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The checkpoint role of Cdc18 in fission yeast

Thesis presented for the degree of

Doctor of Philosophy

University of London

by Naomi Fersht

Cell Cycle Laboratory

Cancer Research UK London Institute

44 Lincoln's Inn Fields, London

Supervisor: Dr Paul Nurse

February 2007

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Declaration

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

Abstract

The highly conserved eukaryotic checkpoints keep tight control over cell cycle progression, arresting the cell in response to incomplete DNA replication or DNA damage. In fission yeast, Rad3 (functional homologue of ATM, mutated in ataxia telangiectasia, and structural homologue of ATR, ataxia telangiectasia and rad3 related) is necessary for activation of both replication and damage checkpoints. However, despite the identification of many checkpoint genes, the actual sequence of upstream events leading to Rad3 activation remains unclear. The aim of my project was to identify and characterise the Rad3-dependent DNA damage/perturbed replication sensors and checkpoint activators.

A genetic screen was carried out in fission yeast, using the working hypothesis that overexpression of these sensors/checkpoint activators would ectopically induce a Rad3-dependent block over mitosis in the absence of DNA damage or disturbed replication. The screen identified several genes of which the DNA replication initiation factor Cdc18/CDC6 had the strongest and most reproducible phenotype. Cdc18 is essential to prevent mitosis during S phase. I chose to concentrate on characterisation of the Rad3-dependent checkpoint role of Cdc18.

The actual level of Cdc18 is important for producing the Rad3-dependent cell cycle block. A stabilised Cdc18 protein, mutated at the conserved CDK (cyclin dependent kinase) consensus sites also caused a Rad3-dependent cell cycle arrest, and was more stable and easier to manipulate than the screen-

derived clone. Genetic crosses demonstrated Cdc18 acts early on in the checkpoint pathway, and through Crb2/Chk1. There was no gross DNA damage or detectable replication intermediates in the presence of elevated or stabilised Cdc18 levels. I also found that artificial depletion of Cdc18 during an S phase block results in loss of the checkpoint but not the replication structures, uncoupling the maintenance of replication forks from the maintenance of the mitotic block.

An unexpected consequence of Cdc18 stabilisation was an increase in the size and variability of chromosome III on pulsed field gel electrophoresis. This localised to an expansion of Sfi1 restriction fragments containing the rDNA repeats.

In conclusion, Cdc18 stabilisation activates a Rad3-dependent checkpoint in the absence of apparent re-replication, which is associated with an expansion of the rDNA repeats on chromosome III. Two models are proposed. In the first, Cdc18 induces low level genome wide replication, that is undetectable but sufficient for checkpoint activation. This leads to increased recombination with unequal crossover events in the rDNA repeats on chromosome III, with subsequent repeat expansion. In the second, the increased levels of Cdc18 directly activate the cell cycle checkpoint independently of the concurrent expansion of chromosome III.

Acknowledgements

I would like to start by thanking my supervisor, Paul Nurse, first for taking on a medic as a PhD student, and then for his continued support and inspiration over the next 6 years. It has been a great privilege to work in his laboratory.

I want to say a very special thank you to all the past and present members of the cell cycle laboratory, especially Trevor Duhig, Nigel Peat and Jacky Hayles. Jacky has been a great friend, always supportive, interested and full of enthusiasm and great ideas (both for science and life in general).

I also want to thank my parents. My mother has made working following the arrival of my two children possible, and I cannot thank her enough for all the love and care. My father was my first, and will always be my greatest, scientific inspiration.

Finally, a massive thank you to my husband, Alister, for sharing the ups and downs of this scientific journey, and to Joseph and Ruby for being such brilliant children.

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This thesis is dedicated to my husband Alister, and to our children Joseph and Ruby.

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List of Abbreviations

anaphase promoting complex (APC)

ataxia telangiectasia (A-T)

autonomously replicating sequence (ars)

base pairs (bp)

Cell Cycle Laboratory (CCL)

cell division cycle (cdc)

clamp complex (CCC)

clamp loader (CCL)

cell untimely torn (cut)

cyclin-dependent kinase (CDK)

cyclin-dependent kinase-activating kinase (CAK)

double-stand break (DSB)

DNA synthesis phase (S phase)

Escherichia coli (E.coli)

ethidium bromide (EtBr)

fluorescent activated cell sorting (FACS)

5'-untranslated region (5'UTR)

forward mutation rate (FMR)

homologous recombination (HR)

Hydroxyurea (HU)

minichromosome maintenance complex (MCM)

mitosis (M)

methyl methanesulfanate (MMS)

mitogen activated protein kinase (MAPK)

non-homologous end joining (NHEJ)

origin recognition complex (ORC)

origin recognition complex from *S. pombe* (Orp)

plating efficiency (P.E.)

pre-initiation complex (pre-IC)

pre-replicative complex (pre-RC)

proliferating cell nuclear antigen (PCNA)

pulsed field gel (PFG)

pulsed field gel electrophoresis (PFGE)

rad9-rad1-hus1 (9-1-1)

replication fork barrier (RFB)

restriction point (R-point)

replication factor C, (RFC)

ribosomal DNA (rDNA)

Saccharomyces cerevisiae (S. cerevisiae)

Schizosaccharomyces pombe (S. pombe)

SMC (structural maintenance of chromosomes)

switch off (S/O)

Thiamine (T)

Two-dimensional (2D)

Tyrosine 15 (Y15)

Publications Arising From This Thesis

Fersht, N, Hermand, D, Hayles, J and Nurse, P. (2007) Cdc18/CDC6 activates the Rad3-dependent checkpoint in the fission yeast. (Submitted).

Notes on Collaborative Work

A number of experiments concerned with the characterisation of the checkpoint role of Cdc18 described in this thesis were done jointly with Dr Damien Hermand (a post-doctoral fellow in the Cell Cycle Laboratory).

These were: the comparison of the Cdc18 protein level in different expression vectors and mutated strains *(Figure 3.2)*; the phosphorylation of Chk1 in the Cdc18 T6A phosphorylation mutant (*Figure 3.3*); the accumulation of Cdc18 in an hydroxyurea (HU) induced cell cycle block *(Figure 3.7)*; the 2D DNA gels demonstrating (1) Cdc18 is not required to maintain replication intermediates in an HU block *(Figure 3.8)*, and (2) the absence of replication intermediates in G2 arrested *Cdc18 T6A* cells *(Figure 4.2).*

I am the corresponding author for, and joint first author of, the work submitted by *Fersht et al.*, (2007).

Chapter 1
Introduction

1.1 General overview of the eukaryotic mitotic cell cycle

Before a cell can divide it must first faithfully copy its DNA so that each daughter cell receives a full complement of genetic information. The eukaryotic cell cycle consists of S phase, mitosis and the gap phases, G1 and G2, and is defined by the ordered temporal progression from G1-S-G2-M (Murray and Hunt 1993). Chromosomal duplication is restricted to S phase (DNA synthesis phase), and chromosomal segregation to M phase (Mitosis). S phase and Mitosis are separated by the gap phases, G1 and G2 (*Figure 1.1*). Eukaryotic checkpoints keep tight control over the order of these events, delaying the cell cycle in response to incomplete DNA replication or DNA damage (Hartwell and Weinert 1989). The damage checkpoint operates in G1, S and G2 to prevent entry into mitosis with damaged DNA. In S phase, the replication checkpoint ensures DNA replication is complete before mitosis and cell division commences (Hartwell and Weinert 1989, Forsburg and Nurse 1994). The spindle checkpoint monitors the separation of chromosomes during mitosis, with cell cycle arrest subsequent to mitotic spindle damage or incorrect attachment of the chromosomes to the mitotic spindle (Amon 1999). Mistakes in these checkpoint controls result in the incomplete transmission of genetic information, with loss of genomic integrity and changes in ploidy. This genomic instability contributes to cancer development in higher organisms and may cause resistance to standard treatments (Hartwell and Kastan 1994).

The fundamental mechanisms of cell cycle progression and checkpoint responses are highly conserved from yeast to humans. The stable haploid cell

cycle of the fission yeast *Schizosaccharomyces pombe* makes it amenable to genetic analysis with well-established molecular biology and cytology techniques (Moreno *et al.* 1991). The mitotic cell cycle and checkpoint controls resemble that of higher organisms with distinct G1-S-G2-M phases, with mammalian homologues identified for the major fission yeast cell cycle and checkpoint genes. It has been used as a model system for the study of several cell biological problems including the mitotic cell cycle. As all the experimental work described in this thesis was carried out in *S. pombe*, I will concentrate on the fission yeast cell cycle.

S. pombe is a unicellular cylindrical eukaryote. It measures 3-4μm in diameter, and 8-15μm in length. Most growth occurs as a consequence of cell elongation, with the cell diameter remaining approximately constant. As a result, the length of a cell reflects how far it has passed through the cell cycle: new daughter cells are the shortest, cells just about to divide are the longest (Mitchison 1971, Fantes and Nurse 1977, Fantes 1977, Mitchison and Nurse 1985).

At key cell cycle stages, cells have to attain certain characteristics to pass through. The first is in late G1, when eukaryotic cells need to decide between two developmental programmes: to commit to progress from the G1 phase of the cell cycle to chromosomal DNA replication; or to exit from the proliferation cycle and differentiate. This decision is dependent on mitogenic stimuli, and is made at the restriction (R)-point in the mammalian cell cycle (Pardee 1974, Zetterberg and Larsson 1985) (reviewed by Zetterberg *et al.* 1995). Higher eukaryotes can also choose to exit the cell cycle altogether and enter a non-dividing state, G0. From G0, they can re-enter the cell cycle at a

later point in time. The R-point is equivalent to "Start" in fission yeast (Nurse and Bissett 1981, Aves *et al.* 1985, Simanis and Nurse 1989). In the presence of adequate nutritional conditions, fission yeast cells grow to the critical cell mass necessary to pass "Start" and undergo DNA replication (Hartwell 1974, Nurse 1975, Nurse 1981). Starved cells undergo G1 arrest, and, if both h+ and h- cells are present, can leave the proliferation cycle to undergo subsequent conjugation and meiosis. Hence, fission yeast can only "differentiate" or initiate alternate pathways of development from pre-Start G1 (reviewed by Woollard and Nurse 1995). The *rum1* gene is crucial in fission yeast to keep the cell in pre-Start G1: cells undergo Start immediately after mitosis in its absence; G2 cells will skip mitosis to get to pre-Start G1 when *rum1* is overexpressed (Moreno *et al.* 1994).

Fission yeast experience a very short G1 before entering S phase, where DNA replication takes place and each chromosome is duplicated to make two sister chromatids (Nurse *et al.* 1976, Nurse and Thuriaux 1977). In eukaryotic cells, sisters remain physically connected from S phase synthesis until mitotic segregation (Uhlmann and Nasmyth 1998). Sister chromatid cohesion is essential for sister separation to opposite poles of the cell at mitosis (Miyazaki and Orr-Weaver 1994). It also enables chromosomal segregation to take place long after replication, by providing the cell with a "memory" of the duplication process. This temporal separation of the eukaryotic cell cycle, with its ability to define which chromatids need to be separated at cell division, distinguishes it from the bacterial cell cycle, where chromosomal segregation follows straight on after DNA replication. The separation of the S and M phases enables both chromosomal condensation, essential for separation of large genomes, and the

two rounds of chromosomal segregation required after a single round of replication in meiosis.

Before entry into mitosis the cells will approximately double their size to reach a critical cell mass. G2 takes up around 70% of total cell cycle time. Late G2 phase is the second key cell cycle stage as cells need to check DNA replication is complete and there is no DNA damage before proceeding into mitosis (the G2-M checkpoint).

Mitosis consists of a sequence of events common to most eukaryotes (Nasmyth 2005). The first phase of mitosis (prophase, prometaphase and metaphase) concerns the amphitelic attachment of sister chromatids (attachment to microtubules from opposing poles of the mitotic spindle). Syntelic attachment (with both sisters attached to the same pole) causes daughter cell aneuploidy. Chromosomes condense in prophase. The nuclear membrane dissolves to mark the start of prometaphase, with proteins attaching to the centromeres to create the kinetocores. Microtubules attach to the kinetocores, and in metaphase the sister chromatids align along the centre of the mitotic spindle. This final key cell cycle stage demands that the chromosomes are correctly aligned and the spindle properly formed (the spindle checkpoint).

The second phase of mitosis concerns the simultaneous destruction of all the connections between sister chromatids and subsequent traction towards the opposing poles in anaphase (Uhlmann *et al.* 1999). In telophase the two daughter nuclei are formed, and the chromosomes disperse. With exit from mitosis the spindle breaks down and a septum forms for cytokinesis, the

splitting of the cell into two daughter cells. As G1 is so short in fission yeast, cytokinesis actually occurs at the subsequent S phase *(Figure 1.1)*.

The mammalian cell cycle stages are similar to those of *S. pombe*. However, there are some important differences. In mammalian cells, G1 and the interpretation of extra-cellular signals is more critical as a decision is needed either to continue proliferation or to exit the cell cycle and differentiate (reviewed by Planas-Silva and Weinberg 1997). Also, dissolution of the nuclear membrane occurs with mitotic entry in mammalian cells, whereas in fission yeast the nuclear membrane does not breakdown and a closed mitosis occurs.

1.2 DNA replication

The fidelity of DNA replication is essential for maintenance of genomic integrity. All eukaryotes accurately and temporally regulate chromosomal replication. Replication control is conserved throughout evolution with homologues of the initiators and regulators found in many organisms (Dutta and Bell 1997).

Eukaryotic chromosomal replication is initiated at multiple sites called replication origins, ars (autonomously replicating sequences), first identified in *S. cerevisiae* (Bell and Stillman 1992). The replication origins direct the formation of the pre-replicative complex (pre-RC) (Kelly and Brown 2000). The pre-RC is made up of a number of proteins: the evolutionarily conserved six subunit ORC (origin recognition complex); CDC6/Cdc18 (Kelly *et al.*, 1993) and CDT1 (cdc10 dependent transcript 1); the heterohexameric MCM (minichromosome maintenance) complex containing MCM2-7. Replication origins are recognised by the ORC, and binding of ORC to origins persists

throughout the cell cycle (Bell and Stillman 1992, Bell *et al.* 1993, Micklem *et al.* 1993, Grallert and Nurse 1996, Leatherwood *et al.* 1996, Lygerou *et al.* 1999). In late mitosis, origins are licensed by a step-wise recruitment of the pre-RC proteins to the ORC platform (*see Figure* 2). When CDC6/Cdc18 and CDT1 have been loaded onto the DNA, they both promote the loading of MCM onto chromatin (Nishitani and Nurse 1995, Coleman *et al.* 1996, Romanowski *et al.* 1996, Donovan *et al.* 1997, Dutta and Bell 1997, Tanaka *et al.* 1997, Donaldson and Blow 1999, Nishitani *et al.* 2000). The pre-RC is essential for replication initiation but needs to assemble further conserved proteins to form the pre-initiation complex (pre-IC) (Kearsey and Labib 1998, Zou and Stillman 2000). Assembly of the pre-ICs takes place throughout S phase in a sequential manner dependent on the timing of origin firing (Zou and Stillman 2000). The initiation factor Dpb11/Cut5 is required, which in turn loads Cdc45 and the DNA polymerases (reviewed by Takeda and Dutta 2005). Cdc45 loading precedes origin firing (DNA unwinding) and DNA polymerase recruitment. Assembly and activation of the pre-IC is governed by Cdc28 and DDK (Dbf4-dependent kinase) activity (Cdc2 and Hsk1/Cdc7 in *S. pombe*) (Brown and Kelly 1998, Johnston *et al.* 2000). The DDK becomes chromatin associated during S phase and phosphorylates the MCM complex (Brown and Kelly 1998, Jares *et al.* 2000).

Following initiation, the MCM proteins are found associated with non-origin DNA progressively more distant from the site of initiation, implying that the MCM complex may be associated with the moving forks (Aparicio *et al.* 1997, Labib *et al.* 2000). The activity of replication origins must be coordinated to ensure that the entire genome is correctly replicated in the absence of any

re-replication of DNA regions. The main control of replication origin activity is by the opposing action of the mitotic CDKs (cyclin dependent kinases and the APC (anaphase promoting complex) ubiquitin ligase. Pre-replication complex origin assembly takes place in the presence of low CDK/ high APC activity, which is only seen at the M/G1 transition. Origin firing occurs with inactivation of the APC plus CDK activation past a threshold (low APC/ high CDK activity) (Kelly and Brown 2000).

In *S. cerevisiae* the CDKs are known to inhibit each pre-RC component (ORC, Cdc6, Cdt1 and MCMs) by different mechanisms to prevent pre-RC assembly, and subsequent origin firing, during S phase, G2 and mitosis. The mitotic CDK, Cdc28, has been shown to bind to Cdc6. Once complexed with Cdc28, Cdc6 is unable to function in the assembly of the pre-RC (Mimura *et al.* 2004).

In fission yeast origin licensing control can be bypassed by: (1) strong overexpression of *cdc18*; (2) moderate co-overexpression of both *cdc18* and *cdt1,* which may allow re-initiation even in G2 cells (Nishitani and Nurse 1995, Yanow *et al.* 2001). The continuous DNA synthesis observed is referred to as re-replication.

Many of the replication machinery components involved in the initiation of DNA synthesis are thought to have roles in preventing mitosis until the completion of S phase in fission yeast. This is called the S-M checkpoint, which will be discussed in further detail later on.

1.3 Cell cycle regulation

The key factors in determining successful cell cycle progression with the complete transmission of correct genetic information to two daughter cells are the:

1) Control mechanisms: the crucial role played by the cyclin dependent kinases (CDKs) and their associated cyclins in triggering chromosomal duplication and segregation.
2) Surveillance mechanisms (the cell cycle checkpoints): the monitoring of the fidelity of chromosomal duplication and segregation.

1.3.1 Cell cycle regulation I: Cell cycle control mechanisms

1.3.1.1 Cyclin Dependent Kinases and Cyclins

In all eukaryotes, the members of the cyclin-dependent kinase (CDK) family play a central role in controlling the onset of S phase and mitosis, and in ensuring that these phases alternate and that there is only one S phase per cell cycle (reviewed by Nurse 1990, Nigg 1995, Nurse 2000). The CDKs are serine/threonine kinases, which bind to their cyclin regulatory subunits to form active heterodimeric complexes.

Mutagenesis screens performed in *S. pombe* looking for the cell division cycle (cdc) phenotype revealed the *cdc2* gene (Bonatti *et al.* 1972, Nurse *et al.* 1976, Nasmyth and Nurse 1981). The Cdc phenotype is characterised by cell cycle arrest and elongation (continued cell growth in the absence of cell division) at the restrictive temperature, but cells grow and divide normally at the permissive temperature. Mutations in *cdc2* were shown to cause defects in

both nuclear division and DNA replication (Nurse and Thuriaux 1980, Nurse 1981). The conserved role of Cdc2 in the eukaryotic cell cycle was demonstrated by the rescue of a *cdc2* mutant, by both *S. cerevisiae Cdc28* and by a human clone of *cdc2* (Beach *et al.* 1982, Lee and Nurse 1987).

Cdc2 is an essential gene. Loss of activity arrests cells both in pre-start G1 and in late G2, whereas overexpression advances entry into mitosis. No complete suppressors of *cdc2* disruption have been identified, leading to the conclusion that a single CDK is responsible for both the G1/S and G2/M transitions in *S. pombe* (Nurse and Thuriaux 1980, Nurse 1981, Nurse 1990, MacNeill and Nurse 1997).

CDK function requires the association of the CDK with a cyclin partner. The cyclins, like the CDKs, are highly conserved throughout eukaryotes, and were first discovered in sea urchin eggs (Evans *et al.* 1983). Cell cycle progression in *S. pombe* is governed by the interaction between its single CDK, Cdc2, and 3 B-type cyclins: Cdc13, Cig1 and Cig2 (Fisher and Nurse 1995). All cyclin protein levels oscillate throughout the cell cycle, and are strictly regulated by both transcription and by ubiquitin-dependent proteolysis (Glotzer *et al.* 1991, Wilkinson *et al.* 1999).

The B-type cyclins contain an N-terminal destruction box, which allows their targeting for ubiquitin-dependent proteolysis. The destruction of a cyclin will inactivate the CDK-cyclin complex (Murray and Kirschner 1989). The ubiquitin-dependent pathway is under the control of the anaphase promoting complex (APC), with cyclin destruction and subsequent CDK inactivation by completion of mitosis. Both Cdc13 and Cig2 contain the characteristic destruction box (but not Cig1), necessary for APC recognition and targeting for

ubiquitin-dependent proteolysis. When a mutant *cdc13* strain with no destruction box is used, cells cannot exit mitosis and an anaphase arrest is seen (Yamano *et al.* 1996, Yamano *et al.* 1998).

Cdc13 is essential for G2/M progression, its levels peaking at the onset of mitosis but undetectable by anaphase. Cells deleted for *cdc13* do not divide and undergo multiple rounds of S phase, which may be visualised as re-replication (Hayles *et al.* 1994). Use of an indestructible Cdc13 mutant strain prevents mitotic exit (Yamano *et al.* 1996). Cdc13 has been shown to co-immunoprecipitate with Cdc2, and the Cdc2-Cdc13 complex is active at entry into mitosis (Booher and Beach 1988, Booher *et al.* 1989, Hagan et al 1988, Moreno *et al.* 1989).

Cig1 lacks the mitotic destruction box and putative ubiquitin interacting motif typical of the other B-type cyclins, suggesting it is not cell cycle regulated. At present there is no clear cell cycle role for Cig1. However, it is involved in the phosphorylation and inactivation of Rum1, with subsequent release of its the inhibitory effect on Cdc2, allowing progression through START into S phase. Hence, Cig1 plays a non-essential part in the G1/S transition (Bueno *et al.* 1991, Moreno *et al.* 1994). It has also been shown to form a complex with Cdc2, with kinase activity peaking in mitosis (Basi and Draetta 1995).

Cig2 is the major cyclin for the G_1/S transition (Bueno and Russell 1993, Martin-Castellanos *et al.* 1996, Mondesert *et al.* 1996). Expression of Cig2, and subsequent associated kinase activity peaks at the onset of DNA replication. In the absence of Cig2, Cdc13 can promote G_1/S progression, but with an observed delay in S phase entry. So, Cdc13 (complexed with Cdc2) is capable of inducing the onset of both S phase and mitosis. This is not a reciprocal

process: although Cig2-Cdc2 can promote the G1/S transition alone, there is no entry into mitosis in the absence of Cdc13 (Bueno and Russell 1993, Fisher and Nurse 1996, Mondesert, McGowan *et al.* 1996).

In higher eukaryotes, there are several CDKs, forming complexes with various cyclins at specific cell cycle stages. In the human cell cycle there are five cyclin groups (A, B, C, D and E) and 4 key CDKs (CDK 1,2,4 and 6). The cyclins act to target the specific CDK to the appropriate phase of the cell cycle. Human CDK1 has the closest homology to *S. pombe* Cdc2, and is the mitotic CDK. CDK1 forms complexes with both Cyclin A and B to regulate the G2/M transition. Both CDK4 and CDK6 have roles in G1, in complexes with the D-type cyclins (Cyclin D1, D2 and D3). CDK2 is the S phase CDK. It is sequentially activated by the E-type cyclins (Cyclin E1 and E2) during the G1/S transition, and by the A-type cyclins (Cyclin A1 and A2) in S phase (Sherr 1993, Reed 1997, Draetta 1994) (reviewed by Ekholm and Reed 2000).

1.3.1.2 The S. pombe Cdc2 cell cycle

Activation of Cdc2 is required for entry into mitosis. As in all eukaryotes this requires active site phosphorylation on threonine 167 and cyclin association (Moreno *et al.* 1989).

In early G1, the CDK inhibitor Rum1 accumulates and specifically inhibits Cdc2. Inactivation of Rum1 allows cells to pass START and proceed into S phase. During S phase and G2, Cdc2 activity is kept low by inhibitory phosphorylation at Y15 by Wee1 and Mik1 (Gould and Nurse 1989, Lundgren *et al.* 1991, Featherstone and Russell 1991, Hayles and Nurse 1995, Russell and Nurse 1986). This is maintained until dephosphorylation by Cdc25 in late G2,

the rate-limiting step for entry into mitosis (Enoch and Nurse 1990). Full activation of the Cdc2/Cdc13 complex will trigger mitotic entry (Russell and Nurse 1986, Millar *et al.* 1991). Mitotic exit is enabled with the degradation of Cdc13, with subsequent inactivation of Cdc2.

Both the initial activation of the CDK, and the regulation of the G1/S and G_2/M transitions by inhibitory phosphorylation and activating dephosphorylation respectively, are highly conserved throughout eukaryotic and mammalian cells (reviewed by Takizawa and Morgan 2000).

There are two models to explain how a cell ensures that: S phase is complete before entry into mitosis; S phase and M alternate; there is only one S phase per cell cycle. The quantitative model of CDK activity in fission yeast proposed that a particular cyclin is irrelevant to function except as a general CDK activator (Fisher and Nurse 1996). CDK activity is initially low in G1. An increase in activity promotes S phase entry and initiation of DNA synthesis. A further increase in activity enables the onset of mitosis, and prevents origin reloading and re-replication. CDK activity falls on exit from mitosis, before rising again for the subsequent S phase. However, the qualitative model proposes cyclin identity may be essential for CDK interaction with appropriate cyclin-specific targets, and are more than just a conformational switch for CDK activity (reviewed by Miller and Cross 2001). It is highly likely that a combination of the two models is occurring.

1.3.1.3 Regulation of CDKs

There are three key biochemical events regulating the activity of the *S. pombe* CDK, Cdc2. The first mechanism is the inhibitory phosphorylation of Cdc2 at

Tyrosine 15 (Y15) by Mik1 and Wee1 during S phase, and dephosphorylation by Cdc25 during G2. In a Wee1 single mutant, mitosis is accelerated but cells remain viable (Nurse and Thuriaux 1980). However, cells will die from mitotic catastrophe in a *wee1⁻ mik1⁻* double mutant (Lundgren *et al.* 1991, Rowley *et al.* 1992, al-Khodairy and Carr 1992). Loss of Cdc25 will result in cells arresting in G2.

Cyclin association and active site phosphorylation on threonine 167 are essential for kinase activity, with the amount of cyclin available strictly cell cycle regulated by both transcription and ubiquitin-dependent proteolysis.

The third key way to regulate Cdc2 activity is by the binding of CDK inhibitor (CDKI) proteins. The major *S. pombe* CDKI is Rum1, isolated as an inducer of re-replication. Rum1 was subsequently shown to inhibit the Cdc2/Cdc13 complex (Moreno, Labib *et al.* 1994) (Correa-Bordes and Nurse 1995). Cells deleted for *rum1* are sterile due to a defect in G1 arrest during nitrogen starvation (Moreno *et al.* 1994).

Cell cycle regulators are frequently mutated in human cancers (reviewed by Malumbres and Barbacid 2001). Alterations seen include cyclin overexpression (mainly cyclin D1 and E1) and loss of CDK inhibitors (such as $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$). However, mutations in CDK are rarely seen. For this reason, cyclin-CDK complexes have been considered as very promising therapeutic targets in human malignancies.

1.3.2 Cell cycle regulation II: Cell cycle surveillance - Mitotic cell cycle checkpoints

The idea that the replication or damage status of DNA during the mitotic cell cycle is "checked", and the information sent via a regulatory pathway to the cycle regulators was proposed by Hartwell and Weinert (Hartwell and Weinert 1989). They identified, in budding yeast, checkpoint mutants that proceeded into mitosis despite the presence of unrepaired DNA damage (Weinert and Hartwell 1988, Hartwell and Weinert 1989). The checkpoint mutants were considered components of the system that monitor and communicate the DNA status. A conserved eukaryotic checkpoint control was established with checkpoint mutants identified in fission yeast and mammalian cells (Savitsky 1995, Lieberman *et al.* 1996, Bentley *et al.* 1996, Weinert 1997, Kostrub *et al.* 1998) *(see Table 1.1 below).*

The DNA damage checkpoint arrests cell cycle progression at the G2/M transition in the presence of damaged DNA. The DNA replication, or S phase, checkpoint has two components. One component is the S-M checkpoint (so called because it ensures S phase and mitosis alternate), the role of which is to prevent mitotic entry until replication is complete (Nyberg *et al.* 2002). Cut (cell ultimately torn) cells are a phenotype of an impaired S-M checkpoint (Stewart and Enoch 1996, Lydall and Weinert 1996). The other component is the intra-S phase checkpoint, which responds to either damage of replicating DNA, or to the presence of replication inhibitors (e.g. hydroxyurea) by slowing down S phase progression (with slower fork movement and the down-regulation of late origin firing to allow sufficient time to repair the DNA) (Paulovich *et al.* 1997, Santocanale and Diffley 1998, Shirahige *et al.* 1998). The intra-S phase

checkpoint prevents irreversible fork collapse, considered DNA damage by the cell with subsequent activation of the DNA damage checkpoint (distinct from, but overlapping with, the intra-S checkpoint during S phase) (Lindsay *et al.* 1998). These major checkpoint pathways have been defined in *S. pombe*. Although all the checkpoints described are distinct, they have many common sensor, transducer and effector molecules.

Table 1.1 The checkpoint proteins

S. pombe	*S. cerevisiae*	Human	Functional domains	Functions	Comment
PI3 kinase				DNA damage/ replication checkpoints	
Rad3	Mec1	ATR	Ser/Thr kinase		Binds to Rad26
Tel1	Tel1	ATM	Ser/Thr kinase		
Rad26	Dcd2	ATRIP	Coiled–coil		Binds to Rad3
RF-C like				DNA damage/	
Rad17	Rad24	RAD17	RF-C	replication checkpoints	Binds to Rad1
PCNA like				DNA damage/ replication checkpoints	
Rad9	Dcd1	RAD9	PCNA-like		Required for Hus1/Rad1 interaction
Rad1	Rad17	RAD1	nuclease		Binds to Rad17/Hus1
Hus1	Mec3	HUS1			Binds to Rad1
BRCT domain					
Rad4/Cut5	Dbp11	TopBP1	BRCT	DNA damage/ replication checkpoints	Binds to Rad9/Rad3
Crb2	Rad9	BRCA1	BRCT	DNA damage checkpoint	Binds to Chk1
Effector kinases					
Cds1	Rad53	CHK2	Ser/Thr kinase FHA domain	DNA replication checkpoint	Binds to Mrc1
Chk1	Chk1	CHK1	Ser/Thr kinase	DNA damage checkpoint	Binds to Crb2
Other proteins					
Mrc1	Mrc1	Claspin		DNA replication checkpoint	Binds to Cds1

A third type of checkpoint, the spindle checkpoint, operates during mitosis to ensure correct alignment of chromosomes on an undamaged spindle. Cell cycle arrest occurs if the chromosomes are not properly attached to the

mitotic spindle or there is mitotic spindle damage (Amon 1999). I will concentrate on the damage and replication checkpoints, and not discuss the spindle checkpoint further. I will first discuss an overview of the damage and replication checkpoints in humans, and then in greater detail in fission yeast.

1.3.2.1 Overview of the human G1/S and G2/M damage checkpoints

The DNA damage response consists of a *signal* (DNA damage) detected by a *sensor* (DNA damage binding protein), which activates the *transducer* system (a protein kinase cascade). This amplifies and diversifies the initial signal by activating the downstream *effectors* of the damage response: simple damage excision, cell cycle arrest to allow repair or apoptosis, and transcriptional control. Key to the damage response in human cells is ATM (mutated in ataxia telangiectasia) (Savitsky *et al.* 1995, Zakian 1995) (reviewed by Abraham 2001, Shiloh 2001). ATM performs two main functions in humans: checkpoint control and telomere maintenance. ATM deficiency leads to the human cancer predisposition and neurodegenerative syndrome Ataxia Telangiectasia. This is an early onset autosomal recessive ataxia associated with characteristic chromosomal aberrations, cell cycle checkpoint defects, cancer susceptibility, and sensitivity to ionizing radiation. Certain types of ATM mutations seem to increase cancer predisposition in heterozygous carriers.

ATM is recruited to and activated at the site of DNA damage. From here it phosphorylates downstream effectors of the damage response (including p53, CHK2 and BRCA1) (*see Figure 1.3*). ATR (ATM and Rad3 related) is another damage surveillance protein. It was discovered when searching for a gene with *ATM* and *rad3* homology in the human genome database (Cimprich *et al.*

1996). ATR mutation, and partial loss of function, is associated with the autosomal recessive Seckel Syndrome, which is clinically similar to A-T (O'Driscoll and Jeggo 2003). ATR and ATM have an overlapping set of targets but are thought to respond to different types of DNA damage. ATM is necessary for the initial rapid phase of the DSB damage response (which lasts 1 to 2 hours). ATR acts at a later stage, to maintain the phosphorylated state of specific substrates. However, the main role of ATR appears to be in response to UV light, stalled replication forks and hypoxia (all of which do not activate ATM). BRCA1 and p53 are phosphorylated by both kinases, but not all substrates are shared, with ATM activating Chk2 and ATR activating Chk1. ATR is thought to be more important during G2-M, and not G1-S (reviewed by (Sancar *et al.* 2004). An important characteristic of ATR is its need for an accessory protein, ATRIP (ATR interacting protein). This is conserved throughout evolution as ATRIP is the functional homologue of *S. pombe* Rad26, which interacts with the ATR homologue Rad3 (Edwards *et al.* 1999).

Both ATM and ATR are members of a family of large PI3-kinase related proteins, characterised by a C-Terminal kinase domain and a long N-terminal region with no clear structure or function. Tel1 (involved in telomere maintenance only) is considered the *S. pombe* homologue of ATM, and Rad3 (involved in checkpoint control and telomere maintenance) the *S. pombe* homologue of ATR.

In fission yeast, six genes have been identified that are involved in the activation of the checkpoint response by detection of stalled forks and DNA damage. They are collectively referred to as the *rad* genes (*rad1, rad3, rad9, rad17, rad26 and hus1*) (Enoch and Nurse 1990, Enoch and Nurse 1991, al-

Khodairy and Carr 1992, Enoch *et al.* 1992, Enoch *et al.* 1993, al-Khodairy *et al.* 1994). Mammalian homologues have been identified for all the *rad* genes (*see Table 1.1*) (Savitsky 1995, Lieberman *et al.* 1996, Bentley *et al.* 1996, Kostrub *et al.* 1998). Cells mutated in these genes are hypersensitive to HU (hydroxyurea), a drug which blocks replication, and also produce a characteristic DNA damage sensitivity phenotype (al-Khodairy and Carr 1992, Enoch *et al.* 1992).

Rad17 is considered a checkpoint specific structural homologue of the replication factor, RFC (a globular protein with a deep groove). The Rad9-Rad1-Hus1 (9-1-1) complex is the checkpoint equivalent of PCNA, forming a PCNA-like structure (a hetero-trimeric ring). RFC and PCNA form part of the replication machinery. RFC acts to load the PCNA onto the DNA. PCNA then recruits Pol∂ for DNA synthesis (Yuzhakov *et al.* 1999). By analogy with eukaryotic DNA replication, Rad17-RFC may load the PCNA-like 9-1-1 complex onto chromatin (reviewed by O'Connell *et al.* 2000). The similarity of the checkpoint proteins to the DNA replication proteins has led to the idea that they are components of DNA-interacting complexes, which act as sensors for DNA damage or replication perturbations. So, Rad17 is the checkpoint specific clamp loader (CCL) and loads the heterotrimeric (9-1-1) clamp complex (CCC) onto the DNA at sites of DNA damage (Melo *et al.* 2001, Zou and Elledge 2001). ATR-dependent phosphorylation of Rad17 following DNA damage is necessary for the checkpoint response.

In human cells, the next step in the damage response is dependent on the cell cycle stage and type of DNA damage. Following DNA damage in G1, ATM acts via Chk2, and ATR via Chk1 (reviewed by Sancar *et al.* 2004) (*Figure*

3.1a). Both Chk1 and Chk2 bring about a rapid G1 arrest via the phosphorylation, and subsequent nuclear extrusion and degradation, of Cdc25A. This prevents Cdc25A from activating Cdk2, resulting in a rapid cell cycle arrest. The G1 arrest is then maintained via p53 phosphorylation and activation (by both ATM/ATR and Chk1/Chk2). The key downstream target of p53 is p21, which acts by binding to and inhibiting the Cdk2-S phase cyclin complex, and prevention of Rb (retinoblastoma) activity (and hence prevention of the E2F-driven transcription of genes necessary for S phase progression) by binding to and inhibiting Cdk4-Cyclin D (Bartek and Lukas 2001).

When DNA damage occurs in G2, either the ATR-Chk1-Cdc25A or ATM-Chk2-Cdc25A pathway is activated, depending on the type of damage, to prevent mitotic entry (*Figure 3.1b*). Chk1 and Chk2 also bring about the phosphorylation and activation of the CDK inhibitor, Wee1.

