sister chromatids are available. Therefore, the checkpoint dependent transcriptional response indirectly plays an important role in maintaining genomic stability.

Chapter II

The DNA Replication Checkpoint Directly Regulates the MBF-Dependent G1/S Transcriptional Program

The gene array experiments were done in the laboratory of Janet Leatherwood (State University of New York Stony Brook). Hybridization was done by Anna Oliva and Adam Rosebrock helped in uploading the array data.

Abstract

The DNA replication checkpoint transcriptionally up-regulates genes that allow cells to adapt to and survive replication stress. Our results show that, in the fission yeast *Schizosaccharomyces pombe*, the replication checkpoint regulates the entire G1/S transcriptional program by directly regulating MBF (aka DSC1), the G1/S transcription factor. Instead of initiating a checkpoint-specific transcriptional program, the replication checkpoint targets MBF to maintain the normal G1/S transcriptional program during replication stress. We propose a mechanism for this regulation, based on *in vitro* phosphorylation of the Cdc10 subunit of MBF by the Cds1 replication-checkpoint kinase. Substitution of two potential phosphorylation sites with phospho-mimetic amino acids suffice to promote the checkpoint transcriptional program, suggesting that Cds1 phosphorylation directly regulates MBF-dependent transcription. The conservation of MBF between fission and budding yeast, and recent results implicating MBF as a target of the budding yeast replication checkpoint, suggest that checkpoint regulation of the MBF transcription factor may be a conserved strategy for coping with replication stress. Furthermore, the structural and regulatory similarity between MBF and E2F, the metazoan G1/S transcription factor, suggests that this checkpoint mechanism may be broadly conserved among eukaryotes.

Introduction

In response to inhibition of DNA replication, the replication checkpoint arrests the cell cycle, stabilizes replication forks and regulates transcription (Carr, 1997; Boddy and Russell, 2001). The transcriptional branch of the checkpoint response upregulates genes thought to be important for cells to survive prolonged replication arrest and to synthesize extra deoxynucleotides. A commonly used trigger of the replication checkpoint is treatment with the ribonucleotide reductase inhibitor hydroxyurea (HU), which prevents deoxynucleotide synthesis, thus preventing DNA replication. HU treatment activates a conserved checkpoint-signaling pathway (Boddy and Russell, 2001). In the fission yeast *Schizosaccharomyces pombe*, the central checkpoint kinase Rad3 (the homolog of the budding yeast Mec1 and metazoan ATM and ATR) activates the S-phase checkpoint effector kinase Cds1 (the homolog of the budding yeast Rad53 and Dun1 and also called Chk2 in mammals), which regulates the downstream targets of the checkpoint (Boddy and Russell, 2001). In the budding yeast *Saccharomyces cerevisiae*, Rad53 and Dun1 are thought to control transcription mainly through the Rfx1/Crt1 transcriptional repressor, which regulates a replication and DNA damage checkpoint specific transcriptional program (Huang et al., 1998). However, the Rfx1 regulatory circuit is not evolutionarily conserved and it is unclear how the replication checkpoint regulates transcription in other eukaryotes.

During a normal fission yeast cell cycle, G1/S transcription is regulated by the MBF/DSC1 transcription factor – containing the proteins Cdc10, Res1, Res2, Rep1 and Rep2 – and its negative regulator Nrm1 (de Bruin et al., 2006; Bahler, 2005). MBF and Nrm1 are conserved in budding yeast, which also contain the paralogous SBF-Whi5

transcription factor-repressor proteins (de Bruin et al., 2004; de Bruin et al., 2006; Costanzo et al., 2004). Furthermore, the MBF/SBF transcription factors and the Nrm1/Whi5 repressors are analogous to the E2F and Rb proteins of metazoans (Cooper, 2006; Costanzo et al., 2004; de Bruin et al., 2004). In each of these systems, expression outside of G1/S is repressed by binding of the repressor to the transcription factor and expression during G1/S is allowed by displacement of the repressor (Frolov and Dyson, 2004; de Bruin et al., 2004; de Bruin et al., 2006; Costanzo et al., 2004). Although the details of regulation of these systems differ, in many cases phosphorylation of either the transcription factor or the repressor is sufficient to activate transcription. As an example, cell cycle regulated phosphorylation of either SBF or Whi5 is sufficient to displace Whi5 and activate SBF-dependent transcription (Costanzo et al., 2004). Likewise, phosphorylation of either Rb or E2F suffices to activate E2F transcription in mammalian cells (DeCaprio et al., 1989; Stevens et al., 2003)

The G1/S transcriptional program of fission yeast comprises about 20 genes expressed in an MBF-dependent manner (Oliva et al., 2005; Peng et al., 2005; Rustici et al., 2004). A number of these genes are also expressed in response to HU treatment, even though most have no obvious checkpoint function (Baum et al., 1997). This correlation leads us to hypothesize that the HU-induced DNA replication checkpoint might regulate transcription by directly regulating MBF. Our hypothesis makes three testable predictions: 1) All MBF-dependent G1/S transcripts should be up-regulated in response to HU in a checkpoint-dependent manner, 2) mutations in MBF components that affect G1/S transcription should similarly affect checkpoint-dependent transcription, and 3) Cds1, the replication-checkpoint effector kinase, should directly regulate MBF activity.

Materials and Methods

Table 2.1 Strains Used in Chapter II

	Genotype	
yFS104	*h+ leu1-32 ura4-D18*	Rhind lab
yFS105	*h- leu1-32 ura4-D18*	Rhind lab
yFS163	*h- leu1-32 ura4-D18 ade6-210 res1::ura4*	Rhind lab
yFS189	*h- leu1-32 ura4-D18 ade6-704 rad3::ura4*	Rhind lab
yFS199	*h- leu1-32 ura4-D18 cds1::ura4*	Rhind lab
yFS397	*h+ leu1-32 ura4-D18 nmt1:cds1-D312E:leu1 mik1-13Myc:kanMX6*	Rhind lab
yFS252	*h- ura4-D18 res2::ura4*	Rhind lab
yFS257	*h- leu1-32 ade6-M216 cdc10-C4*	Rhind lab
yFS502	*h+ leu1-32 ura4-D18 cdc10-8A:kanMX6*	This study
yFS526	*h- leu1-32 ura4-D18 cdc10:kanMX6*	This study
yFS500	*h+ leu1-32 ura4-D18 cdc10-S720E:kanMX6*	This study
yFS527	*h+ leu1-32 ura4-D18 cdc10-T723E:kanMX6*	This study
yFS528	*h+ leu1-32 ura4-D18 cdc10-2E:kanMX6*	This study
yFS532	*h- leu1-32 ura4-D18 cdc10:kanMX6 rad3::ura4*	This study
yFS531	*h- leu1-32 ura4-D18 cdc10:kanMX6 cds1::ura4*	This study
yFS530	*h- leu1-32 ura4-D18 cds1::ura4 cdc10-2E:kanMX6*	This study
yFS529	*h+ leu1-32 ura4-D18 rad3::ura4 cdc10-2E:kanMX6*	This study
yFS493	*h+ leu1-32 ura4-D18 nrm1::kanMX6*	Wittenburg lab
yFS642	*h+ leu1-32 ura4-D18 cdc10-2E nrm1::kanMX6*	This study
yCD60	*h+ leu1-32 ura4-D18 cdc10-4E:kanMX6*	This study
yCD77	*h+ leu1-32 ura4-D18 cdc10-8E:kanMX6*	This study
yCD70	*h+ leu1-32 ura4-D18 cdc10-S720A:kanMX6*	This study
yCD87	*h+ leu1-32 ura4-D18 cdc10-S720EE:kanMX6*	This study
yCD88	*h+ leu1-32 ura4-D18 cdc10-S720ES732E:kanMX6*	This study
yCD91	*h+ leu1-32 ura4-D18 cdc10- S720EES732EE:kanMX6*	This study
yCD93	*h+ leu1-32 ura4-D18-cdc10-S720ET723ES732E:kanMX6*	This study

Cell culture

Strains used in this study are listed in Table 2.1. Cells were grown in YES media at 30˚C, expect for temperature-sensitive (ts) cells, which were grown at 25˚C (Forsburg and Rhind, 2006). HU sensitivity was assayed by spotting 3-fold serial dilutions of cells onto YES plates supplemented with 0, 1 or 3 mM HU and photographing growth after 7 days. Mutants alleles of *cdc10* were made by oligo-mediated site-directed mutagenesis.

A 3' fragment of *cdc10* was amplified with CD42 (TATAGACTAGTACTTCGATCGAAGAACAGAAAAGT) and CD43 (GACTAGTCGGAATTCCCGACTGTTCTTAGCGGCGTATCGG) and cloned into pFA6a-KanMX6 (Wach et al., 1994). Mutants were amplified, along with the 3' kanamycin-resistance marker, using CD44 (GATGCTGACGCTCCTTTTACTGTC) and PP184 (TTCTTTTTCTGTGGCCTCGCTTTCAAGCTGTCATGGACATGCACTGTGAGTCACTCCGTAAAACTAACTTATCTGTGAAGATCTGTTTAGCTTGCCTCGT) and transformed into yFS104 or yFS105. Accurate integration was confirmed by PCR and sequencing.

RNA analysis

RNA was prepared for northern blots and micro-array analysis as previously described (Oliva et al., 2005). Northern blots were probed with random-prime labeled *cdc22*, stripped and reprobed with *adh1*. *cdc22* levels were normalized to *adh1* and then all time courses were normalized to asynchronous wildtype controls included on all gels, such that the 20 minute time point for the wild-time course was set to 1. Micro-array analysis was carried out as previously described, using 3'-biased, ORF PCR product spotted arrays (Oliva et al., 2005). Briefly, total RNA from experimental samples was reverse-transcribed with a poly-dT primer, labeled with Cy3 and co-hybridized with similarly prepared asynchronous, wild type, Cy5-labeled cDNA. Experiments were performed twice; with between 2 and 8 replicate spots per gene per experiment. All data are available at ArrayExpress (www.ebi.ac.uk/arrayexpress). Hierarchical clustering was performed with Cluster (Eisen et al., 1998) and visualized with Java TreeView (jtreeview.sourceforge.net). All quantitations are given as mean ± SEM except as noted.

To identify the cluster in Figure 2.2B, we clustered the data from the 24 wt (yFS105), *rad3*Δ (yFS189) and *cds1*Δ (yFS199) experiments (two experiments each of S and G2, plus and minus HU) for all 821 genes that had at least 2-fold higher signal compare to control asynchronous sample in at least 4 experiments. Of these, only one cluster of 18 genes showed high expression in all S-phase samples and in wild type HU arrested samples, and low expression in all G2 and checkpoint-mutant HU arrested samples. Of these genes, one is a tRNA deaminase downstream of *nrm1* on the opposite strand and was excluded as a likely read-through artifact. To identify the clusters in Figure 2.2C, we clustered the same 24 experiments for the 64 genes that had both a greater than 2-fold difference in signal between wild type G2 and HU-arrested cells and a greater than 2-fold difference in signal between wild type and *rad3*Δ HU-arrested cells.

In vitro kinase assay

The Cdc10 C-terminal 61 codons were PCR amplified and cloned as a GST fusion into the pGEX-3X BamHI and EcoRI sites; site-directed mutations were made by oligo-directed mutagenesis and verified by sequencing. Cds1 *in vitro* kinase assays were performed as previously described (Kai et al., 2001). Briefly, GST-Cdc10, expressed in *E. coli* and purified on GSH beads, was incubated for 15 minutes at 30˚C with γ^{32}P-ATP and Cds1 immunopurified from HU treated *S. pombe*. Labeled protein was resolved by SDS-PAGE and visualized by autoradiography.

Results

All MBF Transcripts are Up-Regulated by the Replication Checkpoint

To test our first prediction, we used a whole-genome ORF micro-array to assay the replication checkpoint transcriptional response. We synchronized wild-type, *rad3*Δ cells – which lack the central checkpoint kinase, and *cds1*Δ cells – which lack the replication-checkpoint effector kinase activated by Rad3, and followed the cultures through a synchronous cell cycle in the absence or presence of HU (Figure 2.1A). We followed the levels of the *cdc22* G1/S transcript, encoding the large subunit of ribonucleotide reductase, by northern blotting in order to identify the peaks of S-phase transcription. Samples were collected from time points in which the untreated cells were in S phase or G2, and RNA was labeled and hybridized to micro-arrays. Using hierarchical clustering, we identified 17 genes that were up-regulated at least 2-fold in G1/S and also up-regulated at least 2-fold in response to HU in a Rad3-dependent manner (Figure 2.1B). All of these genes are MBF-dependent transcripts, defined as genes expressed at G1/S in an MBF-dependent manner in one of three published fission yeast cell-cycle transcription experiments (Oliva et al., 2005; Peng et al., 2005; Rustici et al., 2004). Furthermore, of the 14 core G1/S MBF-dependent transcripts, defined as those identified in at least 2 of the 3 data sets, 11 were identified as transcriptional targets of the replication checkpoint defined as above; the other three were excluded from the cluster because of low amplitude signal. These results suggest that the entire MBF transcriptional program is upregulated by the checkpoint.

Figure 2.1A

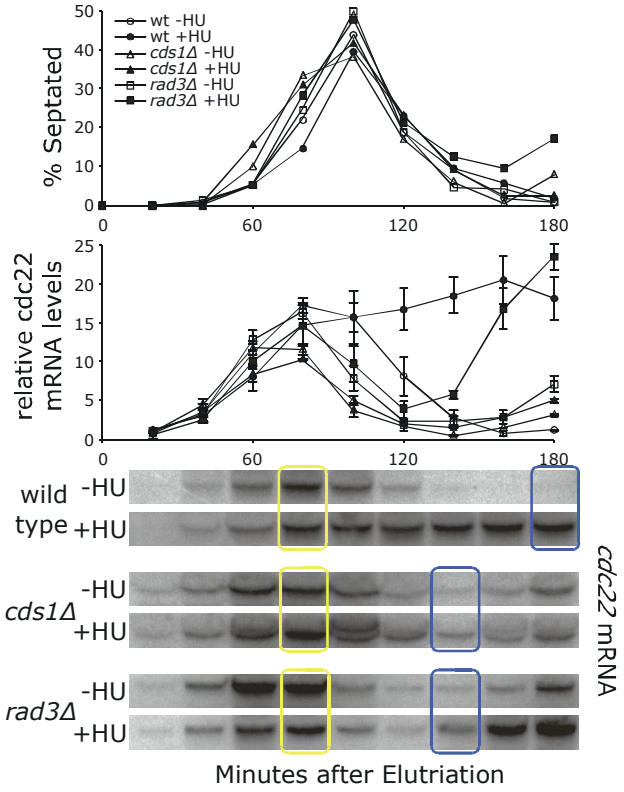

wt -HU
wt +HU
cds1Δ -HU
cds1Δ +HU
rad3Δ -HU
rad3Δ +HU
% Septated
relative cdc22 mRNA levels
wild type
-HU
+HU
cds1Δ
rad3Δ
cdc22 mRNA
Minutes after Elutriation

Figure 2.1 *cdc22* Expression by Northern Blot in Wild Type and the Checkpoint Deficient Strains.

A. Wild-type (yFS105), cds1Δ (yFS199) and rad3Δ (yFS189) cells were synchronized in early G2 by centrifugal elutriation and followed through a synchronous cell cycle in the presence or absence of HU. Samples were taken every 20 minutes for RNA isolation and visual inspection of septation. Northerns were probed with cdc22, stripped and reprobed with adh1 as a loading control. The quantitations represent the mean and SEM of three experiments, normalized to the wild-type 20 minute time point, except for cds1Δ, which is the mean and variance of two experiments. The boxes indicate the points taken for micro-array analysis; yellow are the S-phase time points and blue are the G2 time points. HU-treated rad3Δ cells enter a second round of the cell cycle earlier than untreated cells because, having failed to replicate, they have half as much DNA; because the size at which cells divide is determined by the DNA/cytoplasmic ratio, and because they lack the replication checkpoint G2 arrest, HU-treated rad3Δ cells divide at half the size of untreated cells.

Figure 2.1B

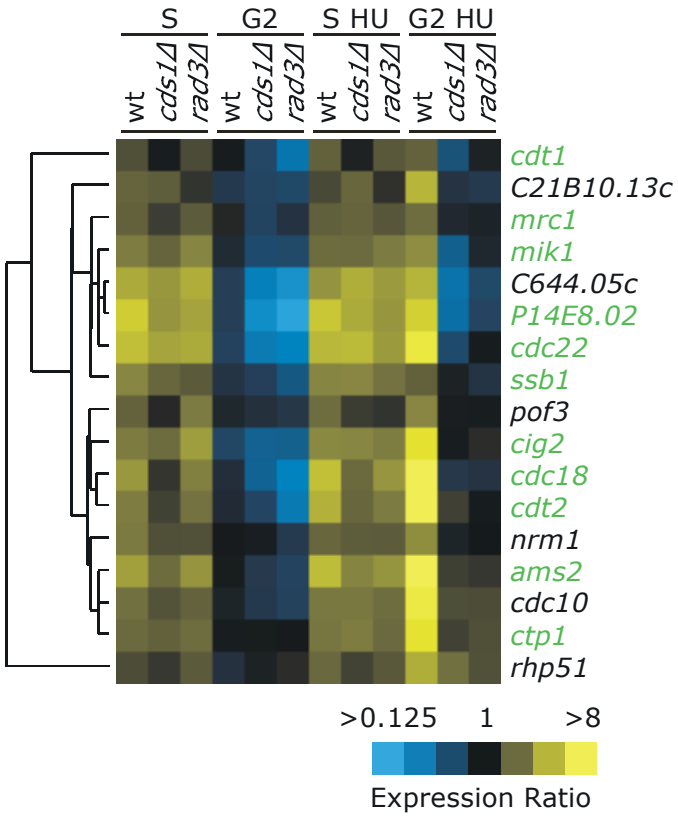

S
G2
S HU
G2 HU
wt
cds1Δ
rad3Δ
cdt1
C21B10.13c
mrc1
mik1
C644.05c
P14E8.02
cdc22
ssb1
pof3
cig2
cdc18
cdt2
nrm1
ams2
cdc10
ctp1
rhp51
>0.125
1
>8
Expression Ratio

Figure 2.1 All MBF-Dependent G1/S Transcripts are Upregulated by The Replication Checkpoint.

B Averaged micro-array data from two experiments for each condition was clustered to identify transcripts that were elevated in G1/S and also elevated in HU in a Rad3-dependent manner. Only these 17 genes meet those criteria. The genes in green were identified as MBF-dependent in two of three previous studies (Oliva et al., 2005; Peng et al., 2005; Rustici et al., 2004). The fold induction in these experiments is lower than in A because the comparison is to asynchronous cells, which have higher levels of MBF-dependent transcripts than the G2 cells used as a baseline in A.

Figure 2.1C

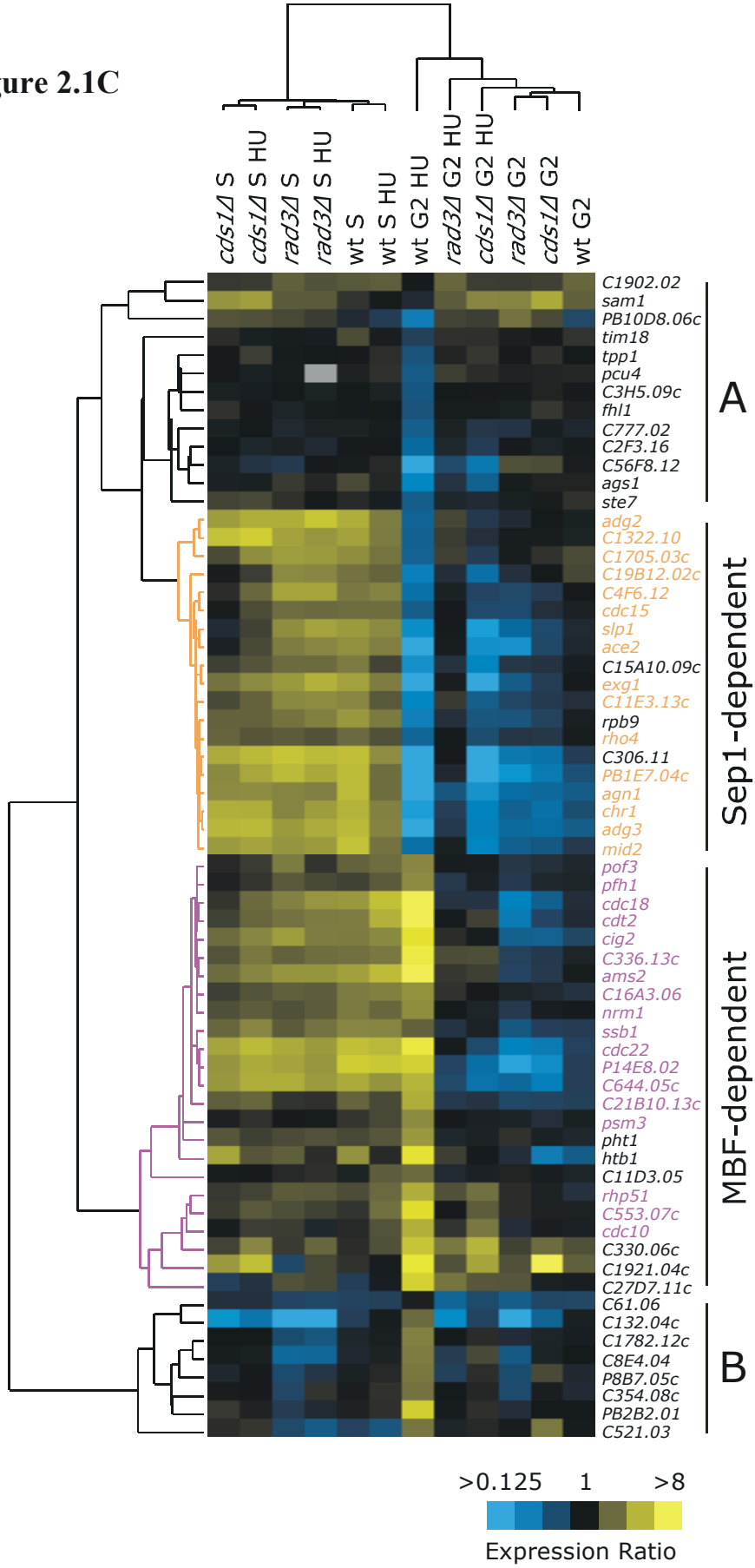

cds1Δ S
cds1Δ S HU
rad3Δ S
rad3Δ S HU
wt S
wt S HU
wt G2 HU
rad3Δ G2 HU
cds1Δ G2 HU
rad3Δ G2
cds1Δ G2
wt G2
C1902.02
sam1
PB10D8.06c
tim18
tpp1
pcu4
C3H5.09c
fhl1
C777.02
C2F3.16
C56F8.12
ags1
ste7
adg2
C1322.10
C1705.03c
C19B12.02c
C4F6.12
cdc15
slp1
ace2
C15A10.09c
exg1
C11E3.13c
rpb9
rho4
C306.11
PB1E7.04c
agn1
chr1
adg3
mid2
pof3
pfh1
cdc18
cdt2
cig2
C336.13c
ams2
C16A3.06
nrm1
ssb1
cdc22
P14E8.02
C644.05c
C21B10.13c
psm3
pht1
htb1
C11D3.05
rhp51
C553.07c
cdc10
C330.06c
C1921.04c
C27D7.11c
C61.06
C132.04c
C1782.12c
C8E4.04
P8B7.05c
C354.08c
PB2B2.01
C521.03
A
Sep1-dependent
MBF-dependent
B
>0.125
1
>8
Expression Ratio

Figure 2.1 All MBF-Dependent G1/S Transcripts are Upregulated by The Replication Checkpoint.

C The same data was filtered to identify transcripts that showed a Rad3-dependent change of at least 2-fold in response to HU. The resulting 64 transcripts were clustered, revealing the four groups described in the text. In this analysis, the experiments were clustered as well. The genes in orange are those previously identified as Sep1-dependent; those in purple have been identified as MBF-dependent (Oliva et al., 2005; Peng et al., 2005; Rustici et al., 2004).

We also wanted to determine if any non-G1/S genes are regulated by the checkpoint in response to HU. We identified 64 genes whose transcript levels showed a Rad3-dependent change (compared to untreated G2) of at least 2-fold in response to HU. Hierarchical clustering identified four groups of genes (Figure 2.1C). One of these groups consists mainly of the MBF-dependent transcripts described above. Another group consists mainly of Sep1-dependent transcripts. These genes, which encode proteins involved in mitosis and cytokinesis, are upregulated during mitosis (Oliva et al., 2005; Peng et al., 2005; Rustici et al., 2004). Presumably, they are indirectly downregulated by the replication checkpoint as a consequence of the G2 arrest, which prevents cells from entering mitosis. This conclusion is bolstered by the fact these genes are also down-regulated in *cds1*Δ cells, which arrest in G2 due to Rad3-dependent activation of Chk1 (another checkpoint effector kinase), but not *rad3*Δ cells, which fail to arrest in G2 and septate even in the presence of HU (Brondello et al., 1999). The last two groups, labeled A and B in Figure 2.1C, comprise genes that are either high or low expression in wild type, HU-treated G2 cells and largely unchanged in all other conditions. We suspect that these signals are spurious for three reasons: 1) the clusters are not enriched in genes of any particular biological functions, 2) these genes show lower signal amplitude and higher variance for the HU-treated sample than genes in the other clusters (Mean SEM 2.3 ± 1.8 fold for clusters A and B versus 4.9 ± 1.6 fold for the other two clusters), suggesting they represent a low level of noise in our analysis, and 3) only 3 of the 21 genes were identified by Chu et al. as HU regulated (Chu et al., 2007). From these

results, we conclude that the replication checkpoint does not directly regulate the levels of any genes other than the MBF-dependent transcripts.

Mutations in MBF Affect Checkpoint-Dependent Transcription

To test our second prediction – that mutations in MBF components affecting G1/S transcription should similarly affect checkpoint-dependent transcription – we examined the checkpoint response in cells lacking Res1 or Res2, redundant DNA-binding subunits of MBF, and in cells carrying *cdc10-C4*, a dominant activating allele of the essential Cdc10 subunit of MBF (McInerny et al., 1995). We find that all three alleles greatly reduce cell cycle regulation of the MBF-dependent genes, reducing the amplitude of *cdc22* regulation from over 15-fold in wild-type cells to about 2-fold in the mutants, with *res2*Δ cells showing constitutively low levels and *res1*Δ and *cdc10-C4* showing constitutively high levels, consistent with previous results (Whitehall et al., 1999; Chu et al., 2007) (Figure 2.2A). As predicted by our hypothesis, *cdc22* is not checkpoint regulated in any of the mutant strains. We see a similar lack of checkpoint regulation across the MBF-dependent transcripts by micro-array analysis (Figure 2.2B). These results show that the checkpoint is unable to regulate transcription in the absence of a functional MBF transcription factor and suggests that MBF activity is regulated by the checkpoint.

Cds1 Phosphorylates MBF

Our final prediction is that Cds1 directly regulates MBF activity, possibly through direct phosphorylation. Our attention was drawn to the 61 C-terminal amino acids deleted

in *cdc10-C4* (McInerny et al., 1995). Since removal of this sequence constitutively activates Cdc10, we imagined that inhibitory Cds1-dependent phosphorylation of the sequence could similarly activate MBF. Furthermore, the sequence contains 7 serines and a threonine, any of which are capable of being phosphorylated by Cds1. To test if this region is a potential Cds1 substrate, we expressed it as a glutathione-S-transferase (GST) fusion in *E. coli* and used it as an *in vitro* kinase substrate for Cds1 immunopurified from HU-arrested fission yeast. We found that HU-activated Cds1 efficiently phosphorylated the wild-type fusion protein, but failed to do so when serine 720 (S720) was mutated to alanine (Figure 2.3A). Approximately 85% of *in vitro* phosphorylation requires S720; the rest requires S732 and/or S736 (Figure 2.3A compare lanes 4,8 and 9). Consistent with these results, S720 and S732 are the only serines in the C-terminus found in R-x-x-S motifs, which are favored by Cds1-related kinases (O'Neill et al., 2002; Seo et al., 2003).

