Exploring the Roles and Mechanisms of Cdc18 in the Cell Cycle of Fission Yeast

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Abstract

The replication factor Cdc18 (Cdc6 in other organisms) is required to initiate DNA replication and the S phase checkpoint, which restrains mitosis until replication is faithfully completed. In this thesis I have investigated these roles within the cell cycle of the single-celled eukaryote *Schizosaccharomyces pombe* (fission yeast), and in addition I have identified putative new roles for this replication factor.

The Cdc18 ATPase loads the MCM complex, a putative DNA helicase, onto chromatin in late mitosis and early G1 phases in preparation for DNA replication. I have shown that the association of Cdc18 with chromatin does not require ATP binding, whilst ATP binding is necessary to initiate an S phase checkpoint signal. ATP binding and hydrolysis are required for MCM loading activity and hence DNA replication. I also present evidence indicating that Cdc18 can restrain mitosis from G1 in the absence of DNA replication, perhaps as part of a complex with the replication factors Cdt1 and the ORC complex.

From my work it appears that Cdc18 is required during S phase for at least two further roles. Firstly, Cdc18 is needed to maintain the S phase checkpoint when DNA replication is affected by perturbations that are likely to involve stalled forks or DNA damage. Secondly, Cdc18 may be required in an unperturbed S phase to prepare cells for entry into or progression through mitosis. This latter role is unlikely to involve DNA metabolism, and may instead be related to chromosome preparation or dynamics.

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List of abbreviations

antibody (Ab)

autonomously replicating sequence (ARS)

base pairs (bp)

Cdk avtivating kinase (CAK)

cell division cycle (cdc)

cyclin dependent kinase (CDK)

deoxynucleoside triphosphate (dNTP)

DNA polymerase (DNA pol)

Drosophila melanogaster (*D. melanogaster*)

gap phase one (G1)

gap phase two (G2)

haemagluttinin antigen (HA)

hydroxyurea (HU)

kilobase (kb)

kilodalton (kD)

megabase (mb)

minichromosome maintenance protein (MCM)

mitosis phase (M phase)

nucleotide triphosphate (NTP)

open reading frame (ORF)

peroxidase-anti-peroxidase

Pyrobaculum aerophilum (*P. aerophilum*)

pre-initiation complex (pre-IC)

pre-replication complex (pre-RC)

proliferating cell nuclear antigen (PCNA)

replication factor C (RFC)

replication protein A (RPA)

ribonucleotide reductase (RNR)

Saccaromyces cerevisiae (*S. cerevisiae*)

Schizosaccharomyces pombe (*S. pombe*)

Sensor I/II (SI/II)

synthesis phase (S phase)

tandem affinity purification (TAP)

temperature sensitive (ts)

untranslated region (UTR)

Walker A (WA)

Walker B (WB)

Xenopus laevis (*X. laevis*)

Chapter 1. Introduction

1.1 The mitotic cell cycle

1.1.1 Overview

The eukaryotic mitotic cell cycle consists of an ordered sequence of events by which one cell gives rise to two identical daughter cells (cell duplication). The fundamental events underlying this process are the duplication and segregation of chromosomes followed by division of the cell, and these events are confined to specific phases of the cell cycle. DNA replication and duplication of chromosomes are restricted to the synthesis phase (S phase) and chromosome segregation occurs during the mitotic phase (M phase). These phases are often separated by two gap phases, named G1 and G2, which generally correspond to periods of cell growth. Models of how the cell cycle operates have been constructed from work carried out in a variety of experimental systems from yeasts to humans, but the fundamental concepts and mechanisms appear to be highly conserved. As the work presented in this thesis is entirely based on experiments carried out in the fission yeast *Schizosaccharomyces pombe*, I will focus on the fission yeast cell cycle, but refer to work in other organisms for comparative purposes.

Commitment to the mitotic cell cycle occurs in G1 at a point called 'Start' in yeasts or the 'Restriction Point' in metazoans. At this point cells assess their ability to properly complete a full mitotic cell cycle, based on a range of factors, from cell size to nutrient status. In higher eukaryotes the decision to pass the restriction point must also consider a host of external signals, including growth factors, attachment factors and developmental cues. The commitment to duplicate is an irreversible decision and cells have no option but to pass through S and M phases before returning to the next commitment point. Cells that are not prepared to commit to duplication can temporarily leave the cell cycle, usually from G1, and pass to a state known as 'quiescence' (or G0). The quiescence state is characterised by a general reduction in a number of active systems involved in cell duplication (Blow

and Hodgson, 2002), but cells in this state are still able to respond to a change in conditions or external cues that would enable them to re-enter the cell cycle. Other fates can await a cell, such as senescence in metazoans (an irreversible exit from the cell cycle) or sexual differentiation in fission yeast.

During S phase DNA replication occurs and cohesion is established between the newly synthesised sister chromatids. Cells then undergo a period of growth (G2) before mitosis. Here we see the formation of a bipolar spindle to which the kinetochores of each sister chromatid become attached. Loss of cohesion between sister chromatids then enables the segregation of each chromatid to opposite poles of the spindle (at the metaphase-anaphase transition). Upon exit from mitosis, the spindle breaks down and cells form a septum which splits the cell in the middle (the process of cytokinesis) giving rise to two new cells with an identical genotype. The length of the G1 and G2 phases can vary greatly between cell types. In *Xenopus laevis,* early fertilised embryos undergo a series of rapid cleavage divisions, and have a modified cell cycle which consists merely of alternating rounds of interphase consisting only of S and M phases (Newport et al., 1985). After the midblastula transition, the G1 and G2 phases appear and the mitotic cell cycle resembles the cycle described above. In the budding yeast *Saccharomyces cerevisiae*, a size control operates in G1 and represses the onset of S phase until cells reach a critical size. Once cells pass Start, they can then initiate mitotic events, such as spindle formation and budding, in parallel to DNA replication. Therefore cells spend only a small, if any, part of the cell cycle in G2. Also, budding yeast cells divide by a mechanism that allows the daughter cell to bud from the mother cell, instead of the symmetrical division that occurs in fission yeast and many mammalian cells (Forsburg and Nurse, 1991a).

The requirement to transmit genetic information accurately from parent to daughter cells raises two fundamental problems for the mitotic cell cycle. Firstly, there is the completion problem. This refers to the need to complete one process before the next process is initiated. The solution to this is the use of checkpoint systems, which restrain one process until an earlier process is

complete (Hartwell and Weinert, 1989). An example of this is the DNA replication checkpoint, which restrains mitosis whilst DNA replication is incomplete. Without such a system a premature mitosis would leave cells with an incomplete chromosome complement. Secondly, there is the alternation problem. This refers to the need to ensure that S phase is followed only by mitosis, and that mitosis is only followed by S phase (Broek et al., 1991). In principle this need is met by ensuring that the completion of one process leads to a loss of competency for that process. This competency is only restored following completion of the alternative process. An example of this is the removal of replication competence as cells progress through S phase. This competence can only be regained as cells exist the subsequent mitosis. Without such controls, cells risk rereplication of DNA and polyploidy. In later sections the two examples used here will be discussed further.

1.1.2 The fission yeast life cycle

Fission yeast cells spend most of the mitotic cell cycle (approximately 70%) in G2 and they approximately double their mass before reaching the critical size required for entry into mitosis (figure 1.1). Fission yeast cells have a very short G1 period and cytokinesis occurs at the same time as the S phase of the following cell cycle. This means that a FACS profile (which plots DNA content against frequency) of a cycling population of cells displays only a 2C DNA peak (DNA replicated). DNA replication can be inhibited using the drug hydroxyurea (HU) which inhibits the enzyme ribonucleotide reductase (RNR) and results in the depletion of free nucleotides available for chain elongation. When replication is inhibited, cytokinesis occurs without chromosome duplication and a 1C DNA peak is produced.

The fission yeast cell is rod shaped (with constant width) and grows bidirectionally from its tips. Inhibition of the cell cycle can occur without inhibition of growth, and in such cdc (cell division cycle) mutants cells become highly elongated (Nurse et al., 1976). This phenomenon can also be seen by a continued rise in population mass with a plateau in cell number. Due to the characterised distribution of cells in the cell cycle, described above, this

cell number plateau can be used to position the block-point of the cell cycle inhibition. If cells become blocked at the G2/M transition (e.g. using a *cdc25-22* temperature sensitive (ts) mutant) then approximately 70% of the population in G2 will not commit to mitosis and cytokinesis. With only M/G1/S phase cells continuing with mitosis and cell division, overall cell number increases in such mutants by approximately 30%. If cells become blocked in the cell cycle at G1/S (e.g. using a *cdc10-v50* ts mutant), then most of the population will divide before reaching the block-point, leading to an approximate cell number doubling.

Pre-start fission yeast cells that are starved of nitrogen sources will exit the cell cycle into quiescence. Haploid cells in this position can conjugate with haploids of opposite mating type to form a diploid zygote (a process called shmooing). In continued starvation conditions, this zygote will undergo pre-meiotic S phase, two meiotic nuclear divisions and sporulation to produce four haploid spores in a zygotic ascus. The diploid zygote can be maintained as a diploid cell if grown on rich medium prior to meiosis. Diploid cells in these circumstances enter a mitotic cell cycle similar to that described for the haploid. Both haploid cells, with one copy of each chromosome (1N), and diploid cells, with two copies of each chromosome (2N), cycle between 1C (DNA unreplicated) and 2C (DNA replicated) states.

1.2 Control of the mitotic cycle-Cyclin Dependent Kinases

The complementary findings of two different approaches, a genetic one and a biochemical one, led to the discovery of the master regulator of the eukaryotic cell cycle: the cyclin-dependent kinase (CDK). Screens for cells that continued to increase their cell size in the absence of cell division (*cdc* mutants) were carried out in both budding yeast (Hartwell et al., 1974) and fission yeast (Nasmyth and Nurse, 1981; Nurse et al., 1976) and led to the isolation of many conditional, temperature-sensitive mutants. The most important mutants were those of *cdc2*, which displayed defects in both DNA replication and nuclear division (Nurse and Bissett, 1981; Nurse and Thuriaux, 1980). A

similar phenotype was observed in mutants of the *CDC28* gene in budding yeast (Hartwell et al., 1974), and complementation analysis showed that ectopic expression of the *CDC28* gene in fission yeast could complement the ts cell cycle arrest of the *cdc2-33* mutant (Beach et al., 1982). Later, a human homolog was cloned by its ability to complement the same ts mutant (Lee and Nurse, 1987). Hence these experiments identified a conserved role for the *cdc2* gene in the eukaryotic cell cycle, and an early indication of gene function came when Cdc2 was shown to be a 34kD phosphoprotein with protein kinase activity against casein (Simanis and Nurse, 1986) and histone H1 (Moreno et al., 1989).

The discovery of Cdc2 via biochemical means began with the identification of a maturation promoting factor (MPF) in the frog *Rana pipiens*. MPF was defined as the cytoplasmic factor from mature oocytes capable of inducing the first meiotic division in G2-arrested immature oocytes (Masui and Markert, 1971; Smith and Ecker, 1971). MPF was also shown to be a mitotic inducer in somatic cells (Kishimoto et al., 1982; Sunkara et al., 1979). MPF was subsequently purified by its ability to induce maturation in *Xenopus* oocytes in the absence of protein synthesis (Lohka et al., 1988). The purified activity consisted of two proteins (45kD and 32kD) which also co-fractionated with histone H1 kinase activity. Given the analogous roles for MPF and Cdc2 for mitotic induction, and their common H1 kinase activity, antibodies against the conserved PSTAIR motif in fission yeast Cdc2 were tested against purified MPF and found to cross-react with the 32kD protein (Dunphy et al., 1988; Gautier et al., 1988). This protein was thus identified as the *Xenopus* homologue of Cdc2, and a homolog of Cdc2 was identified in starfish by a similar analysis (Labbe et al., 1988). Together these findings identified Cdc2/Cdc28 as a component of MPF and established Cdc2 as the key regulator of the onset of mitosis across eukaryotes.

The second protein of MPF was shown to migrate with the same mobility as starfish cyclin (Labbe et al., 1989). Cyclins were first identified in sea urchin eggs as highly unstable proteins that appeared following fertilisation and then

oscillated with each subsequent cleavage division due to periodic proteolysis (Evans et al., 1983). Sequencing and immunoprecipitation experiments confirmed that the Cdc2 partner was a cyclin, and suggested that cyclin proteolysis inactivated MPF (Draetta et al., 1989; Labbe et al., 1989). Cdc2 and its homologs were subsequently known as cyclin dependent kinases (CDKs).

Higher eukaryotes possess several different CDKs, each required for specific processes within the cell cycle. For the remainder of this section, however, I shall concentrate on the fission yeast Cdc2 kinase and its regulation. The regulation of Cdc2, in common with other CDKs, is based upon three mechanisms: association with cyclins, phosphorylation status and inhibiton by CDK inhibitors. A summary of these mechanisms, based on the ensuing discussion, is given in figure 1.2.

1.2.1 Cyclins

The first cyclin to be identified in fission yeast was Cdc13. Overexpression of Cdc2 suppressed the *cdc13-117* ts mutant (Booher and Beach, 1987), and *cdc13* was cloned by its ability to rescue the same ts mutant (Booher and Beach, 1988; Hagan et al., 1988). Deletion of *cdc13* prevents entry into mitosis, indicating that both Cdc2 and Cdc13 are required for mitosis (Booher and Beach, 1988; Hagan et al., 1988). These proteins were shown to form a stable complex and that Cdc13 association was required for mitotic kinase activity (Booher et al., 1989; Moreno et al., 1989). Cdc13 levels fluctuate throughout the cell cycle, peaking at mitotic entry, at the same time as the peak of Cdc2 mitotic kinase activity (Booher et al., 1989; Moreno et al., 1989). At the metaphase to anaphase transition Cdc13 is ubiquitinated and targeted for proteolysis by the 26S proteasome (Yamano et al., 1998). This mechanism is dependent upon recognition of a destruction box in the N-terminus of Cdc13 by the anaphase promoting complex (APC). Removal of this destruction box leads to stabilisation of Cdc13 and anaphase arrest (Yamano et al., 1996).

The fact that Cdc13 is only required for mitotic entry indicated that another cyclin (or cyclins) could act as the Cdc2 co-factor for DNA replication. Subsequently, two further cyclins were cloned: *cig1* and *cig2*. The former was cloned by a homology-based screen (Bueno et al., 1991) whilst the latter was cloned by three groups via three different approaches (Bueno and Russell, 1993; Connolly and Beach, 1994; Obara-Ishihara and Okayama, 1994). The Cig1 associated kinase activity is activated at mitosis with similar kinetics to the Cdc2/Cdc13 kinase, but a clear role has yet to be established for the Cdc2/Cig1 kinase (Basi and Draetta, 1995). The levels of Cig2 transcript, protein and kinase activity all peak at the onset of DNA replication, indicating that Cig2 may play a central role in initiating S phase in partnership with Cdc2 (Connolly and Beach, 1994; Mondesert et al., 1996). Cells deleted for *cig2* do display a delay over S phase entry (Bueno and Russell, 1993; Mondesert et al., 1996) but are nonetheless viable. Both Cdc13 and Cig1 are able to substitute for Cig2 (Fisher and Nurse, 1996; Mondesert et al., 1996) suggesting functional redundancy amongst these cyclins. This has led to the development of the 'quantitative model' (Fisher and Nurse, 1996) in which the level of kinase activity, and not the cyclin partner, regulates cell cycle progression. This model is discussed further in section 1.2.4.

Two other cyclins have been identified in fission yeast although less is known about their function. The Puc1 cyclin plays a role in cell cycle exit by inhibiting G1 arrest in response to nitrogen starvation, possibly by downregulating Rum1, an inhibitor of Cdc2 (Forsburg and Nurse, 1994; Forsburg and Nurse, 1991b; Martin-Castellanos et al., 1996). The Rem1 cyclin is only expressed in meiosis and is required for pre-meiotic S phase in the absence of Cig2 (Malapeira et al., 2005).

1.2.2 Phosphorylation of CDKs

Cdc2 is phosphorylated at two sites, tyrosine-15 (Y15) and threonine-167 (T167). A CDK activating kinase (CAK) is required for T167 phosphorylation and Cdc2 activation, possibly working via stabilisation of the interaction

between Cdc2 and cyclins (Gould et al., 1991; Ross et al., 2000). Two CAKs, Csk1 and Mcs6, have so far been identified (Lee et al., 1999).

The Y15 residue is phosphorylated during interphase and dephosphorylation is required for entry into mitosis (Gould and Nurse, 1989). The peak of dephosphorylation at Y15 correlates with the peak of Cdc2 kinase activity (Moreno et al., 1989). Tyrosine-15 is located within the ATP binding domain of Cdc2 and its phosphorylation is likely to directly inhibit Cdc2 kinase activity.

The phosphorylation state of Y15, and hence the activity of Cdc2, is determined by the balance between the activity of inhibitory kinases and activating phosphatases. Cdc2 is phosphorylated at Y15 in cells blocked at G2/M in a *cdc25-22* arrest, but is then rapidly dephosphorylated when *cdc25* function is restored and cells enter mitosis (Gould and Nurse, 1989). Activation of the kinase is dependent on the *cdc25* gene, and Cdc25 protein levels peak at the G2/M boundary, implicating Cdc25 as the phosphatase that activates Cdc2 through dephosphorylation on tyrosine 15 (Moreno et al., 1989; Moreno et al., 1990). A human tyrosine phosphatase was shown to dephosphorylate Y15 and trigger mitosis in fission yeast (Gould et al., 1990). This phosphatase could also substitute for Cdc25 function, linking Y15 dephosphorylation to Cdc25 activity. Purified human Cdc25 was used to dephosphorylate and activate a Cdc2-CyclinB complex from starfish oocytes arrested in G2 (Strausfeld et al., 1991), whilst Cdc25 from *Drosophila melanogaster* was shown to activate *Xenopus* MPF via dephosphorylation of Cdc2 (Kumagai and Dunphy, 1991). Together, these results support a conserved role for Cdc25 as an activator of the Cdc2 kinase by dephosphorylating the inhibitory tyrosine residue.

The main kinase responsible for Y15 phosphorylation is Wee1. The Wee1 kinase is a dual specificity kinase which targets both serine and tyrosine residues (Featherstone and Russell, 1991; Parker et al., 1992). Wee1 phosphorylates Y15 of Cdc2 when complexed with a cyclin, and this

phosphorylation inhibits the histone H1 kinase activity of Cdc2 (Parker et al., 1992). Another kinase, Mik1, has also been shown to phosphorylate Cdc2 at Y15 (Lee et al., 1994), but a null mutant does not affect cell cycle progression unless Wee1 function is also compromised (Lundgren et al., 1991).
The Cdc25 and Wee1 proteins are themselves subject to regulation. For example, the localisation of Cdc25 varies during the cell cycle; Cdc25 is retained in the cytoplasm during G1 and S phase by a 14-3-3 protein, and is then released and imported into the nucleus during G2 (Zeng and Piwnica-Worms, 1999). The Wee1 kinase is negatively regulated by the Nim1/Cdr1 and Cdr2 proteins (Breeding et al., 1998; Kanoh and Russell, 1998; Parker et al., 1993; Russell and Nurse, 1987) and Nim1/Cdr1 is itself negatively regulated by Nif1 (Wu and Russell, 1997). Regulation of Cdc25, Wee1 and Mik1 by the DNA replication and damage checkpoints will be described in section 1.5.

1.2.3 CDK inhibitors

The activity of CDKs is also regulated by a class of proteins known as CDK inhibitors (CKIs). In mammalian cells, a vast array of CKIs control the activities of various CDK/cyclin complexes, but these will not be addressed here. The best understood CKI in fission yeast is the Rum1 protein. Rum1 is required for G1 arrest in low nitrogen conditions or in the absence of Cdc10 function (Moreno et al., 1994; Moreno and Nurse, 1994). The *cdc10-129 rum1Δ* double mutant enters a fatal mitosis at restrictive temperature, suggesting that Rum1 is required to restrain mitosis from G1. This checkpoint role is likely to operate pre-Start, before the inhibitory Y15 phosphorylation of Cdc2 by Wee1 (Hayles and Nurse, 1995). Rum1 specifically inhibits Cdc13/Cdc2 kinase activity through direct interaction with the complex, and it also promotes the proteolysis of Cdc13 in early G1-arrested cells (Correa-Bordes and Nurse, 1995; Correa-Bordes and Nurse, 1997; Martin-Castellanos et al., 1996; Moreno and Nurse, 1994).

Rum1 protein levels are sharply periodic, peaking at early G1 (Benito et al., 1998), and its mRNA is stabilised in response to nitrogen starvation (Daga et

al., 2003). During S phase and G2, Rum1 is phosphorylated by Cdc2, and this targets the protein for ubiquitin-mediated proteolysis (Benito et al., 1998; Jallepalli et al., 1998; Kominami and Toda, 1997). It is not clear which of the Cdc2/Cig1 (Benito et al., 1998; Correa-Bordes and Nurse, 1997) or Cdc2/Puc1 (Martin-Castellanos et al., 2000) complexes are required for Rum1 phosphorylation. Sic1 plays an analogous role in maintaining a low level of Cdc28/Cyclin B kinase activity in G1 in budding yeast (Schwob et al., 1994). Sic1 and Rum1 functionally substitute for each other, demonstrating functional homology (Sanchez-Diaz et al., 1998). Sic1 too is targeted for proteolysis following CDK phosphorylation (Feldman et al., 1997; Schwob et al., 1994; Skowyra et al., 1997; Verma et al., 1997a; Verma et al., 1997b).

The other CKI identified in fission yeast is the Suc1 protein. Although little is known about its specific function, it inhibits Cdc13/Cdc2 kinase activity and is required as an additional mechanism to promote mitotic exit (Basi and Draetta, 1995). Cells depleted of Suc1 accumulate Cdc13 and Cdc13/Cdc2 kinase activity causing cells to arrest with condensed chromosomes. Suc1 has also been shown to inhibit MPF activity in *Xenopus* egg extracts (Dunphy et al., 1988).

1.2.4 The Cdc2 kinase cycle

In fission yeast, the cell cycle is controlled by a single CDK that becomes complexed to different cyclin partners during the cell cycle. The partial redundant nature of the cyclins, as introduced in 1.2.1, led to a consideration of how Cdc2 can promote alternating rounds of DNA replication and mitosis (Fisher and Nurse, 1996). Cdc2/Cdc13 (in the absence of Cig1 and Cig2) can promote ordered progression through the entire cell cycle, initiating both DNA replication and mitosis. In contrast, Cig2, and only partially Cig1, are capable of promoting DNA replication in the absence of Cdc13 but neither can promote mitosis. This suggests that the requirement for CDK activity to promote DNA replication is less specialised than that required for the onset of mitosis. These results led to the quantitative model (Fisher and Nurse, 1996; Stern and Nurse, 1996), in which the fission yeast cell cycle is driven by a

single oscillation in Cdc2 kinase activity (figure 1.3). The model predicts that a low level of kinase activity is required to prepare for DNA replication, and that an increase to a moderate level of kinase activity promotes the initiation of DNA replication. A further increase in kinase activity is then required to bring about mitosis, and the moderate and high levels of kinase activity are sufficient to prevent a further round of replication. Cyclin proteolysis upon exit from mitosis then re-sets the cycle back to low kinase activity in G1.

Validation of this model is supported by studies disrupting the alternation of S and M phases. Depletion of Cdc13 in G2 induces fission yeast cells to undergo multiple, discrete rounds of DNA replication without intervening mitoses (Hayles et al., 1994). In the absence of Cdc2/Cdc13 in G2, cells fail to accumulate enough kinase activity to undergo mitosis and instead re-set the cell cycle back to G1 and promote another round of S phase. This re-replication is dependent on the presence of Cig1 or Cig2 to support DNA replication. Further, overexpression of Rum1 also induces extensive re-replication by directly inhibiting Cdc2/Cdc13 kinase activity (Moreno and Nurse, 1994), and this rereplication also requires either Cig1 or Cig2 to initiate replication (Martin-Castellanos et al., 1996). Conversely if Cdc13 and Cdc2 are overexpressed in G1 cells prior to Start, the increase in mitotic kinase activity will induce mitosis in the absence of DNA replication (Hayles et al., 1994). The mechanisms by which moderate to high Cdc2 kinase activity inhibits DNA replication are addressed in section 1.4.

1.3 Initiation of DNA replication

In this section I shall describe the factors and processes required to prepare for and initiate DNA replication in fission yeast (figure 1.5). During the period of low Cdc2 kinase activity (late mitosis and early G1) cells prepare for replication via formation of pre-replication (pre-RC) complexes at origins of replication. Cdc2 and Hsk1 kinase activity is then required for the recruitment of further replication factors to the pre-RC and to initiate DNA replication. The principles of this system are highly conserved in eukaryotes.

The concept of the pre-RC stemmed from studies analysing nuclease digestion of the *ARS1* origin of replication in budding yeast. Nuclease protection was seen throughout the cell cycle, provided by the origin recognition complex (ORC), but an extended footprint in G1 disappeared as cells progressed to G2 (Bell and Stillman, 1992; Diffley and Cocker, 1992; Diffley et al., 1994). Extensive work since has shown that the additional factors loaded onto origins in G1 are the Cdc6 (Cdc18 in fission yeast) and Cdt1 proteins, and a complex of mini-chromosome maintenance (MCM) proteins. Disruption of this complex upon initiation of replication, and inhibition of pre-RC formation by CDK activity, prevents another round of replication until cells pass through mitosis.

Preparation for and progression through S phase in fission yeast is dependent upon *cdc10*. A complex of Cdc10, Res1, Res2 and Rep2 (sometimes known as MBF) acts as a transcriptional activator of several factors required for DNA replication (Aves et al., 1985; Ayte et al., 1995; Caligiuri and Beach, 1993; Lowndes et al., 1992; Miyamoto et al., 1994; Nakashima et al., 1995), such as the pre-RC components Cdc18 and Cdt1 (Hofmann and Beach, 1994; Kelly et al., 1993), and Cdc22, the large subunit of RNR (Lowndes et al., 1992; Maqbool et al., 2003). Cdc10 is also required for the expression of Cig2, the putative S phase cyclin, and in turn active Cdc2/Cig2 is a repressor of Cdc10 function (Ayte et al., 2001; Obara-Ishihara and Okayama, 1994). It is likely that Cdc2/Cig2 inhibition by Rum1 association and Y15 phosphorylation allows pre-RC formation in G1. The appearance of Cdc2/Cig2 kinase activity would then trigger S phase and repress Cdc10-mediated transcription of S phase factors.

1.3.1 Origins of replication

The sites at which DNA replication is initiated are referred to as origins of replication initiation (ORI). These origins have traditionally been identified by their ability to confer stability to plasmids in a transformation assay (autonomously replicating sequences or ARSs) and 2D gel electrophoresis demonstrated that initiation of replication occurred from within these

sequences. The replication origins of budding yeast have been well characterised (Newlon and Theis, 1993; Theis and Newlon, 1997; Theis and Newlon, 2001). The ARS elements in budding yeast are approximately 100-150bp in size and share several common features. They consist of four elements that are sufficient for ARS activity: an A element, containing an 11bp AT-rich consensus sequence (ACS), and three adjacent, partially redundant B elements. Approximately 400 budding yeast origins have been mapped on a genomic scale, using chromatin immunoprecipitation (ChIP) of the ORC and MCM components of the pre-RC (Wyrick et al., 2001) and by detecting sites of early DNA duplication on oligonucleotide microarrays (Raghuraman et al., 2001).

A number of fission yeast ORIs have been isolated and characterised (Clyne and Kelly, 1995; Dubey et al., 1996; Dubey et al., 1994; Maundrell et al., 1988; Okuno et al., 1997; Okuno et al., 1999; Zhu et al., 1994). From these findings it is clear that fission yeast origins are larger and less well conserved than their budding yeast counterparts. They are typically 0.5-1kb in length and do not have recognisable consensus elements. They do, however, possess high AT content and often contain stretches of asymmetric A or T residues. A unique feature of the fission yeast ORC complex is its binding to origins via an N-terminal AT-hook binding domain of Orc4 (Chuang and Kelly, 1999; Kong and DePamphilis, 2001; Lee et al., 2001). This domain recognizes the structure of AT-rich stretches through the minor groove of DNA without the requirement of a specific nucleotide sequence. It has been suggested that several ORC binding sites are collectively required for efficient origin firing (Kong and DePamphilis, 2002; Takahashi et al., 2003). Cooperation between ORC complexes to attain the critical concentration to trigger replication would require a minimal length of AT-rich DNA, which is consistent with the 0.5–1kb length of fission yeast origins.

More recently, two different approaches have been taken to map and characterize fission yeast origins on a genomic scale. In the first instance the genome was scanned for 0.5kb-1kb stretches with high AT content (75% for

0.5kb to 72% for 1kb) (Segurado et al., 2003). This identified 385 ORIs at an average spacing of 33kb. Twenty of these ORIs were selected at random and subjected to 2D electrophoresis, and 18 displayed replication intermediates, indicative of origin activity. This collection of origins also failed to display true consensus sequences. Interestingly, similar to the case in budding yeast, the frequency of origins at the mating type locus, centromeres and subtelomeric regions was significantly higher than the average frequency across the genome. This suggests that origins or/and some component(s) of the pre-RC may be important for processes other than DNA replication (such as regulation of chromatin structure). Another approach to mapping origins in fission yeast has been to locate areas of early DNA duplication using DNA microarrays (Christian Heichinger, unpublished data). This method has isolated approximately 375 origins with considerable overlap with those identified by the bioinformatic approach. It has also revealed a range in the efficiency of firing within the origins, with an average efficiency of 29%. Efficient origins identified by this method show a high AT content (72% or more) and at least two groups of five or more asymmetric AT hook binding motifs (Maher and Nathans, 1996). This analysis suggests, however, that other factors, such as local chromatin structure, also influence origin selection and efficiency. In both approaches all origins are located in intergenic regions. A recent paper demonstrates that the features shared by characterized origins of replication, namely AT-richness and asymmetric strand composition, were common to many intergenic regions, and that at least half of intergenic regions have potential origin activity (Dai et al., 2005). Thus fission yeast may make use of a relatively promiscuous DNA binding motif to direct binding of ORC to common features in the genome. Consequently, origin selection may be a rather stochastic phenomenon and it is possible that origin selectivity may be in part governed by epigenetic phenomena such as the state of chromatin in these intergenic regions. Perhaps the ability of these AT-rich intergenic regions to function as origins correlates with the status of promoters of the encompassing genes.

The origins identified in higher eukaryotes are even larger and more diverse in nature than those of fission yeast (DePamphilis, 1999), and will not be addressed in detail here. In brief, metazoan ORIs are similar to those of fission yeast in their lack of consensus sequences and in their preference to localize to intergenic regions close to promoters. A striking difference, however, is that many replication origins in mammals are associated with GC-rich islands (Delgado et al., 1998).

1.3.2 The ORC complex

The identification of ARS elements that function as replication origins in budding yeast led to the discovery of the proteins that bind directly to origin sequences, and these Orc proteins (Orc1-6) defined the origin recognition complex (ORC) (Bell and Stillman, 1992; Diffley and Cocker, 1992). Mutation or deletion of any subunit of the ORC complex prevents DNA replication and is lethal (Bell et al., 1993; Bell et al., 1995; Foss et al., 1993; Fox et al., 1995; Hardy, 1996; Hori et al., 1996; Li and Herskowitz, 1993; Liang et al., 1995; Loo et al., 1995; Micklem et al., 1993). The ORC complex remains associated with origins throughout the cell cycle (Diffley et al., 1994; Liang and Stillman, 1997; Rowley et al., 1995) and serves to recruit Cdc6 (and possibly Cdt1) to replication origins in preparation for S phase (Liang et al., 1995; Piatti et al., 1995; Tanaka et al., 1997; Weinreich et al., 1999).

A homologous ORC complex has been identified in fission yeast and consists of six Orp proteins (ORC subunits from *S. pombe*) which are all essential for DNA replication and to restrain mitosis in the absence of DNA replication (Chuang and Kelly, 1999; Gavin et al., 1995; Grallert and Nurse, 1996; Ishiai et al., 1997; Leatherwood et al., 1996; Lygerou and Nurse, 1999; Moon et al., 1999; Muzi-Falconi and Kelly, 1995). Orp4 ties the ORC complex to DNA via its N-terminus which features 9 copies of the AT-hook motif (Chuang and Kelly, 1999; Kong and DePamphilis, 2001; Lee et al., 2001; Moon et al., 1999). It is likely that the ORC complex remains bound to origins throughout the cell cycle, as Orp1 and Orp5 chromatin levels remain constant in a synchronised cell cycle (Lygerou and Nurse, 1999), whilst Orp1 binding to the

ars2004 and ars3002 origins is unaltered throughout the cell cycle (Ogawa et al., 1999). Both Orp1 and Orp2 physically interact with Cdc18 and ORC is likely to recruit Cdc18 (and possibly Cdt1) to replication origins (Chuang et al., 2002; Grallert and Nurse, 1996; Kearsey et al., 2000; Leatherwood et al., 1996; Nishitani et al., 2000).

Homologs of the Orc1-6 proteins have been identified in higher eukaryotes, including *Xenopus*, *Drosophila* and mammals, and these proteins too are essential for DNA replication (reviewed in Bell and Dutta, 2002). The ORC complex has been shown to recruit Cdc6 and Cdt1 in *Xenopus* (Coleman et al., 1996; Maiorano et al., 2000; Romanowski et al., 1996; Rowles et al., 1996) and Cdc6 in human cells (Saha et al., 1998a). A number of studies into the stability and chromatin association of Orc subunits in mammalian cells have provided conflicting views of their regulation. What is clear is that Orc1 is regulated through the cell cycle, possibly as a mechanism to prevent rereplication (see section 1.4). In hamster cells Orc1 is released from chromatin as cells enter S phase and only rebinds upon exit from mitosis, whilst Orc2 remains chromatin associated throughout the cell cycle (Natale et al., 2000). Removal of Orc1 from the chromatin and prevention of re-binding in G2/M appears to depend upon CDK-mediated phosphorylation and ubiquitination, although Orc1 is not degraded here (Li and DePamphilis, 2002; Li et al., 2004). In human cells Orc1 is phosphorylated as cells enter S phase and this targets the protein for polyubiquitination and degradation by the proteasome (Mendez et al., 2002; Tatsumi et al., 2003; Tatsumi et al., 2000). Orc1 is then expressed and targeted to chromatin as cells exit from mitosis. RNAi of Orc1 suggests that Orc1 tethers other Orc subunits to the DNA, and that removal of ORC from the chromatin prevents MCM association with origins (Ohta et al., 2003).

1.3.3 Cdc18

Cdc18 was cloned simultaneously by two independent means, as a suppressor of the temperature sensitive (ts) mutants *cdc10-129* and *cdc18-K46* (Kelly et al., 1993). It was later shown that *cdc18* transcription is dependent upon

Cdc10, and hence the transcript appears in late mitosis and disappears during S phase (Baum et al., 1998). The protein itself is restricted to the G1 and S phases of the cell. Phosphorylation at six sites by Cdc2 in mitosis and during S/G2 targets the protein for ubiquitination and proteolysis mediated by the proteasome (Jallepalli et al., 1997; Jallepalli et al., 1998; Kominami et al., 1998; Kominami and Toda, 1997; Wolf et al., 1999). Cdc18 is a highly unstable protein, and its half life in a cycling population has been estimated at approximately 5 minutes (Muzi Falconi et al., 1996).

Cdc18 is an essential protein, and its deletion leads to an inability to initiate DNA replication (Kelly et al., 1993). In addition, cells lacking Cdc18 enter a lethal mitosis in the absence of replication, suggesting that Cdc18 is required for both DNA replication and for coordinating the completion of S phase with the onset of mitosis. Further studies have demonstrated that the replication function of Cdc18 requires its ability to load the MCM complex onto chromatin prior to initiation (Kearsey et al., 2000; Nishitani et al., 2000). This key role for Cdc18 is highlighted by the induction of rereplication of the genome upon overexpression of the wild-type protein (Nishitani and Nurse, 1995). This overexpression is effective from G2 and is potentiated by the co-overexpression of Cdt1 (Yanow et al., 2001).