1.1.1.2 The replication checkpoint

DNA replication is initiated at multiple origins of replication throughout S phase (Kelly and Brown 2000). Replication fork termination occurs by meeting a converging fork. However, replication forks will slow down or stall in the presence of DNA damage and replication inhibitors or on meeting a natural replication pause site, a Replication Fork Barrier (RFB) (Rothstein *et al.* 2000). DNA damage may also produce a RFB. Replication stress may lead to genomic instability and cancer development in higher organisms. As mentioned previously, the replication checkpoint consists of the S-M checkpoint (delaying mitosis until S is complete) and the intra-S checkpoint (slowing down S phase progression in the presence of DNA damage). In their absence, replication

stress results in irreversibly collapsed forks, and inappropriate entry into mitosis. The essential DNA replication proteins and the non-essential Rad checkpoint proteins are both required for replication checkpoint function.

As eukaryotes initiate replication at multiple origins, the collapse of a single fork will not result in a region of unreplicated DNA, but the collapse of 2 converging forks may do so (Zhou and Elledge 2000). Stalled replication forks are stabilized (with the replicative machinery protecting the nascent ends of the replicating DNA) and collapsed forks are restarted by recombination. Homologous Recombination is thought to play a key role in replication with the re-activation of stalled forks (Kraus *et al.* 2001).

Four cellular responses are seen in response to replication perturbation: inhibition of late origin firing; stabilization of active replication forks; delay in entry into mitosis; and the slowing of fork progression on damaged templates (Kelly and Brown 2000). The checkpoint proteins are all necessary for the first three, but not for the fork slowing. The intra-S phase checkpoint acts via ATR/Rad3 and Chk1/Cds1 to inhibit late origin firing in the presence of DNA damage and replication stress (Paulovich and Hartwell 1995). So, the intra-S checkpoint acts during normal replication to prevent the formation of irreversible DNA structures at the replication fork. This maintains the fork in a state that is "active" and ready for replication to re-start when appropriate. The S phase checkpoint is important even during normal replication.

When replication forks stall in S-phase, the intra-S phase checkpoint is activated, and ATR, Claspin, Rad17-RFC and the 9-1-1 complex are recruited. As in the G1 arrest, ATR acts via phosphorylation and activation of Chk1, which then brings about an S phase delay via phosphorylation of Cdc25A and

inhibition of Cdk2 activity (*Figure 1.4a*). However, if DNA damage (such as DSBs) occurs in S phase, ATM acts instead, via H2AX, MDC1 and p53, to activate Chk2, with subsequent inhibition of Cdk2 via Cdc25A *(Figure1.4b)*.

The damage sensors for the intra-S phase checkpoint are a combination of checkpoint and repair proteins. As described above, a standard checkpoint response (via ATM-Chk2-Cdc25A-Cdk2) is seen, plus a second pathway that is dependent on the phosphorylation of SMC1 by ATM (aided by the repair proteins BRCA1, FANC1 and NBS1) (reviewed by Lehmann 2005)).

The SMC (structural maintenance of chromosomes) proteins are highly conserved throughout evolution. They form a superfamily of proteins with similar structures, all having a SMC core plus associated non-SMC proteins. Three heterodimeric SMC complexes have been identified, all with roles in repair and the response to DNA damage: cohesin (SMC1, SMC3), which also holds sister chromatids together and is necessary for homologous recombination; condensin, which also condenses chromosomes at mitosis; and SMC5-6. Rad50, which is required for meiotic recombination and recombination repair, is also necessary for the establishment of the intra-S phase checkpoint. Its structure resembles that of the SMC proteins

1.1.1.3 DNA damage and replication checkpoints in S. pombe

Eukaryotic cell cycle checkpoints are highly conserved. I would now like to discuss the current model for the damage and replication checkpoints in *S. pombe*.

1.1.1.3.1 The rad genes

In humans the two key checkpoint genes are *ATM and ATR*, the *S. pombe* homologues being *tel1* and *rad3* respectively. In *S. pombe*, the *rad3* gene plays a central checkpoint role, functioning upstream with the other *rad* genes to send the damage/ stalled replication signal to the effector kinases, Chk1 and Cds1. They bring about a cell cycle arrest by inhibitory phosphorylation of the CDK, Cdc2, via the inhibition of Cdc25 and the activation of Wee1 and Mik1 *(Figure 1.5)*. Rad3 is required for both the initiation and maintenance of a replication block, but for only the initiation of the response to DNA damage (Martinho *et al.* 1998).

The non-essential *rad* checkpoint genes are *rad3, rad26, rad17, rad1, rad9 and hus1*. Mutants in these genes are unable to respond to DNA damage or perturbations in replication. Rad3 forms a complex with Rad26, the complex then functioning as a PI3-related kinase. Rad17, with its four small subunits, has limited homology to Replication factor C (RFC) and the PCNA sliding clamp loader (Griffiths *et al.* 1995). Rad1 has structural similarity to exonuclease, plus a structural motif similar to the sliding clamp protein PCNA. It forms a complex with Rad9 and Hus1, the 9-1-1 complex, independently of a checkpoint signal (*Figure 1.6)*. The 9-1-1 complex is conserved in budding yeast and humans, and has been proposed to provide processivity for DNA-repair and replication enzymes (Thelen *et al.* 1999).

When DNA damage or replication fork stalling occurs, at least two complexes are loaded independently onto the DNA: Rad3-Rad26, and the Rad17-dependent 9-1-1 (consistent with their respective homologies to RFC and PCNA). Rad3-Rad26 appears to be at the head of a phosphorylation

cascade immediately upstream of the 9-1-1 complex. This organization is maintained for both the G2/M and S-M checkpoints. The Rad3 association with Rad26, and subsequent Rad3-dependent phosphorylation of Rad26 appears independent of all the other checkpoint proteins and may be considered an initial response to DNA damage (Edwards *et al.* 1999).

However, when DNA damage occurs in S phase (the intra-S phase checkpoint), the Rad17 and 9-1-1 complexes appear to be required for the Rad3-dependent phosphorylation of Rad26. It has been proposed that the RFC and PCNA-like complexes act upstream of Rad3 in the intra-S phase checkpoint pathway, and may be necessary for to load Rad3 onto the DNA and for its subsequent function (O'Connell, Walworth *et al.* 2000) (*Figure 1.6*). In response to DNA damage, Hus1 and Rad1 associate in the 9-1-1 complex in a Rad3-dependent manner, and Hus1 undergoes Rad3-dependent phosphorylation (al-Khodairy and Carr 1992, Kostrub *et al.* 1998). The Rad3-dependence of all these phosphorylation events sets out a hierarchy among the Rad proteins.

1.1.1.3.2 The checkpoint signal transducers

Two proteins containing BRCT-domains, Crb2/Rhp9 and Mrc1 (mediator of the replication checkpoint), are also necessary for the checkpoint response. Cells lacking Crb2 fail to arrest in the presence of UV or radiation damage, but the replication checkpoint remains intact. Crb2 interacts with Chk1 and Rad4 in response to DNA damage (Saka *et al.* 1997) *(Figure 1.7)*. When DNA damage occurs in G2, Rad3 phosphorylates Crb2, which then binds to Chk1 to bring it into close contact with Rad3 (Mochida *et al.* 2004). However, Rad3

phosphorylates Mrc1 following replication stalling in S phase, which then binds to Cds1 to create close contact between Rad3 and Cds1 (Tanaka and Russell 2004). It has been suggested that the checkpoint mediators distinguish the different cell cycle phases (and hence the need for Mrc1 or Crb2) by nuclear localisation and/or CDK-dependent phosphorylation (Esashi and Yanagida 1999). Cdc2 phosphorylates Crb2, during both a normal cell cycle and in response to DNA damage. It has been shown that non-phosphorylatable Crb2 mutants do not re-enter the cell cycle after DNA damage, raising the possibility of a feedback regulation mechanism between the checkpoint and the cell cycle machinery (Esashi and Yanagida 1999).

The Rad4/Cut5 protein functions in both the S-M and the G2/M checkpoint pathways. It has been grouped with all the other checkpoint *rad* genes upstream of *cds1* and *chk1*, as both the phosphorylation of Chk1 in response to DNA damage and Cds1 kinase activity is Rad4-dependent. Phosphorylation of Rad9, Rad26 and Hus1 in response to either DNA damage or stalled DNA replication is independent of Rad4/Cut5 checkpoint function. Rad4 it is a BRCT scaffold protein and is thought to act with Rad3, Rad26 and Rad17 to effect the checkpoint response. In the model proposed by Harris et al, Rad4 acts to bring together the multimeric complex of Rad3-Rad26-9/1/1 on the DNA (Harris *et al.* 2003) (*Figure 1.7*). In the event of DNA damage, Rad4 anchors Crb2, which in turn links Chk1 to the region. Hence, Rad4 is the link between the Rad3-dependent cascade and Chk1 phosphorylation and activation. Rad4 is also essential for DNA replication during a normal cell cycle, and the replication checkpoint (Saka and Yanagida 1993, Saka *et al.* 1994). Rad4 is thought to tether Cds1 when replication stalls. However, there is

debate as to whether or not this occurs via Mrc1 (Harris *et al.* 2003, Tanaka and Russell 2004).

Chk1 and Cds1 are the link between the Rad proteins and the mitotic machinery controls (Walworth *et al.* 1993, Murakami and Okayama 1995). Rad3 phosphorylates and activates the Cds1 kinase after a DNA replication block and the Chk1 kinase after DNA damage, though redundancy has been observed (Lindsay *et al.* 1998, Kelly and Brown 2000). Cds1 also plays an essential role in recovery from the S phase arrest. In the presence of an HU induced S-phase block, cells lacking Cds1 lose viability (Walworth *et al.* 1993, Murakami and Okayama 1995).

Cds1 is structurally similar to *S. cerevisiae* Rad53 with its forkhead-associated (FHA) N-terminal domain. Rad53 has two FHA domains and is required for both the damage and replication checkpoints. Rad9 binds to the central FHA domain of Rad53, whilst the N-terminal FHA domain is involved in the replication checkpoint cascade. The activation and phosphorylation of Cds1, in response to stalled replication or DNA damage is S phase specific, and protein phosphorylation correlates with kinase activation. This makes Cds1 phosphorylation a useful marker for the S-M checkpoint (Lindsay *et al.* 1998).

Chk1 is primarily the transducer of the damage checkpoint, but also appears to have a distinct role in the replication checkpoint and is essential for recovery from an S phase block. A HU-induced replication block requires the activation of the intra-S phase checkpoint, acting via Cds1, to prevent irreversible fork collapse. In the absence of Cds1, collapsed forks occur which are considered DNA damage by the cell, with subsequent activation of the DNA damage checkpoint and Chk1-induced cell cycle arrest. Hence Chk1 can

substitute partially for Cds1 in an HU block (Lindsay, Griffiths *et al.* 1998). The phosphorylation, and activation, of Chk1 is *rad* gene dependent and occurs in response to DNA damage during G2. The Rad3-dependent serine 345 phosphorylation of Chk1 is directly linked to Chk1 kinase activity, and is therefore considered a useful biochemical marker of DNA damage checkpoint pathway activation (Capasso *et al.* 2002). Overexpression of Chk1 produces a mitotic delay (Walworth *et al.* 1993, Walworth and Bernards 1996). Genetic and 2-hybrid analyses have demonstrated a Chk1-Crb2 interaction, but Chk1 lacks an obvious FHA domain (Saka *et al.* 1997).

Chk1 and Cds1 send the checkpoint signal to the cell cycle machinery. Activation of the fission yeast CDK, Cdc2, is required for entry into mitosis. As in all eukaryotes this requires active site phosphorylation on threonine 167 and cyclin association. Inhibitory phosphorylation on tyrosine 15 (Y15) by Wee1 and Mik1 maintains the complex in an inactive state until dephosphorylation occurs via Cdc25. This is the rate-limiting step for entry into mitosis (Enoch and Nurse 1990, Lundgren *et al.* 1991).

Chk1 and Cds1 act in two ways to inhibit Cdc2 activity and produce cell cycle arrest. The first is by the phosphorylation and inactivation of Cdc25 (Furnari *et al.* 1999). The co-immunoprecipitation of Chk1 and Cdc25 has been shown in vivo (Furnari *et al.* 1997). The Cdc25 phosphorylation also allows physical association with Rad24, leading to nuclear exclusion. Rad24, and Rad25, encode 14-3-3 proteins, which bind to signalling molecules including phosphatases (Ford *et al.* 1994). The loss of Cdc25 activity (by both phosphorylation and spatial re-organisation) results in a decrease in CDK activity. The second way in which Chk1 and Cds1 bring about cell cycle arrest

is through the activation of Wee1 and /or Mik1, which then directly inhibit Cdc2 by Y15 phosphorylation. Cdc25 appears to be the major target of the S-M checkpoint, with supporting activation of Mik1 and Wee1. However, Wee1 and Mik1 do not appear to play a role in the G2 damage checkpoint, which uses inhibition of Cdc25 alone to effect a cell cycle block (Enoch and Nurse 1990). This suggests that the two pathways are mechanically distinct. Checkpoint activation may be mimicked by the overexpression of Cdc25, the inactivation of Wee1 or Mik1, or by the use of a Cdc2 Y15 mutant that uncouples S phase and mitosis (Enoch and Nurse 1991).

In fission yeast, there is an additional S phase checkpoint sub-pathway. When the S phase checkpoint fails to stabilise stalled forks in an HU block (for example in a Cds1 mutant), they are thought to be converted into structures recognised as DNA damage with resultant activation of the Chk1-mediated damage pathway (Lindsay *et al.* 1998).

1.1.3 Human checkpoints, the cell cycle and cancer

I have outlined above the major components and pathways of the cell cycle checkpoints in both mammalian cells and in fission yeast. In humans, their appropriate function is essential for the maintenance of genomic integrity and prevention of changes in ploidy. Failure to arrest the cell cycle in these situations contributes to cancer development and may cause resistance to standard treatments (Hartwell and Kastan 1994). DNA damage is a relatively common event and may result in mutation, cancer and death of the cell or the organism. The DNA damage response enables the cell to either cope with the damage or to activate apoptosis (programmed cell death). The damage

response includes: removal of the DNA damage; activation of the damage checkpoint and subsequent cell cycle arrest, allowing time for repair and hence preventing the transmission of damaged/incompletely replicated DNA; and apoptosis to remove seriously damaged or deregulated cells.

I have already discussed the association between ATM and ATR deficiencies and cancer predisposition. Both the RB protein and p53 act to restrain cell cycle progression. P53 is regulated by MDM2 (inhibits p53) and $p19^{ARF}$ (inhibits MDM2). Amplification of *MDM2*, and loss of *p53* or of $p19^{ARF}$ are observed in many human cancers, with inactivating p53 mutations the most common genetic alteration seen overall (in 50% of human cancers) (Hollstein *et al.* 1991, Levine *et al.* 1991, Oliner *et al.* 1992). The function of p53 may also be abolished by nuclear extrusion or by interaction with viral proteins (in cervical cancer, the oncoprotein E6 binds and inactivates Rb, E7 binds and inactivates p53) (Scheffner *et al.* 1991, Lechner *et al.* 1992). The p53 pathway is probably inactivated in most tumours, which also has treatment implications. Radiotherapy, and many standard chemotherapy regimes, utilise the p53 pathway to kill tumour cells. The Li-Fraumeni syndrome is caused by a germline mutation in *p53* (the same phenotype is seen with a germline mutation in *Chk2*) and is characterised by multiple early onset tumours, such as breast and sarcoma (Li and Fraumeni 1969, Bell *et al.* 1999).

The retinoblastoma gene was the prototype for Knudson's two-hit hypothesis and tumour suppressor genes (Knudson 1971). Loss or inactivation of Rb is a frequent event in human tumours, but is classically associated with childhood retinoblastoma and osteosarcoma. Overexpression of cyclin D1, either by gene amplification or loss of the inhibitory gene $p16^{INK4a}$, will

phosphorylate Rb with subsequent release of the E2F transcription factor. Loss of the tumour suppressor gene *p21* also results in inappropriate E2F release and S phase entry.

1.4 Mechanisms of DNA repair

DNA damage ranges from covalent base modification to the DNA double strand break (DSB). There are many DNA repair pathways, each with structure specific DNA recognition subunits. The DSB is considered the most dangerous form of cellular damage. The two ends of broken DNA may become separated, leading to inappropriate recombination and problems with repair. This major threat to genomic integrity is also specifically generated in certain circumstances in higher eukaryotes, such as V(D)J recombination in B and T cells to generate immunoglobulin diversity. V(D)J recombination is tightly controlled, but mistakes may lead to development of lymphoma (malignancies of B and T cells). For example, the poor prognosis Burkitts B cell lymphoma occurs when inappropriate recombination places the *c-myc* oncogene next to immunoglobulin heavy chains. Inappropriate DSB repair events frequently lead to carcinogenesis in higher eukaryotes by both oncogene activation and tumour suppressor genes loss (Khanna and Jackson 2001).

The detection of DSBs triggers downstream pathways, such as DNA repair and checkpoint activation. There are two distinct and complementary eukaryotic DSB repair mechanisms: Non-homologous end joining (NHEJ) and homologous recombination (HR) (Critchlow and Jackson 1998). Both mechanisms are highly conserved throughout eukaryotes, with fission yeast

homologues identified for the key genes involved, but are used to differing extents in different organisms. Simple eukaryotes, such as fission and budding yeast, use predominantly HR to repair radiation-induced DSB. This is because *S. pombe* spends most of its time in G2 with a sister chromatid available as a repair template. NHEJ is generally inefficient in *S. pombe,* but is used in G1 arrested cells (Ferreira and Cooper 2004). However, NHEJ predominates in DSB repair in the mammalian cell cycle, although HR is also important in S phase and G2 (when sister chromatids are present).

1.4.1 NHEJ

NHEJ uses direct ligation of the broken ends with minimal or no regard for homology. It is therefore error prone and often introduces sequence deletions. Central to NHEJ in all eukaryotes is the heterodimeric Ku protein. This is made up of two subunits, Ku70 (69kDa in humans) and Ku80 (83kDa in humans), which bind directly and sequence independently to the DSB. In vertebrates, the ku protein then recruits and activates the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). This 465kDa protein has C-terminal homology to the PI3-kinase like family, which includes the Rad3 homologues ATM and ATR. It forms the DNA-PK holoenzyme with the Ku heterodimer, and has protein serine/threonine kinase activity once bound to DSB. There is no DNA-PK in *S. pombe* (Manolis *et al.* 2001). Its likely substrates are the XRCC4-ligase IV complex and RFA2, both of which are recruited and loaded onto the DNA ends by Ku (Plumb *et al.* 1999). This facilitates NHEJ, with DNA Ligase IV bringing about the DNA end ligation.

Radiosensitivity to DSB inducing agents is seen with inactivation of the NHEJ proteins in higher eukaryotes, but not in fission yeast due to HR being the dominant repair pathway. Loss of DNA-PKcs or Ku function is associated with increased rates of lymphomas in mice.

1.4.2 Homologous recombination (HR)

In HR, the damaged chromosome invades an intact homologous DNA strand to retrieve the genetic information it requires for accurate repair. This usually results in error-free, non-mutagenic DSB repair. HR involves the "*Rad52* epistasis group" of genes, identified by genetic analysis of the budding yeast, *S. cerevisiae*: *Rad50, Rad51, Rad52, Rad54, Rad55, Rad57, Rad59, Mre11* and *Xrs2*. Mammalian and *S. pombe* homologues of all the "*Rad52*" group have been identified and defects in these genes lead to radiosensitivity (Wood *et al.* 2001). Rad50 has similarity to the SMC (structural maintenance of chromosomes) proteins that are involved in sister chromatid cohesion.

There are several HR pathways all involving strand invasion (reviewed by Raji and Hartsuiker 2006). The classical Szostak model describes resection of the DSB in the 5' to 3' direction to produce a 3' overhang, which then invades undamaged DNA to find a homologous region (Szostak *et al.* 1983). Rad51 (*S. pombe* Rhp51), central to all the HR pathways, catalyses strand exchange events, with the formation of two Holliday junctions. The Holliday junctions are considered a central intermediate in HR, and the critical intermediate in cross-over formation (Holliday 1964, Szostak *et al.* 1983, Paques and Haber 1999). They are then resolved by cleavage and ligation to give two intact DNA molecules.

The recombination mediator proteins, Rad52 and Rad54, assist Rad51. There are two *S. pombe* homologues of Rad52: Rad22 (involved in mating type switching and DNA repair) and Rti1. They interact with each other and with Rhp51 and Rhp54 (homologue of Rad54). Rhp55 and Rhp57 are the *S. pombe* homologues of Rad55 and Rad57.

When HR is used for DSB repair, Holliday junctions must be resolved without the occurrence of crossovers. This is necessary to avoid abnormal chromosomal re-arrangements and loss of heterozygosity, both of which can lead to cell death and cancer in higher eukaryotes. Several pathways ensure resolution of HJ with prevention of cross-overs. The heterodimeric enzyme Mus81-Eme1 has been proposed as a Holliday junction resolvase in human cells and fission yeast (Interthal and Heyer 2000, Boddy *et al.* 2000, Boddy *et al.* 2001, Chen *et al.* 2001). It now appears that Mus81-Eme1 is responsible for the vast majority of crossovers occurring during meiosis in *S. pombe* (Osman *et al.* 2003, Smith *et al.* 2003).

Also central in Holliday junction resolution in *S. pombe* is the Rqh1 helicase, deletion of which results in increased recombination events (Stewart *et al.* 1997). It has high similarity to the hBLM DNA helicase, mutations in which cause the autosomal recessive disorder Bloom's syndrome, characterised by immunodeficiency and increased risk of malignancy (secondary to increased sister chromatid exchange and increased genomic instability).

HR is also involved in DNA replication, in the bypassing of Replication Fork Barriers (natural pause sites) and the restarting of stalled replication forks (McGlynn and Lloyd 2002). In *S. pombe*, mutation of the HR genes may lead to

problems in DNA replication, and recombination intermediates are thought to be commonly associated with ongoing DNA replication (Segurado *et al.* 2002).

1.5 The specific aims of this thesis

Checkpoint responses and the checkpoint genes are highly conserved in evolution with mammalian homologues identified for the major fission yeast checkpoint genes. However, the actual sequence of upstream events leading to activation of Rad3 is unclear with the identity of the DNA damage/stalled replication sensors and initial checkpoint activators remaining uncertain. I, therefore, set out to find new genes or a new role for known genes involved in checkpoint activation upstream of Rad3. I aimed to then characterise their Rad3-dependent checkpoint function.

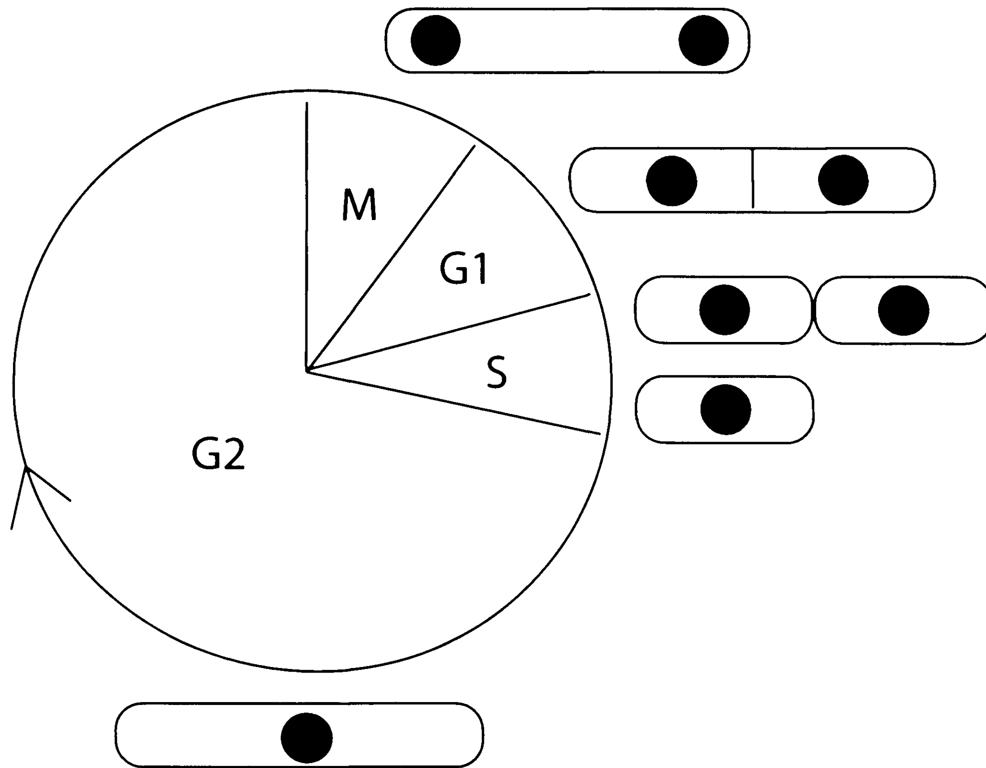


Figure 1.1: Schematic representation of the fission yeast cell cycle
G2 comprises about 70% of the cell cycle, whereas M, G1 and S take up about 10% each.

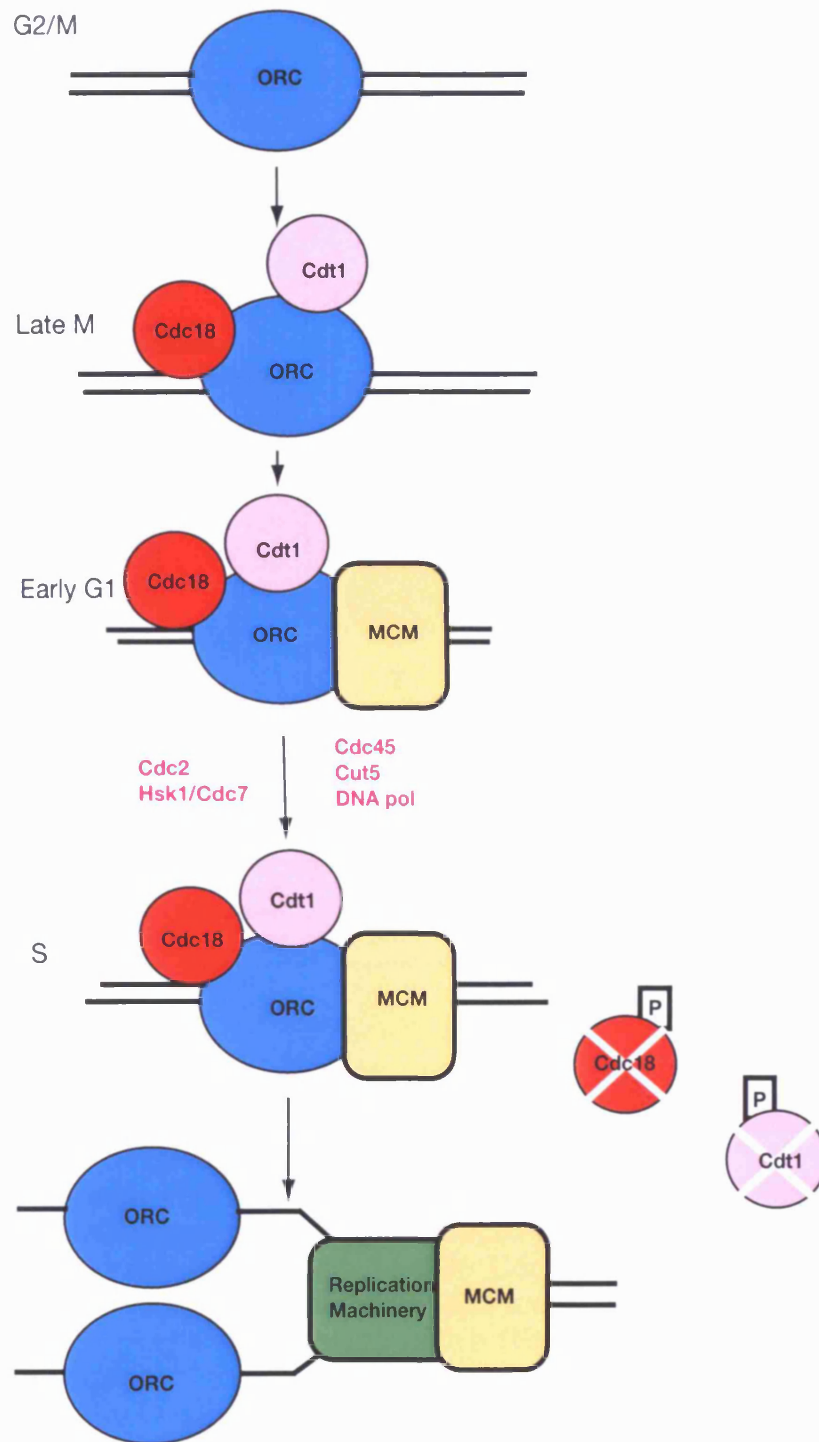


Figure 1.2: Assembly and activation of the pre-replicative (pre-RC) and pre-initiative (pre-IC) complexes

In late mitosis (low CDK/ high APC activity) Cdc18 and Cdt1 associate with ORC, and then in early G1 form the pre-RC by loading MCM. The pre-IC is formed in S phase with the loading of Cdc45, Cut5 and the DNA polymerases under the control of the CDK Cdc2 and the DDK Hsk1/Cdc7. Origin firing then occurs (high CDK/ low APC activity), with dissociation of Cdc18 and Cdt1 from chromatin. Cdc18 and Cdt1 are degraded to prevent DNA re-replication.

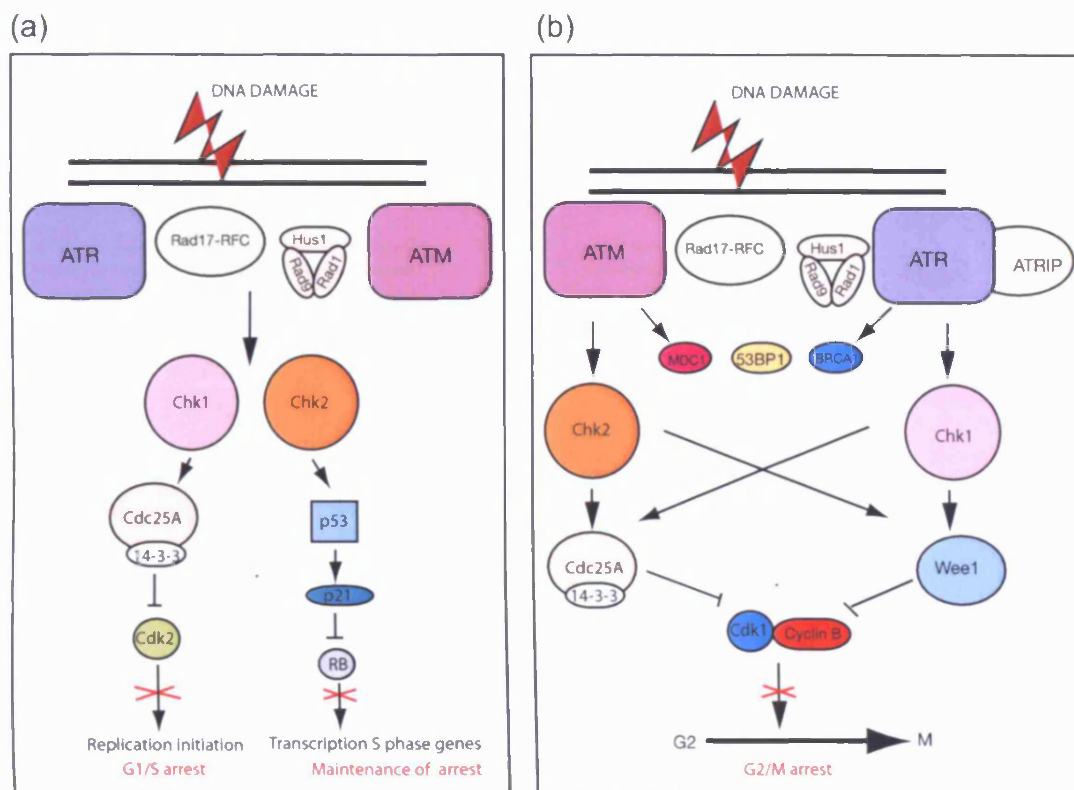


Figure 1.3: Human G1/S and G2/M damage checkpoints
ATM and ATR are recruited to and activated at the site of DNA damage from where they activate an overlapping set of downstream targets.
(a) After DNA damage in G1, ATM/ATR phosphorylates Rad17, Rad9, p53 and Chk1/Chk2, which then phosphorylate Cdc25 leading to inactivation and nuclear extrusion via association with 14-3-3. Cdc25 can no longer activate Cdk2, causing inhibition of DNA replication. p53 mediates maintenance of the G1/S arrest via p21-mediated inhibition of the RB protein, with subsequent inhibition of transcription of S phase genes.
(b) After DNA damage in G2, ATM/ATR act via mediator proteins to phosphorylate and activate Chk1/Chk2. The downstream targets are Cdc25- and Wee1-mediated inhibition of Cdk1, preventing entry into mitosis.

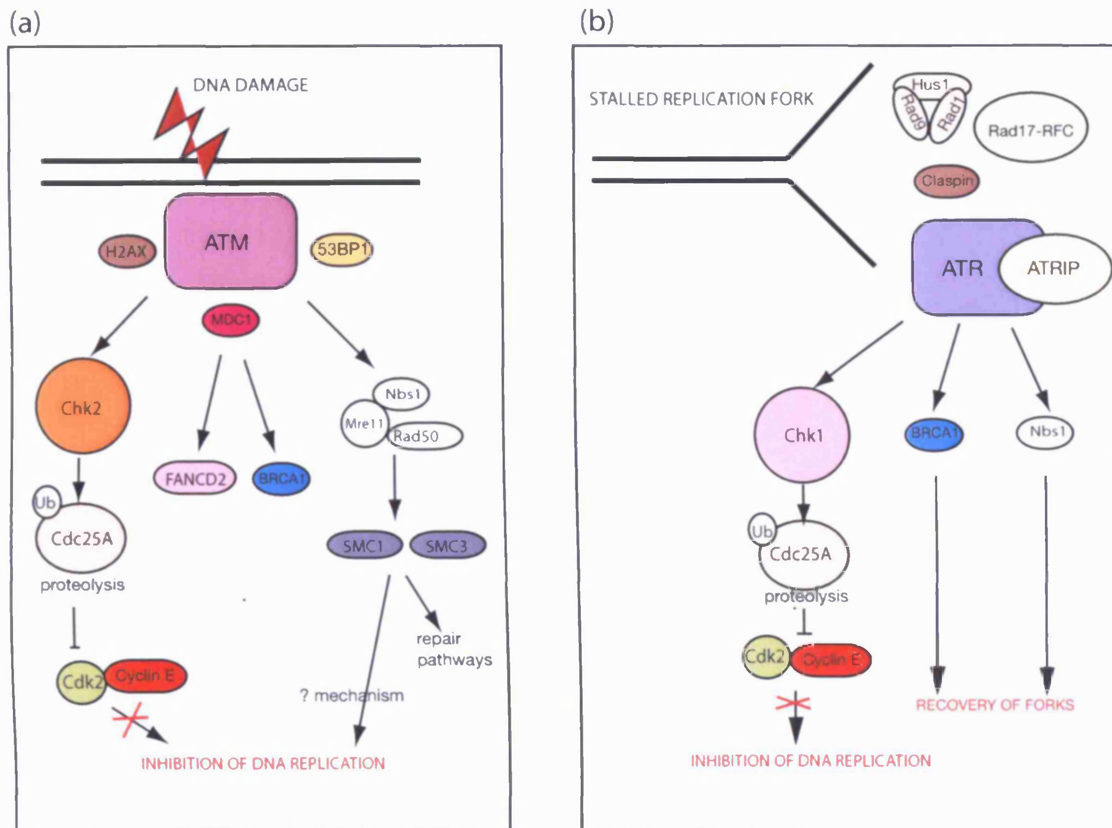


Figure 1.4: Human replication checkpoints
(a) DNA damage in S phase inhibits DNA replication by two ATM-activated parallel pathways: via Chk2-mediated ubiquitin-dependent degradation of Cdc25A, leaving Cdk2 in its inhibited form and unable to initiate replication.
(b) The stalled fork recruits ATR-ATRIP, Rad17-RFC, 9-1-1 and Claspin. ATR phosphorylates Chk1, leading to inactivation of Chk1 via ubiquitin-dependent degradation of Cdc25A and S phase delay. ATR-mediated activation of repair pathways leads to recovery of stalled and collapsed forks.