We next investigated the *in vivo* relevance of Cdc10 C-terminal phosphorylation in the checkpoint. We made a series of site-directed mutant constructs to test whether phosphorylation is necessary or sufficient for checkpoint regulation of MBF *in vivo*. These mutations were used to replace the wild-type copy of *cdc10* at its endogenous locus. To test if Cdc10 C-terminal phosphorylation is necessary, we mutated all 7 serines and the threonine to alanine or glycine. We found that this allele, *cdc10-8A*, has no significant defect in checkpoint regulation of transcription (Figure 2.3B). The fact that replication checkpoint control of MBF seems to be intact in *cdc10-8A* is consistent with the hypothesis that phosphorylation of either Cdc10 or the Nrm1 MBF repressor, which binds to and inhibits MBF in G2 (de Bruin et al., 2006), is sufficient for checkpoint regulation of MBF.

Figure 2.2A

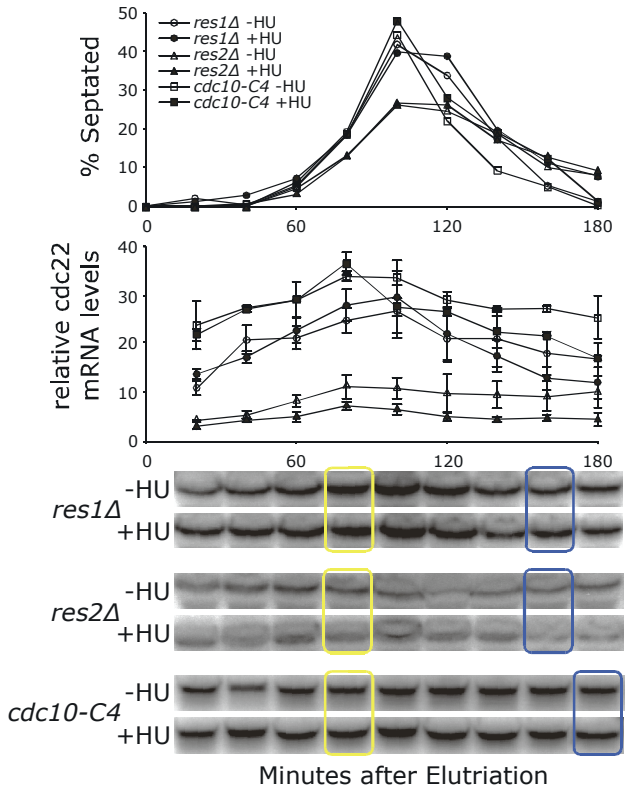


Figure 2.2B

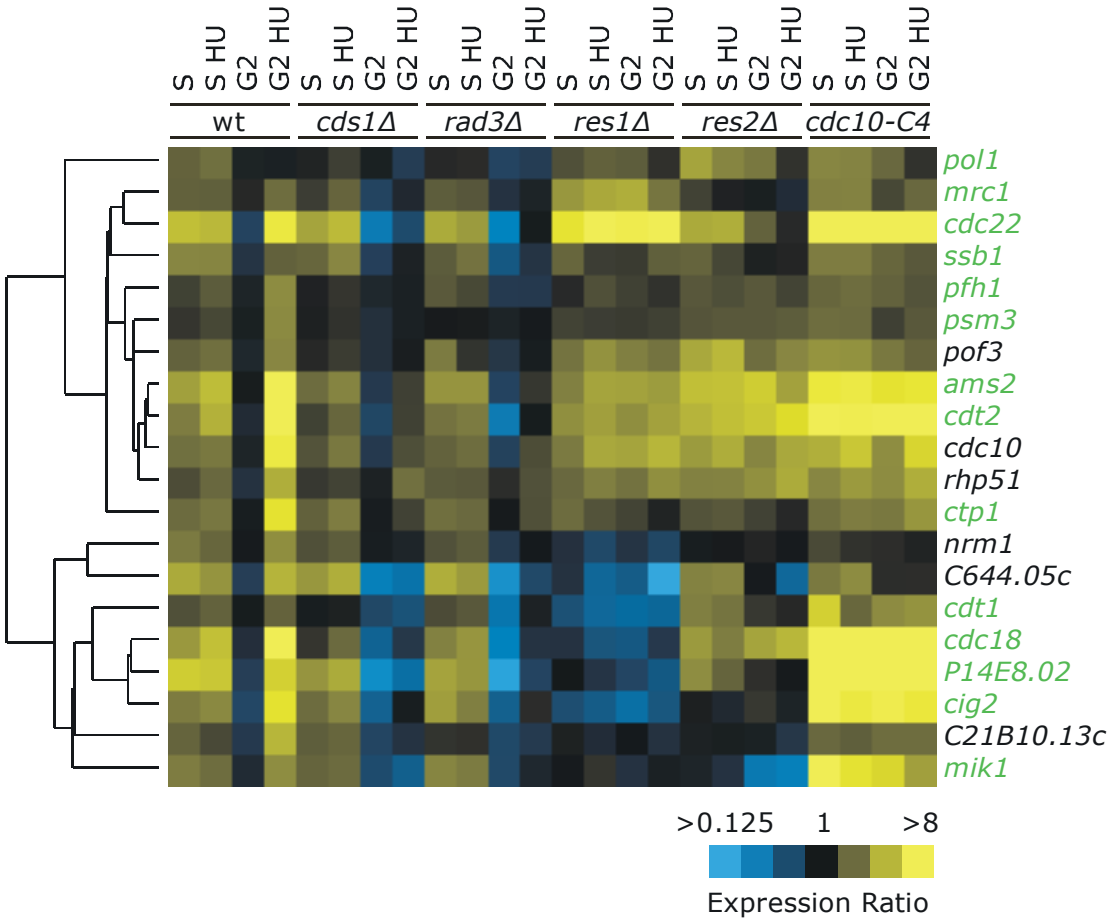


Figure 2.2 Mutations in Subunits of MBF aAect Checkpoint-Dependent Transcription.

A Cell cycle and *cdc22* transcriptional profiles of *res1*Δ (yFS163), *res2*Δ (yFS252) and *cdc10-C4* (yFS257) cultures were analyzed as in Figure 2.1A. The quantitations for *res1*Δ represent the mean and SEM of three experiments; the quantitations for *res2*Δ and *cdc10-C4* represent the mean and variance of two experiments.

B Data for the 14 core MBF-dependent genes, depicted in green, and the 6 other MBF-dependent genes identified in Figure 2.1B were clustered across all the represented experiments.

Phosphomimetic Mutations of Cdc10 are Sufficient to Induce Constitutive MBF Transcription

To test if Cdc10 C-terminal phosphorylation is sufficient for checkpoint regulation of transcription, we made serine to glutamate mutations, which imperfectly mimic phosphorylation. We analyzed a variety of mutant combinations and obtained positive results with S720, the major *in vitro* phosphorylation site, and T723, which is in a T-x-x-D context, a putative Cds1 recognition motif (Durocher et al., 2000). We expect mutations that mimic checkpoint phosphorylation to cause constitutive expression of MBF-dependent transcripts. Indeed, the double S720E T723E mutant, which we call *cdc10-2E*, and *cdc10-8E* both show constitutive *cdc22* and overall MBF-dependent transcript levels comparable to wild-type checkpoint induced levels (Figure 2.3B,C and F). Furthermore, the levels of *cdc22* and overall MBF-dependent transcripts in *cdc10-2E* are not markedly increased by HU treatment. The single S720E mutation shows no significant constitutive increase in MBF-dependent transcript levels; the single T723E mutation shows a partial, approximately 2-fold increase in MBF-dependent transcript levels in the absence of HU (Figure 2.3B,C). From these results we conclude that phosphorylation of both S720 and T723 is most likely sufficient for checkpoint regulation of transcription.

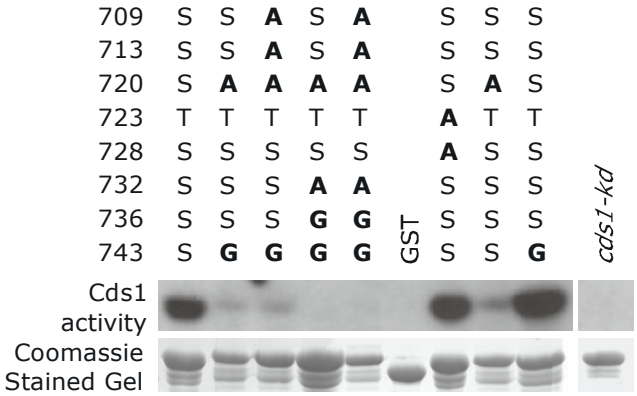
The lack of a checkpoint-transcriptional phenotype for *cdc10-8A* and the constitutive checkpoint-transcriptional phenotype of *cdc10-2E* is consistent with the possibility of redundant phosphorylation of Cdc10 and Nrm1, either of which would suffice to disrupt Nrm1 inhibition of Cdc10 and activate MBF-dependent transcription. This model predicts that Nrm1 should have no function in *cdc10-2E* cells. To test this

prediction, we compared *cdc22* transcript levels in *cdc10-2E*, *nrm1*Δ and *cdc10-2E nrm1*Δ cells in both synchronous and asynchronous cultures. As predicted, *cdc10-2E* and *nrm1*Δ show equivalently high levels of *cdc22* transcripts and the deletion of *nrm1* in a *cdc10-2E* background does not further elevate *cdc22* levels (Figure 3E and data not shown).

cdc10-2E Confers Resistance to HU

The replication checkpoint has three known functions: the role investigated here in maintaining the G1/S-phase transcriptional program, a well understood role in preventing mitosis, and a less well understood role in stabilizing stalled replication forks (Boddy and Russell, 2001). To investigate the importance of checkpoint-mediated transcription relative to the other functions, we built strains that lack all three functions (*rad3*Δ) or just the transcription and fork stabilization functions (*cds1*Δ) and induced constitutive checkpoint signaling in these backgrounds with *cdc10-2E*. We tested these strains for sensitivity to moderate levels of HU and found that elevated levels of MBF-dependent transcripts make cells significantly more resistant to this treatment (Figure 2.4). Specifically, the restoration of checkpoint-induced levels of MBF-dependent transcripts allows *rad3*Δ cells to survive at 1 mM HU and *cds1*Δ cells to survive at 3 mM HU, conditions they cannot survive normally. These results indicate that checkpoint-mediated transcriptional response plays an important adjunct role in the survival of replication stress, in addition to the known role of the checkpoint in the maintenance of replication fork stability and the prevention of premature mitosis (Desany et al., 1998; Enoch and Nurse, 1990).

Figure 2.3A



Potential Phosphorylation Sites on Cdc10

		709	713	720	723	728	732	736	743
		S	S	S	T	S	S	S	S
Mutant	2A			A	A				
	2E			E	E				
	8A	A	A	A	A	A	A	A	A
	8E	E	E	E	E	E	E	E	E

Figure 2.3B

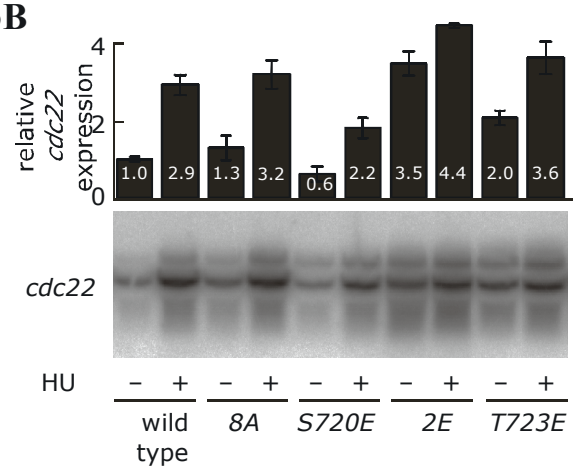


Figure 2.3C

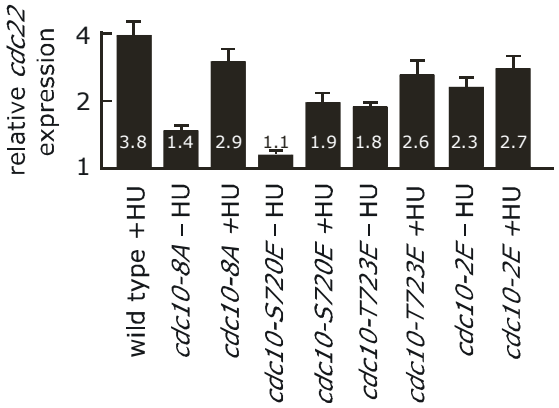


Figure 2.3D

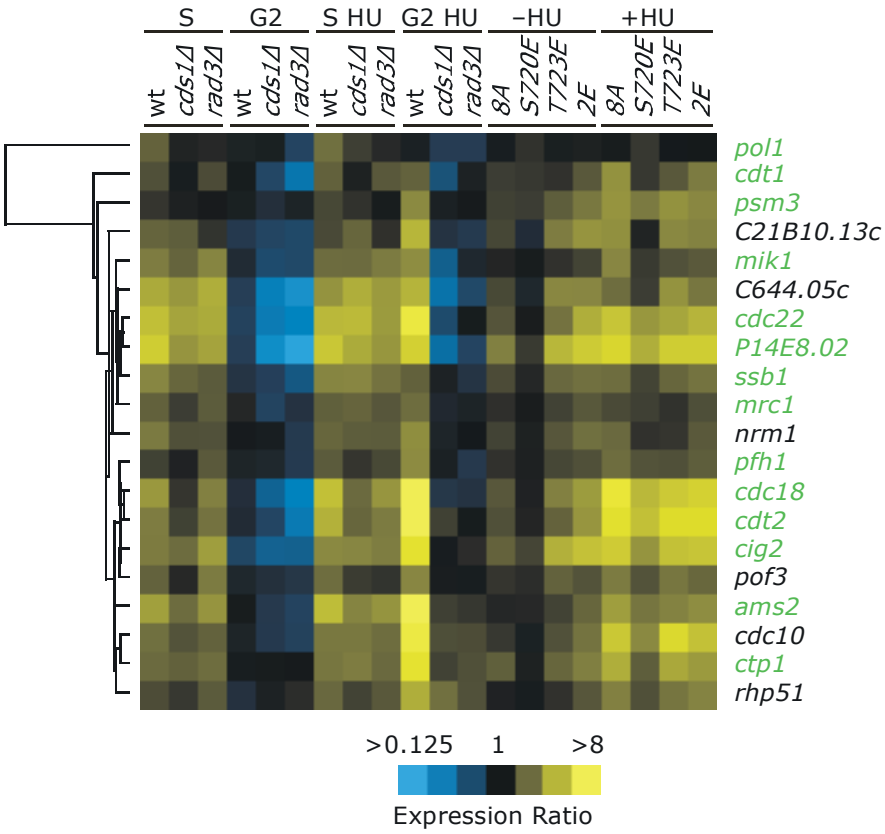


Figure 2.3E

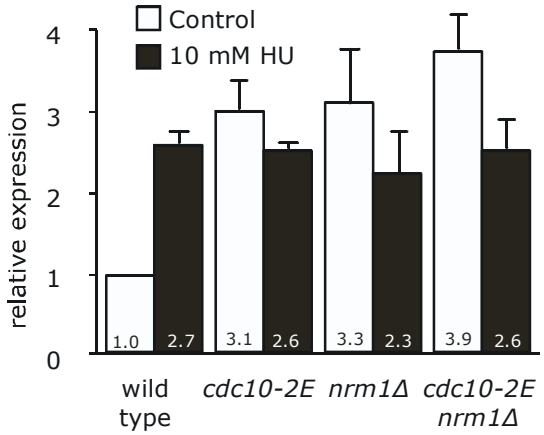


Figure 2.3F

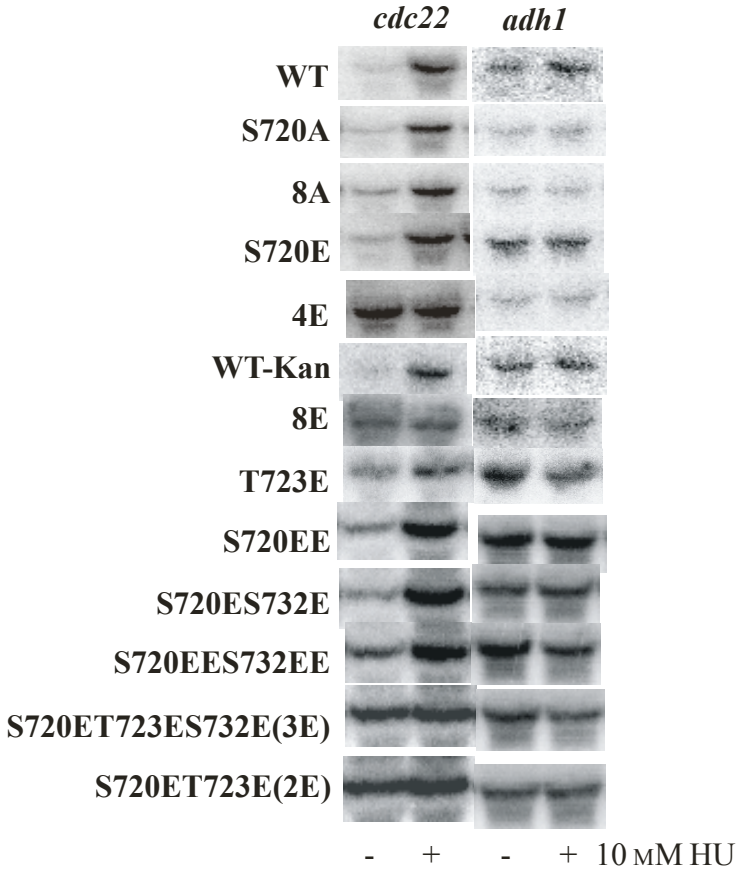


Figure 2.3 Phospho-mimetic Substitutions of Cds1 Phosphorylation Sites in the C-Terminus of Cdc10 Suffice to Promote the Checkpoint Transcriptional Program.

A The 61 C-terminal amino acids of Cdc10 fused to GST, and the indicated S to A and S to G mutations, were used as *in vitro* substrates for Cds1 kinase immunopurified from wild-type (yFS105) or *cds1-D312E* (yFS397, *cds1* kinase dead) cells treated for 4 hours with 10 mM HU.

B Northern analysis of *cdc22* transcript levels in asynchronous wild-type (yFS105), *cdc10-8A* (yFS502), *cdc10-S720E* (yFS500) *cdc10-2E* (yFS528) and *cdc10-T723E* (yFS527) cells untreated or treated with 10 mM HU for 4 hours. The quantitation represents the mean and SEM of between 3 and 6 experiments, normalized to *adh1* and untreated wild type. The amplitude of the induction is lower than in Figure 2.1A because the data is normalized to asynchronous wild-type cells, instead of G2 wild-type cells.

C Quantitation of the array data in (D). Average expression level of the 20 genes relative to asynchronous wild-type cells is shown.

D Data for the 20 genes examined in Figure 2.2B were clustered across the all the represented experiments. The data is the average of two independent experiments.

E Northern analysis of *cdc22* transcript levels in asynchronous wild-type (yFS105), *cdc10-2E* (yFS528), *nrm1*Δ (yFS528) and *cdc10-2E nrm1*Δ (yFS528) cells untreated or treated with 10 mM HU for 4 hours.

F Northern analysis of *cdc22* transcript levels in asynchronous wild-type (yFS105), *cdc10-S720A* (yCD70), *cdc10-8A* (yFS502), *cdc10-S720E* (yFS500), *cdc10-4E* (yCD60), *cdc10-WT-kan* (yCD117), *cdc10-8E* (yCD77), *cdc10-T723E* (yFS527), *cdc10-S720EE* (yCD87), *cdc10-S720ES732E* (yCD88), *cdc10-S720EES732EE* (yCD91), *cdc10-S720ET723ES732E* (yCD93) and *cdc10-2E* (yFS528) cells untreated or treated with 10 mM HU for 4 hours.

Figure 2.4

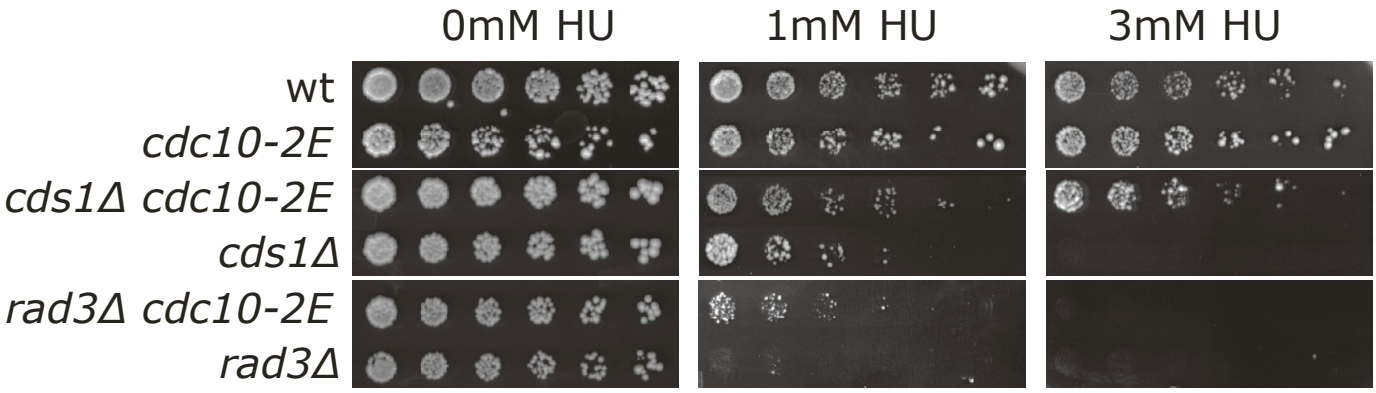

0mM HU
1mM HU
3mM HU
wt
cdc10-2E
cds1Δ cdc10-2E
cds1Δ
rad3Δ cdc10-2E
rad3Δ

Figure 2.4 The Role of Checkpoint-Dependent Transcription in Surviving Chronic Replicative Stress.

Wild-type (yFS526), *cdc10-2E* (yFS528), *cds1*Δ (yFS531), *cds1*Δ *cdc10-2E* (yFS530), *rad3*Δ (yFS532), *rad3*Δ *cdc10-2E* (yFS529) cells were three-fold serially diluted on to YES plates supplemented with 0, 1 or 3 mM HU and photographed after 7 days of growth.

Discussion

Our results demonstrate that the fission yeast replication checkpoint regulates the MBF G1/S transcription factor to maintain the normal G1/S transcriptional program during replication stress. This conclusion is supported by three lines of evidence. First, all MBF transcripts, and only MBF transcripts, are up-regulated by the checkpoint in response to HU arrest (Figure 2.1). Second, mutations in the Res1, Res2 and Cdc10 MBF subunits affect checkpoint-regulated transcription in the same manner they affect normal G1/S transcription (Figure 2.2). Third, phosphomimetic mutations of sites of *in vitro* Cds1 phosphorylation, in the allele we call *cdc10-2E*, cause constitutive G1/S transcription *in vivo* (Figure 2.3). In addition, restoring a sustained high level of G1/S transcription with *cdc10-2E* to *rad3*Δ and *cds1*Δ cells, which normally lack checkpoint-induced transcription, modestly increases their resistance to HU, demonstrating the *in vivo* relevance of the response (Figure 2.4). Chu *et al*. reported that the over expression of the MBF subunit Rep2 partially rescues the HU sensitivity of *cds1*Δ and *rad3*Δ cells, much as *cdc10-2E* does (Chu et al., 2007). We speculate that over expression of Rep2 may phenocopy the constitutive expression seen in *cdc10-2E* and suppress HU sensitivity by the same mechanism.

Our results also shed light on the roles of the Res1 and Res2 DNA binding subunits of MBF. Res1 and Res2 have been proposed to be activating and repressing subunits, respectively (Baum et al., 1997). Our array data shows that the situation is more complicated, with each protein required for the activation and repression of a different subset of genes (Figure 2.2B). For instance, *cdc22* is up regulated in *res1*Δ cells, while *cdc18* is down regulated.

Our results suggest that Cds1 regulates MBF by phosphorylating the C-terminus of Cdc10; however, we have been unable to detect such phosphorylation *in vivo*. To detect checkpoint-dependent *in vivo* phosphorylation of Cdc10, we tried a number of approaches, including western blots with a variety of 1D PAGE systems and conditions, 2D IEF/SDS-PAGE, phospho-epitope affinity purification and mass spectroscopy, all with only negative results. In particular, although we have been able to detect phosphorylation of Cdc10, we have found no evidence for checkpoint-dependent phosphorylation nor C-terminal specific phosphorylation. We believe two factors may have contributed to our inability to detect *in vivo* checkpoint-dependent phosphorylation of Cdc10. The first is that Cdc10 is multiply phosphorylated throughout the cell cycle and therefore the addition of one or two extra checkpoint-dependent phosphates may not greatly affect its overall phosphorylation state nor its mobility in a polyacrylamide gel (Simanis and Nurse, 1989). The second is that sites on Cdc10 phosphorylated by Cds1 during the checkpoint may also be phosphorylated during normal S phase. Use of normal regulatory phosphorylation sites by checkpoint kinases is certainly the case for Cdc25 in the G2 DNA damage checkpoint. The sites phosphorylated on Cdc25 during the G2 checkpoint are the same sites used by other kinases to regulate the G2/M transition in the normal cell cycle (Peng et al., 1998). Therefore, G2 checkpoint activation does not increase the phosphorylation of Cdc25; it merely maintains normal G2 phosphorylation in a checkpoint-dependent manner. If Cdc10 were regulated in a similar manner, we would not expect to see a checkpoint-dependent change in phosphorylation.

Our analysis of *cdc10-2E* suggests that phosphorylation of the Cdc10 C-terminus is sufficient to activate G1/S transcription. However, the fact that replication checkpoint

control of MBF is intact in *cdc10-8A*, which cannot be phosphorylated by Cds1 on its C-terminus, shows that such phosphorylation is not necessary for checkpoint regulation. We hypothesize that phosphorylation of either Cdc10 or the Nrm1 MBF repressor, which binds to and inhibits MBF in G2 (de Bruin et al., 2006), is sufficient for checkpoint regulation of MBF. In the paralogous SBF-Whi5 system, Whi5 repression of SBF is relieved by CDK phosphorylation, but phosphorylation of either Whi5 or SBF is sufficient to disrupt binding (Costanzo et al., 2004; de Bruin et al., 2004). Likewise, Cds1 phosphorylates Nrm1 in response to HU (de Bruin et al., 2006), which may suffice for checkpoint regulation in the *cdc10-8A* cells. This model suggests that, while not necessary for checkpoint regulation, phosphorylation of Cdc10 is sufficient for checkpoint regulation by disrupting the binding and inhibition of MBF by Nrm1. Consistent with this model, *cdc10-2E* and *nrm1*Δ have similar and non-additive transcriptional phenotypes (Figure 2.3E).