The Cdc18/Cdc6 protein is a member of the large family of ATPases known as ATPases associated with various cellular activities (AAA), and shares significant homology with certain ORC subunits, especially Orc1 (Neuwald et al., 1999). Archaea do not have any obvious homologs of the ORC complex, but do possess Cdc6-like proteins, raising the possibility that archaeal Cdc6 may play a dual role in both origin recognition and MCM recruitment (Liu et al., 2000). Cdc18/Cdc6 also shows significant homology with clamp loading proteins such as replication factor C (RFC). This has led to the suggestion that these enzymes catalyse analogous reactions; the ATP-dependent loading of ring-shaped molecules onto DNA (Perkins and Diffley, 1998). The primary sequence of Cdc18 is shown in figure 1.4. Highlighted are the six Cdc2 phosphorylation sites, five of which are located in the N-terminus, along with

the Walker A (WA), Walker B (WB), Sensor I (SI) and Sensor II (SII) motifs that are typical of AAA ATPases. These motifs are addressed in further detail in chapter 3. The structure of a Cdc6-like protein from the archaeon *Pyrobaculum aerophilum* has been solved by crystallography to 2Å resolution (Liu et al., 2000). Two domains of Cdc6 generate a two-lobed, cashew-shaped molecule. Between these domains the protein binds Mg.ATP using the Walker and Sensor motifs.

Cdc6 is required for DNA replication in a range of other systems (Coleman et al., 1996; Piatti et al., 1995; Yan et al., 1998). ChIP studies in budding yeast have shown that Cdc6 is required to recruit the MCM complex to replication origins in G1 (Aparicio et al., 1997; Tanaka et al., 1997). In *X. laevis*, Cdc6 is required for the association of MCM proteins with chromatin (Coleman et al., 1996). As with fission yeast, the depletion of Cdc6 in budding yeast leads to a lack of replication and aberrant entry into mitosis (Piatti et al., 1995).

1.3.4 Cdt1

Cdt1 was originally identified in fission yeast as a protein induced by the Cdc10 transcription factor (Hofmann and Beach, 1994). Cdt1 is an essential protein, required for DNA replication and the inhibition of mitosis in the absence of replication. As with Cdc18, Cdt1 is present and chromatin-bound in G1/S and is required for the loading of the MCM complex onto chromatin (Nishitani et al., 2000). Cdt1 and Cdc18 are recruited to chromatin independently, but these proteins physically interact via the C-terminus of Cdc18 (Nishitani et al., 2000). Although Cdt1, like Cdc18, is degraded by the proteasome, this degradation does not appear to be regulated by Cdc2 (Damien Hermand, unpublished data).

A Cdt1 homolog has been identified in budding yeast which interacts with the MCM complex (Tanaka and Diffley, 2002). The nuclear accumulation of Cdt1 and the MCM complex is interdependent, and is restricted to G1 (dependent upon CDK inactivity). The Cdt1 homolog in *D. melanogaster* (named DUP) is required for DNA replication in embryos and for the endoreduplication and

amplification of chorion genes that take place in follicle cells during *Drosophila* oogenesis (Whittaker et al., 2000). In addition, DUP is required for the checkpoint preventing mitosis until completion of S phase, as DUP mutants enter mitosis with unreplicated DNA. The DUP protein colocalizes with Orc1 and Orc2, suggesting that it associates with replication origins (Austin et al., 1999).

Extensive work has been carried out with the *Xenopus* and human Cdt1 proteins. Cdt1 is required to load the MCM complex onto chromatin and for DNA replication in *Xenopus* egg extracts (Maiorano et al., 2000). Cdt1 chromatin association is dependent upon the ORC complex but not upon Cdc6 association. A recent study suggests, however, that a strict sequential assembly of Cdc6 and then Cdt1 onto chromatin is required for replication competency (Tsuyama et al., 2005). Cdt1 is degraded in S phase via ubiquitin mediated proteolysis (Arias and Walter, 2005).

The human homolog of Cdt1 has been identified and cloned, and this protein is restricted to G1 (Nishitani et al., 2001; Wohlschlegel et al., 2000). Cdt1 is essential for the loading of MCM proteins onto chromatin and for DNA replication (Rialland et al., 2002). Whilst it is clear that Cdt1 is removed by the proteasome in S phase (Nishitani et al., 2004), the means by which this occurs remains controversial. One likely mechanism is Cdk2/4-mediated phosphorylation which promotes ubiquitination by the SCF(Skp2) ubiquitin ligase (Li et al., 2003; Liu et al., 2004). However, other mechanisms must exist, as mutant forms of Cdt1 that do not stably associate with Skp2 are still degraded in S phase to the same extent as wild-type Cdt1 (Takeda et al., 2005).

Higher eukaryotes possess an additional control element over Cdt1 activity. This element is the Geminin protein, first isolated in *Xenopus* as a factor destroyed upon mitotic exit that was capable of inhibiting DNA replication (McGarry and Kirschner, 1998). Geminin was subsequently shown to bind Cdt1 and to inhibit the loading of MCM proteins onto chromatin (Tada et al.,

2001; Wohlschlegel et al., 2000). Given this situation it was perhaps surprising to see that Cdt1 and Geminin are mostly not coexpressed in interphase cells (Nishitani et al., 2001; Wohlschlegel et al., 2000). It may be that Geminin has a redundant role in S and G2 to inhibit any Cdt1 that may escape degradation. Proteasome inhibitors stabilise human Cdt1 in S and an interaction between Cdt1 and Geminin was detected in these circumstances (Nishitani et al., 2001). What seems clear is that Geminin is not required to promote the proteolysis of Cdt1 (Nishitani et al., 2004).

1.3.5 The MCM complex

The MCM proteins, Mcm2-7, were first isolated in budding yeast from mutants defective in the maintenance of mitotically stable minichromosomes (Maine et al., 1984). Highly conserved homologs have since been identified in fission yeast and metazoans, and each component is essential for the initiation and completion of DNA replication (Dutta and Bell, 1997; Kelly and Brown, 2000; Tye, 1999). Work with *Xenopus* egg extracts showed that the Mcm2-7 proteins function as a complex that is critical for DNA replication to occur (Chong et al., 1995; Kubota et al., 1997; Kubota et al., 1995; Madine et al., 1995), and the MCM complex is a component of the pre-RC (see previous sections). Once the MCM complex has been loaded onto DNA, none of the other components of the pre-RC complex are required for the continued association of the MCMs with chromatin (Donovan et al., 1997; Hua and Newport, 1998; Rowles et al., 1999). In fission yeast (Kearsey et al., 2000; Maiorano et al., 1996; Okishio et al., 1996; Pasion and Forsburg, 1999; Sherman and Forsburg, 1998) and metazoan cells (Lei and Tye, 2001) the MCM proteins are localised in the nucleus throughout the cell cycle, with their chromatin association dependent upon Cdc6 and Cdt1 function in M/G1. In contrast, the MCM complex in budding yeast, in association with Cdt1, is excluded from the nucleus after S phase in a CDK-dependent manner (Labib et al., 1999; Tanaka and Diffley, 2002).

While the exact biochemical function of the MCM complex remains uncertain, evidence suggests that it is the replicative helicase that unwinds DNA ahead of

the replication fork. Using ChIP, MCMs appear to travel with the replication forks (Aparicio et al., 1997; Tanaka et al., 1997) and genetic data shows that MCMs are required for fork progression as well as replication initiation (Labib et al., 2000). Biochemical and structural studies also lend weight to the idea of MCMs acting as a replicative helicase. The MCM complex has been purified from fission yeast and shown by electron microscopy to form a heterohexameric complex with a globular shape and central cavity that could assemble around the DNA duplex (Adachi et al., 1997). A complex of Mcm4/6/7 has been shown in fission yeast and mammalian cells to possess weak 3'-5' helicase activity *in vitro* (Ishimi, 1997; Lee and Hurwitz, 2000; Zou and Van Houten, 1999) and the Mcm4/6/7 complex from human cells forms a toroidal structure with six lobes and a large central cavity (Sato et al., 2000). In *Xenopus* egg extracts the unwinding of plasmid DNA requires an intact MCM complex and ATP hydrolysis (Shechter et al., 2004). A number of studies have also analysed the single MCM protein within the archaeon *Methanobacterium thermoautotrophicum.* This protein forms a double hexamer structure with a large central cavity, and possesses robust 3'-5' helicase activity with processitivity above 500bp (Chong et al., 2000; Kelman et al., 1999; Pape et al., 2003; Shechter et al., 2000). It has not yet been shown, however, that this MCM protein is required for DNA replication.

1.3.6 From pre-RC to replication

The transition from the pre-RC to replication elongation is a two-step process involving the recruitment of a number of additional factors to replication origins, to form the pre-initiation complex (pre-IC), followed by unwinding of DNA and DNA synthesis. A crucial component of this pre-IC in fission yeast is Cdc45, which is essential for DNA replication (Miyake and Yamashita, 1998). Cdc45 and its cofactor Sld3 associate with chromatin after pre-RC formation and are required to recruit DNA polymerase (pol) α onto the DNA (Gregan et al., 2003; Nakajima and Masukata, 2002; Uchiyama et al., 2001). Sld3 is also required for Cdc45 function in budding yeast (Kamimura et al., 2001) and Cdc45 (also known as Sna41) is highly conserved and essential for replication in eukaryotes from budding yeast to *Xenopus* and humans (Hardy,

1997; Hopwood and Dalton, 1996; Kukimoto et al., 1999; Mimura and Takisawa, 1998; Owens et al., 1997; Saha et al., 1998b). Cdc45 has been shown to recruit a range of replication factors to chromatin in these organisms, including the replication factors pol α, pol ε, RPA and PCNA (Kukimoto et al., 1999; Mimura et al., 2000; Mimura and Takisawa, 1998; Zou and Stillman, 2000). In addition to the recruitment of replication factors Cdc45 appears to have two other functions for DNA replication. Firstly, Cdc45 may be required for unwinding of DNA at initiation of replication (Walter and Newport, 2000). Secondly, Cdc45 is likely to function as a replicative helicase in concert with the MCM complex. Cdc45 travels with replication forks in budding yeast (Aparicio et al., 1999; Tercero et al., 2000) and forms a complex with the MCM proteins during S phase in *Xenopus* egg extracts (Masuda et al., 2003; Pacek and Walter, 2004). Furthermore, inhibition of either Cdc45 or the MCM complex prevents chain elongation and the Cdc45/MCM complex shows helicase activity *in vitro*.

A number of additional factors are required for Cdc45 chromatin association in S phase. Cdc23 (Mcm10 or Dna43 in other organisms) is bound to chromatin throughout the cell cycle and is essential for replication initiation and elongation (Aves et al., 1998; Gregan et al., 2003). Cdc23 is required to recruit Cdc45 to chromatin but is unlikely to be required for maintenance of Cdc45 on the DNA during elongation (Gregan et al., 2003). Instead, the role of Cdc23 in DNA elongation appears to be as a cofactor for the primase/pol α complex (Fien et al., 2004; Yang et al., 2005). In budding yeast and *Xenopus*, Mcm10 chromatin binding is dependent upon pre-RC formation and is restricted to S phase (Ricke and Bielinsky, 2004; Sawyer et al., 2004; Wohlschlegel et al., 2002). Here, too, Mcm10 is required to recruit Cdc45. One study in budding yeast shows that Mcm10 is also required for stabilisation of pol α during S phase (Ricke and Bielinsky, 2004).

In fission yeast Cut5 (also known as Dpb11) is also required for the recruitment of Cdc45 and initiation of DNA replication (Dolan et al., 2004; Saka and Yanagida, 1993). This function appears to be conserved in budding

yeast and *Xenopus*, where direct interactions with pol ε have also been reported (Araki et al., 1995; Hashimoto and Takisawa, 2003; Masumoto et al., 2000). A novel set of proteins, known as the GINS complex, has been isolated in budding yeast and *Xenopus* that is also necessary for the recruitment of Cdc45 to chromatin (Kubota et al., 2003; Takayama et al., 2003). The loading of this ring-like structure is dependent upon the pre-RC and Cut5, and the GINS complex appears to travel with replication forks in association with Cdc45 and the MCM complex during S phase. Another protein required for the initiation of DNA replication is Drc1 (Sld2 in budding yeast), which interacts with Cut5 (Noguchi et al., 2002; Wang and Elledge, 1999).

The transition from pre-RC to pre-IC is also dependent on two kinase activities: the S-phase CDK and DDK (Dbf4-dependent kinase), Cdc2 and Hsk1 in fission yeast respectively. Whilst the requirement for these two kinase activities for the recruitment of initiation factors to chromatin is well documented (reviewed in Bell and Dutta, 2002), the targets of CDK/DDK in this positive replication role are poorly defined. Drc1 has been identified as a target of the S-CDK in fission and budding yeasts, and this phosphorylation is required for interaction with Cut5 and S phase progression (Masumoto et al., 2002; Noguchi et al., 2002). In addition, Cdc28 phosphorylates pol ε and this is likely to be required for DNA replication (Kesti et al., 2004). Members of the MCM complex are phosphorylated by DDK in a range of organisms, although the functional significance of this has yet to be determined (Brown and Kelly, 1998; Hardy, 1997; Jiang et al., 1999; Lee et al., 2003; Lei et al., 1997; Masai et al., 2000).

1.4 Mechanisms that restrict S phase to once per cell cycle

As discussed in section 1.2.4, Cdc2 kinase activity in S phase and G2 is required to prevent rereplication of the genome. This mechanism appears to function through Cdc18 and Orp2. Cdc2 activity from S to M is responsible for repressing *cdc18* transcription, and for the phosphorylation of Cdc18 which targets the protein for proteolysis. The importance of this regulation is

shown by the observation that overexpression of Cdc18 induces rereplication (Nishitani and Nurse, 1995). Importantly, though, elimination of the Cdc2 phosphorylation sites from Cdc18 is not sufficient to induce rereplication, suggesting that other mechanisms exist within the cell to prevent rereplication (Jallepalli et al., 1997; Lopez-Girona et al., 1998). One of these mechanisms might be the modification of Orp2. Orp2 is a phosphoprotein which is hypermodified by Cdc2 from S phase to mitosis (Leatherwood et al., 1996; Lygerou and Nurse, 1999; Vas et al., 2001). This phosphorylation is not required for DNA replication but is likely to be important in preventing rereplication of the genome: removal of the Cdc2 phosphorylation sites from Orp2 potentiates the rereplication caused by overexpression of wild-type Cdc18 or Cdc18 lacking its own phosphorylation sites (Vas et al., 2001). Whilst Cdt1 degradation at G1/S does not appear to be regulated by Cdc2 (Damien Hermand, unpublished data), Cdc2 kinase does inhibit *cdt1* transcription, and the removal of Cdt1 as cells enter S phase may also play a role in preventing rereplication. Overexpression of Cdt1 potentiates the rereplication induced by overexpression of Cdc18 (Yanow et al., 2001). Taken together, these results suggest that multiple, overlapping mechanisms exist within the cell to prevent rereplication within the fission yeast cell.

A system of overlapping mechanisms also appears to operate in budding yeast to prevent rereplication. Phosphorylation of Cdc6 by Cdc28 in S phase targets the protein for ubiquitin-mediated proteolysis (Drury et al., 2000; Elsasser et al., 1999) and Cdc6 function is also restricted by direct association with Cdc28 (Mimura et al., 2004). Cdt1 and the MCM complex, in association with each other, are removed from the nucleus during S and G2 in a CDK-dependent manner (Labib et al., 1999; Nguyen et al., 2000; Tanaka and Diffley, 2002). In common with fission yeast, Orc2 (and Orc6) is phosphorylated by the S-phase CDK in S/G2 in budding yeast (Nguyen et al., 2001). Indeed, only when all three of these CDK-mediated mechanisms are abrogated can one induce rereplication in budding yeast (Nguyen et al., 2001).

The role of CDKs in preventing rereplication in metazoans is less clear-cut

than it is in yeasts. As described in section 1.3.2, Orc1 is removed from chromatin at S phase in metazoans (and subsequently degraded in human cells). Although inhibition of Orc1 abrogates MCM chromatin association (Ohta et al., 2003), evidence that Orc1 regulation is important for preventing rereplication is lacking. Regulation of Cdc6 does not seem to form part of the metazoan control system, as this protein is bound to chromatin throughout most of the cell cycle (Alexandrow and Hamlin, 2004; Coverley et al., 2000; Mendez and Stillman, 2000; Oehlmann et al., 2004). Instead, the main route by which metazoans prevent rereplication during S phase and G2 appears to be by downregulation of Cdt1 activity. As described in section 1.3.4, Cdt1 is degraded at G1/S and inhibited in S/G2 by Geminin in higher eukaryotes. The importance of these regulations is demonstrated by the fact that disruption of either gives rise to rereplication. Overexpression or stabilization of Cdt1 in *Xenopus* (Arias and Walter, 2005; Li and Blow, 2005; Maiorano et al., 2005), *Drosophila* (Thomer et al., 2004) or human cells (Vaziri et al., 2003) gives rise to extensive rereplication. Similarly, depletion of Geminin leads to rereplication in the same *Xenopus* (Li and Blow, 2005), *Drosophila* (Quinn et al., 2001) and human systems (Melixetian et al., 2004; Zhu et al., 2004). Disrupting both systems has a synergistic effect in *Xenopus* (Li and Blow, 2005). Collectively, these results indicate that, in most metazoan cells, the main feature that prevents rereplication is the absence of Cdt1 activity, which can occur despite any potential CDK-dependent inhibitory phosphorylation of other pre-RC components.

1.5 The DNA replication and damage checkpoints

As alluded to earlier, cells face an intrinsic challenge of ensuring that one cell cycle process is only initiated after a previous process has been completed. The solution to this 'completion problem' is a collection of cell cycle checkpoints, whereby an uncompleted cell cycle event sends an inhibitory signal to later events (Hartwell and Weinert, 1989). The components of the checkpoint systems of cells are not required for normal progression through the cell cycle *per se*, but are called into action when a process is delayed or perturbed. Cell cycle checkpoints operate at three points in eukaryotes: at the

boundaries of G1/S, G2/M and metaphase/anaphase. In this section I shall concentrate on the mechanisms which control the G2/M transition in fission yeast, and specifically those which inhibit entry into mitosis when DNA replication is incomplete (replication checkpoint) or DNA damage is present (damage checkpoint). Throughout this thesis I will refer to the collective inhibition of mitosis when DNA is either incompletely replicated or damaged as the S phase checkpoint. The S phase checkpoint blocks entry into mitosis by inhibitory phosphorylation of Cdc2 at tyrosine-15 (Enoch et al., 1991; Enoch and Nurse, 1990; Rhind et al., 1997; Rhind and Russell, 1998; Rowley et al., 1992a).

Six so-called 'Rad' proteins are required for the S phase checkpoint: Rad1, Rad3, Rad9, Rad17, Rad26 and Hus1 (al-Khodairy, 1992; al-Khodairy et al., 1994; Enoch et al., 1992; Jimenez et al., 1992; Rowley et al., 1992b). Rad1 has homology to PCNA (Thelen et al., 1999) and Rad17 has a limited homology to RFC (Griffiths et al., 1995). The similarities between checkpoint and replication proteins suggests that the former constitute complexes which interact with DNA to act as sensors for detecting DNA damage or impediments to replication. Indeed, complex formation within this set of proteins has been reported. The Rad9, Rad1 and Hus1 proteins form a heterotrimeric ring called the 9-1-1 complex (Griffith et al., 2002; Kostrub et al., 1998), Rad17 forms a heteropentameric ring with the four RFC subunits (Griffith et al., 2002; Shimada et al., 1999) whilst Rad3 associates with Rad26 (Edwards et al., 1999) (figure 1.6). The Rad17-RFC complex is bound to chromatin throughout the cell cycle (Griffiths et al., 2000) and is thought to load the 9-1-1 complex onto damaged DNA (Caspari and Carr, 2002). In addition to these Rad protein sensors in the S phase checkpoint, a number of replication factors may also be involved in relaying a signal of damaged or incompletely replicated DNA. Deletion of any of a range of factors required for initiation of DNA replication (e.g. DNA polymerase α) results in an inability to initiate an S phase checkpoint (reviewed in Murakami and Nurse, 2000) but the interplay between the replication and Rad proteins in this sensor role is poorly understood.

Downstream of the sensor components are the transducers, Rad3-Rad26, Chk1 and Cds1, and Rad3-dependent Chk1 and Cds1 phosphorylation is essential for checkpoint activation (Rhind and Russell, 2000). Cds1 activation occurs in response to inhibition of DNA replication, although Chk1 can also fulfil this role in the absence of Cds1 (Boddy et al., 1998; Lindsay et al., 1998; Zeng et al., 1998), whilst Chk1 is activated by DNA damage (Walworth et al., 1993). Divergence of the replication and damage checkpoint signals from Rad3 to Cds1 or Chk1 requires specialised adaptor proteins. Cds1 activation by Rad3 in response to replication inhibition is mediated by Mrc1 (Tanaka and Russell, 2001; Tanaka and Russell, 2004) whereas Crb2 is required for Rad3 phosphorylation of Chk1 in response to DNA damage (Mochida et al., 2004; Saka et al., 1997). Rad3 has also been shown to phosphorylate Rad26 in response to DNA damage independently of the other Rad proteins (Edwards et al., 1999). This suggests that Rad26 phosphorylation may be an initial response to DNA damage, but the functional significance of this phosphorylation is not yet known.

The Cds1 and Chk1 kinases cause Cdc2 inhibition by phosphorylating Mik1, Wee1 and Cdc25 (Baber-Furnari et al., 2000; Boddy et al., 1998; Furnari et al., 1997; Nurse, 1997; O'Connell et al., 1997; Rhind and Russell, 2001; Zeng et al., 1998). Phosphorylation of Mik1 and Wee1 activates these Cdc2 Y15 kinases, whilst phosphorylation of Cdc25 inactivates this Cdc2 Y15 phosphatase. In addition, Cdc25 phosphorylation results in association with Rad24, a member of the 14.3.3 protein family, and this leads to exclusion from the nucleus (Ford et al., 1994; Furnari et al., 1999; Lopez-Girona et al., 1999; Zeng and Piwnica-Worms, 1999).

The components and complexes of this checkpoint system are highly conserved across eukaryotes (see table 1.1), and will not be described in detail here (for a review see Nyberg et al., 2002). There are, however, a few significant differences in some organisms that are worth noting. In budding yeast, the S phase checkpoint signal does not inhibit entry into mitosis via

Cdc28 tyrosine phosphorylation, but rather prevents transition from metaphase to anaphase (Amon et al., 1992; Sorger and Murray, 1992; Stueland et al., 1993). Rad53 activation leads to the inhibition of Cdc5, a positive factor in the metaphase/anaphase transition (Cheng et al., 1998; Glover et al., 1998; Nigg, 1998), whilst Chk1 stimulation leads to the activation of Pds1, an inhibitor of the transition (Cohen-Fix and Koshland, 1997; Paciotti et al., 1998; Sanchez et al., 1999; Yamamoto et al., 1996). There are also some important differences in the use of PIKKs (phosphoinositide 3-kinase related kinases) as transducers in the checkpoint pathway. In mammalian cells two such kinases are used, ATR and ATM. ATR is required for transduction of signals involving single stand DNA breaks and replication inhibition, whereas ATM is required for transduction of signals involving double strand breaks (reviewed in Nyberg et al., 2002). In yeasts, the ATR homologs Rad3 and Mec1 are used to convey all S phase checkpoint signals, whilst the ATM homologs Tel1 are involved in telomere metabolism (Matsuura et al., 1999; Ritchie et al., 1999).

Table 1.1 Components of the S phase checkpoint system in eukaryotes

Protein function	Fission yeast	Budding yeast	Mammals
Sensors	Rad9	Ddc1	Rad9
	Rad1	Rad17	Rad1
	Hus1	Mec3	Hus1
	Rad17	Rad24	Rad17
Adaptors	Crb2	Rad9	BRCA1
	Mrc1	Mrc1	Claspin
Transducers	Rad3	Mec1	ATR
	(Tel1)	(Tel1)	ATM
	Rad26	Ddc2	ATRIP
	Chk1	Chk1	Chk1
	Cds1	Rad53	Chk2

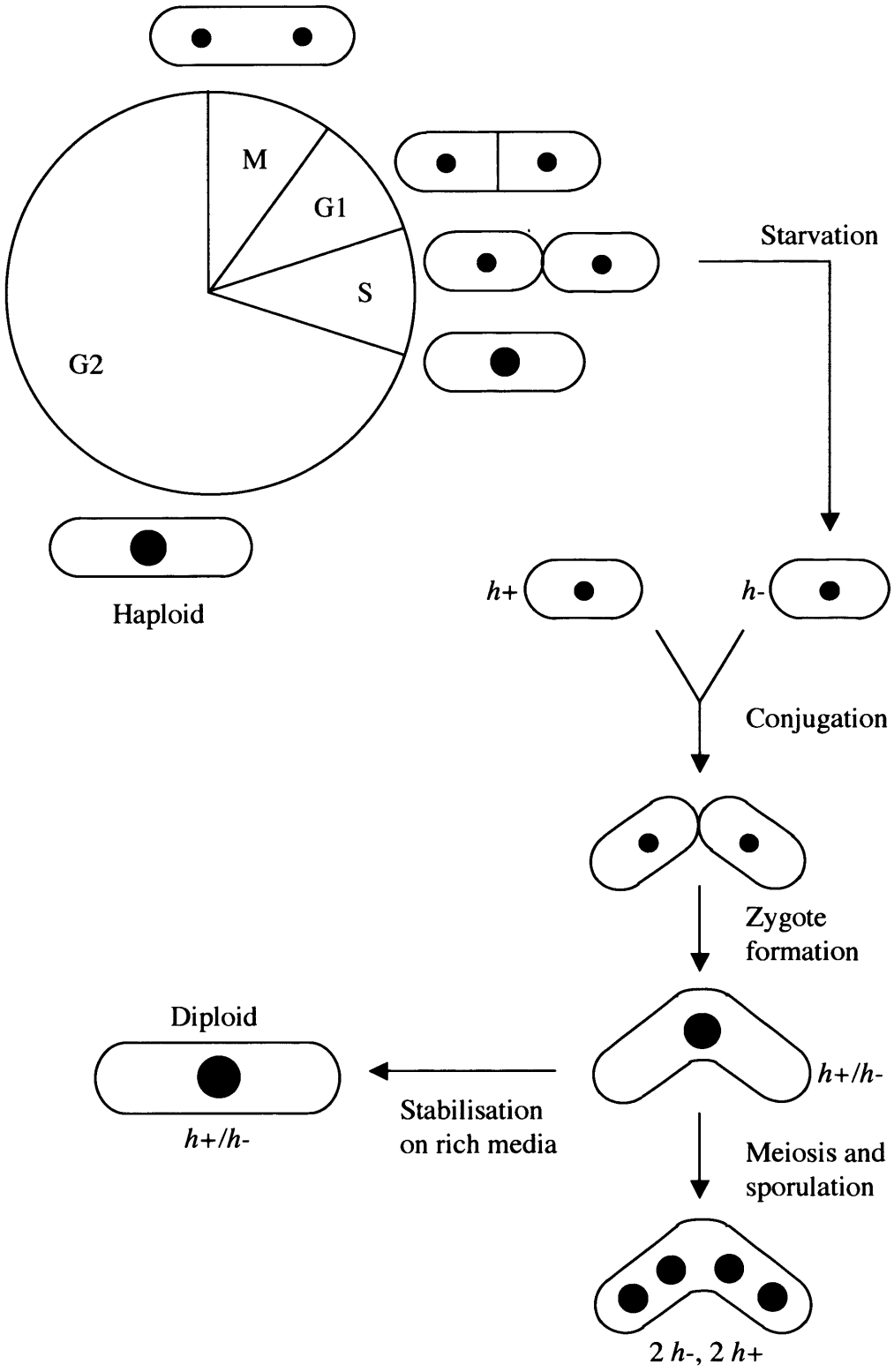


Figure 1.1 The fission yeast life cycle

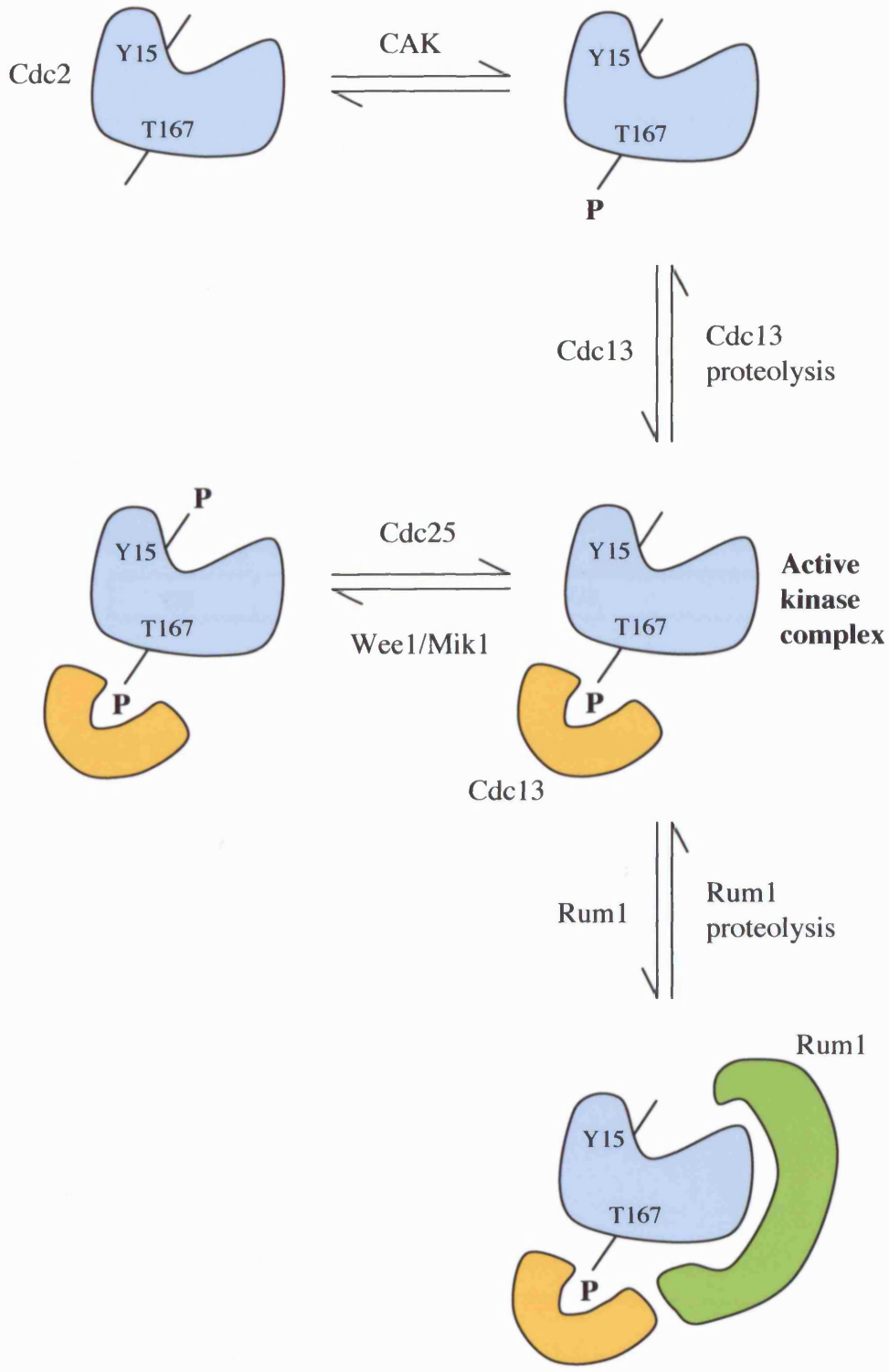


Figure 1.2 Summary of Cdc2 kinase control in fission yeast

Note that Cdc2 is also active for DNA replication when associated with the Cig1 and Cig2 cyclins. CAK=CDK Activating Kinase.

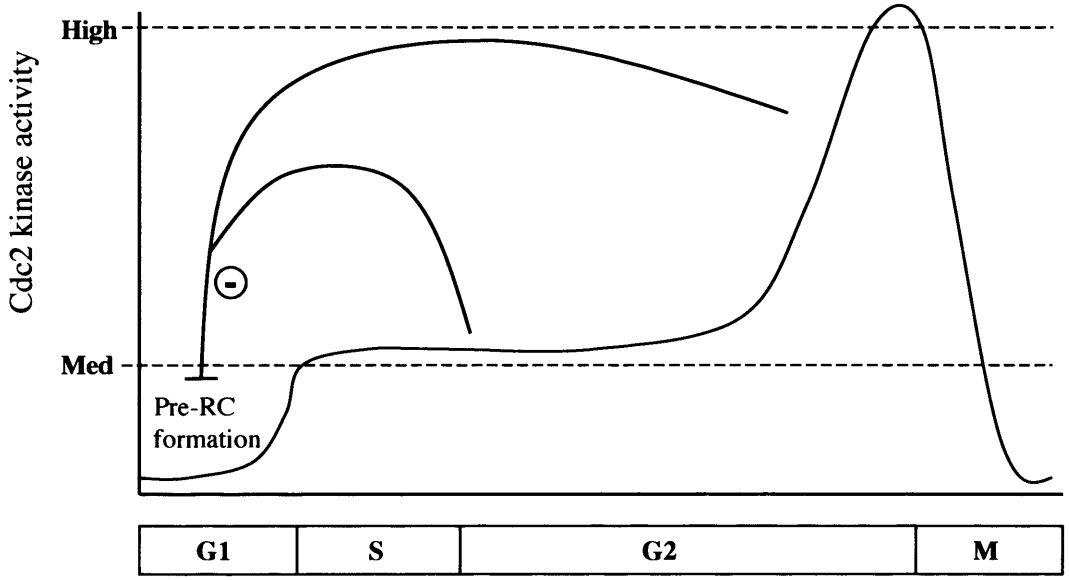


Figure 1.3 The quantitative model of Cdc2-mediated cell cycle progression based on a Cdc2 kinase cycle

Cells prepare for DNA replication when Cdc2 kinase activity is low in G1. A medium level of kinase activity (Med) triggers S phase whereas a high level of activity (High) is required to trigger mitosis. Both medium and high levels of kinase activity prevent the reformation of pre-RCs.

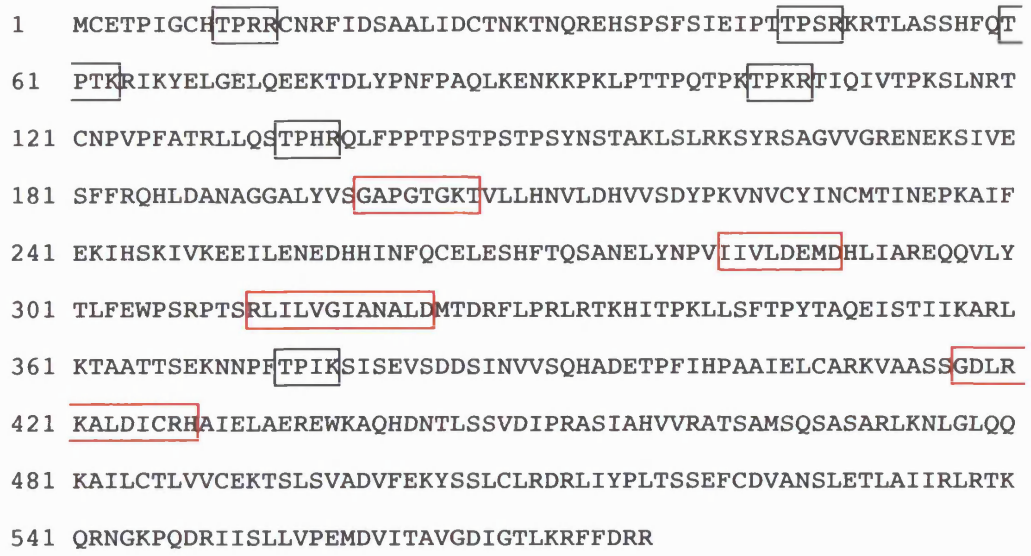


Figure 1.4 Primary structure of the Cdc18 protein

Black boxes represent consensus Cdc2 phosphorylation sites. Red boxes represent, from N-terminus to C-terminus, the Walker A motif (consensus GXXGXGKT), the Walker B motif (consensus HyHyHyHyDEXD), the Sensor I motif (consensus HyHyXXXN) and the Sensor II motif (consensus GDXR), where Hy=hydrophobic residue.