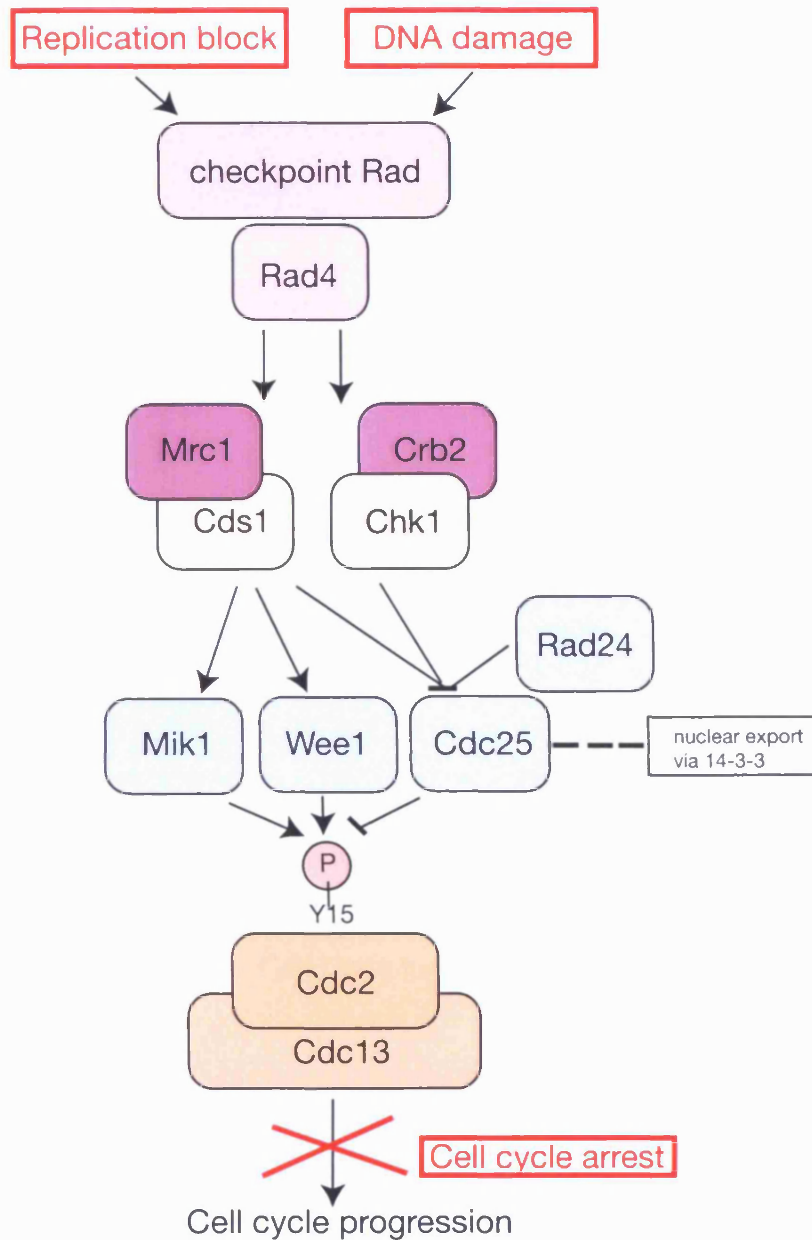


Figure 1.5: An overview of the replication and damage checkpoint pathways in *S. pombe*
Both pathways act through the *rad* genes to inhibit the activity of Cdc2 and impose a cell cycle block. The replication checkpoint uses the Mrc1/Cds1 pathway to activate Wee1 and Mik1 and inhibit Cdc25. The damage checkpoint acts via Crb2/Chk1 to inhibit Cdc25.

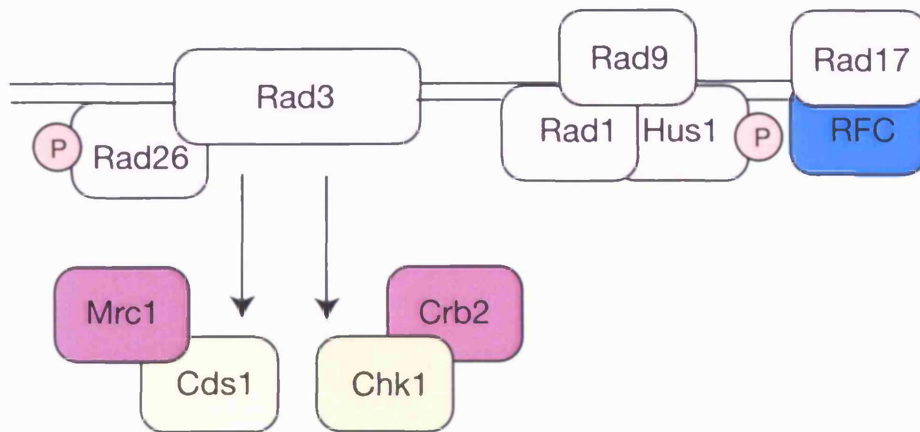


Figure 1.6: Events following DNA damage or replication fork stalling
Rad3 associates with and phosphorylates Rad26. Rad3 also phosphorylates Hus1. Rad17-RFC (the "clamp loader') is thought to load the 9-1-1 complex (the "sliding clamp") onto the DNA. They are all required for the checkpoint signal transmission to Mrc1/Cds1 and to Crb2/Chk1.

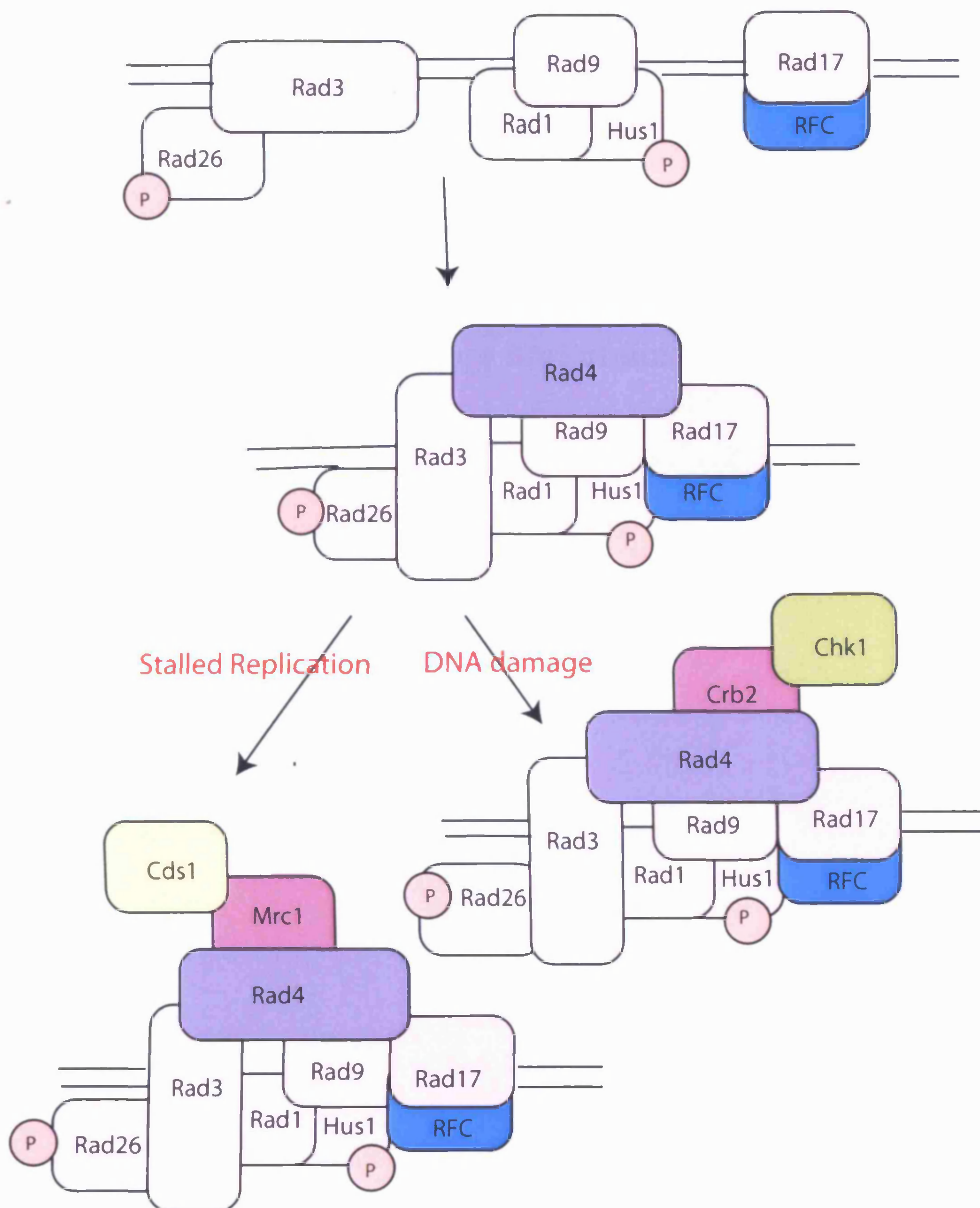


Figure 1.7: The scaffold protein Rad4 is the link between the Rad3-dependent upstream cascade and the appropriate downstream signal transducer activation

Rad4 acts to bring together the multimeric complex of Rad3/Rad26/9-1-1 on the DNA. If DNA damage activated the checkpoint response, Rad4 then anchors Crb2, which then anchors Chk1. During a replication block, Rad4 tethers Cds1, possibly via Mrc1.

Chapter 2

Identification of Rad3-dependent DNA damage/perturbed replication sensors and checkpoint activators

2.1 Introduction

Six genes have been identified in fission yeast that are involved in activating the checkpoint response by an S phase block (the S-M checkpoint) or DNA damage, and are collectively referred to as the checkpoint *rad* genes (*rad1, 3, 9, 17, 26* and *hus1*) (Enoch and Nurse 1990, Enoch *et al.* 1991, Enoch *et al.* 1992, al-Khodairy and Carr 1992, Enoch *et al.* 1993, al-Khodairy *et al.* 1994). Cells mutated in these genes are hypersensitive to DNA damage and to Hydroxyurea (HU), an inhibitor of DNA replication. Central to the damage and replication checkpoint responses is Rad3, *S. pombe* homologue of ATR, encoding a protein with structural and functional similarities to ATM. Rad3 phosphorylates and activates the Cds1 kinase after a DNA replication block, and phosphorylates and activates the Chk1 kinase after DNA damage. However, Chk1 can also substitute for Cds1 in a DNA replication block (Lindsay *et al.* 1998) (Kelly and Brown 2000). Checkpoint activation blocks the onset of mitosis by decreasing the activity of the mitotic Cdc2/cyclin complexes by Cdc2 Y15 phosphorylation. Cds1 also plays a role in recovery from the S phase arrest; in an HU-induced S phase block cells lacking Cds1 lose viability (Walworth *et al.* 1993, Murakami and Okayama 1995, Lindsay *et al.* 1998, Boddy *et al.* 1998, Murakami and Nurse 2000, Boddy and Russell 2001).

Both Chk1 and Cds1 phosphorylation and activation are dependent on the presence of Rad3 and the other checkpoint Rad proteins. However, the actual sequence of upstream events leading to activation of Rad3 is unclear, with the identity of the DNA damage/incomplete replication sensors and initial

checkpoint activators remaining uncertain. I set out to find new genes or a new role for known genes involved in checkpoint activation upstream of Rad3. I postulated that overexpression of such genes might ectopically activate the replication and/or damage checkpoint in the absence of DNA damage or stalled replication. In the presence of Rad3 the checkpoint would be activated leading to cell elongation, but in the absence of Rad3 cells would grow normally. Thus checkpoint activation and cell cycle arrest should be: ***dependent*** on Rad3; but ***independent*** of DNA damage and DNA replication intermediates. Under normal circumstances the identified ectopic checkpoint activator would respond to DNA damage/ replication perturbations, but also not produce damage or replication intermediates itself that would lead to secondary checkpoint activation.

My approach was two-fold:

(1) To identify and test suitable candidate genes which would activate the checkpoint in a Rad3-dependent manner

(2) To screen for genes capable of ectopic activation of the checkpoint in a Rad3-dependent manner

2.2 Results

2.2.1 Candidate genes

The first approach was to identify possible candidates for the roles of DNA damage sensors and checkpoint activators. It was hypothesised that genes involved in detection of DNA double strand breaks (DSB) and subsequent

activation of the repair pathways may also be involved upstream in the damage checkpoint.

Key candidates for activating a Rad3-dependent damage checkpoint in fission yeast were selected: Ku70 and Ku80 are the *S. pombe* homologues of the human heterodimeric Ku protein subunits, Ku70 and Ku80. They bind directly to DSB during DNA repair by NHEJ; Rad22 (*S. pombe* homologue of human Rad52), known to bind to DSB in fission yeast (Kim *et al.* 2000); and Tel1 (*S. pombe* homologue of human ATM: ataxia telangiectasia mutated, and a Rad3 paralogue). ATM performs two main functions in humans: checkpoint control and telomere maintenance. The clinical picture is of an early onset autosomal recessive ataxia associated with characteristic chromosomal aberrations, cell cycle checkpoint defects, cancer susceptibility, and sensitivity to ionizing radiation. There are two *S. pombe* homologues of ATM: Rad3 (involved in checkpoint control and telomere maintenance) and Tel1 (involved in telomere maintenance only). A *tel1*$^{-}$ mutant strain has no obvious phenotype (Matsuura *et al.* 1999).

pRep41X *ku70* (a gift from M. Ferreira) and pRep1 *tel1* (a gift from A. Matsura) were received. *Rad22* was subcloned into pRep3X. The sequence of the putative *S. pombe* homologue of *hKu80* (*ku80*) was obtained from the Sanger Centre *S. pombe* blast server and the gene obtained by PCR from a *S. pombe* genomic library. BamH1 and Sal1 restriction enzyme sites were added allowing the *ku80* gene to be inserted into pRep3X. Sequencing confirmed the gene was full length with no mutations, deletions or insertions.

All 4 plasmids (pRep41X *ku70,* pRep1 *tel1,* pRep3X *rad22 and* pRep3X *ku80)* were transformed into a *leu-132 S. pombe* strain using the modified

Lithium Acetate protocol (see Chapter 7 Materials and Methods). The transformed cells were plated out and left to grow for 3 days at 32°C under selective conditions (the omission of leucine from the media selects for cells containing the LEU2 marked *nmt1* plasmids) in the presence of 15μM thiamine to suppress gene expression. The genes were induced by replica-plating onto selective media minus thiamine. The cells were examined by light microscopy at 24 hours for cell elongation due to checkpoint activation. A hundred colonies were screened per gene. However, none of the candidates produced an elongated phenotype and were not pursued further (*see Table 2.1*).

Table 2.1 Screening of candidate genes

Gene	Overexpression vector	Number elongated	% elongated
ku70	pRep41X	0/100	0
ku80	pRep3X	0/100	0
rad22	pRep3X	0/100	0
tel1	pRep1	0/100	0

2.2.2 Genetic Screening for Rad3-dependent cell cycle arrest

The second approach used was screening of *S. pombe* gene libraries.

2.2.2.1 S. pombe cDNA library overexpression screen

Two different *S. pombe* cDNA libraries were used: the Edgar/Norbury library *(B.Edgar and C.Norbury unpublished)* which uses the pRep3X vector containing the thiamine repressible full strength *nmt1* promoter (Maundrell 1990) and the *LEU2* marker; and a new Gateway compatible Lifetech library constructed from total *S. pombe* RNA derived from mitotic, meiotic and schmooing cells in a 2:1:1 ratio, within a Gateway modified version of the pRep4X vector (created by

T.Duhig in this laboratory*)*. This vector contains the same *nmt1* promoter as pRep3X but has the *ura4* marker in place of *LEU2*.

The genetic screen was then performed as described in the schematic (*Figure 2.1a*). The libraries were transformed into *h+ rad3^{ts} leu1-32 ura4-D18 ade6-704* (Martinho *et al.* 1998) using the modified Lithium Acetate protocol (see Chapter 7 Materials and Methods). The *rad3^{ts}* strain has a specific mutation in the *rad3* gene A2217V (alanine mutated to valine). At the permissive temperature (25°C) the *rad3^{ts}* strain behaves like *wildtype rad3^{+}*, but at the restrictive temperature (36°C) it exhibits the damage sensitivity phenotype of a *rad3Δ* deleted strain. Briefly, the transformed cells were plated out and left to grow for 5 days at 25°C under selective conditions in the presence of 15μM thiamine to suppress gene expression. They were then replica-plated onto selective media containing Phloxin B in the absence of thiamine to allow expression of the gene controlled by the *nmt1* promoter. Phloxin B was used as cells that die, for example if undergoing cell cycle arrest, take up the dye. Such colonies appear a much darker pink due to the Phloxin B accumulated in cells. Each plate was replica-plated in duplicate to enable observation of the effects of gene overexpression on the same colonies at both the permissive and restrictive temperatures (i.e. in the presence and absence of Rad3 function).

Screening was carried out after 24 hours (as it takes up to 12 hours at 32°C, and around 18 hours at 25°C, after the removal of thiamine to induce gene expression by the *nmt1* promoter). Initially, screening was done by eye at 25°C looking for the dark pink colonies due to increased Phloxin B uptake.

These colonies were then viewed under the light microscope to identify those with elongated cells. Where this was the case, the equivalent colony on the 36°C plate was viewed under the light microscope looking for normal growth. Colonies that showed elongation at 25°C and normal growth at 36°C (*see Table 2.2 below*) were picked and re-screened. If the phenotype persisted, plasmid recovery was performed. The 14 recovered plasmids were transformed back into the *rad3^{ts}* strain to determine which were responsible for the Rad3-dependent arrest phenotype, as some colonies may contain several different plasmids following library transformation. The phenotype maintaining plasmids were then transformed into and the genes overexpressed in *h- leu1-32 ura4D-18* and *h- rad3-136 leu1-32 ura4D-18* (behaves as a *rad3*Δ delete) strains at 25°C, 32°C and 36°C to exclude a temperature effect. As the initial screen had been performed at 25°C and 36°C, I had to ensure that the observed effects on cell growth were consequent to gene overexpression and not temperature. Plasmids producing the desired phenotype were fully sequenced to identify the gene, and to check the gene was full length with no mutations, deletions or insertions.

1) Edgar/Norbury *S. pombe* cDNA library: 50,000 colonies were screened; six colonies had the appropriate phenotype; sequencing of recovered plasmids found all six contained full-length *spd1* (S phase delaying protein).

2) Gateway compatible Lifetech *S. pombe* cDNA library: 80,000 colonies were screened; eight colonies had the desired phenotype; sequencing of the recovered plasmids revealed *cig1* (from 3 different plasmids from 3

separate colonies), *tel1* (from 2 different plasmids from 2 separate colonies), *cdc18*, *MCM7*, and *sty1*.

2.1.1.2 Cdc18

Cdc18/CDC6 is a key factor initiating DNA replication in eukaryotes. On transformation back into the *rad3^{ts}* strain, pRep4X *cdc18* strongly maintained the phenotype of cell elongation in the presence of Rad3 at 25°C, and wildtype growth in the absence of Rad3 at 36°C (Figure 2.2a). Sequencing showed the gene to be full-length with no mutations, insertions or deletions. There was a 70bp leader sequence. The pRep4X *cdc18* plasmid was then transformed into a *rad3^{+} leu1-32 ura4D-18* strain and into the *rad3-136 leu1-32 ura4D-18* (behaves as *rad3^{-})* strain using the modified Lithium Acetate protocol. The transformed cells were plated out and left to grow for 3 days at 32°C on selective media containing 15μM thiamine to suppress gene expression. The colonies were then replica-plated in triplicate onto selective media minus thiamine (to induce gene expression) and put at 25°C, 32°C and 36°C. They were screened after 24 hours by light microscopy to exclude temperature-sensitive elongation. In the presence of the Rad3 protein, elongated cells were observed at all three temperatures (*Figure 2.2b*). However, in the absence of Rad3 wild-type growth occurred at all 3 temperatures (*Figure 2.2c*). I concluded that the *cdc18*-induced elongation observed was Rad-dependent and not temperature-sensitive.

Table 2.2 cDNAs recovered from genetic screening

Gene	Function	Number of plasmids	Phenotype maintained on rad3ts retransformation	Temperature effect?	Human homologue (%homology): Function
cdc18	DNA replication initiation	1	yes	no	*CDC6* (33%): DNA replication initiation
cig1	B type cyclin	3	yes	yes	*Cyclin A2* (35%): At G1/S transition
spd1	RNR inhibitor	6	yes	no	None found
tel1	PI3K in telomere length control	2	no	not done	*ATM/ATR* (29%): Checkpoint and telomere length control
MCM7	MCM complex subunit	1	no	not done	*MCM7* (48%): DNA helicase subunit
sty1	MAPK (stress pathway)	1	very weak	not done	*MAPK14* (54%): stress response

2.1.1.3 Spd1

Six clones that produced a Rad3-dependent cell cycle arrest were found to contain the *spd1* (S phase delayed) gene. Spd1 was originally isolated in an overexpression screen looking for cell cycle inhibitors (Woollard *et al.* 1996). *Spd1* is a non-essential gene encoding a cell cycle-regulated protein, the levels of which peak in G1, decline in S-phase and then return in G2. Overexpression causes both a post-Start G1 arrest and a G2 arrest. The G2 arrest acts via Rad3. The G1 arrest is independent of all known checkpoint proteins but requires Cdc22. *Cdc22* encodes the large subunit (with *suc22* encoding the small subunit) of the essential ribonucleotide reductase (RNR) enzyme, which catalyses deoxyribonucleoside triphosphate (dNTP) formation for DNA replication and repair. The deletion of *spd1* produces an acceleration through G1 into S. However, Spd1 is not essential for the checkpoint response (Borgne and Nurse 2000, Liu *et al.* 2003, Hakansson *et al.* 2006).

I proposed that because Spd1 is not essential for the checkpoint response it may be acting as an amplifier of the upstream checkpoint signal (Borgne and Nurse 2000). This could manifest as damage tolerance in the absence of Spd1. Damage tolerance was screened for by comparing the UV–C, MMS, Bleomycin (which behaves as a radiomimetic) and HU survival curves for *spd1Δ::ura4* and *wildtype S. pombe* strains. One thousand log phase cells per plate of each strain were plated onto medium containing increasing concentrations of Bleomycin (from 0-6000μu/ml), MMS (from 0-0.06%) and HU (from 0-25mM). Cells from both strains plated out onto YE5S medium only were exposed to increasing doses of UV-C (0-600J/m^2). The cells were than

incubated at 32°C for three days before colonies were counted and survival curves calculated and plotted. The UV-C (Figure 2.3a), Bleomycin (Figure 2.3b), MMS (Figure 2.3c), and HU survival (Figure 2.3d) curves for the two strains were essentially identical, providing no evidence for increased damage tolerance in the absence of Spd1.

If Spd1 is not acting to amplify the checkpoint signal why do we see activation of the Rad3-dependent checkpoint? Other possible explanations considered were: Spd1 is directly damaging the cells (but that does not explain the Rad3-independent G1 arrest); there is functional redundancy between *spd1* and other checkpoint genes; Spd1 actually functions in other damage pathways, such as the stress pathway.

It has recently been proposed that Spd1 is an inhibitor/regulator of the RNR enzyme. This model assumed a direct interaction between Spd1 and Suc22 proteins (Liu *et al.* 2003). Spd1 inhibits RNR activity by anchoring the Suc22 subunit inside the nucleus during G1. With the fall of Spd1 levels in S phase, Suc22 is released into the cytoplasm to form the active enzyme complex with Cdc22, and produce the dNTPs required for replication. However, Hakansson *et al.* have demonstrated Spd1 binding to the Cdc22 subunit in the cytoplasm and propose a more direct inhibition of the RNR enzyme (Hakansson *et al.* 2006). This suggests that overexpression of Spd1 may produce a post-Start G1 arrest that is Rad-independent by depletion of dNTPs required for S phase. On deletion of *spd1*, activation of the RNR enzyme is no longer dependent on the S phase decline of Spd1 protein levels, explaining the acceleration seen from G1 into S.

It has been suggested that Chk1 may directly or indirectly modify Spd1 in response to DNA damage (Liu *et al.* 2003). Chk1 could be acting as a signal from the damage pathway to ensure degradation of Spd1 and release of dNTPs for use in DNA repair.

2.1.1.4 Cig1

Cig1 encodes a non-essential *S. pombe* B-type cyclin, but lacks the mitotic destruction box and putative ubiquitin interacting motif typical of this class (implying it is not cell cycle regulated). Cig1 overexpression produces elongation with a 1C DNA content (Bueno *et al.* 1991). A clear role for Cig1 in the cell cycle is yet to be found, but it is involved in the inactivation of Rum1 by phosphorylation. This releases the inhibitory effect of Rum1 on Cdc2, allowing progression through START into S phase (Moreno *et al.* 1994). It has 35% homology to human cyclin A2.

All three *cig1* plasmids recovered from the screen were transformed back into the *rad3^{ts}* strain, and all showed the same phenotype *(Figure 2.4a).* Sequencing confirmed a full-length gene in all 3 plasmids, with no mutations, deletions or insertions. Screening to exclude a temperature effect, by looking at *cig1* overexpression in *wildtype* and in the *rad3-136* strain at 25°C, 32°C and 36°C, was performed as described in *2.2.2.2.*. In the *rad3^{-}* background cells were: slightly elongated at 25°C; abnormal/curved at 32°C; and wild-type at 36°C (*Figure 2.4b*). In a *rad3^{+}* background cells were: elongated at 25°C; elongated and curved at 32°C; and wild-type at 36°C (*Figure 2.4c*). So, in the presence of Cig1 overexpression, cells appear elongated at 25°C and wild-type

at 36°C regardless of the Rad3 background status. I concluded that the apparent Cig1 induced Rad3-dependent elongation was in fact a Cig1 induced temperature-sensitive elongation.

2.1.1.5 Others (MCM7, Tel1, Sty1)

Tel 1 had previously been tested as one of the candidate genes and showed no elongation at 25°C. It encodes a phosphotidyl inositol (PI3) kinase involved in telomere length control in parallel with the Rad3-dependent checkpoint pathway. It has 29% homology with both human ATM and ATR (ataxia telangiectasia related). It was not possible to retransform one of the *tel1* plasmids into *rad3^{ts}*, whilst the other *tel1* plasmid failed to maintain the desired phenotype on retransformation into *rad3^{ts}*.

MCM7 is a component of the MCM complex required for DNA replication. It has 48% homology to human MCM7, a subunit of the DNA helicase. MCM7 failed to show cell elongation at 25°C on retransformation into *rad3^{ts}*.

Sty1 encodes a mitogen-activated protein kinase (MAPK) transmitting osmotic stress and other stress signals. It has 54% homology to human MAPK 14, a serine-threonine protein kinase involved in signalling the response to cytokines and physiological stimuli and triggers apoptosis in response to stress. There was only very weak effect on retransformation into *rad3^{ts}*, with cell elongation at 25°C seen in only 1% of colonies.

2.1.1.6 S. pombe genomic library Screen

A genomic library screen was performed as described in the schematic (*Figure 2.1b*). A size selected multi-copy genomic library (a gift from Tamagawa: consisting of large genomic fragments 11-12kb, with the *LEU2* marker) was transformed into the *rad3^{ts}* strain using the modified Lithium Acetate protocol. The transformed cells were plated out and left to grow for 3 days at 36°C (so that genes causing a Rad3-dependent arrest would be able to grow normally initially in the absence of Rad3) under selective conditions. Each plate was replica-plated in duplicate to enable observation of the effects of gene expression on a single colony at both the permissive and restrictive temperatures. 60,000 colonies were screened. No colonies were found with the desired phenotype of a Rad3-dependent arrest.

2.1.1.7 Human cDNA library overexpression screen

A Gateway compatible Lifetech human cDNA library within a Gateway modified version of the pRep4X vector was screened by the same protocol for the *S. pombe* cDNA libraries.

This was a preliminary screen only and was of 108,600 colonies. None gave the desired phenotype of a Rad3-dependent arrest.

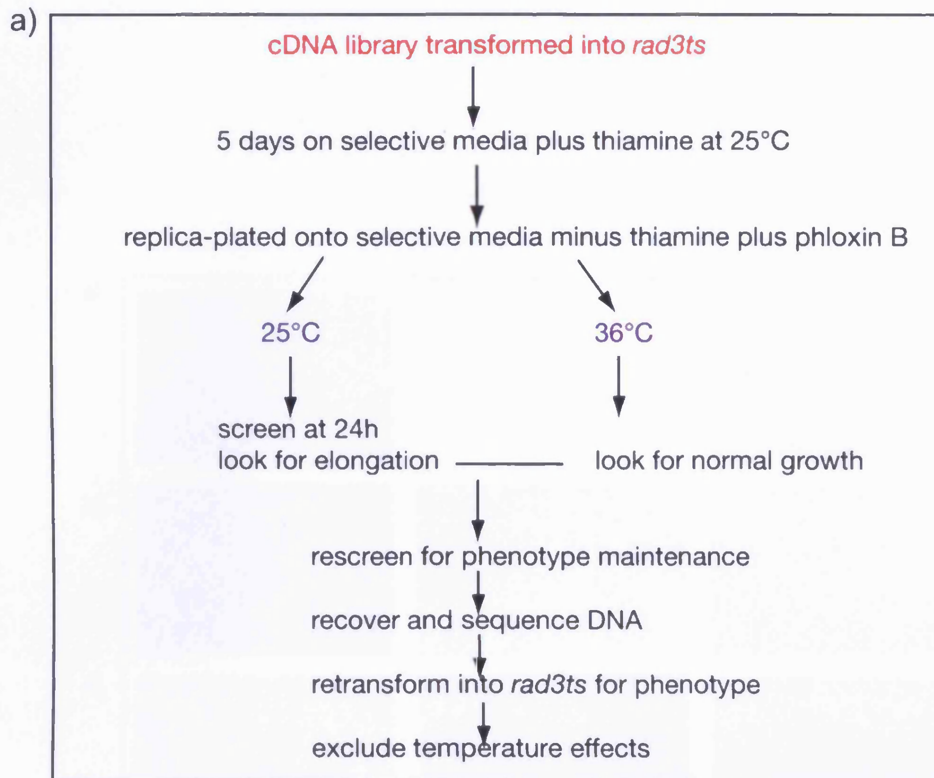
2.3 Discussion

I had set out to identify Rad3-dependent DNA damage/ perturbed replication sensors and checkpoint activators. All the candidate genes initially considered did not demonstrate such a role. Genetic screening of cDNA libraries

suggested Rad3-dependent checkpoint roles for Cdc18, Spd1, Cig1, Sty1, MCM7 and Tel1. However, Spd1 had no apparent direct checkpoint effect and the decision was made not to study it further. Cig1 was shown to have a temperature-sensitive rather than a Rad3-dependent effect on cell elongation. Sty1 was not studied further as only a very weak phenotype was seen on re-transformation. Despite the failure of MCM7 and Tel1 to reproduce the Rad3-dependent phenotype, which excluded them from further experiments during this work, they should be considered again in the future due to their close relationships respectively with Cdc18 and Rad3.

In view of the persistently strong phenotype seen with Cdc18 in comparison with the other genes obtained from the screen, I decided to work on the further characterisation of the Rad3-dependent checkpoint role of Cdc18.

a)



b)

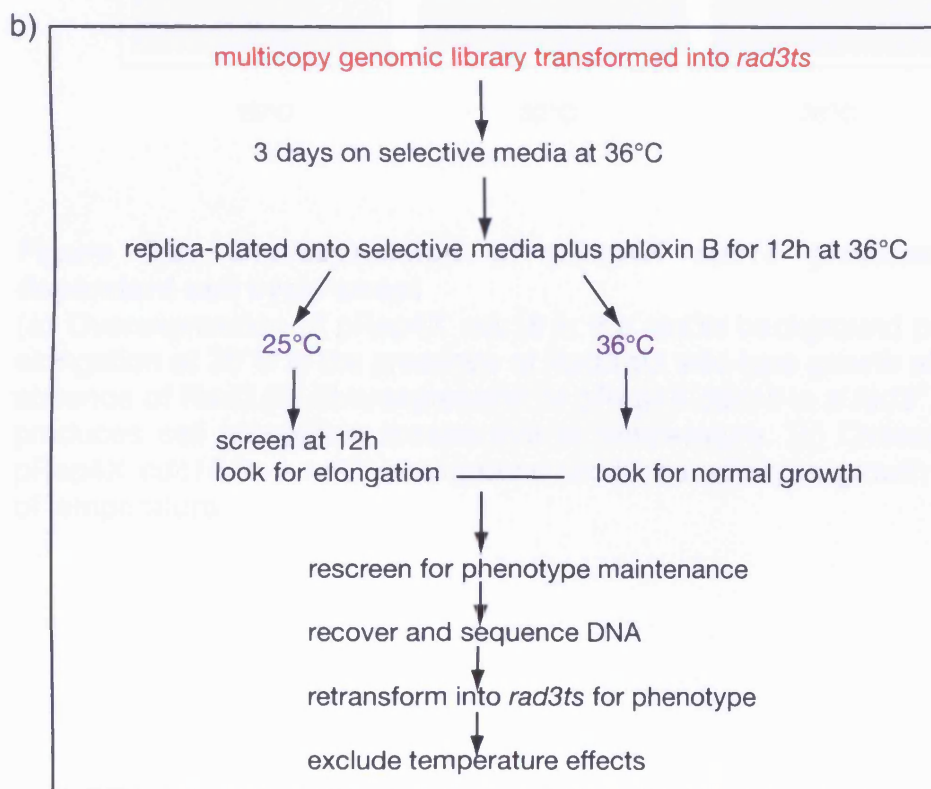


Figure 2.1: Genetic screening for activators of a Rad3-dependent cell cycle arrest
(a) Schematic for cDNA library screen (b) Schematic for multicopy genomic library screen.

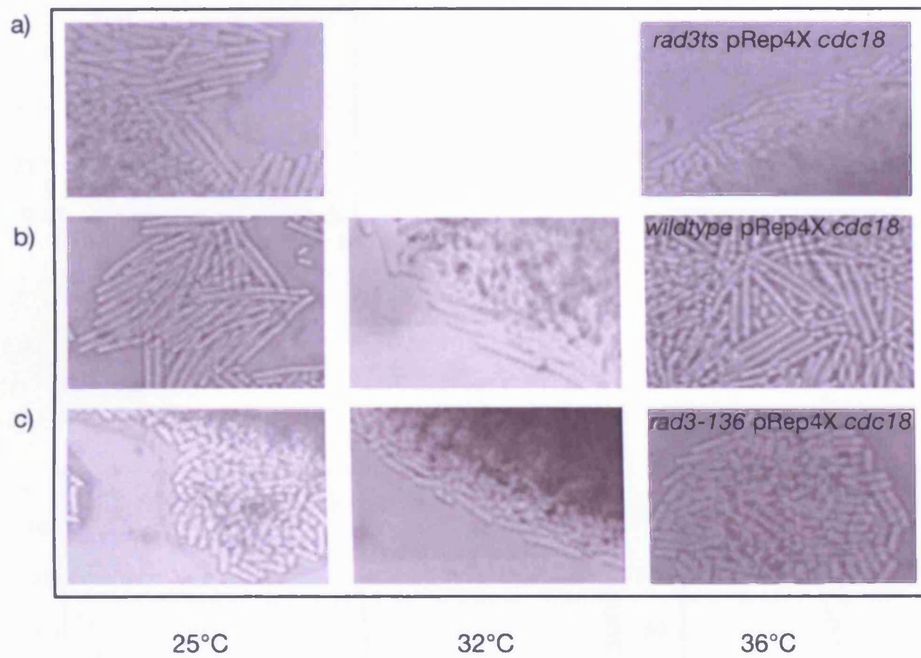


Figure 2.2: Overexpression of pRep4X *cdc18* produces a Rad3-dependent cell cycle arrest

(a) Overexpression of pRep4X *cdc18* in the *rad3ts* background produces cell elongation at 25°C in the presence of Rad3 but wild-type growth at 36°C in the absence of Rad3.(b) Overexpression of pRep4X *cdc18* in a *rad3*$^+$ background produces cell elongation irrespective of temperature. (c) Overexpression of pRep4X *cdc18* in a *rad3*$^-$ background produces wild-type growth irrespective of temperature.