Our analysis of the role of MBF in the fission yeast replication checkpoint reveals a very different regulatory logic from that employed by budding yeast. The major transcriptional response to replication stress in budding yeast is a checkpoint-specific, Rfx1-dependent induction of the *RNR* genes, which are induced to ten-fold higher levels during an HU arrest than during a normal S phase (Huang et al., 1998). This apparently budding yeast specific transcriptional response is in marked contrast to the fission yeast strategy of simply maintaining normal G1/S transcription, and may explain why budding yeast can tolerate ten-fold higher levels of HU than fission yeast. However, genomic analysis of the budding yeast replication stress response and recent work on budding yeast Nrm1 suggest that MBF is a conserved checkpoint target in yeast (Gasch et al.,

2001; de Bruin et al., 2006; de Bruin et al., 2008). Therefore, the checkpoint regulation of MBF may be conserved in budding yeast, but largely obscured by the more dramatic Rfx1-dependent response.

The fission yeast regulatory mechanism places some genes under checkpoint control that have no obvious checkpoint function, such as the origin licensing genes *cdc18* and *cdt1*. However, it also assures the continued expression of the three genes known to be important of the survival of replication stress: *cdc22* – encoding the large subunit of ribonucleotide reductase, *mik1* – encoding a mitosis-inhibiting kinase (Christensen et al., 2000; Rhind and Russell, 2001) and *mrc1* – encoding the Cds1 mediator (Tanaka and Russell, 2001). In addition, several other genes that have plausible roles in replication stress are also regulated, including *ssb1* – encoding the large subunit of the RPA single-strand DNA binding protein, *rph51* – encoding the Rad51 recombinase, *ctp1* – encoding a subunit of the MRN recombinational repair complex (Limbo et al., 2007); and *pfh1* – encoding a putative repair helicase.

It has long been recognized that the G1/S transcription factors – MBF in fission yeast, MBF and SBF in budding yeast and E2F in metazoa – share common domain structures, but the similarity of their cell cycle regulation has only recently been appreciated (Cooper, 2006; Costanzo et al., 2004; de Bruin et al., 2006; de Bruin et al., 2004). In particular, MBF appears to be regulated by Nrm1 much in the same way E2F is regulated by Rb. Because HU treatment of primary human cells causes extensive phosphorylation of Rb and because Chk2, the human homolog of Cds1, can phosphorylate and activate E2F, we propose that checkpoint regulation of transcription

through Nrm1/Rb-MBF/E2F is likely be conserved across eukaryotes (DeCaprio et al., 1989; Stevens et al., 2003).

Appendix 1

Phosphomimetic mutations of Cdc10 by glutamic acid (E) and glutamine (Q) both are sufficient to induce constitutive MBF transcription

An alternative approach to phosphorylation site mapping is glutamic acid substitution mutagenesis. The rationale for this analysis is based on the assumption that glutamic acid imperfectly mimics the structure of a phosphorylated serine or threonine residue (Figure 2.5). It is an imperfect substitution of a phosphorylation event because a serine/threonine to glutamic acid substitution adds a single negative charge whereas phosphorylation adds two negative charges. In contrast to alanine substitution, glutamic acid substitution has the potential to directly assess the effects of modification at particular sites without the need for additional phosphorylation *in vitro* or in cells.

In order to determine if Cdc10 C-terminal phosphorylation is sufficient for checkpoint regulation of transcription, we made serine to glutamate mutations. We analyzed a variety of mutant combinations (Figure2.3F). We found that substitution of S720 and T723 (*cdc10-2E*) with glutamic acid were sufficient for transcriptional deregulation. *cdc10-2E* shows constitutive *cdc22* and overall MBF-dependent transcript levels comparable to wild-type checkpoint induced levels. Furthermore, the levels of *cdc22* and overall MBF-dependent transcripts in *cdc10-2E* are not markedly increased by HU treatment.

We expect mutations that mimic checkpoint phosphorylation to cause constitutive expression of MBF-dependent transcripts. Indeed, in order to have controls for glutamic acid substitution, we also replaced serine and threonine residues with glutamine or Q. Its side chain is an amide formed by replacing the side-chain hydroxyl of glutamic acid with

an amine functional group. It can therefore be considered the amide of the acidic amino acid glutamic acid. Results from substitution with glutamine will provide the evidence that the negative charge introduced by kinase is the necessary moiety that leads to change in biological function (Table 2.2).

We observed *cdc10-2Q*, shows constitutive *cdc22* expression by northern blot analysis similar to *cdc10-2E*. In addition, the level of *cdc22* is not increased by HU treatment (Figure 2.6). This observation leads us to hypothesize that the mechanism by which kinase regulates biological function is by changing the conformation of a protein. The change in conformation does not necessarily coming from addition of the negative charge; any bulky shaped amino acid, which is large enough to provide the limitation for a partner protein to bind, could serve the same purpose. We assume that adding charge is a common evolutionary trend in protein function regulation, as adding charge by an active enzyme adds specificity along with addition of bulky shape.

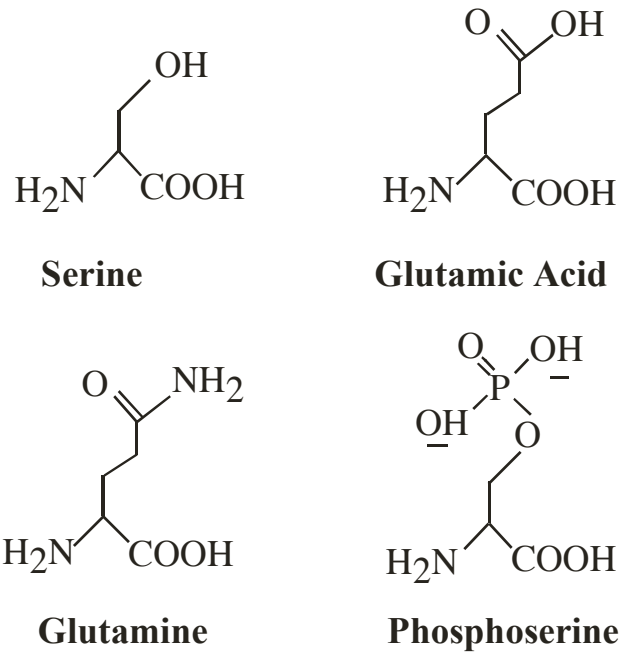


Figure 2.5 Structure of Serine, Glutamic acid, Glutamine and Phosphoserine.

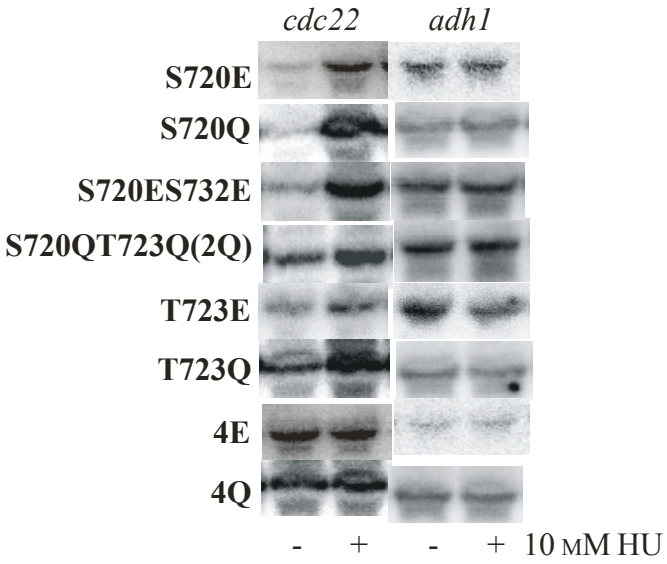


Figure 2.6 Northern blot.

Figure 2.6 Northern Blot Analysis of *cdc22* Transcript Levels in Asynchronous Cells.

cdc10-S720E (yFS500), *cdc10-S720Q* (yCD89), *cdc10-S720ES732E* (yCD88), *cdc10-S720QT723Q* (yCD95), *cdc10-T723E* (yFS527), *cdc10-T723Q* (yCD92), *cdc10-4E* (yCD60) and *cdc10-4Q* (yCD90) cells untreated or treated with 10 mM HU for 4 hours. Northern blots were probed with *cdc22*, stripped and reprobed with *adh1* as a loading control.

Table 2.2 Strains Used in Appendix 1

	Genotypes	
yCD89	*h+ leu1-32 ura4-D18 cdc10-S720Q:kanMX6*	This study
yCD92	*h+ leu1-32 ura4-D18 cdc10-T723Q:kanMX6*	This study
yCD95	*h+ leu1-32 ura4-D18 cdc10-S720QS723Q:kanMX6*	This study
yCD60	*h+ leu1-32 ura4-D18 cdc10-4E*	This study
yFS500	*h+ leu1-32 ura4-D18 cdc10-S720E:kanMX6*	This study
yFS527	*h+ leu1-32 ura4-D18 cdc10-T723E:kanMX6*	This study
yCD90	*h+ leu1-32 ura4-D18 cdc10-4Q:kanMX6*	This study
yCD88	*h+ leu1-32 ura4-D18 cdc10-S720ES732E:kanMX6*	This study

Appendix 2

In vivo phosphorylation of Cdc10 in response to DNA damage

Previously we determined that Cdc10 is a target of Cds1 *in vitro*. We were also able to identify two potential phosphorylation sites in Cdc10 by phosphomimetic substitution of serine and threonine with glutamic acids. The phosphomimetic substitution of S720E and T723E was sufficient to deregulate MBF dependent S-phase transcription.

In order to determine if Cdc10 is phosphorylated *in vivo*, we examined at the Cdc10 protein levels by western blot using an anti-myc antibody in response to HU treatment. We were unable to see a significantly slow migrating band in HU treated cells. We tried different salt concentrations, and different reducing agents in the lysis buffer. We also tried different gel conditions. However we were unable to improve the band shift (Figure 2.7A, B and C).

In order to test if phosphorylated Cdc10 is easier to detect by MMS treatment, we treated the cells with 0.03% MMS and visualized Cdc10-myc by western blot. We were able to observe a slower migrating band, comparatively more prominent in MMS treated cells than HU treated cells (Figure 2.7D). We hypothesized that MMS induced damage might be modifying Cdc10, leading to the appearance of a slower migrating band. This hypothesis was based on the observation that slower migrating phosphorylated Rad26 is only detectable in bleomycin treated cells not with HU treated cells (Wolkow and Enoch, 2002).

To test if Cds1 kinase plays a role in modifying Cdc10, we made myc tagged *cdc10 cds1*Δ. We repeated the experiment with or without HU or MMS and blotted with antibody against the myc tag. The slow migrating band was still present in *cdc10cds1*Δ cells suggesting that slow migrating band was not due to modification by Cds1 (Figure 2.7E).

To determine if the slower migrating band was due to phosphorylation of Cdc10, we treated the cell lysate with varying concentrations of alkaline protein phosphatase. The phosphatase treatment did not remove the slow migrating band, suggesting that the modification is not due to phosphorylation (Figure 2.7F). In addition, we tested the ability of Cdc10 to bind 14-3-3 proteins. 14-3-3 proteins function at several key points in the G1/S and G2/M transitions by binding to regulatory proteins and modulating their function. In most cases, the association with 14-3-3 proteins requires a specific phosphorylation of the protein ligand and mediates cell cycle arrest. 14-3-3 binding may lead to cytoplasmic sequestration of the protein ligand (Zhang et al., 1997; Henriksson et al., 2002). In order to determine if Cdc10 protein is able to bind 14-3-3 protein, we tested the ability of bacterially expressed GST tagged 14-3-3 protein (Rad24) to interact with myc tagged Cdc10 protein from yeast lysate. We expressed GST alone as a negative control. We purified GST and GST-Rad24 protein with GSH beads. Washed beads were incubated for an hour with Cdc10-myc cell lysate made from untreated cells or cells treated with 10mM HU for 4 hours or treated with 0.03% MMS for 4 hours. Washed beads were boiled in SDS sample buffer and run on 10% SDS-PAGE, transferred and developed with antibodies against myc-tag. We observed very weak interaction between Cdc10 and Rad24 in cells treated with HU and MMS. However, these results need to be

confirmed by repeating the experiment or adjusting the protocol and adding a positive control (Figure 2.7G).

Finally we tried Phostag, a reagent recently shown to facilitate phosphorylation specific band shift. Phostag is a dinuclear metal complex (1,3-bis [bis(pyridine-2-ylmethyl)amino]propan-2-olato dizinc (II) complex that acts as a novel phosphate-binding tag molecule (Kinoshita-Kikuta et al., 2007). The Phos-tag molecule has a vacancy on two metal ions that is suitable for accessing a phosphomonoester dianion (R-OPO_3^{2-}) as a bridging ligand. A Mn(II) homologue (Mn^{2+}-Phos-tag) can capture R-OPO_3^{2-}anions, such as phosphoserine and phosphotyrosine, at alkaline pH (9). We utilized this molecule to detect the Cdc10 in vivo phosphorylation. SDS-PAGE was conducted with Phos-tag added in the separation gel (10μM to 30μM). A western blot was performed to detect myc tagged Cdc10 protein. We observed minor shift of Cdc10 proteins. However the reproducibility was very poor and we were unable to see checkpoint dependent shift with Phos-tag molecule (Figure 2.7 H and I).

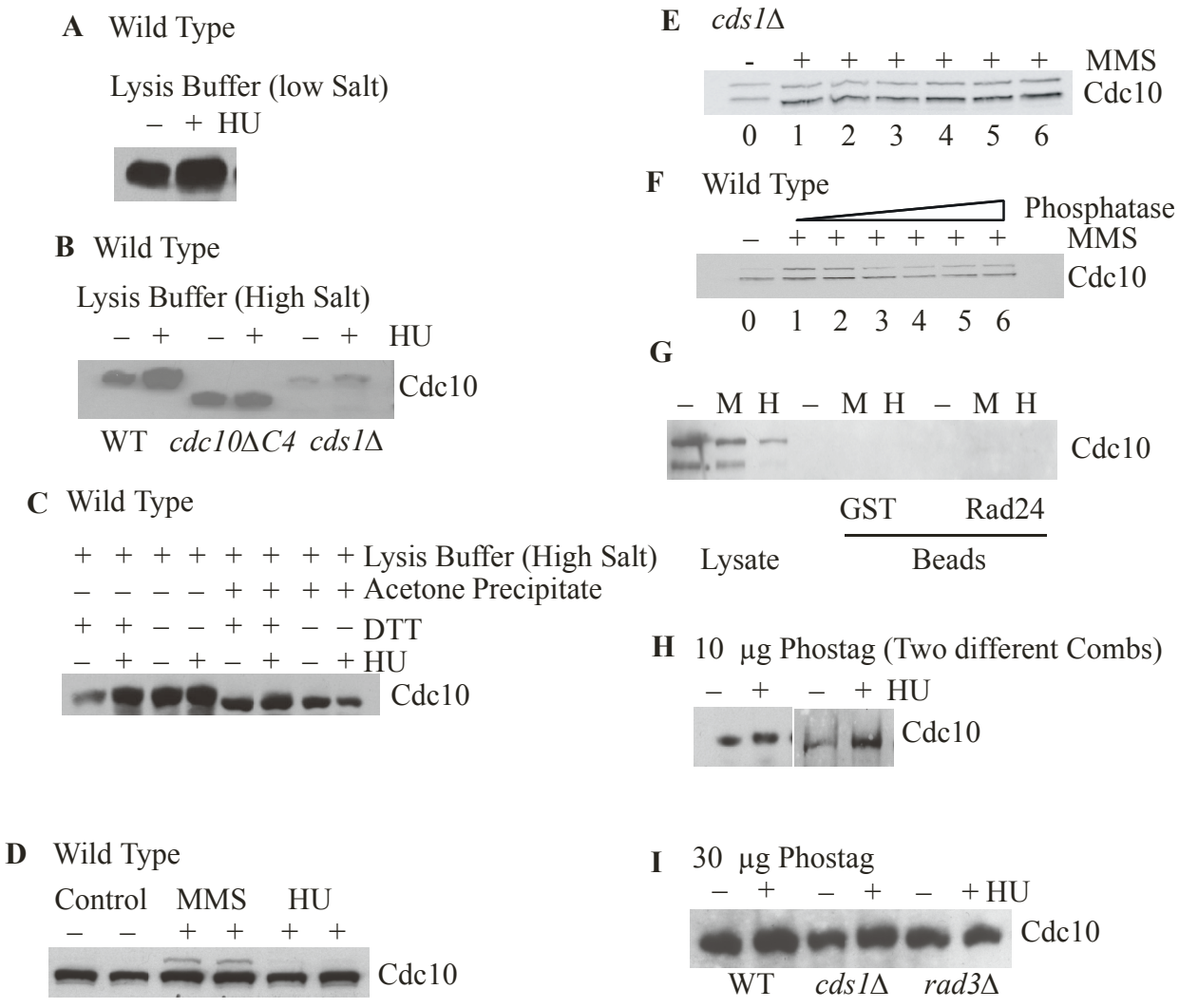


Figure 2.7 In vivo phosphorylation of Cdc10.

Figure 2.7 *In vivo* Phosphorylation of Cdc10.

A Asynchronous wild type cells (yPP3) 20 OD cells treated with 10 mM HU for 4 hours. Cells were lysed with 200 μL of buffer. Boiled with 2X SDS sample buffer. Protein concentration was determined by BCA method and equivalent concentration of protein was loaded in a 6% acrylamide gel. Directly conjugated HRP-myc antibodies was used to detect Cdc10 protein level. Lysis buffer: 150 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA pH 8.0, 10% glycerol, 1% NP-40, 50 mM NaF, 5 μg/ml PLA mix (pepstatin, leupeptin, Aprotinin), 1 mM PMSF and 1 mM Na_2VO_3.

B Asynchronous wild type cells (yPP3), *cdc10ΔC4* (yCD3) and *cds1::ura4* (yCD18) 20 OD cells treated with 10 mM HU for 4 hours. Cells were lysed with 200 μL of buffer. Boiled with 2X SDS sample buffer. Protein concentration was determined by BCA method and equivalent concentration of protein was loaded in a 6% acrylamide gel. Directly conjugated HRP-myc antibodies was used to detect Cdc10 protein level. Lysis buffer: 150 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA pH 8.0, 10% glycerol, 1% NP-40, 50 mM NaF, 5 μg/ml PLA mix (pepstatin, leupeptin, Aprotinin), 1 mM PMSF and 1 mM Na_2VO_3.

C Asynchronous wild type cells (yPP3) 20 OD cells treated with 10 mM HU for 4 hours. Cells were lysed with 200 μL of buffer. Incubated 90 minutes with TBP (Tributylphosphine), and iodoacetamide. Half of each sample was boiled with 2X SDS sample buffer (loaded 10 μL). Another set (100μL) was precipitated with acetone at room temperature for 1 hour. The pelleted precipitate dissolved in 100μL 2X SDS sample buffer (loaded 5 μL). Lysis buffer: Protein extraction reagent type 4 (Sigma C0356)

containing 7 M urea, 2 M thiourea, 40mM trizma base and 1% C7BzO pH 10.4 (Sigma C 0356), 5 mM Tributylphosphine (Sigma T 75670). Directly conjugated HRP-myc antibody was used to detect Cdc10 protein level.

D Asynchronous wild type cells (yPP3) 20 OD cells treated with 10 mM HU or 0.03% MMS for 4 hours. Cells were lysed with 200 μL of buffer. Protein concentration was determined by BCA method, boiled with 2X SDS sample buffer and equivalent concentration of protein was loaded in a 6% acrylamide gel. Directly conjugated HRP-myc antibodies was used to detect Cdc10 protein level. Lysis buffer: 150 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA pH 8.0, 10% glycerol, 1% NP-40, 50 mM NaF, 5 μg/ml PLA mix (pepstatin, leupeptin, Aprotinin), 1 mM PMSF and 1 mM Na_2VO_3.

E Asynchronous cds1Δ cells (yPP3) 5 OD cells treated with 0.03% MMS for 6 hours. Cells were lysed with 200 μL of buffer. Protein concentration was determined by BCA method, boiled with 2X SDS sample buffer and equivalent concentration of protein was loaded in a 6% acrylamide gel. Directly conjugated HRP-myc antibodies was used to detect Cdc10 protein level. Lysis buffer: 150 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA pH 8.0, 10% glycerol, 1% NP-40, 50 mM NaF, 5 μg/ml PLA mix (pepstatin, leupeptin, Aprotinin), 1 mM PMSF and 1 mM Na_2VO_3.

F Asynchronous wild type cells (yPP3) 5 OD cells treated with 0.03% MMS for 4 hours. Cells were lysed with 200 μL of buffer. Phosphatase reaction contains 5 μL of lysate, phosphate buffer, 10mM $MnCl_2$, 120 U to 600 U of phosphatase and incubated at 30^0C for 30 minutes. Protein concentration was determined by BCA method. Boiled with 2X SDS sample buffer and equivalent concentration of protein was loaded in a 6%

acrylamide gel. Directly conjugated HRP-myc antibodies was used to detect Cdc10 protein level. Lysis buffer: 150 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA pH 8.0, 10% glycerol, 1% NP-40, 50 mM NaF, 5 μg/ml PLA mix (pepstatin, leupeptin, Aprotinin), 1 mM PMSF and 1 mM Na_2VO_3.

G Asynchronous wild type cells (yPP3) 20 OD cells treated with 10 mM HU (H) or 0.03% MMS (M) for 4 hours. Cells were lysed with 200 μL of buffer. Bacterially expressed GST and GST tagged Rad24 were purified with GSH beads. Washed beads were incubated with untreated or treated (HU or MMS) cell lysate for 1 hour. Washed beads were boiled with 2X SDS sample buffer and loaded, and 5 μL of lysate was run as control. Directly conjugated HRP-myc antibodies was used to detect Cdc10 protein level.

H Asynchronous wild type cells (yPP3) 20 OD cells treated with 10 mM HU for 4 hours. Cells were lysed with 200 μL of buffer and boiled with 2X SDS sample buffer. Protein concentration was determined by BCA method and equivalent concentration of protein was loaded in a 7.5% acrylamide gel with 10 μM Phos tag reagent and 20 μM $MnCl_2$ in seperation gel. $MnCl_2$ was removed from gel by washing in buffer 1 (25mM Tris, 192mM glycine, 10% Methanol and 1 mM EDTA) and in buffer 2 (25mM Tris, 192 mM glycine and 10% methanol) before transfer. Directly conjugated HRP-myc antibodies was used to detect Cdc10 protein level.

I Similar as section 2.7 H with 30 μM Phos tag reagent used in separation gel.

Table 2.3 Strains Used in Appendix 2

	Genotypes	
yPP3	*h- leu1-32 ura4-D18 cdc10-13myc:kanMX6*	This study
yCD3	*h- leu1-32 ura4-D18 ade-6-210cdc10ΔC4-13myc:kanMX6*	This study
yCD18	*h- leu1-32 ura4-D18 cdc10-13myc:kanMX6cds1::ura4*	This study
yCD100	*h- leu1-32 ura4-D18 cdc10-13myc:kanMX6rad3Δ::ura4*	This study

Chapter III

The Role of Checkpoint Induced S- phase Transcription in Cell Survival

Abstract

Checkpoint activation during S phase modulates MBF (MCB binding factor) dependent gene transcription. Our results show that both the replication checkpoint and the S-phase DNA damage checkpoint activate MBF regulated S-phase gene transcription and that this coordinated transcriptional response is beneficial for survival during replication stress. Previously we have shown that there are about 20 MBF regulated genes affected during the checkpoint activation. Some of the genes have obvious beneficial functions such as *mik1, mrc1* and *cdc22*. However, there are also some MBF regulated genes with potentially contradictory functions for survival, such as *cdc18* and *cdt1*. In this chapter, I focused on testing the role of three MBF transcripts with predicted beneficial role for survival during stress.

I demonstrate that the beneficial role of the transcriptional response during checkpoint activation involves at least three major MBF transcripts: *cdc22, mrc1* and *mik1*. Mrc1 dependent stabilization of stalled forks is crucial for survival during S phase arrest. In addition, the ability of cells to prevent mitosis in a Mik1-dependent manner contributes significantly to cell survival during S-phase DNA damage. Our data also suggest that the level of Cdc22 is a determining factor for replication checkpoint activation and that when over-expressed *cdc22* can alleviate the effects not only of hydroxyurea (HU) but also of methyl methanesulfonate (MMS).

Introduction

In response to inhibition of DNA replication, the replication checkpoint arrests the cell cycle before mitosis, stabilizes stalled replication forks and regulates S-phase transcription (Carr, 1997; Boddy and Russell, 2001). The transcriptional branch of the checkpoint response up-regulates genes thought to be important for cells to survive prolonged replication arrest and is conserved among all eukaryotes.

A commonly used trigger of the replication checkpoint is hydroxyurea (HU). Hydroxyurea is a competitive inhibitor of ribonucleotide reductase, which interferes with deoxyribonucleotide synthesis, and as a result, forks stall and activate the replication checkpoint, also known as the S-M checkpoint. Another common trigger for S-phase checkpoint activation is methyl methanesulfonate (MMS), which methylates DNA at adenine and guanine residues generating bulky adducts that are difficult for polymerase to read through and causing activation of the S-phase DNA damage checkpoint. The HU induced replication checkpoint stabilizes stalled replication forks. The MMS induced S-phase DNA damage checkpoint slows replication. It is still unclear whether the mechanisms and targets of these checkpoints are the same. Ionizing radiation induced double stranded breaks do not slow replication and cells finish replication with damaged DNA and activate the Chk1 mediated G2 DNA damage checkpoint.

In all eukaryotes, checkpoints are activated by sensor kinases: ATM/ATR in metazoans, Rad3 in fission yeast and Mec1 in budding yeast. The effector kinases directly downstream of these sensor kinases are Chk1/Chk2 in metazoans, Cds1/Chk1 in fission yeast and Rad53 in budding yeast (Boddy and Russell, 2001).

In the budding yeast *Saccharomyces cerevisiae*, the effector kinase Rad53 responds to replication arrest as well as MMS induced DNA damage (Huang et al., 1998; Zhu and Xiao, 2001). Downstream of Rad53 is the Dun1 kinase, which is also activated in response to DNA damage and is required for the transcriptional response (Zhou and Elledge, 1993). The replication checkpoint induces transcription primarily through the alleviation of Rfx1/Crt1 transcriptional repression (Huang et al., 1998). Rfx1-dependent induction of the *RNR* gene induces up to ten-fold higher levels of deoxyribonucleotides during an HU arrest than during a normal S phase. The Rfx1-dependent transcriptional program is not evolutionary conserved in other eukaryotes and may explain why budding yeast can tolerate ten-fold higher levels of HU than fission yeast (Zhou and Elledge, 1992).

Unlike budding yeast, fission yeast does not have a checkpoint specific transcriptional response. Instead, the replication checkpoint up-regulates all genes normally expressed during S phase, many with no obvious checkpoint function. In chapter II we have shown that the checkpoint dependent S-phase kinase Cds1 directly targets MBF, the transcription factor complex responsible for S-phase gene transcription. I specifically identified two phosphorylation sites on Cdc10 (a subunit of the MBF complex), which cause constitutive transcription of MBF dependent genes when replaced with phosphomimetic glutamate. Therefore, instead of inducing transcription of only *cdc22*, the gene product needed to make more ribonucleotide reductase in response to HU, fission yeast activates all MBF dependent genes by targeting Cdc10 (Dutta et al., 2008). The regulation of S phase gene transcription by the replication checkpoint is

conserved among metazoans where the replication dependent checkpoint regulates E2F1 to regulate the transcription of S phase genes (Cooper, 2006).

There are about 20 MBF regulated genes that are upregulated in response to activation of the checkpoint. Among these genes are three whose functions are quite obvious and are predicted to be required for checkpoint-induced survival. *cdc22*, encoding the large subunit of ribonucleotide reductase, catalyzes the reduction of ribonucleotides to deoxyribonucleotides, which is the first and rate limiting step in the pathway for the production of deoxyribonucleotides needed for DNA synthesis (Gordon and Fantes, 1986). *mik1* encodes a mitosis inhibiting kinase, that phosphorylates Cdc2 in parallel with the Wee1 kinase and keeps Cdc2 inactive (Rhind and Russell, 2001). *mrc1* encodes the mediator of replication checkpoint kinase Cds1 and is required for Cds1 activation(Alcasabas et al., 2001).