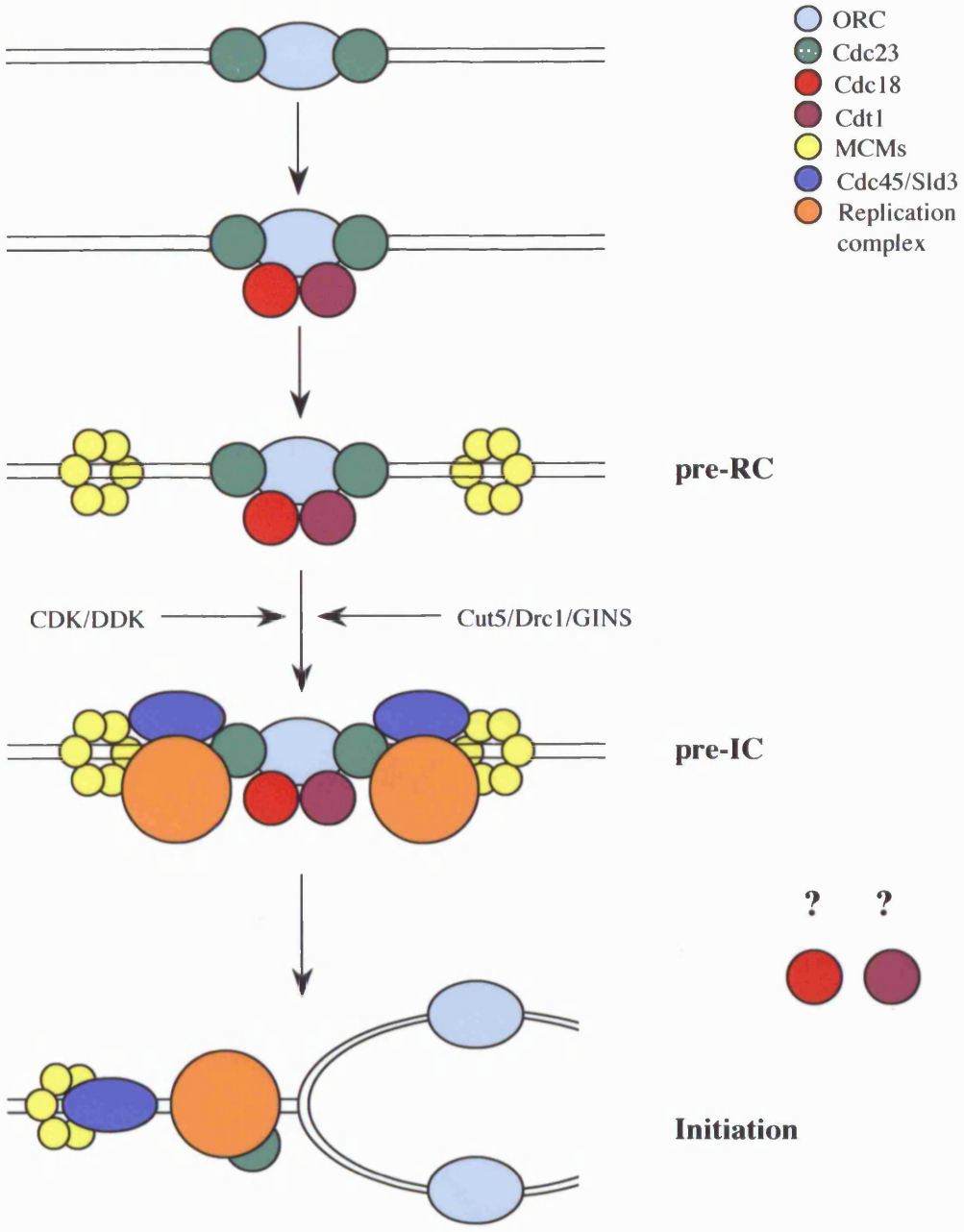


Figure 1.5 A model for the initiation of DNA replication in fission yeast

The replication complex contains, amongst other entities, DNA polymerases (pol) α and ε, primase, RFC and PCNA. Cdc45 recruits DNA pol α to the pre-IC, and then likely travels along the DNA in association with the MCM complex. Cdc23 is required for Cdc45 recruitment and for primase/DNA pol α function during elongation (and is hence pictured travelling with the replication complex). It is not known if Cdc18 and/or Cdt1 form part of the pre-IC, or if the proteins are present during S phase.

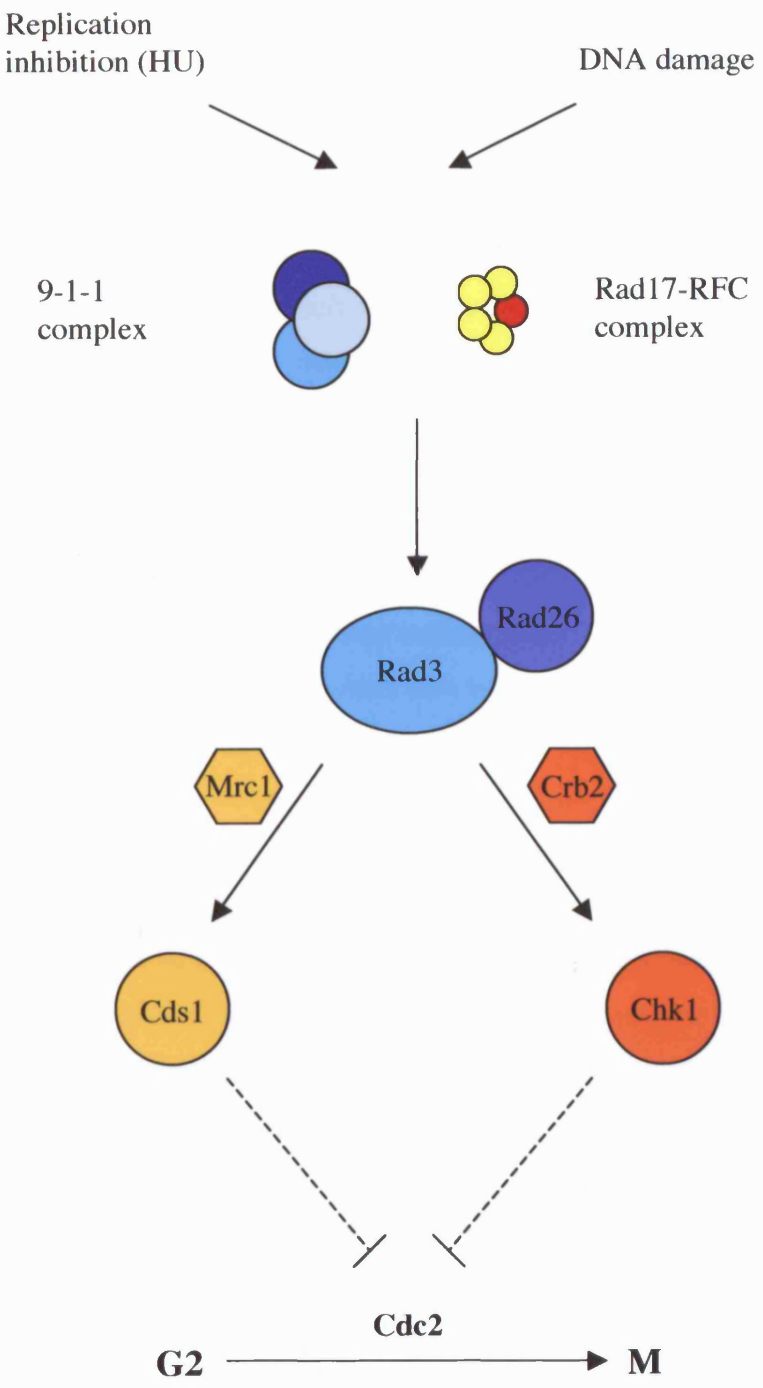


Figure 1.6 Summary of the DNA replication and damage checkpoints

Signals generated by incomplete replication diverge from Rad3 to Cds1 via Mrc1, whilst signals from DNA damage diverge from Rad3 to Chk1 via Crb2.

Chapter 2. Generation, analysis and screening of temperature sensitive (ts) *cdc18* mutants

2.1 Introduction

This project aimed to produce a bank of temperature sensitive (ts) *cdc18* mutants that could be used to isolate Cdc18-interacting factors via suppression screening. Isolation of genes (from a cDNA library) capable of rescuing the mutant phenotypes could reveal novel interactions between Cdc18 and other proteins.

Prior to this project, one ts *cdc18* mutant existed in the lab, namely *cdc18-K46* (Nasmyth and Nurse, 1981). This mutant fails to grow at restrictive temperature and arrests at the end of S phase with 2C DNA that can not be segregated (chromosomes do not enter a pulsed field gel and cells cut in the absence of *rad3*) (Kelly et al., 1993). By randomly mutating *cdc18* it was hoped that a number of ts mutants could be found that displayed a range of phenotypes at restrictive temperature. Such phenotypes might involve a complete destabilisation of the protein (giving the null phenotype of *cdc18*Δ), a replication deficient protein capable of initiating the S phase checkpoint (giving a 1C arrest), or a stable form of the protein that might ectopically activate the *rad3* pathway or cause re-replication. In addition, a strategy for creating a replication deficient ts mutant by site-directed mutagenesis was devised. Glycine 317 (G317) is a highly conserved residue in Cdc6/Cdc18 proteins, and a specific alteration of the corresponding residue in budding yeast Cdc6 (G260D) gives a ts 1C cell cycle arrest with failure to load Mcm5 onto replication origins (Feng et al., 2000). Hence the equivalent alteration (G317D) was attempted in fission yeast.

A total of 13 new ts *cdc18* mutants were isolated, each showing one of three different phenotypes. Mutants representing each class of phenotype were screened for suppressors from a fission yeast overexpression cDNA library,

but only Cdc18 itself was found to be able to suppress the mutant phenotype at restrictive temperature.

2.2 Results

2.2.1 Generation of ts *cdc18* mutants

Both random and site-directed mutagenesis of *cdc18* was used to produce ts *cdc18* mutants. The target construct used was cloned genomic *cdc18* with the *ura4* gene inserted after the *cdc18* ORF (figure 2.1). Mutagenic PCR amplification of the *cdc18-ura4* region gave fragments containing randomly mutated DNA. The mutagenic PCR reaction (Fromant et al., 1995) uses a non-proofreading polymerase, $MnCl_2$ to reduce stringency at the active site and unequal concentrations of dNTPs (see materials and methods). For site-directed mutagenesis, a mutant construct containing *cdc18-G317D* (pOH1) was obtained using a Stratagene QuickChange site-directed mutagenesis kit and complementary mismatched primers at the mutation site. High fidelity PCR amplification of the new *cdc18-ura4* region gave fragments containing a G317D version of *cdc18*. Mutated *cdc18-ura4* fragments were purified using a QIAquick PCR purification kit and transformed into *ura4Δ* cells. Uridine prototrophs, produced from integration of the *cdc18-ura4* fragment (assumed to be at the genomic locus of *cdc18*), were selected at 25°C. The integrants were then screened at 36.5°C for temperature sensitivity. The *cdc18-G317D* integrant was temperature sensitive, as predicted. Of approximately 20,000 integrants produced from the random mutagenesis procedure, 12 showed an inability to form colonies at the restrictive temperature and were selected.

A further screen was carried out for strains that were sensitive to HU, in either a temperature-dependent manner or not. Integrants were replica-plated to YE5S medium for 24 hours before replica plating to YE5S, YE5S with 12mM HU, and YE5S with 12mM HU at 36.5°C. Wild-type cells subjected to such treatment eventually leak through the HU block and form colonies, whereas checkpoint-deficient cells (e.g. *rad3Δ*) do not tolerate such treatment and die. None of the integrants produced from the random mutagenesis procedure were sensitive to HU at 25°C or at 36.5°C (although the above 12 isolates

demonstrating basic temperature sensitivity failed to grow on HU at 36.5°C, as expected).

Microscopic inspection demonstrated that all 13 ts strains were highly elongated at 36.5°C (figure 2.2A), and hence the temperature sensitivity wa due to cell cycle arrest. To check that the temperature sensitivity was *cdc18*-specific, and that no other ts mutations existed within the cell, strains were backcrossed to a wild-type *ura4Δ* strain. The resultant progeny showed a tight association of temperature sensitivity with the *ura4* marker in all cases, and the 50:50 segregation of the *ura4* marker showed that one integration event had occurred within the ts mutants. In order to show that the temperature sensitivity was due to a defect in *cdc18* function, strains were transformed with a wild-type copy of *cdc18* (expressed using a constitutive and thiamine-repressible *nmt81* promoter). In the absence of thiamine, these transformants were capable of forming colonies at the restrictive temperature (figure 2.2B, C), showing that the sensitivity of the isolated stains was due to a defect of *cdc18*.

2.2.2 General characterisation of ts *cdc18* mutants

In order to assess the point of the cell cycle at which the ts mutants arrested, cell number and FACS analysis was performed. Exponentially growing cultures of the mutants were shifted from 25° to 36.5°C, and samples taken every hour. Cell number analysis revealed cell cycle arrest after an approximate doubling in cell number for all mutants (figure 2.3A). These data position the arrest point early in the cell cycle, from G1 to late S phase (see 1.1.2). FACS analysis revealed that the mutants arrested with 1C DNA (G1 or early S arrest), 2C DNA (late S), or 2C DNA following a slow S phase (indicated by a transient 1C peak or spreading of the 2C peak to <2C regions) (figure 2.3B, table 2.1). It should be noted that the FACS profiles in these circumstances can drift towards the right as they persist in the block, probably due to an autofluorescence artefact as a consequence of cell elongation (Sazer and Sherwood, 1990). Thus the mutants showed a varying ability to initiate DNA replication (giving three different classes of phenotype), but in all cases

a G1 or S phase checkpoint activation was induced. In order to investigate this activation further, the mutant alleles were crossed into a *rad3*Δ background. When these double mutants were shifted to restrictive temperature the cells did not elongate, but instead entered a lethal mitosis, giving the cut phenotype (figure 2.2D). On agar plates a cut phenotype can be seen by the production of very small, rounded cells that often appear brighter than wild-type cells (probably due to the lack of a nucleus in some cases). These results demonstrate that the arrest shown by the ts mutants is one caused by incomplete or aberrant DNA replication which signals to the Rad3 pathway.

Colony PCR and subsequent sequencing was used to confirm the location of the integration event and to analyse the sequence of the mutant alleles. Colony PCR uses genomic DNA from cells inoculated directly into the reaction as a template. Colony PCR here was performed using a *ura4* primer (R6) and a primer complementary to a region of *cdc18* 5'-flanking sequence that was not amplified by the original PCR reactions (275, see figure 2.1). In this way, PCR products will only be produced from mutant copies of *cdc18* integrated at the genomic locus. All 13 ts mutants gave such products. By combining this data with the results of the genetic backcrosses it is possible to conclude that the mutants contain a single, mutant copy of *cdc18* at the genomic locus that is linked to *ura4*. Sequencing of the colony PCR products, across the entire *cdc18* ORF (in both forward and backward directions) identified a number of conservative and substitution mutations within the *cdc18* alleles (table 2.1).

Table 2.1 Summary of new ts *cdc18* mutants

cdc18 allele	Arrest phenotype	Amino acid substitutions
G317D	1C	G317D
3.2	1C	I109L, I260S, K481E, T486A, I551V
3.6	2C	S55C, S159C, K162N, V197G
3.9	1C	G317D, S552G
D8	2C, slow S	I379F, Q392R, V459D, D499N, S506P, C523S, L532P
B2.1	2C, slow S	D326V, K357I, V560A
B3.1	2C, slow S	V284T, K474Q
F2.1	2C, slow S	T101I, S311K. Q479L, K545R
H1.2	2C, slow S	S520A, A526T, I535F, Q541L
H2.3	2C	D219E, N443D, S494T, K540E, L553S
I1.2	2C	E434V, V560A
K3.2	2C	N153S, N190K, V197A, H290L, L322I, I455N
L2.1	2C	I243F, N275S, Y300F

Perhaps surprisingly, one mutant allele (3.9) produced from random mutagenesis contained the exact amino acid substitution designed for the site-directed mutant. Given the identical phenotypes of *cdc18-G317D* and *cdc18-3.9*, it is reasonable to assume that the G317D substitution is the active change (that which gives rise to the mutant phenotype) of the two substitutions of *cdc18-3.9*. As this mutant was obtained before the site-directed mutant, this allele has been used to investigate the phenotype given by the G317D change. These investigations, and the structural significance of the G317D substitution, are presented in chapter 3.

Using the sequence data of the remaining mutants to correlate structure with function is hampered by the presence of multiple amino acid substitutions (2-7) throughout the protein sequence of the ts alleles. In all cases there are two

or more changes of side chain type (e.g. from aromatic to basic, which I will call 'effective' changes). Of 45 changes only two pairs of substitutions affect the same residue, and there is a wide range in the types of residues involved in the substitutions. There are no direct changes to any of the *cdc2* phosphorylation sites, and the proportion of changes in and around the WA, WB, SI and SII motifs (11%) is close to that expected at random (14%). (Approximately 73 residues, 14% of the primary sequence, constitute these motifs.)

It may be possible, however, to implicate particular mutations in the presented phenotype if we analyse those effective changes that affect residues conserved or similar across eukaryotes (from alignment of *S. cerevisiae, S. pombe, X. laevis* and *H. sapiens* Cdc18/Cdc6 sequences) (figure 2.4 and table 2.2). For instance, in *cdc18-3.2*, which arrests with 1C DNA, K481E represents an effective change to a residue conserved across these species In addition, T486A alters a residue that features a hydroxyl-type side chain across the same organisms. Neither of these residues lies within a previously characterised domain of the protein, but K481 does lie within an QQK motif that is highly conserved. Mutation of the entire QQK motif (to a run of three alanines) results in a completely non-functional form of the protein (Liu et al., 2000). This QQK motif is affected in the *cdc18-F2.1* mutant (Q479L), but this strain also contains a change to the conserved residue S311, which lies within the Sensor I domain. The Sensor I domain is also affected in *cdc18-B2.1* (D326V) whilst the WB domain is altered in *cdc18-B3.1* (V284T). This analysis still provides a limited extrapolation of the sequence data, given that, in many cases, there are still two possible active mutations (or none at all), and the alterations involving known domains (e.g. Sensor I) result in different arrest phenotypes. However, the identification of conserved residues in uncharacterised regions, that give a mutant phenotype upon mutation, may allow further investigation into the structure and function of Cdc18.

Table 2.2 Mutations likely to be active in the mutant phenotypes of the ts mutants. Where no putative active mutations have been identified, all effective mutations alter non-conserved residues.

cdc18 allele	Arrest phenotype	Putative active mutations	Discussion
G317D	1C	G317D	See chapter 3
3.2	1C	K481E T486A	Conserved residue, QQK motif Hydroxyl side-chain
3.6	2C	None identified	
3.9	1C	G317D	See chapter 3
D8	2C, slow S	Q392R V459D	Conserved residue Aliphatic side-chain
B2.1	2C, slow S	D326V	Conserved residue, SI motif
B3.1	2C, slow S	V284T	Conserved residue, WB motif
F2.1	2C, slow S	S311K Q479L	Conserved residue, SI motif Conserved residue, QQK motif
H1.2	2C, slow S	S520A I535F	Conserved residue Aliphatic side-chain
H2.3	2C	None identified	
I1.2	2C	None identified	
K3.2	2C	I455N	Aliphatic side-chain
L2.1	2C	I243F	Conserved residue

2.2.3 Overexpression suppressor screening of ts *cdc18* mutants

Suppressor screening was performed using the laboratory fission yeast cDNA library. The cDNA for this library was produced from total RNA collected from mitotic, meiotic and shmooing cells (with 2:1:1 ratio). The library construct consists of a cDNA cloned behind the *nmt1* promoter, a selectable marker (e.g. budding yeast LEU2+) and a fission yeast ARS. The *nmt1* promoter provides constitutive high overexpression of its attached ORF in the

absence of thiamine (its repressor) and provides significant expression even in the presence of thiamine.

Three ts mutants were used for suppression screening, one from each class of arrest phenotype: 3.9 (1C arrest), 3.6 (2C arrest) and F2.1 (2C after slow S). For each screen, approximately 10^9 cells were transformed with 10μg of cDNA library and plated onto selective medium containing thiamine at 25°C. Approximately 2 x 10^5 transformants were obtained from each transformation. Transformants were replica-plated to selective medium with and without thiamine at 25°C for 48 hours before replica-plating to selective medium with and without thiamine at 36.5°C. For *cdc18-F2.1*, an additional screen was performed using a restrictive temperature of 34.5°C. After 2-4 days plates at restrictive temperature were inspected for colony growth and viable transformants were picked and patched to selective medium with thiamine at 25°C. Between 46 and 96 colonies, all on medium lacking thiamine, were selected in each screen. Each clone was then subject to colony PCR to test for the presence of *cdc18* cDNA. By using one primer complementary to library vector sequence (CO8) and one primer internal to the *cdc18+* ORF (R5) it was possible to identify transformants carrying *cdc18* plasmid. Positive clones (typically 80%) were discarded, and plasmid was extracted from the remaining clones. These plasmids were cloned using *E.coli* DH5-α and sequenced from the 5' end using vector primer. The resultant sequences were mapped onto Artemis, a program that represents the annotated genome sequence of fission yeast. All of the sequences mapped to the *cdc18* locus. With one exception, all clones contained a full-length *cdc18* ORF with a 5' UTR in the range of 70-130bp (mode of 75, mean of 92). The remaining clone contained a *cdc18* ORF lacking the coding sequence for the first 80 amino acids. Hence no factors other than Cdc18 itself were found that could suppress the temperature sensitivity of the ts *cdc18* mutants when overexpressed.

2.3 Discussion

The random and site-directed mutagenesis procedures used in this project successfully produced a bank of ts *cdc18* mutants. All of the mutants arrested

early in the cell cycle at restrictive temperature, and showed a varying ability to initiate DNA replication (broadly defining three classes of mutant). In all cases the absence of replication (1C arrest) or aberrant replication (2C arrest) triggers an S phase checkpoint signal to halt the cell cycle. In the absence of Rad3, this signal can not be sent and cells proceed to a catastrophic mitosis (giving the cut phenotype). As wild-type Cdc18 can rescue the defects seen at restrictive temperature, it is reasonable to assume that the defects represent a loss of function (recessive defects). The previously isolated ts *cdc18* mutant, *cdc18-K46*, would fall into the third class of mutant created in this study. This mutant gives a *rad3*-dependent late S phase arrest that can be overcome in the presence of wild-type Cdc18.

It is interesting to consider that the isolated bank consisted entirely of *cdc* mutants. Firstly, this suggests that structural changes capable of completely destabilising the protein (and giving a null phenotype) would be lethal at a range of temperatures. Secondly, it suggests that changes which are capable of stabilising the protein (possibly giving re-replication) may be lethal at a range of temperatures. Alternatively, it may be that the chances of simultaneously affecting all of the sites responsible for destabilising the protein (e.g. the *cdc2* phosphorylation sites) in the random mutagenesis procedure are too small.

It is also interesting to reflect on the phenotypes of the bank of mutants produced. The mutants arresting with 1C DNA at restrictive temperature represent a novel ts phenotype in *cdc18* mutants. In these cases, the initiation function of Cdc18 has been removed without affecting the ability of Cdc18 to initiate the S phase checkpoint signal. This contrasts with the phenotype of *cdc18*Δ, where cells fail to undergo DNA replication and proceed immediately into a lethal mitosis. Hence these mutants allow significant insight into both the initiation and checkpoint functions of Cdc18, and will be investigated further in chapters 3 and 4.

The mutants arresting with 2C DNA at late S present a less clear picture. A traditional explanation is that the initiation function of Cdc18 is not

completely ablated, but is faulty in some way. Faulty initiation may allow bulk replication (taking the DNA content to 2C) but leaves chromosomes in a state unfit for mitosis (causing Rad3 checkpoint activation). Quite how this would happen mechanistically is unclear, especially as these alleles are recessive with respect to wild-type (from the complementation data). An alternative explanation may be that Cdc18 plays a role within S phase aside from its initiation and checkpoint roles, and this is considered further in chapter 5.

The isolation of *cdc18* clones from the cDNA library that suppressed each of the screened ts mutants demonstrates that the screening procedure was effectively set-up. It was disappointing, and somewhat surprising, that no factor other than Cdc18 was capable of suppressing the mutant phenotypes when overexpressed. The mutants used displayed a range of initiation problems, and the use of a semi-restrictive temperature (34.5°C) should have aided the activity of weak suppressors. Whilst the level of expression of inserts from the strong *nmt1* promoter is very high, it must be noted that the screens were conducted both with and without its repressor, thiamine. In the presence of thiamine a significant expression is still achieved without the possible deleterious effect of overexpression of certain factors. In addition, in the absence of thiamine high overexpression of Cdc18, which we would normally expect to give rereplication, provided viable complementation. This suggests that factors within the library have an intrinsic range of expression levels, probably due to variations in UTR regions. Nonetheless, future screening attempts may require a different expression vector for the cDNA library. In addition, it may be that other mutants within the broad phenotypic classes, carrying different mutations, are more amenable to the screening procedures employed. Specific attention could be directed towards proteins displaying putative surface substitutions. A final consideration might be to attempt to remove the *cdc18* clones from the cDNA library, prior to screening, in order to reduce the work required to identify non-*cdc18* constructs obtained from the screen.

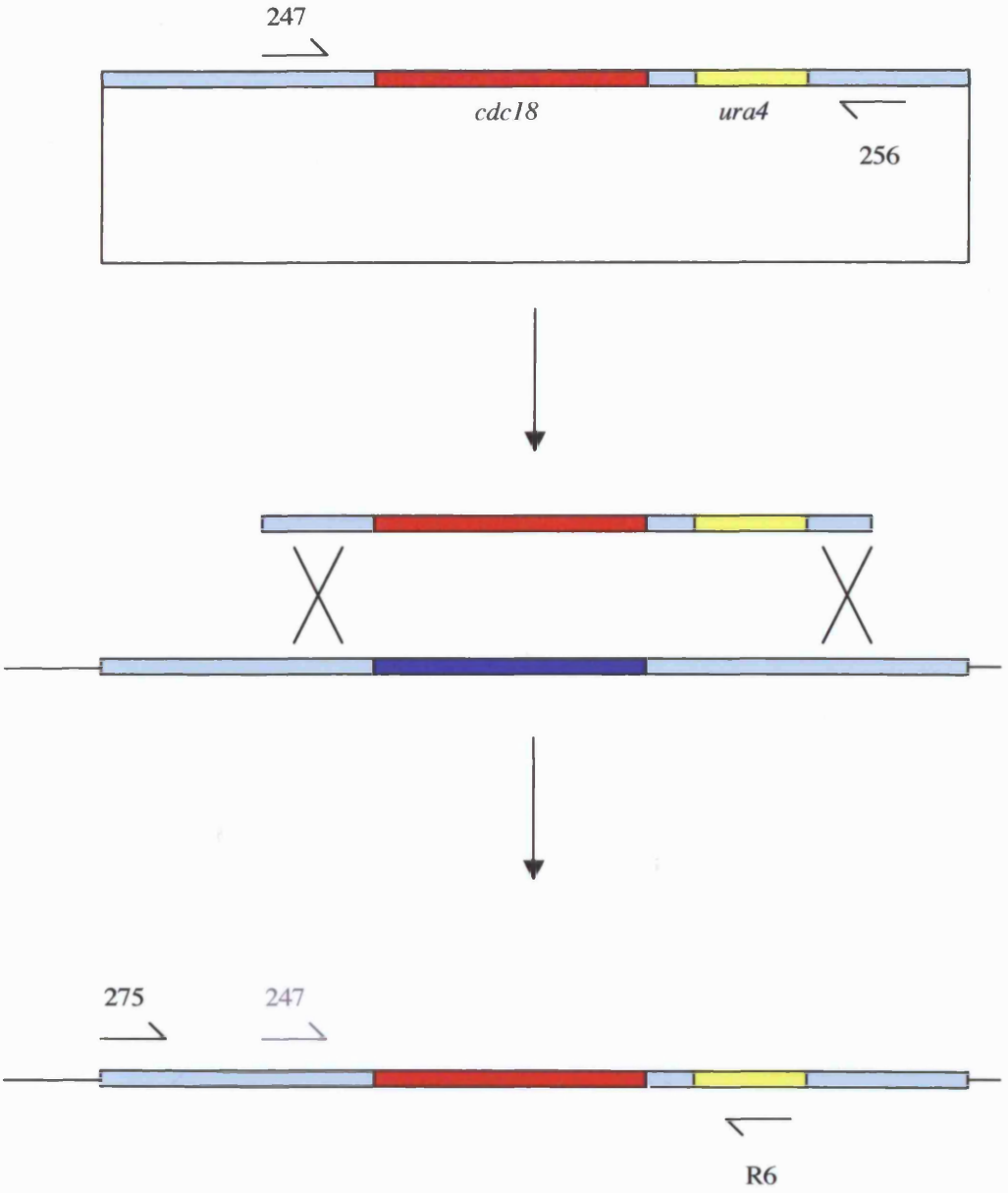


Figure 2.1 Outline of ts mutant construction

Cloned *cdc18-ura4* (in pDH17) or *cdc18-G317D-ura4* (in pOH1) were amplified using primers 247 and 256. Resultant PCR products were integrated into the genome (shown here by homologous recombination to replace the genomic *cdc18* ORF, in blue). Resultant uridine prototrophs were subjected to colony PCR using primers 275 and R6.

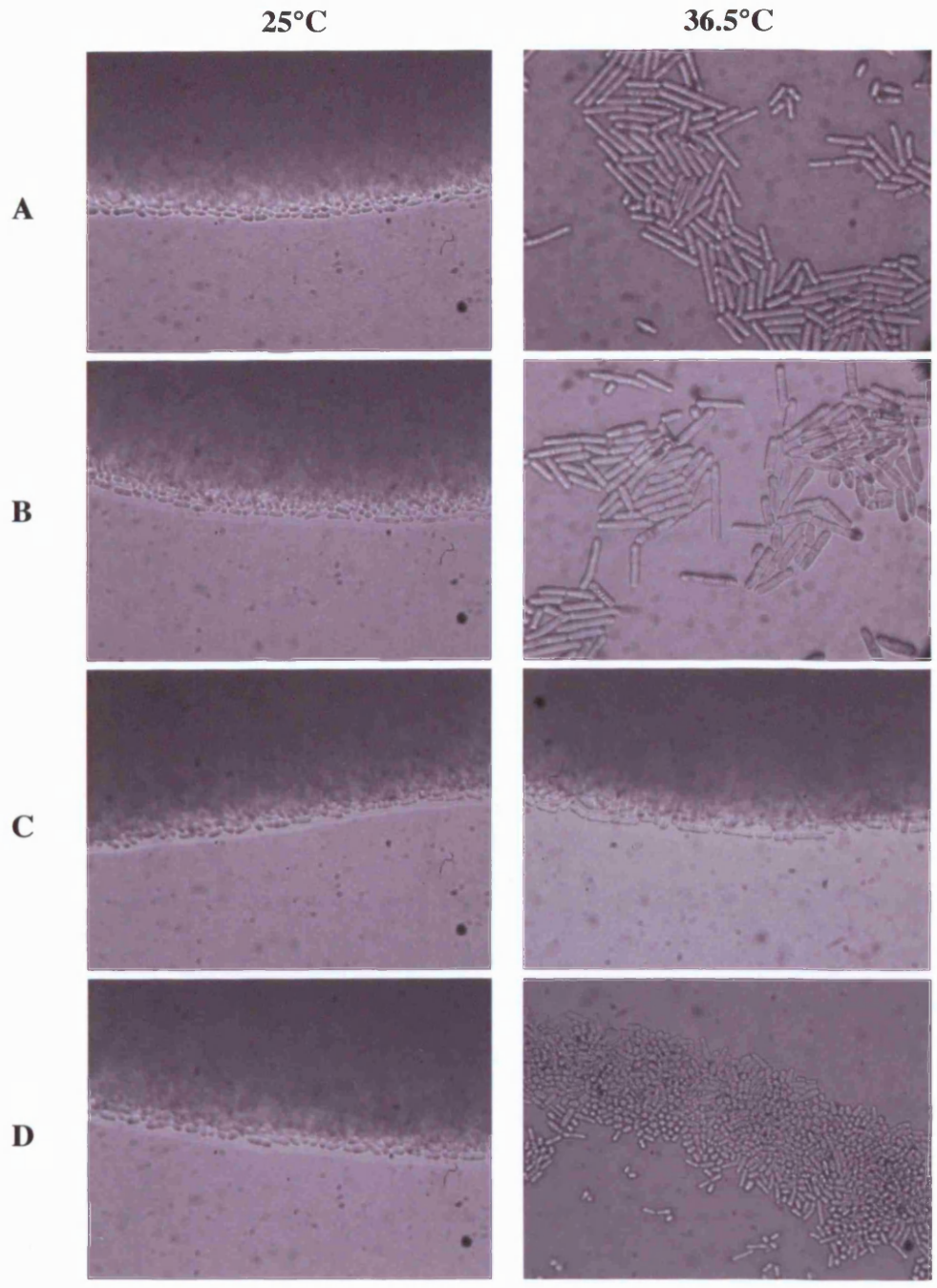


Figure 2.2 Visual phenotypes of ts *cdc18* mutants as illustrated by *cdc18-F2.1*

A) At 25°C, *cdc18-F2.1* forms colonies and cell size is wild-type. At 36.5°C there is no cell division so there is no colony formation and cells are highly elongated. B) *cdc18-F2.1* transformed with an empty vector on selective medium. The phenotypes at both temperatures are identical to those of the mutant alone. C) *cdc18-F2.1* transformed with *pnmt81-cdc18*. At At 36.5°C there is now colony formation. D) *cdc18-F2.1* in a *rad3*Δ background. At 36.5°C cells now enter a lethal mitosis, giving a cut phenotype.