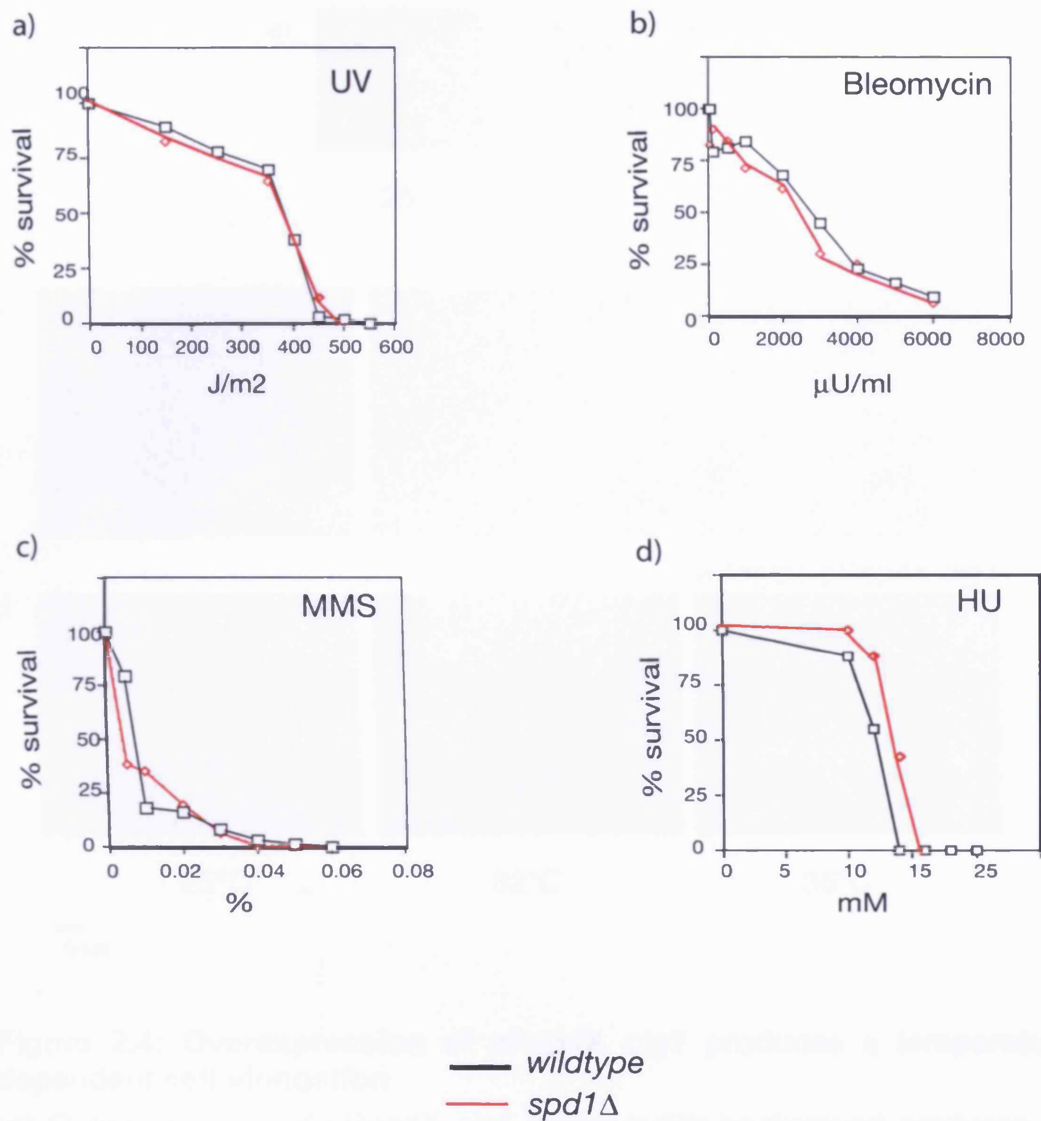


Figure 2.3: Spd1 does not amplify the checkpoint signal
Survival curves were obtained for *wildtype* and a *spd1Δ* strain in the presence of increasing (a) UV (b) Bleomycin (c) MMS and (d) HU. There was no obvious effect of the absence of Spd1.

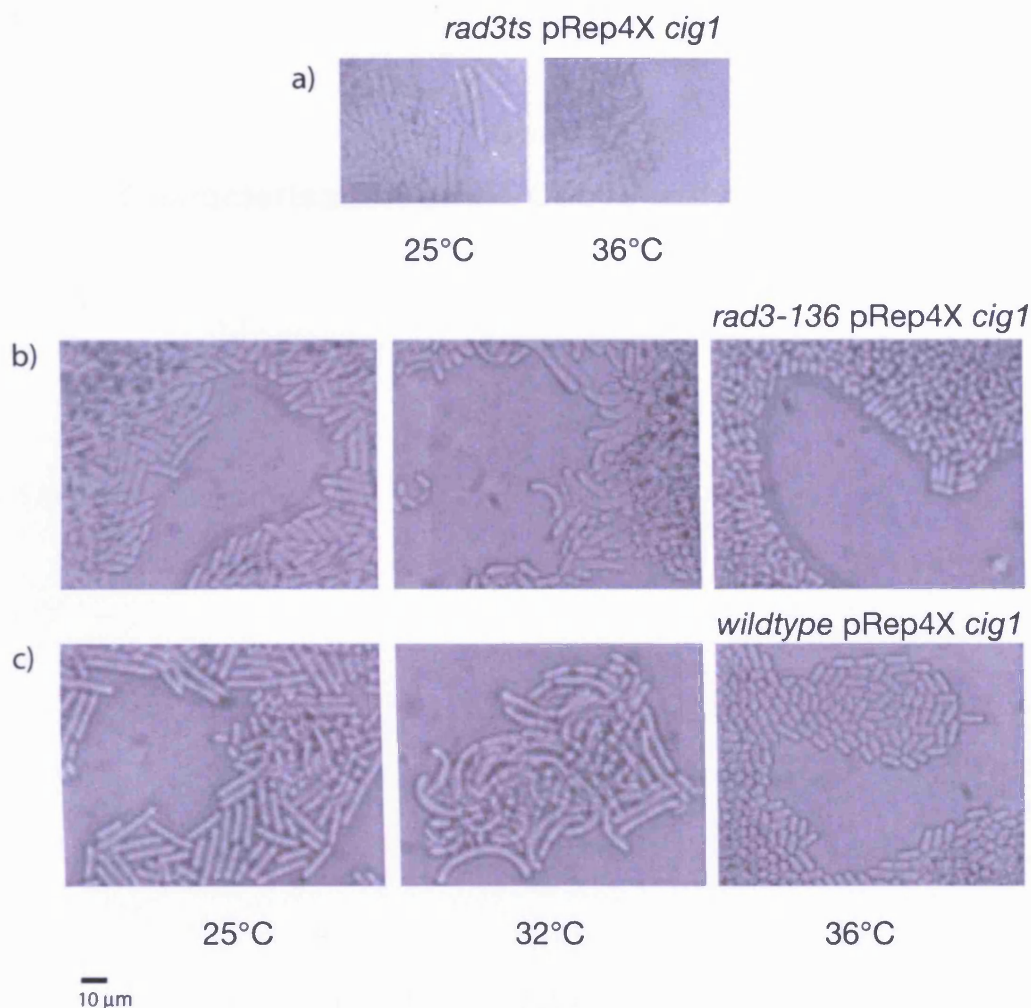


Figure 2.4: Overexpression of pRep4X *cig1* produces a temperature-dependent cell elongation

(a) Overexpression of pRep4X *cig1* in the *rad3ts* background produces cell elongation at 25°C in the presence of Rad3 but wild-type growth at 36°C in the absence of Rad3 (b) Overexpression of pRep4X *cig1* in a *rad3*$^{+}$ background produces slightly elongated cells at 25°C, abnormal/curved cells at 32°C, and wild-type growth at 36°C. (c) Overexpression of pRep4X *cig1* in a *rad3*$^{-}$ background produces elongated cells at 25°C, elongated and curved cells at 32°C, and wild-type growth at 36°C.

Chapter 3

Characterisation of the Cdc18-activated checkpoint I

3.1 Introduction

A genetic screen looking for upstream elements of the DNA damage and replication checkpoints revealed a Cdc18-activated Rad3-dependent cell cycle arrest. Cdc18 is the fission yeast homologue of human CDC6. It is an essential gene, and was cloned simultaneously as a multi-copy suppressor of *cdc10-129*^{ts} and by complementation of the *cdc18-k46* mutant (Kelly *et al.* 1993). It is a key initiator and regulator of DNA replication in eukaryotes, co-ordinating alternate rounds of S phase and mitosis (Nishitani and Nurse 1995). Eukaryotic replication is initiated at specific DNA sequences called replication origins (Kelly and Brown 2000) *(Figure 1.2).* They are recognised by the ORC (origin recognition complex) (Bell and Stillman 1992, Bell *et al.* 1993, Micklem *et al.* 1993, Grallert and Nurse 1996, Leatherwood *et al.* 1996, Lygerou *et al.* 1999). As cells reach the end of mitosis, origins are licensed by a step-wise recruitment of proteins to the ORC platform. Cdc18, along with Cdt1, is loaded onto the DNA from where they recruit the heterohexameric complex composed of the 6 minichromosome maintenance (MCM 2-7) proteins, and form the pre-replicative complex (Kelly *et al.* 1993, Hofmann and Beach 1994, Piatti *et al.* 1995, Nishitani and Nurse 1995, Muzi Falconi *et al.* 1996, Cocker *et al.* 1996, Tanaka *et al.* 1997, Detweiler and Li 1997, Donovan *et al.* 1997, Kearsey and Labib 1998, Maiorano *et al.* 2000, Nishitani *et al.* 2000). The MCM complex is considered to be the replicative helicase, travelling in front of the replication machinery opening up the DNA during S phase (Aparicio *et al.* 1997, Labib *et al.* 2000).

It is essential the entire eukaryotic genome is replicated completely, and that this is restricted to once per cell cycle. In fission yeast, Cdc18 regulation is crucial to prevent re-replication of DNA with a single cell cycle. Cdc18 expression is tightly cell cycle regulated, both transcriptionally and post-translationally. Transcription, under the control of Cdc10, starts in mitosis, peaks at G1/S and then is repressed during S phase and G2 (Kelly *et al.* 1993). However, Cdc18 does not accumulate during mitosis due to the presence of the mitotic CDK, Cdc2/Cdc13, which phosphorylates Cdc18, targeting it for ubiquitin-dependent degradation (Jallepalli *et al.* 1997, Jallepalli *et al.* 1998). A physical interaction between the amino-terminus of Cdc18 and CDK has been described and both Cdc2-Cig2 and Cdc2-Cdc13 phosphorylate Cdc18 *in vitro* (Brown *et al.* 1997, Lopez-Girona *et al.* 1998). With the drop in mitotic CDK activity in anaphase, Cdc18 levels rise and peak at G1/S.

The phosphorylation of Cdc18 by CDK is conserved but does not always lead to degradation. In humans and in Xenopus, nuclear export of phosphorylated Cdc18 occurs (Petersen *et al.* 1999, Elsasser *et al.* 1999, Fujita *et al.* 1999, Calzada *et al.* 2000, Drury *et al.* 2000).

This critical control of the timing of the initiation of DNA replication can be bypassed by strong overexpression of Cdc18 or a moderate co-overexpression of both Cdc18 and Cdt1, which allows re-initiation even in G2 cells (Nishitani and Nurse 1995, Yanow *et al.* 2001). The continuous DNA synthesis results in increased DNA content and giant nuclei, as visualised on fluorescent microscopy and FACS analysis, and is referred to as re-replication. It is thought that aberrant replication occurs in this situation, and the whole genome might not be fully re-replicated (Nishitani and Nurse 1995). Cdc18 overexpression

leading to re-replication has been shown to induce: (1) a Rad3-dependent mitotic block, with the likely mechanism of the structures generated by re-replication activating the S-M checkpoint; (2) a Rad3-independent block attributed to a direct interaction of the N-terminus of Cdc18 with and subsequent inhibition of Cdc2 (Greenwood *et al.* 1998).

Cdc18 is required to induce and maintain the S phase checkpoint. In its absence cells are unable to initiate DNA replication and proceed into mitosis with a <1C DNA content and generate a "cut" phenotype (*c̲ell u̲ntimely t̲orn*: cytokinesis takes place without prior completion of mitosis, resulting in the cleavage of an undivided nucleus by the septum) (Kelly *et al.* 1993). However, this S phase checkpoint role is not known to be Rad3-dependent.

3.2 Results

3.2.1 Stabilisation of Cdc18 activates a Rad3-dependent checkpoint in the absence of apparent re-replication

3.2.1.1 Description of pRep4X cdc18

The cDNA from the *cdc18*-containing plasmid, obtained from the overexpression screen using the Gateway compatible *S. pombe* cDNA library, was fully sequenced. This confirmed the presence of the full-length *cdc18* gene, with a 70 base pair 5'-untranslated region (5'UTR) and no mutations, deletions or insertions. The 5'UTR may affect expression and consequent phenotype. This could explain the different behaviours of studied *cdc18* cDNAs: the *cdc18* cDNA isolated as a multi-copy suppressor of Cdc10, which was missing the 5'UTR and the first 139 nucleotides of the coding sequence,

did not induce re-replication (Kelly *et al.* 1993); continuous DNA synthesis is observed in the *cdc18* cDNA with a 86 base-pair 5'UTR (Nishitani and Nurse 1995); and my newly isolated *cdc18* cDNA, which induces a Rad3-dependent checkpoint activation, in the absence of apparent re-replication as assessed by both microscopic observation and FACS analysis (*Figure 3.1a*). Another explanation for the different behaviours was the use of the pRep4X vector rather than the pRep3X, which produces a lower level of Cdc18 overexpression *(see section 3.2.2 below and Figure 3.2).*

The Rad3-dependence of the cell cycle arrest seen with overexpression of pRep4X *cdc18*, was investigated further by measurement of the plating efficiency (P.E.) of cells overexpressing Cdc18 in the presence and absence of Rad3. P.E. is the ratio of colonies grown to actual number of cells plated, and reflects the viability of the strain used. Cultures of *wildtype* (*rad3$^+$*) and *rad3-136* (a non-functional mutation that behaves as a *rad3$^-$* (Nasim and Smith 1975)) strains transformed with pRep4X *cdc18,* were grown to mid log phase in the presence of 15μM thiamine to suppress gene expression by the *nmt1* promoter. The cells were then filtered and washed three times to remove the thiamine. A thousand cells from each strain were plated out onto media with or without thiamine, and incubated for 3 days at 32°C before the number of colonies was counted and the P.E. calculated (*see Table 3.1*). When the promoter was repressed there was no difference seen in the number of colonies produced (P.E: 77% rad3$^+$ versus 75% rad3$^-$). After switching on Cdc18 overexpression, the P.E. fell to 2.5% in the presence of Rad3, but there was no effect on P.E. in the absence of Rad3 (which remained at 75%). I concluded that: Cdc18 overexpression activates a Rad3-dependent loss of cell viability;

elevated levels of Cdc18 *per se* cause no gross cellular damage, as assessed by normal cell viability and P.E in the absence of Rad3.

Table 3.1 Plating efficiency of 1000 cells

Promoter	*rad3*$^+$ pRep4X *cdc18*	*rad3*$^-$ pRep4X *cdc18*
OFF	77%	75%
ON	2.5%	74%

3.2.1.2 Description of Cdc18 T6A

The *cdc18* cDNA, obtained from the screen described in Chapter 2, produced a Rad3-dependent cell elongation on overexpression. However, the behaviour of the pRep4X *cdc18* was not reliably reproducible, with observation of incomplete penetrance. The degree/uniformity of cell elongation observed following the replica-plating of *rad3ts* pRep4X *cdc18* colonies to 25°C after the removal of thiamine (activating the Cdc18-induced Rad3-dependent cell cycle arrest) varied considerably between and even within colonies. Therefore, other means of stabilising Cdc18 levels to produce a Rad3-dependent cell cycle arrest were investigated. Phosphorylation of the 6 CDK consensus sites by Cdc2 regulates the stability of the Cdc18 protein (Nishitani and Nurse 1995, Jallepalli *et al.* 1997, Greenwood *et al.* 1998, Lopez-Girona *et al.* 1998, Baum *et al.* 1998), and over-expression of a mutant lacking these phosphorylation sites results in increased re-replication (Jallepalli *et al.* 1997). The endogenous *cdc18* gene was replaced in a *rad3*^{ts} strain by a *cdc18 T6A* mutant gene encoding a protein with all 6 CDK sites mutated from threonine to alanine (at positions: 10, 46, 60, 104, 134 and 374) expressed by the *cdc18* promoter. This prevents phosphorylation by Cdc2 thus inhibiting Cdc18 proteolysis and producing stable

high Cdc18 levels. The *cdc18 T6A* mutant strain was only viable in the absence of Rad3. Incubation of this strain at 25°C resulted in cell elongation, however at 36°C cells grew normally (*Figure 3.1b*). No re-replication was observed on FACS analysis and no enlarged nuclei (typical of DNA re-replication) were observed (*Figure 3.1c*). I concluded that the Cdc18 phosphorylation mutant activates a Rad3-dependent cell cycle arrest, in the absence of apparent DNA re-replication.

Of note, two different *cdc18* phosphorylation mutant parent strains were used in this thesis: one with the *cdc18 T6A* mutant gene tagged with the *S. cerevisiae* marker *LEU2* (which complements the *S. pombe leu1-32* mutation); and one with a *kan^r TAP-tagged cdc18 T6A* mutant gene. All strains derived from crosses using these two parent *cdc18 T6A* strains are clearly described in Tables 7.1 and 7.2 in Chapter 7 Materials and Methods. Table 3.2 below describes the 4 most frequently used *cdc18 T6A* strains.

Table 3.2 *Cdc18 T6A* mutant strains

Genotype	
h+ rad3^{ts} cdc18-T6A LEU2 ade6-704 leu1-32 ura4-D18	CCL1
h+ rad3^{ts} cdc18-T6A-TAP kan^r ade6-704 leu1-32 ura4-D18	CCL2
h+ cdc25-22^{ts} rad3Δ::ura4 cdc18-T6A kan^r leu1-32 ura4-D18	CCL3
h+ rad3^{ts} cdc18-T6A LEU2 ade6-704 leu1-32	CCL4

3.2.2 Level of Cdc18 appears important for effect

To test whether the level of Cdc18 is important for cell elongation in the absence of re-replication, Western blotting was performed to look at the Cdc18 protein expression levels in *rad3+* pRep3X *cdc18*, *rad3^{ts}* pRep4X *cdc18*, *rad3^{ts} cdc18 T6A-TAP* (CCL2) and *rad3+ (Figure 3.2).* This showed: Cdc18 levels are

apparently lower in the Cdc18 phosphorylation mutant than when it is overexpressed in the pRep vectors; there is a apparent higher level of Cdc18 overexpression in the pRep3X than in pRep4X. This suggests the possibility that the actual level of Cdc18 may determine its effects: (1) re-replication occurring at higher levels (as seen with pRep3X *cdc18*); (2) Rad3-dependent arrest in the absence of apparent re-replication at lower levels (as seen with pRep4X *cdc18* and *cdc18 T6A*). I speculated that lower levels of Cdc18 overexpression/stabilization might induce the S-M checkpoint without re-replication. Cdc18 T6A is more stable and reproducible, probably as a result of only moderately elevated Cdc18 levels. I therefore used this mutant for further characterisation of the Cdc18 induced Rad3-dependent checkpoint.

3.2.3 Cdc18 acts at the beginning of the checkpoint pathway and via Rad3/Chk1/Crb2

3.2.3.1 Cdc18 acts via Rad3

My results so far have suggested that stabilisation or elevated levels of Cdc18 sends the S-M checkpoint signal in the absence of DNA replication intermediates. If true, then Rad3 inactivation would abrogate the signal and release the cells from the cell cycle block. To test this possibility, an asynchronous population of log phase *rad3*^{ts} *cdc18 T6A* (CCL1) cells was blocked for 4 hours at 25°C. The temperature was then raised to 36°C to inactivate *rad3* (see schematic in *Figure 3.3a).* Samples were taken every 20 minutes from release for FACS analysis and for calculation of the septation index and the percentage of binucleated cells present. As predicted, cells were synchronously released from the cell cycle block *(Figure 3.3a).* This experiment

demonstrates that Rad3 is required to not only to initiate and but also to maintain the checkpoint signal generated by high level of Cdc18.

3.2.3.2 Cdc18 acts via rad3/chk1/crb2

The block and release procedure described in 3.2.3.1 was also used to test activation of the downstream effector checkpoint kinases Cds1 and Chk1. A *rad3*^{ts} *cdc18 T6A chk1-HA* strain was used. However, after Rad3 inactivation for 1 hour at 36°C (see schematic in *Figure 3.3b*), the culture was shifted back to 25°C for 2 hours to re-impose the mitotic block. Protein extract samples were taken every 20 minutes for 3 hours after release, and the phosphorylation status of Cds1 and Chk1 analysed by Western blot. After shift back to 25°C when Rad3 was active, only a small proportion of the Cds1 protein pool showed the altered mobility indicative of its phosphorylated active form (data not shown) (Lindsay *et al.* 1998). However, most of Chk1 was converted to a slow migrating form corresponding with its phosphorylation and activation (Walworth and Bernards 1996) *(Figure 3.3b)*, suggesting that Chk1 is likely to be the main effector kinase responsible for the block.

Genetic crosses were performed to see whether the other *rad* checkpoint genes were also required for the block. Inactivation of *chk1, rad9, hus1, rad17, rad1, rad26* and *crb2* suppressed the elongated phenotype, cells looked wild-type, and were able to grow and divide normally. In all cases backcrossing recovered the *rad3*^{ts} *cdc18 T6A* phenotype, confirming its presence in the checkpoint mutant background. Deletion of either *cds1* or its activating partner *mrc1* demonstrated they were not required for the cell cycle block, because cells still became elongated in their absence *(Table 3.3 and Figure 3.4)*. Cdc18

therefore appears to be activating the Chk1/Crb2 damage arm of the Rad3-dependent checkpoint rather than acting through the Cds1/Mrc1 replication arm.

Table 3.3 Genetic crosses

Checkpoint mutant	Phenotype with *cdc18 T6A*	*rad3ts cdc18 T6A* phenotype seen on backcross
rad3ts 25°C	elongated	*
36°C	wild-type	
rad3Δura4	wild-type	Yes
rad3ΔkanR	wild-type	Yes
rad26Δura4	wild-type	Yes
rad26Δkanr	wild-type	Yes
chk1Δura4	wild-type	Yes
chk1Δkanr	wild-type	Yes
chk1Δura4 cds1Δura4	wild-type	Yes
crb2Δura4	wild-type	Yes
crb2Δkanr	wild-type	Yes
rad9Δura4	wild-type	Yes
rad9ΔkanR	wild-type	Yes
hus1 1-14	wild-type	Yes
hus1ΔkanR	wild-type	Yes
rad17 h-21	wild-type	Yes
cds1Δura4	elongated	*
cds1ΔkanR	elongated	*
mrc1Δura4	elongated	*

*not backcrossed as had elongated phenotype

I conclude that an elevated level of Cdc18 acts upstream of Rad3 to activate the S-M checkpoint through the Chk1 kinase, and that components of the *rad* checkpoint gene network are required to maintain the cell cycle block.

3.2.4 Checkpoint genes are not required to maintain DNA damage intermediates seen on two-dimensional (2D) DNA gel electrophoresis.

Neutral-neutral two-dimension DNA gel electrophoresis, followed by Southern blotting and probing with a fragment of interest, is a technique used to detect replication or recombination intermediates within a specific region of the genome (Friedman and Brewer 1995). Typical structures seen are: the "simple Y" (corresponding to passive replication of a fragment); the "bubble arc" (corresponding to origin firing); and, the "X-spike" (corresponding to recombination and/or termination intermediates) (*Figure 3.5a*). I wanted to see if DNA damage was represented on a 2D gel as a specific structure, and, would such a structure play a role in upstream checkpoint signalling.

3.2.4.1 DNA damage produces a specific 2D gel intermediate

Neutral-neutral 2D DNA gel analysis of wild-type cells treated with 0.02% MMS (a DNA damaging agent) revealed a structure not seen with HU treated wild-type cells. An exponentially growing *wildtype* culture (of the *972 h-* strain) at 32°C was split at t=0h; 11mM HU was added to one half; 0.02% MMS to the other. At 5 hours samples were taken from both cultures for preparation of genomic DNA for 2D gel electrophoresis. Southern blots of the gels were probed with origin and non-origin containing fragments of rDNA. The rDNA replication origin is *ars 3001*, which has been mapped to a 600 bp sequence in the upstream non-transcribed regions of the rDNA repeats. All origin activity is confined to this sequence, with no activity detected in other regions of the 10.4Kb rDNA repeat. The origin probe recognises *ars 3001*, demonstrating the "simple Y" and the "bubble arc", as well as the "X-spike". The non-origin probe

recognises a fragment of the rDNA repeat spanning the 5.8S and 25S genes, downstream of *ars 3001*. It reveals the "simple Y" of passive replication and the "X-spike" only *(Figure 3.5b)*.

An additional structure was found at the top of the X spike in the MMS treated cells only. This "X" like structure could be a DNA damage intermediate (*Figure 3.5b*). It was hypothesised that this intermediate structure could function as part of the DNA damage checkpoint, either as an upstream signal or as a downstream target.

3.2.4.2 The damage intermediate may be cell cycle phase dependent

The next step was to determine whether the "X" structure could be produced by DNA damage at any point in the cell cycle, or whether it is specific to a particular cell cycle phase. *Wildtype* cells were blocked in S phase by adding 11mM HU to an exponential culture at 32°C. After 3.5h 0.02% MMS was added. Samples were taken at 3.5h and 5.5h for preparation of genomic DNA for 2D gel electrophoresis. Cells were blocked in G2 using a *cdc25-22^{ts}* strain, exponentially growing cells at 25°C were shifted to 36.5°C for 3.5 hours (G2 block confirmed by FACS analysis, DAPI staining and by absence of replication intermediates on the 2D gel) and then 0.02% MMS was added. Samples were again taken at 3.5h and 5.5h for preparation of genomic DNA for 2D gel electrophoresis. Both gels were blotted and probed with the 3.4Kb non-origin rDNA fragment.

The "X" structure was present after the addition of MMS in both S phase and G2 blocked cells (*Figure 3.6a*). This may be interpreted as either the damage intermediate being produced in G2 cells (and not cell cycle phase

dependent), or as leak through the block with damage subsequently occurring in S phase cells (and therefore S phase dependent). The Y arc seen in the damaged G2 cells may represent strand invasion as part of the repair process and not replication intermediates. I concluded that the "X" structure may be cell cycle phase specific. S phase cells will be most sensitive to the effects of an alkylating agent such as MMS, explaining the strong presence of the structure in asynchronous and S phase cells, but its weak signal in G2 cells.

3.2.4.3 Presence of the damage intermediate is not rad checkpoint gene-dependent

If the structure is either a downstream target of, or a component of, the damage checkpoint, its presence would be dependent on the *rad* checkpoint genes. To test this, 0.02% MMS was added to exponentially growing cultures of *rad3Δ, chk1Δ, cds1Δ, cds1Δchk1Δ, hus1-14, rad1Δ, rad17-,* and *rad9Δ strains* at 32°C. Samples were taken for preparation of genomic DNA for 2D gel electrophoresis at 0h and 5h. Southern blotting of the gels and probing with the 3.4Kb non-origin rDNA fragment demonstrated the presence of the structure in all the Rad checkpoint mutants after exposure to MMS, although it was rather weak in some mutants *(Figure 3.6b).*

In conclusion, the structure does not appear to be a downstream target of the damage checkpoint. However, it may play an upstream role signalling the presence of DNA damage at any stage in the cell cycle with subsequent activation of the damage and/or the repair pathways (either the non homologous end joining or homologous replication repair pathways).

3.2.5 *Cdc18 sends the checkpoint signal but is not required for fork maintenance*

Cdc18 is required to induce and maintain the S phase checkpoint. In the absence of Cdc18, cells cannot initiate DNA replication and proceed into mitosis with a 1C DNA content, and "cut" cells are seen (Kelly *et al.* 1993).

3.2.5.1 Cdc18 accumulates in an HU block

To investigate what happens to Cdc18 levels during an HU-induced S phase block, a *cdc25-22 cdc18-TAP* strain was synchronized in late G2 by incubation at 36.5°C for 4 hours and then released in the absence or presence of HU for 3 hours. Samples were taken every 20 minutes following release for: (1) FACS analysis of DNA content to follow progression through the cell cycle; (2) Western blot analysis of total protein lysates using anti-Cdc18 antibodies *(Figure 3.7a)*. In the absence of HU, Cdc18 levels peaked with the first S phase and then disappeared. However, Cdc18 levels remained high throughout the HU-induced S phase block. This suggests that Cdc18 accumulates in an HU block.

3.2.5.2 This accumulation is Rad3-dependent

The experiment described in section 3.2.5.1 was repeated using a *cdc25-22 rad3Δura4 cdc18-TAP* strain to see if the accumulation of Cdc18 in an HU block required the presence of Rad3. The cells were again synchronized in late G2 by incubation at 36.5°C for 4 hours and then released in the presence of HU for 3 hours. Samples were again taken every 20 minutes for FACS and Western blot analysis. In the absence of Rad3, Cdc18 levels peaked and then fell,

implying that Cdc18 accumulation during an HU block is dependent upon the presence of Rad3 and the Rad3-dependent checkpoint (Figure 3.7b).

3.2.5.3 Cdc18 is required to maintain the checkpoint response but is not needed to maintain the replication fork

A *cdc18* switch off (S/O) strain (in which wild-type level Cdc18 expression is controlled by the thiamine-repressible nmt81 promoter) was used to further explore the role of Cdc18 in the HU checkpoint response (see timeline in *Figure 3.8).* A 32°C exponentially growing culture was split at t=-4h and 11mM HU was added to both cultures. At t=-1h one culture received thiamine at a concentration of 5µl/ml to switch off *cdc18* expression (this takes 1 hour). At t=0h both cultures were followed for the next 6 hours in a continued HU block. Hourly samples were taken for FACS analysis, and to monitor cell phenotype using DAPI and calcoflor staining. After 4 hours in the presence of thiamine, the *cdc18-S/O* strain started to accumulate cells with aberrant mitosis including "cut" cells *(Figure 3.8b),* indicating that the artificial depletion of Cdc18 leads to loss of the mitotic block imposed by the checkpoint response.

It has been proposed previously that replication forks play an essential role in checkpoint activation (Tercero *et al.* 2003). Also, inactivation of both ORC (Orp1) and Cdc18 results in disappearance of replication forks and loss of the checkpoint (Murakami *et al.* 2002). The above experiment was repeated to look at what happens to the replication intermediates on the depletion of Cdc18 alone during an HU block. At 2h, 4h and 6h samples were taken for preparation of genomic DNA for two-dimensional gel electrophoresis. The 2D DNA gels were Southern blotted and probed with the *ars 3001* origin rDNA fragment.

Replication forks were seen at similar intensity in both the presence (*Figure 3.8a*) and absence of Cdc18 (*Figure 3.8b*). This suggests that although Cdc18 stabilization is essential to maintain the checkpoint response and prevent aberrant entry into mitosis, it is not required to maintain the stalled replication forks. It also implies that that the aberrant mitotic entry is not a consequence of replication fork collapse. These results uncouple the roles of Cdc18 in fork assembly and in checkpoint activation.

3.3 Discussion

The key conclusions from the results in Chapter 3 are:

1) Elevated levels of Cdc18 activate a Rad3-dependent checkpoint arrest
2) The checkpoint acts through the Rad3/Chk1/Crb2 "damage" pathway and not the Rad3/Cds1/Mrc1 "replication" pathway
3) Rad3 is required to initiate and to maintain the checkpoint signal generated by a high level of Cdc18
4) Cdc18 acts early on in this checkpoint pathway
5) Wild-type Cdc18 is required for checkpoint control (eliminate Cdc18 and the checkpoint is lost), even when the checkpoint is activated by other means (such as HU), and this Cdc18 accumulation is also Rad3-dependent
6) Cdc18 is not required to maintain stalled replication forks in an HU block

A late step for S phase activation is Cdc2/Cig2 activity, which also phosphorylates Cdc18, targeting it for by ubiqitin-dependent degradation. The S phase checkpoint blocks mitosis via Rad3 in the presence of replicating DNA

to ensure mitosis does not occur until S phase is completed. I have shown that on removal of Cdc18 from cells blocked in S phase, cells enter aberrantly into mitosis. As the presence of Cdc18 is essential for the checkpoint response, the checkpoint is dependent on the inhibition of the cyclin-cdk complex activity and hence prevention of Cdc18 destruction. On checkpoint activation, Rad3 inhibits Cdc2, leading to a decrease in Cdc18 phosphorylation and subsequent degradation.

This could result in the generation of a positive feedback loop, in which the initial stabilisation of Cdc18 is in turn enforcing the checkpoint pathway by further Rad3 inhibition of Cdc2 and subsequent further stabilization of Cdc18 levels (*Figure 3.9a*). So, in the presence of a replication block, such as that induced by HU, the positive feedback loop is put into action. However, this is lost in the absence of Cdc18, and aberrant mitotic entry observed. The Cdc18 phosphorylation mutant has the same effect as a replication block. With no Cdc2-induced Cdc18 degradation possible, the checkpoint is enforced and the cells don't enter mitosis (*Figure 3.9b*).

I hypothesized that this loop is only activated when a certain threshold is exceeded, with wild-type Cdc18 levels on the DNA being too low to activate the checkpoint response during a normal S phase (due to their rapid degradation by the CDK complex). This would allow the cell to discriminate between local noise activating Rad3, and the necessity to amplify the response and institute a complete arrest. The overexpression and stabilisation of Cdc18 in these experiments are exceeding the set threshold and activating the checkpoint.

I also propose that, in light of the evidence that Cdc18 is not required to maintain the stalled replication forks during S phase, it is actually acting as an S

phase sensor, letting the cell know that replication is ongoing and to hold off from mitosis.

The 2D DNA gels looking at the effects of DNA damage showed a damage intermediate (the "X" spike). This may be a visualisation of a Rad3-dependent checkpoint activator, generated in response to DNA damage, acting in a similar way to Cdc18 stabilisation.

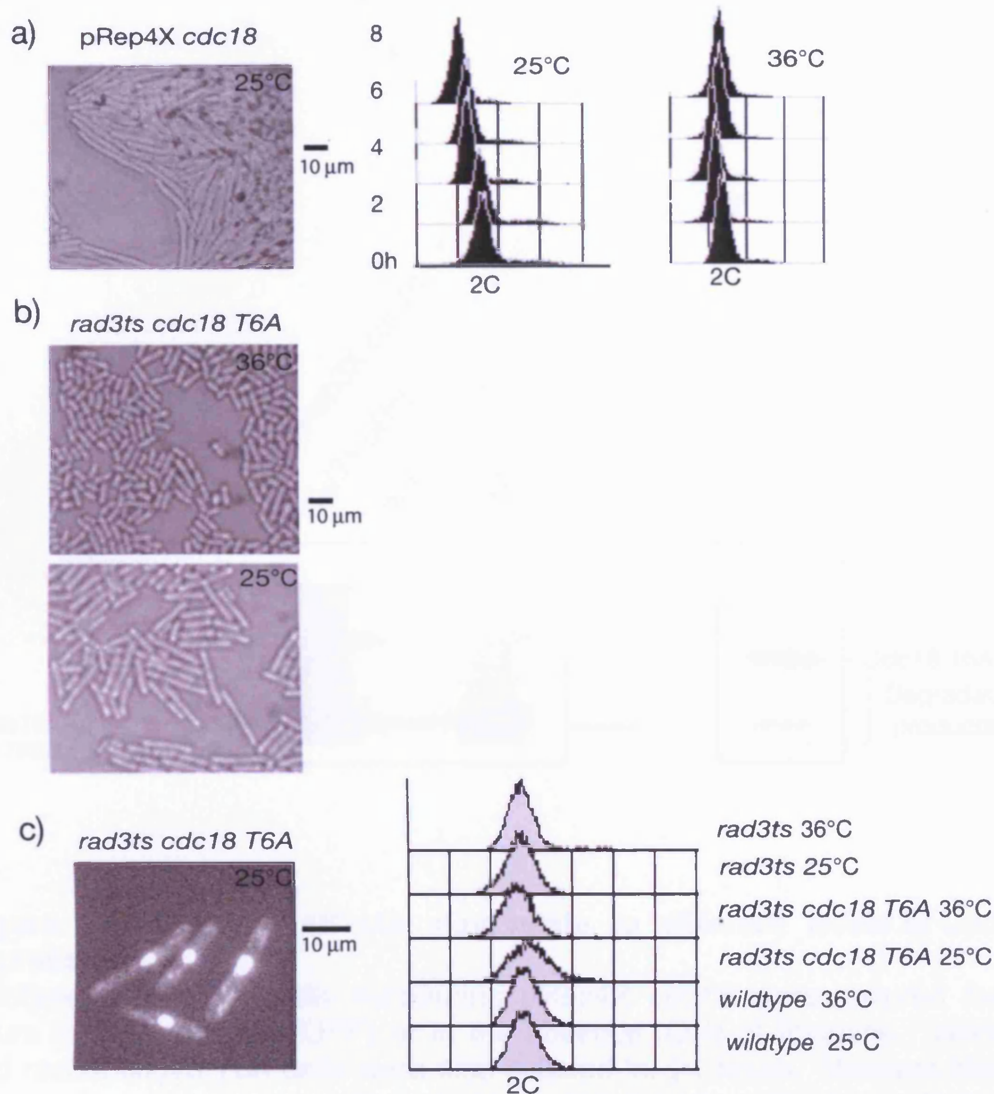


Figure 3.1: Elevated levels of Cdc18 induce a Rad3-dependent cell cycle arrest with no evidence of re-replication on microscopy or FACS analysis

(a) Left panel: cell elongation is observed when *rad3ts* pRep4X *cdc18* cells grown at 36°C in the presence of thiamine (OFF), are replica-plated to 25°C in the absence of thiamine (ON). Right panel: *rad3ts* pRep4X *cdc18* cells were grown for 24 hours at 36°C in the presence of thiamine (OFF), the thiamine was then washed out and the cells resuspended in the absence of thiamine (ON). The culture was then split, and the cells were grown for 22 hours at either 36°C (*rad3*$^-$) or 25°C (*rad3*$^+$), before samples were taken two hourly for FACS analysis. Note no re-replication on FACS at both temperatures. (b) *Rad3ts cdc18 T6A* cells exhibit wild-type growth at 36° C (top panel), but elongate after 6 hours at 25°C (bottom panel). (c) There is no evidence of re-replication in elongated *rad3ts cdc18 T6A* cells on light microscopy (left panel) or FACS analysis (right panel).