In addition, the following genes that have plausible roles in replication stress are also regulated: *cdc18* is involved in activation and maintenance of Cds1 kinase activity (Murakami et al., 2002), *ssb1* encodes the large subunit of RPA single strand DNA binding protein, *rhp51* encodes the Rad51 recombinase for homologous recombination (Parker et al., 1997), *pof3* has a role in substrate recognition in the Skp1-Cullin-1/Cdc53-F Box (SCF) ubiquitin ligase complex and is required for the maintenance of telomere length, transcriptional silencing at the telomere and for chromosome segregation (Katayama et al., 2002), and, *ams2* is required for proper chromosome segregation via regulation of CENP-A localization to the centromere (Chen et al., 2003a; Chen et al., 2003b)

I hypothesize that increasing the transcription of the genes that are directly or indirectly linked to different aspects of recovery from replication stress or DNA damage is beneficial for cell survival. To address this hypothesis, I used various mutant strains compromised for checkpoint induced transcription with different kinds of DNA damaging agents in mediating cell survival. In this chapter, first I show that checkpoint activation by S-phase DNA damage as well as replication checkpoints during S phase modulates the transcription of MBF dependent S-phase genes. In contrast, the S-phase transcriptional program is not affected during activation of the G2 DNA damage checkpoint. Second, our results indicate that the checkpoint dependent transcriptional response is beneficial for cell survival during acute exposure to HU, MMS and IR and chronic exposure to HU. Third, the requirement for checkpoint functions is dependent on the stages of cell cycle and kind of damages. Finally our data suggest that the beneficial role of transcription during replication stress is mediated at least in part by *cdc22, mik1* and *mrc1*.

Materials and Methods

Table 3.1: Strains were Used in Chapter III

The following strains used in Chapter III		
yFS625	*h- leu1-32 ura4-D18 cdc10:kanMX6*	This study
yFS626	*h+ leu1-32 ura4-D18 cdc10-2E*	This study
yFS627	*h- leu1-32 ura4-D18 cdc10:kanMX6 cds1::ura4*	This study
yFS628	*h- leu1-32 ura4-D18 cdc10:kanMX6 rad3::ura4*	This study
yFS629	*h- leu1-32 ura4-D18 cds1::ura4 cdc10-2E*	This study
yFS630	*h+ leu1-32 ura4-D18 rad3::ura4 cdc10-2E:kanMX6*	This study
yFS632	*h+ leu1-32 ura4-D18 mik1::ura4, nmt:pyp3(kan)*	This study
yFS620	*h+ leu1-32 P3nmt:GFP-cdc22(kan)*	Hiroshi M
yFS643	*h+ ade6-704mik1::leu rad3::ura4cdc10-2E:kanMX6*	This study
yFS644	*h+ mik1::leu cds1::ura4cdc10-2E:kanMX6*	This study
yFS645	*h+ mrc1:kanMX6 rad3::ura4 cdc10-2E:kanMX6*	This study
yFS646	*h+ mrc1: ura4 cds1::ura4cdc10-2E:kanMX6*	This study
yFS647	*h+ leu1-32P3nmt:GFP:cdc22(kan) rad3::ura4*	This study
yFS648	*h+ leu1-32P3nmt-GFP:cdc22(kan) cds1::ura4*	This study
yFS135	*h- leu1-32 ura4-D18 mik1::LEU2*	Rhind lab
yFS624	*h- leu1-32 ura4-D18 mrc1:kanMX6*	Russell Lab
yFS198	*h- leu1-32 ura4-D18 chk1::ura4*	Rhind lab

Cell culture

Cells were grown in YES (yeast extract supplement) medium at 30˚C (Forsburg and Rhind, 2006), with the exception of yFS632, yFS647, yFS648 and yFS620 that were grown in LUAH (Edinburgh minimal media with leucine, uracil, adenine and histidine) supplemented with thiamine as needed. The expression of *nmt1* (no message in thiamine, commonly used thiamine repressible promoter) driven genes was induced by growing cells in LUAH without thiamine for 16-18 hours at 30˚C. We used centrifugal elutriation to synchronize cells (Beckman coulter Ananti J-20XP). HU (10 mM) was added to the medium immediately after elutriation. Chronic HU sensitivity was assayed by spotting 3-fold serial dilutions of cells onto YES plates supplemented with 0, 1 or 3 mM HU and photographing growth after 7 days. For acute sensitivity assays, cells were incubated with

either 10 mM HU or 0.03% methyl methanesulfonate (MMS) and plated on YES/LUAH. For ionizing radiation (IR), a Faxitron Cabinet X-ray system Model RX-650 was used. Colonies were counted after 7 days. Experiments were performed at least three times and all quantitations are given as mean ± SEM except as noted.

RNA analysis

RNA was prepared for northern blots, probed with random-prime labeled (Stratagene) *cdc22*, stripped and reprobed with *adh1* (Oliva et al., 2005). *cdc22* levels were normalized to adh1 and then all time courses were normalized to asynchronous wild-type controls included on all gels. The 20 minute time point for the wild type time course was set to 1. Experiments were performed at least three times and all quantitations are given as mean ± SEM except as noted.

Flow cytometry methods

Asynchronous cells were grown to an OD 1.0, collected before adding 10 mM HU (time 0), then collected 4 hours after HU treatment. Cells were washed once, resuspended in fresh media, and collected every 20 minutes thereafter. Collected samples were fixed in 70% ethanol for 24 hours before processing for FACs. Isolated nuclei were prepared for flow cytometry (Kommajosyula and Rhind, 2006) and analyzed on a FACScan flow cytometer (Becton-Dickinson). The average S phase progression was calculated by measuring the mean of the S-phase peak as a percentage of the position between the means of the 1C and 2C contents.

Results

cdc22 Transcription is Maintained during Activation of the S-Phase DNA Damage Checkpoint

Blocking replication by HU upregulates S-phase gene transcription. In order to determine if other S-phase checkpoint triggers induce a transcriptional response, we followed the expression of *cdc22* using northern blot analysis in synchronous populations of wild type cells in response to MMS. Cells were synchronized in G2 by elutriation and treated with 10 mM HU or 0.015% MMS. MMS was added 60 minutes after elutriation as adding MMS at time 0 delays cells in G2 (our unpublished observation). As shown in Figure 3.1A and 3.1B, both HU and MMS treated cells maintained *cdc22* mRNA level when arrested in S phase. However, neither checkpoint induced S-phase transcription in G2 prior to entry into S phase.

Ionizing Radiation Induced G2 DNA Damage Checkpoint does not Activate MBF Dependent Transcription

In order to determine if the checkpoint induced transcription is also upregulated during activation of G2 DNA damage checkpoint we synchronized cells in G2 by centrifugal elutriation. We followed septation index as well as *cdc22* transcript level by northern blot every 20 minutes after elutriation (Figure 3.1). Cells treated with 200 Gy of ionizing radiation septated 40 minutes later than untreated cells (120 minutes and 160 minutes in control and treated sample, respectively). We observed a similar S-phase peak of *cdc22* level 40 minutes later in-irradiated cells than untreated cells (100 minutes and 140 minutes control and treated, respectively). However, we failed to observe any

measurable peak of *cdc22* transcripts during G2. Therefore, our data suggest that MBF dependent transcription is activated by the checkpoint only during S phase and not during G2.

Requirement of the Checkpoint Functions is Different in S phase and in G2

Activation of the replication checkpoint leads to three major outcomes. The checkpoint is known to a) regulate fork stability (FS), b) prevent mitosis (M) and c) regulate S phase gene transcription (T). To test the functions required for survival during activation of different kinds of checkpoints (replication, S-phase DNA damage and G2 DNA damage), we analyze survival of varieties of mutants deficient in checkpoint dependent functions. A list of mutants made is shown in Table 3.2. The MBF transcription in all newly built strains was determined by *cdc22* RNA level by northern blot in response to HU and MMS (Figure 3.2).

The cells deficient in all three-checkpoint functions such as *rad3*Δ (FS-M-T-) are sensitive to HU, MMS and IR (Figure 3.3A). However in these cells the sensitivity relative to wild type is greater in MMS than HU and IR. Relative sensitivity is defined by comparing the sensitivity of mutant with sensitivity of wild type strain at same time in case of HU and MMS and at same dose in case of IR. For example, if sensitivity of wild type cells = 0.8 and sensitivity of *rad3*Δ = 0.2, the relative sensitivity of *rad3*Δ will be 0.2/0.8=0.25. The relative sensitivity of *rad3*Δ cells is about 0.006 in MMS as compared to about 0.1 in HU and IR (Figure 3.3E). These data might suggest that the G2 DNA damage checkpoint dependent repair is crucial when cells finish replication with mismatched nucleotides.

Table 3.2 Three Functions of the Checkpoint

	Transcription	G2 Checkpoint	Fork Stability
Wild Type	+	+	+
nmt-pyp3mik1Δ	+	-	+
cds1Δcdc10-2E	+	+	-
cds1Δ	-	+	-
rad3Δcdc10-2E	+	-	-
rad3Δ	-	-	-

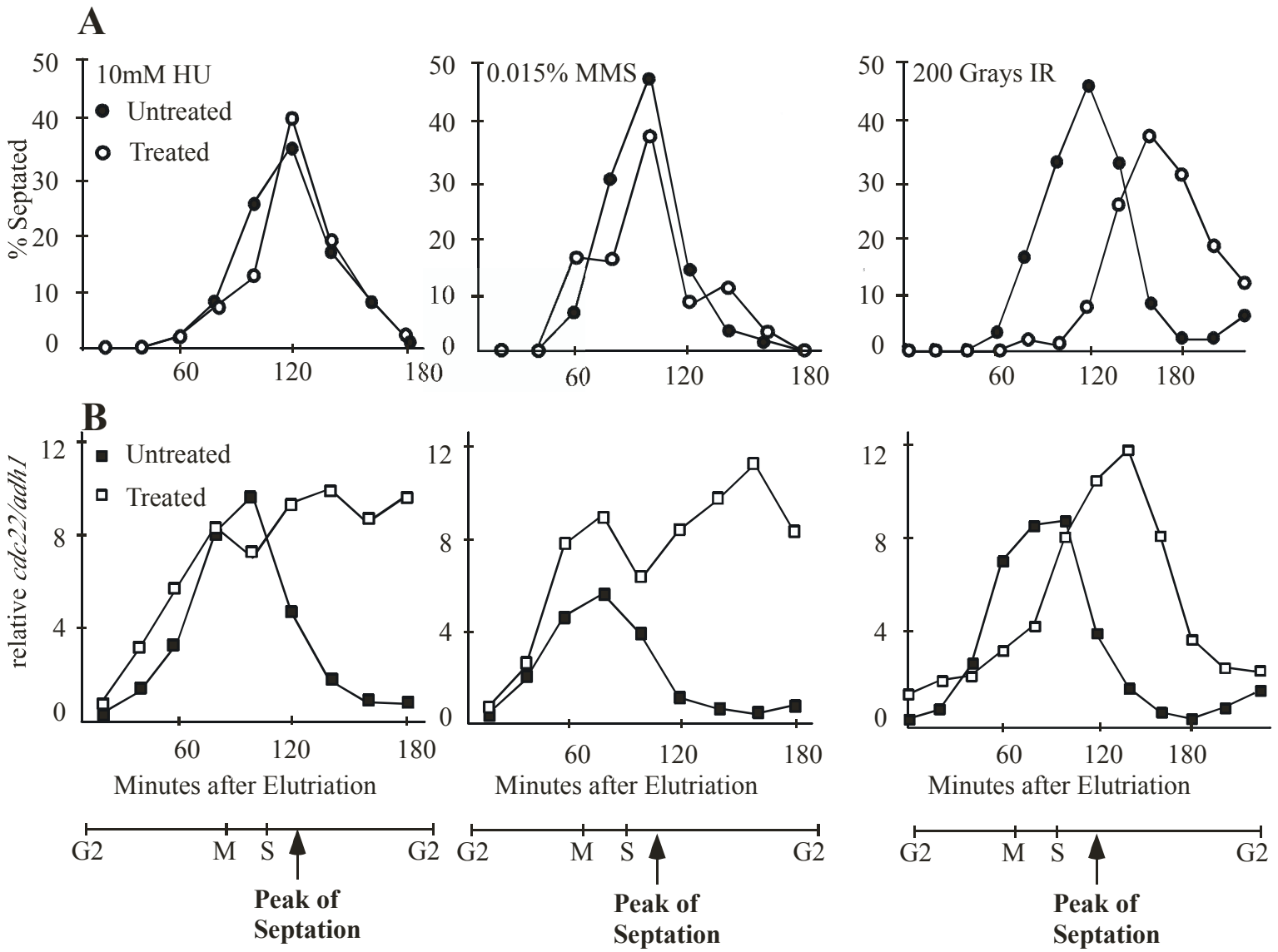

A
10mM HU
Untreated
Treated
0.015% MMS
200 Grays IR
% Septated
B
Untreated
Treated
relative cdc22/adh1
Minutes after Elutriation
Minutes after Elutriation
Minutes after Elutriation
G2
M
S
G2
Peak of Septation
Peak of Septation
Peak of Septation

Figure 3.1

Figure 3.1. *cdc22* mRNA is Upregulated only during Activation of the Replication Checkpoint and S-phase DNA Damage Checkpoint.

Wild type (yFS625) cells were synchronized in early G2 by centrifugal elutriation and followed through a synchronous cell cycle in the presence or absence of 10 mM HU added right after elutriation or in the presence of 0.015% MMS added 60 minutes after elutriation. I used 200 grays of ionizing radiation to activate the G2 DNA damage checkpoint at time 0. Samples were taken every 20 minutes for RNA isolation and visual inspection of septation. Northern blots were probed with *cdc22*, stripped and reprobed with *adh1* as a loading control.

A) Septation index of cells treated with HU, MMS or IR.

B) *cdc22* mRNA levels by northern blot in cells treated with 10 mM HU, 0.015% MMS or 200 Grays of ionizing radiation.

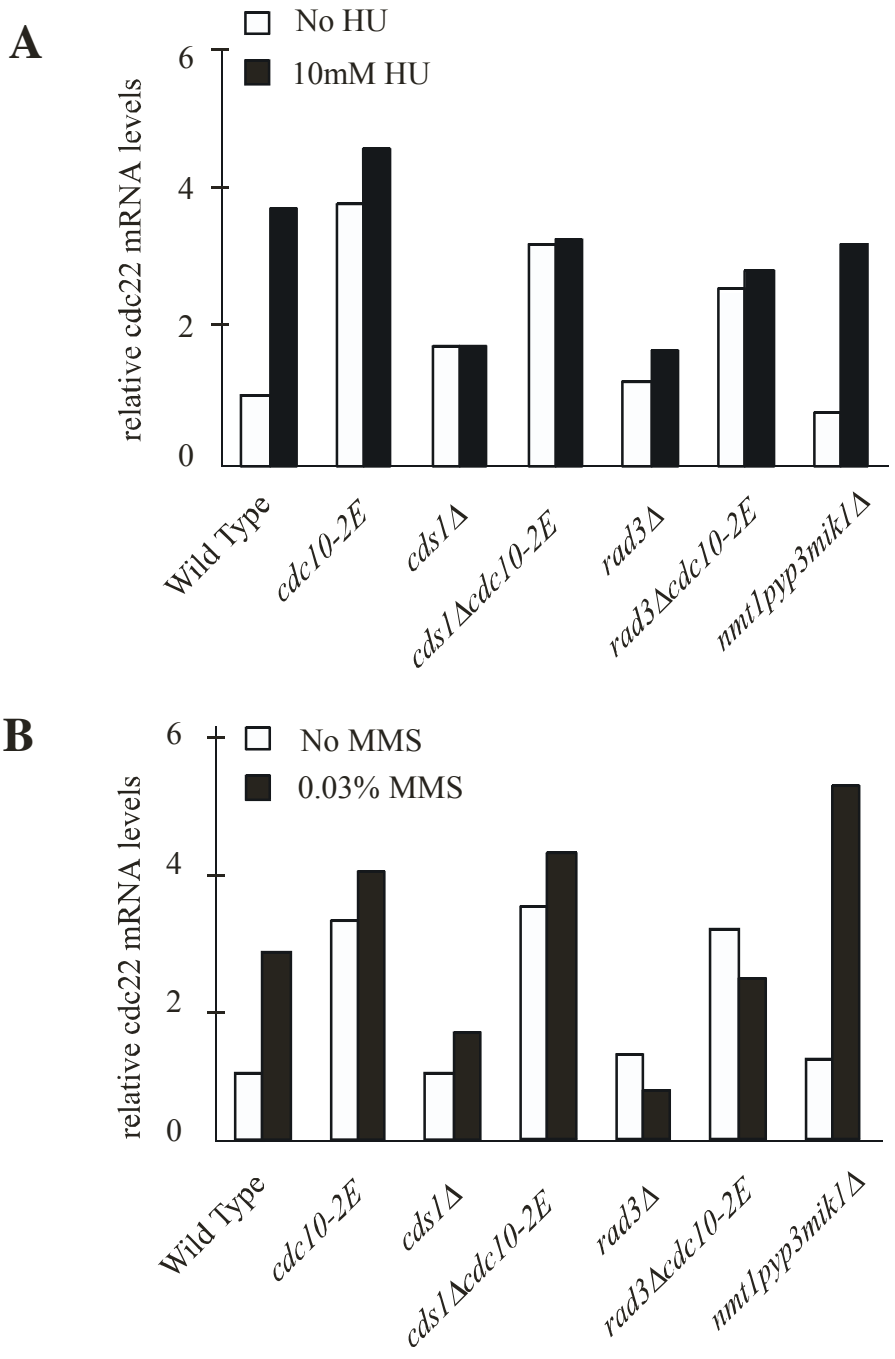

A
No HU
10mM HU
relative cdc22 mRNA levels
6
4
2
0
Wild Type
cdc10-2E
cds1Δ
cds1Δcdc10-2E
rad3Δ
rad3Δcdc10-2E
nmt1pyp3mik1Δ
B
No MMS
0.03% MMS
relative cdc22 mRNA levels
6
4
2
0
Wild Type
cdc10-2E
cds1Δ
cds1Δcdc10-2E
rad3Δ
rad3Δcdc10-2E
nmt1pyp3mik1Δ

Figure 3.2

Figure 3.2. *cdc22* mRNA Transcription is Upregulated in the Checkpoint Dependent Manner.

Northern blot analysis of *cdc22* transcript levels in asynchronous wild-type (yFS625), *cdc10-2E* (yFS626) *rad3*Δ (yFS628), *rad3*Δ *cdc10-2E* (yFS630), *cds1*Δ (yFS627), *cds1*Δ *cdc10-2E* (yFS629) and *nmt1: pyp3 mik1*Δ (yFS632) cells untreated or treated with 10 mM HU (A) or 0.03% MMS (B) for 4 hours. The quantitation represents the *cdc22* level normalized to *adh1* and untreated wild type.

The cells unable to prevent mitosis such as *chkl*Δ (effector kinase for G2 DNA damage checkpoint) are sensitive to IR but not to HU and MMS, suggesting ionizing induced double stranded breaks are repaired by activating G2 DNA damage checkpoint. (Figure 3.3A).

The cells unable to stabilize forks or induce transcriptional regulation such as *cds1*Δ (FS-M+T-) are very sensitive to replication stress (in HU) (Figure 3.3A). However, these *cds1*Δ (FS-M+T-) cells survive better if they are able to finish replication (e.g. in MMS), suggesting activation of G2 DNA damage and repair of damaged DNA is a possibility at this stage (Figure 3.3A) (Sommariva et al., 2005).

Mrc1 has two functions: it plays an important role in transmitting checkpoint signaling during S phase and it has an important checkpoint-independent role during replication (Alcasabas et al., 2001). Mrc1 may negatively regulate Cdc45 and MCM helicase to render stalled forks capable of resuming replication (Nitani et al., 2006). We observed that *mrc1*Δ (FS-M+T+) is very sensitive in HU, MMS and in IR. Sensitivity of *mrc1*Δ cells in HU and MMS was expected, as Mrc1 is critical for stabilizing replication forks. However, we observed that in MMS relative sensitivity of *mrc1*Δ cells is significantly greater than *cds1*Δ (0.17 and 0.6 in *mrc1*Δ and *cds1*Δ respectively). This observation supports the idea that Mrc1 plays checkpoint independent role in response to DNA damage (Figure 3.3E).

Stabilization of Stalled Forks is Critical for Survival during Activation of the Replication Checkpoint

To investigate the importance of checkpoint-mediated transcription relative to the other two functions, we built strains that lack one or more functions but maintain the other functions of checkpoint activation (Table 3.2). First, we compared *cdc10-2E* (FS+ M+ T+), *rad3*Δ (FS- M- T-), and *rad3*Δ *cdc10-2E* (FS- M- T+), cells. In HU challenge, *rad3*Δ cells are extremely sensitive and adding constitutive transcription in *rad3*Δ *cdc10-2E* (FS- M- T+), made measurable effect on survival (Figure 3.3B). Second, we compared *cdc10-2E* (FS+ M+ T+), *cds1*Δ (FS- M+ T-), and *cds1*Δ *cdc10-2E* (FS- M+ T+) double mutants. We found that *cds1*Δ cells are very sensitive to HU and that adding constitutive transcription in *cds1*Δ *cdc10-2E* cells made a measurable change in survival. However, *cds1*Δ *cdc10-2E* (FS- M+ T+) cells are still very sensitive to HU compared to *cdc10-2E* cells (FS+ M+ T+). These data show that stabilization of stalled forks is the most important function needed to survive during replication stress (Figure 3.3B).

Prevention of Mitosis is Crucial When Cells Finish Replication with Damaged DNA

We also tested the requirement of checkpoint functions during the activation of the S-phase DNA damage checkpoint by measuring cell survival in MMS. *rad3*Δ (FS- M- T-), cells are extremely sensitive in MMS, however *cds1*Δ (FS- M+ T-) are only moderately sensitive to MMS (Figure 3.3C). MMS causes reduction in fork progression rate but does not stop replication forks like HU treatment. Therefore MMS treated cells finish replication. If cells are able to activate the G2 DNA damage checkpoint (*cds1*Δ FS- M+ T-) the chance of survival is greater compared with cells, which cannot prevent mitosis (*rad3*Δ FS- M- T-).

Unlike HU, MMS induced damage doesn't stop replication completely, therefore cells finish replication with a slower fork speed and are able to activate the G2 DNA damage checkpoint. Activation of the G2 DNA damage checkpoint leads to better relative survival of *rad3*Δ *cdc10-2E* (FS- M- T+) double mutant in MMS than in HU. This difference can be seen in relative sensitivity of *rad3*Δ *cdc10-2E* and *rad3*Δ mutants (Figure 3.3F). In HU, *rad3*Δ *cdc10-2E* cells survived only 1.5-fold compare to *rad3*Δ cells (relative sensitivity 0.15/0.1) . However, in MMS, *rad3*Δ *cdc10-2E* cells survived 16 times better than *rad3*Δ cells (relative sensitivity 0.11/0.006) (Figure 3.3F). These data suggest that, transcriptional regulation might have an indirect role in preventing mitosis contributing better survival of cells when treated with MMS (Figure 3.3F).

Checkpoint Dependent Prolonged S Phase Transcription is Beneficial for Cell Survival during Chronic Stress

I tested the same strains for sensitivity to moderate levels of chronic HU and found that elevated levels of MBF-dependent transcripts make cells significantly more resistant to this treatment. Specifically, the restoration of checkpoint-induced levels of MBF-dependent transcripts in *rad3*Δ *cdc10-2E* and *cds1*Δ *cdc10-2E* cells allow these cells to survive at 1mM HU better than *rad3*Δ and *cds1*Δ cells alone (Figure 3.4B).

To determine the relative contribution of preventing mitosis in cell survival compared to other two function of checkpoint, we built a strain where G2 DNA damage checkpoint is compromised. The strain with compromised G2 DNA damage checkpoint was made by removing Mik1 kinase as well as by over expressing pyp3 phosphatase (*nmt1: pyp3mik1*Δ*)*. Mik1 is mitosis inhibiting kinase, which phosphorylates Cdc2 in

parallel with Wee1 kinase. Pyp3 is a protein phosphatase substitutes for cdc25 when over-expressed and dephosphorylates Cdc2 (Millar et al., 1992)(Rhind et al 1998). Mik1 and Cdc25 both are the G2 DNA damage checkpoint targets. Therefore *nmt1: pyp3mik1*Δ cells do not arrest in G2 upon activation of checkpoints.

I observed that *cds1*Δ cells are more vulnerable to HU than *nmt1:pyp3 mik1*Δ cells indicating that stabilizing stalled forks is more crucial than preventing mitosis during S phase arrest (Figure 3.3A and 3.4A). These results indicate that checkpoint-mediated transcriptional response plays an important adjunct role in the survival of replicative stress, in addition to the known role of the checkpoint in the maintenance of replication fork stability and the prevention of premature mitosis (Desany et al., 1998; Enoch et al., 1992) (Figure 3.4B).