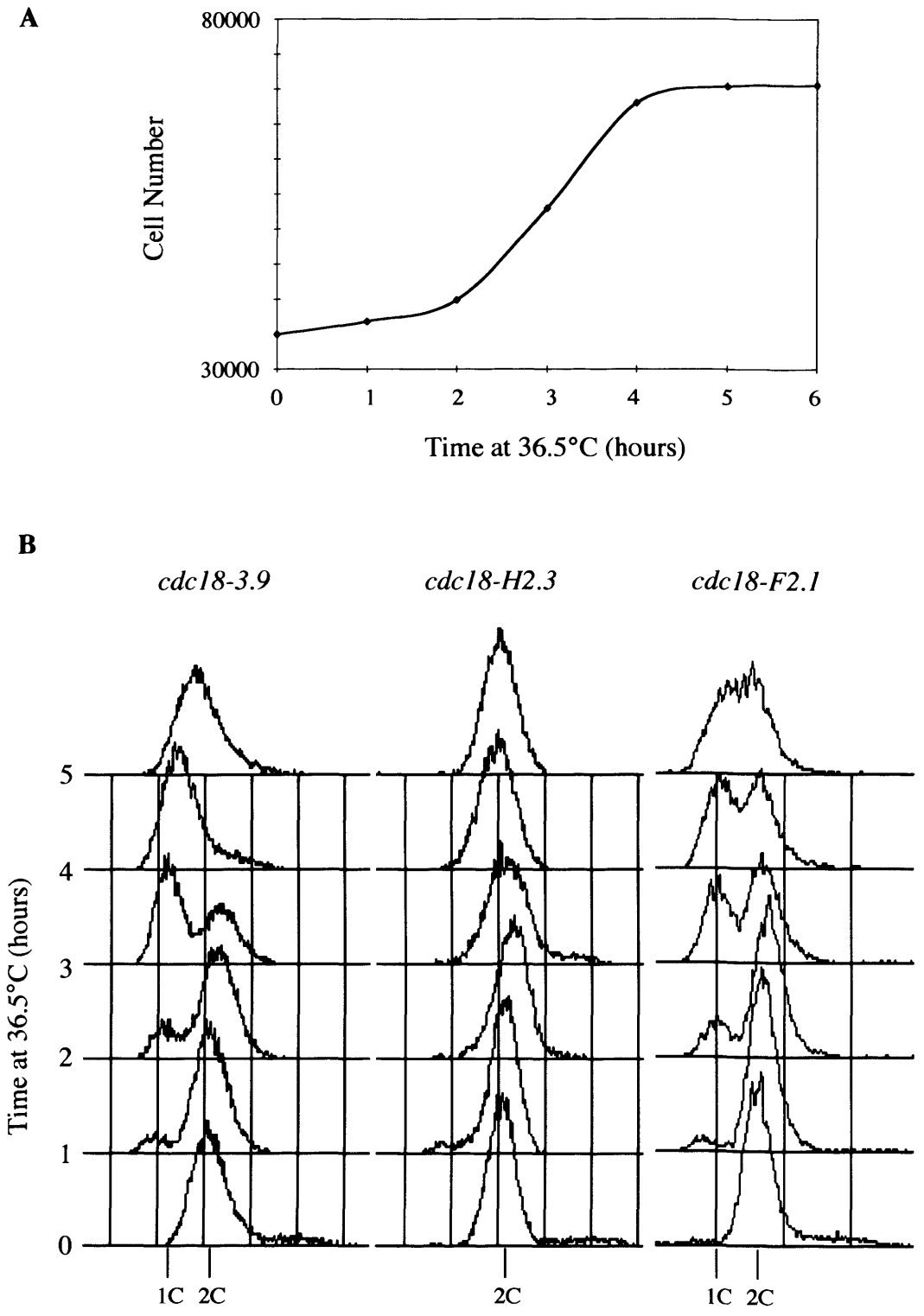


Figure 2.3 Phenotypes of ts *cdc18* mutants by cell number and FACS

A) As illustrated by *cdc18-H2.3*, cell number plateaus after doubling at 36.5°C. B) Cells arrest with 1C DNA (illustrated by *cdc18-3.9*), 2C DNA (illustrated by *cdc18-H2.3*) or 2C DNA after a slow S phase (as illustrated by *cdc18-F2.1*) at 36.5°C.

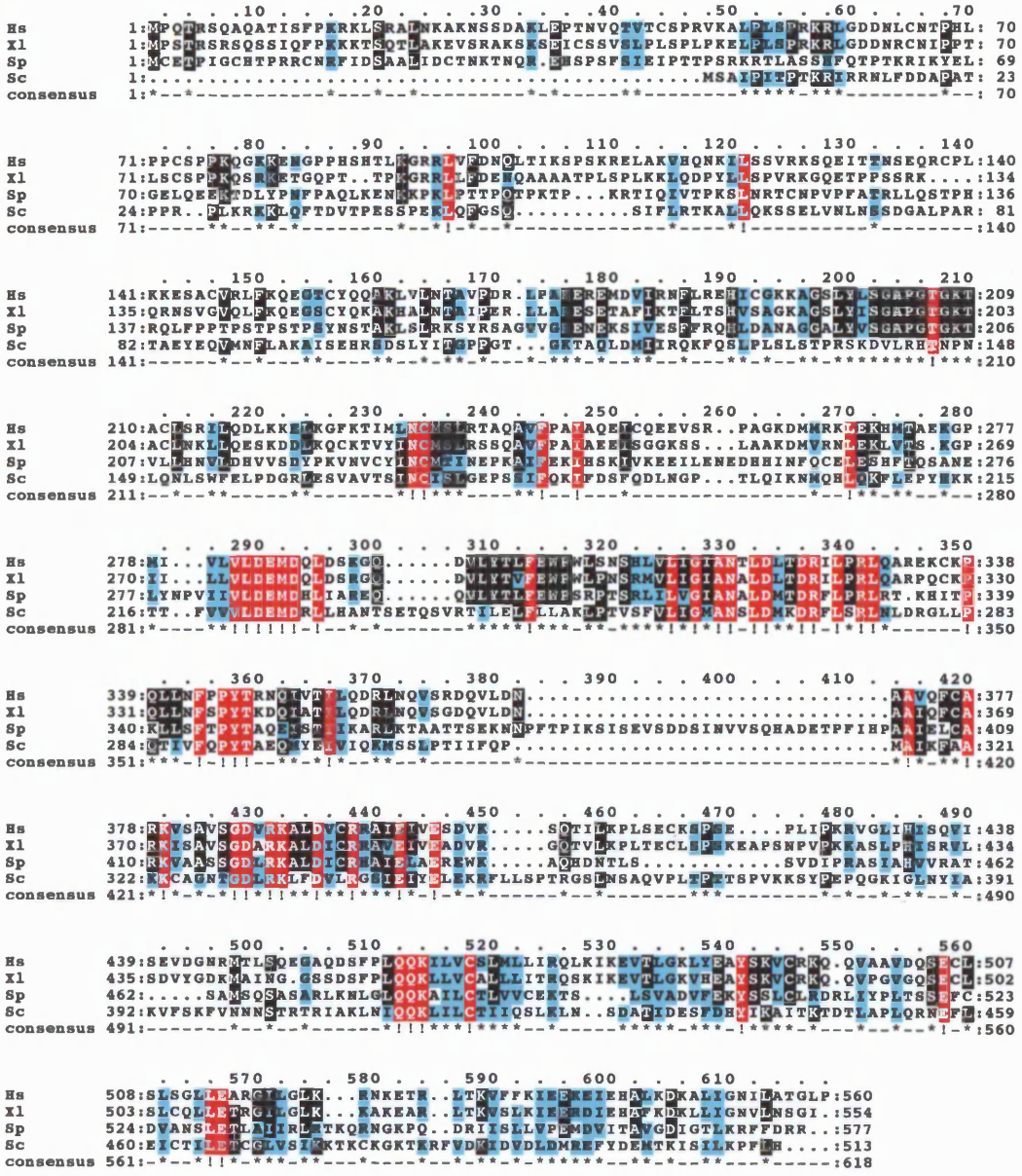


Figure 2.4 Multiple sequence alignment of Cdc6 proteins

Cdc6 proteins from *S. cerevisiae* (Sc), *S.pombe* (Sp), *X. laevis* (Xl) and *H. sapiens* (Hs). Red highlighting denotes identity between all four sequences, black highlighting denotes identity between three of the sequences, and green highlighting denotes similarity between at least three sequences.

Chapter 3. Detailed analysis of the WA and WB motifs of Cdc18

3.1 Introduction

The WA and WB motifs of ATPases have been implicated in the processes of ATP binding and hydrolysis respectively (Neuwald et al., 1999). A number of *in vivo* and *in vitro* studies have been carried out on the conserved regions of Cdc18 and its homologues, including the WA and WB motifs. In several organisms a model of nucleotide metabolism by this protein has been formed, yet some discrepancies, and differences between organisms, still exist.

The largest volume of work has been effected using budding yeast. Mutation of the conserved lysine residue of the WA motif (K114E) leads to a loss of MCM loading and hence replication, although the protein is still able to restrain mitosis (Weinreich et al., 1999). In this paper the mutant protein is still able to bind chromatin, which conflicts with other evidence which shows that the mutation prevents chromatin association *in vivo* and disrupts interaction with Orc1 *in vitro* (Wang et al., 1999). Mutation of the conserved glutamate residue of the WB motif (E224G) reduces MCM loading with concomitant defects in replication (Perkins and Diffley, 1998). These data suggest that ATP binding is not required for the checkpoint function of Cdc6, but binding and hydrolysis is required for MCM loading and replication. In addition to the WA and WB motifs, the Sensor I (SI) and Sensor II (SII) motifs have been implicated in Cdc6 function. As previously described, one particular mutation in SI (G260D) gives rise to a temperature-dependent loss of MCM loading and defects in replication (Feng et al., 2000). A number of other mutations in SI (e.g. N263A) and SII (e.g. R332A) give rise to temperature-dependent replication defects along with decreased MCM loading capability (Schepers and Diffley, 2001). Overexpression of the E224G (WB) and N263A (SI) forms of the protein, but not the K114E (WA) form, leads to a dominant inhibition of growth, characterised by incomplete replication (Perkins and Diffley, 1998). This suggests a common role for the WB and SI

motifs in Cdc6 function. A partial trypsin digestion assay has shown that wild-type Cdc6 can alter the conformation of the ORC complex (Mizushima et al., 2000). This activity is inhibited by mutation to the WA motif (K114E), mutation to the SI and SII motifs (N263A andR332E), and incubation with ATPγS (Mizushima et al., 2000; Takahashi et al., 2002). Taken together these results suggest that ATP hydrolysis enables MCM loading at origins by modifying the structure of ORC. ATP binding does not seem to be required for chromatin binding or checkpoint signalling.

The biochemical functions of the WA and WB motifs of human Cdc6 have been well studied *in vitro* (Herbig et al., 1999). Recombinant Cdc6 can bind and hydrolyse ATP, and interacts physically with itself and with human Orc1. Mutation of the WA motif (K208A) prevents ATP binding but does not affect its association with Orc1. Mutation of the WB motif (E285Q) prevents ATP hydrolysis without affecting ATP binding or association with Orc1. Microinjection of either form into G1 cells either prevents replication (WA) or inhibits its completion (WB).

In *Xenopus* egg extracts too it appears as though ATP hydrolysis is required for its replication function, although here it seems that ATP binding is required for association of Cdc6 with replication origins (Frolova et al., 2002). Mutation of the WA motif (K202E) prevents the association of Cdc6 with the chromatin, concomitant with a lack of MCM loading and DNA replication. Mutation of the WB motif (E227G) does not significantly affect chromatin association, but does decrease MCM loading and greatly inhibits DNA replication. Both mutant proteins inhibit DNA replication when added to extract before the wild-type protein. DNA replication is supported, however, if either mutant protein is added to extracts at the same time as wild-type protein. In addition, DNA replication is supported if the WA and WB proteins are added to extracts together. These results provide compelling evidence that Cdc6 acts as a multimer in *vivo*, and suggest that ATP binding and hydrolysis occur *in trans* between Cdc6 subunits within the complex. This is supported by the detection of physical interaction between Cdc6 subunits in a number of

papers (Frolova et al., 2002; Herbig et al., 1999; Saha et al., 1998a). Multimerisation of Cdc6 is also consistent with the dominant negative effects of certain mutant proteins in the systems described above.

In fission yeast, the WA and WB motifs are also essential for Cdc18 function. Mutation of the WA motif (K205A) results in a completely non-functional protein that is unable to initiate replication or a checkpoint signal (DeRyckere et al., 1999). Mutation of the WB motif (DE286AA) has been reported by the same laboratory to give two phenotypes: either a slow S phase with an intact checkpoint response (DeRyckere et al., 1999) or a slow S phase with a partial checkpoint response (Liu et al., 2000). Mutation of the SI motif (N320A, D323A) gives a non-functional protein, whereas mutation of the SII motif (D418A, R420A, K421A) causes a late S phase arrest (Liu et al., 2000), implicating these motifs in Cdc18 function. In this chapter I have used previously constructed and novel strains to expand our understanding of the WA and WB motifs in fission yeast. Neither the WA nor the WB domain is required for Cdc18 chromatin association. An intact WA motif is required for checkpoint activation, MCM loading and DNA replication. The WB and SI domains are required for MCM loading and DNA replication but not for checkpoint activation. These results suggest that Cdc18 associates with chromatin in any nucleotide state. The binding of ATP then enables Cdc18 to initiate a checkpoint signal, whilst hydrolysis enables origin licensing and thus DNA replication.

The overexpression of Cdc18 gives rise to large scale rereplication of the fission yeast genome (Muzi Falconi et al., 1996; Nishitani and Nurse, 1995). This effect is equalled, if not bettered, by the overexpression of a C-terminal fragment of Cdc18 (Greenwood et al., 1998). As the N-terminus (only) of Cdc18 physically interacts with Cdc2, this result demonstrates that rereplication induced by Cdc18 overexpression is not dependent upon the direct inhibition of Cdc2. In this chapter I show that rereplication induced by the C-terminus requires intact WA and WB motifs. This indicates that overexpression of Cdc18 gives rise to rereplication by directly reloading

MCM proteins, and not by an indirect effect, such as stimulating Rad3-dependent inhibition of Cdc2.

3.2 Results

3.2.1 Development of a chromatin association assay

A number of questions relating to the functions of Cdc18 required an analysis of the localisation of various proteins to chromatin. Therefore I developed a chromatin association assay, which was based upon that published previously (Lygerou and Nurse, 1999). A number of adaptations of this protocol were made in order to optimise the yield and purity of the chromatin associated protein fraction, and the final adjusted procedure is given in the materials and methods. In brief, the protocol firstly prepares spheroplasts for each sample, using lysing enzymes to remove the cell wall in the presence of osmotic support (1.2M sorbitol). These spheroplasts are then lysed by removing this osmotic support and adding triton detergent. Spinning the total lysate gives a pellet, containing chromatin (amongst other entities), and a soluble fraction. Treating the pellet with DNaseI, in the presence of 0.5M NaCl and 1% NP40, releases DNA-bound proteins. Hence upon further spinning one is left with a pellet and a supernatant containing proteins formerly associated with DNA: this is the chromatin associated protein fraction (figure 3.1A).

A new strain was constructed for the assay development, named the COT strain (*cdc25-22 orp1-HA cdc18-TAP*). Cultures of COT, growing exponentially at 25°C, were used as samples. The positive and negative controls used for the chromatin fraction were Orp1 and α-Tubulin respectively. Orp1 has been shown to bind chromatin constitutively throughout the cell cycle (Lygerou and Nurse, 1999) and α-Tubulin is a cytoplasmic protein. Cdc18 was detected using a PAP antibody that recognises its TAP tag, Orp1 was detected using an anti-HA monoclonal antibody and α-Tubulin was detected using and anti-Tubulin monoclonal antibody. Volume equivalents of soluble (S), chromatin (C) and final pellet (P) fractions were run on Westerns using a variety of treatments of the initial pellet (figure 3.1B). In all cases a large proportion of total Cdc18 and Orp1 is located in the soluble

fraction. This likely represents a combination of soluble protein within the cell and protein that has been lost from the chromatin during the procedure (and hence has not been used as an indicator of *in vivo* soluble levels). A similarly large proportion of these proteins can be found in the initial pellet, and this represents chromatin-bound protein along with protein in unlysed cells and protein that had formed insoluble aggregates (Nishitani and Nurse, 1995). At optimised conditions, approximately 50% of these proteins are released from the initial pellet using DNaseI in 0.5M NaCl (with or without 1% NP40). Extremely low amounts of Cdc18 or Orp1 are found in the chromatin fraction without such treatment, or by using 1% NP40 alone. None of the treatments increase the very low level of α-Tubulin released into the chromatin fraction.

Further verification and development of the technique involved analysing a synchronous mitotic cell cycle using a *cdc25-22* block and release experiment with the COT strain. Cells were blocked at the G2/M transition by incubation at 36.5°C for 3.5 hours. Shifting the temperature of the culture down to 25°C released cells into a synchronous cell cycle, which was followed every 20 minutes for the next 3 hours. One half of the culture was untreated and one half was released in the presence of 12mM HU. In the untreated sample S phase can be located between 80 and 120 minutes post release (as indicated by 4C DNA and the rise in septation index, figure 3.2A, B). Both Cdt1 (detected using a polyclonal anti-Cdt1 antibody) and Cdc18 can be seen to associate with chromatin prior to S phase and to dissociate after S phase (figure 3.2C), as previously reported (Nishitani et al., 2000). In addition, it has been shown that whilst the total amount of Mcm4 remains constant throughout the cell cycle, binding of low mobility Mcm4 to chromatin follows a similar periodicity to that of Cdc18/Cdt1 (Nishitani et al., 2000), and this is reproduced here (Mcm4 detected using a polyclonal anti-Mcm4 antibody). For the rest of the thesis my description of Mcm4 loading refers to the association of these low mobility forms of Mcm4 with chromatin.

In the presence of HU, cells arrest early in S phase. Cells septate in the absence of chromosomal duplication and hence a 1C DNA peak appears at

120 minutes. Within the HU block Cdc18, but not Cdt1, remains present (figure 3.2D) as previously observed (Damien Hermand, unpublished data). In addition, Cdc18 and Mcm4 remain associated with chromatin, and these results are considered further in chapter 4. It is interesting to note that the level of Cdc18 at its peak in a synchronous S phase is equal to the level of Cdc18 in an HU block (figure 3.2E). It is still possible, however, that levels of Cdc18 are elevated still further in a prolonged incubation in HU.

Cdc18 can sometimes be seen on these Western blots as a closely spaced doublet, demonstrating the variability of mobility due to phosphorylation (Jallepalli et al., 1997). It is interesting that the doublet is apparent in both total and chromatin extracts, showing that phosphorylation of Cdc18 does not remove it from chromatin (although it may prevent re-binding). Throughout this thesis tagged versions of Cdc18, but not the native species, are often separated into different phospho-forms on the 7.5% gels used throughout.

Whilst the use of HA tagged Orp1 as a chromatin loading control was successfully employed in the development of the assay, an alternative loading control was sought that did not require the introduction of the tagged *orp1* gene into each strain of interest. An anti-Orp2 polyclonal was generously donated by the Russell laboratory, which is effective in recognising Orp2 without needing to affinity purify the serum (figure 3.3A). By using Orp1-HA from the previous experiments as a loading control, it was possible to assess Orp2 chromatin binding throughout the cell cycle. Although the phosphorylation status of Orp2 varies throughout the cell cycle, as reported (Vas et al., 2001), the total amount of Orp2 bound to chromatin (confirmed by densitometry) does not (figure 3.3B). In addition, HU treatment does not alter Orp2 loading. Thus Orp2 was subsequently used as a loading control for the chromatin association assay.

3.2.2 A functional WA domain is required for DNA replication and checkpoint function but not for chromatin binding

A series of strains had been constructed previously within the laboratory (Greenwood, 2000) to assess the function of mutant Cdc18 proteins. The first of these strains contains a wild-type copy of *cdc18*, integrated into the genome behind the thiamine-repressible *nmt81* promoter (*nmt81-cdc18+*, or the 'switch-off' (SO) strain). This strain grows exponentially at 32°C in the absence of thiamine, as the level of Cdc18 produced from the *nmt81* promoter is sufficient to support growth without inducing re-replication. Upon thiamine addition transcription of the wild-type gene is repressed and cells rapidly accumulate with a 1C DNA content (figure 3.4A). Cells then proceed without delay into a lethal mitosis, giving the cut phenotype, as observed by DAPI staining of cellular DNA (figure 3.4B). For this thesis a cut phenotype is recorded if any of the following characteristics are observed:

a). uninucleate cells with a septum lying upon or cutting the nucleus.
b). septated cells with a nucleus in one side of the cell only or fragmented DNA in both sides.
c). anucleate cells.

The cut phenotype can often be seen on a FACS profile as a shift to DNA contents less than 1C, or on cell number analysis as an increase in cell number following cell cycle arrest. However, as septation does not always lead to cell division in these circumstances (especially in longer cells) such measures do not provide accurate quantification of the cut phenotype.

The next strain in the series had been used to show that the WA domain is absolutely required for Cdc18 function (in agreement with DeRyckere et al., 1999). Here, an HA-tagged version of *cdc18*, containing a mutation of the conserved WA lysine residue (K205A), and expressed from the endogenous *cdc18* promoter, is integrated into the switch-off strain. The resultant strain (*cdc18-WA-HA;* SO), grows exponentially at 32°C in the absence of thiamine. Upon thiamine addition cells rapidly accumulate with a 1C DNA content and

proceed into a lethal mitosis, giving the cut phenotype (figure 3.4A, B). The rate of accumulation of 1C DNA and cut cells is very similar to that seen in the absence of the WA protein (SO strain), demonstrating that the WA version of Cdc18 is not capable of either initiating DNA replication or initiating an S phase checkpoint. I was interested to find out if the lack of function of the WA protein over this timecourse is due to an inability to bind chromatin. Wild-type Cdc18 can be resolved from the HA-tagged Cdc18-WA on a 7.5% gel and detected using a polyclonal anti-Cdc18 antibody (figure 3.4C). Chromatin-bound, wild-type Cdc18 is present in cycling and HU-blocked cells, but is only just detectable an hour after the addition of thiamine. Cdc18-WA was found to be chromatin-bound throughout the experiment and in cells blocked with HU. Whilst Cdc18-WA can bind chromatin, it fails to load Mcm4 in the absence of wild-type protein. Thus an intact WA domain (and hence presumably ATP binding) is required for MCM loading, DNA replication and the S phase checkpoint, but not for Cdc18 chromatin association.

For all remaining Western blots, α-Tubulin (total fractions) and Orp2 (chromatin fractions) have been used as loading controls. Probing with other antibodies was only carried out once the loading of Tubulin or Orp2 was equal in all lanes. For most of the remaining figures these loading controls have not been shown, but loading controls have been included where the relative levels of other factors are being discussed.

3.2.3 Functional WB and Sensor I domains are required for MCM loading and DNA replication

Defining the role of the WB domain of Cdc18 has been more difficult than defining that of the WA domain. In the same switch-off system as that described above, a mutation of one of the two acidic residues of the WB motif (E287G) was made previously (*cdc18-E287G-HA;* SO) (Greenwood, 2000). This strain grows exponentially at 32°C in the absence of thiamine. Upon thiamine addition transcription of the wild-type gene is repressed and cells arrest at the end of S phase, as indicated by a plateau of cell number after an approximate doubling and 2C DNA (figure 3.5A). The absence of cut cells at

5 hours demonstrates that the S phase checkpoint is intact. Cdc18-E287G was found to be chromatin-bound throughout the experiment and in cells blocked with HU (figure 3.5B). Association of Mcm4 with the chromatin is seen in cycling and HU-blocked cells, but not at the block point.

These results suggest either that the WB domain is not required for the initiation or checkpoint functions of Cdc18, or that the WB mutation used has not completely abrogated its function. In order to investigate this, the strain was forced to enter an HU block with wild-type *cdc18* repressed. The experiment used here employs two HU blocks, one to synchronise the culture and one to analyse the strain's behaviour in the absence of wild-type *cdc18* transcription. Cells were initially synchronised at early S phase by incubation in HU for 3 hours (figure 3.6A). HU was then washed out and thiamine added to the culture to repress transcription of wild-type *cdc18*. An hour later, when DNA replication is complete and cells are entering G2, HU is added again. Two to three hours later cells accumulate with 1C DNA at early S phase. Figure 3.6B shows that cells enter this second HU block with only Cdc18-E287G bound to the chromatin. In these circumstances a significant amount of Mcm4 is loaded on to chromatin (approximately 40% of that loaded in the presence of wild-type Cdc18, as determined by densitometry). The residual ability of the Cdc18-E287G protein to load MCMs onto chromatin may explain the entry of cells into S phase when only the Cdc18-E287G protein is present. In addition, this residual activity suggests that the single WB mutation introduced into the protein does not completely abrogate its ATP hydrolysis function. Further evidence for these conclusions is provided in section 3.2.4.

A more clear-cut picture was obtained using another previously constructed strain. In this strain a midT-tagged version of *cdc18*, containing a double mutation in the WB motif (DE286AA), is integrated behind the endogenous promoter. A wild-type copy of the gene is also present within the cell on a plasmid, under control of the *nmt81* promoter (*cdc18-DE286AA-midT; pnmt81-cdc18* or, more concisely, *cdc18-DEAD-midT;* SO) (Liu et al., 2000). This strain grows exponentially at 32°C in the absence of thiamine. Published

results show that, upon thiamine addition, cells arrest with a 2C DNA content after a slow S phase. In addition, the S phase checkpoint signal is lost after approximately 5 hours. In my hands, however, a different phenotype is observed (figure 3.7A). Upon thiamine addition cells accumulate with 1C DNA and replication is absent throughout the timecourse. In addition, the S phase checkpoint is maintained for longer (12% cuts at 5 hours in comparison to 42% in the switch-off strain or 34% in the published results for this mutant). Figure 3.7B shows that Cdc18-DEAD is bound to chromatin in cycling cells, in HU-blocked cells and throughout the timecourse. (Note that I was unable to resolve the midT-tagged mutant protein from its wild-type counterpart, and hence the former is followed using a polyclonal anti-midT antibody.) Commensurate with the lack of replication, the Cdc18-DEAD protein is unable to load Mcm4 onto chromatin. These results demonstrate that an intact WB domain (and presumably ATP hydrolysis) is not required for chromatin binding or for S phase checkpoint initiation, but is required for MCM loading and hence replication.

The arrest phenotype produced by the Cdc18-DEAD protein is reminiscent of that seen with the ts mutant protein Cdc18-3.9 at restrictive temperature, as described in chapter 2. At 36.5°C this strain accumulates with 1C DNA and initiates the S phase checkpoint (cut cells are absent until after the 5 hour timepoint) (figure 3.8A). The mutant protein is bound to chromatin at the block point, but it cannot load Mcm4 (figure 3.8B). As a positive control for Mcm4 loading the same strain was incubated in HU at 25°C. As discussed in chapter 2, the active mutation in this protein is G317D. Glycine 317 lies within the Sensor I (SI) region of the protein. The Sensor regions of AAA-type proteins are commonly believed to either aid the process of ATP hydrolysis, or to transduce ATP hydrolysis into catalytic or/and conformational change (reviewed in Neuwald et al., 1999). The *P. aerophilum* structure enables us to see that the Sensor I region lies within close proximity to the co-ordinated Mg.ADP moiety and the WA, WB and Sensor II motifs (figure 3.9) (Liu et al., 2000). These results suggest that an intact SI domain is required for Mcm4 chromatin association and thus DNA replication, most

likely by enabling ATP hydrolysis or by translating ATP hydrolysis into loading activity. The same conclusions can be reached in budding yeast, where the analogous G260D mutation also confers an inability to load MCM protein (Mcm5) onto replication origins (Feng et al., 2000).

The results from this section have further consequences for the S phase checkpoint role of Cdc18. Cdc18 that can bind ATP, but not hydrolyse it, is capable of sending an S phase checkpoint. The ability to initiate such a signal without loading Mcm4 onto chromatin, and hence without replication, suggests that Cdc18 itself (or processes dependent upon it but not its initiation function) is responsible for checkpoint activation. In addition, it suggests that DNA replication intermediates are not necessarily required for the S phase checkpoint (although they may still be able to stimulate or/and potentiate such a signal). This will be discussed further in chapter 4.

3.2.4 Functional WA and WB domains are required for rereplication caused by Cdc18 overexpression

It has been shown that overexpression of the C-terminus of Cdc18 (amino acids 150-577) causes rereplication to the same extent as overexpression of the full length protein (Greenwood et al., 1998). Overexpression of the N terminus (1-141) does not induce rereplication, but does cause cell cycle arrest via direct inhibition of Cdc2. I was interested to find out if the rereplication caused by overexpression of the C-terminus of Cdc18 required the activity of the WA and WB domains. This could indicate whether the ability of the C-terminus to cause rereplication depends upon its ability to load MCM proteins onto the DNA.

For this analysis I used a plasmid containing a copy of the C-terminus of Cdc18 behind the *nmt1* promoter (*pnmt1-cdc18-C*). A Stratagene QuickChange site-directed mutagenesis kit, and appropriate mutagenic primers, were used to introduce a number of mutations into the C-terminal ORF. These mutations affected the WA motif (K205A) or the WB motif (D286G, E287G and DE286AA). Wild-type cells were transformed with these

constructs, including the intact C-terminal plasmid, and cultured at 32°C in selective medium containing thiamine. Cells were then filtered, washed and resuspended in selective medium lacking thiamine and cultured at 32°C for 20 hours. After this time, overexpression of Cdc18-C had caused large-scale rereplication (figure 3.10). Approximately 75% of cells had a DNA content of 4C or more, with DNA contents rising up to 32C. In contrast, no rereplication was observed following overexpression of Cdc18-C-K205A or Cdc18-C-DE286AA. This demonstrates that intact WA and WB domains are required for rereplication induced by overexpression of Cdc18-C. Hence the MCM loading activity of Cdc18 is required for rereplication when this protein is overexpressed. Interestingly, an intermediate level of rereplication was induced by Cdc18-C-E287G. This is consistent with the observation that Cdc18-E287G (at wild-type levels) demonstrates residual Mcm4 loading activity (section 3.2.3). In addition, Cdc18-C-D286G is incapable of causing any level of rereplication when overexpressed. This suggests that D286 is the more important acidic residue in the WB domain for ATP hydrolysis.

3.3 Discussion

The Cdc6 protein has been shown to possess ATPase activity in a human *in vitro* system, and here its WA and WB motifs are responsible for ATP binding and hydrolysis respectively (Herbig et al., 1999). It is thus reasonable to assume that mutations to the WA and WB motifs in other systems will abrogate or remove these respective biochemical activities. In this chapter, specific mutations to the WA, WB and SI motifs have enabled the construction of a model for nucleotide metabolism by Cdc18. Cdc18 can associate with chromatin irrespective of its nucleotide state, but ATP binding is absolutely required for both its checkpoint and initiation functions. Checkpoint activation does not require ATP hydrolysis, but hydrolysis is required for MCM loading and DNA replication. The SI domain plays an essential role in either enabling ATP hydrolysis or in translating hydrolysis into catalytic activity.

This model has strong similarities with the Cdc6 models in other organisms. In most cases it appears that chromatin association is not regulated by nucleotide binding, with the exception of Cdc6 in *Xenopus*, which requires ATP binding for chromatin association. In all cases the binding and hydrolysis of ATP appears to be required for the loading of the MCM complex onto chromatin, a prerequisite for DNA replication. Whilst many mutations of the WB motif gives rise to reduced MCM loading and impaired DNA replication (often giving rise to unfaithful duplication of part or all of the genome), the DE286AA mutation in fission yeast eliminates MCM loading and replication is apparently absent. The requirement for an intact SI motif in fission yeast for MCM loading is consistent with results in budding yeast, where SI mutations also impair MCM loading and DNA replication.

The relationship between nucleotide status and checkpoint function has only been assessed in fission yeast and budding yeast. ATP binding is required for checkpoint function in the former but not the latter yeast.

Despite significant homology between the eukaryotic and archaeal Cdc6-like proteins, the function and mechanism of Cdc6 may differ dramatically between the two kingdoms. The Cdc6-like factor in *Sulfolobus solfataricus* recruits MCM proteins to DNA containing bubble and fork structures, but this activity does not require DNA association or an intact WA motif (De Felice et al., 2004).

The results in this chapter have also suggested that rereplication caused by Cdc18 overexpression depends upon the ability of the overexpressed factor to load MCM proteins onto chromatin. This implies that when Cdc18 is expressed at high levels it can load significant amounts of MCM proteins without the need for increased Cdt1 levels. This elevated MCM loading escapes the normal controls which prevent rereplication and stimulates replication of regions that have already been duplicated. These results are consistent with the findings that Cdc18-induced rereplication requires functional Mcm4 (Kearsey et al., 2000). In addition these results are consistent

with findings in budding yeast, where ts mutant *cdc6-3* displays re-initiation of replication, increased DNA content and constant MCM chromatin association (Liang and Stillman, 1997). Interestingly, the C-terminus of Cdc18 is still able to induce rereplication when it carries the E287G mutation, but not when it carries the D286G mutation. This suggests that the conserved aspartate residue in the WB motif is the more important acidic residue for ATP hydrolysis. It is the glutamate residue of the WB motif that has been targeted in all other systems to understand Cdc6 function, and this may explain the leaky phenotypes produced by such WB mutant proteins.

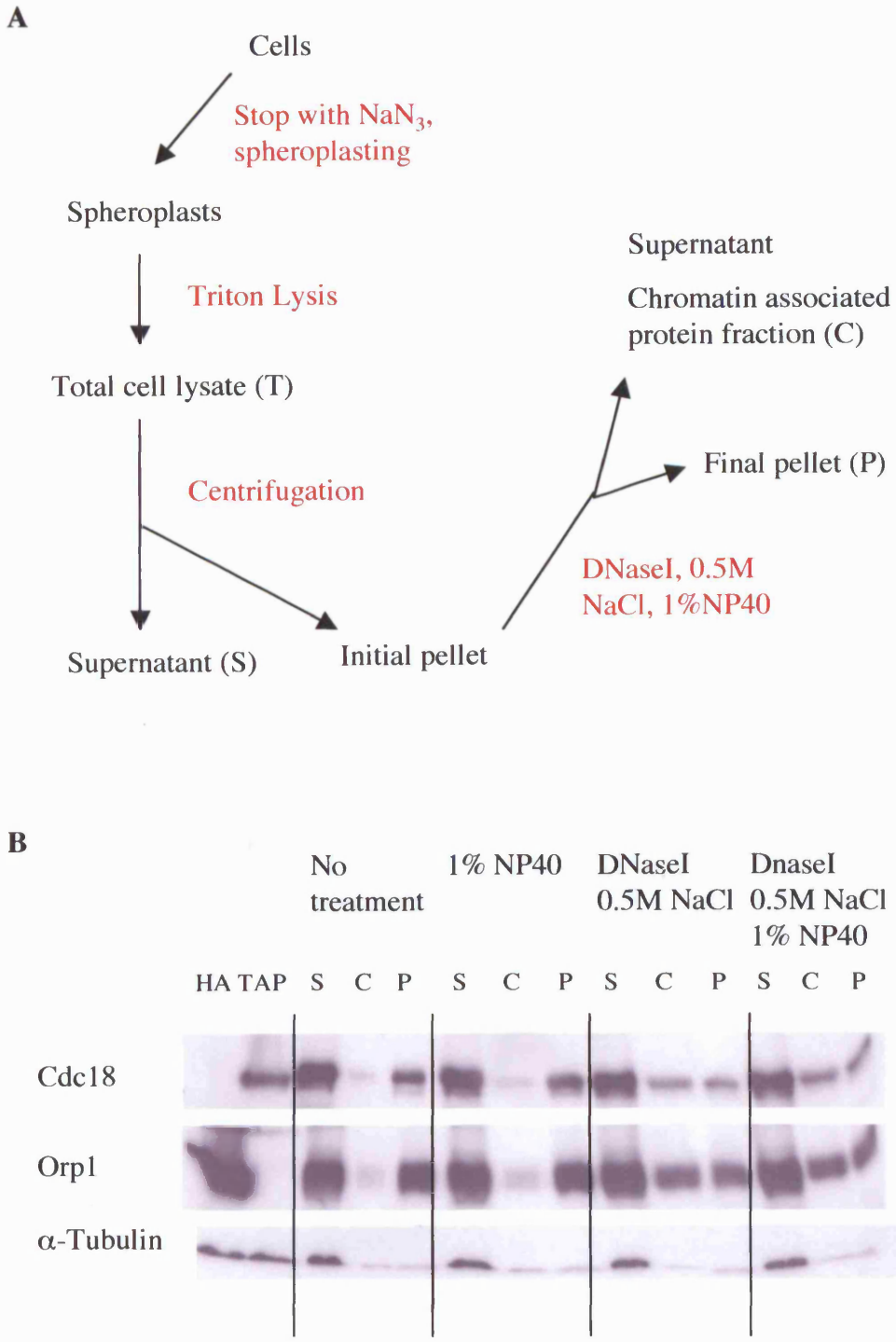
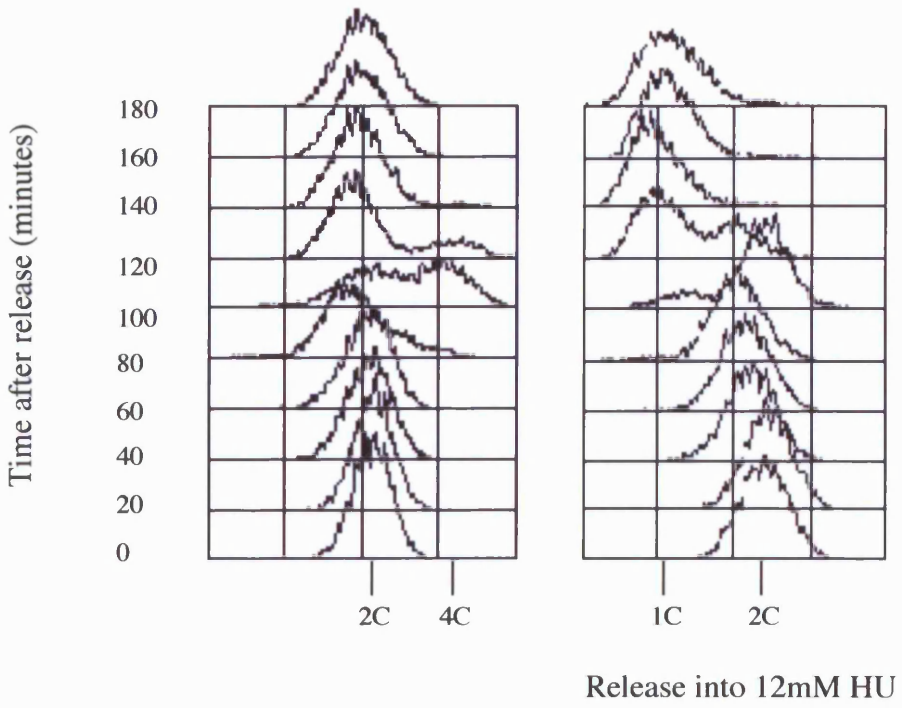


Figure 3.1 Development of the chromatin association assay

A). Summary of chromatin association protocol.