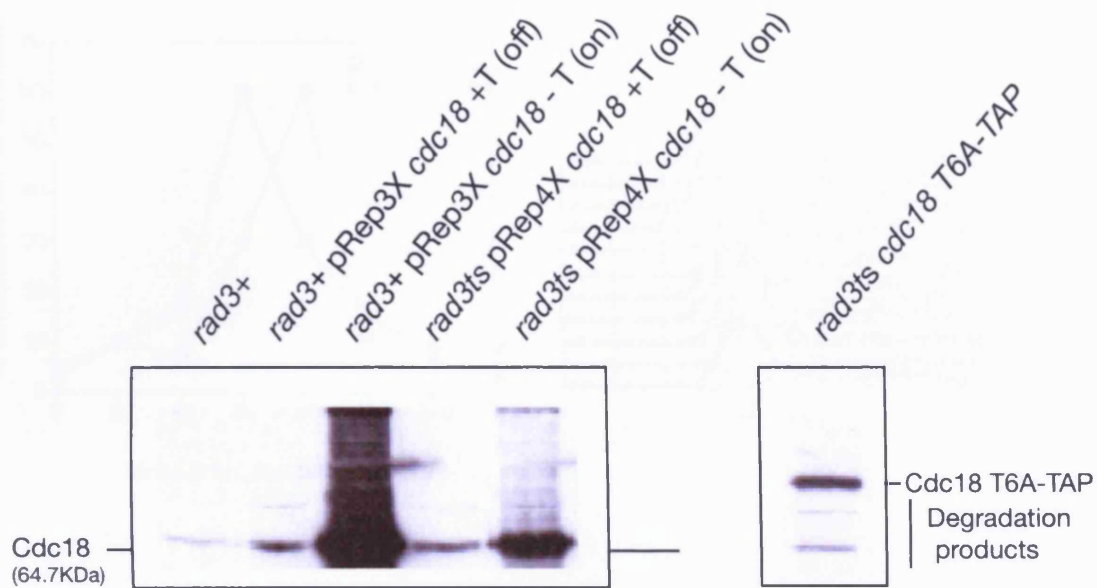


Figure 3.2: Different effects may relate to different levels of Cdc18 expression

Wildtype and *rad3ts* cells harbouring pRep4X *cdc18* were cultured for 24 hours in the presence (OFF) or in the absence (ON) of thiamine. *Wildtype* and *rad3ts cdc18 T6A* cells were also cultured for 24 hours. Western blotting was performed probing with polyclonal anti-Cdc18 antibodies. Note the Cdc18-T6A phosphorylation mutant protein runs at a higher molecular weight due to the attached TAP tag.

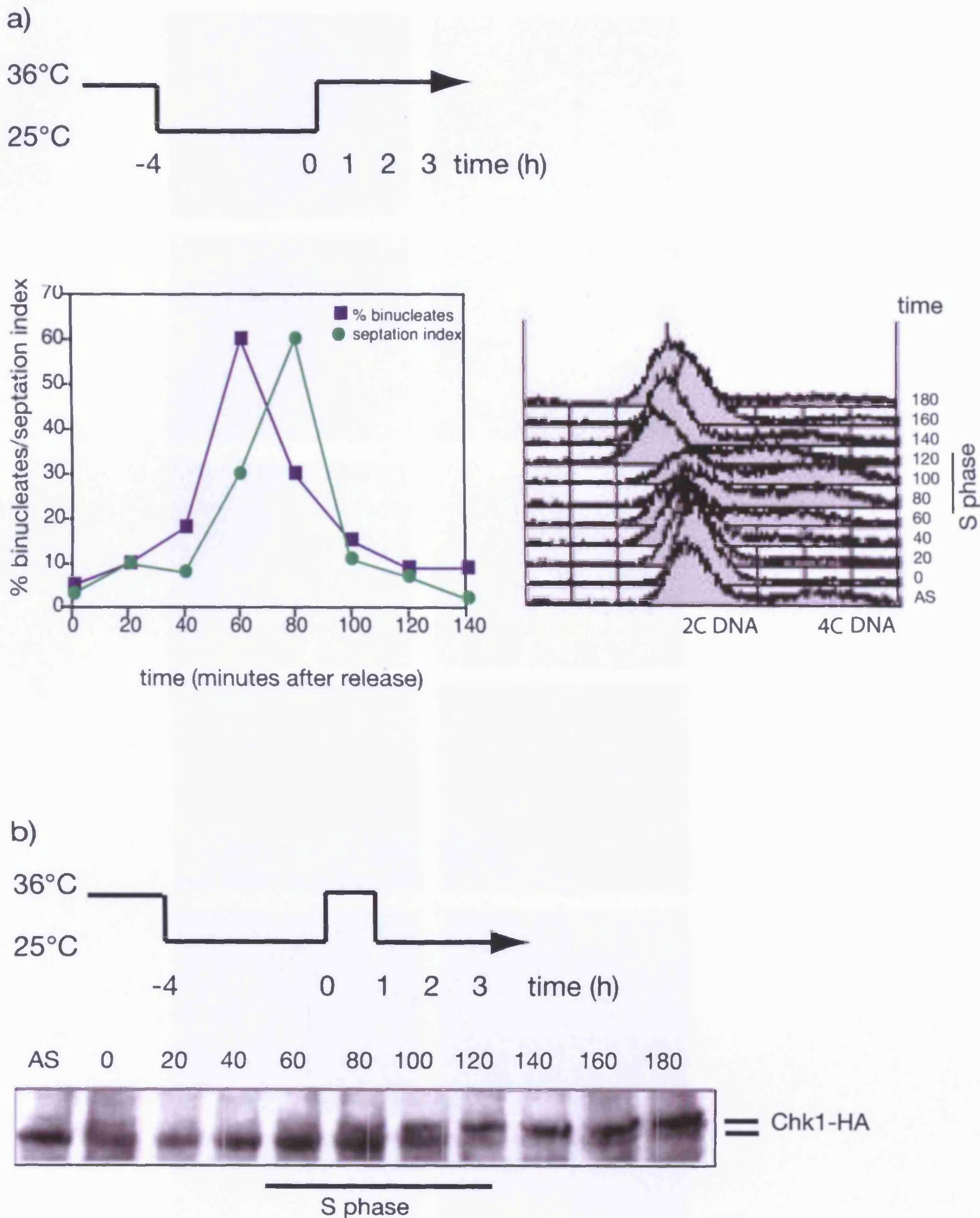


Figure 3.3: Mutation of the CDK consensus sites of Cdc18 results in a Rad3-dependent cell cycle arrest and Chk1 activation

(a) A *rad3ts cdc18 T6A* strain was synchronized using a Cdc18-activated Rad3-dependent block at 25°C (see schematic). The synchronous release was confirmed by FACS analysis (bottom right) and the percentage of binucleated cells and the septation index (bottom left). (b) A *rad3ts cdc18 T6A chk1-HA* culture was synchronized as in (a) but shifted back to 25°C (see schematic) after 1 hour to re-impose the mitotic block. Samples were taken for western blotting using anti-HA antibodies. Note that most of Chk1 is converted to a slower migrating form corresponding with its phosphorylation and activation.

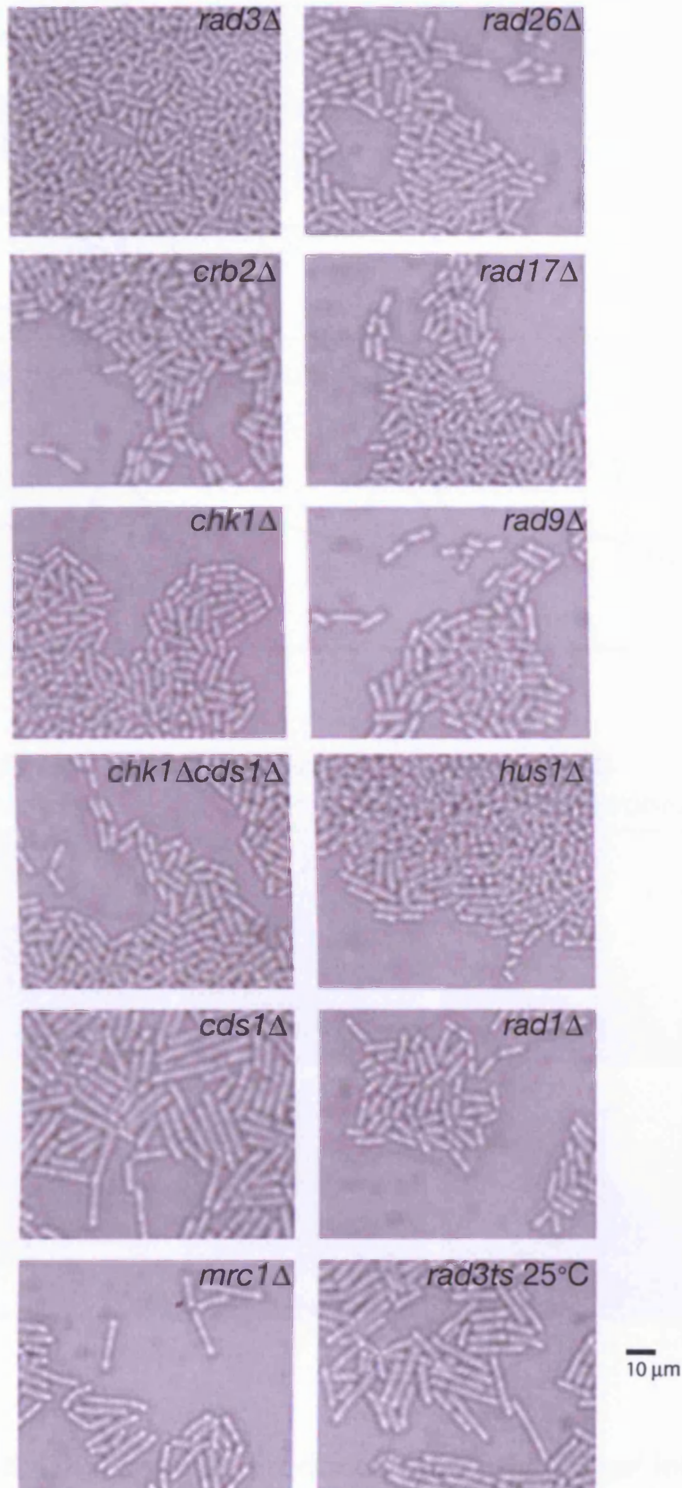
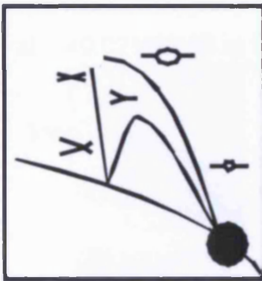
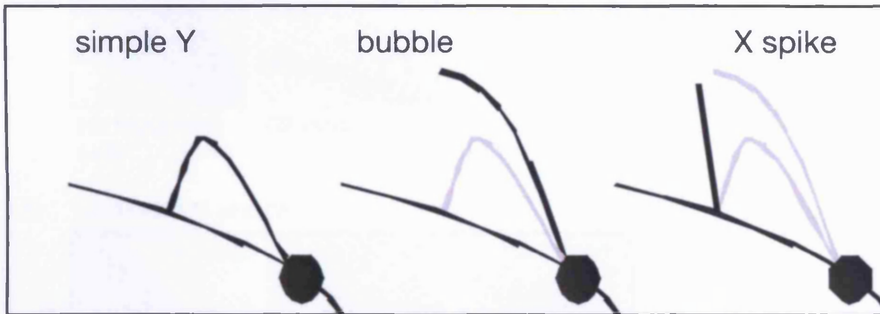


Figure 3.4: The Cdc18-activated Rad3-dependent cell cycle arrest acts through the Chk1/Crb2 pathway and not Cds1/Mrc1
The *cdc18 T6A* phosphorylation mutant was expressed in the known essential checkpoint pathway mutant backgrounds, and elongation or wild-type growth screened for using light microscopy.

a)





b)

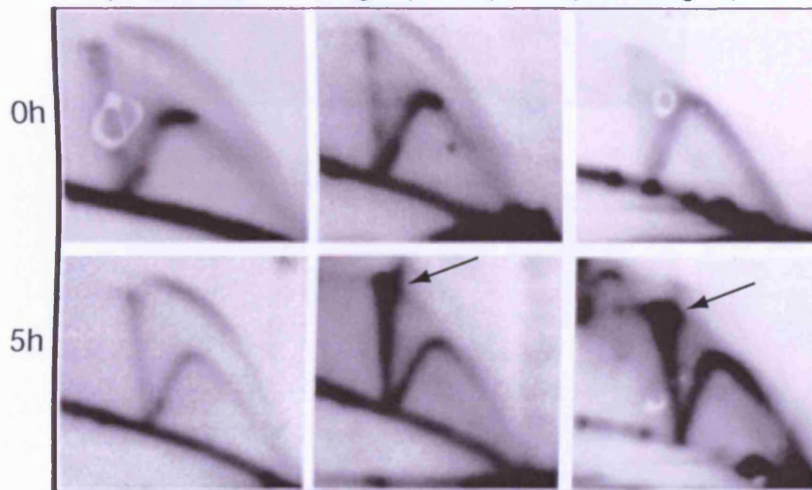


Figure 3.5: DNA damage produces a specific 2D gel intermediate
(a) Schematic of the replication intermediates detectable by 2D gel electrophoresis. (b) 2D gels of DNA extracted from wildtype cells treated with HU (left panel) and MMS (middle panel) and probed for with ars3001,and treated with MMS (right panel) and probed for with a fragment which recognises a non-origin sequence downstream of ars3001.
The arrow is pointing towards the "X" like stucture.

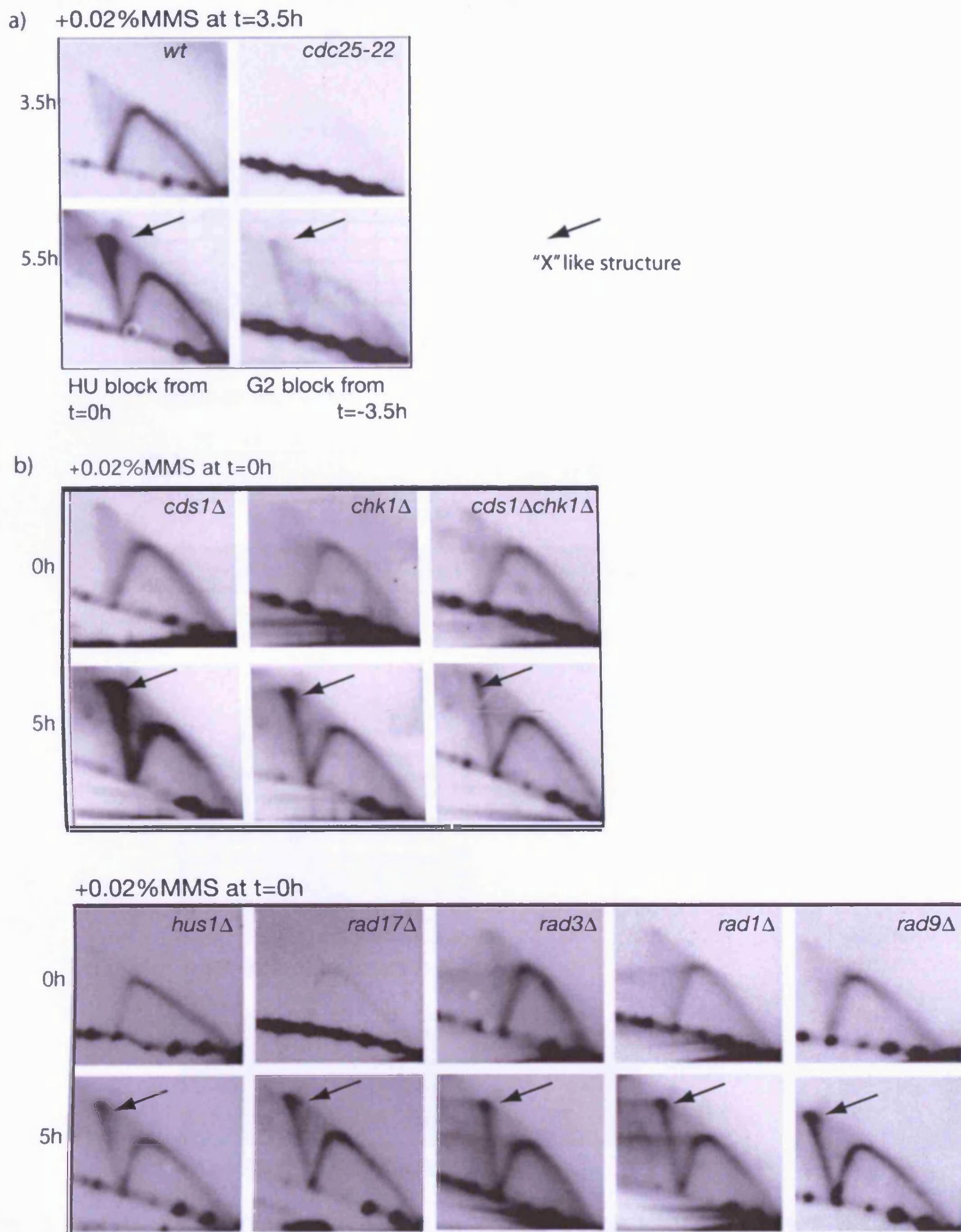


Figure 3.6: The damage intermediate may be cell cycle phase specific and is not dependent on the *rad* checkpoint gene network
(a) The damage intermediate was seen on 2D gel electrophoresis of wild type cells treated with MMS in an HU-induced S-phase block (left panel) and in a G2 arrest (right panel). (b) The damage intermediate was present on 2D gel electrophoresis of the *rad* checkpoint genes treated with MMS. All blots were probed for with a fragment recognising a non-origin sequence downstream of ars3001.

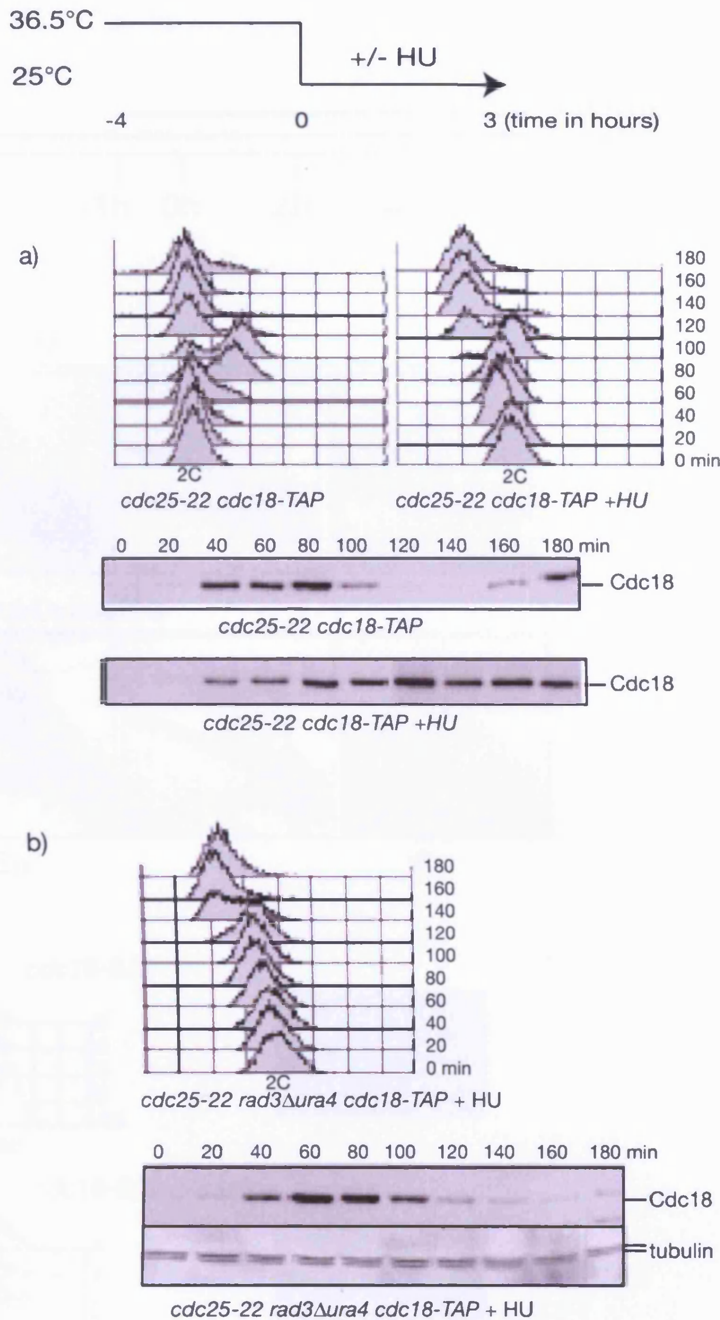
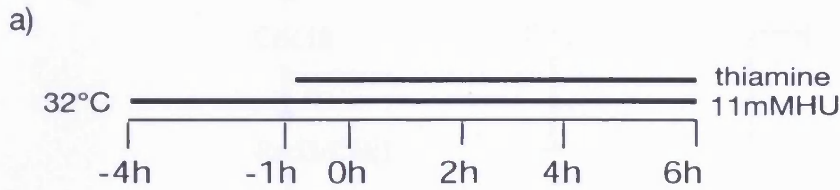
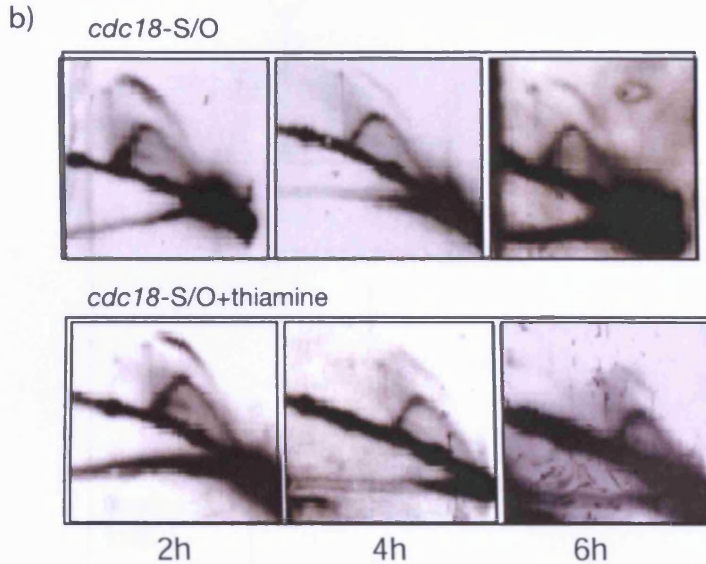


Figure 3.7: Cdc18 accumulates in a Rad3-dependent manner in an HU block

(a) A *cdc25-22 cdc18-TAP* strain was synchronised in late G2 by incubation at 36°C for 4 hours and then released into the absence or the presence of HU (see schematic). After release at the permissive temperature of 25°C, samples were taken every 20 minutes for 3 hours and cell cycle progression was followed by flow cytometry analysis (top). Western blot analysis of total protein lysates from each samples were performed using anti-Cdc18 antibodies (bottom). Note the accumulation of Cdc18 in the HU block, and that the cells start to leak through the block after 120 minutes. (b) The G2 block and release into HU described in (a) was repeated in the absence of Rad3 using a *cdc25-22 rad3Δura4 cdc18-TAP* strain. FACS analysis (top panel) demonstrated that no S phase occurs, and there is no accumulation of Cdc18 (bottom panel, plus note presence of tubulin control).

a)





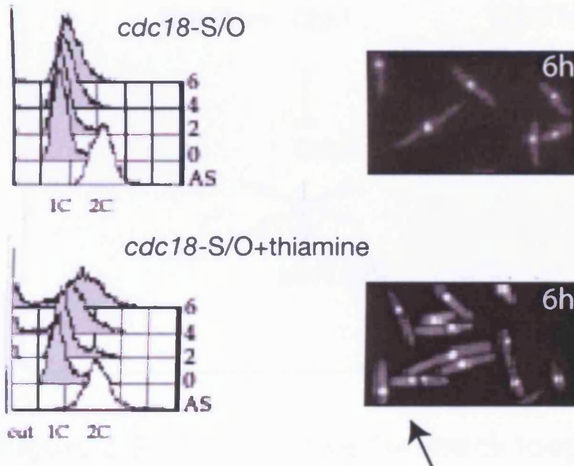


Figure 3.8: Cdc18 is not required for replication fork stabilisation

(a) A *cdc18* switch off strain (S/O) was grown at 32°C and HU added for 10 hours. At time = -1h, thiamine was added to half of the culture and cells grown for another 6h (see schematic). (b) 2D DNA gels of genomic DNA extracted from *cdc18 S/O* in the absence (*cdc18*$^{+}$) or presence (*cdc18*$^{-}$) of thiamine were probed for ars 3001. Note the presence of replicating structures at all timepoints in both strains. (c) Left panel: FACS analysis of the samples from (b). Right panel: At 6 hours the proportion of aberrant mitotic cells were determined by DAPI staining. Note the cut cells in the presence of thiamine (*cdc18*$^{-}$) (bottom panel).

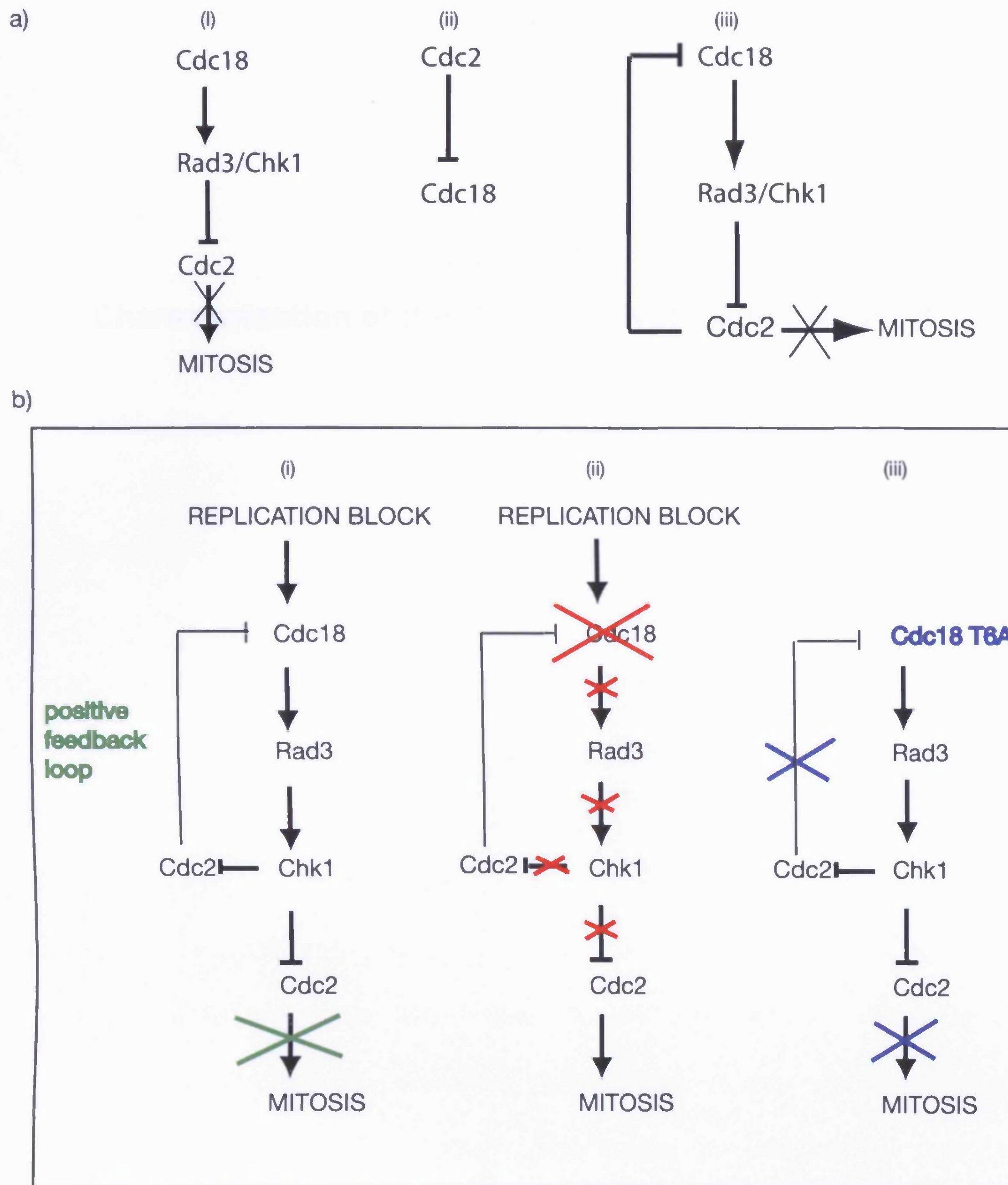


Figure 3.9: The positive feedback loop model for the Cdc18-activated Rad3-dependent checkpoint

(a) A positive feedback loop amplifies the Cdc18-activated Rad3-dependent delay into an absolute arrest, by stabilizing Cdc18 levels due to inhibition of Cdc2-directed Cdc18 degradation: (i) Cdc18 induces a Rad3-dependent inhibition of Cdc2 and consequent inhibition of mitosis; (ii) Cdc2 inhibits Cdc18 by phosphorylation which targets it for degradation; (iii) So, Cdc18-directed inhibition of Cdc2 acts to prevent its own degradation, increasing Cdc18 levels and enforcing the Rad3-dependent arrest. (b) (i) A replication block activates the positive feedback loop. (ii) This is dependent on the presence of Cdc18. (iii) The phosphorylation mutant will also activate the positive feedback loop.

Chapter 4

Characterisation of the Cdc18-activated checkpoint II

4.1 Introduction

I have shown in Chapters 2 and 3 that an increase in the level of Cdc18 activates a Rad3-dependent cell cycle block. When the *cdc18* cDNA is overexpressed by the strong *nmt1* promoter within the pRep4X vector, the subsequent checkpoint effects observed are secondary to the increased levels of Cdc18. I also attributed the Rad3-dependent cell cycle block observed with the phosphorylation mutant to increased Cdc18 levels, although it is possible that the phosphorylation mutant has other effects beyond increasing Cdc18 protein levels. It is known that phosphorylation destabilises Cdc18, so from now on I will refer to stabilisation, as well as increase, of Cdc18 levels when discussing the phosphorylation mutant.

This Cdc18-induced, Rad3-dependent arrest appears to utilise the Chk1/Crb2 "damage" pathway and not the Cds1/Mrc1 "replication" pathway. However, a Rad3-dependent accumulation of wild-type Cdc18 is a necessity for checkpoint control in an HU-induced S phase arrest. These two observations were linked in the positive feedback loop model for the Rad3-dependent checkpoint role of Cdc18 put forward in Chapter 3 (*Figure 3.9*). I proposed that an initial stabilisation of Cdc18 levels enforced the checkpoint pathway by Rad3-mediated inhibition of Cdc2 (preventing Cdc2-mediated Cdc18 phosphorylation and targeting for ubiquitin-dependent degradation) and subsequent further stabilisation of Cdc18 levels. I also considered the idea that a threshold level of Cdc18 must be exceeded for checkpoint activation, allowing the cell to distinguish between local noise and the need for cell cycle arrest.

I next wanted to characterise further the actual nature of the Cdc18-induced block. I also wished to establish whether the increased Cdc18 protein level itself activates the checkpoint directly or via a secondary effect. The stabilisation of Cdc18 levels may cause: 1) low level re-replication undetectable on FACS analysis or with nuclear DAPI staining and fluorescent microscopy; 2) DNA damage. These would both subsequently activate a Rad3-dependent cell cycle block.

4.2 Results

4.2.1 The Cdc18 phosphorylation mutant (Cdc18 T6A) produces a spectrum of Rad3-dependent cell cycle effects

In Chapter 3 (3.2.3.1) I described how the Cdc18-activated Rad3-dependent arrest could be used under specific conditions to synchronise *rad3*^{ts} *cdc18 T6A* cells, implying the presence of an absolute cell cycle block in liquid culture. To quantitate this further, the *rad3*^{ts} *cdc18 T6A* was streaked out onto YE5S media and left to form colonies for 3 days at 36°C. The colonies were then replica-plated in duplicate to 36°C and 25°C. By 5h widespread cell elongation was seen at 25°C with wild-type growth in the corresponding colonies at 36°C. However, by 24h the cells at 25°C had reverted to wild-type behaviour, suggesting adaptation to the effects of the mutant Cdc18.

The cell length at division is the most sensitive way to measure a delay in entry into mitosis. Therefore, I chose this method to quantify the transient nature of the cell cycle block. Asynchronous log phase populations of both *rad3*^{ts} *cdc18 T6A* and control *rad3*^{ts} cells were blocked for 7 hours at 25°C in

liquid culture (supplemented minimal media). At hourly timepoints, live cells were sampled and stained with calcoflor before imaging on the fluorescent microscope. For each sample, 12 cells with a septum were identified and photographed. Their length at septation was measured using a standard calibration bar (Image J programme). After 7 hours at 25°C the mutant *cdc18 T6A* septated cells were found to be 60% longer than the wild-type septated cells (22.8μM versus 13.9μM); there were no differences in length at division at t=0h (*Figure 4.1)*. This implies the presence of a transient mitotic block.

4.2.2 There is no evidence of Cdc18-induced DNA re-replication causing checkpoint activation

In 3.2.1 I described the absence of apparent re-replication in the presence of pRep4X *cdc18* overexpression or the Cdc18 phosphorylation mutant, as assessed by DAPI staining of the nucleus or by FACS analysis. FACS analysis was repeated on samples taken hourly for 5 hours from asynchronous log phase cultures of *rad3^{ts}* and *rad3^{ts} cdc18 T6A* at 36°C. Both strains maintained a 2C DNA content throughout the timecourse with no evidence of DNA re-replication (*Figure 4.2a*).

4.2.2.1 The checkpoint signal is sent in the absence of detectable replication intermediates on 2-dimensional DNA gel electrophoresis

When *cdc18* is under the control of the pRep3X thiamine repressible promoter, its overexpression produces re-replication in G2 arrested cells demonstrated by increased DNA content on FACS analysis and by the presence of replication intermediates on 2-D DNA gel electrophoresis (Yanow *et al.* 2001). I therefore

looked for the presence or absence replication intermediates on a 2-D gel in G2 arrested *cdc18 T6A* cells. The mutant Cdc18 T6A protein remains elevated throughout the cell cycle as it cannot be phosphorylated and subsequently degraded (*personal communication - D.Hermand*). An asynchronous log phase culture of the *cdc25-22^{ts} rad3Δura4 cdc18 T6A* strain, grown overnight at 25°C, was arrested at the G2/M transition by shifting to 36.5°C for 8 hours (*see timeline in Figure 4.2b*). Samples were taken two hourly for cell number analysis using a Coulter Counter (*Figure 4.2d)* and hourly for FACS analysis (*Figure 4.2e*), to confirm the G2 arrest. At 0h and 3h (when the cells are blocked in G2, and before any leak through may occur) samples were taken for preparation of genomic DNA for 2D gel electrophoresis. The Southern blot of the gel obtained was probed for with the origin fragment of the rDNA repeat. Replication intermediates were visualised in the asynchronous 0h culture, but these had all disappeared in the G2 arrested cells *(Figure 4.2c).* I concluded that the mutant Cdc18, in a *rad3Δ* background, is not causing detectable re-replication.

4.1.3 There is no evidence of gross Cdc18-induced DNA damage causing checkpoint activation

Because Chk1 is the main effector kinase for the DNA damage checkpoint, I tested whether it was the elevated Cdc18 protein level itself sending the checkpoint signal or whether it was inducing DNA damage with subsequent cell cycle arrest. This can be looked for on a gross level by first assessing the effect of the presence of the mutant protein on culture generation time and the timing of S phase initiation and completion. The next step was to look at the

results of non-lethal DNA damage such as forward mutation rate.

4.1.1.1 Basic physiological parameters

4.1.1.1.1 Generation time is not altered in the Cdc18 T6A mutant

In Chapter 3.2.1.1 it was noted that Cdc18 overexpression in the absence of Rad3 had no effect on the plating efficiency. To look more closely at cell growth, asynchronous exponentially growing cultures of *rad3^{ts}* and *rad3^{ts} cdc18 T6A* were followed for 5 hours (300 minutes) at 36°C. Samples were taken every 20 minutes for cell number processing using a Coulter Counter. The optical density (OD) was measured hourly at 595nm on a Spectrophotometer. The Cdc18 T6A mutant protein had no detectable effect on exponential cell growth, with the generation time for both control and mutant strains at 36°C being 165 minutes, calculated from the logarithmic plots of the cell number measurements (*Figure 4.3a*).

4.1.1.1.2 S phase initiation and completion is not altered in the Cdc18 T6A mutant

To determine any effects on S phase, synchronized log phase cultures of both mutant and wildtype Cdc18 in a *rad3Δura4* background were prepared using a *cdc25-22^{ts}* block and release procedure (*see timeline in Figure 4.3b*). The strains were cultured overnight at 25ºC, and then synchronised in late G2 using a 4 hour incubation at 36.5ºC. Cell number samples were taken every 60-90 minutes during the block to ensure its effect *(Figure 4.3d).* After the cells were released from the G2 block by shifting back to 25ºC, samples were taken and

fixed every 20 minutes for the next 3 hours and cell cycle progression followed by FACS analysis (*Figure 4.3c),* cell number and septation rate (the septa were visualised and counted by fluorescent microscopy after calcoflor staining) *(Figure 4.3e).* No differences were observed in the timing of S phase initiation and completion despite the known presence of the mutant protein at an elevated level during S phase and persisting throughout G2. I concluded that the elevated levels of Cdc18 did not affect the timing of S phase initiation or completion.