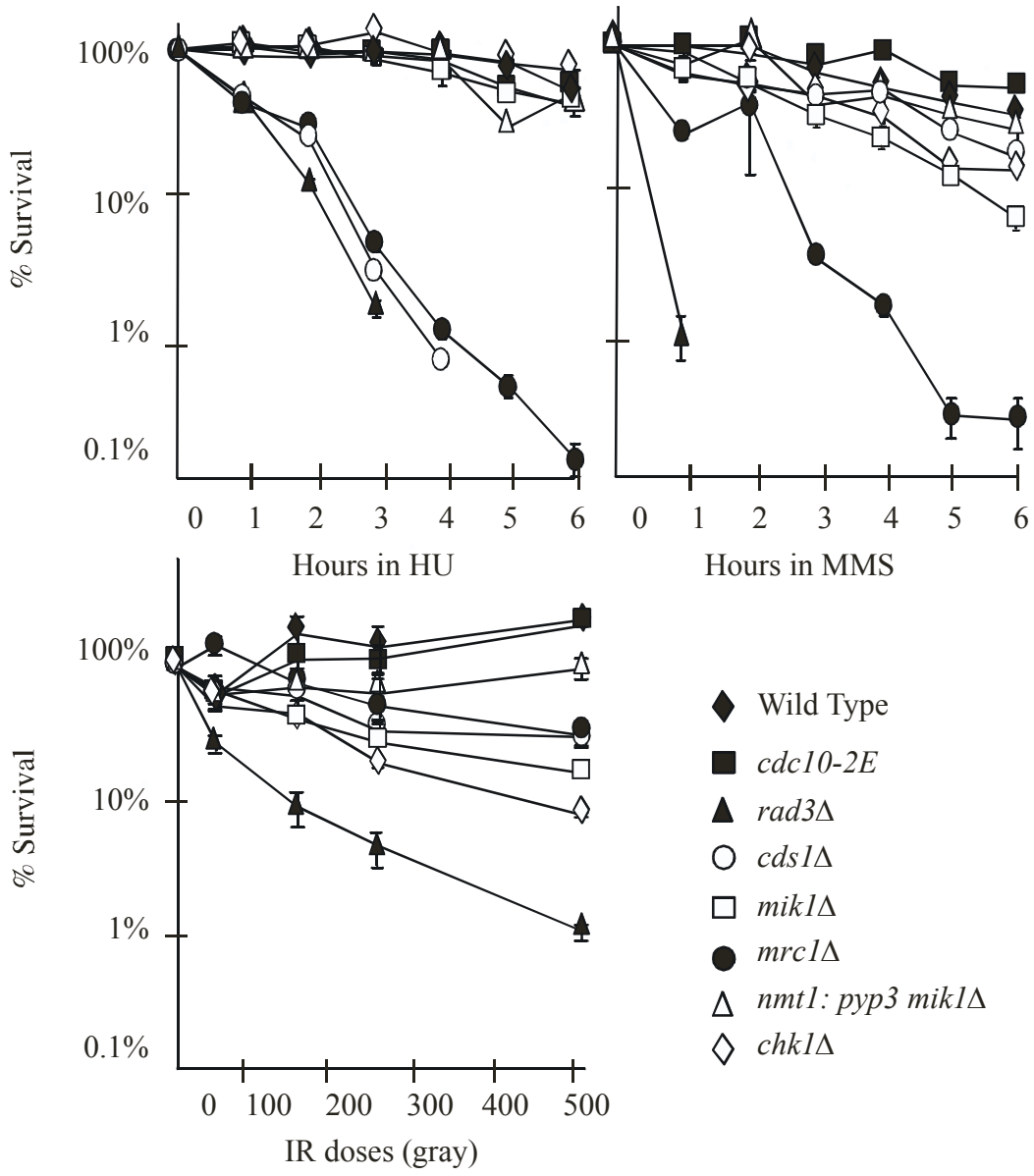

100%
10%
1%
0.1%
% Survival
0 1 2 3 4 5 6
Hours in HU
0 1 2 3 4 5 6
Hours in MMS
100%
10%
1%
0.1%
% Survival
0 100 200 300 400 500
IR doses (gray)
Wild Type
cdc10-2E
rad3Δ
cds1Δ
mik1Δ
mrc1Δ
nmt1: pyp3 mik1Δ
chk1Δ

Figure 3.3A

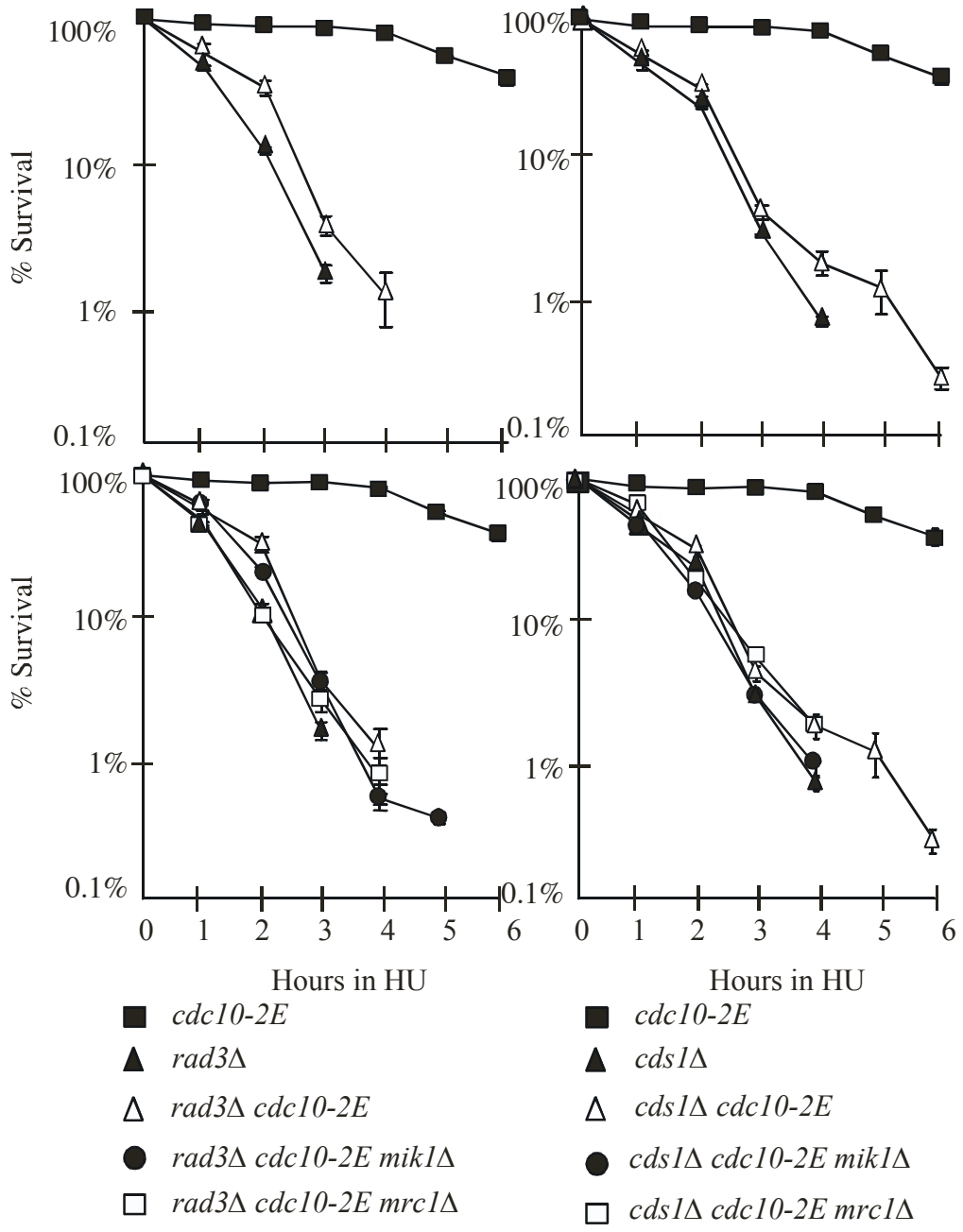

100%
10%
1%
0.1%
% Survival
0 1 2 3 4 5 6
Hours in HU
cdc10-2E
rad3Δ
rad3Δ cdc10-2E
rad3Δ cdc10-2E mik1Δ
rad3Δ cdc10-2E mrc1Δ
cds1Δ
cds1Δ cdc10-2E
cds1Δ cdc10-2E mik1Δ
cds1Δ cdc10-2E mrc1Δ

Figure 3.3B

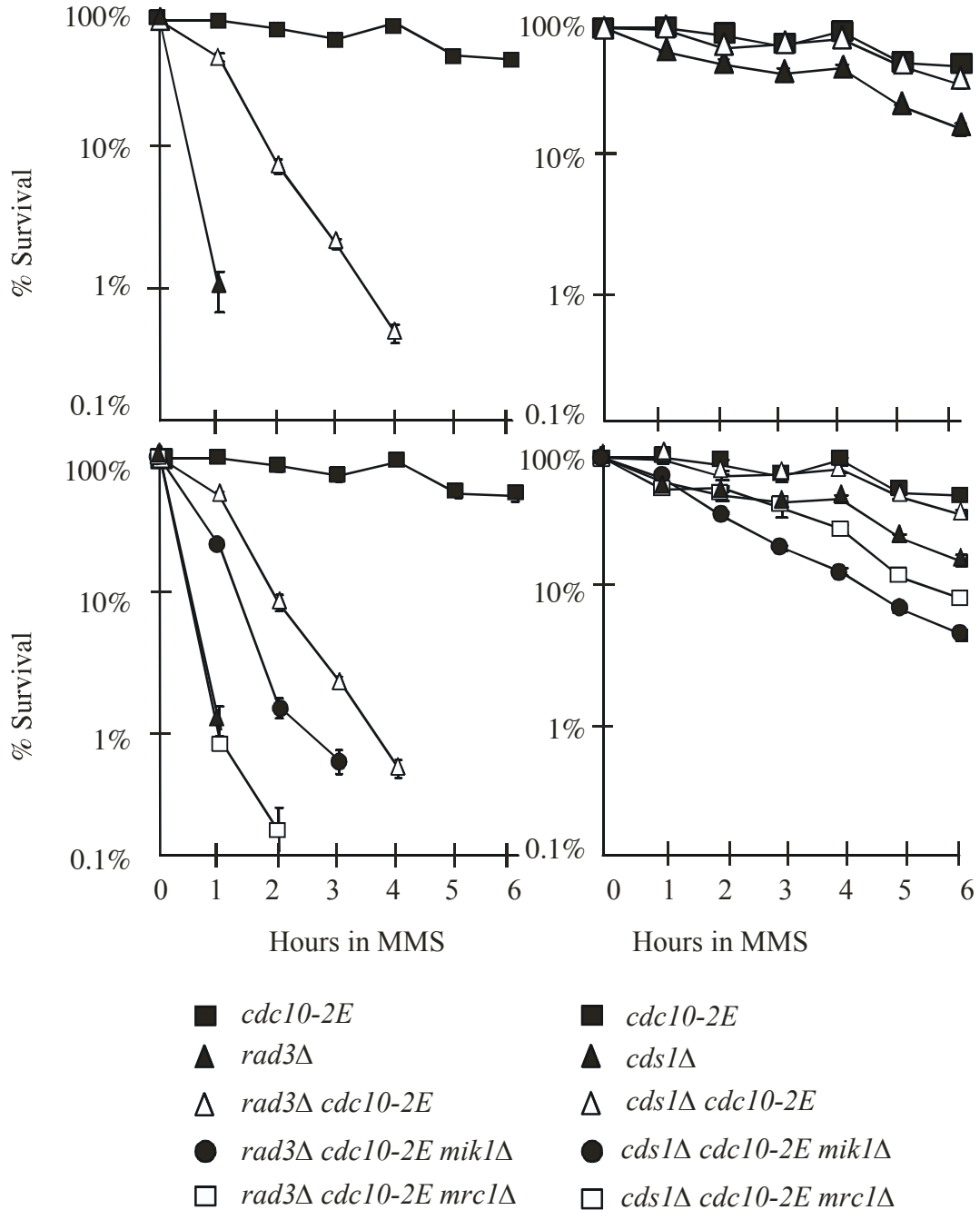

100%
10%
1%
0.1%
% Survival
0 1 2 3 4 5 6
Hours in MMS
cdc10-2E
rad3Δ
rad3Δ cdc10-2E
rad3Δ cdc10-2E mik1Δ
rad3Δ cdc10-2E mrc1Δ
cds1Δ
cds1Δ cdc10-2E
cds1Δ cdc10-2E mik1Δ
cds1Δ cdc10-2E mrc1Δ

Figure 3.3C

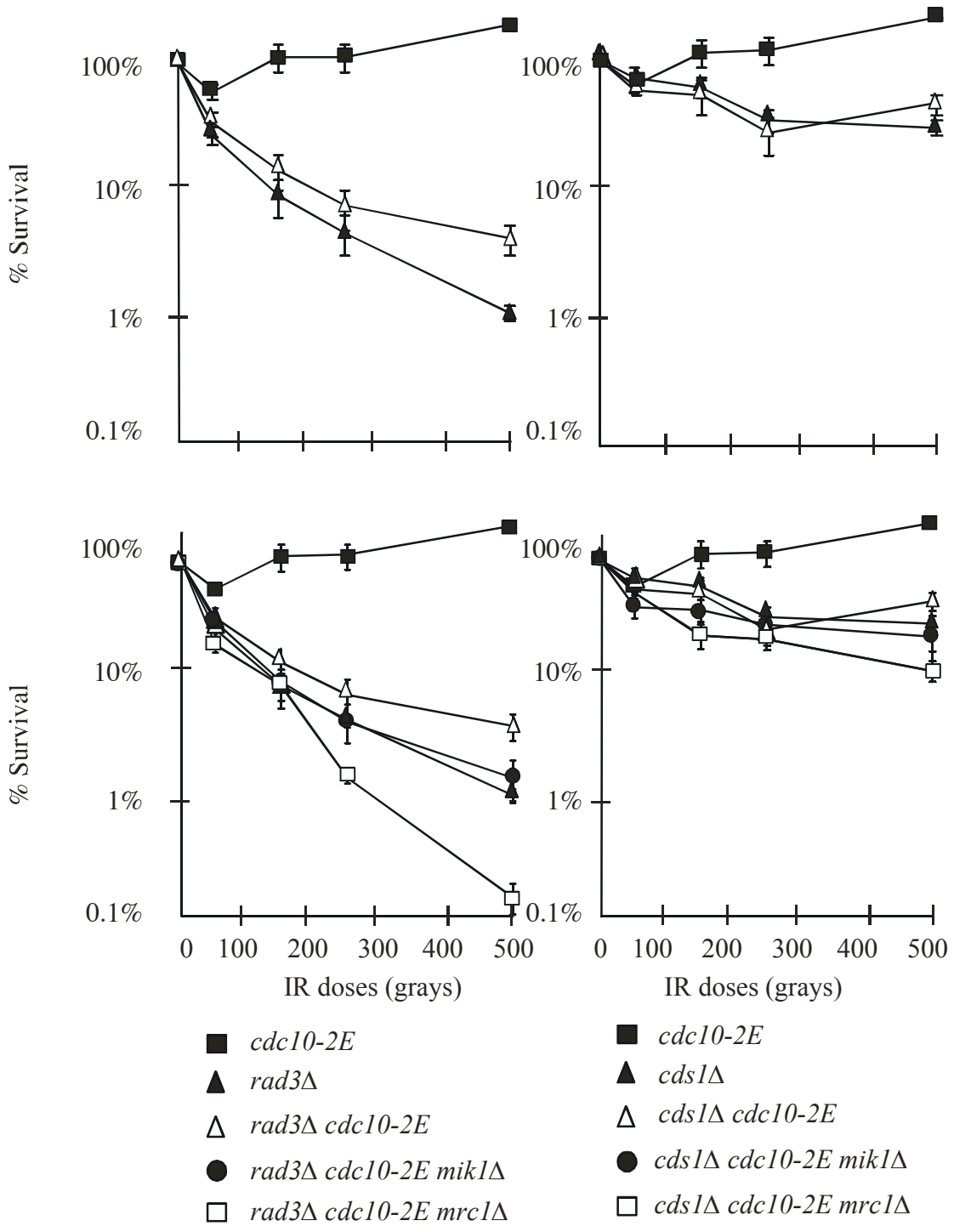

100%
10%
1%
0.1%
% Survival
0 100 200 300 400 500
IR doses (grays)
cdc10-2E
rad3Δ
rad3Δ cdc10-2E
rad3Δ cdc10-2E mik1Δ
rad3Δ cdc10-2E mrc1Δ
cds1Δ
cds1Δ cdc10-2E
cds1Δ cdc10-2E mik1Δ
cds1Δ cdc10-2E mrc1Δ

Figure 3.3D

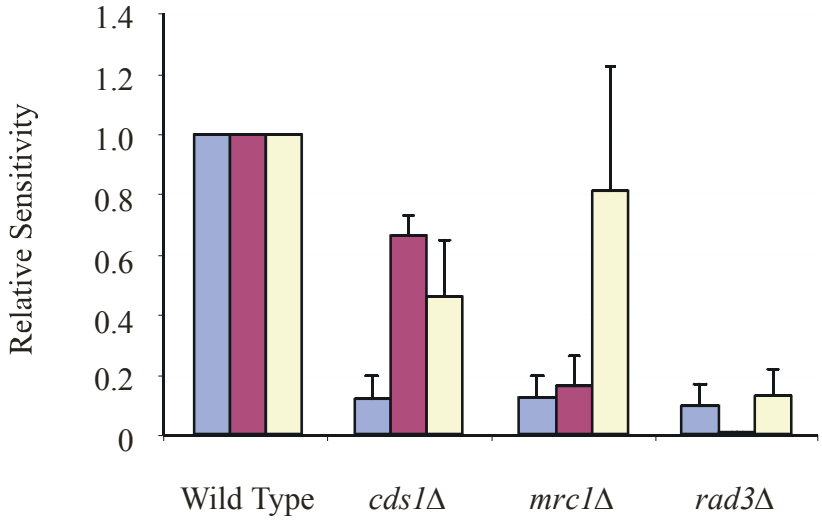


Figure 3.3E

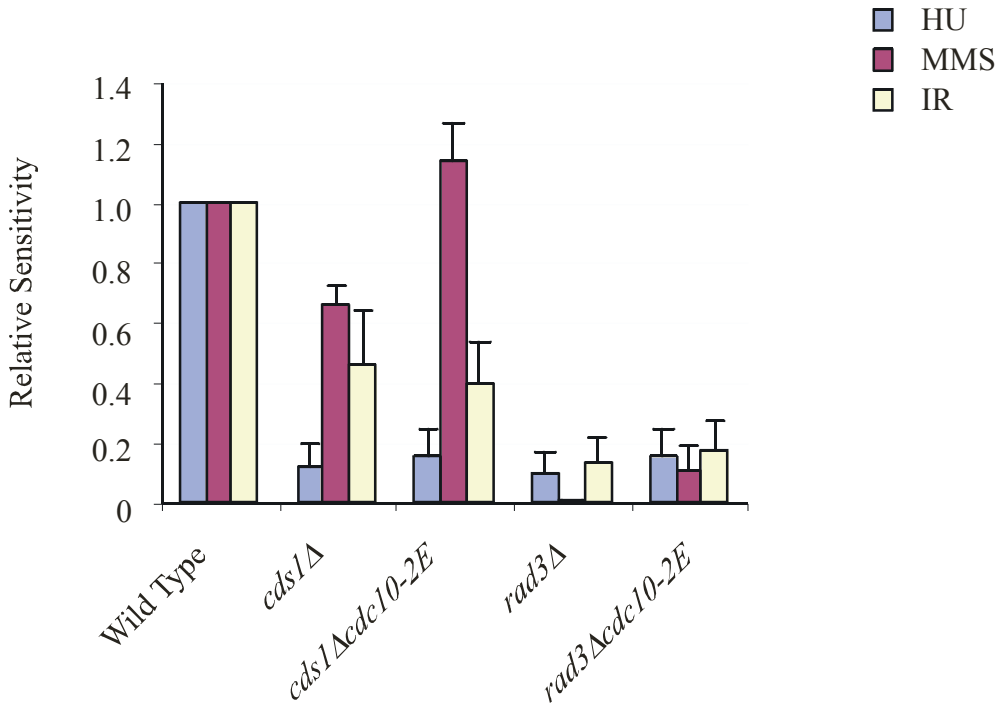


Figure 3.3F

Figure 3.3. Importance of Transcription during Activation of the Replication Checkpoint, S-phase DNA Damage Checkpoint and G2 DNA Damage Checkpoint.

Wild-type (yFS625), *cdc10-2E* (yFS626), *cds1*Δ (yFS627), *cds1*Δ *cdc10-2E* (yFS629), *rad3*Δ (yFS628), *rad3*Δ *cdc10-2E* (yFS630), *nmt1: pyp3 mik1*Δ (yFS632), *rad3*Δ *cdc10-2E mik1*Δ (yFS 643), *cds1*Δ *cdc10-2E mik1*Δ (yFS644), *rad3*Δ *cdc10-2E mrc1*Δ (yFS 645)*, cds1*Δ *cdc10-2E mrc1* (yFS 645), *mik1*Δ *(*yFS135), *mrc1*Δ (yFS624), and *chk1*Δ (yFS198) cells were treated with 10 mM HU, or 0.03% MMS for various time periods or 0-500Gy of ionizing radiation. The percentage survival was calculated by dividing the number of viable cells at each time point by the number of viable cells before the addition of HU, MMR or IR (time zero). The quantitation represent the mean and SEM of three experiments.

A Importance of checkpoint functions during acute stress. This figure represents sensitivity of wild type cells and checkpoint mutants in response to HU, MMS and IR.

B Survival during acute HU. Constitutive transcription in checkpoint deficient mutant is beneficial during acute exposure to HU.

C Survival during acute MMS. Constitutive transcription in checkpoint deficient mutant is beneficial during acute exposure to MMS.

D Survival during ionizing radiation induced damage. Constitutive transcription in checkpoint deficient mutant is beneficial during acute exposure to IR.

E and F Relative sensitivity of mutant to wild type cells. The relative sensitivity was calculated by comparing mutant sensitivity divided by wild type sensitivity at same

time (HU and MMS) and at same dose (IR). The graph represent the mean and SEM of six time points for HU and MMS. For example, if sensitivity of wild type cells = 0.8 and sensitivity of *rad3*Δ = 0.2, the relative sensitivity of *rad3*Δ will be 0.2/0.8=0.25.The quantitation for IR represnting mean and SEM of four dosages.

MBF Transcripts with Potential Beneficial Functions

Once we established that the MBF dependent transcription is beneficial for cell survival during stress, we wanted to identify potential MBF transcripts responsible for providing protective function leading to better survival. We focused on three MBF targets. First we focused on *mik1*. The MBF target *mik1* is involved in inhibitory phosphorylation of Cdc2 in response to IR (Rhind and Russell, 2001). We hypothesized that MBF target *mik1* must be taking part in preventing mitosis and contributing to better survival during stress. Second, we focused on *mrc1*. Mrc1 plays a dual role in cell cycle. It is a part of a fork protection complex and is known to negatively regulate Cdc45 during forks restart (Nitani et al., 2006). Mrc1 is also known to be a target of the checkpoint and an adaptor protein for activating Cds1, restricting activity of Cds1 to S phase. Because *mrc1* is also a MBF target we expected *mrc1* to play a major role in recovery during stress. Finally, we know that MBF transcript *cdc22* is a HU target. The transcription of *cdc22* is absolutely necessary to make sure stalled forks restart; so *cdc22* must be contributing a major part in survival during stress. Therefore, we decided to test the requirement of these three MBF transcripts in survival of cells during stress.

Role of Mik1

In order to determine the role of *mik1* dependent prevention of mitosis in contributing to the beneficial role of transcription in *rad3*Δ *cdc10-2E* cells, I made *rad3*Δ *cdc10-2E mik1*Δ strain. *rad3*Δ *cdc10-2E mik1*Δ cells are as sensitive as *rad3*Δ alone in IR

induced damage (Figure 3.3D). These data suggest that better survival in *rad3*Δ *cdc10-2E* is contributed by *mik1*. However, *rad3*Δ *cdc10-2E mik1*Δ cells are worse than *rad3*Δ *cdc10-2E* but better than *rad3*Δ cells treated with MMS (Figure 3.3C), suggesting partial contribution of *mik1* to survival during activation of the S-DNA damage checkpoint. High transcription during activation of the replication checkpoint showed less significant effect on survival of *rad3*Δ cells in HU than in MMS (Figure 3.3B). These data suggest that *mik1* plays an important role in preventing mitosis during activation of the G2 DNA damage checkpoint and activation of the S-phase DNA damage checkpoint (IR and MMS) and plays a less significant role in preventing mitosis in cells arrested in S phase due to activation of the replication checkpoint (HU).

MBF Target mik1 Delays Catastrophic Mitosis

Based on our observations, *rad3*Δ *cdc10-2E* and *cds1*Δ *cdc10-2E* cells are able to tolerate more HU than *rad3*Δ and *cds1*Δ alone, both in acute and in chronic exposure to HU. In order to determine if the rescue of *rad3*Δ *cdc10-2E* resulted from a delay in catastrophic mitosis, we followed the septation index in these cells. Catastrophic mitosis, also known as the cut phenotype, is an unequal segregation of chromosome as cells try to divide with unreplicated DNA. In fission yeast this phenomenon can be visualized under the microscope by the appearance of septa in small cells with a nucleus in only one half. I treated G2 synchronized cells with 10mM HU and followed septation index for two consecutive cell cycles. I observed that *rad3*Δ cells treated with HU proceed to lethal mitosis right after finishing the first cell cycle (Figure 3.5). The *rad3*Δ *cdc10-2E* double mutant cells proceed to lethal mitosis approximately at the same time, but the percent of

cut cells were reduced significantly. Similarly, I found that *cds1*Δ cells arrest in HU for the first 6 hours, and then these cells also proceed to lethal mitosis and *cds1*Δ *cdc10-2E* cells arrested longer than *cds1*Δ alone (Figure 3.5). To determine the direct role of *mik1* in delaying mitosis in *rad3*Δ *cdc10-2E* and *cds1*Δ *cdc10-2E* strains followed the septation index in these strains for two cycles in HU. These triple mutants showed catastrophic mitosis similar to *rad3*Δ and *cds1*Δ alone suggesting the delay in mitosis in *rad3*Δ *cdc10-2E* and *cds1*Δ *cdc10-2E* double mutant is mediated by *mik1* dependent inhibitory phosphorylation of Cdc2 (Figure 3.5).

Role of Mrc1 in Survival during Acute Stress

We observed a measurable benefit of having constitutive transcription in both *rad3*Δ *cdc10-2E* and *cds1*Δ *cdc10-2E* when exposed to acute or to chronic HU. We knew that the MBF target *mrc1* has a role in stabilizing stalled forks during normal replication. To test if elevated *mrc1* plays an additional role in stabilizing stalled forks during replication stress, we made *rad3*Δ *cdc10-2E mrc1*Δ. We noticed in HU and MMS treatment, *rad3*Δ *cdc10-2E mrc1*Δ cells are as sensitive as *rad3*Δ alone, suggesting an important role for *mrc1* during HU and MMS induced damage (Figure 3.3 B and C).

Role of MBF Target mrc1 in Recovery from Replication Stress

It has been shown that Mrc1 negatively regulates Cdc45 and MCM helicase to render stalled forks capable of resuming replication (Nitani et al., 2006). Because we have seen that in HU, *cds1*Δ *cdc10-2E mrc1*Δ cells are as sensitive as *cds1*Δ alone

(Figure 3.3B), we hypothesized that the improved HU resistance of *cds1*Δ *cdc10-2E* cells is due to an improved ability to resume replication compared to *cds1*Δ cells. In order to determine the role of *mrc1* in recovery from HU block, I treated asynchronous cultures with 10 mM HU for 4 hours. I washed the cells, resuspended in fresh media and collected samples for FACS every 20 minutes for 220 minutes (Figure 3.6A). The wild type cells and *cdc10-2E* cells finished replication within 40 minutes after release from HU block (Figure 3.6A). I observed that all the mutants have different degrees of difficulties finishing replication. *cds1*Δ *cdc10-2E* cells progress in S phase more than *cds1*Δ cells. I also observed that S- phase progression in *cds1*Δ *cdc10-2E mik1*Δ cells is similar to *cds1*Δ. However the S-phase progression in *cds1*Δ *cdc10-2E mrc1*Δ cells is worse than S-phase progression in *cds1*Δ alone (Figure 3.6A). These data suggest that *mrc1* plays an important role in recovery from stalled forks in addition to checkpoint independent function.

Inability to Restart Replication Resulted in Asymmetric segregation of DNA in Mitosis

Our data show that the beneficial role of having high transcription in *cds1*Δ *cdc10-2E* is partly mediated by *mrc1*. Since *mrc1* has role in restarting replication, cells might have difficulties resuming replication in absence of *mrc1* function leading to cell death. To test if more cells are dying in *cds1*Δ *cdc10-2E mrc1*Δ compared to *cds1*Δ *cdc10-2E* cells, we determine the appearance of a sub-G1 peak, represents dying cells containing less than 1C genome content. We observed that appearance of sub-G1 peak (20%) 120 minutes after HU release in *cds1*Δ cells. The appearance of the sub-G1 peak in *cds1*Δ *cdc10-2E* cells is delayed 100 minutes and less cells enter sub G1 (7%). The

percent of sub-G1 cells increased in *cds1*Δ *cdc10-2E mrc1*Δ to similar extent as *cds1*Δ (22%) (Figure 3.6B).

Over Expression of cdc22 Encoding Large Subunit of RNR Confers Resistance to HU

To determine the role of *cdc22* in checkpoint activation and survival, I constructed a mutant where *cdc22* is under regulation of *nmt1* (no message in thiamine) promoter, assuming in the presence of thiamine, the promoter will turn off the *cdc22* transcription. However, we were unable to turn off *cdc22* under this condition. We rationalized that a weaker promoter might be appropriate and we are constructing *cdc22* under weaker *nmt* promoter such as *nmt 41* and *nmt 81*; that work is ongoing.