B). Release of Cdc18 and Orp1 from the initial pellet requires treatment with DNaseI in 0.5M NaCl. Total extracts were used as antibody controls: HA= *cdc25-22 orp1-HA*, TAP= *cdc25-22 cdc18-TAP*.

A



B

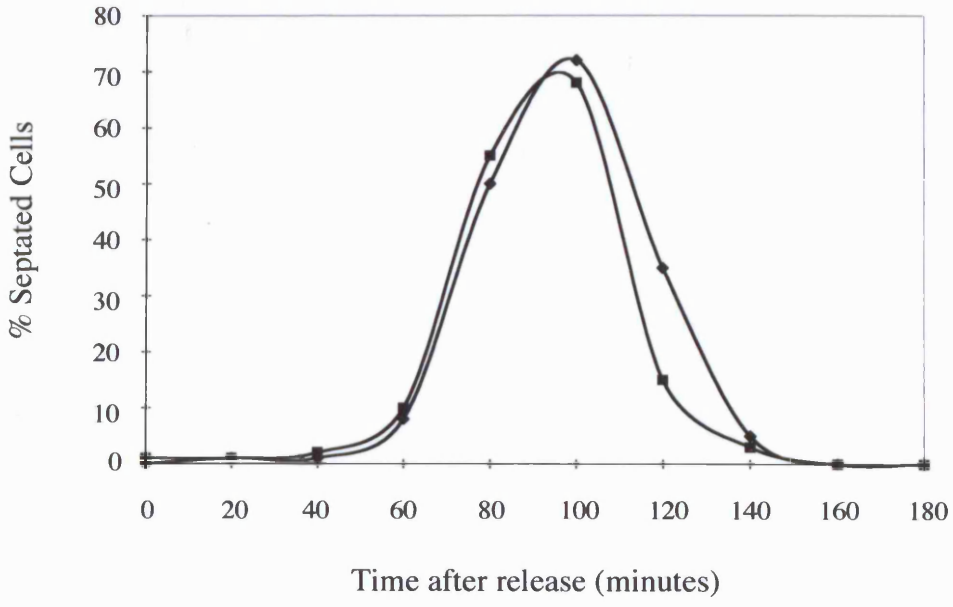


Figure 3.2 Chromatin association of proteins in a synchronous cell cycle

A) FACS profiles of cells released into a synchronous mitotic cell cycle, in the absence or presence of HU. B) Septation index of the two cell populations: without HU (■) and with HU (◆).

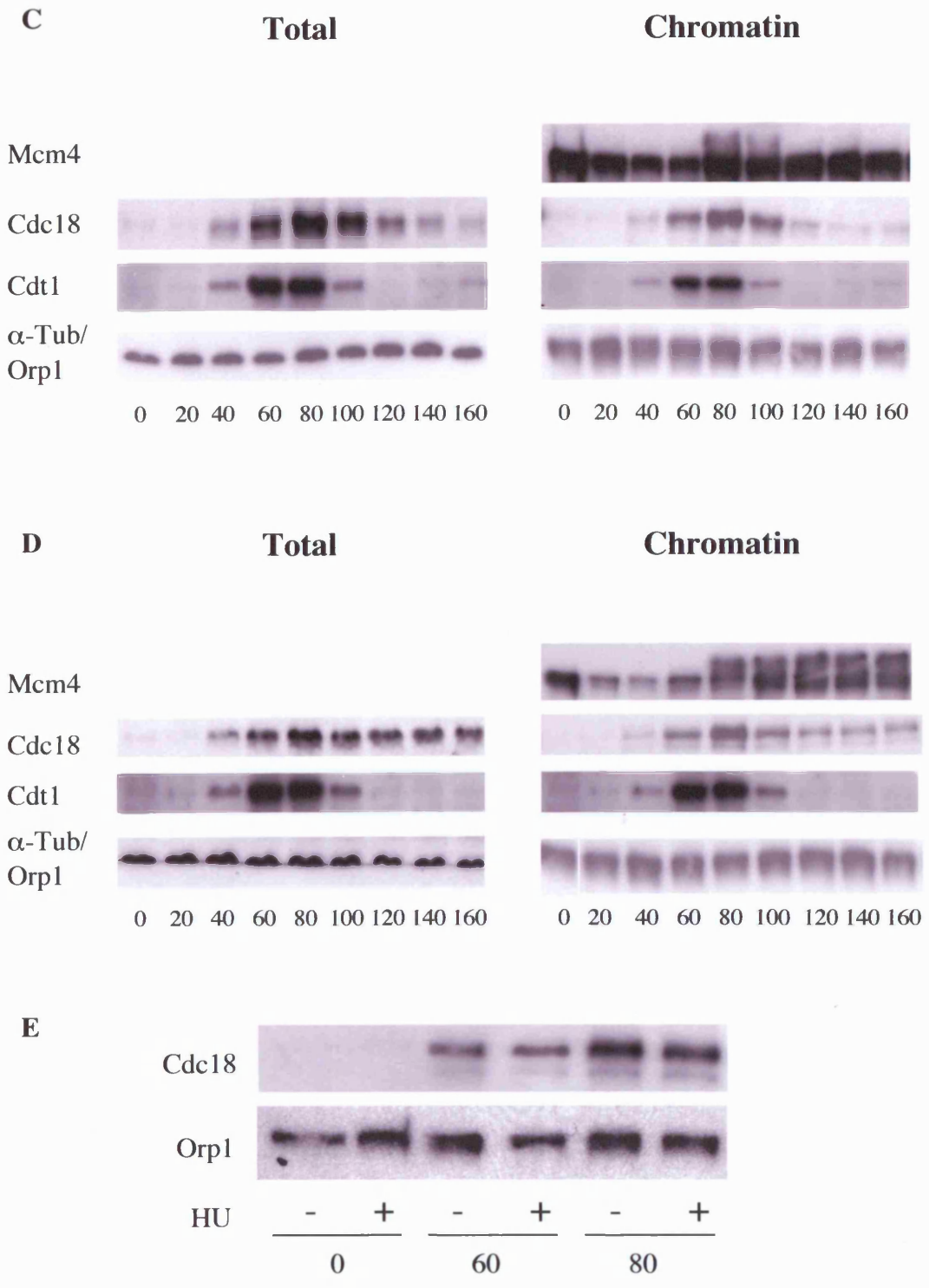


Figure 3.2 continued

C) Analysis of proteins in total and chromatin extracts in a synchronous cell cycle. All numbers refer to time after release from *cdc25-22* block in minutes. α-Tubulin and Orp1 have been used as loading controls for total and chromatin blots respectively. D) The same analysis with HU added at release. E) Comparison of Cdc18 levels with or without HU at various stages of the cell cycle.

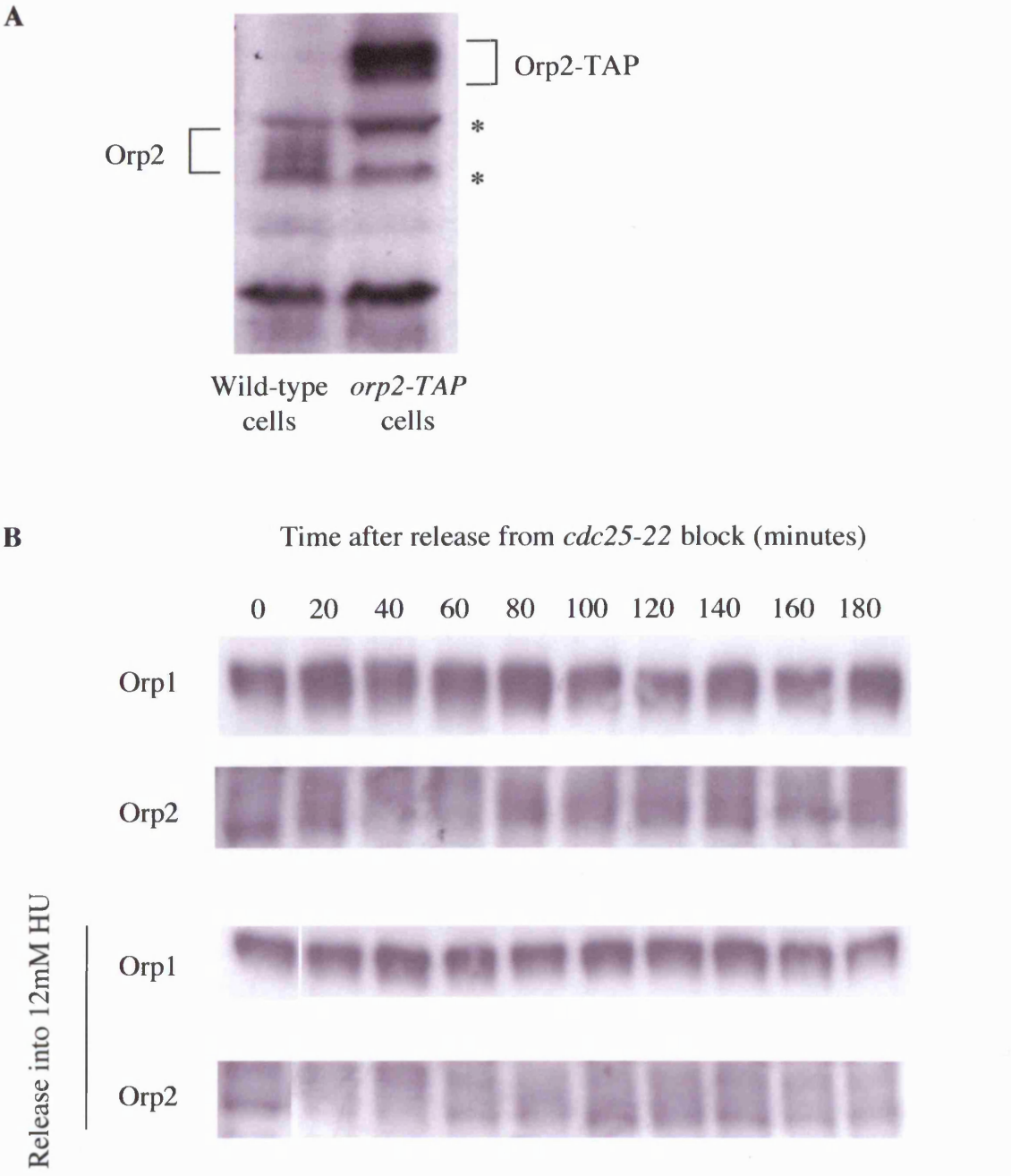


Figure 3.3 Orp2 is constitutively bound to chromatin throughout the cell cycle

A) Polyclonal anti-Orp2 antibody recognises Orp2 isoforms, at the predicted size of ~65kDa, between two cross-reacting bands (*). Total cell extracts have been used for this Western blot, with approximately 50μg protein loaded per lane. B) The total amount of Orp2 bound to chromatin is unchanged throughout the cell cycle or with HU treatment.

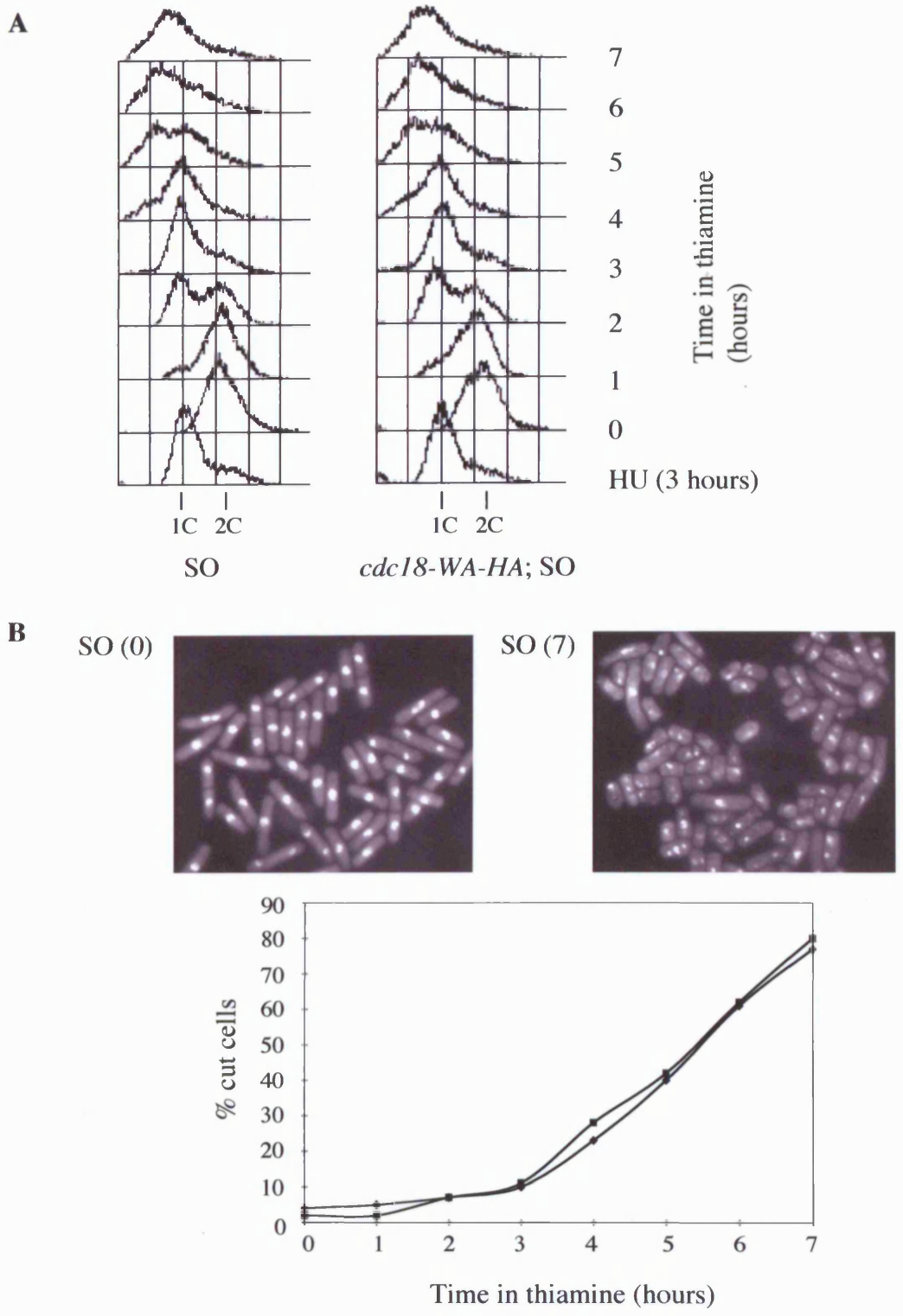


Figure 3.4 A functional WA domain is required for DNA replication and checkpoint function but not for chromatin binding.

A) FACS profiles of the described strains in the presence of HU or thiamine. B) Illustration of the cut phenotype as observed from DAPI staining, and the rate of appearance of cut cells in the timecourse for the SO strain (◆) and the *cdc18-WA-HA*; SO strain (■).

C

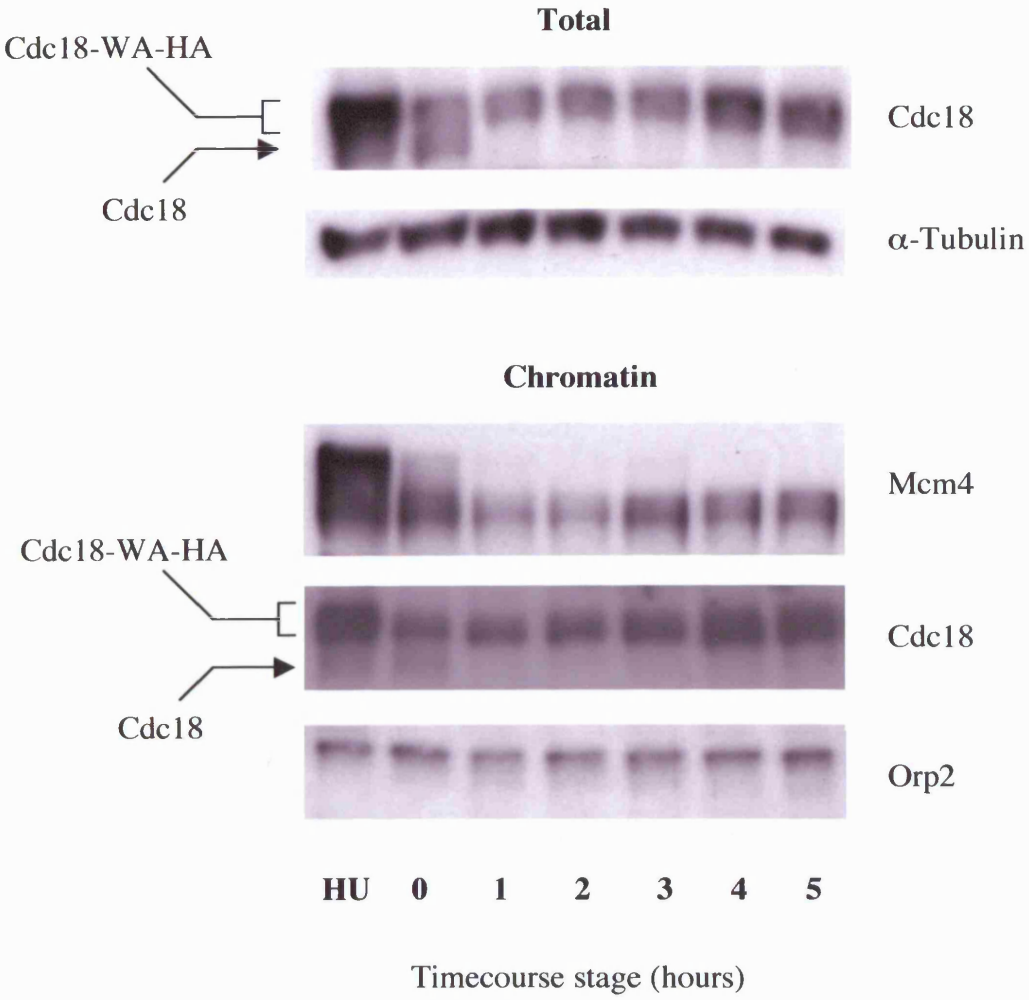


Figure 3.4 continued

C) Cdc18-WA can bind chromatin but can not load Mcm4. Note that the level of wild-type Cdc18 produced from the *nmt81* promoter is significantly lower than the level of Cdc18-WA produced from the endogenous promoter. In addition, the level of Cdc18 produced from the endogenous promoter increases upon HU treatment, whereas that of Cdc18 produced from the constitutive *nmt81* promoter does not.

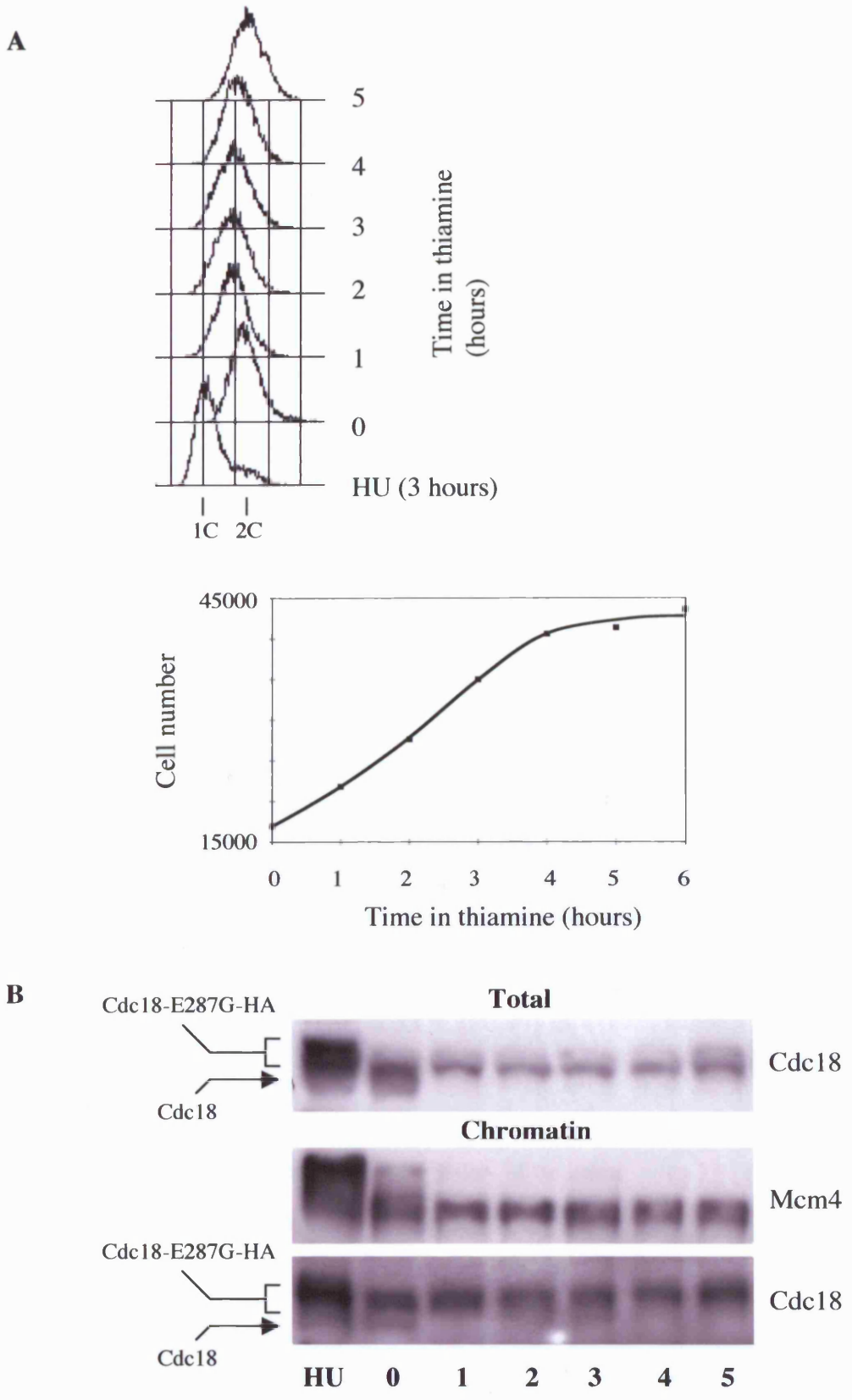


Figure 3.5 Cdc18-E287G binds chromatin and causes a late S phase block

A) In the presence of thiamine *cdc18-E287G-HA*; SO arrests at late S with 2C DNA. B) Cdc18-E287G binds to chromatin and there is no Mcm4 loaded at the block point.

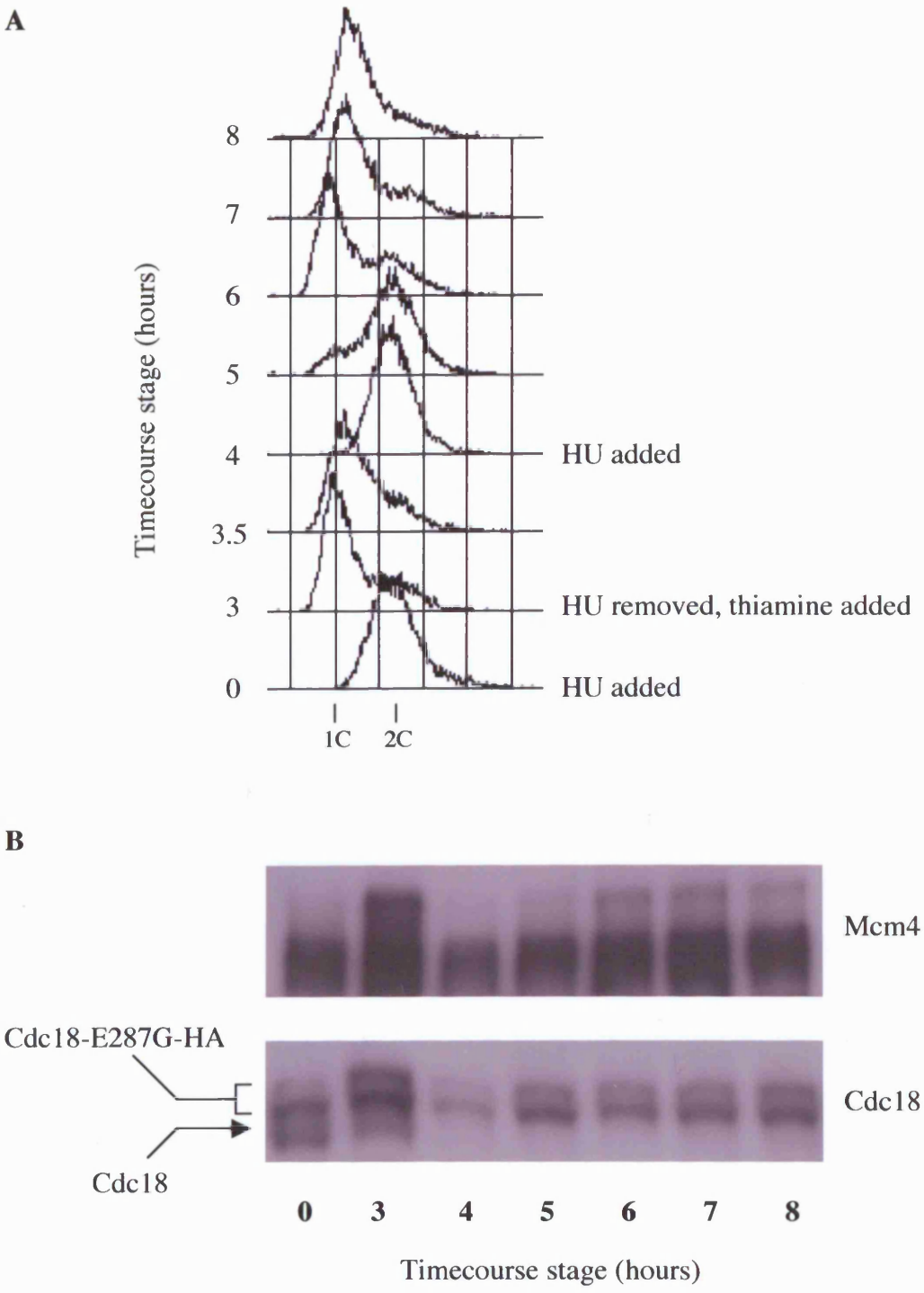


Figure 3.6 Cdc18-E287G has the ability to load Mcm4 onto chromatin

A) FACS profile of the *cdc18-E287G-HA*; SO strain throughout the described timecourse. B) Western blots of chromatin fractions from the timecourse. In an HU block in the apparent absence of wild-type Cdc18 (6-8 hours), Cdc18-E287G can bind chromatin and loads a significant amount of Mcm4 onto chromatin.

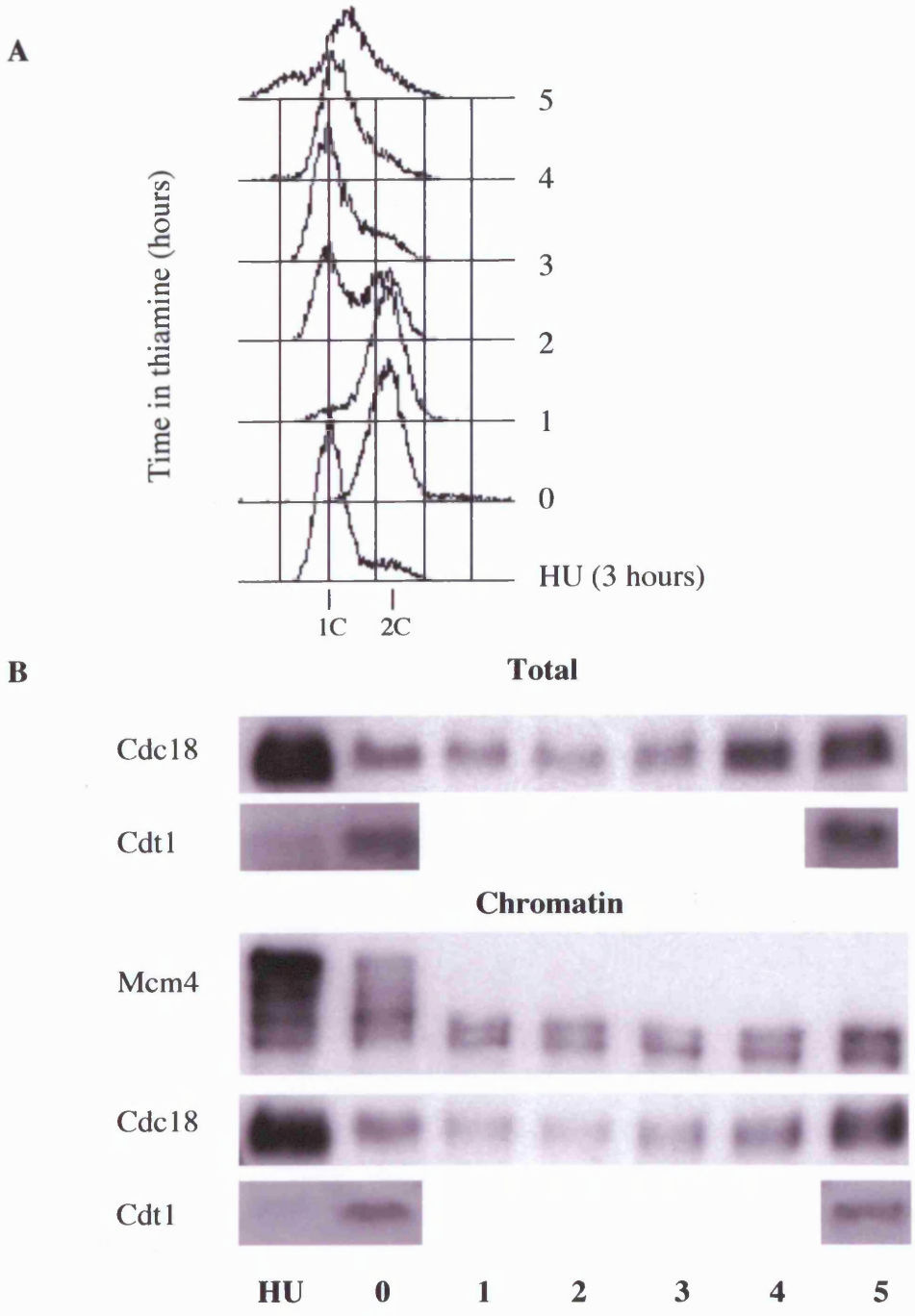


Figure 3.7 Cdc18-DEAD can not load Mcm4 onto chromatin and hence can not initiate replication

A) In the presence of thiamine *cdc18-DEAD-midT;* SO arrests with 1C DNA content. At 5 hours the S phase checkpoint begins to decay, with 12% of the cells displaying the cut phenotype. B) The Cdc18-DEAD protein can bind chromatin but can not load Mcm4. Note that Cdt1 is present and chromatin bound at the block point, locating the block to a position prior to the HU block point. This will be discussed further in chapter 4.

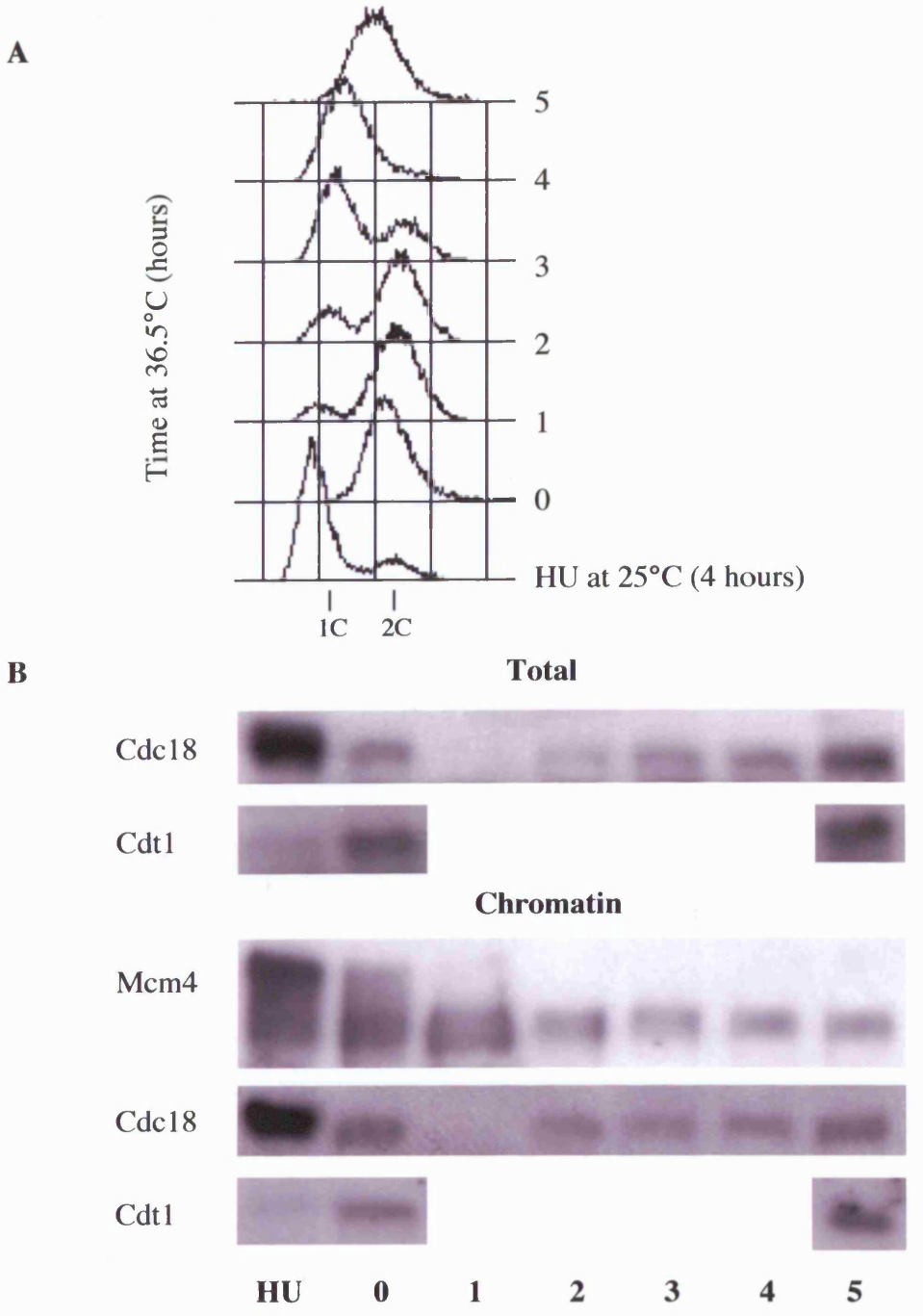


Figure 3.8 Cdc18-3.9 can not load Mcm4 onto chromatin and hence can not initiate replication at 36.5°C

A) At 36.5°C ts mutant *cdc18-3.9* arrests with 1C DNA content. The shift of the peak to the right between 4 and 5 hours is due to cell elongation. In an extended timecourse, the peak continues to drift to the right, past the 2C mark, in close correlation with increased cell length. B) The Cdc18-3.9 protein can bind chromatin but can not load Mcm4 at 36.5°C. Note that Cdt1 is present and chromatin bound at the block point, locating the block to a position prior to the HU block point. This will be discussed further in chapter 4.