4.1.1.2 Forward mutation rate

If Cdc18 is activating the checkpoint via low level DNA damage, then it may increase the observed spontaneous mutation rate. A forward mutation will change a wild-type gene to a mutant form. The forward mutation of *ura4*$^+$ to *ura4*$^-$ results in 5FOA resistance (5-FOA-R). This forward mutation rate (FMR) has been previously reported to be 1 per 10^7 cells (Kai and Wang 2003), and is easily detected and calculated as *ura4+* strains, unlike *ura4-* strain, cannot grow in the presence of 5FOA. This is because the nontoxic 5FOA compound is converted to toxic 5-fluorouracil in yeast strains expressing a functional *ura4* gene. A mutagenesis assay was performed to see if there was any increase in the FMR in the presence of the Cdc18 T6A mutant. *Rad3ts ura4+* and *rad3ts ura4+ cdc18 T6A* strains were grown to mid log phase, and 1x10^8 cells of each plated out onto YE5S media containing 5FOA for 3 days at 36°C. After 3 days incubation at 36°C the number of 5FOA-R colonies were counted. The FMR per 10^7 cells was 3.6 for *rad3*^{ts}*ura4*$^+$ and 2.8 for *rad3*^{ts}*ura4*$^+$*cdc18 T6A* (*see Table 4.1 below*). There are two possible explanations for this in the presence

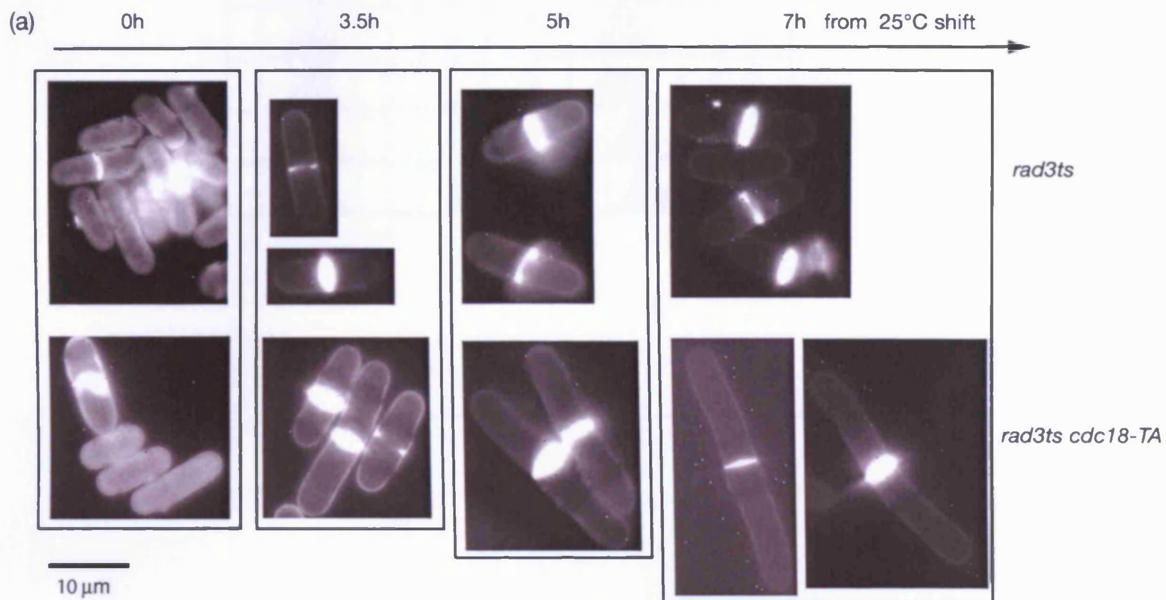
of only a historic control: 1) elevated levels of Cdc18 do not significantly affect the spontaneous mutation rate; 2) *cdc18* and *rad3* may both cause an increase in the spontaneous mutation rate, but are epistatic so no significant difference is seen between the two strains.

Table 4.1 Cdc18 T6A does not increase the forward mutation rate (FMR)

	Total cells plated onto 5FOA	Total ura4$^-$ colonies	FMR per 10^7 cells
rad3^{ts} ura4$^+$	3.72×10^8	135	3.6
rad3^{ts} ura4$^+$ cdc18 T6A	3.16×10^8	89	2.8

4.2 Discussion

From the results in Chapters 3 and 4, I conclude that stablisation of Cdc18 levels can lead to a transient S-M checkpoint activation, blocking onset of mitosis in the absence of detectable replication intermediates or DNA over-replication. However, as only a single replication origin was looked at on the 2D gels, I would need to perform either quantitative Southern blotting or microarray analysis of replication origins to be really sure of the absence of replication intermediates. Cdc18 acts upstream of Rad3 to bring about Chk1 phosphorylation and activation, and the *rad* checkpoint gene network is necessary for this cell cycle block. Cds1 and Mrc1 are not required for the checkpoint. In the absence of Rad3, cells containing elevated Cdc18 levels have the same generation time and cell cycle parameters as those containing wild-type Cdc18, and there are no gross effects on mutation rate. It appears that the increased level of Cdc18 transiently activates the S-M checkpoint through Chk1, with no other obvious effects on the cell cycle.



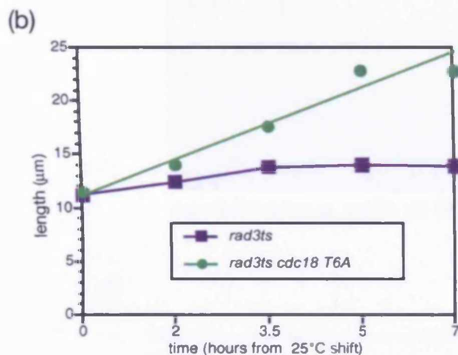


Figure 4.1: Cdc18 induces a transient mitotic block
Asynchronous log phase populations of *rad3ts cdc18 T6A* and *rad3ts* were blocked for 7 hours at 25°C. (a) At hourly timepoints, live cells were sampled and stained with calcoflor. (b) At each timepoint, the cell length of 12 septated cells was measured. After 7 hours at 25°C the mutant *cdc18 T6A* septated cells were 60% longer than wild-type (22.8μM versus 13.9 μM). Note there was no difference in length at division at t=0h.

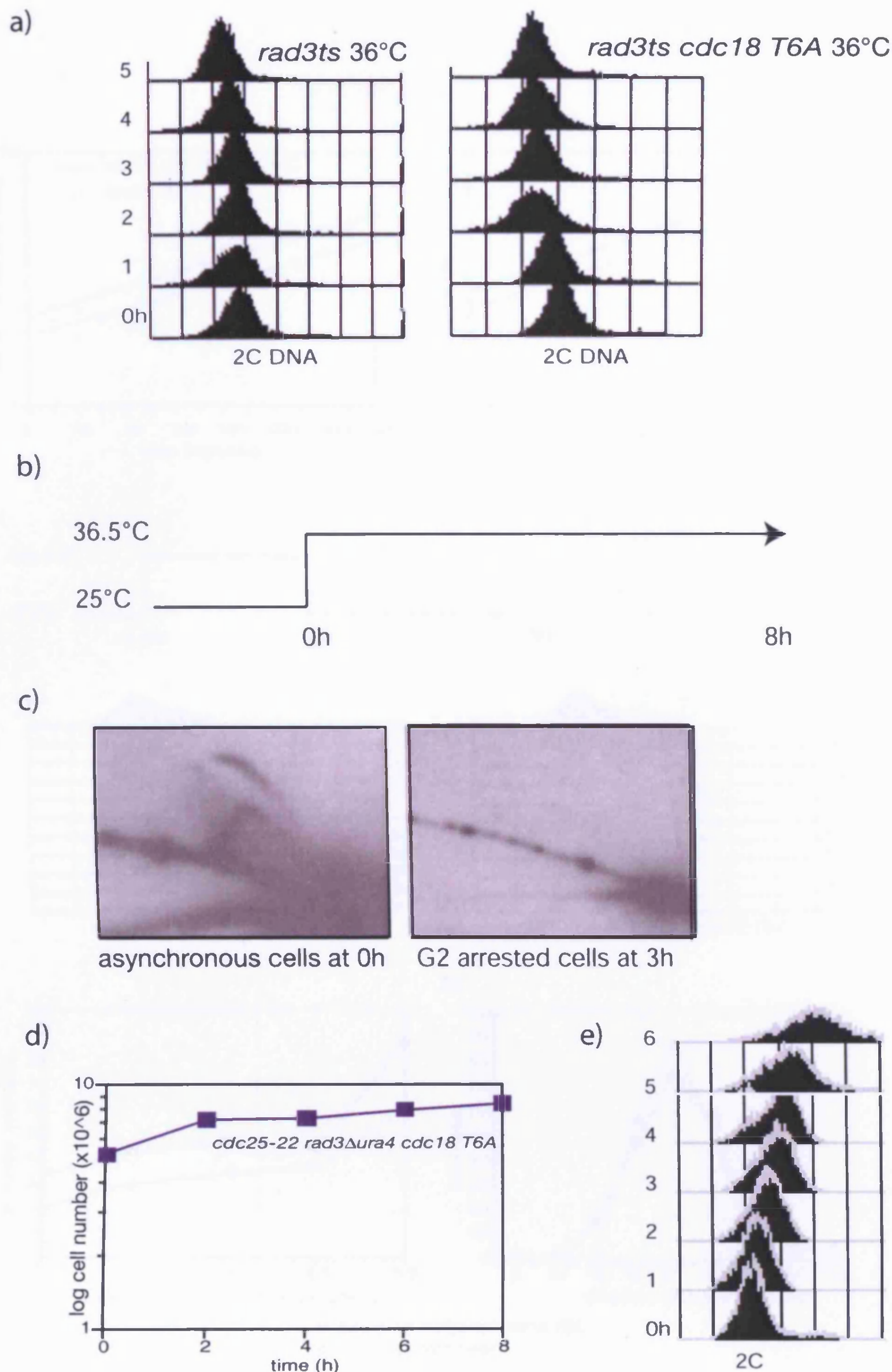


Figure 4.2: There is no detectable re-replication in the presence of the Cdc18 T6A phosphorylation mutant

(a) There is no evidence of re-replication on hourly FACS analysis of asynchronous log phase cultures of *rad3ts* and *rad3ts cdc18 T6A* at 36°C. (b) Schematic: A G2 block was induced using *cdc25-22ts rad3Δura4 cdc18 T6A*

(c) Asynchronous (AS) and G2 block 2D gels (at 0h and 3h respectively) were probed with non-origin rDNA. Note the disappearance of replication intermediates at 3h. (d) G2 arrest confirmed by cell number (left panel) and FACS analysis (right panel).

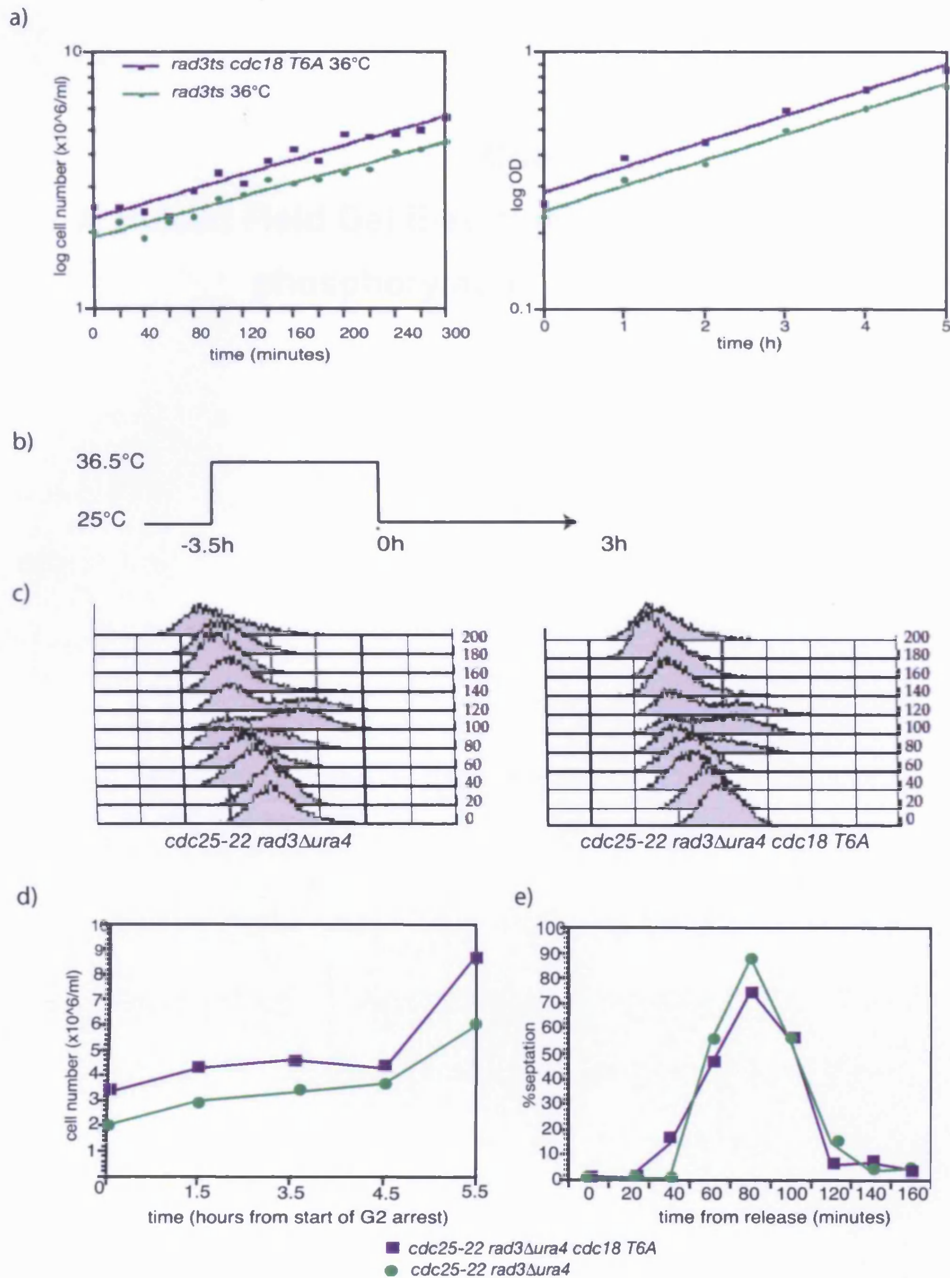


Figure 4.3: There is no evidence of gross Cdc18-induced DNA damage causing checkpoint activation

(a) There is no difference in generation time (calculated from cell number) between *rad3ts* and *rad3ts cdc18 T6A* at 36°C. (b) Schematic for G2 block and release. (c) No differences seen in the timing of S phase initiation and completion on FACS analysis. (d) This was confirmed on cell number and septation index measurements.

Chapter 5

A Pulsed Field Gel Electrophoretic analysis of the Cdc18 phosphorylation mutant, Cdc18 T6A

5.1 Introduction

I have shown that the Cdc18 phosphorylation mutant, Cdc18 T6A, is capable of initiating a spectrum of Rad3/Chk1-dependent checkpoint responses, from a G2 delay to absolute cell cycle arrest. This appears likely to be a direct effect of the elevated Cdc18 protein levels, with no re-replication seen by FACS or detection of replication intermediates on 2-D DNA gels of G2 arrested cells. Because Chk1 is the main effector kinase for the DNA damage checkpoint, I carried out preliminary experiments looking for evidence of a Cdc18-induced damage mediated pathway. These showed no evidence of gross DNA damage. The next step was to use pulsed field gel electrophoresis (PFGE) as a more sensitive means of assessing DNA damage and also to confirm the absence of re-replication.

Fission yeast contains three chromosomes, (5.7Mb, 4.6Mb and 3.5Mb in size), which can be separated and visualised by PFGE. Replication intermediates do not migrate through a pulsed field gel (PFG) and stay in the well (Grallert and Nurse 1996). Hence, there is no chromosomal entry into the gel during an HU block, a synchronised S phase, or in the presence of ongoing re-replication. Damage causing double stranded breaks in the DNA is seen as a failure to resolve the chromosomes plus a smear in the lower end of the gel (Miller and Cooper 2003).

5.2 Results

5.2.1 Chromosome III does not appear to enter the PFG in the presence of the Cdc18 T6A mutant

I wanted to look for evidence of DNA damage or re-replication in the presence of the Cdc18 phosphorylation mutant using PFGE. I first repeated previous experiments to demonstrate what happens to resolution of the wild-type *S. pombe* chromosomes in the presence of both an HU replication block and a MMS-induced DNA damage. An exponentially growing *wildtype* strain at 32°C was split into 4 equal cultures at t=0h. Hydroxyurea (at 11mM concentration) was added immediately to culture B. At 2 hours, 0.002% MMS and 0.005% MMS was added to cultures C and D respectively. At 3 hours, samples were taken for the preparation of agarose plug embedded genomic DNA for PFGE. The gel was run for 72 hours and the DNA visualised with Ethidium Bromide (EtBr) (*Figure 5.1*). In the presence of HU, stalled replication forks prevented entry of chromosomes into the gel. As the concentration of MMS increased, the chromosomes were not resolved and DSBs appeared as a smear in the kilobase (Kb) region of the gel.

Cultures were set up of the *cdc18 T6A* mutant strains and their appropriate controls: *rad3^{ts}* and *rad3^{ts} cdc18 T6A* (CCL1) at 36°C; *cdc25-22^{ts} rad3Δ* and *cdc25-22^{ts} rad3Δ cdc18 T6A* (CCL3) at 25°C (asynchronous cycling cells) and also at 36.5°C (blocked for 3.5h before samples taken to ensure cells were arrested in G2). Samples were taken in mid-log phase for the preparation of agarose plug embedded genomic DNA for PFGE. The gel was run for 72

hours before staining with EtBr. In the *cdc18 T6A* mutant strain, chromosomes I and II entered the gel normally. There was no evidence of DNA damage in the form of DSBs. However, there was no or little entry of chromosome III into the gel in either cycling (in both *rad3*^{ts} and *cdc25-22*^{ts} *rad3Δ* mutant backgrounds, the latter not shown) or G2 arrested cells when Cdc18 T6A was present. All 3 chromosomes entered the gel as usual in the control strains *(Figure 5.2a)*.

5.1.2 Chromosome III is present in the PFG as a smear over the size range 3.5-greater than 5.7Mb

There are two reasons why chromosome III is not seen: 1) It is not entering the PFG; 2) It is present in the PFG, but at a different size. To check this, the PFG obtained in 5.2.1 was Southern blotted and probed with both a 3.4Kb fragment of non-origin rDNA and with an 800bp central portion of the adenine 6 gene (both specific to chromosome III). This revealed chromosome III was present in the gel, but as a smear extending from 3.5Mb up to greater than 5.7Mb *(Figure 5.2b).* The fact that all 3 chromosomes are able to enter the gel supports the interpretation that there is no ongoing Cdc18-induced re-replication which could activate the checkpoint.

5.1.3 Chromosome III does not resolve into a discrete band at any stage in the cell cycle

I hypothesised that, as the *cdc18 T6A* strain appears to grow normally with wild-type physiological parameters in the absence of Rad3, the changes seen in chromosome III are resolved to a discrete band with each round of mitosis. I therefore wanted to see what happened to chromosome III as it progressed

through the cell cycle. A synchronized mid log-phase culture of the mutant Cdc18 in a *rad3Δura4* background (CCL3) was prepared using the *cdc25-22* block and release procedure described in 4.1.1.1.2.. Samples were taken hourly during the 36.5ºC block for light microscopy (looking for cell elongation) and cell number determination to confirm the G2 arrest. On release, samples were taken every 20 minutes for 3 hours to monitor progression through S phase (using septation index and FACS analysis) and for the preparation of agarose plug embedded genomic DNA for PFGE (*Figure 5.3a*). The PFG was run for 72 hours before staining with EtBr. Chromosomal gel entry was reduced for all 3 chromosomes as expected around the 80 minute timepoint, corresponding with S phase and the presence of replication intermediates. Chromosome III was not visualised with EtBr staining at any point in the cell cycle *(Figure 5.3b)*. Southern blotting and probing for chromosome III with non-origin rDNA revealed a smear that was present throughout the cell cycle *(Figure 5.3c)*. The presence of the smear was reduced around the time of S phase. I concluded that the abnormality present in chromosome III persists throughout the cell cycle, but like chromosomes I and II does not enter the PFG during S phase.

5.1.4 Removal of the Cdc18 T6A mutant allows resolution of chromosome III in a discrete band

If it was the presence of Cdc18 T6A de-stabilising chromosome III, then elimination of Cdc18 T6A should allow the size of chromosome III to be stabilised. The *cdc18 T6A* mutant gene was crossed out of a *cdc25-22 rad3Δ cdc18 T6A* strain (CCL3), selecting for *wildtype*, *cdc25-22, rad3Δ* and *cdc25-22*

rad3Δ strains by random spore analysis. In each case, 50% of the cells should have chromosome III from the *cdc18 T6A* parent, and 50% from the non-*cdc18 T6A* parent. Samples were taken from exponentially growing cycling cells from the cross-derived strains for preparation of agarose plug embedded genomic DNA. The DNA was analysed with PFGE *(Figure 5.4a)* followed by Southern blotting and rDNA repeat probing *(Figure 5.4b).* In 17 strains analysed (not all data shown) chromosome III was now found to form a discrete band with an approximate 1:1 split between a normal sized chromosome III (in 9 out of the 17) and an increased (greater than 5.7Mb) sized chromosome III (in 8 out of the 17). This shows an approximately Mendelian inheritance of the normal:increased sized chromosome III from the parent strains.

I selected four of these strains (two with a large chromosome III and two with a normal sized 3.5Mb chromosome III) for further analysis. Each strain was cultured both overnight and for 30 generations before processing exponentially growing cells for PFGE. Southern blotting and rDNA probing demonstrated that after 30 generations of growth the normal size chromosome IIIs remains unchanged, but in contrast the larger chromosome IIIs returned closer to a normal size *(Figure 5.4c).* This implies the maintenance of an enlarged chromosome III requires the continued presence of Cdc18 T6A. If Cdc18 T6A is removed then the enlarged chromosomes gradually revert back to a normal size.

5.1.5 Chromosome III remains abnormal in the presence of Cdc18 T6A mutant after meiosis

I then looked at the strains derived from the cross performed in 5.2.4 that still contained the *cdc18 T6A* mutant. Samples were taken from exponentially growing cycling cells from the cross-derived strains for preparation of agarose plug embedded genomic DNA. The DNA was analysed with PFGE *(Figure 5.5a)* followed by Southern blotting and rDNA repeat probing *(Figure 5.5b).* In all 15 of these strains chromosome III remained smeared as in the parent strain (not all data shown). In 12 strains chromosome III was large (up to 7Mb in size) and smeared. In 3 strains it was close to wild-type size (around 3.5Mb) and starting to smear.

I conclude that the Cdc18 T6A mutant induces variable changes in the size of chromosome III. These changes disappear with removal of the mutant Cdc18.

5.1.6 The ribosomal rDNA repeat is expanded in chromosome III in the presence of the Cdc18 T6A mutant

All eukaryotes contain the genes encoding the ribosomal DNA (rDNA) as a tandem array of repeated units. The total number of rDNA repeats per genome varies greatly between given organisms, and is maintained at this appropriate level. However, variations in repeat number have been observed with both expansion and contraction. *S. cerevisiae* contains up to 150 copies of rDNA on chromosome XII. The phenomenon of rDNA repeat expansion has been observed in *S. cerevisiae*, in mutants defective for the essential subunits of the RNA polymerase I (pol I) transcription factor (TF) UAF. Pol II was used instead

of Pol I to transcribe chromosomal rDNA, with a consequent increase in repeat number, suggesting the ability to use recombination to alter rDNA repeat number (Oakes *et al.* 1999). However, excessively high recombination events may also be harmful: accumulation of extra-chromosomal rDNA circles is toxic and may cause senescence (Sinclair and Guarente 1997). In fission yeast there are 100-150 of these 10.4Kb units (1-1.5Mb total), located at both ends of chromosome III (27% at one end, 73% at the other). This makes the rDNA *ars3001* the most abundant replication origin in the genome.

To determine if there were changes in the repeat number of rDNA, an enzymatic digest of *cdc18 T6A* chromosomes in agarose plugs was performed and the size of the restriction digest fragments examined.

5.1.6.1 Sfi1 digest of chromosome III demonstrates expansion of the restriction fragments containing the rDNA repeats

The restriction enzyme Sfi1 cuts chromosome III into 4 discrete bands: A (915Kb containing 73% of the rDNA repeats); B (383Kb); C (1900Kb); D (242Kb containing 27% of the rDNA repeats) *(Figure 5.6a).* I carried out an Sfi1 digest of DNA embedded agarose plugs containing the *cdc18 T6A* mutant chromosomes (CCL1) and of their appropriate controls. PFGE was carried out under two different conditions (see Chapter 7 Materials and Methods): over 24 hours using the standard conditions used after enzymatic digest, to resolve smaller fragments (in the kilobase Kb range) and over 72 hours using the standard conditions used for whole chromosome analysis, to resolve larger fragments (in the megabase Mb range). Southern blotting and probing for rDNA in the first run (looking in the Kb range) showed the loss of both rDNA

containing bands in the presence of the Cdc18 T6A mutant (*Figure 5.6b*). It should be noted that the ethidium staining of the Cdc18 T6A digest was faint, raising the possibility that the rDNA bands were below the detection level. However, the second run (looking in the Mb range) clearly demonstrated a smear extending from 915Kb to about the 2Mb region of the gel in the presence of the Cdc18 T6A mutant (*Figure 5.6c*), confirming the presence and expansion of the rDNA repeats.

I concluded that there is expansion in the restriction fragments containing the rDNA repeats within chromosome III in the presence of the Cdc18 T6A phosphorylation mutant protein. The same digests were then probed with *ade6* (data not shown). There was no change seen in the size of the central *ade6* containing restriction fragment (fragment C), implying that the expansion seen is specific to the rDNA containing restriction fragments.

5.1.6.2 The expansion of chromosome III may be limited to the rDNA repeats

I looked at the effects of the presence of the Cdc18 T6A phosphorylation mutant on a *S. pombe* linear minichromosome strain (*Chr16*). This contains a 530Kb central region of chromosome III with no rDNA repeats, and is stably maintained in addition to the 3 regular chromosomes (Niwa *et al.* 1986). The minichromosome strain was crossed with *rad3^{ts} cdc18 T6A* (CCL1), and a *rad3^{ts} cdc18 T6A Chr16* strain selected by random spore analysis. Samples were taken in mid-log phase from *wildtype*, *Chr16* and *rad3^{ts} cdc18 T6A Chr16* strains cultures at 36°C, for the preparation of agarose plug embedded genomic DNA for PFGE. As before, in the presence of the Cdc18 T6A mutant the intact chromosome III could not be visualised with EtBr staining (*Figure 5.7a*), but the

530Kb minichromosome entered the PFG normally (Figure 5.7a and b). This supports the finding that there is expansion in the restriction fragments containing the rDNA repeats on chromosome III, suggesting that this expansion may be occurring within the rDNA repeats themselves.

5.1.7 The Cdc18 T6A phosphorylation mutant may not enhance recombination genome wide

The Cdc18 T6A mutant is affecting the size of chromosome III, with the expansion of the restriction fragments containing the rDNA repeats. I hypothesised that the phosphorylation mutant was promoting recombination with unequal cross-over events, leading to an increase in size of the rDNA containing restriction fragment. Decreased numbers of rDNA repeats were not observed. This may be due to: selection against cells containing less rDNA genes; or the unequal cross-over mechanism only producing rDNA gene amplification, as described in the transcription-induced cohesin dissociation model of rDNA amplification (Kobayashi and Ganley 2005). In this model, suppression of the *S. cerevisiae* silencing gene *Sir2* allows transcription with subsequent displacement of cohesin. This results in unequal sister chromatid templates for DSB repair and amplification of rDNA copy number.

To investigate whether the unequal crossing-over phenomenon seen with Cdc18 T6A is genome wide or restricted to the rDNA repeats I looked for evidence of a generalised increase in recombination events.

5.1.7.1 The Cdc18 phosphorylation mutant may not enhance meiotic recombination

Leu1 and the mating type locus, *mat1* (which determines whether a strain is h^+ or h^-), are located 140Kb (physical separation) apart on chromosome II. Genetic separation is measured in centimorgans (where one centimorgan is equal to a 1% probability of a recombinant between the two genes). One centimorgan may range from a 3 to 24Kb in physical separation, due to variation in the recombination frequencies between any two genes over the whole genome. The genetic separation between *leu1* and *mat1* has been estimated previously at 14 centimorgans (Kohli *et al.* 1977).

An h^+ *cdc25-22 rad3Δura4 cdc18 T6A* leu^+ strain was crossed with an h^- *cdc25-22 rad3Δura4 cdc18 T6A* leu^- strain. Spores were plated out, and at 5 days the resulting colonies replica-plated in duplicate onto selective media for leucine and onto sporulation plates. They were scored for leu^+/leu^- at 24 hours, and for h^+/h^- after staining with iodine at 72 hours (*see Table 5.1 below*). Out of 74 colonies scored, only seven recombinants were seen (three h^+ leu^- and four h^- leu^+). This translates to a recombination frequency of 9.5%, equating with a genetic separation of 9.5 centimorgans between *leu1* and *mat1*. In the absence of the appropriate controls (h^+ *cdc25-22 rad3Δura4* leu^+ crossed with h^- *cdc25-22 rad3Δura4* leu^-, and h^+ leu^+ crossed with h^- leu^-), I can only propose that as this value was not significantly different to that expected historically (14 centimorgans) that Cdc18 T6A may not increase recombination levels during meiosis.

Table 5.1 Cdc18 T6A may not cause increased meiotic recombination

	leu-	leu+
h+	**3**	**34**
h-	**33**	**4**
Recombinants	**3/36**	**4/38**
Recombination frequency	**(7/74) 9.5%**	
Genetic separation	**9.5 centimorgans**	

5.1.1.2 The Cdc18 phosphorylation mutant does not enhance mitotic recombination genome wide

I then looked for evidence of genome wide increased levels of mitotic recombination in the presence of Cdc18 T6A. *Cdc2-56^{ts}* is a temperature sensitive mutant of the *cdc2* gene: *cdc2^{+}* (but with a wee phenotype) at 25°C, and *cdc2^{-}* (with an elongated phenotype) at 36°C. It is able to revert spontaneously to the *cdc2^{+}* phenotype at a considerably higher frequency ($4x10^{-6}$ versus $2x10^{-8}$) than other *cdc2* alleles studied. This high reversion frequency was shown to result from the duplication of the chromosomal region encompassing the mutant *cdc2* gene. One end of the duplicated region mapped to a 5S ribosomal RNA gene. There are about 30 5S rRNA genes dispersed throughout the *S. pombe* genome. It was proposed that unequal crossing over between two 5S rRNA genes led to the *cdc2* duplication seen in the revertants (Carr *et al.* 1989).

I hypothesized that if Cdc18 T6A enhances recombination with unequal crossing over throughout the *S. pombe* genome, then it would induce an even higher *cdc2-56^{ts}* spontaneous reversion frequency. The *cdc2-56^{ts} and rad3Δura4 cdc18 T6A* strains were crossed, and *cdc2-56^{ts} rad3Δura4* and *cdc2-56^{ts} rad3Δura4 cdc18 T6A* strains selected for by random spore analysis. I

measured the spontaneous reversion frequencies of *cdc2-56^{ts} rad3Δura4* and two *cdc2-56^{ts} rad3Δura4 cdc18 T6A* strains. Approximately $5x10^7$ colonies were plated out for each strain at the permissive temperature of 25°C. After 5 days the colonies were replica-plated to 36°C. At 24 hours the number of revertants were counted, as only these cells would be able to form colonies at 36°C. The reversion frequencies were calculated (*see Table 5.2 below*) with no increase seen in the presence of the mutant Cdc18.

Table 5.2 Cdc18 T6A does not enhance genome wide mitotic recombination

strain	Cell number plated	Number of revertants	Reversion frequency
cdc2-56^{ts} rad3Δura4	$7x10^7$	10446	$15x10^{-5}$
cdc2-56^{ts} rad3Δura4 cdc18 T6A	$13.3x10^7$	7856	$6x10^{-5}$

I concluded that it is unlikely that there is any enhanced genome wide meiotic or mitotic recombination in the presence of Cdc18 T6A. Any increased recombinatorial activity appears to be limited to the restriction fragments containing the rDNA repeats. In fact the phosphorylation mutant appeared to reduce the reversion frequency.

5.2 Discussion

I have established that moderately elevated levels of Cdc18 can lead to a transient Rad3/Chk1-dependent S-M checkpoint activation, blocking onset of mitosis in the absence of detectable replication intermediates and DNA over-

replication, with no gross effects on cell cycle time or mutation rate. However, one unexpected consequence of an elevated Cdc18 level is to increase the size of chromosome III, with expansion of the Sfi1 restriction fragments containing the rDNA repeats.

Why is the chromosomal expansion seen limited to the rDNA repeats? The total number of chromosomal rDNA repeats is maintained at the appropriate level for an organism, and rapidly restored following contraction or expansion (Kobayashi *et al.* 1998). Genes found in such a repeated structure have a high frequency of recombination events, and are considered unstable. There are several processes that stimulate recombination in the rDNA repeats. These are: 1) The presence of the recombination hotspot, *HOT1* (a DNA sequence enhancing genetic exchange around that region); 2) Collisions between replication forks and RNA polymerases (Voelkel-Meiman *et al.* 1987); 3) The presence of replication fork barriers (RFBs) (also known as pause sites), which block replication forks from moving into the adjacent rDNA repeat in a direction opposite to that of rDNA transcription (Brewer and Fangman 1988). RFBs are a highly conserved feature of rDNA, with barriers being found at the 3' end of the rRNA genes in a number of organisms including *S. cerevisiae,* where they were first identified (Brewer *et al.* 1992). *S. pombe* rDNA repeats contain four closely spaced polar replication barriers. It has been proposed in *S. cerevisiae,* that expansion and contraction of the rDNA tandem repeats is coupled with DNA replication, with fork blockage at RFBs directly stimulating recombination via the formation of DNA DSB (Kobayashi *et al.* 2004).

Recombination regulatory mechanisms are present in the rDNA repeats to protect against possible harmful effects. A Fob1-dependent system is

present is *S. cerevisiae* (Merker and Klein 2002). Fob1 binds to the RFB site and is necessary for RFB activity (Brewer, Lockshon *et al.* 1992). It is also required for HOT1 activity in a recombination test system outside the rDNA (Kobayashi and Horiuchi 1996). Mutations in *Fob1* decrease recombination, however mutations in the *Sir2* gene increase recombination in the chromosomal rDNA.

Sir2 is required for the transcriptional silencing of three budding yeast chromosomal regions: the silent mating-type locus; the telomere regions; and the rDNA (reviewed by (Rusche *et al.* 2003)). Sir2 is a NAD+ dependent histone deacetylase, and plays an essential role in forming heterochromatin (the higher repressive order of chromatin) (Smith *et al.* 2002). Pol II cannot access heterochromatin, with subsequent silencing and suppression of rDNA recombination (Fritze *et al.* 1997). Sir2 has been shown to be required for the efficient association of the cohesin subunit, Scc1, to rDNA (Kobayashi, Horiuchi *et al.* 2004); cohesin is essential for sister chromatid cohesion, and I will review this topic before discussing further the results.

In eukaryotic cells, sister chromatids remain physically connected from S phase synthesis until anaphase separation (reviewed in (Nasmyth *et al.* 2000). Sister chromatid cohesion is essential for amphitelic separation of sisters to opposite poles of the cells at mitosis, and acts as a "memory" that permits chromosomal segregation to take place long after completion of duplication. Cohesin is a multi-subunit protein complex essential for sister cohesion in yeast and vertebrates (Toth *et al.* 1999). There are four cohesin subunits (Smc1, Smc3, Scc1 and Scc3), all required for establishing and maintaining the attachment between sisters.

Separation at the metaphase-anaphase transition is triggered by proteolytic cleavage of the Scc1 cohesin subunit by separase, a protease activated by the destruction of its inhibitory chaperone, securin (separin). This is mediated via the APC (anaphase promoting complex) (a ubiquitin protein ligase) and *S. pombe* Slp1/ *S. cerevisiae* CDC20. Separins exist in all eukaryotes. Human separin is overexpressed in many tumour cells, with increased levels thought to predispose to missegregation and genomic instability (Zou *et al.* 1999).

The spindle assembly checkpoint operates during mitosis to ensure the sister chromatids are correctly aligned on an undamaged spindle before separation into two identical daughter cells. It is vital that separase is not activated until the alignment of every single chromosome on the mitotic spindle is complete. In most eukaryotic cells, lagging or unaligned chromosomes transmit a signal via their unattached kinetochores. This promotes the formation of a form of Mad2, Mad2-1, which inhibits the APC via sequestration of Slp1/CDC20, with subsequent inhibition of the B-type cyclin and securin proteolysis. This is turn prevents activation of separase. However, when the sister chromatids are attached ampitelically and under traction, there are no unattached kinetochores. This promotes the activation of Slp1/CDC20 and the APC with subsequent destruction of securin, and consequent activation of separase (Zachariae and Nasmyth 1999).