Next, I over expressed *cdc22* driven under the strong *nmt1* promoter to determine whether over expression of *cdc22* can rescue lethality associated with deficiency in checkpoint activation in *rad3*Δ and *cds1*Δ cells. I observed that over expression of *cdc22* rescued lethality associated with *rad3*Δ and *cds1*Δ in HU and in MMS completely (Figure 3.7A). However, for IR induced damage, over expression of *cdc22* in *rad3*Δ *nmt1:cdc22* and *cds1*Δ *nmt1:cdc22* rescued lethality similar to *rad3*Δ *cdc10-2E* and *cds1*Δ *cdc10-2E* which confirms the limited role of *cdc22* during G2 (Figure 3.7A). I also tested the ability of these strains to survive chronic exposure to HU. Both *rad3*Δ *nmt1:cdc22* and *cds1*Δ *nmt1:cdc22* grew similar to wild type cells even in 3 mM HU (Figure 3.7B). Because there was a difference between the beneficial role of transcription in *rad3*Δ*, cds1*Δ*, rad3*Δ *cdc10-2E* and *cds1*Δ *cdc10-2E*, I hypothesized that the level of *cdc22* induction could be different in *cdc10-2E* and *nmt1: cdc22*. I determined the

expression of *cdc22* in *nmt1:cdc22* compared to wild type expression using northern blot (Figure 3.7C). I observed that the expression of *cdc22* in *nmt1:cdc22* strain is at least 10 times higher than asynchronous wild type cells (Figure 3.7C).

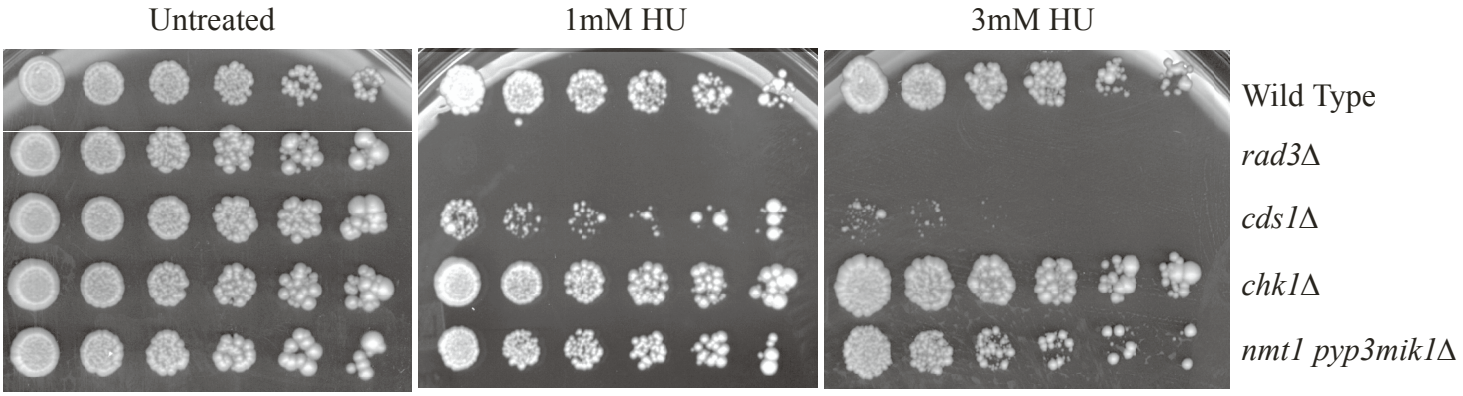


Figure 3.4A

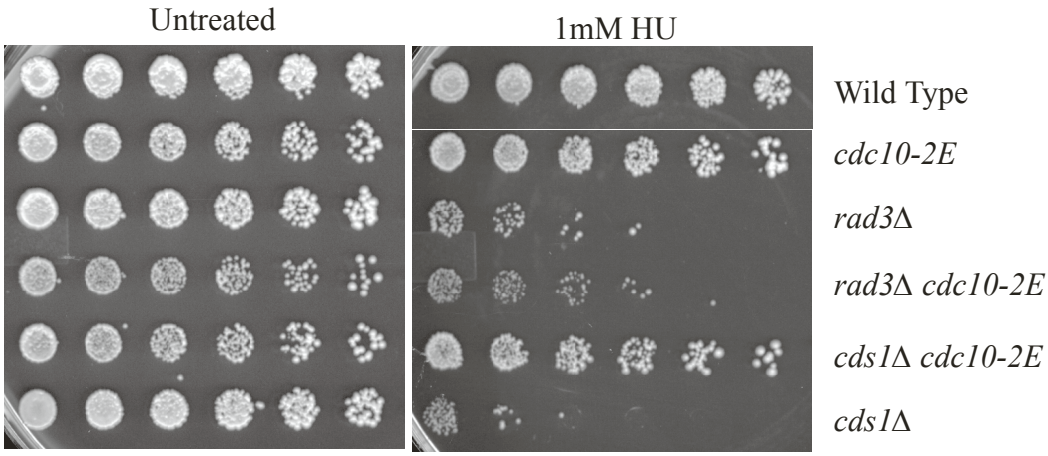


Figure 3.4B

Figure 3.4. The Role of the Checkpoint-Dependent Transcription in Surviving Chronic Replicative Stress.

Wild-type (yFS625), *cdc10-2E* (yFS626), *cds1*Δ (yFS627), *cds1*Δ *cdc10-2E* (yFS629), *rad3*Δ (yFS628), *rad3*Δ *cdc10-2E* (yFS630), *nmt1: pyp3 mik1*Δ (yFS632), and *chk1*Δ (yFS198) cells were three-fold serially diluted on to YES plates supplemented with 0, 1 or 3 mM HU and photographed after 7 days of growth.

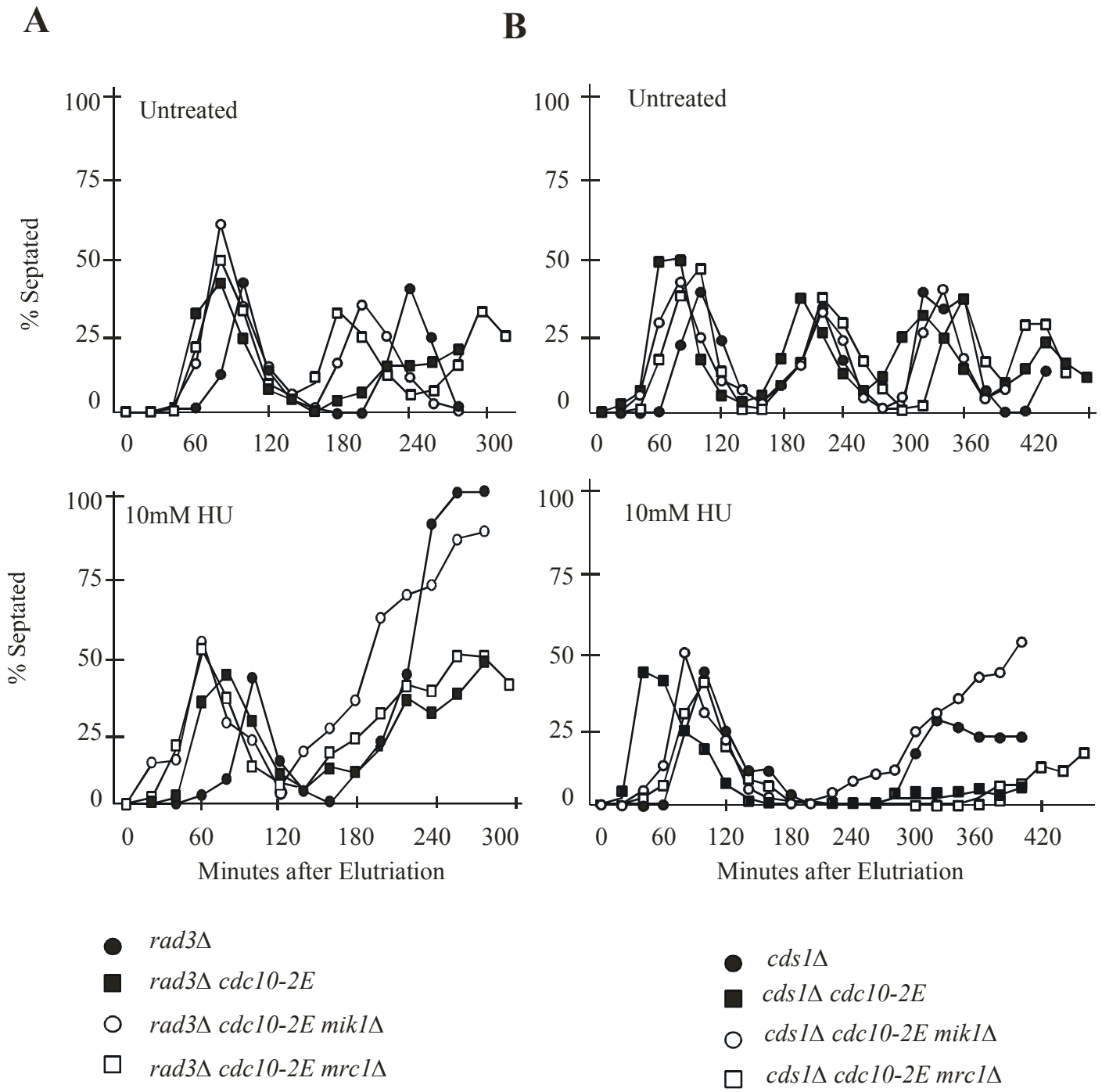

A
B
Untreated
10mM HU
% Septated
Minutes after Elutriation
rad3Δ
rad3Δ cdc10-2E
rad3Δ cdc10-2E mik1Δ
rad3Δ cdc10-2E mrc1Δ
cds1Δ
cds1Δ cdc10-2E
cds1Δ cdc10-2E mik1Δ
cds1Δ cdc10-2E mrc1Δ

Figure 3.5

Figure 3.5. Role of *mik1* in Reducing Cut Phenotype in *rad3*Δ*cdc10-2E* and *cds1*Δ *cdc10-2E* Cells.

*rad3*Δ (yFS628), *rad3*Δ *cdc10-2E* (yFS630), *rad3*Δ *cdc10-2E mik1*Δ (yFS 643), *cds1*Δ *cdc10-2E mik1*Δ (yFS644), *rad3*Δ *cdc10-2E mrc1*Δ (yFS645) and *cds1*Δ *cdc10-2E mrc1*Δ (yFS 646) cells were synchronized in early G2 by centrifugal elutriation and followed through a synchronous cell cycle in the presence or absence of 10 mM HU. Samples were taken every 20 minutes for visual inspection of septation.

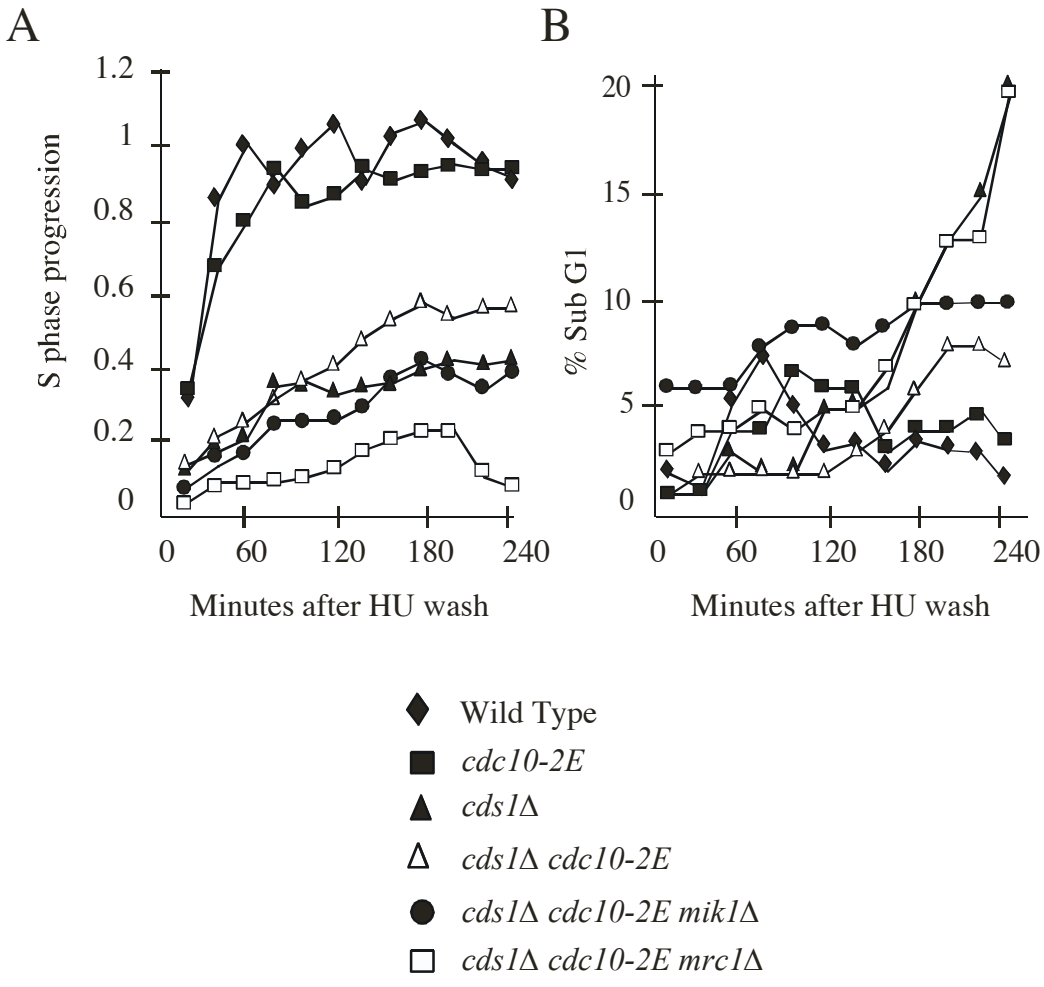

A
B
S phase progression
1.2
1
0.8
0.6
0.4
0.2
0
0
60
120
180
240
Minutes after HU wash
% Sub G1
20
15
10
5
0
0
60
120
180
240
Minutes after HU wash
Wild Type
cdc10-2E
cds1Δ
cds1Δ cdc10-2E
cds1Δ cdc10-2E mik1Δ
cds1Δ cdc10-2E mrc1Δ

Figure 3.6

Figure 3.6. *mrc1* plays a Major Role in Recovery from Replicative Stress.

Wild-type (yFS625), *cdc10-2E* (yFS626), *cds1*Δ (yFS627), *cds1*Δ *cdc10-2E* (yFS629), *rad3*Δ (yFS628), *rad3*Δ *cdc10-2E* (yFS630), *rad3*Δ *cdc10-2E mrc1*Δ (yFS645) and *cds1*Δ *cdc10-2E mrc1*Δ (yFS 646), *rad3*Δ *cdc10-2E mik1*Δ (yFS 643), *cds1*Δ *cdc10-2E mik1*Δ (yFS644) asynchronous cells were treated with 10 mM HU for 4 hours, washed, resuspended in fresh media and collected every 20minutes. A. S-phase progression was determined by measure DNA content by FACs. B. Sub G1 peak was determined by measuring cells containing less than 1C genome content by FACs.

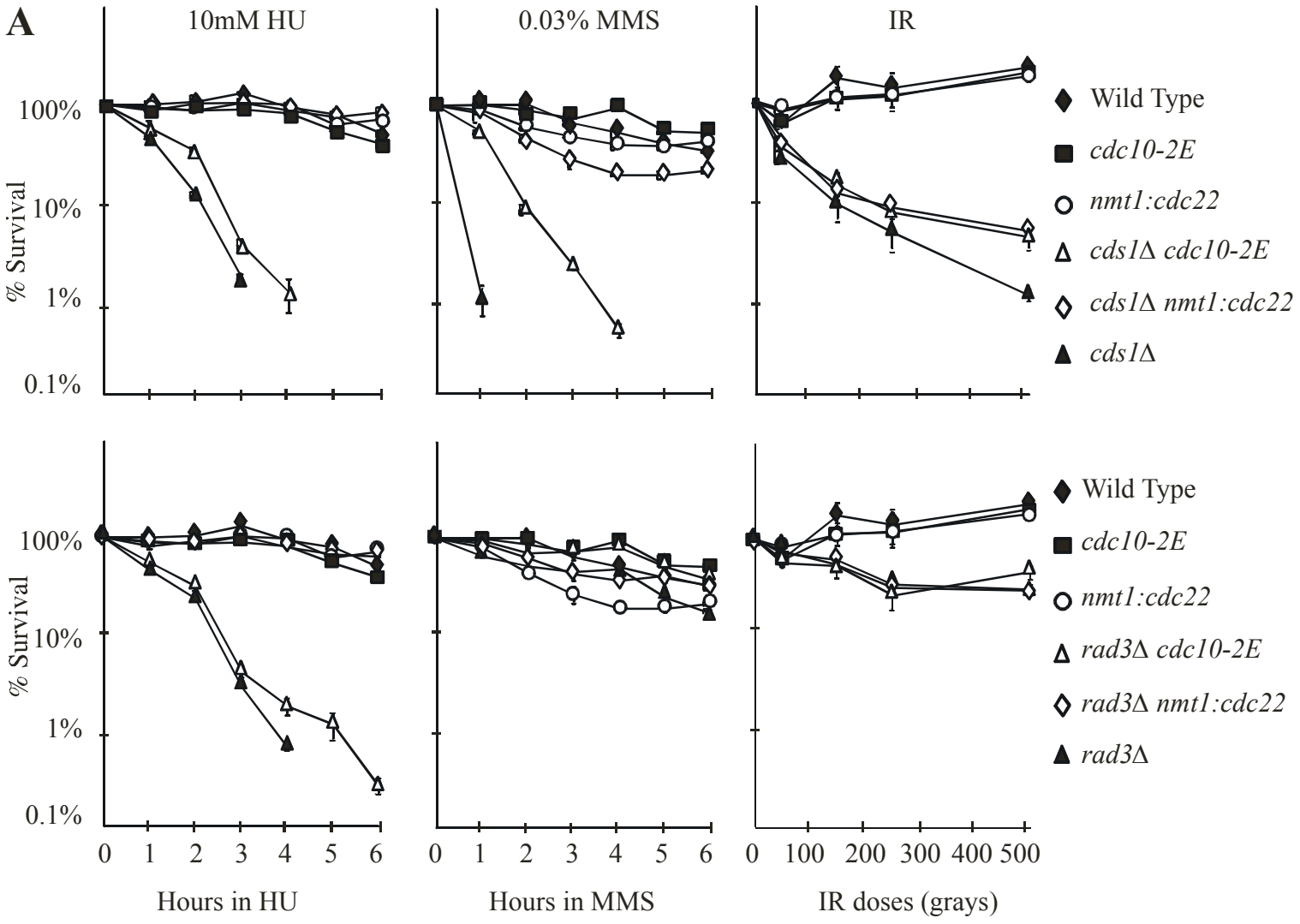

A
10mM HU
0.03% MMS
IR
100%
10%
1%
0.1%
% Survival
Wild Type
cdc10-2E
nmt1:cdc22
cds1Δ cdc10-2E
cds1Δ nmt1:cdc22
cds1Δ
Wild Type
cdc10-2E
nmt1:cdc22
rad3Δ cdc10-2E
rad3Δ nmt1:cdc22
rad3Δ
0 1 2 3 4 5 6
0 1 2 3 4 5 6
0 100 200 300 400 500
Hours in HU
Hours in MMS
IR doses (grays)

Figure 3.7A

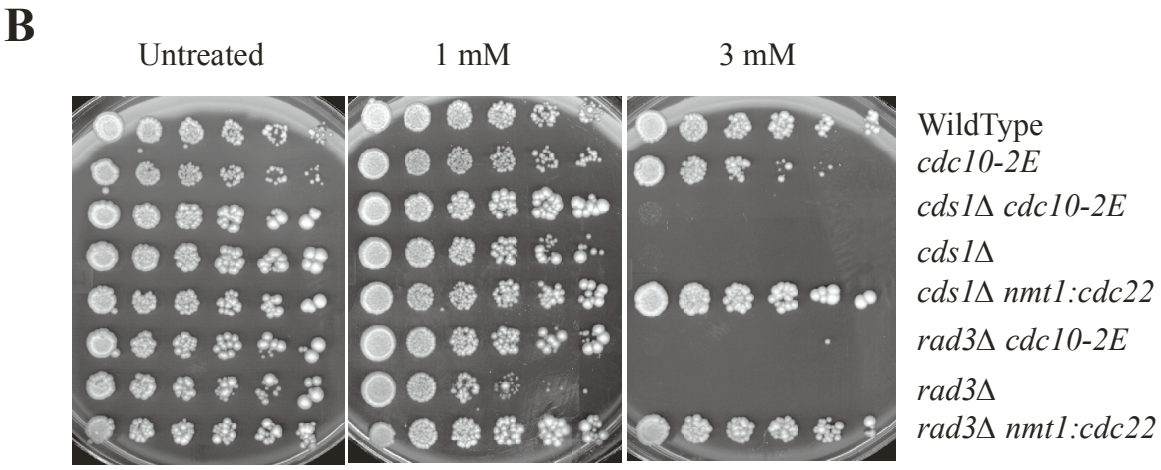


Figure 3.7B

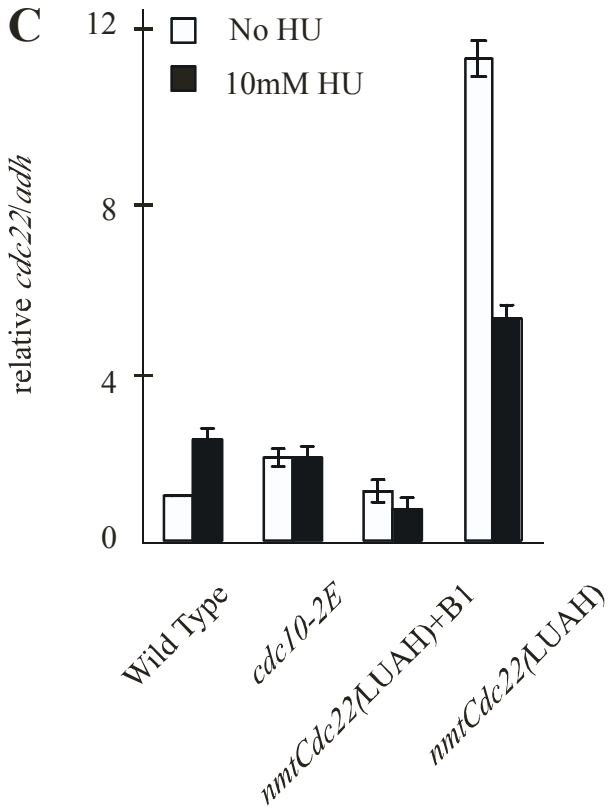


Figure 3.7C

Figure 3.7: Overexpression of *cdc22* Confers HU Resistance.

A Overexpression of *cdc22* rescues cells exposed to acute HU, MMS and IR induced damage. Wild-type (yFS625), *cdc10-2E* (yFS626), *cds1*Δ (yFS627), *rad3*Δ (yFS628), *cds1*Δ *nmt1:cdc22* (yFS648) and *rad3*Δ *nmt1:cdc22* (yFS647) cells were treated with 10 mM HU, or 0.03% MMS or 0-500Gy of ionizing radiation. The relative percentage survival was calculated by dividing the number of viable cells at each time point by the number of viable cells before the addition of HU, MMS or IR (time zero).

B Overexpression of *cdc22* rescue cells in chronic HU induced damages. Wild-type (yFS625), *cdc10-2E* (yFS626), *cds1*Δ (yFS627), *rad3*Δ (yFS628), *cds1*Δ *nmt1:cdc22* (yFS648) and *rad3*Δ*nmt1:cdc22* (yFS647) cells were treated with 0, 1and 3 mM HU and the growth at 30^0C was photographed after 7 days.

C Relative expression of *cdc22* in acute HU (10 mM) was determined by northern blot analyses with *cdc22* as a probe and normalized with *adh1* expression in wild type (yFS625), *cdc10-2E* (yFS626), *nmt1:cdc22* (yFS620) in YES, *nmt1:cdc22* (yFS620) in LUAH+B1, *nmt1:cdc22* (yFS620) in LUAH for 17 hours**.**

Discussion

Our results demonstrate that activation of the fission yeast S-phase checkpoints trigger MBF dependent S-phase gene transcription. Checkpoint dependent coordinated transcription of S-phase genes during replication stress is beneficial to cells under replication stress. The beneficial role of the transcriptional response is important for survival and is contributed in part by *cdc22*, *mrc1* and *mik1*. Four lines of evidence support these conclusions. First, MBF dependent gene transcription is restricted to only during S phase and can be activated both by HU induced replication checkpoint and by MMS induced S-phase DNA damage checkpoint. In contrast, activation of the G2 DNA damage checkpoint by IR does not modulate MBF dependent transcription (Figure 3.1). Second, our data provide evidence that the transcriptional response is beneficial for cell survival in both acute and chronic stress (Figures 3.3 and 3.4). Third, I showed that the function of two checkpoint-dependent transcripts contribute to survival during stress: *mrc1* and *mik1* (Figure 3.5 and 3.6). Finally, our data suggest that positive regulation of *cdc22* not only provides an opportunity to reduce the extent of damage by broken stalled forks but that high levels of *cdc22* could provide protection against DNA damage caused by MMS (Figure 3.7).

Both MMS and HU affect cells in S phase; however, the mechanisms by which they do so are different. The process cells use to cope with the stress produced by HU and damage caused by MMS could be overlapping but may not be completely identical. Ionizing radiation on the other hand, introduces double stranded breaks in DNA but is unable to slow replication and arrests cells in the G2 by activating G2 DNA damage checkpoint.

I noticed that cells deal with HU induced replication arrest differently than MMS induced damage. The beneficial role of transcription is more significant in MMS induced DNA damage. I hypothesized that since MMS induced S-phase checkpoint slows replication but cells do finish replication and arrest in G2, the unresolved replication structures activate the Chk1 dependent DNA damage checkpoint. During activation of G2 DNA damage checkpoint, cells not only have time but also have a sister chromatid to repair the damages.

Our observations suggest that during replication stress by HU or MMS, stabilizing stalled forks is more crucial for survival than preventing mitosis. I speculate that when cells are arrested in S phase, unless they are able to maintain stalled forks, the forks won't be able to restart and finish replication. Collapsed forks will not be able to finish replication and activating the DNA damage checkpoint will not improve viability. On the other hand, during IR induced damage, which activates the G2 DNA checkpoint, preventing mitosis is more important than stabilizing forks as these cells are in G2 and have already finished replication. Repairing damaged DNA is enough to rescue the cells. The fact that cells lacking fork-stabilizing function such as *cds1*Δ cells are no worse than *cds1*Δ *cdc10-2E* double mutants when experiencing IR induced DNA damage also validates the conclusion that stabilizing forks is not very important during IR induced damage. Therefore, the checkpoint dependent transcriptional response appears to have evolved to protect cells from stress during S phase.

Sensitivity of *mrc1*Δ cells in HU and MMS suggest the crucial role of *mrc1* during normal replication and during S phase arrest. Transcription of MBF target *mrc1* not only stabilizes stalled forks, but also helps resume replication during S-phase arrest as

shown by *cds1*Δ *cdc10-2E*. Therefore *mrc1,* a component of the fork protection complex serves as a link between normal replication and checkpoint activation by being a mediator of Cds1 activation (Alcasabas et al., 2001).