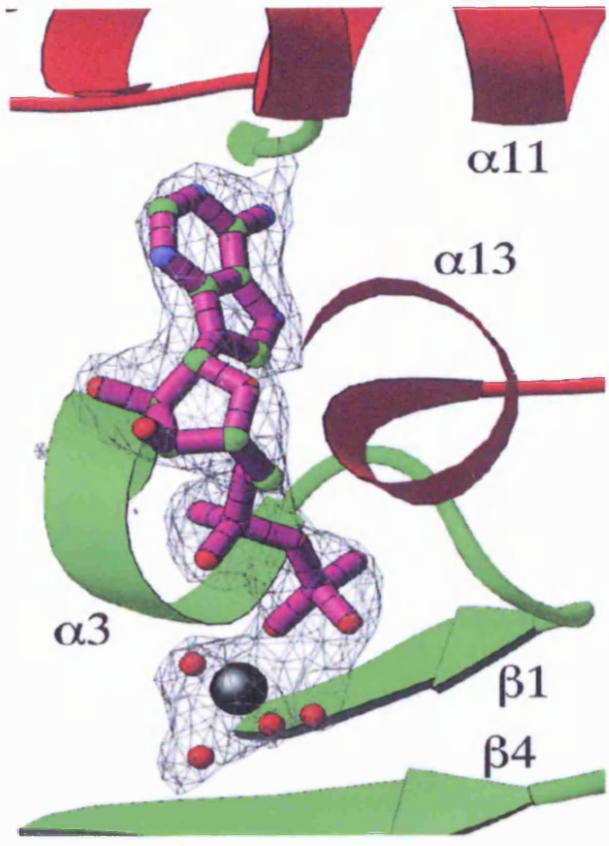


Figure 3.9 Structure of the active site of *P. aerophilum* Cdc6

The ADP (purple) and Mg (black) components lie opposite the β sheet (β4) of the Sensor I domain. β4 contains I164, the equivalent residue to G317 in fission yeast. Also shown here are β1/α3 of the WA domain and α13 of the Sensor II domain.

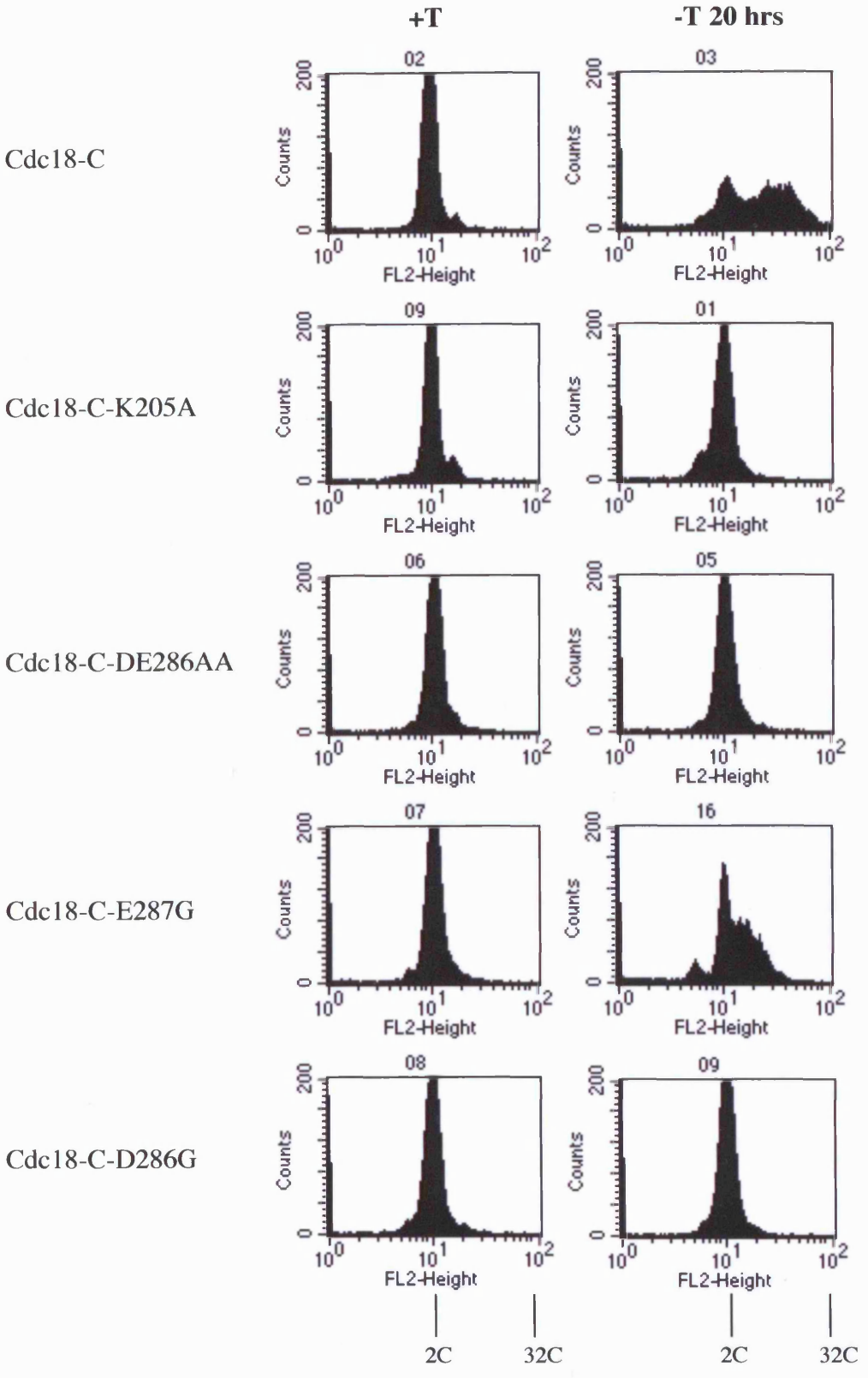


Figure 3.10 Rereplication caused by overexpression of Cdc18-C requires intact WA and WB motifs

Logarithmic FACS profiles for transformants in thiamine (+T) or 20 hours after thiamine removal (-T 20 hrs).

Chapter 4. Analysis of the S phase checkpoint function of Cdc18

4.1 Introduction

Depletion of the Cdc6 protein in fission yeast and budding yeast leads to a loss of DNA replication and aberrant entry into mitosis (Kelly et al., 1993; Piatti et al., 1995). This demonstrates that in these organisms the Cdc6 protein is required to initiate a replication checkpoint signal as well as to initiate DNA replication. The same phenotype is demonstrated in deletion mutants of other replication factors, leading to the generally accepted idea that the replication intermediates resulting from the actions of the initiation factors are responsible for the replication checkpoint signal. Certainly, Cdc6 does not appear to be required for DNA replication after the loading of MCM proteins onto replication origins (Donovan et al., 1997; Hua and Newport, 1998; Labib et al., 1995; Rowles et al., 1999). However, recent evidence demonstrates that Cdc6 persists in the cell following replication initiation, and that it plays a direct role in checkpoint activation or maintenance. Cdc6 is present in G2 in *Drosophila* (Crevel et al., 2005), and in human cells and *Xenopus* egg extracts Cdc6 is chromatin-bound throughout S and G2 (Alexandrow and Hamlin, 2004; Coverley et al., 2000; Mendez and Stillman, 2000). Overexpression of Cdc6 in G2 in human cells blocks entry into mitosis in a Chk1-dependent manner (Clay-Farrace et al., 2003). In *Xenopus* egg extracts, Chk1 activation in response to replication inhibition (using aphidicolin) is dependent upon Cdc6 (Oehlmann et al., 2004).

Recent work within the laboratory has also provided strong evidence for a direct role for Cdc18 in the S phase checkpoint. Moderate overexpression or stabilisation of Cdc18 gives rise to ectopic activation of Rad3 and cell cycle arrest, without apparent induction of rereplication (Damien Hermand and Naomi Fersht, unpublished data). Crucially, it has been shown that the S phase checkpoint is lost from cells blocked with HU when *cdc18* transcription is turned off (Damien Hermand, unpublished data). This demonstrates that

Cdc18 is required to maintain the S phase checkpoint in these circumstances. In contrast, the S phase checkpoint is not lost in HU blocked cells when *cdt1* transcription is suppressed.

In this chapter I present evidence suggesting that Cdc18 is present and chromatin-bound throughout S phase. In addition, I demonstrate that Cdc18 is required to maintain the S phase checkpoint in the cell cycle arrests imposed by *cdc17-K42* and *pol1-1* ts mutants, as well as in an HU block. The mechanism of Cdc18 checkpoint function is explored; checkpoint function appears to require nucleotide binding and association with chromatin, but does not apparently require replication intermediates.

4.2 Results

4.2.1 Cdc18 is present in a range of cell cycle blocks

Cell synchronisation experiments have shown that Cdc18 accumulates in the cell in G1 and S phases of the cell cycle, and disappears as cells go into G2 (Nishitani and Nurse, 1995). From figure 3.2 it appears that Cdc18 protein levels peak early in S phase. To further assess the natural history of Cdc18 in the cell cycle, cells were synchronised at various points using a variety of ts mutants (in addition to using HU). The mutants used, and the points in the cell cycle where they arrest at restrictive temperature, are discussed herein (and summarised in table 4.1).

The *cdc10* gene is required for S phase. The Cdc10 protein is a transcriptional activator for a number of genes required for DNA replication, including *cdc18* and *cdt1* (Baum et al., 1998; Hofmann and Beach, 1994; Kelly et al., 1993) The ts *cdc10* mutants, such as *cdc10-v50*, arrest with 1C DNA and mitosis is restrained from G1 in a *rum1*-dependent manner (Moreno et al., 1994; Moreno and Nurse, 1994).

The *orp1* gene encodes Orp1, part of the ORC complex required for DNA replication. The *orp1-4* mutant arrests with 1C DNA and uninucleate cells. Chromosomes at the block point do not enter a pulsed-field gel, suggesting the

presence of replication intermediates, and an early S phase block (Grallert and Nurse, 1996). However, 2D gel analysis failed to identify replication intermediates at the block point, suggesting a late G1 arrest (Synnes et al., 2002). To support this, the checkpoint induced by the arrest is dependent upon Chk1 (not Cds1, the component responsible for the S phase checkpoint in an HU block), and Cdt1 is present at the block point (figure 4.1).

The *cdc23* gene is essential for DNA replication (Gregan et al., 2003) and the *cdc23-M36* mutant arrests at late S phase: cell number plateaus after doubling and cells display 2C DNA at the arrest (Liang and Forsburg, 2001). By the same analysis, late S phase blocks are caused by the *pol1-1* and *cdc17-K42* mutants (D'Urso et al., 1995, al-Khodairy and Carr, 1992). *Pol1* encodes DNA polymerase α, which initiates leading and lagging strand synthesis in conjunction with primase (Baker and Bell, 1998). *Cdc17* encodes DNA ligase, which ligates the Okazaki fragments of the lagging strand (Waga and Stillman, 1994).

In the final two mutants, *cdc25-22* and *cdc27-P11*, cells arrest with 2C DNA. Cell number plateaus after only an approximate 30% increase in cell number, indicating a G2/M arrest. Cdc25 activates Cdc2 at the G2/M transition, whilst Cdc27 encodes a subunit of the DNA polymerase δ (pol δ) complex (MacNeill et al., 1996). This complex is involved in a number of DNA metabolism processes, including replication, repair and recombination (Bermudez et al., 2002). It is not clear why the *cdc27-P11* mutant arrests at G2/M (rather than in S phase). It may be that pol δ is responsible for repair or recombination processes in an unperturbed G2.

Table 4.1 Summary of ts mutants used in this study

Ts mutant	Protein product	Arrest point
cdc10-v50	Cdc10 transcription factor	G1
orp1-4	Orp1	G1/S
cdc23-M36	Cdc23	Late S
pol1-1	DNA polymerase α	Late S
cdc17-K42	DNA ligase	Late S
cdc27-P11	DNA polymerase δ subunit	G2
cdc25-22	Cdc25	G2

Each ts mutant was crossed into a *cdc18-TAP* background prior to analysis. Exponentially growing cultures of the mutants were shifted from 25°C to 36.5°C, and cell number followed for 6 hours. For strains arresting early in the cell cycle (G1 or S), samples for total and chromatin protein were taken 5 hours post shift. For strains arresting late in the cell cycle (G2/M), samples were taken 3 hours post shift. Samples were also collected for wild-type cells (with *cdc18-TAP*) in a cycling population (36.5°C) and in an HU block (5 hours at 36.5°C). Total and chromatin-bound protein for each cell-cycle status is shown in figure 4.1.

As previously observed, Cdc18 and Cdt1 are present (and chromatin-bound) in a cycling population. Cdc18 levels (and Mcm4 chromatin binding) increase upon treatment with HU, whereas Cdt1 disappears in such circumstances. As expected, Cdc18 and Cdt1 are absent when their transcriptional activator, Cdc10, is inactivated. These proteins are also absent from the G2/M block imposed by the *cdc25-22* mutant, although Cdc18 can be detected at much higher exposure. This either represents a genuine and very low-level expression of Cdc18 at the block-point, or is perhaps a consequence of leakage of a small proportion of the population into G1.

At the *orp1-4* arrest point both Cdc18 and Cdt1 are present and chromatin-bound (although not at the levels seen in an HU block). In addition, Mcm4 is

not bound to chromatin, supporting the idea that the arrest point lies at late G1 as opposed to early S phase. This latter result is in agreement with an *in situ* chromatin assay which showed that Mcm4 loading is absent in the *orp1-4* mutant (Kearsey et al., 2000). These data collectively suggest that the Orp1 defect in these cells does not interfere with the binding of the loading factors, Cdc18 and Cdt1, to replication origins, but rather alters the platform onto which the MCM proteins are normally loaded. Alternatively, it may be that the amount of Cdc18/Cdt1 loaded onto origins, or the manner in which they bind to ORC, is insufficient to allow MCM loading.

All of the induced late S phase arrests lack Cdt1, or Mcm4 chromatin binding, as expected. However, Cdc18 is present and chromatin-bound in all cases (at a level similar to that seen in an *orp1-4* block). This finding has two possible interpretations. Either Cdc18 is present and chromatin-bound throughout S phase, or Cdc18 expression is induced in late S phase blocks in response to incomplete or damaged replication.

Also of interest is the picture presented at the G2/M block caused by the *cdc27-P11* mutant. Cdt1 and chromatin-associated Mcm4 are absent, but Cdc18 is present (and chromatin-bound) to the level seen in the late S phase blocks. This could mean one of two things. Firstly, it may be that Cdc18 is normally present in G2 cells, at a point prior to the *cdc25-22* block. Given that all data produced thus far in fission yeast shows that Cdc18 levels drop as cells complete S phase, and rise again as cells enter the next cell cycle, this seems unlikely. More likely is that Cdc18 is being induced in response to the problems experienced when Cdc27 is inactivated. If, indeed, a G2/M arrest is caused by failure of pol δ to repair damage normally experienced in G2 for example, then Cdc18 may be produced as a checkpoint or repair response. In support of this, Cdc18 transcript level has been shown to double in response to DNA damaging agents (Chen et al., 2003), and that this induction is greatly increased if cells are synchronized in G2 first (Watson et al., 2004). It would be interesting to ascertain if the Cdc18 protein itself is induced in response to DNA damage in G2.

The presence of Cdc18 after initiation of replication could, in principle, put the cell at risk of rereplication. However, a number of controls exist to prevent rereplication in the cell (reviewed in Nishitani and Lygerou, 2002). The removal of Cdt1 at the G1/S transition, for example, would enable Cdc18 to exist in the cell at later points in the cell cycle without negating genome stability.

4.2.2 Cdc18 is required for maintenance of the S phase checkpoint

The inability of cells to establish an S phase checkpoint in the absence of *cdc18* has been well characterised (Kelly et al., 1993). In recent times this checkpoint role has been expanded in two important ways. Firstly, it appears as though, contrary to popular belief, this initiation function is due to the protein itself, and not necessarily reliant on the replication intermediates produced by the initiation function of Cdc18 (section 3.2.3 and explored further in 4.3.5). Secondly, the S phase checkpoint is lost from cells blocked in HU when *cdc18* transcription is turned off (Damien Hermand, unpublished data). This demonstrates that Cdc18 is required to maintain the S phase checkpoint in these circumstances. The presence of Cdc18 in the G1 and S phase blocks analysed in the previous section suggested that Cdc18 might play a universal role in the maintenance of the S phase checkpoint. As such, the requirement for Cdc18 in the checkpoints induced by the *cdc17-K42* and *pol1-1* mutants was analysed.

To replicate the requirement for Cdc18 to maintain an HU-induced checkpoint, wild-type and switch-off (SO) strains were arrested in 12mM HU for 3 hours at 32°C (figure 4.2A). At this point thiamine was added to the cultures to repress *nmt* transcription. In the continued presence of HU, the SO strain enters mitosis and produces cut cells (figure 4.2B). The wild-type strain also eventually cuts in a prolonged HU incubation, but at a rate much lower than that seen in the SO strain. Thiamine addition significantly reduces the levels of Cdc18 in the SO strain but not in the wild-type strain (figure 4.2A).

The *nmt81-cdc18+* gene was then crossed into the *cdc17-K42* background, producing the *cdc17-K42;* SO strain. Both the *cdc17-K42* and *cdc17-K42;* SO strains were arrested at late S phase by shifting to 36.5°C (figure 4.3A). At this point thiamine was added to the cultures to repress *nmt* transcription. At continued restrictive temperature, the *cdc17-K42;* SO strain enters mitosis and produces cut cells (figure 4.3B). The *cdc17-K42* strain also eventually cuts at restrictive temperature, but at a rate much lower than that seen in the *cdc17-K42;* SO strain. Thiamine addition significantly reduces the levels of Cdc18 in the *cdc17-K42;* SO strain but not in the original *cdc17-K42* strain (figure 4.3A).

Next, the *nmt81-cdc18+* gene was then crossed into the *pol1-1* background, producing the *pol1-1*; SO strain. Both the *pol1-1* and *pol1-1*; SO strains were arrested at late S phase by shifting to 36.5°C (figure 4.4A). At this point thiamine was added to the cultures to repress *nmt* transcription. At continued restrictive temperature, the *pol1-1*; SO strain enters mitosis and produces cut cells (figure 4.4B). The *pol1-1* strain also eventually cuts at restrictive temperature, but at a rate lower than that seen in the *pol1-1*; SO strain. Thiamine addition significantly reduces the levels of Cdc18 in the *pol1-1*; SO strain but not in the original *pol1-1* mutant (figure 4.4A).

These results show that Cdc18 is important for maintaining the cell cycle arrests caused by the *cdc17-K42* and *pol1-1* mutants in addition to that caused by HU treatment. This suggests that Cdc18 is universally required for the S phase checkpoint. There are likely to be a range of structures produced in such circumstances, from stalled forks to unprocessed Okazaki fragments and DNA damage. Hence Cdc18 may be required to restrain mitosis when replication is perturbed by a range of insults.

4.2.3 Maintenance of the S phase checkpoint requires nucleotide binding

In chapter 3 it was shown that an intact WA domain, and hence probably nucleotide binding, is required to initiate an S phase checkpoint. As it is now

apparent that Cdc18 is required to maintain the S phase checkpoint, I was interested to know if nucleotide binding or/and hydrolysis was required for this checkpoint maintenance. To this end, the approach used in section 4.2.2 was applied to the following strains: *cdc18-WA-HA*; SO, *cdc18-DEAD-midT; SO* and *cdc18-3.9* (figure 4.5). These strains were arrested in 12mM HU (3 hours at 32°C for the SO strains and 4 hours at 25°C for the ts mutant) before addition of thiamine (SO strains) or shift to 36.5°C (ts mutant). In the continued presence of HU, *cdc18-WA-HA*; SO enters mitosis and produces cut cells at a rate very similar to that seen with the SO strain. In contrast, *cdc18-DEAD-midT; SO* and *cdc18-3.9,* like the wild-type strain, cut at a much lower rate in prolonged HU incubation.

These results show that the WA domain of Cdc18 is required to maintain the S phase checkpoint. In contrast, the WB and Sensor I domains are not, suggesting that ATP hydrolysis is not required for checkpoint maintenance. However, it is possible that ATP hydrolysis in the WB and Sensor I mutants used here has been stimulated by wild-type activity in the initial HU block, if binding and hydrolysis occurs *in trans* within Cdc18 complexes. Either way, though, proteins which are bound to nucleotide (ATP or ADP) are capable of maintaining the S phase checkpoint, whereas a protein that can not bind nucleotide (WA mutant) can not maintain the checkpoint. Hence maintenance of the S phase checkpoint requires nucleotide binding.

4.2.4 Cdc18 checkpoint function correlates with Cdc18 chromatin binding

Another important question from the studies presented so far is whether the checkpoint function of Cdc18 requires localisation to chromatin. If chromatin binding is essential, then we should see Cdc18 bound to chromatin in all cases where an S phase checkpoint has been induced. Indeed, as shown by figure 4.1, Cdc18 is bound to chromatin in all S phase cell-cycle arrests tested. In addition, the checkpoints initiated by the WB and Sensor I mutants correlate with Cdc18 chromatin association. This contrasts with a lack of an absolute correlation between the checkpoint and Cdt1/Mcm4 chromatin association. In

another ts *cdc18* mutant, *cdc18-3.6*, a late S phase arrest correlates with Cdc18 chromatin binding but not with Cdt1/Mcm4 chromatin binding (figure 4.6). Hence a positive checkpoint signal correlates with Cdc18 chromatin association. However, if chromatin-bound Cdc18 sends the checkpoint signal then this activity requires nucleotide binding: the Cdc18-WA protein binds chromatin yet does not initiate a checkpoint (section 3.2.2).

To explore this further I analysed a checkpoint active form of Cdc18, the TA mutant (Damien Hermand, unpublished). In the Cdc18-TA protein the acceptor threonine residue in all six *cdc2* phosphorylation sites has been mutated to alanine, and these changes greatly increase the stability of the protein. Cells containing Cdc18-TA are viable only in the absence of Rad3 (in *rad3Δ*, or *rad3-ts* at 36.5°C); ectopic activation of Rad3 by Cdc18-TA causes cell cycle arrest. In a *cdc18-TA rad3-ts* strain a shift from 36.5°C to 25°C induces a G2/M arrest, suggesting that Cdc18-TA is present in G2 cells and can send a checkpoint via Rad3 from this position (Damien Hermand, unpublished). I was interested to see if Cdc18-TA was bound to chromatin in G2 cells.

For this analysis a *cdc18-TA rad3Δ cdc25-22* strain was synchronised at G2/M by incubating at 36.5°C for 3.5 hours. Shifting to 25°C released these cells into a synchronous cell cycle, and samples were taken at 0 (G2/M), 80 (S) and 120 (G2) minutes post release (figure 4.7). A control strain of *cdc18+ rad3Δ cdc25-22* was subjected to the same procedure, and Cdc18 in total and chromatin extracts was detected using anti-Cdc18 polyclonal antibody. At the G2/M block levels of both Cdc18-TA and Cdc18 are very low. The levels of Cdc18-TA in cycling cells and during S phase are several fold higher than wild-type levels, as expected from the increased stability of the mutant protein. At 120 minutes cells have completed S and have entered G2. Here, wild-type protein has disappeared, whereas Cdc18-TA persists and is chromatin-bound. Hence a form of Cdc18 that causes a G2/M arrest when Rad3 is present binds chromatin in G2. These results lead to several conclusions. Firstly, we again see a correlation between Cdc18 chromatin

binding and its ability to send an S phase checkpoint. Secondly, the results illustrate the multi-layered control over rereplication that exist within the cell. In the mutant strain, Cdc18 levels in S phase are elevated, and the amount of Cdc18 in G2 approximately equals that seen in S phase of a wild-type strain. Despite this, there is no evidence of rereplication, demonstrating that mechanisms such as the removal of Cdt1 and phosphorylation of Orp2 are sufficient to prevent rereplication even in the presence of fairly large increases in Cdc18 level. Lastly, the results have implications for the control of Cdc18 chromatin binding. The presence of Cdc18-TA on chromatin in G2 suggests either that it persists on chromatin during replication (and its removal depends on direct degradation) or that Cdc18-TA can rebind chromatin in G2 having been removed during S. In the latter case, as well as targeting the protein for degradation, Cdc18 phosphorylation may prevent its rebinding to chromatin. (Note that phosphorylation does not remove Cdc18 from the chromatin-see section 3.2.1).

Whilst S phase checkpoint activation and maintenance correlates with Cdc18 chromatin binding, it has not been possible to demonstrate conclusively that chromatin association is required for Cdc18 checkpoint function. Such conclusions would require identifying a form of Cdc18 that does not bind to chromatin, which so far has not been identified. In this instance two approaches would be used. If a non-binding form of Cdc18 was found that could still send a checkpoint then we could conclude that the soluble form of Cdc18 would be sufficient for checkpoint function. If a non-binding form was found that could not send a checkpoint, then we should attempt to reattach it to the chromatin. Restoration of the checkpoint would then show that the chromatin-bound form of Cdc18 is responsible for checkpoint function.

4.2.5 Cdc18 checkpoint function appears not to depend upon replication intermediates.

As we saw in chapter 3, mutant forms of Cdc18 (Cdc18-DEAD and Cdc18-3.9) that are capable of binding but not hydrolysing ATP can initiate an S phase checkpoint. In these circumstances it seems likely that replication

intermediates are absent: cells show a 1C DNA peak, Mcm4 is not loaded onto chromatin, and Cdt1 is still present (which at least places the arrest point prior to the HU block point). This would suggest that Cdc18 is able to initiate a checkpoint response irrespective of its loading function which allows replication.

To explore this further, *cdc18-3.9* was subjected to analysis by pulsed-field gel electrophoresis (PFGE). This electrophoresis technique can separate whole fission yeast chromosomes according to their size (figure 4.8). Entry of chromosomes into such gels is retarded by certain structural changes to the DNA, such as chromosome circularisation or the presence of replication intermediates. For instance, chromosomes from cells arrested in S phase by HU do not enter a pulsed field gel, and remain in the loading well. Chromosomes that are structurally unstable or fragmented, such as those prepared from *cdc17-K42* at restrictive temperature, appear on the gel as a smear or a fast migrating band. The chromosomes from the ts mutant *cdc18-3.9* enter the gel at permissive temperature, but are retained in the loading well at restrictive temperature. This suggests the presence of replication intermediates at the ts arrest point that has been studied, in disagreement with the previous data. It may be that this result is misleading, however. As discussed in section 4.2.1, the *orp1-4* ts mutant shows a 1C arrest with Cdc18 and Cdt1, but not Mcm4, bound to chromatin. In this case, as with *cdc18-3.9*, chromosomes do not enter a pulsed field gel at restrictive temperature (Grallert and Nurse, 1996). However, 2D gel-electrophoresis failed to detect any replication intermediates from the early *ars3001* origin in these circumstances (Synnes et al., 2002). It may be that replication intermediates are absent from these cells, but that pre-initiation structures are present (perhaps as a consequence of Cdc18/Cdt1 origin binding) which prevents gel entry. In any case, further analysis of *cdc18-3.9* is needed to fully answer the question of whether Cdc18 sends a checkpoint in the absence of replication intermediates. Such analysis could include looking for the relevant structures by 2D gel electrophoresis or by scanning for amplified regions by DNA microarray analysis. These methods have their own disadvantages however.

For instance, the former is unlikely to have the sensitivity required to find a low number of intermediates across the entire genome, whereas the latter may not be able to recognise the scenario whereby intermediates exist but no DNA synthesis has occurred.

4.3 Discussion

Given the short length of S phase in fission yeast (approximately 20 minutes at 25°C) it has been difficult to assess the behaviour of proteins during this phase of the cell cycle using synchronised cultures. The picture presented in section 4.2.1, using cells arrested at various points of the cell cycle, suggests that, like in metazoan cells, Cdc18 is present and chromatin-bound throughout S phase. It is still possible, however, that Cdc18 is induced in the late S phase arrests analysed in response to stalled or damaged replication. In either case, the presence of Cdc18 in these arrests is required to maintain the checkpoint induced in these circumstances. As Cdc18 is required for checkpoint maintenance at early S (e.g. HU block) and late S (e.g. *cdc17-K42*) it seems that Cdc18 plays a universal role in maintenance of the S phase checkpoint, restraining mitosis whenever replication is perturbed by stalled forks or damage. These results are consistent with the requirement for Cdc6 in aphidicolin-induced Chk1 activation in *Xenopus* (Oehlmann et al., 2004). The presence of Cdc18 in the G2/M arrest imposed by *cdc27-P11* suggests that Cdc18 may even be involved in the response to DNA damage outside of S phase. The roles of the Cdc6 protein as a positive (replication initiation) and a negative (S phase checkpoint) regulator of the cell cycle may explain why both overexpression and underexpression of Cdc6 has been observed in human tumours (Karakaidos et al., 2004; Robles et al., 2002).

Cdt1 is not required for checkpoint maintenance in HU (Damien Hermand, unpublished data), and indeed this factor is not present in any of the cell cycle arrests positioned after replication initiation. The absence of Cdt1 following initiation could enable the continued presence of Cdc18 (to perform a checkpoint role, for example) without the risk of rereplication. MCM loading is also apparently absent at the late S phase blocks, suggesting that chromatin-

bound MCMs are not required for checkpoint maintenance. Indeed, in the *Xenopus* work it was shown that the requirement of Cdc6 for Chk1 phosphorylation does not depend on its ability to load Mcm2-7 onto chromatin.

The inability of the WA mutant protein to maintain the S phase checkpoint in HU-blocked cells suggests that ATP binding is required both to initiate and to maintain the S phase checkpoint. The WB and SI mutants, however, do maintain this checkpoint, demonstrating that Cdc18 forms bound to nucleotide are capable of initiating and maintaining the S phase checkpoint. It may be that Cdc18-ATP initiates the S phase checkpoint, and that Cdc18-ADP maintains the checkpoint after ATP hydrolysis.

In all cases studied, a positive checkpoint signal correlates with Cdc18 chromatin association, suggesting that Cdc18 effects its checkpoint function from the DNA. Such a model would appear to be the most likely, as localisation to the chromatin would allow Cdc18 to detect incompletely replicated or damaged DNA, and to initiate the appropriate signal. Indeed, it appears as though Cdc18 is required to anchor Rad3 to the chromatin in response to HU (Damien Hermand, unpublished data). In *Xenopus* egg extracts, however, aphidicolin-induced Chk1 activation does not require association of Cdc6 with chromatin (Oehlmann et al., 2004). This may reflect fundamental differences between the two species, although much of the Cdc6 protein in the extracts remains nuclear, where it may still be able to detect directly structures such as stalled replication forks. If, indeed, Cdc6 chromatin association during S phase in *Xenopus* is not required for its checkpoint function, then this may reflect an additional role for Cdc6 (aside from its initiation and checkpoint functions). This idea is addressed further in chapter 5.

Demonstrating that Cdc18 chromatin association is required for its checkpoint function will rely upon removing Cdc18 from the DNA. This could involve disrupting the ORC complex, or altering Cdc18 itself. The sole ts Orp mutant

available is *orp1-4*, and Cdc18 still binds to chromatin in this mutant background (section 4.2.1). One approach to altering Cdc18 may be to target its C-terminal winged-helix domain (Liu et al., 2000). The winged-helix domain is responsible for DNA recognition in a number of transcription factors. Two important features of the motif are the recognition helix, which binds in the major groove of DNA, and a ββ wing which contacts the phosphodiester backbone. Whilst Cdc18 interacts with the ORC complex (Grallert and Nurse, 1996; Leatherwood et al., 1996) it may be that Cdc18 also interacts directly with DNA via this winged-helix domain. However, mutations to either the recognition helix or the ββ wing result in late S phase arrest (Liu et al., 2000). This suggests that these mutations do not prevent chromatin association, as it seems highly unlikely that MCM loading and DNA replication (albeit erroneous) can occur without Cdc18 interacting with replication origins. Mutation of the nearby QQK motif, however, does lead to a completely non-functional protein (Liu et al., 2000), and it would be interesting to see if this mutant protein can still associate with chromatin.

Although more work is required, it appears as though Cdc18 can initiate the S phase checkpoint without initiating DNA replication (and hence without replication intermediates). This suggests that Cdc18, perhaps as part of the pre-replicative complex, can inhibit mitosis in the absence of replication, as well as when replication is perturbed by stalled forks or damage. It is still likely that DNA structures provide the signal for Rad3 activation within S phase, and such a signal is likely to be stronger than that provided by the pre-initiation complex; ts mutants arresting within S phase maintain a checkpoint response for longer than those arresting with 1C DNA (data not shown). Within S phase, Cdc18 appears to function as a signalling molecule in the checkpoint response; repression of *cdc18* transcription in an HU block leads to aberrant entry into mitosis yet replication intermediates are still visible (Damien Hermand, unpublished data).