So, separation of sister chromatids at the metaphase-anaphase transition is triggered by separase cleaving the Scc1 cohesin subunit. The silencing gene, *Sir2,* is thought to act (either directly or indirectly) with cohesin to decrease the frequency of unequal sister chromatid recombination

(Kobayashi, Horiuchi *et al.* 2004). The majority of recombination repair events seen do not lead to changes in rDNA repeat number, and are based on equal sister chromatid repair.

Kobayashi *et al* proposed a transcription-induced cohesin dissociation model of rDNA repeat amplification, involving Sir2 and E-Pro (an RNA *Pol II* promoter) (Kobayashi and Ganley 2005). In wild-type cells: Sir2 represses E-Pro activity; there is cohesin association throughout the rDNA; replication forks pause at the RFB, producing DSBs which are repaired by equal sister chromatid recombination; there is no change in rDNA copy number. In the absence of Sir2; E-Pro is active, transcription displaces cohesin; unequal sister chromatids are used as templates for recombinational repair; expansion of the rDNA repeats is seen.

Increased levels of Cdc18 may increase the rDNA repeat number in a mechanism involving cohesin. Cohesin loading in *Xenopus* has been shown to be dependent on the formation of the pre-replicative complex; Cdt1, MCM2-7, ORC and CDC6 (Takahashi *et al.* 2004). The increased levels of Cdc18 present after S phase, and specifically during mitosis, in the Cdc18 T6A phosphorylation mutant could be interfering with cohesin association, leading to mis-alignment, unequal sister chromatid recombination and rDNA repeat expansion.

There are three possible explanations for a Rad3-dependent checkpoint activation with an associated expansion in number of the rDNA repeats: 1) Cdc18 is inducing genome wide replication at a low level, which is not detectable by FACS analysis or by 2D DNA gels, but is sufficient to activate the checkpoint. In the rDNA repeats on chromosome III, the presence of a low

level of replication bubbles may result in recombination with unequal cross-over events (as a result of replication fork blockage at the RFB, with DSB formation) with a subsequent increase in size of the chromosomal rDNA copy number; 2) Cdc18 is inducing recombination only in the rDNA repeats, and this leads to checkpoint activation; 3) The increased level of Cdc18 may be directly activating the cell cycle checkpoint, in a manner which occurs independently of the Cdc18 effects on the size of chromosome III or on inducing DNA replication. With this model, activation of the Rad3/Chk1-dependent S-M block is a direct consequence of elevated Cdc18 levels, and is not due to effects on DNA replication, recombination or damage. Support for this view is given by the fact that cells containing Cdc18 T6A and lacking Rad3 appear to grow normally, which would not be the case if damaging changes were occurring to the DNA. It may be that the Cdc18 T6A mutant protein may also have other effects independent of Cdc18 stabilization because the protein cannot be phosphorylated. The observed expansion of the rDNA repeats may be a phenotype peculiar to the presence of the mutant Cdc18 protein, completely independent of the Cdc18-induced Rad3-dependent checkpoint response.

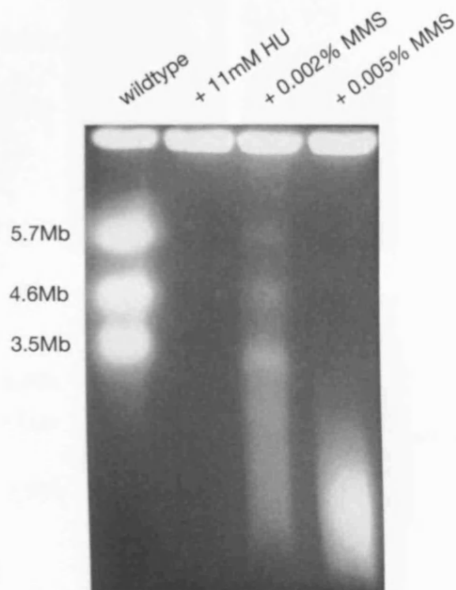


Figure 5.1: PFGE controls using an exponentially growing wildtype strain at 32°C
PFGE was performed on wildtype cells (lane 1), wildtype cells plus 11 mM HU for 3h (lane 2), wildtype cells plus 0.002% MMS for 1h (lane 3) and wildtype cells plus 0.005% MMS for 1h (lane 4). The DNA was visualised with Ethidium Bromide. Note resolution of the 3 *S. pombe* chromosomes (lane 1), the failure of chromosomal entry in the presence of HU (lane 2), and the loss of chromosomal resolution/appearance of DSB with increasing %MMS (lanes 3 and 4).

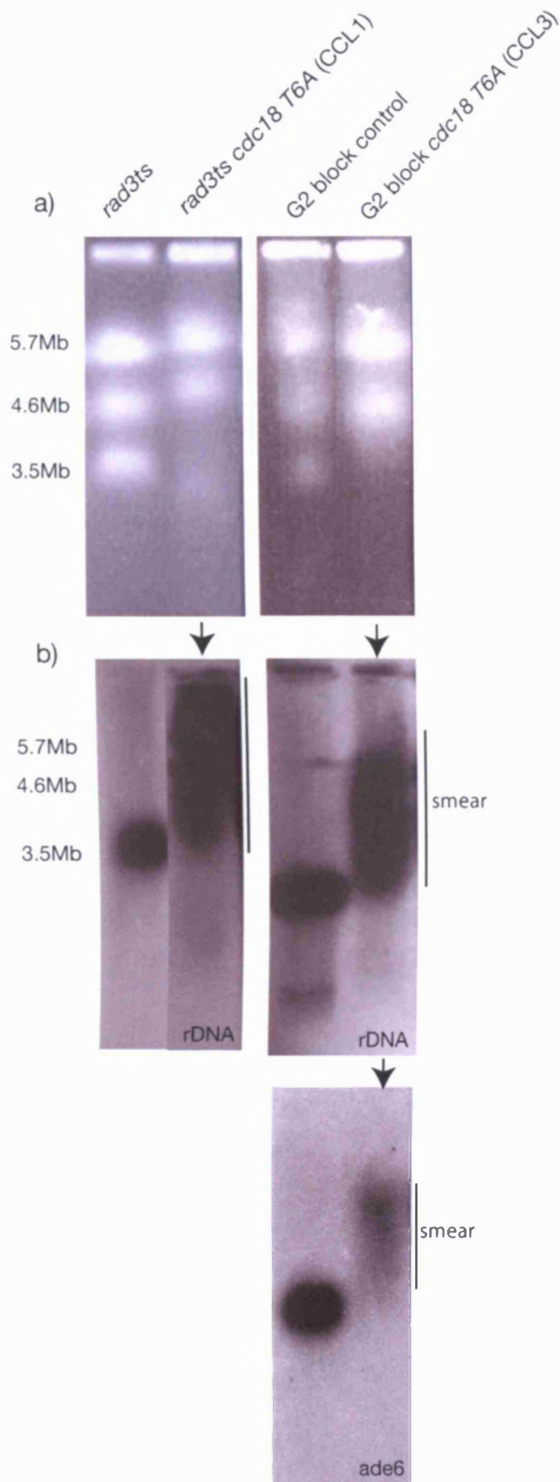


Figure 5.2: The Cdc18 phosphorylation mutant affects the mobility of chromosome III

(a) Chromosome III is not visualised in the presence of Cdc18 T6A on ethidium bromide staining, either in asynchronous cycling cells (left panel) or G2 arrested cells (right panel), but it is present in the controls. (b) Southern blotting and probing with non-origin rDNA (for a fragment downstream of ars3001) (top panel) and ade6 (bottom panel) demonstrate chromosome III is present in the gel as a smear.

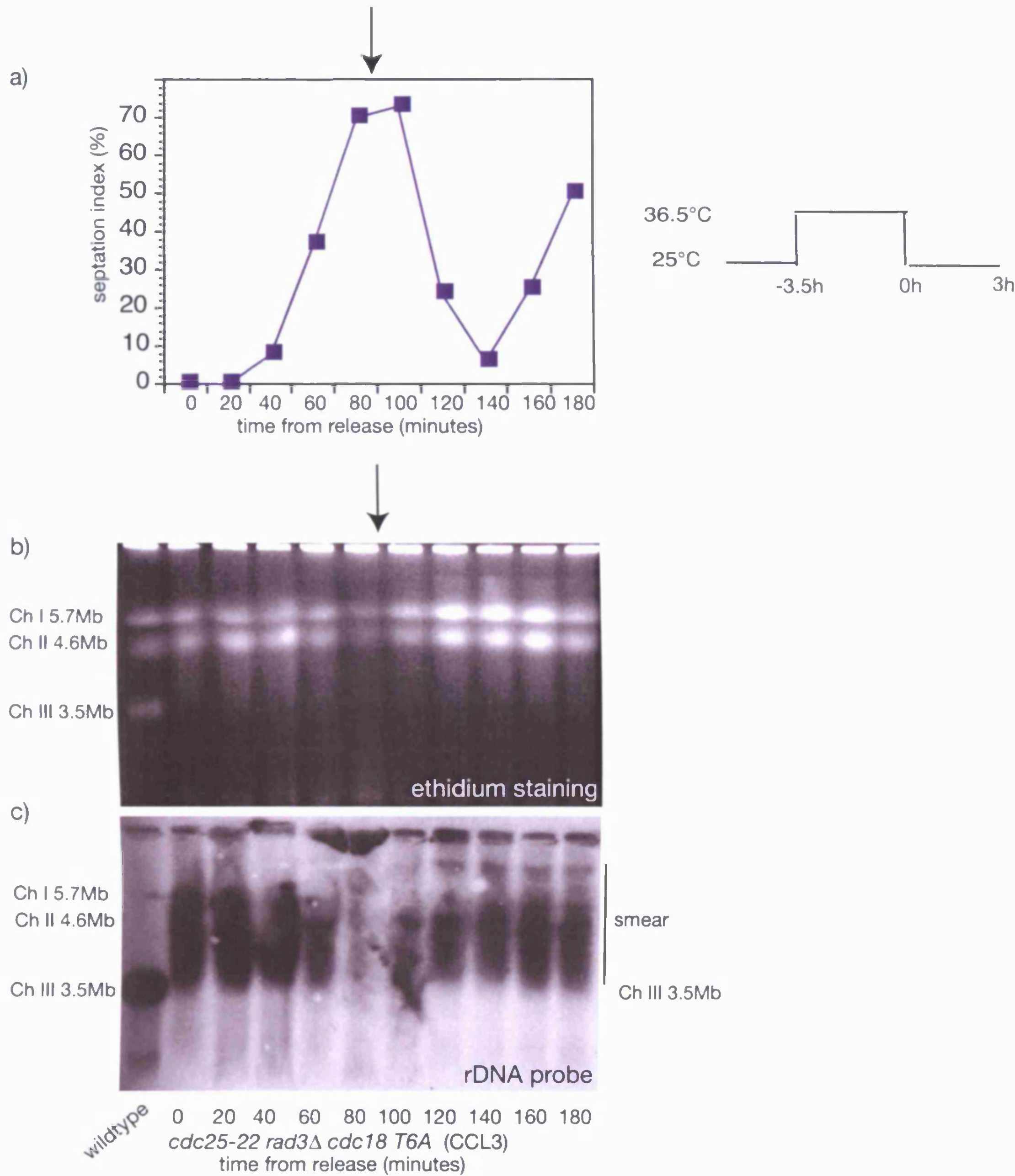


Figure 5.3: There is no change in the behaviour of chromosome III throughout the cell cycle in the presence of the Cdc18 phosphorylation mutant

(a) *Cdc25-22 rad3Δ ura4 cdc18 T6A* cells were synchronised in G2 by growing overnight at 25°C and then shifting to 36.5°C for 3.5h (schematic). Cells were then released by shifting back to 25°C and samples taken for septation rate (left panel) and (b) PFGE (top panel) every 20 minutes for 3h. Note the absence of chromosome III on ethidium bromide staining. (c) Southern blotting and probing for chromosome III with non-origin rDNA. Note the smear present throughout the cell cycle.

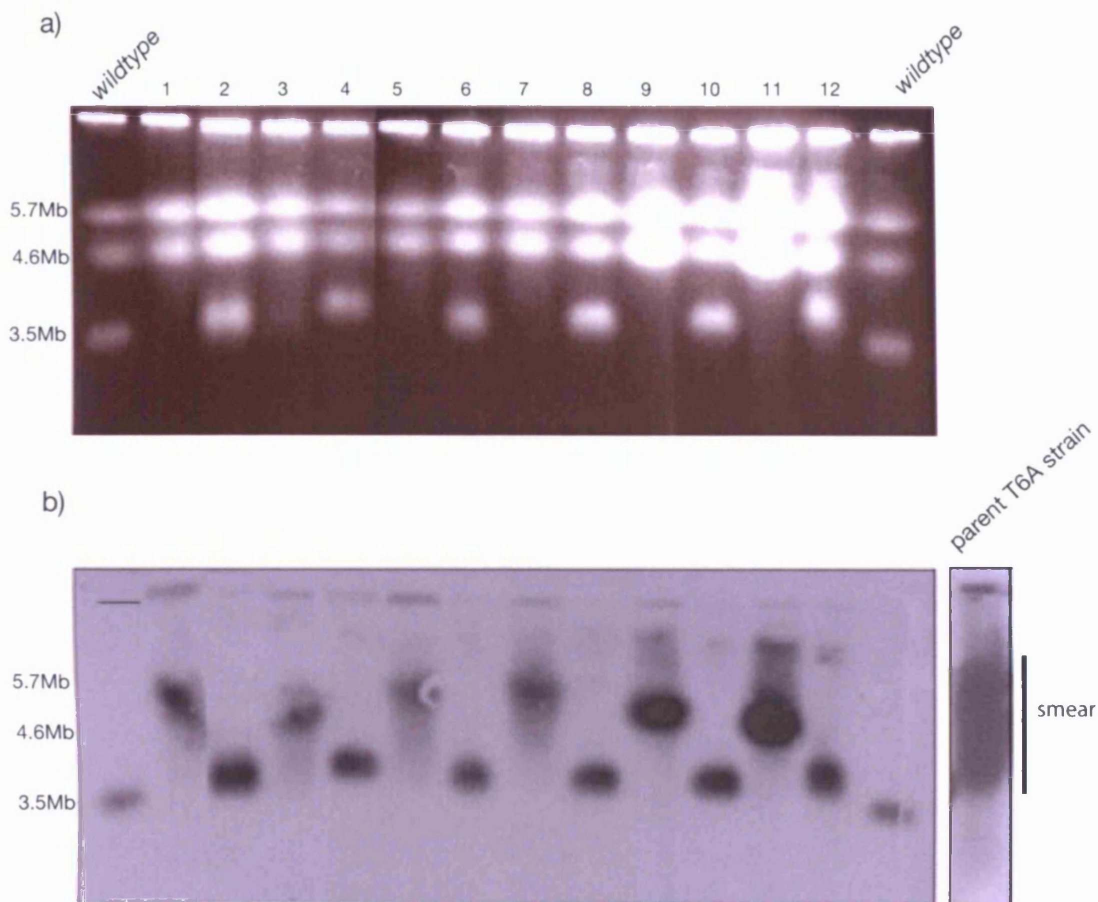


Figure 5.4: The changes in the size and variability of chromosome III disappear with the removal of the Cdc18 phosphorylation mutant
(a) Ethidium bromide stained PFG of cross-derived strains not containing *cdc18 T6A*. Note absence of chromosome III in approximately half of the strains. (b) Southern blotting and probing of (a) for chromosome III with with non-origin rDNA. Note ratio of normal:enlarged chromosome IIIs (6:7).

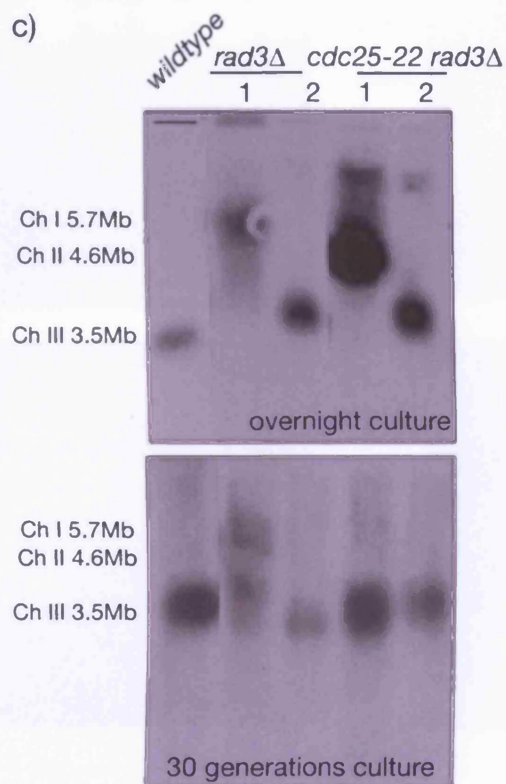


Figure 5.4c

(c) Southern blotting and probing for chromosome III with non-origin rDNA in four selected strains from (a). Top panel shows overnight culture, bottom panel shows 30 generation culture. Note that after 30 generations the larger chromosomes IIIs returned closer to a normal size.

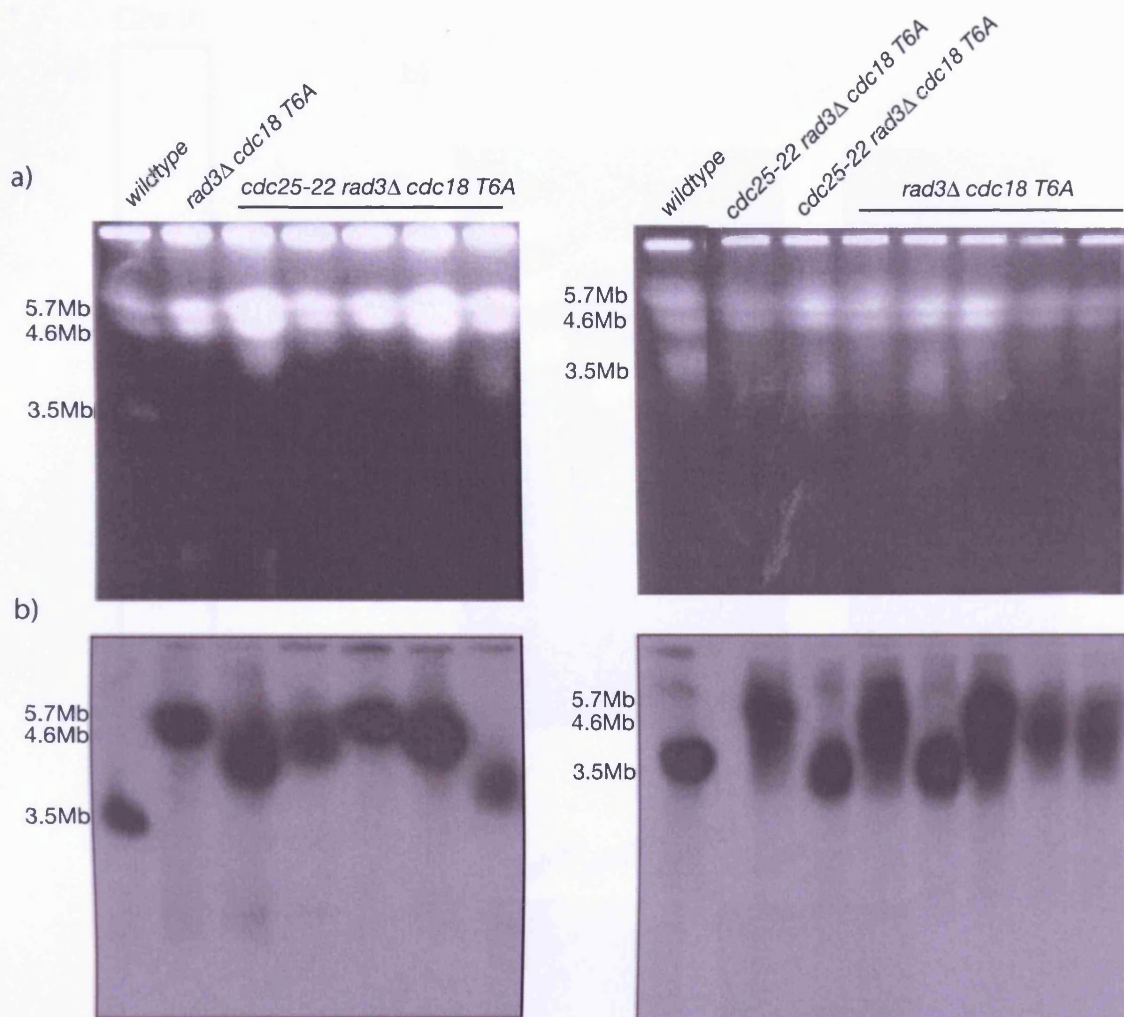


Figure 5.5: Chromosome III remains abnormal in the presence of the Cdc18 T6A phosphorylation mutant after meiosis

(a) Samples were taken for PFGE from exponentially growing cycling cells from the cross-derived strains that contained *cdc18 T6A*. Note the absence or reduction in the level of chromosome III on ethidium bromide staining. (b) Southern blotting and non-origin rDNA repeat probing. Note that chromosome III is present as a smear in all strains (but is more discreet when it approaches wildtype size).

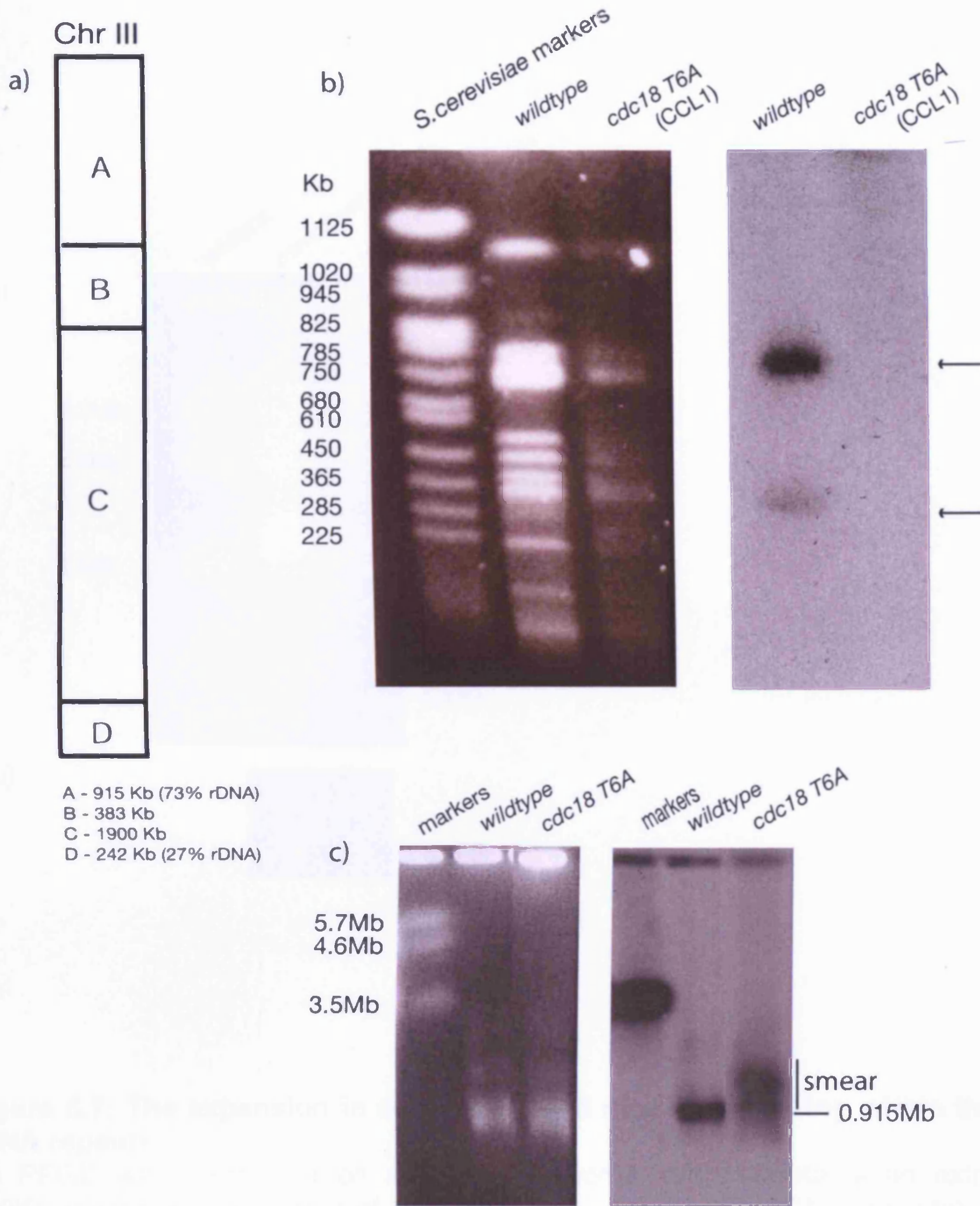


Figure 5.6: There is an expansion in the restriction fragments containing the rDNA repeats within chromosome III in the presence of Cdc18 T6A
(a) Schematic of digestion of chromosome III with the restriction enzyme Sfi1. (b) Agarose plugs containing wild type Cdc18 and Cdc18 T6A chromosomes underwent Sfi1 digest prior to PFGE under conditions to resolve fragments in kilobase (Kb) range (left panel). The gel was then Southern blotted and probed for chromosome III with non-origin rDNA (right panel). Note the absence of the rDNA containing restriction fragments. (c) As in (b) except PFGE was run under conditions to resolve fragments in megabase (Mb) range. Note rDNA containing fragments present as a smear in the 1-2Mb range.

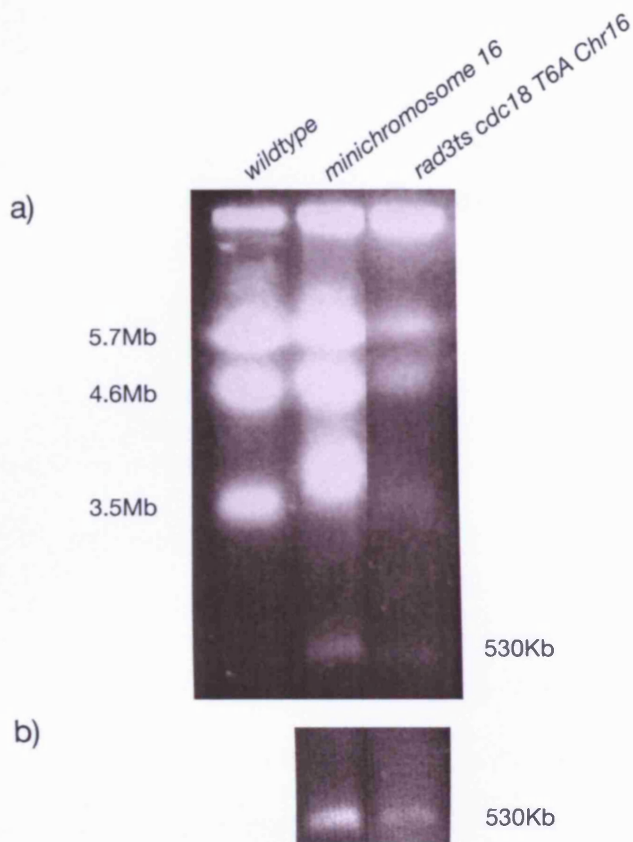


Figure 5.7: The expansion in chromosome III may be occurring within the rDNA repeats

(a) PFGE was performed on a minichromosome strain (contains an extra 530Kb centromeric fragment of chromosome III, that has no rDNA repeats) in the absence and presence of Cdc18 T6A. In both cases ethidium bromide staining demonstrated the presence of the minichromosome within the PFG. (b) Isolated enhancement of the Kb gel range to demonstrate the presence of the minichromosome more clearly.

Chapter 6
General Discussion

6.1 Cdc18 induces a Rad3-dependent checkpoint arrest

6.1.1 An overexpression screen, looking for Rad3-dependent activators of the DNA damage/replication checkpoints, suggests a new role for Cdc18/CDC6

Despite the identification of many genes involved in the checkpoint response to DNA damage or stalled/perturbed replication, the actual sensors and primary pathway activators remain unknown. Central to the G2/M and S-M checkpoints in fission yeast is Rad3, homologue of the human PI3-kinase related protein ATR (ataxia telangiectasia and rad3 related). ATR and its ortholog, ATM (ataxia telangiectasia) (homologue of *S. pombe* Tel1), are two of the key damage checkpoint genes in humans. Rad3 works upstream with the *rad* gene network to send the checkpoint signal to the effector kinases: Chk1 and Cds1 (Walworth *et al.* 1993, Savitsky 1995, Lieberman *et al.* 1996, Bentley *et al.* 1996, Kostrub *et al.* 1998, Lindsay *et al.* 1998, Martinho *et al.* 1998).

I set out to identify new genes, or a new role for known genes, involved in checkpoint activation upstream of Rad3. I postulated that overexpression of such genes might ectopically activate either the replication and the damage checkpoints, or both. In the presence of Rad3 the checkpoint would be activated leading to lethality, but in the absence of Rad3 cells would grow normally. Thus checkpoint activation and cell cycle arrest would be Rad3-dependent, but independent of DNA damage and DNA replication intermediates.

The search for such Rad3-dependent activators of the DNA damage/replication checkpoints found 6 candidate genes: *cdc18*, *spd1*, *cig1*, *sty1*, *MCM7* and *tel1*. However, as Cdc18 had the strongest and most reproducible phenotype I elected to concentrate on the characterisation of the Cdc18-induced Rad3-dependent checkpoint. For these studies I used a *cdc18 T6A* strain (encoding a protein with all 6 CDK sites mutated from threonine to alanine preventing phosphorylation by Cdc2) as this produced a more predictable, stable and reproducible Rad3-dependent arrest than Cdc18 overexpression using the thiamine-repressible *nmt1* promoter.

Cdc18/CDC6 is an essential gene with established roles in the initiation of DNA replication, and in the induction and maintenance of the S phase checkpoint. In the absence of Cdc18, cells inappropriately enter mitosis and a *cut* phenotype is observed (Kelly *et al.* 1993, Nishitani and Nurse 1995). Re-replication only occurs with strong overexpression of Cdc18 (Nishitani and Nurse 1995, Yanow *et al.* 2001), and this induces both a Rad3-dependent mitotic block (the presence of replication structures leading to S-M checkpoint activation), and a Rad3-independent block (attributed to a direct interaction of the N-terminus of Cdc18 with and subsequent inhibition of Cdc2) (Bueno and Russell 1992, Nishitani and Nurse 1995, Greenwood *et al.* 1998). However, Cdc18 is not known to activate a Rad3-dependent checkpoint response in the absence of replication intermediates (see Table 6.1 below).

Table 6.1 Summary of effects of overexpression/stabilisation of Cdc18 levels in different constructs

Construct	Effects				
	Re-replication	**Mitotic block**	**Rad3-dependent block**	**Transient block**	**Changes in size/variability chromosome III**
pRep3x *cdc18* (Nishitani and Nurse 1995)	Yes	Yes	No	No	Not known
pRep3X C-terminus *cdc18* (Greenwood *et al.* 1998)	Yes	Yes	Yes	No	Not known
pRep3X N-terminus *cdc18* (Greenwood *et al.* 1998)	No	Yes	No	No	Not known
pRep4x *cdc18* (This thesis)	No	Yes	Yes	No	Not known
cdc18 T6A (This thesis)	No	Yes	Yes	Yes	Yes

In this thesis I have demonstrated that increased levels or stabilisation of Cdc18 activate a Rad3-dependent checkpoint arrest, and that the *rad* checkpoint gene network is necessary for this cell cycle block. Rad3 is essential for initiation and maintenance of the checkpoint signal. Wild-type Cdc18 is required for checkpoint control, even when the checkpoint is induced by other means, such as HU. However, Cdc18 is not required to maintain stalled replication forks in an HU block. Cdc18 operates early on in the checkpoint, and acts mainly through the Rad3/Chk1/Crb2 "damage" pathway. It does not require the presence of a functioning Rad3/Cds1/Mrc1 "replication" pathway. I found no evidence of gross DNA damage or effects on the cell cycle, and no evidence of replication intermediates.

However, an unexpected consequence of stabilization of Cdc18 levels was to increase the size of chromosome III, with expansion of the Sfi1 restriction fragments containing rDNA repeats. I confirmed that the changes

seen in chromosome III were a consequence of exposure to the presence of mutant Cdc18 protein.

6.1.2 How does stabilisation of Cdc18 levels produce a Rad3-dependent arrest?

I considered three possible scenarios to explain the observation of a transient checkpoint activation in the presence of elevated Cdc18 levels: (1) A direct effect of Cdc18, occurring independently of any other effects of Cdc18 in the cell such as changes in chromosome III; (2) An indirect effect of Cdc18 consequent to low level activity of a known mechanism, such as endoreplication, or of an unknown mechanism that results in the increased size of chromosome III; and (3) Both direct and indirect effects acting independently to activate the checkpoint.

In a normal S phase, Cdc2 phosphorylates Cdc18 which becomes targeted for ubiquitin-mediated proteolysis (Brown *et al.* 1997, Jallepalli *et al.* 1997, Kominami and Toda 1997Jallepalli *et al.* 1998, Lopez-Girona *et al.* 1998, Kominami *et al.* 1998, Wolf *et al.* 1999). This results in most of Cdc18 being degraded by end of S phase, allowing cells to proceed into G2 and M. In Chapter 3, I put forward the idea that Cdc18 acts as a marker that "S phase is in progress". This proposal stemmed from the observation that although Cdc18 is essential for checkpoint activation in an HU block, it is not required to maintain stalled replication forks. I hypothesised that once Cdc18 has played its role in replication initiation, it remains physically associated with the DNA transmitting information that replication is ongoing until sister chromatid duplication is complete. In its absence, cells inappropriately enter mitosis but

replication forks do not collapse.

I also proposed a positive-feedback loop model for the Cdc18-activated Rad3-dependent checkpoint. Cdc2 is the target of the S-M checkpoint, and its Rad3-dependent inactivation blocks mitosis (*Figure 3.10a(i))*. Cdc2 also phosphorylates Cdc18 targeting it for degradation (*Figure 3.10a(ii))*. The checkpoint requires Rad3-dependent Cdc2 inhibition to prevent Cdc18 destruction. I considered the possibility that the positive feedback loop, in which the initial stabilisation of Cdc18 enforces the checkpoint pathway by further Rad3 inhibition of Cdc2, subsequently leads to further stabilisation of Cdc18 levels (*Figure 3.10a(iii)*). In the presence of a replication block, such as that induced by HU, the positive feedback loop is put into action. However, this is lost in the absence of Cdc18, and aberrant mitotic entry observed. The Cdc18 phosphorylation mutant has the same effect as a replication block. With no Cdc2-induced Cdc18 degradation possible, the checkpoint is enforced and cells don't enter mitosis (*Figure 3.10b*).

It may be that the S-M checkpoint is active during a normal S phase at an extremely low level, with a monitoring mechanism in place to maintain and amplify the checkpoint when required. *S. pombe* Cds1 activity is undetectable in an undisturbed S phase (Lindsay, Griffiths *et al.* 1998), but ATR is present during normal DNA replication and regulates the timing of origin firing in *Xenopus* egg extracts (Shechter *et al.* 2004). I proposed that wild-type Cdc18 levels are too low to activate a strong checkpoint response during a normal S phase, and that the feedback loop has a threshold which enables discrimination between local noise and a true requirement for checkpoint activation. This threshold is exceeded at the levels of Cdc18 produced during either *nmt1*-

controlled overexpression or in the phosphorylation mutant, resulting in Rad3-dependent checkpoint activation. The idea of a threshold may also explain the spectrum of effects seen, from a G2 delay to an absolute arrest with features of transience and incomplete penetrance.

Checkpoints involve a sensor detecting a particular structure, and then activating the effectors of checkpoint activation. There are many potential structural candidates that could activate the checkpoint in the event of DNA damage. However, another possibility is that the replication machinery may have a role as a signaler of a replication block. In support of this idea, I found that the original overexpression screen produced not only Cdc18 but also MCM7 as Rad3-dependent checkpoint activators; MCM7 is part of the MCM complex, which is thought to function as the replicative DNA helicase (Kearsey and Labib 1998). The replication machinery is conveniently sited to perform this secondary function of Rad3-dependent checkpoint activation. The MCM proteins remain nuclear throughout S phase (Mendez and Stillman 2000), and are necessary for ongoing DNA synthesis (Labib *et al.* 2000). The MCM complex is re-localized and exported from the nucleus on completion of S phase. This would fit with a putative role in preventing mitosis until replication is complete, by providing an “S phase in progress” signal. Human MCM7 has been shown to interact with ATRIP, which binds and activates the ATR kinase (the human homologue of Rad3), leading to the proposal that excess hMCM7 might activate the intra-S phase checkpoint (Cortez *et al.* 2004, Tsao *et al.* 2004).