Our data provide evidence that the MBF target *mik1* plays direct role in delaying mitosis by preventing Cdc2 activity (Christensen et al., 2000). These data are consistent with the observation that constitutively expressed Mik1 kinase in *cdc10*Δ*C4* (a dominant allele of *cdc10* resulting in constitutive over expression of MBF regulated genes) plays a role in increased cell length in division (Ng et al., 2001).

Our data show that ten fold over expression of *cdc22* rescued not only acute HU treated cells but also acute MMS treated *rad3*Δ *nmt1:cdc22* and *cds1*Δ *nmt1:cdc22* cells. The protection with high *cdc22* levels I observed in *rad3*Δ *nmt1:cdc22* and *cds1*Δ *nmt1: cdc22* is significantly greater than the protection observed with 4 fold increase in *cdc22* levels seen in *cdc10-2E* allele or HU treated wild type cells (Figure 3.7). These data indicate that the lethality associated with absence of the replication checkpoint and S-phase DNA damage checkpoint is not because the cells undergo lethal mitosis; rather it is because of their inability to complete replication properly. These data also suggest that if fission yeast had an independent RNR regulation as budding yeast has, fission yeast could tolerate high levels of HU like budding yeast.

Our result with MBF transcription in response to IR in synchronous cells also provides an explanation for the conclusion of Watson *et al.* that ionizing radiation induces MBF transcription (Watson et al., 2004). In their experiments, Watson *et al.* observed induction of MBF transcripts such as *cdc22, cdt1, cdt2* and *cdc18* 160 minutes after irradiation of G2 synchronous cells (Watson et al., 2004). They interpreted this

increase as evidence that S-phase transcription is modulated by IR treatment in G2. Likewise, based on our synchronous *cdc22* northern data, I observed that *cdc22* transcription is high in cells at 160 minutes after irradiation (Figure 3.1). However, our septation data provide evidence that at 160 minutes after irradiation, cells are in S phase. Our experiments done on synchronous cells provide evidence that the MBF dependent transcription peak observed by Watson *et al*. was due to cells entering S phase after exit from a G2 arrest. Therefore, our data show that MBF dependent transcription is not regulated outside S phase.

Our data do not rule out the possible role of other S-phase transcripts that might have some direct or indirect roles that are crucial for survival. For example the role of Rad51 in homologous recombination (Jang et al., 1996), the role of RPA in recovery (Parker et al., 1997), and the role of Cdc18 in activating and maintaining Cds1 kinase activity could play additional roles in survival during stress (Murakami et al., 2002). Further experiments are needed to address the roles of these proteins in different aspects of recovery.

Appendix 3

Role of Res1 in Regulating MBF

In order to determine the role of checkpoint dependent S-phase transcription alone we wanted to build a strain in which only S phase transcription would be affected, leaving the other two checkpoint functions preventing mitosis and stabilization of fork intact. Because our *cdc10-8A* strain still has checkpoint dependent regulation, we focused on Res1, which is a member of the MBF complex (Caligiuri and Beach, 1993; Tanaka et al., 1992). Res1 was originally described as a positive regulator of the MBF complex since in *res1*Δ cells, *cdc18* mRNA was constitutively down regulated (Baum et al., 1997). We decided to use *res1*Δ cells as a model strain of constitutively low S-phase transcription in our study. However, when we looked at the *cdc22* expression by northern blot, we observed that it is highly expressed in *res1*Δ cells. Based on this observation, we hypothesized that the role of Res1 could be more complex than originally thought. We looked at all of the S phase genes by micro-array, and we observed that genes were differentially regulated in *res1*Δ cells (Figure 2.2B). *cdc18* mRNA was constitutively expressed at low levels throughout the cycle as observed by Baum *et al*. and *cdc22* mRNA level was very high; both were consistent with our observation by northern blot. These cells express high level of *mrc1* but a very low level of *mik1, cdc18* and *cig2* (Dutta et al 2008). So the mechanism by which Res1 regulates transcription of S phase genes could be different at different promoters.

Recently, Nrm1 has been identified as a negative regulator of the MBF complex and shown to be a direct *in vivo* target of Cds1 (de Bruin et al., 2008). We hypothesized if we introduced the serine to alanine mutation in both Cdc10 as well as in Nrm1, the double mutant should not have any checkpoint dependent regulation. There are thirteen potential Cds1 phosphorylation sites on Nrm1 that have been identified by mass spectroscopy. Different combinations of serine to alanine substitution mutations are currently in progress in collaboration with Curt Wittenburg and Rob de Bruin. We tested four different Nrm1 mutants. *nrm1-2A* was made by substituting serine 203 and serine 206 with alanine. *nrm1-3A* was made by substituting serine 251, 264 and 330 were substituted with alanines. *nrm1-4A* was made by substituting serine 57, T116, S174, S237 with alanines. *nrm1-8A* was made by substituting undisclosed serine/threonine residues with alanines. We made double mutants of *nrm1* mutants with *cdc10-8A* or *cdc10-S720A* and followed the *cdc22* expression by northern blot. However, all the double mutants tested showed normal checkpoint regulation of MBF transcription (Figure 3.8A). Currently, the Wittenberg lab is working on building *nrm1-13A* and also trying to determine the phosphorylation sites by substituting serines with glutamic acids. If the sites that are sufficient or *nrm1* dependent regulation of transcription is (are) identified, we would like to make double mutant with our *cdc10-2A* and determine the effect on S-phase transcription by northern blot analysis.

When *res1*Δ cells are challenged with acute HU, MMS or IR, we observed that these cells are not sensitive to HU or IR but are sensitive to MMS (Figure 3.8B). We hypothesized that these cells are resistant to HU because these cells are proficient in

cdc22 transcription. The sensitivity of *res1*Δ cells to MMS could be due to inability to prevent mitosis, as the *mik1* levels in these cells are very low. However, these cells are proficient in activating the G2 DNA damage checkpoint, which should be enough for survival. Additionally, disruption of the G2 DNA damage checkpoint (by deleting *mik1* and over expressing *pyp3* under *nmt1*) in a *res1*Δ background rescues the lethality associated with differential transcription of S-phase genes. We were unable to explain the mechanism underlying this observation. These observations led us to conclude that *res1*Δ strains cannot be used in our transcriptional studies to represent as control strains with constitutively low S phase transcription.

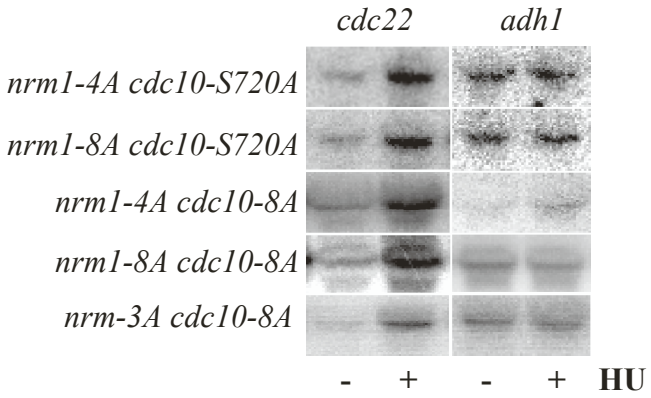

cdc22
adh1
nrm1-4A cdc10-S720A
nrm1-8A cdc10-S720A
nrm1-4A cdc10-8A
nrm1-8A cdc10-8A
nrm-3A cdc10-8A
- + - + HU

Figure 3.8 A

Figure 3.8A Northern Analysis of *cdc22* Transcript Levels in Asynchronous Cells.

nrm1-4A cdc10-S720A (yCD80), *nrm1-8A cdc10-S720A* (yCD85), *nrm1-4A cdc1-8A* (yCD79), *nrm1-8A cdc10-8A* (yCD126), and *nrm1-3A cdc10-2A* (yCD137) cells untreated or treated with 10 mM HU for 4 hours. Northerns blot were probed with *cdc22*, stripped and reprobed with *adh1* as a loading control.

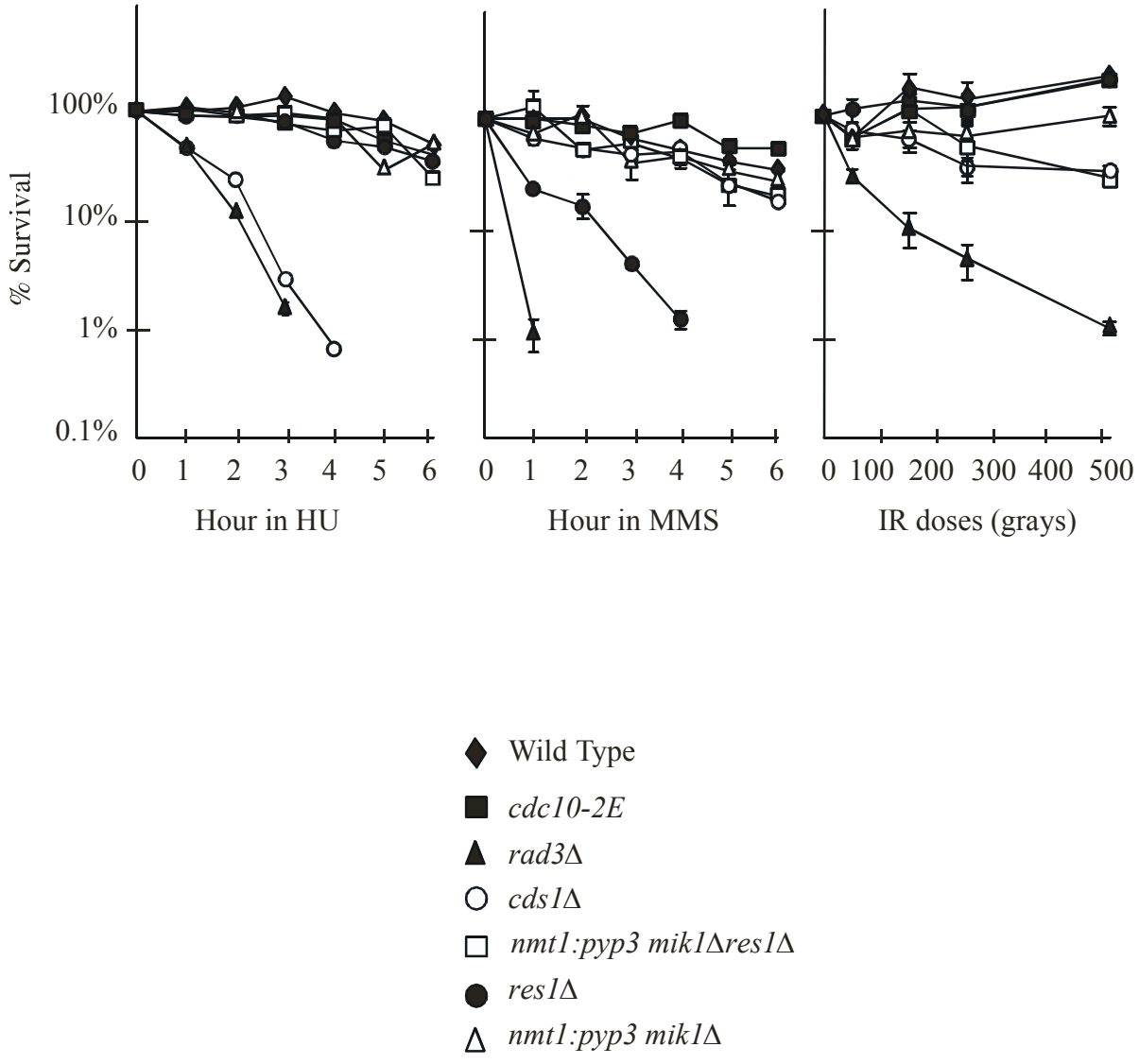

% Survival
100%
10%
1%
0.1%
0 1 2 3 4 5 6
Hour in HU
0 1 2 3 4 5 6
Hour in MMS
0 100 200 300 400 500
IR doses (grays)
Wild Type
cdc10-2E
rad3Δ
cds1Δ
nmt1:pyp3 mik1Δres1Δ
res1Δ
nmt1:pyp3 mik1Δ

Figure 3.8B

Figure 3.8B. Importance of Transcription during Activation of the Replication Checkpoint, the S-phase DNA Damages Checkpoint and the G2 DNA Damage Checkpoint.

Wild-type (yFS625), *cdc10-2E* (yFS626), *rad3*Δ (yFS628), *cds1*Δ (yFS627), *res1*Δ *(*yFS163), *nmtpyp3mik1*Δ (yFS632), *nmtpyp3mik1*Δ*res1*Δ (yFS631), cells were treated with 10 mM HU, or 0.03% MMS or 0-500Gy of ionizing radiation. The relative percentage survival was calculated by dividing the number of viable cells at each time point by the number of viable cells before the addition of HU, MMR or IR (time zero).

Table 3.3 Strains used in Appendix 3

	Genotypes	
yCD80	*h+ leu1-32 ura4-D18 cdc10-S720A:kanMX6nrm1-4A:kanMX6*	This Study
yCD85	*h+ leu1-32 ura4-D18 cdc10-S720A:kanMX6nrm1-8A:kanMX6*	This Study
yCD79	*h+ leu1-32 ura4-D18 cdc10-8A:kanMX6nrm1-4A:kanMX6*	This Study
yCD126	*h+ leu1-32 ura4-D18 cdc10-8A:kanMX6nrm1-8A:kanMX6*	This Study
yCD137	*h+ leu1-32 ura4-D18 cdc10-2A:kanMX6nrm1-3A:kanMX6*	This Study
yFS625	*h- leu1-32 ura4-D18 cdc10:kanMX6*	This study
yFS626	*h+ leu1-32 ura4-D18 cdc10-2E*	This study
yFS627	*h- leu1-32 ura4-D18 cdc10:kanMX6 cds1::ura4*	This study
yFS628	*h- leu1-32 ura4-D18 cdc10:kanMX6 rad3::ura4*	This study
yFS163	*h- leu1-32 ura4-D18 ade6-210 res1::ura4*	Rhind lab
yFS631	*h- leu1-32 res1::ura4 mik1::ura4 nmt:pyp3(kan)*	This study
yFS632	*h+ leu1-32 ura4-D18 mik1::ura4, nmt:pyp3(kan)*	This study

Chapter IV

Discussion and Future Direction

It is well established that the replication checkpoint modulates transcription. In budding yeast, this checkpoint dependent transcriptional response is independent from normal S phase periodic transcription. HU arrest activates a unique regulatory pathway that controls the activation of *RNR* alone (Huang et al., 1998). In fission yeast there is no such unique regulatory pathway for maintaining *RNR* alone in response to HU. Baum et al, 1997 offered evidence that replication stress probably modulates the transcriptional machinery that controls normal S phase gene transcription. 1) HU treatment upregulates many S phase genes and 2) the up regulation of S phase genes is dependent on Cdc10, a major component of MBF. Based on these observations, I hypothesized that the fission yeast the replication checkpoint might be targeting the MBF complex directly to deal with replication stress during HU arrest. Our hypothesis predicts three outcomes. First, all S phase transcripts should be affected. Second, any mutation that interferes with normal MBF function would interfere with checkpoint-induced transcription. And finally the replication checkpoint kinase Cds1would be directly involved in modification of one or more subunits of the MBF complex.

In Chapter II of this study, I focused on the mechanism by which the checkpoint might be regulating MBF. In Chapter III, I discussed how the transcriptional response could be important during replicative stress. I also showed that at least three MBF dependent transcripts play major roles in cell survival in response to HU, MMS and IR.

My data demonstrate that the fission yeast replication checkpoint regulates the MBF G1/S transcription factor to maintain the normal G1/S transcriptional program during replication stress. I provided three lines of evidence supporting our hypothesis. First, using gene arrays, I showed that all MBF transcripts and only MBF transcripts are upregulated by the checkpoint in response to HU arrest (Figure 2.1B). During the progress of my work the same set of genes was identified as being S-phase specific by three other independent studies (Oliva et al., 2005; Peng et al., 2005; Rustici et al., 2004). Second, mutations in the Res1, Res2 and Cdc10 subunits affect checkpoint-regulated transcription in the same manner they affect normal G1/S transcription (Figure 2.2B). Third, I showed that phosphomimetic mutations of sites phosphorylated by Cds1 *in vitro*; in the allele I call *cdc10-2E*, cause constitutive G1/S transcription *in vivo* (Figure 2.3B). In addition, restoring a sustained high level of G1/S transcription with addition of *cdc10-2E* to *rad3*Δ and *cds1*Δ cells, which normally lack checkpoint-induced transcription, modestly increases their resistance to HU, demonstrating the *in vivo* relevance of the response (Figure 2.4).

These results also shed light on the roles of the Res1 and Res2 DNA binding subunits of MBF. Res1 and Res2 have been proposed to be activating and repressing subunits, respectively (Baum et al., 1997). My array data show that the situation is more complicated (Figure 2.2B). For instance, *cdc22*, *mrc1, cdt2* transcripts are up-regulated in *res1*Δ cells, while *cdc18, mik1* and *cig2* transcripts are down-regulated. In *res2*Δ cells different genes are also differentially regulated. My data suggest that the DNA binding members of the MBF complex could be working differently at different promoters. In metazoans, several forms of E2F protein and the DP protein exist. Binding of E2F/DP has

been shown to both activate and repress the transcription of some genes during G1 (Weintraub et al., 1992; Weintraub et al., 1995; Zwicker et al., 1996). Neither Res1 nor Res2 are regulated transcriptionally or translationally during the cell cycle (Baum et al., 1997). However it is possible that a transcript is regulated differently depending on posttranslational modification of either Res1 or Res2. In addition, it is still unknown if these subunits are localized at different compartments depending of the cell cycle stages or cellular stress. Nonetheless it is clear that the roles of both Res1 and Res2 are complicated in MBF transcription and vary depending on the gene.

My results suggest that Cds1 regulates MBF by phosphorylating the C-terminus of Cdc10; however, I have been unable to detect such phosphorylation *in vivo*. I know that Cdc10 is multiply phosphorylated throughout the cell cycle and therefore the addition of one or two extra phosphates may not greatly affect its overall phosphorylation state or its mobility on a polyacrylamide gel (Simanis and Nurse, 1989).

Although I have tried many different approaches to detect *in vivo* phosphorylation of Cdc10, there are alternative approaches that could be used. It is possible to develop specific monoclonal antibodies against phosphorylated Cdc10 and could be use for western blot to detect *in vivo* checkpoint dependent phosphorylation. Monoclonal antibodies against phospho epitopes have been shown to be very useful to detect phosphorylation in many different proteins that failed to be resolved in traditional SDS PAGE. However, the limitation for this approach would be interference of antibody recognition of one phosphoserine by the presence of another closely spaced phosphoserine.

Alternatively, phosphoamino acid analysis or peptide mapping and two-dimensional gel electrophoresis could be used. It has been shown that Cdc10 is phosphorylated throughout the cell cycle (Simanis and Nurse, 1989). However, checkpoint dependent Cdc10 phosphorylation using *in vivo* labeling has not been done. In addition, if there are distinct checkpoint dependent bands, mass spectroscopy could be used too to detect checkpoint dependent Cdc10 phosphorylation.

It could also be possible that the sites on Cdc10 phosphorylated by Cds1 during the checkpoint may also be phosphorylated during normal S phase. If that is the case, using phospho-specific antibodies or *in vivo* labeling will not be sufficient to differentiate between checkpoint dependent and checkpoint independent phosphorylation of Cdc10. If that were the case, to be able to dissect the checkpoint dependent phosphorylation from checkpoint independent phosphorylation, it would be necessary to identify the kinase necessary for normal cell cycle dependent MBF transcription.

Both *rad3*Δ and *cds1*Δ cells are defective in checkpoint dependent maintenance of S-phase transcription without affecting normal S-phase dependent transcription. This observation leads us to speculate that another unidentified kinase is active during normal S phase to regulate MBF function. Cdc2, an obvious candidate for such response is not needed for regulation of MBF during normal S phase (Baum et al., 1997). However, data from budding yeast suggest Pho85/Pcl as another potential candidate.

In budding yeast, Pcl1 and Pcl2 were identified as G1 cyclins that had G1 periodic activity and associate with the Pho85 kinase. In the absence of Cdc28 or G1 cyclins Cln1 and Cln2, either Pcl1 or Pcl2 is required for G1 progression (i.e. a *cln1*Δ *cln2*Δ *pcl1*Δ *pcl2*Δ quadruple mutant is inviable and arrests in G1) (Siegmund and

Nasmyth, 1996). Consistent with the requirement for Pcl1-Pho85 or Pcl2-Pho85 kinase activity under these conditions, Pho85 is required for G1 progression in the absence of Cln1 and Cln2 (Espinoza et al., 1994; Measday et al., 1994; Measday et al., 1997). In addition, Pcl2-Pho85 kinase activity peaks in G1 phase (Measday et al., 1994). These data suggest that Pho85 might be involved in regulating START/G1 transition by regulating MBF dependent transcription.

There is no study addressing the role of Pho85 in fission yeast. Based on the observation in budding yeast, it is tempting to speculate Pho85 could be a possible kinase regulating MBF. One possible approach would be to make a *pho85*Δ strain in *cdc2* temperature sensitive background and determine the S-phase transcription at non-permissive temperature.

Analysis of *cdc10-2E* suggests that phosphorylation of Cdc10's C-terminus is sufficient to activate G1/S transcription. However, the fact that replication checkpoint control of MBF is intact in *cdc10-8A*, which cannot be phosphorylated by Cds1 on its C-terminus, shows that such phosphorylation is not necessary for checkpoint regulation. I hypothesize that phosphorylation of either Cdc10 or the Nrm1 MBF repressor, which binds to and inhibits MBF in G2 (de Bruin et al., 2006), is sufficient for checkpoint regulation of MBF. If this is the case, the double mutants made with *cdc10-8A* and *nrm1-13A* would be next step to test our hypothesis. I tried different combination of mutations in *nrm1-2A, nrm1-4A* and *nrm1-8A* made by Rob de Bruin in the Curt Wittenburg lab with *cdc10-2A* and *cdc10-8A* to explore the possibility of transcriptional deregulation. However, I was unable to find sites on *nrm1* that lead to constitutive low MBF transcription when combined with *cdc10-2A* or with *cdc10-8A* in response to HU (Figure

3.8A). Thirteen potential phosphorylation sites on Nrm1 have been identified by mass spectroscopy. However, alanine substitution of all thirteen sites has not yet been made. If *nrm1-13A* was available, it would be easy to make *nrm1-13A cdc10-8A* and *nrm1-13A cdc10-2A* double mutant and look for MBF dependent transcription. In addition, phosphomimetic substitutions of potential phosphorylation sites on Nrm1 are also needed to corroborate our hypothesis, as we have shown with *cdc10-2E*.

In the second part of my research, I demonstrated that fission yeast modulates S-phase transcription if cells are arrested in S phase by either HU or MMS. We speculate that preventing mitosis might not be enough for survival when S phase is under stress and additional steps are needed to ensure the stalled forks are protected. By comparing wild type and *cds1*Δ *cdc10-2E* cells, I observed that the *cds1*Δ *cdc10-2E* cells are still very sensitive even though these cells are proficient in G2 DNA damage checkpoint and transcriptional response. These data provide direct evidence that the stabilization of the replication forks is the most important step for survival during replication stress.

Although the checkpoint dependent transcription is less important than fork stability, the over expression of *cdc22* do rescue the lethality associated with replication stress. We hypothesized that this is because in presence of excess nucleotide produced by *cdc22* over expression, replication forks do not arrest in the first place. This hypothesis is supported by the fact that in presence of high level of *cdc22*, the HU arrested cells do not activate Cds1 kinase activity (Rhind N personal communication).

The constitutive S-phase transcription in *rad3*Δ *cdc10-2E* suppresses the lethality of *rad3*Δ in MMS more significantly than in HU. I propose that since the MMS treated cells finish replication, *mik1* dependent delay in Cdc2 activation might provide time to

repair MMS induced damage. The fact that the cells treated with MMS finish replication may also explain why *cds1*Δ cells are not sensitive to MMS but extremely sensitive to HU.

As a part of my work I identified three S-phase transcripts that are important for survival during replication stress. One of them is *cdc22*. My data show over expression of *cdc22* by ten fold rescued not only acute HU treated cells but also rescued acute MMS treated *rad3*Δ *nmt1: cdc22* and *cds1*Δ *nmt1: cdc22* cells. The protection due to a 10 fold increase in *cdc22* level I observed in *rad3*Δ *nmt1: cdc22* and *cds1*Δ *nmt1: cdc22* is significantly higher than the protection I observed with 4 fold increase in *cdc22* expression seen in *rad3*Δ *cdc10-2E* and *cds1*Δ *cdc10-2E* allele in HU treated cells (Figure 3.7). Therefore these data suggest that if fission yeast had an independent RNR regulation similar to budding yeast, fission yeast could tolerate high levels of HU arrest like budding yeast.

An alternative explanation for not achieving similar level of survival benefit with *cdc10-2E* compared to the *nmt1: cdc22* allele in checkpoint deficient background is the presence of transcripts in *cdc10-2E* that could be potentially harmful during replication stress. For example, *cdt1* and *cdc18* are under MBF regulation and are constitutively up-regulated in presence of *cdc10-2E* allele. Both *cdt1* and *cdc18* play important roles during origin firing. During replication stress in response to HU or MMS, high transcription of *cdt1* and *cdc18* could be triggering re-replication. I propose to make *rad3*Δ *cdc10-2E nmt1:cdc22* and *cds1*Δ *cdc10-2E nmt1:cdc22* mutants and compare the survival of these strains relative to *rad3*Δ *cdc10-2E, rad3*Δ *nmt1:cdc22, cds1*Δ *cdc10-2E* and *cds1*Δ *nmt1:cdc2*2 mutants. The difference in survival between *rad3*Δ *cdc10-2E nmt1:cdc22*

and *rad3*Δ *cdc10-2E* will uncover any detrimental effect that might be contributed by *cdc10-2E* allele during stress.

However, why providing excess nucleotide should rescue MMS treated cells is not clear. In the presence of high levels of RNR, the HU arrested cells do not activate Cds1 kinase activity (Rhind N personal communication), which explains why cells survive in HU when *cdc22* is overexpressed. However the mechanism by which overexpression of *cdc22* rescues the lethality associated with MMS induced damage is not clear. I hypothesized that providing extra nucleotide might help cells finish the replication comparatively earlier than with normal level of nucleotides. This hypothesis could be tested by looking at cell cycle progression by FACS. In addition, Cds1 kinase activity could be determined in *rad3*Δ *nmt1:cdc22* and *cds1*Δ *nmt1:cdc22* cells treated with MMS.

We cannot rule out the possibility that other MBF targets are important during replication stress, specifically the role of Rad51 and RPA in homologous recombination in G2. It could be possible to look at the role of *rad51* by using *rad3*Δ *cdc10-2E rad51*Δ and *cds1*Δ *cdc10-2E rad51*Δ cells. In addition one could determine the role of RPA using allele of RPA, *rad11 D223Y* shown to be required for survival during HU, MMS, UV, γ irradiation. It will be useful to compare the survival of *rad3*Δ *cdc10-2E rad11 D223Y* and *cds1*Δ *cdc10-2E rad11 D223Y* cells (Parker et al 1997; Kibe T et al 2007; Ono Y et al 2003)

G1/S transcriptional regulation is a conserved mechanism in yeast and metazoans (Costanzo et al., 2004; de Bruin et al., 2006; de Bruin et al., 2004; Cooper, 2006). The Rfx1 dependent pathway in budding yeast is an exception, which over shadowed the

effect of MBF regulation in budding yeast. However, why cells up regulate whole S phase transcriptional machinery in response to replication stress is still open for debate. The conservation of the G1/S regulation among yeast and metazoans suggest that this pathway provides the necessary transcriptional regulation that cells need during replication stress. It is probably easier to modulate an existing pathway that serves all the necessary function for survival during replication stress than evolving a new pathway. For example, the adaptor protein needed for Cds1 activation is Mrc1. *mrc1* is a MBF target and available only during S phase, which explain why Cds1 is active only during S phase. Mrc1 is also a part of fork protection complex and actively involved during fork pausing during normal replication. Therefore linking *mrc1* in fork stabilizion and checkpoint activation serves the important step in the evolution MBF regulation by checkpoint.