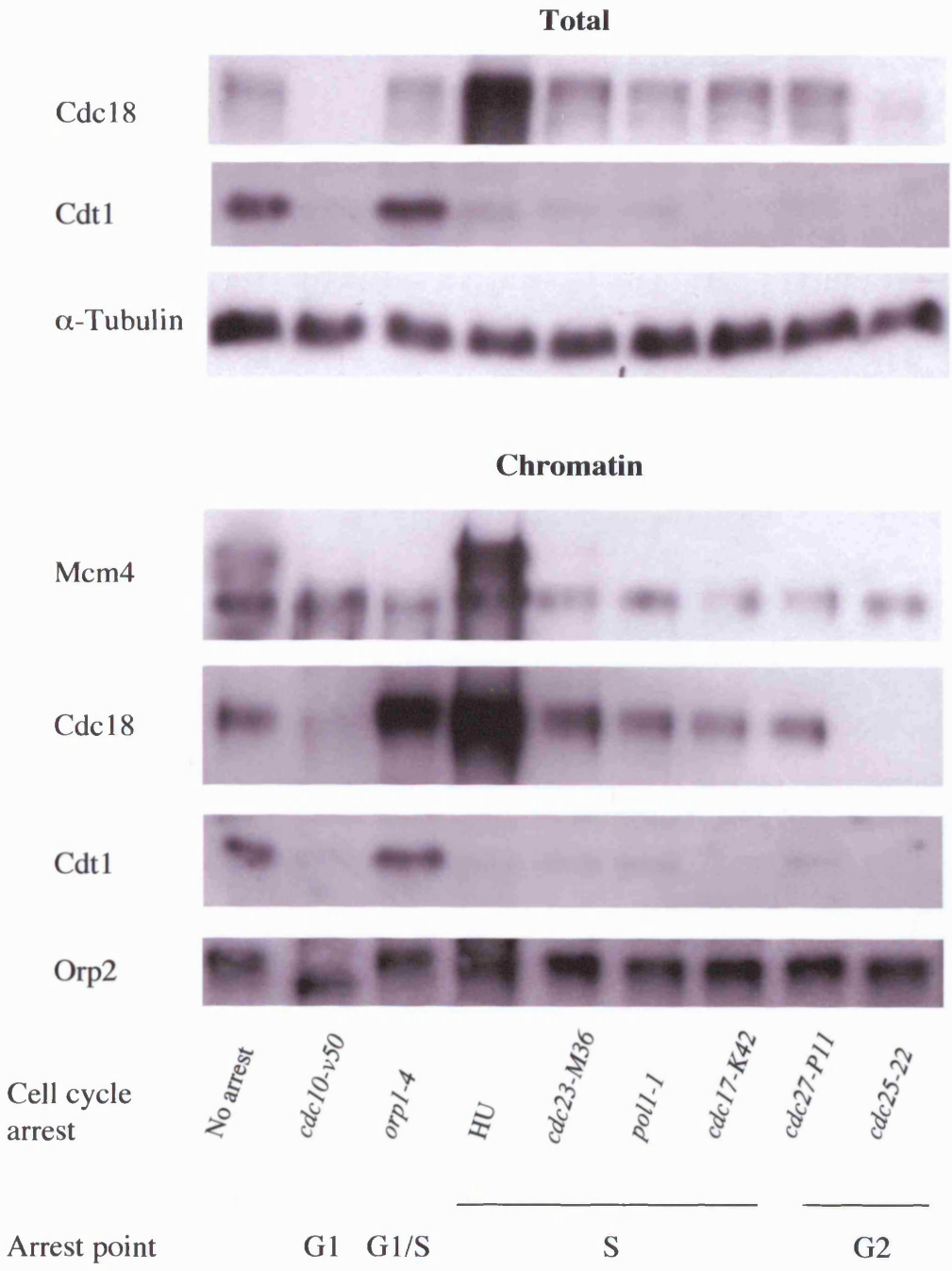


Figure 4.1 Analysis of proteins in a range of cell cycle arrests

Cdc18 is detected using a PAP antibody. α-Tubulin and Orp2 loading controls for total and chromatin blots are shown.

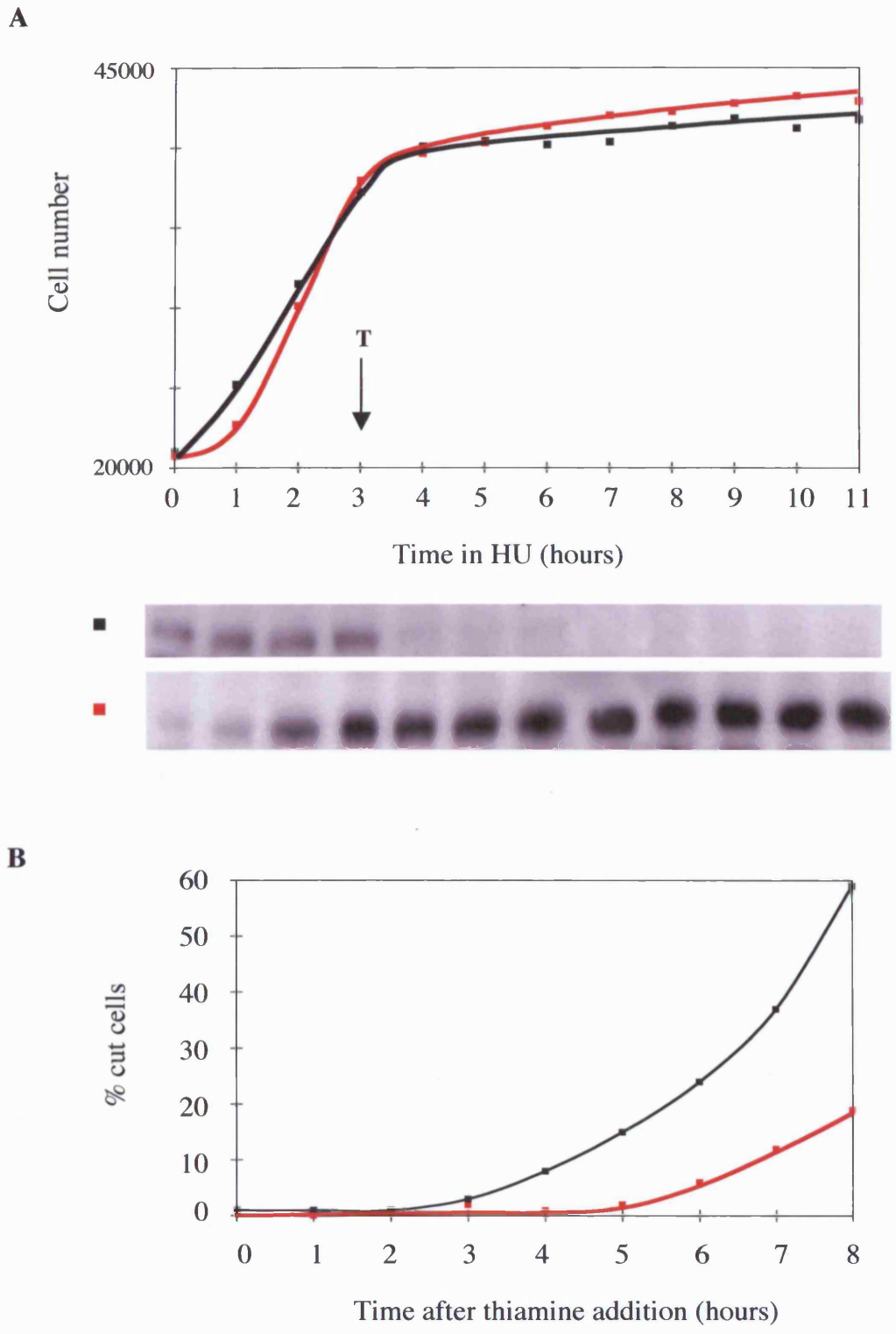


Figure 4.2 Cdc18 is required for checkpoint maintenance in an HU block

A) Cell number and Cdc18 levels throughout the experiment for SO strain (■) and wild-type strain (■). **T**=thiamine addition. B) The appearance of cut cells after the addition of thiamine to the same strains.

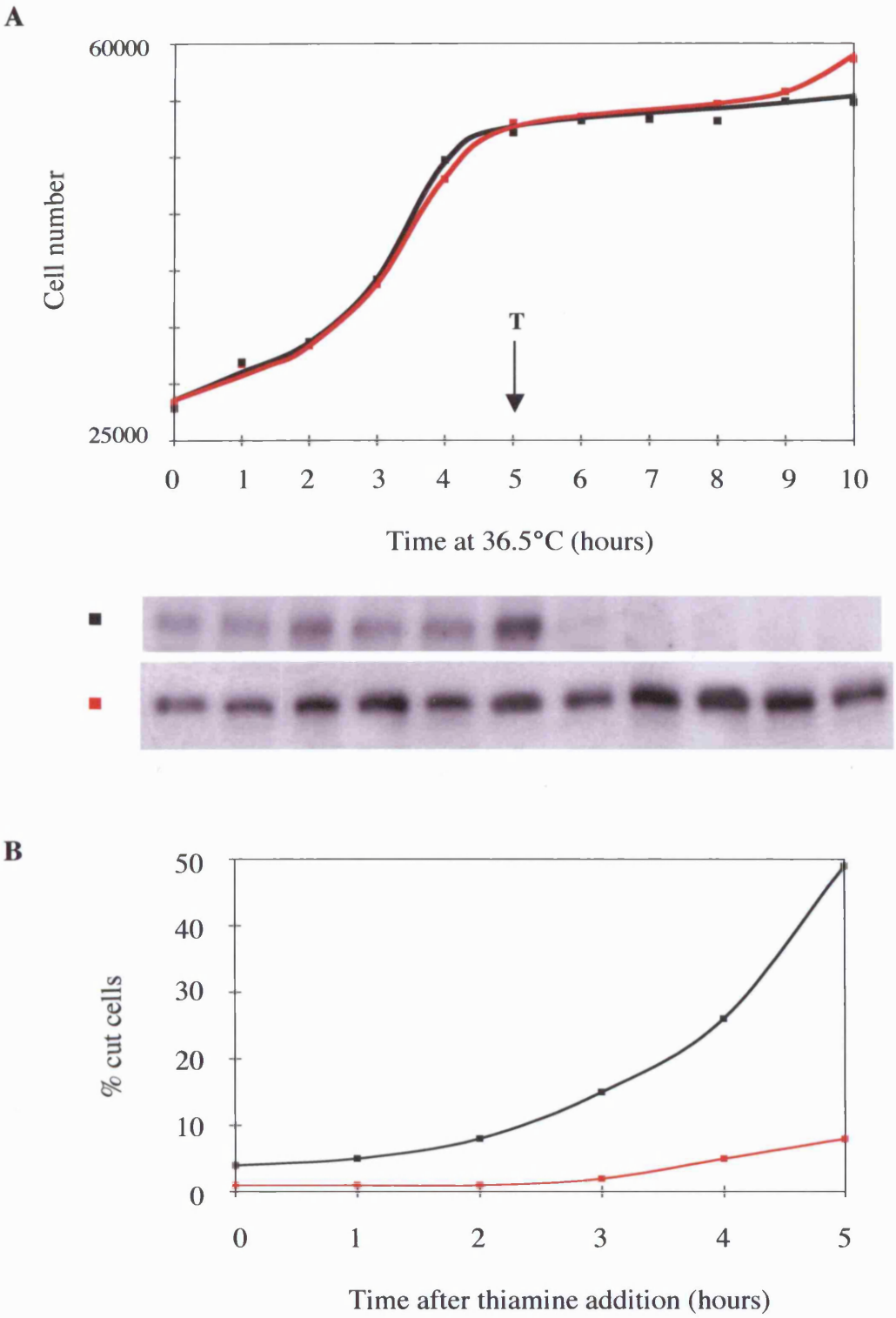


Figure 4.3 Cdc18 is required for checkpoint maintenance in a *cdc17-K42* block

A) Cell number and Cdc18 levels throughout the experiment for *cdc17-K42*; SO (■) and *cdc17-K42* (■). **T**=thiamine addition. B) The appearance of cut cells after the addition of thiamine to the same strains.

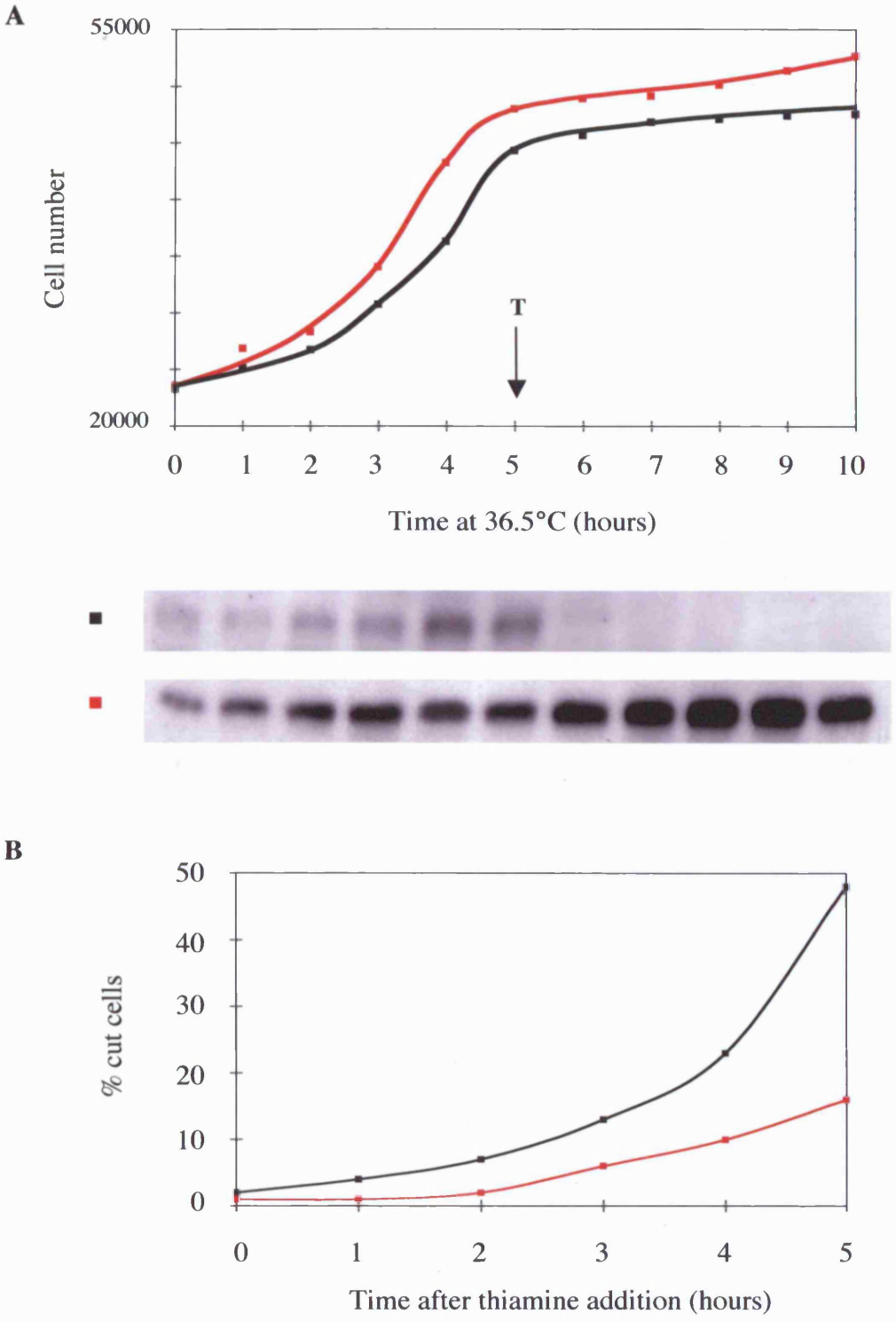


Figure 4.4 Cdc18 is required for checkpoint maintenance in an *pol1-1* block

A) Cell number and Cdc18 levels throughout the experiment for *pol1-1*; SO (■) and *pol1-1* (■). **T**=thiamine addition. B) The appearance of cut cells after the addition of thiamine to the same strains.

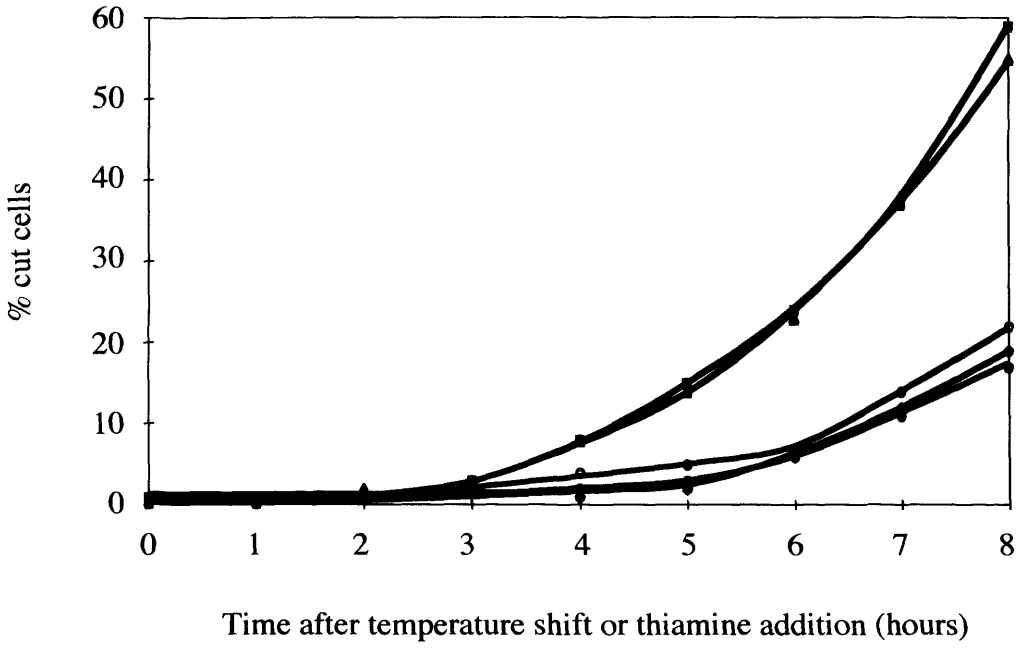
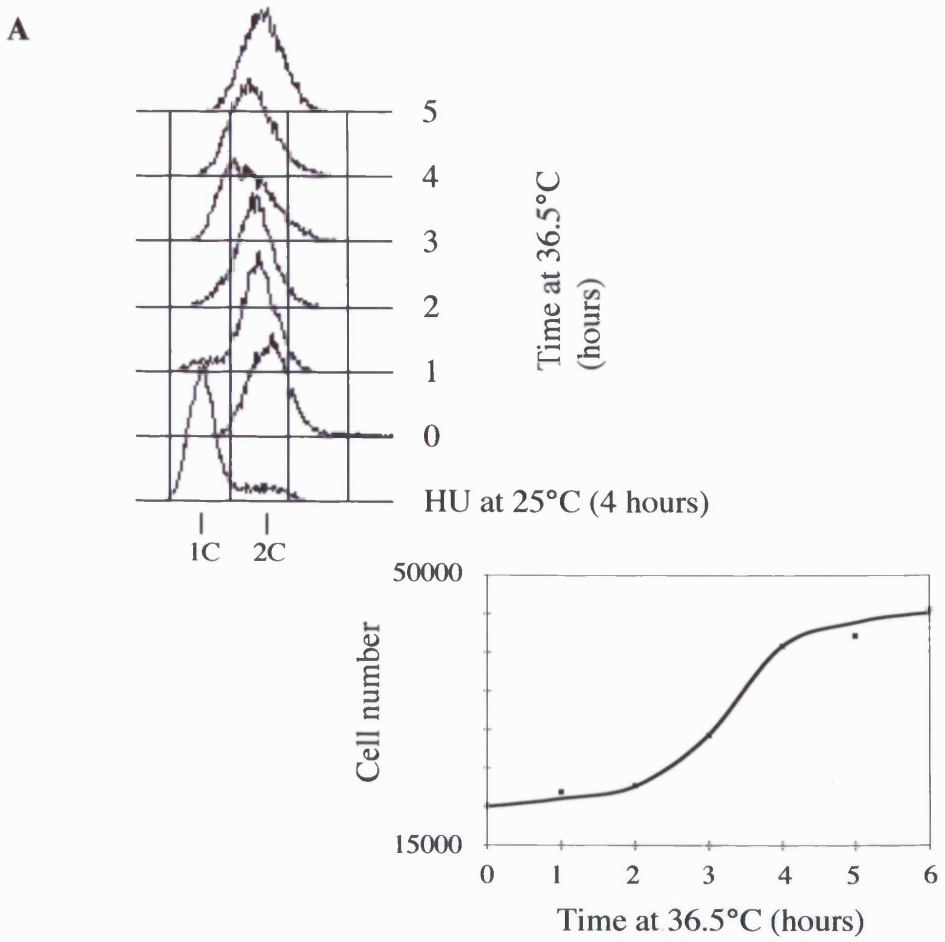


Figure 4.5 Checkpoint maintenance in HU requires an intact WA motif

Rate of appearance of cut cells in HU following shift to 36.5°C (*cdc18-3.9*, ◆) or addition of thiamine (SO, ■, *cdc18-WA-HA*; SO, ▲, *cdc18-DEAD-midT*; SO, o, wild-type, ●).



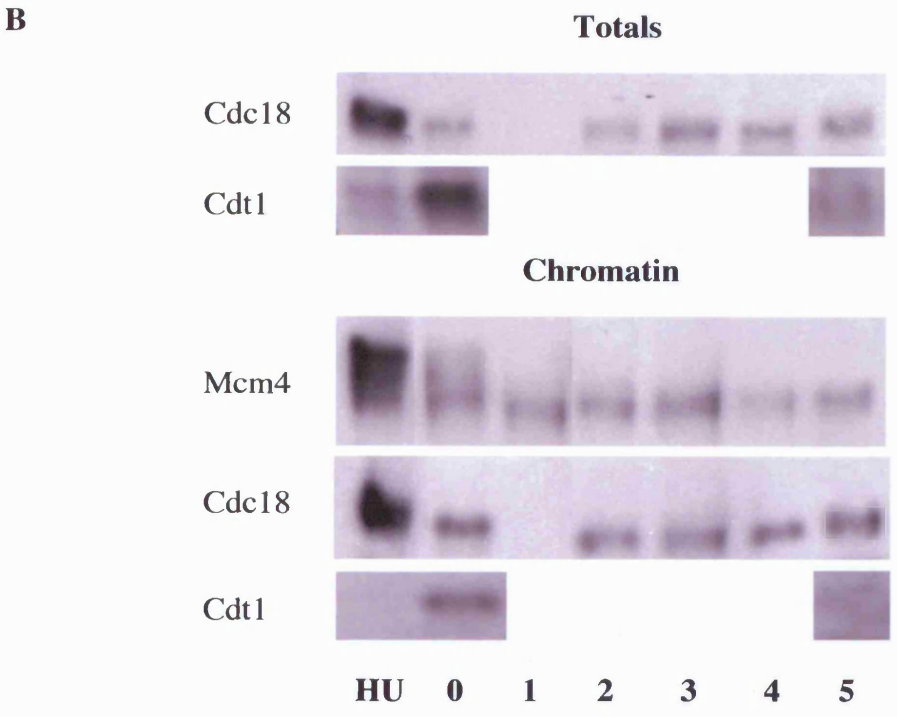


Figure 4.6 Cdc18-3.6 binds chromatin and causes a late S phase arrest

A) At 36.5°C *cdc18-3.6* arrests at late S with 2C DNA. B) Cdc18-3.6 binds to chromatin and there is no Cdt1 or Mcm4 loaded at the block point.

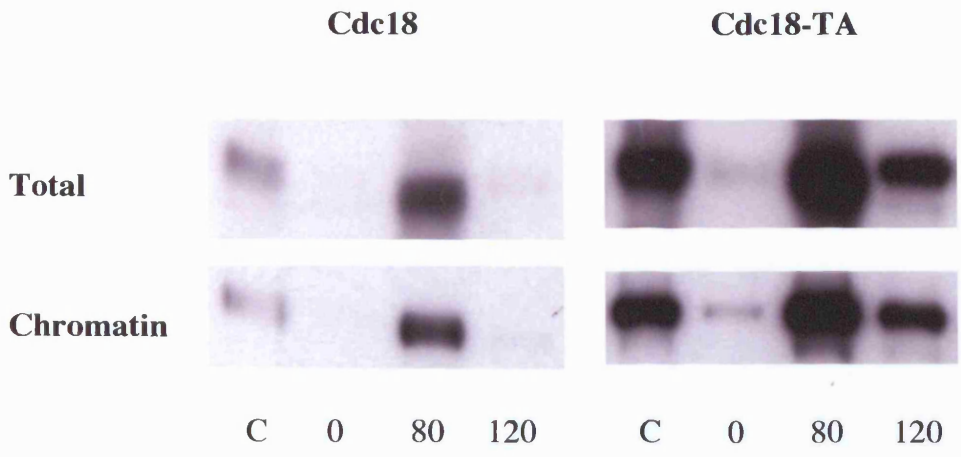


Figure 4.7 Cdc18-TA is present and chromatin-bound in G2 cells.

Total and chromatin-bound levels of wild-type Cdc18 and Cdc18-TA in a *cdc25-22* block and release experiment. C=cycling cells. All numbers refer to time after release from the *cdc25-22* block in minutes.

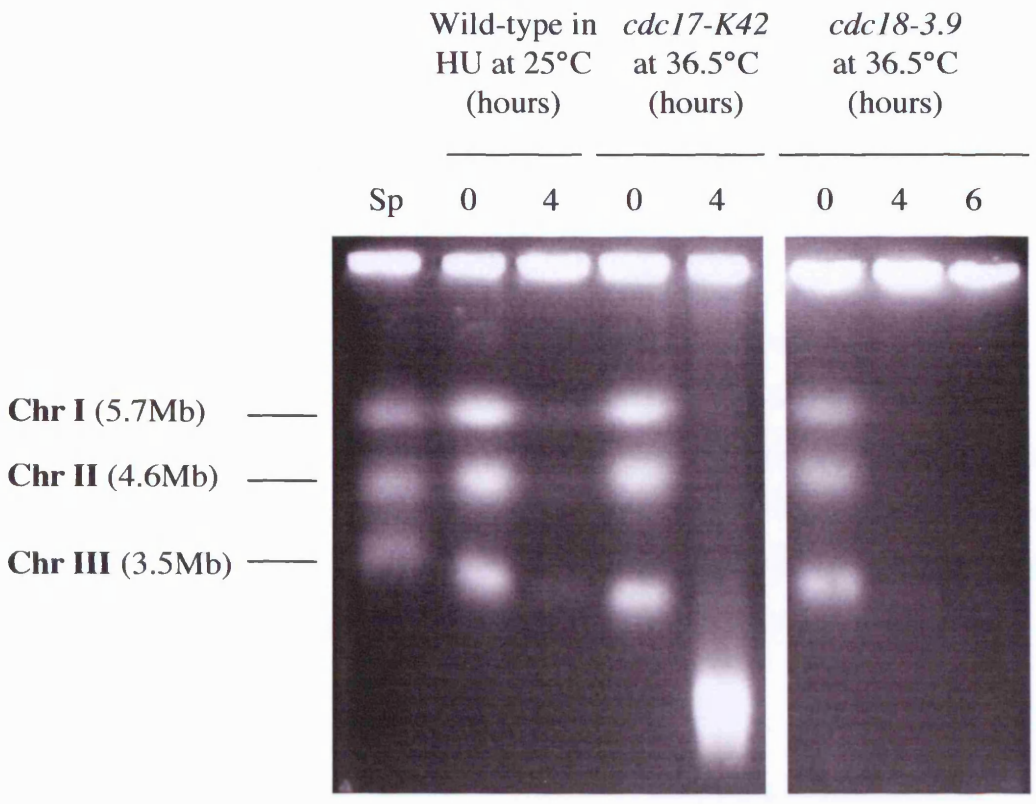


Figure 4.8 Chromosomes from *cdc18-3.9* do not enter a pulsed field gel at restrictive temperature

Chromosomes from the indicated strains were separated on a 0.8% gel. Approximately 8 x 10^7 cells are loaded per lane. Sp=commercially prepared fission yeast marker chromosomes (Sigma 170-3633).

Chapter 5. Investigation of a third role for Cdc18 in the cell cycle

5.1 Introduction

As discussed in chapter 2, a number of ts *cdc18* mutants were isolated that arrest at late S phase with 2C DNA. Two possible explanations for this phenotype are as follows. Firstly it may be that the initiation function of Cdc18 is not completely removed, but is faulty in some way. Faulty initiation may allow bulk replication but leaves chromosomes in a state unfit for mitosis (causing Rad3 checkpoint activation). An alternative explanation may be that Cdc18 plays a role within S phase aside from its initiation and checkpoint roles.

In order to investigate this second possibility, I adapted a procedure first employed to position the point of function of a number of ts genes (Nasmyth and Nurse, 1981). In this 'sequencing' experiment the point of function of a gene is assessed in relation to the HU arrest point. In principle, cells are arrested in HU at permissive temperature before shifting to restrictive temperature and removing HU. A continuation of the cell cycle (as indicated by a rise in cell number) demonstrates that the temperature-dependent defect lies prior to the HU arrest point. A continued cell-cycle arrest (as indicated by no rise in cell number) demonstrates that the temperature-dependent defect lies after the HU arrest point (but prior to cell division). In these experiments the ts *cdc18* mutant *cdc18-H2.3* sequences after the HU block point, suggesting a role for Cdc18 post initiation. In this chapter I will describe how further experiments show that this ts allele carries two distinct recessive defects, one of which sequences after HU and is not related to its checkpoint function. This defect does not apparently affect replication or the structural integrity of the DNA, but still causes a cell cycle arrest, most likely at G2/M. Hence Cdc18 may play a role in S phase that is required for cell cycle progression at a later stage. The possible identity of this function is discussed.

5.2 Results

5.2.1 *Cdc18-H2.3* sequences after HU

In the original sequencing experiment by Nasmyth and Nurse, cells were released from an HU block at the same time as shifting to restrictive temperature. I was concerned that this approach may not allow enough time for inactivation of the ts protein before cells continued through S phase. In addition, I was concerned that restrictive conditions used, 36°C in yeast extract, were not sufficient to completely hold a ts arrest. Hence a revised sequencing experiment was devised, as detailed in figure 5.1A. Strains are initially arrested in 12mM HU for four hours at 25°C. Cells are then incubated at 36.5°C for one hour, in minimal medium, prior to HU wash out, and cell number followed for five hours after.

Wild-type cells complete DNA replication within one hour of HU wash out and progress through the cell cycle: cell number doubles by four hours and continues to rise thereafter (figure 5.1B, C). In contrast, as expected, cell number does not increase for the ts mutant *cdc17-K42* despite a doubling of DNA content. As previously discussed, *cdc17* encodes DNA ligase, which ligates the Okazaki fragments of the lagging strand at the end of S phase. In the replication-deficient ts mutant *cdc18-3.9*, arresting in HU at 25°C bypasses the MCM loading deficiency and cells complete replication and continue through the cell cycle after HU wash out. However, this mutant does arrest in the next cell cycle (whilst at restrictive temperature), as shown by the appearance of a 1C peak and plateau of cell number after an approximate doubling. Finally, ts mutant *cdc18-H2.3*, which arrest at late S with 2C DNA in a shift-up experiment, was subjected to the sequencing experiment. Although DNA replication was apparently complete one hour after HU wash out, cell number failed to increase, indicative of continued cell cycle arrest. Hence *cdc18-H2.3* sequences after HU. This result initially suggests that Cdc18 may play a role in cell cycle progression post initiation, although it is possible that this role is specifically required in response to HU.

Next, the sequencing experiment was adapted to remove the temperature shift or to shift cell temperature two hours after HU wash out (Figure 5.2A). In both cases, replication is complete two hours after HU removal, and cell number increases thereafter (figure 5.2B, C). As expected, cell number increase at the lower, permissive temperature is delayed in comparison to that seen at restrictive temperature. Hence *cdc18-H2.3* is resistant to HU at 25°C, although we can not rule out a sensitivity to HU at 36.5°C. Importantly, if Cdc18 does have a role in cell cycle progression post initiation, then this role is likely to be completed within S phase: shifting *cdc18-H2.3* to restrictive temperature as cells enter G2 does not inhibit cell number increase (until the next cell cycle).

5.2.2 *Cdc18-H2.3* is a recessive allele

I was interested to know if *cdc18-H2.3* was a recessive allele. To this end, a stable diploid was constructed containing a wild-type copy of *cdc18* and the *cdc18-H2.3* allele (heterozygous mutant diploid). A homozygous wild-type diploid strain was used as a control. In both these strains, shifting to 36.5°C led to an exponential rise in cell number (data not shown). In addition, when these strains were subjected to the sequencing experiment, cell number increased at very similar rates after HU wash out (figure 5.3). These results demonstrate that the *cdc18-H2.3* allele is recessive with respect to wild-type in both the shift-up and sequencing experiments. Further confirmation of this was obtained using the *cdc18-H2.3* haploid transformed with wild-type *cdc18* behind the *nmt81* and *nmt41* promoters (*cdc18-H2.3; pnmt81-cdc18* and *cdc18-H2.3; pnmt41-cdc18*). In both transformants, shifting to 36.5°C led to an exponential rise in cell number (data not shown). Further, cell number increases for these transformants after HU removal in the sequencing experiments (figure 5.3). As *cdc18-H2.3* is a recessive allele, the cell cycle arrests seen in the shift-up and sequencing experiments can be best understood as a loss of function of Cdc18.

5.2.3 *Cdc18-H2.3* has two distinct defects

Further insight into the *cdc18-H2.3* mutant was provided by pulsed field gel analysis. When *cdc18-H2.3* is shifted to restrictive temperature its chromosomes fail to enter a pulsed field gel, indicative of structural alterations

to the DNA (such as replication intermediates) (figure 5.4A). In contrast, chromosomes from *cdc18-H2.3* subjected to the sequencing procedure do enter the gel following completion of replication at 6 hours (figure 5.4B). This suggests that *cdc18-H2.3* has two distinct defects; one which produces incomplete DNA structures when presented prior to initiation, and one that does not when presented post initiation.

Further evidence for two defects was gained by introducing the *cdc18-H2.3* allele into a *rad3ts* background (*cdc18-H2.3 rad3ts*). When this double mutant is shifted to 36.5°C cells rapidly enter a lethal mitosis and cut (75% cuts 6 hours after the shift). A different picture is presented in the sequencing experiment however. In the sequencing experiment, the *rad3ts* strain progresses through the cell cycle after HU wash out (figure 5.5). Only a small proportion of cells (12%) display the cut phenotype at the end of the experiment which may be due to the loss of Rad3 signalling occurring before HU removal. In some cells this leads to (lethal) mitosis before the cell can complete DNA replication. In contrast to *rad3ts*, cell cycle arrest is maintained in the double ts mutant (*cdc18-H2.3 rad3ts*), and a similar number of cut cells are seen at the end of the experiment (12%).

These results show that *cdc18-H2.3* has two distinct defects. The first, presented by a shift-up experiment, affects DNA replication such that incomplete DNA structures are produced. These structures prevent entry of the chromosomes into a pulsed field gel and induce a Rad3-dependent checkpoint. In the absence of Rad3 function cells display the cut phenotype, which could reflect an attempt to segregate unfinished sister chromatids. The second defect, presented by the sequencing experiment, does not appear to lead to incomplete replication. Chromosomes at this block point do enter the gel, and cells do not cut in the absence of Rad3 function. Additionally, the absence of any increase in cell number for *cdc18-H2.3 rad3ts* in the sequencing experiment also shows that the mutant Cdc18 protein is not ectopically activating the Rad3 pathway in these circumstances (consistent with the recessive nature of the allele). Furthermore, this shows that the cell cycle

arrest caused by *cdc18-H2.3* in the sequencing experiment does not work via the *rad* pathway.

5.2.4 Cdc18 effects its third role during S phase for transition later in the cell cycle

To explore these experiments further, levels of Cdc18 protein in total extracts were followed at particular points throughout the sequencing experiment (figure 5.6). In wild-type cells, Cdc18 levels increase upon treatment with HU (0 to 4 hours). Shifting to 36.5°C causes a decrease in Cdc18 level (5 hours). Completion of replication and entry into G2 (at 6 hours) leads to removal of Cdc18, which rises again in the next cell cycle (8 hours). A similar pattern is seen with ts mutant *cdc18-3.9*. Entry into G2 at 6 hours leads to the removal of Cdc18, but at 8 hours cells have arrested at the 1C (ts) block point in the next cell cycle. Here, significant levels of Cdc18 are seen, consistent with results from figure 3.8. The same pattern is also seen in *cdc18-H2.3*, with the significant difference that Cdc18 appears to be absent at the block point (6 and 8 hours). The cell number and FACS data from 5.2.1 suggests that the block point for *cdc18-H2.3* in the sequencing experiment lies between late S phase and mitosis. As Cdc18 appears to be present in late S phase blocks (chapter 4), the lack of Cdc18 at 6/8 hours suggests that the post-HU arrest point induced by *cdc18-H2.3* lies in G2 or early M (and not within S phase).