6.1.3 Cdc18/CDC6 plays a central role in S phase progression and is crucial for checkpoint control.

The importance of Cdc18/CDC6 is reflected in the tightness of its regulation, both transcriptionally and post-translationally. Cdc18 is expressed during mitosis and G1, with transcription repressed in G2 and S phase (Kelly *et al.* 1993, Muzi Falconi *et al.* 1996, Baum *et al.* 1998). The second layer to Cdc18/CDC6 regulation is phosphorylation and targeted degradation by Cdc2. The quantitative model of CDK activity in fission yeast can be used to understand the Cdc18 protein levels throughout the cell cycle (Fisher and Nurse 1996). These authors proposed that CDK activity must be low prior to S phase (which equates to high levels of Cdc18 during assembly of the pre-replicative complex). CDK activity then reaches a high level to promote S phase (with Cdc18 levels falling, but sufficient for an "S phase in progress" signaling role). An even higher threshold is reached to promote mitosis and inhibit replication (hence Cdc18 is not present in G2 and M), until CDK activity falls on exit from mitosis (allowing Cdc18 to re-accumulate ready for the next round of replication).

6.1.4 How does the increase in the size of chromosome III seen with the stabilisation of Cdc18 levels fit into this story?

Expansion in the size of chromosome III occurs in the Sfi1 digestion fragments containing the rDNA repeats. Recombination between the rDNA repeated structure occurs at high frequency, and the number of repeats is therefore unstable (Kobayashi *et al.* 1992). The phenomenon of repeat expansion has

broad biological and medical implications, and is involved in several inherited genetic disorders such as Fragile X syndrome and Huntington's disease.

In Chapter 5, I proposed several models to explain this observation in the context of a Cdc18-induced Rad3-dependent checkpoint. The first model supposed that Cdc18 was inducing low level genome wide replication that was undetectable but sufficient for checkpoint activation. This resulted in increased recombination with unequal crossover events in the rDNA repeats on chromosome III, leading to subsequent repeat expansion. The second model suggested that the increased levels of Cdc18 were directly activating the cell cycle checkpoint independently of the concurrent expansion of chromosome III. The third model suggested that Cdc18 was inducing recombination at the rDNA repeats only, not genome wide, and this could activate the checkpoint. I also considered the possibility that the expansion of chromosome III is a phenotype peculiar to the phosphorylation mutant.

I also discussed, in Chapter 5, what specific properties of the rDNA repeats could lead to expansion, and discussed several processes that stimulate recombination in the rDNA. Previously, it has been thought that the repetitive nature of all expandable elements can lead to occasional strand slippage during DNA replication (reviewed by Mirkin 2006). If unrepaired, this may convert to an expanded repeat after a second round of replication. But such strand slippage is likely to lead to only a limited increase in repeats, rather than the large scale expansion seen in chromosome III in the presence of the phosphorylation mutant. Human disorders, such as Fragile X syndrome and Huntington's disease, are characterised by DNA repeat expansion. A threshold length for expansion (100-120bp) has been demonstrated, and is associated

with the formation of unusual DNA secondary structures (hairpins, triplexes etc) by DNA unwinding or strand separation, as is seen with DNA replication.

The whole process of repeat expansion (of both the rDNA repeats and of the small DNA repeats involved in some human hereditary disorders) is tied up with the two processes of replication and recombination. We know that Cdc18 is a key player in DNA replication and is bound to chromatin throughout S phase. As I discussed at the end of Chapter 5, increased levels of Cdc18 may lead to expansion of the rDNA repeat number in a cohesin-related mechanism. Cohesin loading in *Xenopus* has been shown to be dependent on the formation of the pre-replicative complex, including Cdt1, MCM2-7, ORC and CDC6 (Takahashi *et al.* 2004). Proper sister chromatid cohesion enables DSB repair by recombination without any net change in the number of rDNA repeats. Cohesin holds the sister chromatids together from DNA synthesis during S phase until anaphase segregation. However, cohesin dissociation leads to sister chromatid misalignment and subsequent expansion of the rDNA repeats. The increased levels of Cdc18 present after S phase in the presence of the T6A phosphorylation mutant may be affecting cohesin association, with subsequent mis-alignment and rDNA expansion. It may be acting through effects on *sir2*, the silencing gene required for the efficient association of the cohesin subunit, Scc1, to rDNA (Kobayashi *et al.* 2004). In the absence of Sir2, expansion of the rDNA repeats is seen (Kobayashi and Ganley 2005).

I also considered and discounted the following mechanisms behind Cdc18-induced expansion of the rDNA containing fragments of chromosome III: the occurrence of "onion skin" re-replication in the DNA (no replication intermediates were detected during a G2 block using 2D DNA gel

electrophoresis); the production of a ring chromosome (this would not have entered the PFG); that telomere elongation rather than rDNA repeat expansion is occurring (telomeres are not thought to elongate above 2Kb in size); the presence of a chromosomal translocation (the concurrent reciprocal event was not observed).

In conclusion, this work has shown that a moderately elevated level of Cdc18, brought about either by use of an ectopic promoter or by modifying its stability, activates the Rad3-dependent checkpoint. I propose this is either a direct effect of Cdc18, or an indirect effect via Cdc18-induced expansion of the rDNA containing restriction fragment of chromosome III.

6.2 The global role of Cdc18/CDC6

There is increasing evidence, both in *S. pombe* and also in higher eukaryotes, that CDC6/Cdc18 may perform two separate functions during the cell cycle. The first is the well-established and extensively characterised role in replication initiation. The second is the emerging role in the regulation of the S-M checkpoint.

As described earlier, in *S. pombe* Cdc18 is targeted for ubiquitin-dependent proteolysis by the Cdc2 cyclin-dependent kinase, resulting in falling Cdc18 levels in S phase and its absence in G2. However, in comparison, the human CDC6 protein is present throughout the cell cycle. Chromatin bound CDC6 persists throughout S phase and G2 before its degradation by the APC during mitosis, suggesting it may have other functions during late S phase until mitosis (Petersen 2000). The majority of the non-chromatin bound soluble

CDC6 is exported from the nucleus during S phase and G2 in a CDK-dependent manner (Petersen *et al.* 1999, Pelizon *et al.* 2000). This relocation of CDC6 is thought to prevent re-replication. However, it has also been proposed that a fraction of CDC6 remains chromatin bound throughout the cell cycle, and that it is the MCM proteins that fluctuate between soluble and chromatin bound forms (Mendez and Stillman 2000).

It is thought that once replication licensing has occurred, CDC6 is not required for the subsequent DNA replication (Rowles *et al.* 1999, Jares *et al.* 2000), but CDC6 may play another role, involving checkpoint activation, later in the cell cycle. In human cells, overexpression of CDC6 inhibits the activation of Cdk1-cyclin B and blocks entry into mitosis in a Chk1-dependent manner, suggesting important roles in both origin licensing and cell cycle progression (Clay-Farrace *et al.* 2003). It has also been shown in *Xenopus* that: CDC6 is displaced from chromatin as a direct consequence of origin licensing; rebinding of CDC6 to chromatin occurs in S phase after replication initiation (as the replication forks progress away from the origin); CDC6 is required for Chk1 activation in response to stalled forks, separate from its role in loading the MCM complex onto chromatin (Oehlmann *et al.* 2004).

Further evidence for the checkpoint role of Cdc18/CDC6 is the relationship with oncogene-induced senescence. This is associated with human pre-cancerous lesions and is considered a barrier to tumourigenesis. CDC6 overexpression has been shown to induce an ATM-dependent senescence in human cells lines (Bartkova *et al* 2006).

It is now also being proposed that CDC6 has third role, unrelated to DNA replication and checkpoint control. CDC6 appears to be critical for the

formation of the meiotic spindle in mouse oocytes (Anger *et al.* 2005). Other DNA replication proteins (ORC and MCM) are also thought to be involved in chromosomal condensation and distribution in mitosis (Pflumm and Botchan 2001, Prasanth *et al.* 2002).

In summary, Cdc18/CDC6 has clearly defined roles in the initiation of replication and in the induction and maintenance of the S phase checkpoint, and an emerging role in mitosis.

6.3 The relationship between cell cycle biology and cancer medicine

I am a Clinical Oncologist, treating cancer patients with a combination of chemotherapy and radiotherapy. Chemotherapy involves the use of cytotoxic and cytostatic drugs, and biological, immunological and genetic therapies. Chemotherapy may also be used to enhance the effects of radiotherapy, by acting synergistically or as a radio-sensitizer. I chose to work on DNA checkpoints as their disruption results in genomic instability, and may lead to cancer in humans. The efficacy of radiotherapy, and of many chemotherapeutic agents, relies on an intact checkpoint pathway. I have found a new checkpoint role for Cdc18, which is either a direct effect or is secondary to low-level replication and subsequent recombination. I would therefore like to discuss the implications of my research with respect to cancer diagnosis and therapy, and to consider the possible clinical role of Cdc18/CDC6.

The basic cell biology of 25 years ago identified signalling molecules that are now being used as therapeutic targets in cancer treatment. The clinical importance of these molecules was not obvious at the time. The cyclin-

dependent kinase pathways have since been targeted, along with the tyrosine kinase receptor pathways. One recent development, with dramatic clinical effects, was the specific inhibition of the Bcr-abl fusion protein (a tyrosine kinase receptor) with imatinib (Glivec®). The Philadelphia chromosome is found in CLL (chronic lymphocytic leukaemia) and represents a t(11:22) translocation placing the *bcr* gene next to the *abl* gene. Imatinib was also found to have dramatic effects in gastro-intestinal stromal tumours (GIST). As a result, more generalised tyrosine kinase inhibitors are being developed with promising clinical results. The more we know and understand about the genetics behind malignancy, the greater the predictive power of behaviour and outcome (known as tumour profiling), and the greater the ability to individualise therapeutic treatments and optimise outcome. Cancer clinicians and cancer scientists need to work together to produce the desired end result: personalised medical treatments for cancer with ever increasing survival rates.

So what is the role of Cdc18/CDC6 in understanding, assessing and treating human cancers? Firstly, Cdc18/CDC6 and the ORC-associated DNA replication factors play a key role in the initiation of replication, which results from the convergence of many, potentially mutagenic, signalling pathways. They are therefore perfectly positioned to give an accurate indication of cell division. Cell proliferation markers have been long used as indicators of dysplasia and malignancy (reviewed by Semple and Duncker 2004). Ki-67 in particular has been used successfully in the diagnosis of breast cancer, prostate cancer, and soft tissue sarcomas, but is not so helpful in other malignancies. However, anti-CDC6 antibodies have been shown to detect a dramatically higher percentage of abnormal cells in cervical cancer and brain

tumour samples than Ki-67 (Williams *et al.* 1998, Ohta *et al.* 2001).

The MCM proteins, closely associated with Cdc18/CDC6 in the pre-replicative complex, are sensitive and specific biomarkers of cell cycle entry. They are currently being used as markers of dysplasia/early cancer in the development of new, and in the improvement of established, minimally invasive screening tests. For example, MCMs are being used in an attempt to improve the sensitivity of the smear test for cervical cancer (Gonzalez *et al.* 2005, Scott *et al.* 2006). Cdc18/CDC6 could be used in the same way.

But CDC6 may play a role in cancer beyond that of a proliferation marker (Gonzalez *et al.* 2006). *Gonzalez et al* concluded that the aberrant expression of CDC6 is oncogenic, by down regulation of the INK/ARF locus. The INK/ARF locus is one of the most frequently inactivated in human cancers, and encodes 3 tumour suppressors: $p15^{INK4b}$, ARF and $p16^{INK4a}$. They proposed a model whereby a conserved DNA regulatory domain, sensitive to CDC6 levels, positively governs the INK4/ARF locus. They compared the CDC6 induced down regulation of tumour suppressors to the silencing of mating type loci in budding yeast. I have already discussed and considered the possible interaction between Cdc18/CDC6 and the silencing gene, *Sir2,* which may underlie Cdc18-enhanced recombination as the putative mechanism behind the observed rDNA repeat expansion.

Cdc18/CDC6 is a universal component of all cells, cancerous or not. Given its key role in replication and this new role in checkpoint control, deregulation of Cdc18/CDC6 carries the risk of severe consequences to the cell and organism. However, this also translates into its potential use as a clinically important therapeutic target, diagnostic tool and prognostic factor.

Chapter 7

Materials and Methods

7.1 Fission yeast physiology and genetics

7.1.1 Gene and protein nomenclature

Throughout this thesis I have followed the genetic nomenclature of *Schizosaccharomyces pombe* reviewed in (Kohli and Nurse 1995). Gene names consist of three lower case letters in italics (e.g. *rad3*). The wild-type gene is indicted by a superscript plus symbol "+" following the gene name (e.g. *rad3*$^{+}$). Gene mutant alleles are represented in italics by either a number, or a combination of numbers and letters, separated from the gene name by a hyphen (e.g. *rad3-136*). A superscript is used to denote a temperature sensitive mutation (e.g. *rad3*^{ts}). Deletions are specified by the symbol delta "Δ" (e.g. *rad3*Δ). If the deleted gene has been replaced by another gene, this further specified by "::" followed by the name of the replacing gene (e.g. *rad3Δ::ura4*$^{+}$). The protein encoded by a specific gene has the same name but not in italics, the first letter upper case and rest in lower case (e.g. Rad3). When genes are expressed from a plasmid the name of the plasmid and a "p" preceeds the gene name (e.g. pRep4X *cdc18*). Strain mating type is indicated as h+ or h-.

S. cerevisiae genes are named as *S. pombe* genes, except a capital letter is used (e.g. *Cdc6*). Human genes are named as *S. pombe* genes, except capitals are used (e.g. *CDC6*).

7.1.2 Strain growth and maintenance

The *S. pombe* strains used in this study are listed in Table 7.1 (previously constructed) and Table 7.2 (constructed for this thesis). All strains were derived

from the wild types *972h-* and *975h+*. Growth conditions and media were used as previously described (Moreno *et al.* 1991). Maintenance and growth of fission yeast strains, storage and waking up of frozen cultures, checking of phenotypes and genetic crosses were performed as described in (Moreno *et al.* 1991, MacNeill and Fantes 1993). When required Hydroxyurea (HU) at 11mM, thiamine (vitamin B1) at 15µM, geneticin (G418) at 0.5mg/ml, or 5-fluoro-orotic acid (5FOA) at 1mg/ml was added.

Table 7.1 Fission yeast strains previously constructed

Genotype		Reference
Wildtype *(972 h+ and 972 h-)*		CCL collection
h- ade6-704 leu1-32		CCL collection
h- rad3^{ts} ade6-704 leu1-32 ura4-D18		T.Carr
h+ rad3^{ts} ade6-704 leu1-32 ura4-D18		T.Carr
h- spd1Δ::ura4 ade6-704 leu1-32 ura4-D18		CCL collection
h- rad3Δ::ura4 ade6-704 leu1-32 ura4-D18		T.Carr
h- rad3-136 leu1-32 ura4-D18		CCL collection
h- leu1-32 ura4-D18		CCL collection
h- cdc25-22		CCL collection
h- chk1Δ::ura4 ura4-D18		CCL collection
h- chk1Δ::ura4 ura4-D18 leu1-32		CCL collection
h- cds1Δ::ura4 leu1-32 ura4-D18		CCL collection
h- chk1Δ::ura4 cds1Δ::ura4 ade6-704 leu1-32 ura4-D18		CCL collection
h+rad1Δ::ura4 leu1-32 ura4-D18		CCL collection
h- rad9Δ::ura4 ade6-704 leu1-32 ura4-D18		CCL collection
h- hus1-14 leu1-32		CCL collection
h- rad17- ade6-704 leu1-32 ura4-D18		CCL collection
h- rad26Δ::ura4 leu1-32 ura4-D18		CCL collection
h- crb2Δ::ura4 leu1-32 ura4-D18		CCL collection
h- mrc1Δ::ura4 ade6-704 leu1-32 ura4-D18		T.Carr
h- mik1Δ::ura4+ wee1-50 ade6-704 leu1-32 ura4-D18		CCL collection
h+ rad3^{ts} cdc18 T6A LEU2 ade6-704 leu1-32 ura4-D18	*CCL1*	CCL collection
h+ rad3^{ts} cdc18 T6A-TAP kan^{r} ade6-704 leu1-32 ura4-D18	*CCL2*	CCL collection
h+ rad3Δ::kan^{r} leu1-32 ura4D-18		CCL collection
h+ chk1Δ::kan^{r} leu1-32 ura4D-18		CCL collection
h+ cds1Δ::kan^{r} leu1-32 ura4D-18		CCL collection
h+ crb2Δ::kan^{r} leu1-32 ura4D-18		CCL collection

h+ rad26Δ::kan^{r} *leu1-32 ura4D-18*		CCL collection
h+ rad9Δ::kan^{r} *leu1-32 ura4D-18*		CCL collection
h+ hus1Δ::kan^{r} *leu1-32 ura4D-18*		CCL collection
h- nmt81 cdc18-td HIS3 ade6-M210 his5 kanR		CCL collection
h- cdc18Δ::ura4 ade6-704 leu1-32 pRep81 *cdc18*		CCL collection
h- cdc2-56^{ts} *leu1-32*		CCL collection
h- nda3-km11^{cs} *leu-132 ura4-D18*		CCL collection
cdc25-22^{ts} *cdc18-TAP kan*^{r} *leu1-32 ura4-D18*		CCL collection
h- cdc25-22^{ts} *rad3Δ::ura4 leu1-32 ura4-D18*		CCL collection
cdc25-22^{ts} *rad3Δ::ura4 cdc18-TAP kan*^{r} *leu1-32 ura4-D18*		CCL collection
h+ cdc25-22^{ts} *rad3Δ::ura4 cdc18 T6A-TAP kan*^{r} *leu1-32 ura4-D18*	*CCL3*	CCL collection
h- his2 ade6-M210 Chr16		T.Toda
rad3^{ts} *cdc18 T6A LEU2 chk1-HA ade6-704 leu1-32 ura4-D18*		CCL collection

Table 7.2 Fission yeast strains constructed for this thesis

Genotype	
rad3^{ts} *ade6-704 leu1-32*	
rad3-136 leu1-32 ura4-D18 pRep4X-cdc18	
rad3-136 leu1-32 ura4-D18 pRep4X-cig1	
leu1-32 ura4-D18 pRep4X-cdc18	
leu1-32 ura4-D18 pRep4X-cig1	
rad3Δ:: ura4 ade6-704 leu1-32 ura4-D18 pRep4X-cdc18	
h- mik1Δ::ura4 ade6-704 leu1-32 ura4-D18	
h+ rad3^{ts} *cdc18-T6A LEU2 ade6-704 leu1-32*	*CCL4*
rad3Δ::ura4 cdc18-T6A LEU2	
chk1Δ::ura4 cdc18-T6A LEU2	
cds1Δ::ura4 rad3^{ts} *cdc18-T6A LEU2*	
chk1Δ::ura4 cds1D::ura4 cdc18-T6A LEU2	
mrc1Δ::ura4 rad3^{ts} *cdc18-T6A LEU2*	
crb2Δ:: ura4 cdc18-T6A LEU2	
rad9Δ::ura4 cdc18-T6A LEU2	
hus1-17 cdc18-T6A LEU2	
rad1Δ::ura4 cdc18-T6A LEU2	
rad17h-21 cdc18-T6A LEU2	
rad26Δ::ura4 cdc18-T6A LEU2	
mik1Δ::ura4 rad3^{ts} *cdc18-T6A LEU2*	
hus1Δ::kan^{r} *cdc18-T6A LEU2*	
rad3Δ::kan^{r} *cdc18-T6A LEU2*	
chk1Δ::kan^{r} *cdc18-T6A LEU2*	
cds1Δ::kan^{r} *rad3*^{ts} *cdc18-T6A LEU2*	
crb2Δ::kan^{r} *cdc18-T6A LEU2*	
rad26Δ::kan^{r} *cdc18-T6A LEU2*	

rad9Δ::kan^r cdc18-T6A LEU2	
hus1Δ::kan^r cdc18-T6A LEU2	
rad3^{ts} cdc18-T6A LEU2 ade6-M210 Chr16	CCL5
cdc2-56^{ts} rad3Δ::ura4	
cdc2-56^{ts} rad3Δ::ura4 cdc18-T6A kan^r	CCL6
nda3-km11^{cs} rad3Δ::ura4	
nda3-km11^{cs} rad3Δ::ura4 cdc18-T6A kan^r	CCL7

7.1.3 Strain construction

Generation of all double mutant strains was by crossing two single mutant strains of opposed mating type, followed by random spore analysis. Random spore analysis was also used to generate mutant strains with additional markers. All the checkpoint mutant *cdc18 T6A* strains and the *minichromosome 16 cdc18 T6A* strain were derived by crossing *h+ rad3^{ts} cdc18 T6A LEU2* with the opposing phenotype mutant strain, followed by random spore analysis at 36°C. This was to allow growth of *cdc18-T6A* checkpoint mutant strains with a lethal phenotype in the presence of *rad3$^+$* that were also *rad3^{ts}*. They were selected for on the basis of prototrophy for leucine (*cdc18-T6A*) and uracil or G418 resistance (for kan^r). They were then replica-plated to 25°C to observe normal growth or elongation.

7.1.4 Transformation of plasmids and libraries

Cells were transformed with plasmid DNA by a modified lithium acetate method (Bahler *et al.* 1998). Approximately 1-2μg of DNA was transformed into 5x10^8 log phase cells with 20μg of herring sperm carrier DNA per transformation. Plasmids were maintained by use of a selectable marker.

7.1.5 Induction of gene expression from the nmt promoter

The thiamine repressible *nmt1* promoter is derived from the *nmt1+* gene, which is required for thiamine biosynthesis (Maundrell 1990). Three versions are available in which the promoter sequences have been mutated to different degrees to give lower levels of expression (Basi *et al.* 1993, Forsburg 1993). They can be ordered by strength: REP1>REP41>REP81. These all have the *LEU2* marker and a Nde1 restriction enzyme site in the polylinker. Plasmids containing *LEU2*, but with Xho1 replacing Nde1 are named 3X, 41X, 81X and can be ordered by strength: REP1=REP3X>REP41X>REP81X. They are also available with different markers e.g. REP4X=REP3X with *ura4* replacing *LEU2*.

The standard thiamine concentration for repression of gene expression from the *nmt* promoters is 5µg/ml, although some low level expression occurs even in the presence of thiamine. Strains containing genes under the control of the *nmt1* promoter were always grown on plates containing 5µg/ml thiamine before replica-plating onto minus thiamine plates to allow induction of gene expression (takes up to 12 hours at 32°C). To induce full expression in liquid culture, cells were grown until log phase in minimal media containing thiamine, filtered and washed three times with thiamine-free media, and then resuspended and grown in the absence of thiamine.

7.1.6 Shift experiments with temperature sensitive mutants

Temperature-sensitive strains were grown either at the permissive temperature of 25°C (*cdc25-22^{ts}*) or the restrictive temperature of 36°C (*rad3^{ts} cdc18-T6A* CCL1) and then shifted as required. The *cdc25-22^{ts}* block and release method of culture synchronisation was performed as described previously (Moreno *et*

al. 1989). Cold-sensitive strains (*nda3-km11*^{cs}) were cultured at the permissive temperature of 32°C or the restrictive temperature of 19°C.

7.1.7 Physiological experiments with Hydroxyurea (HU)

HU inhibits the ribonucleotide reductase enzyme resulting in nucleotide depletion. At a concentration of 11mM it will arrest an entire population of exponentially growing cells in early S phase on plates or in liquid culture.

7.1.8 Flow cytometric analysis (FACS)

Culture samples containing $2x10^6$-10^7 cells were fixed in 70% ethanol and stored at 4°C. Two hundred µl of each sample were then processed for FACS analysis by washing in 3ml of 50mM Na_3Citrate, resuspending in 0.5ml 50mM Sodium Citrate containing 0.1mg RNaseA, and put at 37°C for a minimum of 2 hours. 0.5ml 50mM Sodium Citrate containing 2µg/ml propidium iodide was then added before sonicating for 30 seconds at setting 6 in a Soniprep 150 sonicator (MSE). FACS analysis was performed using a Becton Dickinson FACScan as previously described looking at both DNA content and cell length (estimated by forward scatter) (Sazer and Sherwood 1990).

7.1.9 Cell number determination

A Coulter counter was used for measuring cell number. Cell fixation was by adding 1.6mls of formol saline (0.9% saline, 3.7% formaldehyde) to 0.4ml of culture and storing at 4°C. Before cell number processing and counting, 18mls of ISOTON solution was added and the sample sonicated as in 7.1.8.

7.1.10 Cell length determination

Gross changes in cell length were observed from forward scatter measurements during FACS analysis (7.1.7). Actual cell length was determined by scanning a picture of the cell(s) containing a calibration bar taken at the same resolution. The images were then imported into the programme NIH Image J, enabling the measurement of individual cell lengths.

7.2 Molecular Biology Techniques

7.2.1 General techniques

The following techniques were essentially carried out as previously described (Sambrook *et al.* 1989): DNA digestion by restriction enzymes; DNA gel electrophoresis; preparation of competent bacterial cells for transformation; bacterial transformation with DNA. Qiagen columns (Qiagen) were used for both small (mini-prep) and large (maxi-prep) preparation of DNA from bacteria. PCR reactions were performed in a MiniCycler (MJ Research), conditions for each reaction determined by the DNA template and oligonucleotide primers used. PCR products were purified using a GENECLEAN II Kit (BIO 101).

7.2.2 Construction of plasmids

S. pombe plasmids consist of: a bacterial origin of replication and selectable marker; a yeast selectable marker; and the equivalent of an autonomous replication sequence (ars), responsible for high frequency of transformation. Budding yeast markers used in *S. pombe* are the *LEU2* and *URA3* genes.

Plasmids containing these markers complement the *S. pombe* mutations *leu1*$^-$ and *ura4*$^-$.

7.2.3 Plasmid recovery

Cell cultures (1.4mls) were grown up with selection and the cells collected after microfugation. Added to the resuspended pellet was 0.2 ml of 2% Triton X-100/1% SDS/100mM NaCl/10mM Tris HCl (pH 8.0)/1mM Sodium EDTA. After addition of 0.2ml phenol:chloroform and 0.3 g acid washed glass beads the cells were vortexed for 2 minutes and then microfuged for 5 minutes. The upper aqueous layer was transferred and extracted with 200µl phenol:chloroform. The DNA was then precipitated and 1-5 µl used for transformations of competent *S. pombe* cells.

7.2.4 DNA sequencing

Sequencing was performed using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). DNA was purified using a 1.5ml Qiagen mini-prep column (Qiagen). A PCR reaction was set up containing 5µl DNA/1µl (25ng) primer/9µl Terminator Ready Reaction Mix containing Amplitaq DNA polymerase (Perkin Elmer). The PCR products were then ethanol precipitated and samples run on a 4.8% acrylamide gel and detected using an ABI Prism 377 DNA sequencer. Sequences were analysed using the Applied Biosystems Sequence Navigator Software and matched against the Sanger centre *S. pombe* genome database.

7.2.5 Western Blotting

Cells were boiled for 6 min after being washed once in STOP buffer. Protein extracts were prepared using glass beads in HB and a fastprep machine (Bio101) (Moreno *et al.* 1991). Protein concentration was determined using the BCA kit from Sigma. About 50µg was separated on 10% SDS-PAGE and blotted on nitrocellulose membrane (Amersham Hybond ECL) and detected by ECL (Amersham). The antibodies used were PAP (Sigma) at 1:1000, monoclonal anti-HA (Babco) at 1:1000 and polyclonal anti-Cdc18 antibodies (Nishitani and Nurse 1995) at 1:1000.

7.2.6 Preparation of genomic DNA for two-dimensional gel electrophoresis

Around $8x10^8$ cells were harvested by filtration and washed first with 50mls ice-cold nuclear isolation buffer (NIB) (50mM MOPS pH 7.2, 150mM KAC, 2mM $MgCl_2$) plus 0.1% sodium azide, and then with 50mls NIB. The cell pellets were frozen and stored at -80°C. Genomic DNA was extracted from the cells as described (Wu and Gilbert 1995). The pellets were re-suspended in 3mls NIB with 500µM spermidine and 150µM spermine. The solution was filled with acid–washed glass beads and cells broken by vortexing at 4°C for 15 minutes. Two mls of NIB were added to the extracted supernatant and centrifuged for 10 minutes at 6500g at 4°C. The pellet was re-suspended in 2mls G2 buffer pH 8.0 (800mM Guanidine-HCl, 30mM EDTA, 30mM Tris-HCl, 5% Triton X-100) with 200µg/ml RnaseA, and incubated for 30 minutes at 37°C. Proteinase K at 20mg/ml was then added. After a further 60 minutes of 37°C incubation, the

lysate was centrifuged at 6500g for 10 minutes at 4°C. An equal volume of Qiagen buffer QBT was added to the supernatant and the total volume put through a pre-equilibrated (with 1ml QBT) Qiagen genomic 20/G column. The column was washed 3 times with 1ml Qiagen buffer QC before eluting the DNA in 1.8mls of Qiagen buffer QF, pre-warmed to 50°C. DNA was precipitated with isopropanol, washed in 70% ethanol and re-suspended in TE, with a yield of 15-20µg. The DNA was then digested with 80 units of restriction enzymes (EcoR1 and Kpn1) in a 200µl volume at 37°C for 2.5 hours before ethanol precipitation.

7.2.7 2D gel conditions

Gels were run as described in (Friedman and Brewer 1995). The first dimension conditions were a 0.4% agarose gel in TBE, no ethidium bromide (EtBr), run for 24 hours in TBE at 1V/cm at room temperature. The gel was strained with EtBr to allow visualisation and excision of the lane from 1cm below the fragment of interest to around 7cm above. The excised lane was rotated through 90° and re-cast in a 1.1% agarose gel (with 0.3µg/ml EtBr in TBE) equilibrated at 55°C. The second dimension was then run at 4°C in TBE buffer plus 0.3µg/ml EtBr circulating at 50-100mls/min at 6V/cm to allow the arc of linears to migrate 8-10cm.

7.2.8 Southern blotting for 2D gels

The 2D gel was then washed at room temperature with gentle agitation for 30 minutes in 5 volumes of 0.25N HCl. This was repeated in denaturing solution

(0.5M NaOH, 1M NaCl) and finally in neutralising solution (0.5M Tris pH7.5, 3M NaCl). A Posiblot transfer apparatus (Stratagene) was used to transfer the DNA to a GeneScreen Plus membrane (NEN). A UV Stratalinker (Stratagene) crossed-linked the DNA to the membrane. Hybridisations were performed at 68°C using around $2x10^6$ cpm of a randomly primed probe per ml of QuickHyb hybridisation solution (Stratagene). Membranes were washed 3 times for 20 minutes with 0.1% SDS, 0.1XSSC at 63°C. The membranes were then exposed to BioMax film (Kodak), scanned and processed in Adobe Photoshop.

7.2.9 Probes for 2D gels

To detect non-origin DNA within the tandem rDNA repeats the digested genomic DNA was probed with a 3.4Kb fragment from the rDNA repeats. This was isolated by EcoR1 digest of a plasmid containing the rDNA repeat (YIP32-rDNA), followed by purification with a QIAquick Gel Extraction Kit (Qiagen). The probe was labeled with $\alpha(^{32}P)$dATP (Amersham) using the Stratagene Prime It II labelling kit, followed by removal of non-incorporated nucleotides with a ProbeQuant G-50 Micro Column (Amersham). The cpm/µl was quantified with a scintillation counter.

7.2.10 Preparation of agarose-embedded DNA for pulsed field gel electrophoresis

DNA was prepared as previously described (Ferreira and Cooper 2001). Cultures of cells were washed twice in SP1 buffer (1.2M sorbitol, 50mM citrate/phosphate pH5.6, 40mM EDTA), resuspended at a density of $0.5–1x10^8$

cells/ml, and then treated with SP1 buffer containing 0.6mg/ml Zymolyase-T100 (ICN) for 1 hour at 37°C. Cells were pelleted and gently resuspended in 100μl TSE buffer (0.9M sorbitol, 10mM Tris-HCl pH 7.5, 45mM EDTA) plus one volume of 1% LMP agarose (GIBCO–BRL) in TSE. The cell suspension was placed in 100μl plug moulds (Biorad) and allowed to solidify. Agarose plugs were first incubated for 90 minutes at 50°C in 1% SDS, 0.25M EDTA, 50mM Tris-HCl (pH 7.5) and then for 48 hours at 50°C in 1% lauryl sarcosine, 0.5M EDTA, 10 mM Tris-HCl (pH 9.5) containing 1mg/ml Proteinase K (GIBCO–BRL). Plugs were then washed in TE10X (10mM Tris-HCl pH 7.5, 10mM EDTA) at room temperature, and Proteinase K was inactivated by incubating with 0.04mg/ml PMSF in TE10X for 1 hour at 50°C. The plugs were finally washed twice for 30 minutes in TE10X at room temperature and stored at 4°C.

7.2.11 Digestion of agarose-embedded DNA

For Sfi1 restriction enzyme digest, plugs were pre-equilibrated for 30 minutes in NEB buffer 2 on ice (10mM NaCl, 5mM Tris-HCl pH 7.9, 1mM MgCl2, 0.1mM DTT, 100μg/ml BSA). Buffer was then replaced, and 100 units of Sfi1(NEB) added. The plugs were incubated overnight at 50°C and then washed twice for 30 minutes in TE10X on ice and stored at 4°C.

7.2.12 PFGE conditions

7.2.12.1 For whole chromosome analysis, or to visualise larger fragments after Sfi1 restriction enzyme digest

Before electrophoresis, plugs were equilibrated in 1X TAE running buffer for at least 1 hour. Plugs were loaded in a 0.8% pulsed-field certified grade agarose gel (Biorad) and electrophoresed at 14°C in a CHEF-DR III pulsed-field gel apparatus (Biorad) at 2V/cm for 72 hours, using 3 blocks. Parameters were: Block 1 - switch time 1,200 seconds, 96° included angle, run time 24 hours; block 2 - switch time 1,500 seconds, 100° included angle, run time 24 hours; block 3 - switch time 1,800 seconds, 106° included angle, run time 24 hours. The gel was then stained with EtBr and visualized using a Dual Intensity Transilluminator.

7.2.12.2 Sfi1 restriction enzyme digest analysis

The digested agarose plugs were pre-equilibrated in 0.5xTBE running buffer for at least an hour. Plugs were loaded onto a 1% pulsed-field certified grade agarose gel (Biorad) in 0.5xTBE and electrophoresed at 14°C in a CHEF-DR III pulsed-field gel apparatus (Biorad) at 6V/cm for 24 hours, using 1 block with a switch time of 60-120 seconds and a 120° angle. The gel was then stained with EtBr and visualized using a Dual Intensity Transilluminator.

7.2.13 Southern blotting of PFG

The gels were first washed in 5 volumes 0.25N HCl for 10 minutes. They were then denatured by washing in 0.5M NaOH/1M NaCl for 30 minutes, followed by 30 minutes in neutralising solution (NaCl, Tris-HCl pH7.5, 1mM EDTA). All

washes were at room temperature with gentle agitation. The DNA was transferred to a Duralon UV membrane by capillary action overnight on the bench. A UV Stratalinker (Stratagene) crossed-linked the DNA to the membrane. Hybridisation was performed at 65°C overnight with a randomly primed probe. Membranes were washed sequentially with: 1% SDS, 0.1XSSC at 25°C; 0.1% SDS, 0.1XSSC at 65°C; 0.01% SDS, 0.1XSSC at 65°C. Each wash was for 20 minutes. After a final brief wash in 1% SDS, 0.1XSSC at 25°C the membrane was exposed to BioMax film (Kodak), scanned and processed in Adobe Photoshop.

7.2.14 Probes for PFGE blots

Chromosome III was probed for using a 3.4Kb non-origin rDNA fragment and an 800kb fragment of the adenine 6 gene. The 3.4Kb non-origin rDNA probe was prepared as described in section 7.2.8. The 800bp adenine 6 probe was isolated from plasmid pIRT2 by digestion with EcoR1 and HindIII, followed by purification with a QIAquick Gel Extraction Kit (Qiagen). The probe was then labelled and purified as described in section 7.2.8.

7.3 Microscopy

7.3.1 Visualisation of nuclei by DAPI staining

Five µl of rehydrated 70% ethanol fixed cells were placed on a slide and heat or air dried before adding 5µl of DAPI (1µg/ml DAPI and 1mg/ml p-phenylenediamine antifade in 50% glycerol). Cells were then imaged on the

fluorescent microscope with a 360nm excitation/ 480nm emission filter, and photographs taken which were processed in Adobe Photoshop.

7.3.2 Visualisation of septa with calcoflor

3µl of live cells or rehydrated 70% ethanol fixed cells were air dried on a slide and 3µl of Calcoflor (10mg/ml stock) added. Cells were then imaged on the fluorescent microscope with a 360nm excitation/ 480nm emission filter, and photographs taken which were processed in Adobe Photoshop.

7.3.3 Phase and DIC imaging

3µl of cells were placed on a glass slide and a glass coverslip overlaid. Cells were viewed by DIC or phase microscopy using a Zeiss Axioplan microscope and photographs taken, which were scanned and then processed in Adobe Photoshop.

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