Checkpoint regulation of MBF also up-regulates genes that are not very helpful during replication stress. For example *cdc18* and *cdt1*, which function during origin firing and should not be activated during S-phase arrest. To deal with possibly harmful consequences of MBF regulation, metazoans evolved an apoptosis pathway at G1/S transition. This pathway has evolved to add an additional step to maintain genome integrity in multicellular organisms.

List of abbreviation

ATM	Ataxia telangiectasia
ATR	ATM related protein
RFC	Replication factor C
DSB	Double stranded breaks
HU	Hydroxyurea (Sigma H8627-25G)
MMS	Methyl methane-sulfonate (Sigma M4016-5G)
YES	Yeast extract with supplements
LUAH	Edinburgh minimal media supplemented with leucine, uracil, adenine & histidine
FACS	Florescent activated cell sorter
GST	Glutathione S-transferase
GSH	Glutathione immobilized on beaded agarose (Sigma G4510)

References

Abraham, R. T. (2001). Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev *15*, 2177-2196.

Alcasabas, A. A., Osborn, A. J., Bachant, J., Hu, F., Werler, P. J., Bousset, K., Furuya, K., Diffley, J. F., Carr, A. M., and Elledge, S. J. (2001). Mrc1 transduces signals of DNA replication stress to activate Rad53. Nat Cell Biol *3*, 958-965.

Allen, J. B., Zhou, Z., Siede, W., Friedberg, E. C., and Elledge, S. J. (1994). The SAD1/RAD53 protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. Genes Dev *8*, 2401-2415.

Amon, A., Tyers, M., Futcher, B., and Nasmyth, K. (1993). Mechanisms that help the yeast cell cycle clock tick: G2 cyclins transcriptionally activate G2 cyclins and repress G1 cyclins. Cell *74*, 993-1007.

Andrews, B. J., and Herskowitz, I. (1989). The yeast SWI4 protein contains a motif present in developmental regulators and is part of a complex involved in cell-cycle-dependent transcription. Nature *342*, 830-833.

Baber-Furnari, B. A., Rhind, N., Boddy, M. N., Shanahan, P., Lopez-Girona, A., and Russell, P. (2000). Regulation of mitotic inhibitor Mik1 helps to enforce the DNA damage checkpoint. Mol Biol Cell *11*, 1-11.

Bahler, J. (2005). Cell-cycle control of gene expression in budding and fission yeast. Annu Rev Genet *39*, 69-94.

Bartek, J., Lukas, C., and Lukas, J. (2004). Checking on DNA damage in S phase. Nat Rev Mol Cell Biol *5*, 792-804.

Bartek, J., and Lukas, J. (2001). Pathways governing G1/S transition and their response to DNA damage. FEBS Lett *490*, 117-122.

Baum, B., Wuarin, J., and Nurse, P. (1997). Control of S-phase periodic transcription in the fission yeast mitotic cycle. EMBO J *16*, 4676-4688.

Bean, J. M., Siggia, E. D., and Cross, F. R. (2005). High functional overlap between MluI cell-cycle box binding factor and Swi4/6 cell-cycle box binding factor in the G1/S transcriptional program in Saccharomyces cerevisiae. Genetics *171*, 49-61.

Blasina, A., de Weyer, I. V., Laus, M. C., Luyten, W. H., Parker, A. E., and McGowan, C. H. (1999). A human homologue of the checkpoint kinase Cds1 directly inhibits Cdc25 phosphatase. Curr Biol *9*, 1-10.

Boddy, M. N., and Russell, P. (2001). DNA replication checkpoint. Curr Biol *11*, R953-6.

Breeden, L., and Nasmyth, K. (1987). Cell cycle control of the yeast HO gene: cis- and trans-acting regulators. Cell *48*, 389-397.

Brondello, J. M., Boddy, M. N., Furnari, B., and Russell, P. (1999). Basis for the checkpoint signal specificity that regulates Chk1 and Cds1 protein kinases. Mol Cell Biol *19*, 4262-4269.

Caligiuri, M., and Beach, D. (1993). Sct1 functions in partnership with Cdc10 in a transcription complex that activates cell cycle START and inhibits differentiation. Cell *72*, 607-619.

Carr, A. M. (1997). Control of cell cycle arrest by the Mec1sc/Rad3sp DNA structure checkpoint pathway. Curr Opin Genet Dev *7*, 93-98.

Chehab, N. H., Malikzay, A., Appel, M., and Halazonetis, T. D. (2000). Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. Genes Dev *14*, 278-288.

Chen, D., Toone, W. M., Mata, J., Lyne, R., Burns, G., Kivinen, K., Brazma, A., Jones, N., and Bahler, J. (2003a). Global transcriptional responses of fission yeast to environmental stress. Mol Biol Cell *14*, 214-229.

Chen, E. S., Yanagida, M., and Takahashi, K. (2003b). Does a GATA factor make the bed for centromeric nucleosomes? Cell Cycle *2*, 277-278.

Christensen, P. U., Bentley, N. J., Martinho, R. G., Nielsen, O., and Carr, A. M. (2000). Mik1 levels accumulate in S phase and may mediate an intrinsic link between S phase and mitosis. Proc Natl Acad Sci U S A *97*, 2579-2584.

Chu, Z., Li, J., Eshaghi, M., Peng, X., Karuturi, R. K., and Liu, J. (2007). Modulation of cell cycle-specific gene expressions at the onset of S phase arrest contributes to the robust DNA replication checkpoint response in fission yeast. Mol Biol Cell *18*, 1756-1767.

Cooper, K. (2006). Rb, whi it's not just for metazoans anymore. Oncogene *25*, 5228-5232.

Costanzo, M., Nishikawa, J. L., Tang, X., Millman, J. S., Schub, O., Breitkreuz, K., Dewar, D., Rupes, I., Andrews, B., and Tyers, M. (2004). CDK activity antagonizes Whi5, an inhibitor of G1/S transcription in yeast. Cell *117*, 899-913.

Dalal, S. N., Schweitzer, C. M., Gan, J., and DeCaprio, J. A. (1999). Cytoplasmic localization of human cdc25C during interphase requires an intact 14-3-3 binding site. Mol Cell Biol *19*, 4465-4479.

de Bruin, R. A., Kalashnikova, T. I., Aslanian, A., Wohlschlegel, J., Chahwan, C., Yates, J. R. r., Russell, P., and Wittenberg, C. (2008). DNA replication checkpoint promotes G1-S transcription by inactivating the MBF repressor Nrm1. Proc Natl Acad Sci U S A *105*, 11230-11235.

de Bruin, R. A., Kalashnikova, T. I., Chahwan, C., McDonald, W. H., Wohlschlegel, J., Yates, J. r., Russell, P., and Wittenberg, C. (2006). Constraining G1-specific transcription to late G1 phase: the MBF-associated corepressor Nrm1 acts via negative feedback. Mol Cell *23*, 483-496.

de Bruin, R. A., McDonald, W. H., Kalashnikova, T. I., Yates, J. r., and Wittenberg, C. (2004). Cln3 activates G1-specific transcription via phosphorylation of the SBF bound repressor Whi5. Cell *117*, 887-898.

DeCaprio, J. A., Ludlow, J. W., Lynch, D., Furukawa, Y., Griffin, J., Piwnica-Worms, H., Huang, C. M., and Livingston, D. M. (1989). The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. Cell *58*, 1085-1095.

Desany, B. A., Alcasabas, A. A., Bachant, J. B., and Elledge, S. J. (1998). Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway. Genes Dev *12*, 2956-2970.

Dirick, L., Bohm, T., and Nasmyth, K. (1995). Roles and regulation of Cln-Cdc28 kinases at the start of the cell cycle of Saccharomyces cerevisiae. EMBO J *14*, 4803-4813.

Dirick, L., Moll, T., Auer, H., and Nasmyth, K. (1992). A central role for SWI6 in modulating cell cycle Start-specific transcription in yeast. Nature *357*, 508-513.

Durocher, D., Smerdon, S. J., Yaffe, M. B., and Jackson, S. P. (2000). The FHA domain in DNA repair and checkpoint signaling. Cold Spring Harb Symp Quant Biol *65*, 423-431.

Eisen, M. B., Spellman, P. T., Brown, P. O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A *95*, 14863-14868.

Elledge, S. J. (1996). Cell cycle checkpoints: preventing an identity crisis. Science *274*, 1664-1672.

Enoch, T., Carr, A. M., and Nurse, P. (1992). Fission yeast genes involved in coupling mitosis to completion of DNA replication. Genes Dev *6*, 2035-2046.

Enoch, T., and Nurse, P. (1990). Mutation of fission yeast cell cycle control genes abolishes dependence of mitosis on DNA replication. Cell *60*, 665-673.

Espinoza, F. H., Ogas, J., Herskowitz, I., and Morgan, D. O. (1994). Cell cycle control by a complex of the cyclin HCS26 (PCL1) and the kinase PHO85. Science *266*, 1388-1391.

Falck, J., Petrini, J. H., Williams, B. R., Lukas, J., and Bartek, J. (2002). The DNA damage-dependent intra-S phase checkpoint is regulated by parallel pathways. Nat Genet *30*, 290-294.

Featherstone, C., and Russell, P. (1991). Fission yeast p107wee1 mitotic inhibitor is a tyrosine/serine kinase. Nature *349*, 808-811.

Forsburg, S. L., and Rhind, N. (2006). Basic methods for fission yeast. Yeast *23*, 173-183.

Frolov, M. V., and Dyson, N. J. (2004). Molecular mechanisms of E2F-dependent activation and pRB-mediated repression. J Cell Sci *117*, 2173-2181.

Furnari, B., Rhind, N., and Russell, P. (1997). Cdc25 mitotic inducer targeted by chk1 DNA damage checkpoint kinase. Science *277*, 1495-1497.

Gasch, A. P., Huang, M., Metzner, S., Botstein, D., Elledge, S. J., and Brown, P. O. (2001). Genomic expression responses to DNA-damaging agents and the regulatory role of the yeast ATR homolog Mec1p. Mol Biol Cell *12*, 2987-3003.

Gordon, C. B., and Fantes, P. A. (1986). The cdc22 gene of Schizosaccharomyces pombe encodes a cell cycle-regulated transcript. EMBO J *5*, 2981-2985.

Gould, K. L., and Nurse, P. (1989). Tyrosine phosphorylation of the fission yeast cdc2+ protein kinase regulates entry into mitosis. Nature *342*, 39-45.

Harrington, L. A., and Andrews, B. J. (1996). Binding to the yeast SwI4,6-dependent cell cycle box, CACGAAA, is cell cycle regulated in vivo. Nucleic Acids Res *24*, 558-565.

Hartwell, L. H., and Weinert, T. A. (1989). Checkpoints: controls that ensure the order of cell cycle events. Science *246*, 629-634.

Henriksson, M. L., Francis, M. S., Peden, A., Aili, M., Stefansson, K., Palmer, R., Aitken, A., and Hallberg, B. (2002). A nonphosphorylated 14-3-3 binding motif on exoenzyme S that is functional in vivo. Eur J Biochem *269*, 4921-4929.

Hirao, A., Kong, Y. Y., Matsuoka, S., Wakeham, A., Ruland, J., Yoshida, H., Liu, D., Elledge, S. J., and Mak, T. W. (2000). DNA damage-induced activation of p53 by the checkpoint kinase Chk2. Science *287*, 1824-1827.

Huang, M., Zhou, Z., and Elledge, S. J. (1998). The DNA replication and damage checkpoint pathways induce transcription by inhibition of the Crt1 repressor. Cell *94*, 595-605.

Humphrey, T. (2000). DNA damage and cell cycle control in Schizosaccharomyces pombe. Mutat Res *451*, 211-226.

Ishida, S., Huang, E., Zuzan, H., Spang, R., Leone, G., West, M., and Nevins, J. R. (2001). Role for E2F in control of both DNA replication and mitotic functions as revealed from DNA microarray analysis. Mol Cell Biol *21*, 4684-4699.

Jang, Y. K., Jin, Y. H., Myung, K., Seong, R. H., Hong, S. H., and Park, S. D. (1996). Differential expression of the rhp51+ gene, a recA and RAD51 homolog from the fission yeast Schizosaccharomyces pombe. Gene *169*, 125-130.

Kai, M., Tanaka, H., and Wang, T. S. (2001). Fission yeast Rad17 associates with chromatin in response to aberrant genomic structures. Mol Cell Biol *21*, 3289-3301.

Katayama, S., Kitamura, K., Lehmann, A., Nikaido, O., and Toda, T. (2002). Fission yeast F-box protein Pof3 is required for genome integrity and telomere function. Mol Biol Cell *13*, 211-224.

Kelly, T. J., Martin, G. S., Forsburg, S. L., Stephen, R. J., Russo, A., and Nurse, P. (1993). The fission yeast cdc18+ gene product couples S phase to START and mitosis. Cell *74*, 371-382.

Kinoshita-Kikuta, E., Aoki, Y., Kinoshita, E., and Koike, T. (2007). Label-free kinase profiling using phosphate affinity polyacrylamide gel electrophoresis. Mol Cell Proteomics *6*, 356-366.

Koch, C., Moll, T., Neuberg, M., Ahorn, H., and Nasmyth, K. (1993). A role for the transcription factors Mbp1 and Swi4 in progression from G1 to S phase. Science *261*, 1551-1557.

Koch, C., Schleiffer, A., Ammerer, G., and Nasmyth, K. (1996). Switching transcription on and off during the yeast cell cycle: Cln/Cdc28 kinases activate bound transcription factor SBF (Swi4/Swi6) at start, whereas Clb/Cdc28 kinases displace it from the promoter in G2. Genes Dev *10*, 129-141.

Kommajosyula, N., and Rhind, N. (2006). Cdc2 tyrosine phosphorylation is not required for the S-phase DNA damage checkpoint in fission yeast. Cell Cycle *5*, 2495-2500.

Kowalik, T. F., DeGregori, J., Schwarz, J. K., and Nevins, J. R. (1995). E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. J Virol *69*, 2491-2500.

Krek, W., Ewen, M. E., Shirodkar, S., Arany, Z., Kaelin, W. G. J., and Livingston, D. M. (1994). Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A-dependent protein kinase. Cell *78*, 161-172.

Lee, M. S., Enoch, T., and Piwnica-Worms, H. (1994). mik1+ encodes a tyrosine kinase that phosphorylates p34cdc2 on tyrosine 15. J Biol Chem *269*, 30530-30537.

Limbo, O., Chahwan, C., Yamada, Y., de Bruin, R. A., Wittenberg, C., and Russell, P. (2007). Ctp1 is a cell-cycle-regulated protein that functions with Mre11 complex to control double-strand break repair by homologous recombination. Mol Cell *28*, 134-146.

Lopez-Girona, A., Furnari, B., Mondesert, O., and Russell, P. (1999). Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. Nature *397*, 172-175.

Lowndes, N. F., Johnson, A. L., Breeden, L., and Johnston, L. H. (1992). SWI6 protein is required for transcription of the periodically expressed DNA synthesis genes in budding yeast. Nature *357*, 505-508.

Lowndes, N. F., Johnson, A. L., and Johnston, L. H. (1991). Coordination of expression of DNA synthesis genes in budding yeast by a cell-cycle regulated trans factor. Nature *350*, 247-250.

Martin, K., Trouche, D., Hagemeier, C., and Kouzarides, T. (1995). Regulation of transcription by E2F1/DP1. J Cell Sci Suppl *19*, 91-94.

Matsuoka, S., Huang, M., and Elledge, S. J. (1998). Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science *282*, 1893-1897.

McGowan, C. H. (2002). Checking in on Cds1 (Chk2): A checkpoint kinase and tumor suppressor. Bioessays *24*, 502-511.

McInerny, C. J., Kersey, P. J., Creanor, J., and Fantes, P. A. (1995). Positive and negative roles for cdc10 in cell cycle gene expression. Nucleic Acids Res *23*, 4761-4768.

Measday, V., Moore, L., Ogas, J., Tyers, M., and Andrews, B. (1994). The PCL2 (ORFD)-PHO85 cyclin-dependent kinase complex: a cell cycle regulator in yeast. Science *266*, 1391-1395.

Measday, V., Moore, L., Retnakaran, R., Lee, J., Donoviel, M., Neiman, A. M., and Andrews, B. (1997). A family of cyclin-like proteins that interact with the Pho85 cyclin-dependent kinase. Mol Cell Biol *17*, 1212-1223.

Melo, J., and Toczyski, D. (2002). A unified view of the DNA-damage checkpoint. Curr Opin Cell Biol *14*, 237-245.

Millar, J. B., Blevitt, J., Gerace, L., Sadhu, K., Featherstone, C., and Russell, P. (1991). p55CDC25 is a nuclear protein required for the initiation of mitosis in human cells. Proc Natl Acad Sci U S A *88*, 10500-10504.

Millar, J. B., Lenaers, G., and Russell, P. (1992). Pyp3 PTPase acts as a mitotic inducer in fission yeast. EMBO J *11*, 4933-4941.

Miyamoto, M., Tanaka, K., and Okayama, H. (1994). res2+, a new member of the cdc10+/SWI4 family, controls the 'start' of mitotic and meiotic cycles in fission yeast. EMBO J *13*, 1873-1880.

Murakami, H., Yanow, S. K., Griffiths, D., Nakanishi, M., and Nurse, P. (2002). Maintenance of replication forks and the S-phase checkpoint by Cdc18p and Orp1p. Nat Cell Biol *4*, 384-388.

Nevins, J. R. (1998). Toward an understanding of the functional complexity of the E2F and retinoblastoma families. Cell Growth Differ *9*, 585-593.

Ng, S. S., Anderson, M., White, S., and McInerny, C. J. (2001). mik1(+) G1-S transcription regulates mitotic entry in fission yeast. FEBS Lett *503*, 131-134.

Nitani, N., Nakamura, K., Nakagawa, C., Masukata, H., and Nakagawa, T. (2006). Regulation of DNA replication machinery by Mrc1 in fission yeast. Genetics *174*, 155-165.

O'Neill, T., Giarratani, L., Chen, P., Iyer, L., Lee, C. H., Bobiak, M., Kanai, F., Zhou, B. B., Chung, J. H., and Rathbun, G. A. (2002). Determination of substrate motifs for human Chk1 and hCds1/Chk2 by the oriented peptide library approach. J Biol Chem *277*, 16102-16115.

Obara-Ishihara, T., and Okayama, H. (1994). A B-type cyclin negatively regulates conjugation via interacting with cell cycle 'start' genes in fission yeast. EMBO J *13*, 1863-1872.

Oliva, A., Rosebrock, A., Ferrezuelo, F., Pyne, S., Chen, H., Skiena, S., Futcher, B., and Leatherwood, J. (2005). The cell cycle-regulated genes of Schizosaccharomyces pombe. PLoS Biol *3*, e225.

Osborn, A. J., Elledge, S. J., and Zou, L. (2002). Checking on the fork: the DNA-replication stress-response pathway. Trends Cell Biol *12*, 509-516.

Painter, R. B., and Young, B. R. (1980). Radiosensitivity in ataxia-telangiectasia: a new explanation. Proc Natl Acad Sci U S A *77*, 7315-7317.

Parker, A. E., Clyne, R. K., Carr, A. M., and Kelly, T. J. (1997). The Schizosaccharomyces pombe rad11+ gene encodes the large subunit of replication protein A. Mol Cell Biol *17*, 2381-2390.

Parker, L. L., Atherton-Fessler, S., and Piwnica-Worms, H. (1992). p107wee1 is a dual-specificity kinase that phosphorylates p34cdc2 on tyrosine 15. Proc Natl Acad Sci U S A *89*, 2917-2921.

Peng, H., Zalkow, L. H., Abraham, R. T., and Powis, G. (1998). Novel CDC25A phosphatase inhibitors from pyrolysis of 3-alpha-azido-B-homo-6-oxa-4-cholesten-7-one on silica gel. J Med Chem *41*, 4677-4680.

Peng, X., Karuturi, R. K., Miller, L. D., Lin, K., Jia, Y., Kondu, P., Wang, L., Wong, L. S., Liu, E. T., Balasubramanian, M. K., and Liu, J. (2005). Identification of cell cycle-regulated genes in fission yeast. Mol Biol Cell *16*, 1026-1042.

Qin, X. Q., Livingston, D. M., Kaelin, W. G. J., and Adams, P. D. (1994). Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. Proc Natl Acad Sci U S A *91*, 10918-10922.

Rhind, N., Furnari, B., and Russell, P. (1997). Cdc2 tyrosine phosphorylation is required for the DNA damage checkpoint in fission yeast. Genes Dev *11*, 504-511.

Rhind, N., and Russell, P. (1998). Tyrosine phosphorylation of cdc2 is required for the replication checkpoint in Schizosaccharomyces pombe. Mol Cell Biol *18*, 3782-3787.

Rhind, N., and Russell, P. (2001). Roles of the mitotic inhibitors Wee1 and Mik1 in the G(2) DNA damage and replication checkpoints. Mol Cell Biol *21*, 1499-1508.

Russell, P., and Nurse, P. (1986). cdc25+ functions as an inducer in the mitotic control of fission yeast. Cell *45*, 145-153.

Russell, P., and Nurse, P. (1987). Negative regulation of mitosis by wee1+, a gene encoding a protein kinase homolog. Cell *49*, 559-567.

Rustici, G., Mata, J., Kivinen, K., Lio, P., Penkett, C. J., Burns, G., Hayles, J., Brazma, A., Nurse, P., and Bahler, J. (2004). Periodic gene expression program of the fission yeast cell cycle. Nat Genet *36*, 809-817.

Seo, G. J., Kim, S. E., Lee, Y. M., Lee, J. W., Lee, J. R., Hahn, M. J., and Kim, S. T. (2003). Determination of substrate specificity and putative substrates of Chk2 kinase. Biochem Biophys Res Commun *304*, 339-343.

Shiloh, Y. (2003). ATM and related protein kinases: safeguarding genome integrity. Nat Rev Cancer *3*, 155-168.

Siegmund, R. F., and Nasmyth, K. A. (1996). The Saccharomyces cerevisiae Start-specific transcription factor Swi4 interacts through the ankyrin repeats with the mitotic Clb2/Cdc28 kinase and through its conserved carboxy terminus with Swi6. Mol Cell Biol *16*, 2647-2655.

Simanis, V., and Nurse, P. (1989). Characterization of the fission yeast cdc10+ protein that is required for commitment to the cell cycle. J Cell Sci *92*, 51-56.

Slansky, J. E., and Farnham, P. J. (1996). Introduction to the E2F family: protein structure and gene regulation. Curr Top Microbiol Immunol *208*, 1-30.

Sommariva, E., Pellny, T. K., Karahan, N., Kumar, S., Huberman, J. A., and Dalgaard, J. Z. (2005). Schizosaccharomyces pombe Swi1, Swi3, and Hsk1 are components of a novel S-phase response pathway to alkylation damage. Mol Cell Biol *25*, 2770-2784.

Stevens, C., Smith, L., and La Thangue, N. B. (2003). Chk2 activates E2F-1 in response to DNA damage. Nat Cell Biol *5*, 401-409.

Taba, M. R., Muroff, I., Lydall, D., Tebb, G., and Nasmyth, K. (1991). Changes in a SWI4,6-DNA-binding complex occur at the time of HO gene activation in yeast. Genes Dev *5*, 2000-2013.

Tanaka, K., Okazaki, K., Okazaki, N., Ueda, T., Sugiyama, A., Nojima, H., and Okayama, H. (1992). A new cdc gene required for S phase entry of Schizosaccharomyces pombe encodes a protein similar to the cdc 10+ and SWI4 gene products. EMBO J *11*, 4923-4932.

Tanaka, K., and Russell, P. (2001). Mrc1 channels the DNA replication arrest signal to checkpoint kinase Cds1. Nat Cell Biol *3*, 966-972.

Wach, A., Brachat, A., Pohlmann, R., and Philippsen, P. (1994). New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae. Yeast *10*, 1793-1808.

Watson, A., Mata, J., Bahler, J., Carr, A., and Humphrey, T. (2004). Global gene expression responses of fission yeast to ionizing radiation. Mol Biol Cell *15*, 851-860.

Weintraub, S. J., Chow, K. N., Luo, R. X., Zhang, S. H., He, S., and Dean, D. C. (1995). Mechanism of active transcriptional repression by the retinoblastoma protein. Nature *375*, 812-815.

Weintraub, S. J., Prater, C. A., and Dean, D. C. (1992). Retinoblastoma protein switches the E2F site from positive to negative element. Nature *358*, 259-261.

White, S., Khaliq, F., Sotiriou, S., and McInerny, C. J. (2001). The role of DSC1 components cdc10+, rep1+ and rep2+ in MCB gene transcription at the mitotic G1-S boundary in fission yeast. Curr Genet *40*, 251-259.

Whitehall, S., Stacey, P., Dawson, K., and Jones, N. (1999). Cell cycle-regulated transcription in fission yeast: Cdc10-Res protein interactions during the cell cycle and domains required for regulated transcription. Mol Biol Cell *10*, 3705-3715.

Wolkow, T. D., and Enoch, T. (2002). Fission yeast Rad26 is a regulatory subunit of the Rad3 checkpoint kinase. Mol Biol Cell *13*, 480-492.

Xu, M., Sheppard, K. A., Peng, C. Y., Yee, A. S., and Piwnica-Worms, H. (1994). Cyclin A/CDK2 binds directly to E2F-1 and inhibits the DNA-binding activity of E2F-1/DP-1 by phosphorylation. Mol Cell Biol *14*, 8420-8431.

Yang, J., Winkler, K., Yoshida, M., and Kornbluth, S. (1999). Maintenance of G2 arrest in the Xenopus oocyte: a role for 14-3-3-mediated inhibition of Cdc25 nuclear import. EMBO J *18*, 2174-2183.

Zeng, Y., and Piwnica-Worms, H. (1999). DNA damage and replication checkpoints in fission yeast require nuclear exclusion of the Cdc25 phosphatase via 14-3-3 binding. Mol Cell Biol *19*, 7410-7419.

Zhang, S. H., Kobayashi, R., Graves, P. R., Piwnica-Worms, H., and Tonks, N. K. (1997). Serine phosphorylation-dependent association of the band 4.1-related protein-tyrosine phosphatase PTPH1 with 14-3-3beta protein. J Biol Chem *272*, 27281-27287.

Zhou, Z., and Elledge, S. J. (1992). Isolation of crt mutants constitutive for transcription of the DNA damage inducible gene RNR3 in Saccharomyces cerevisiae. Genetics *131*, 851-866.

Zhou, Z., and Elledge, S. J. (1993). DUN1 encodes a protein kinase that controls the DNA damage response in yeast. Cell *75*, 1119-1127.

Zhu, Y., Takeda, T., Whitehall, S., Peat, N., and Jones, N. (1997). Functional characterization of the fission yeast Start-specific transcription factor Res2. EMBO J *16*, 1023-1034.

Zhu, Y., and Xiao, W. (2001). Two alternative cell cycle checkpoint pathways differentially control DNA damage-dependent induction of MAG1 and DDI1 expression in yeast. Mol Genet Genomics *266*, 436-444.

Zwicker, J., Liu, N., Engeland, K., Lucibello, F. C., and Muller, R. (1996). Cell cycle regulation of E2F site occupation in vivo. Science *271*, 1595-1597.