Taken together, the data from the previous sections suggest that Cdc18 plays a third role within the cell cycle, aside from its replication initiation and checkpoint functions. This role is performed either in a normal S phase, or in response to HU, and its product is required for progression through G2/M. This role is unlikely to involve the production of faithfully duplicated DNA, but may instead be involved in faithful chromosome duplication or dynamics. It would be interesting to define the arrest point of *cdc18-H2.3* in the sequencing experiment more clearly, and to find out which checkpoint system restrains cell cycle progression in these circumstances.

5.2.5 Cdc18 is detectable after transcriptional repression in an HU block

The findings of this chapter are in apparent disagreement with the conclusions from a previous set of experiments (Muzi Falconi et al., 1996). In this paper the sequencing experiment was applied to a switch-off strain. Cells were arrested in HU before thiamine addition to repress *cdc18* transcription. HU was then removed, and cells progressed through the cell cycle until they reached the following S phase. From those data it was concluded that Cdc18 plays no role in the cell cycle after initiation of replication. I used the SO strain described in chapter 3 to perform a similar sequencing experiment (figure 5.7A). Cells were arrested in 12mM HU for 3 hours at 32°C. Thiamine was then added to the culture and HU removed an hour later. Within another hour DNA replication was complete and cells progressed through the cell cycle, as shown by the appearance of a 1C DNA peak (7 hours). Cdc18 levels were followed prior to the removal of HU in this experiment (figure 5.7B). Whilst thiamine addition greatly reduces the level of Cdc18 in the HU block, Cdc18 is not completely removed. Densitometric analysis was conducted on the Western blots using the Image J program. Allowing for a higher background staining in the 4 hour lane for the Cdc18 Western blot, and normalising for α-Tubulin levels, I estimate the level of Cdc18 at 4 hours to be 5-10% of that present at 3 hours. Note that Cdc18 appears to persist at low levels for up to 3 hours after thiamine addition in a prolonged HU incubation (figure 4.2A). Thus while sequencing with the SO strain initially suggests that Cdc18 plays no role in the cell cycle after the initiation of replication, it may be that the inability to completely remove the protein compounds the experiment. Whilst the level of Cdc18 produced in SO systems, in the presence of thiamine, is insufficient to support its replication or checkpoint function, it may be sufficient to support the putative third role described in the previous sections.

5.3 Discussion

The original sequencing experiment performed with *cdc18-H2.3* was designed to identify the cause of the late S phase arrest phenotype at restrictive

temperature. In the end, both of the two alternative possibilities put forward for this were found to exist. The Cdc18-H2.3 protein does seem to lack accurate initiation function, such that bulk replication can occur but in a manner which leaves the DNA unfit for segregation. Perhaps the protein loads the MCM complex onto DNA in a way which leads to mutagenic replication (via the MCM complex itself or via the subsequent erroneous loading of other factors, such as non-proofreading polymerases). In addition to this initiation defect, Cdc18-H2.3 seems to lack a function required in S phase (or in response to HU) for progression later on in the cell cycle. These two defects are consistent with the behaviour of the ts mutant in a shift-up experiment. Upon shift to restrictive temperature, only about 10% of cells, located in S phase at the time, will be affected by the loss of the putative third role for Cdc18. Only these cells will arrest in G2/M, and will not contribute to cell number increase throughout the experiment. The rest of the cell population (mostly G2 cells) will progress to the initiation-defect block point, and will divide during the timecourse, giving rise to an approximate doubling in cell number. In all cases a 2C DNA content is produced.

The nature of the S phase function effected by Cdc18 is unclear. The results presented here suggest that Cdc18 is required in S, or in response to HU, for cell cycle progression. However, the defect leaves chromosomal DNA intact and cells remain blocked in the absence of Rad3 function, suggesting that Cdc18 is responsible for processes outside of DNA metabolism (replication, recombination and repair). In *Xenopus* egg extracts, Cdc6 rebinds to origins once replication forks have moved away from the sites of initiation (Oehlmann et al., 2004). Whilst the continued presence of Cdc6 is required for its checkpoint function, re-binding to chromatin is not. This raises the possibility that Cdc6 re-binds to origins in order to perform some other function, apart from its replication and checkpoint functions.

It may be that Cdc18 is required for higher order processing of chromosomes, such as condensation or cohesion. A novel ts *cdc18* mutant (*cdc18-641*) is suppressed by separase and the condensin subunits SMC2 and SMC4 (Yuasa

et al., 2004). These factors also suppressed other ts mutants involved in DNA metabolism, such as DNA topoisomerase III (separase and SMC2) and Orc5 (separase and both SMCs). These results suggest a relationship between DNA replication and chromosome dynamics. The five subunit condensin complex is required for chromosomal condensation (Sutani et al., 1999), and has been implicated in S phase checkpoint control (Aono et al., 2002). Defects in any of the condensin subunits inhibit Cds1 activation in response to HU, an analogous scenario to the loss of checkpoint maintenance in HU when Cdc18 levels are reduced. Whilst the putative third function of Cdc18 is unlikely to relate to its checkpoint function, it may well relate to the condensation activity of the condensing complex. Also of possible relevance is the recent finding that, in *Xenopus*, recruitment of cohesins to chromosomes requires fully licensed chromatin and is dependent on ORC, Cdc6, Cdt1 and Mcm2-7 (Takahashi et al., 2004). Loading of condensin or cohesin complexes onto chromosomes would be consistent with the MCM loading function of Cdc18, and its homology with other clamp loaders such as RFC (Perkins and Diffley, 1998). Interestingly, Cdc18 has been found to interact with Swi6 in a 2-hybrid screen (Louise Chretien, unpublished data). Amongst the roles for Swi6 in the cell are the recruitment of cohesins to chromatin and establishment of heterochromatin.

An alternative possibility is that Cdc18 is required to load the mitotic kinase, Cdc2-Cdc13, onto chromosomes in order to promote entry into mitosis. This CDK complex is recruited to origins during S phase in an ORC-dependent manner, and this recruitment is required to prevent rereplication (Wuarin et al., 2002). It may be that Cdc18 is required for this Cdc2 loading activity, which is in turn required for both origin suppression and for enabling Cdc2 mitotic function (such as initiating chromosome condensation). Whatever the role of Cdc18 highlighted in this chapter, its action is supported by protein levels insufficient to provide replication and checkpoint activities. This suggests that the number of sites at which Cdc18 operates or the number of reactions catalysed by the protein is small.

A number of new investigations are required to expand the possibility that Cdc18 has a third role in the fission yeast cell cycle. Of great importance is to understand the point of the cell cycle at which the ts mutant *cdc18-H2.3* arrests in the sequencing experiment. To this end a GFP-tagged version of α-Tubulin should be introduced into this mutant, and fluorescence microscopy used to ascertain the microtubular structures present at the block. A high proportion of mitotic spindle structures would indicate an early mitotic block, for example. In addition, it is important to find out which checkpoint system (if any) restrains the cell at the block point. A candidate system would be the spindle checkpoint, which prevents the metaphase-anaphase transition until all chromosomes have established a bipolar attachment with the spindle.
In order to test this, the behaviour of *cdc18-H2.3* in the sequencing experiment in a spindle checkpoint deficient background (e.g. *bub1Δ*) should be analysed.

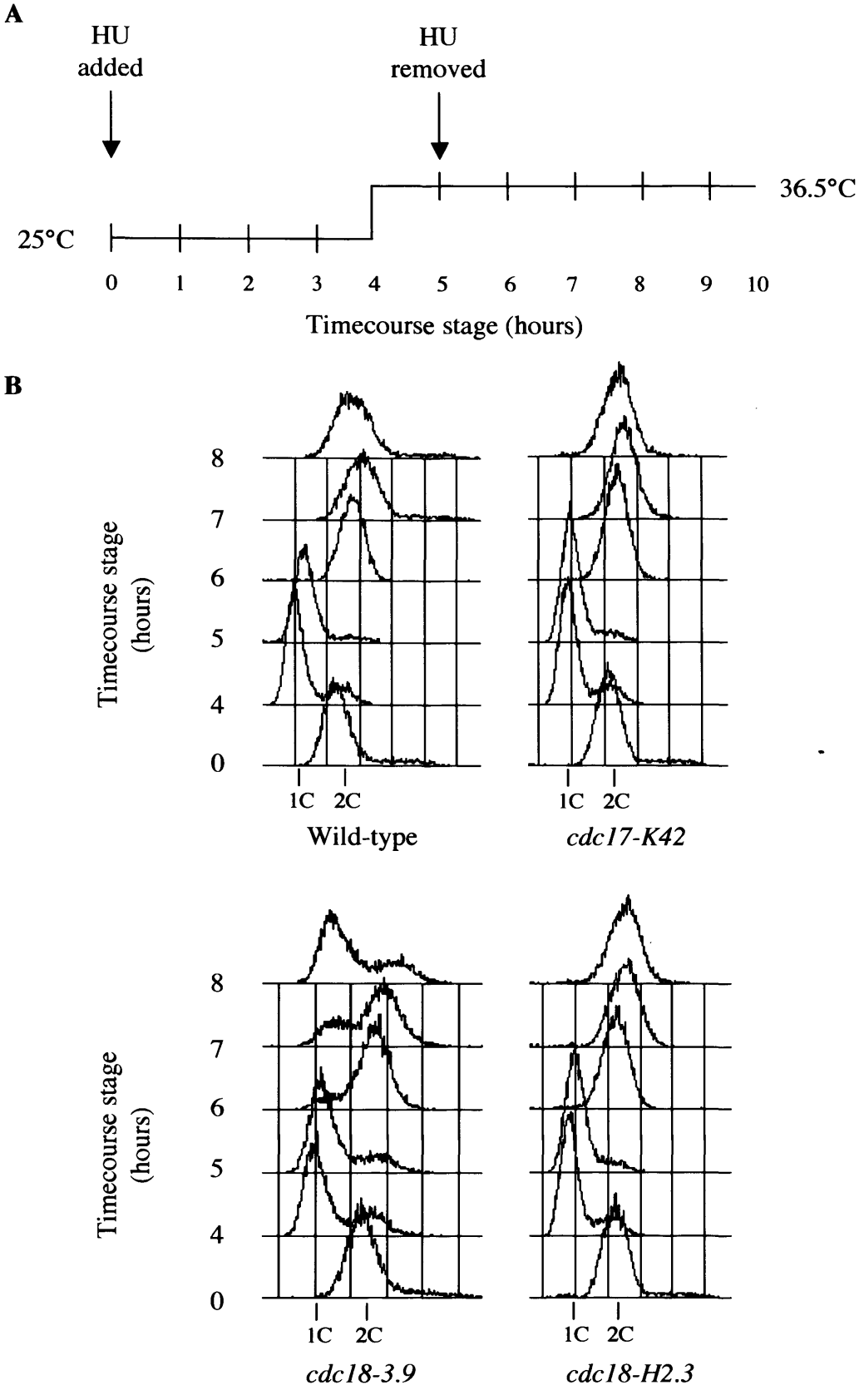


Figure 5.1 *Cdc18-H2.3* sequences after HU

A) Outline of sequencing experiment. B) FACS profiles of the indicated strains during the sequencing experiment.

C

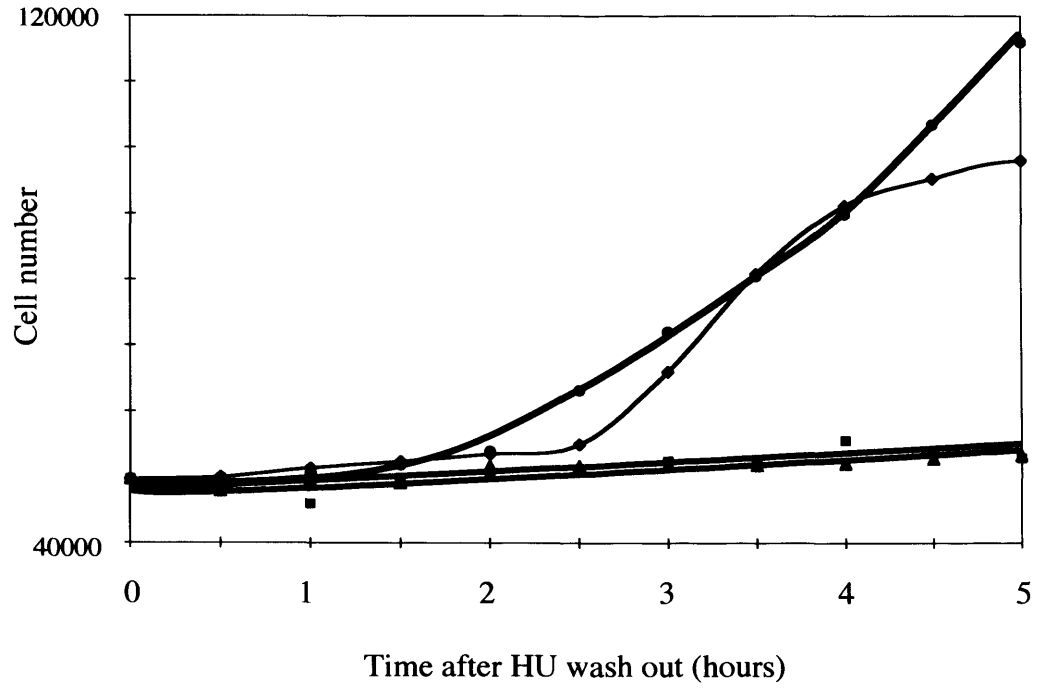


Figure 5.1 continued

C) Cell number after HU wash out for wild-type (●), *cdc17-K42* (▲), *cdc18-3.9* (◆) and *cdc18-H2.3* (■).

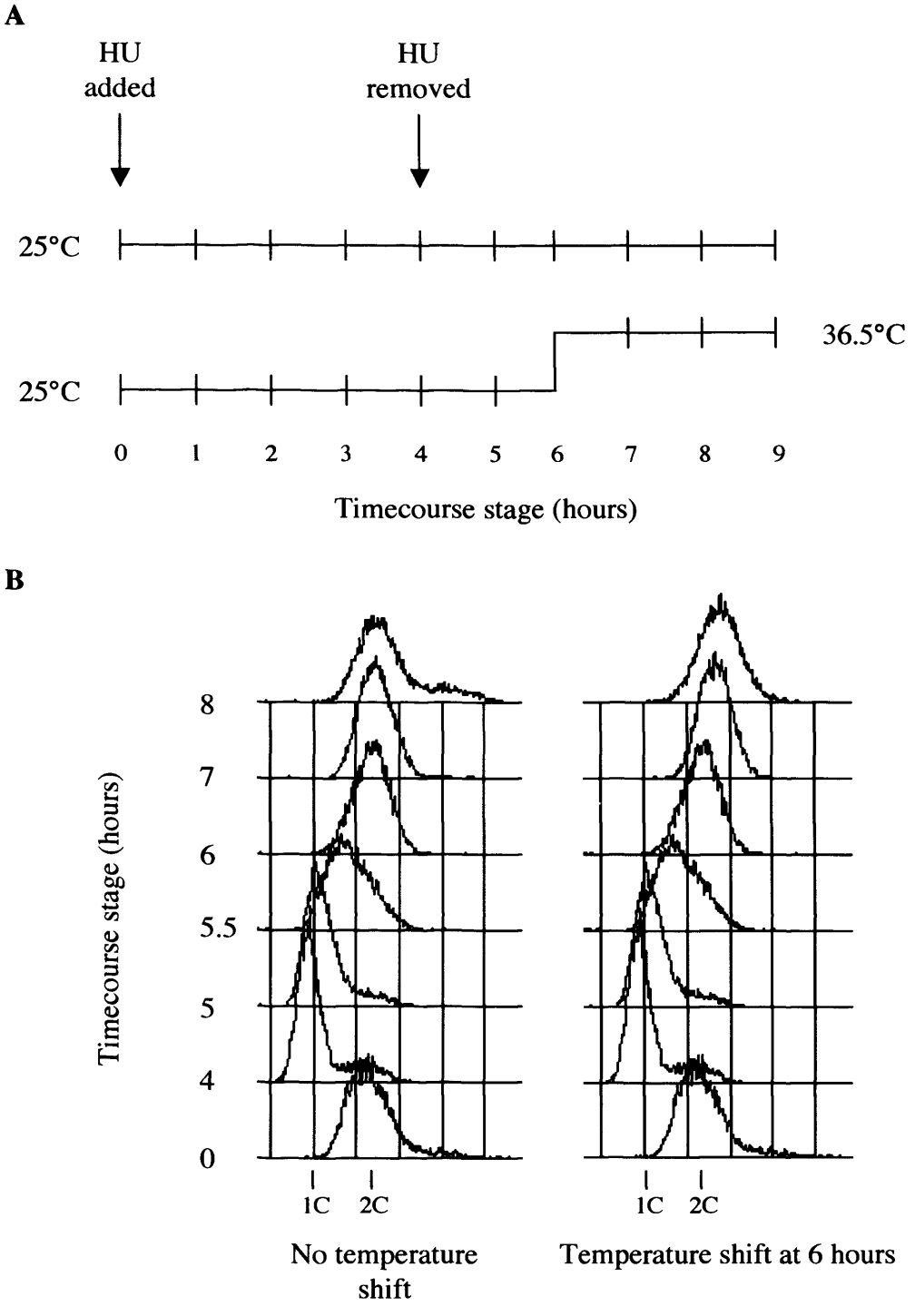


Figure 5.2 Adapted *cdc18-H2.3* sequencing experiments

A) Outline of adapted experiments with no temperature shift or shift following HU wash out. B) FACS profiles for *cdc18-H2.3* in these experiments.

C

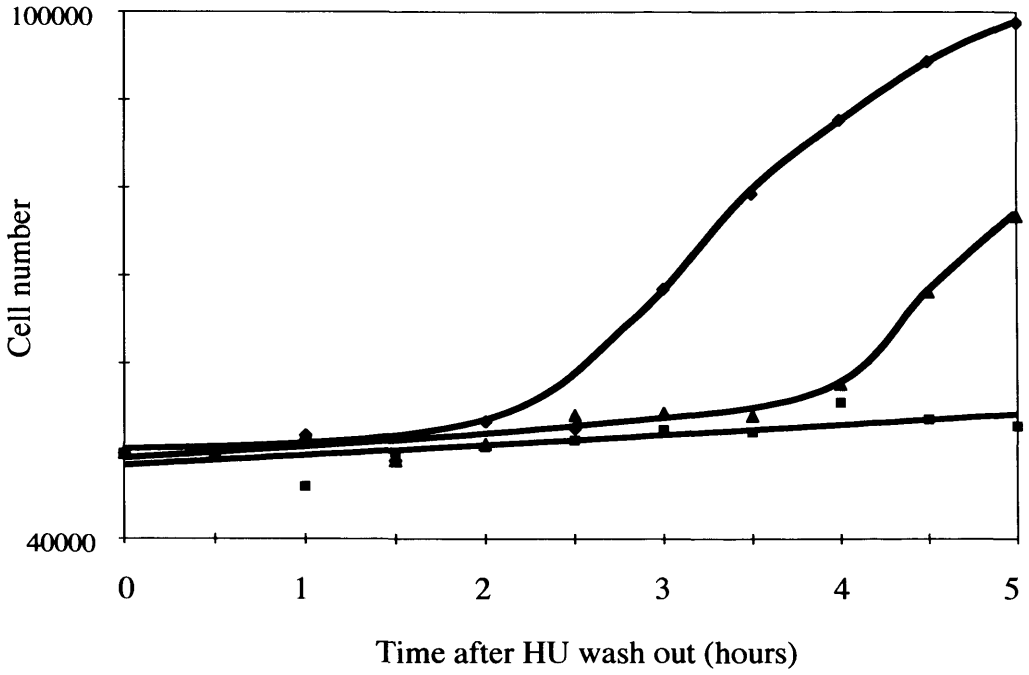


Figure 5.2 continued

C) Cell number after HU wash out for *cdc18-H2.3* without heat shift (▲), or with shift two hours after wash out (◆). Data from the original sequencing experiment is also shown (■).

A

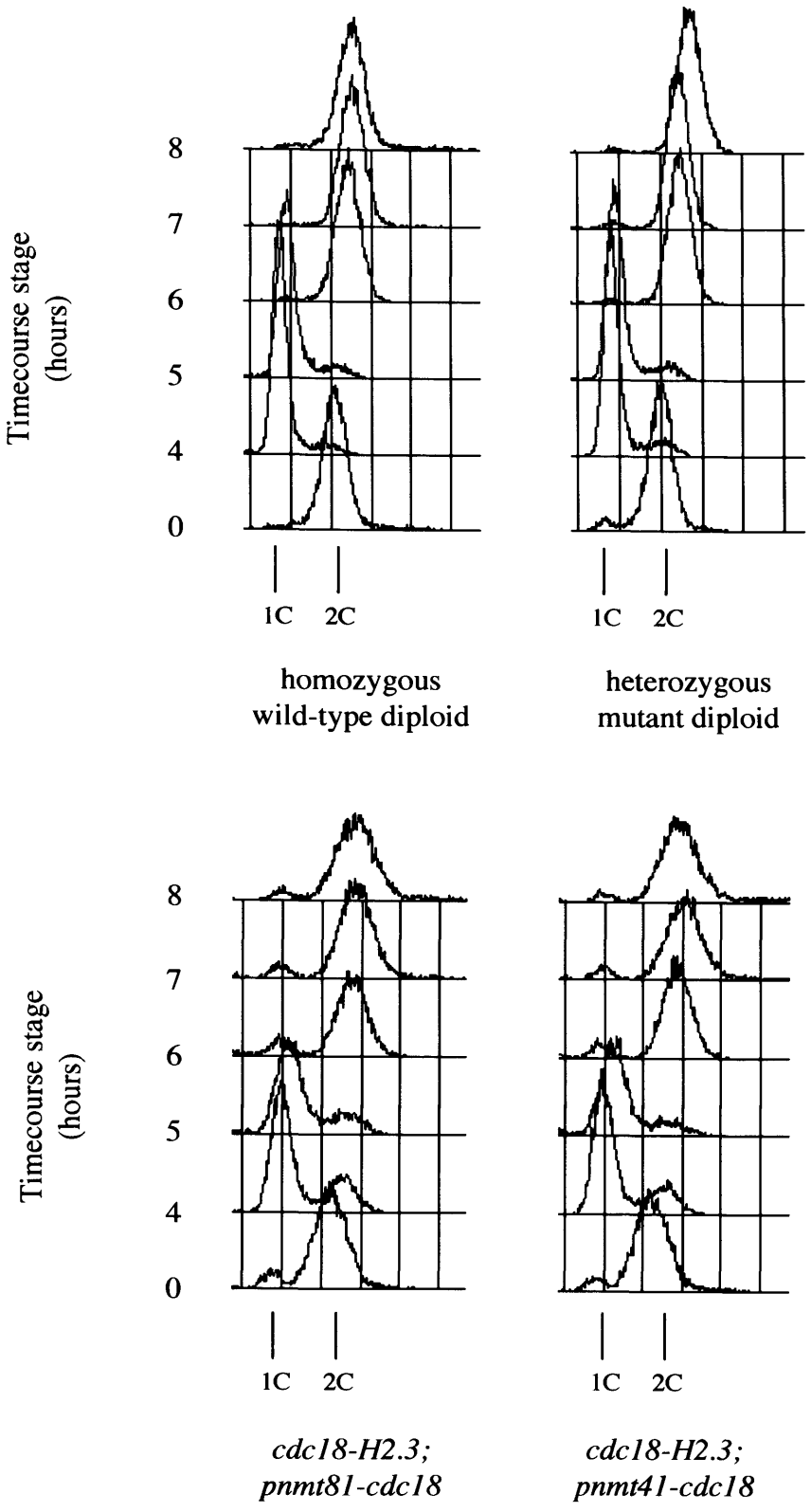


Figure 5.3 *Cdc18-H2.3* has a recessive defect in the sequencing experiment

A) FACS profiles of the indicated strains during the sequencing experiment.

B

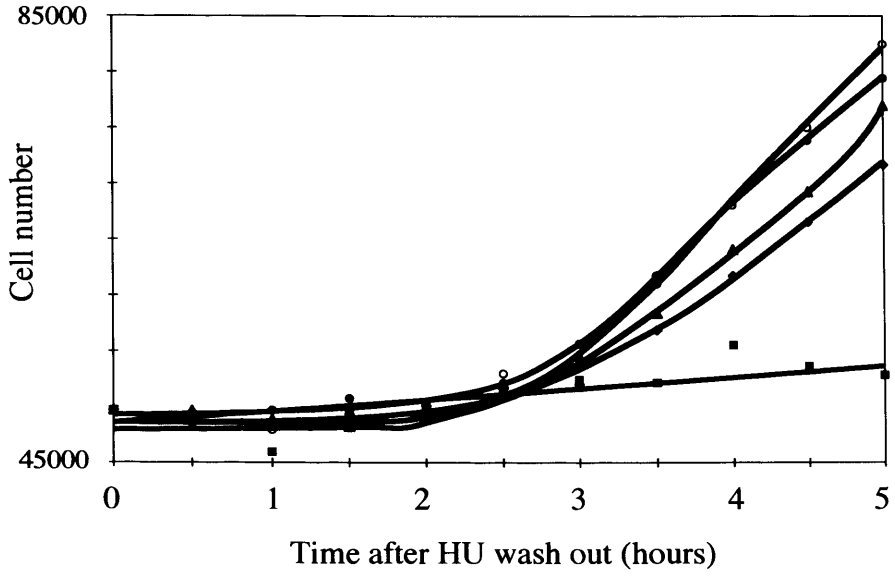


Figure 5.3 continued

B) Cell number after HU wash out for *cdc18-H2.3* (■), homozygous wild-type diploid (O), heterozygous mutant diploid (●) *cdc18-H2.3; pnmt81-cdc18* (▲), and *cdc18-H2.3; pnmt41-cdc18* (◆).

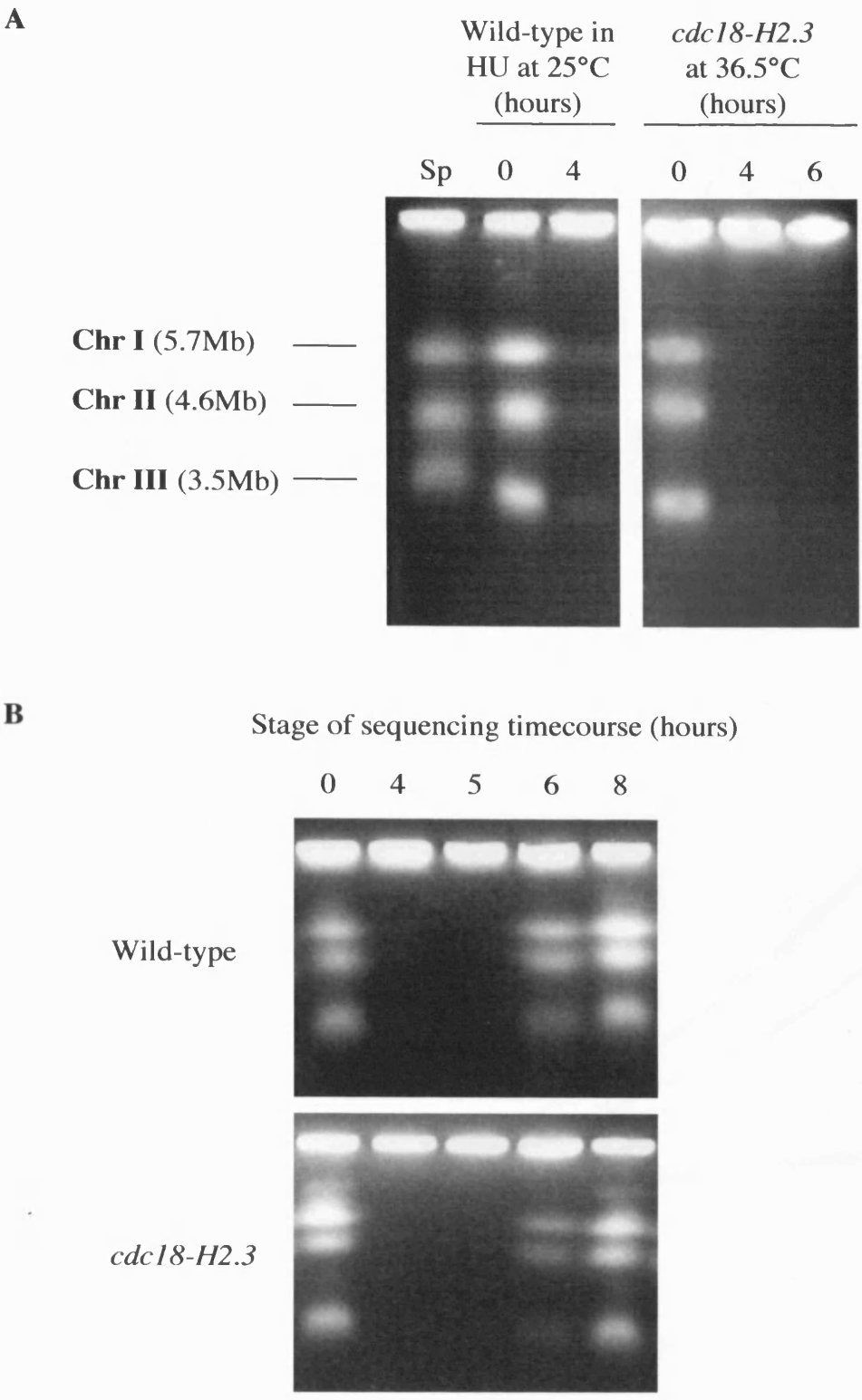


Figure 5.4 Pulsed field gel analysis of *cdc18-H2.3* arrest states

Chromosomes from the indicated strains were separated on a 0.8% gel. Approximately 8 x 10^7 cells are loaded per lane. A) Chromosomes from *cdc18-H2.3* shifted to 36.5°C do not enter the gel. B) Chromosomes from wild-type and *cdc18-H2.3* enter the gel at 36.5°C in the sequencing experiment.

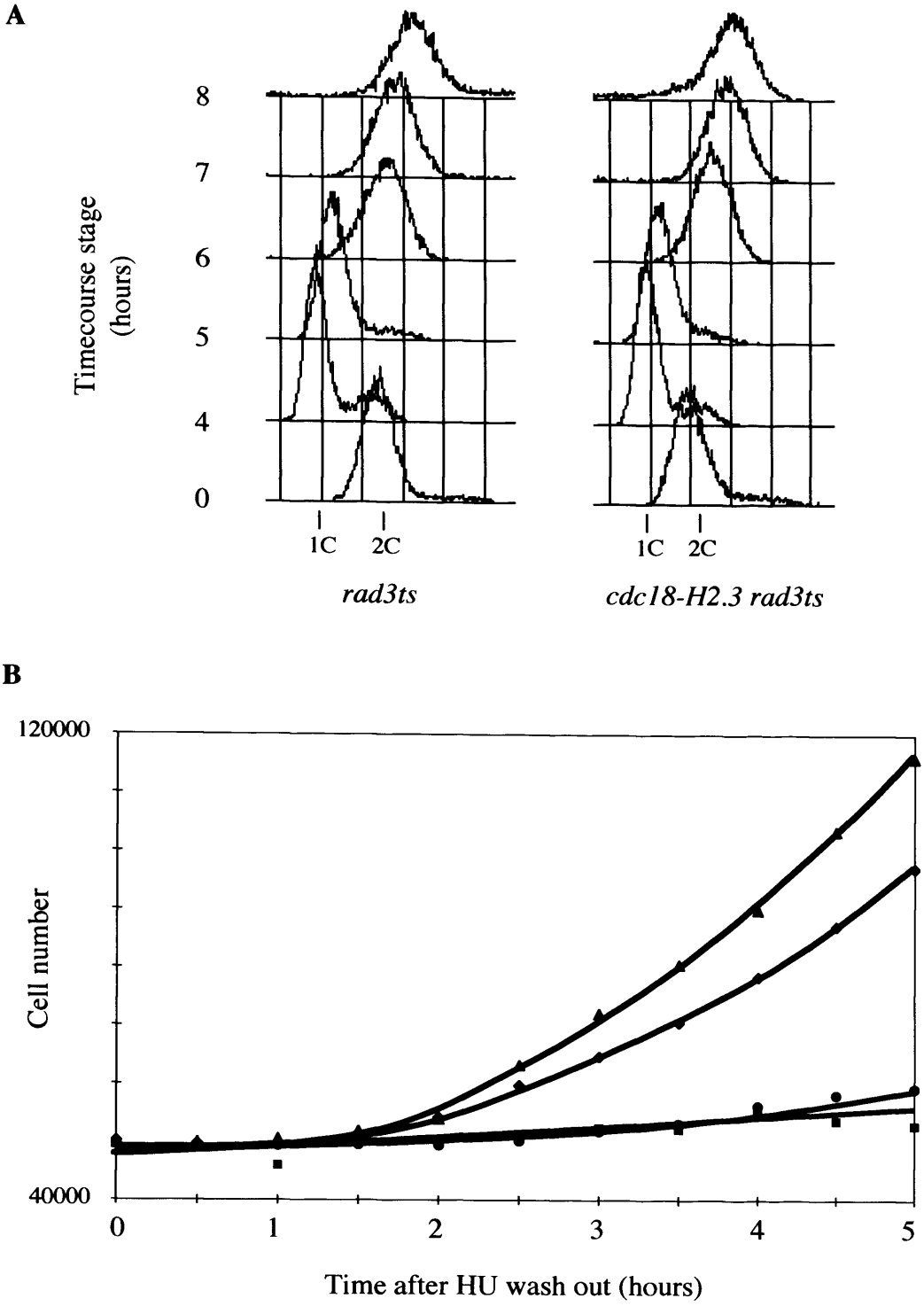


Figure 5.5 *Cdc18-H2.3* post-HU arrest is maintained in the absence of Rad3 function

A) FACS profiles of the indicated strains during the sequencing experiment. B) Cell number after HU wash out for wild-type (▲), *cdc18-H2.3* (■), *rad3ts* (◆) and *cdc18-H2.3 rad3ts* (●).

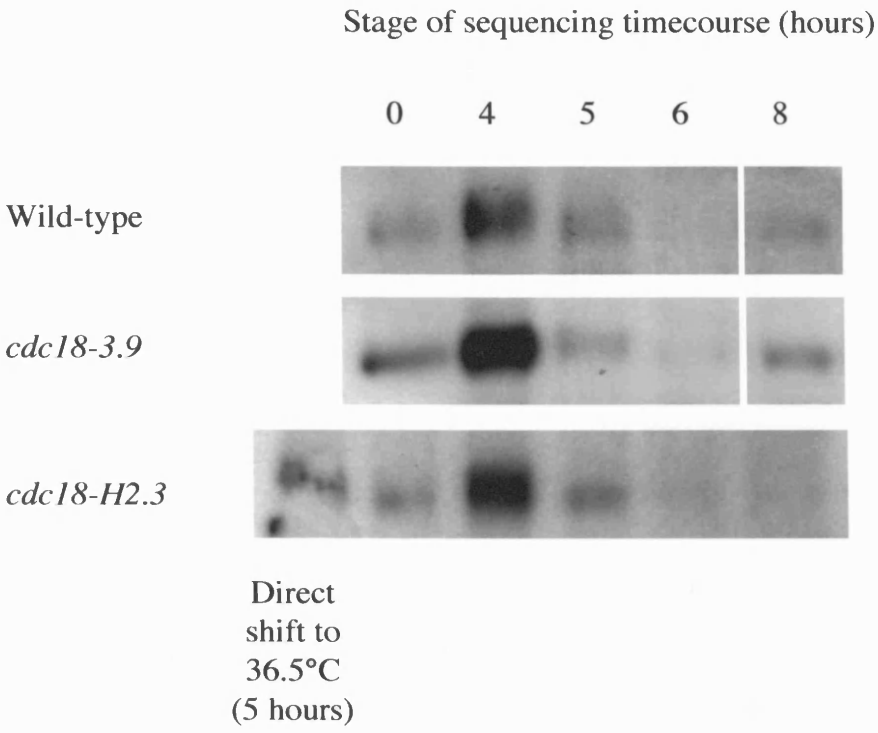
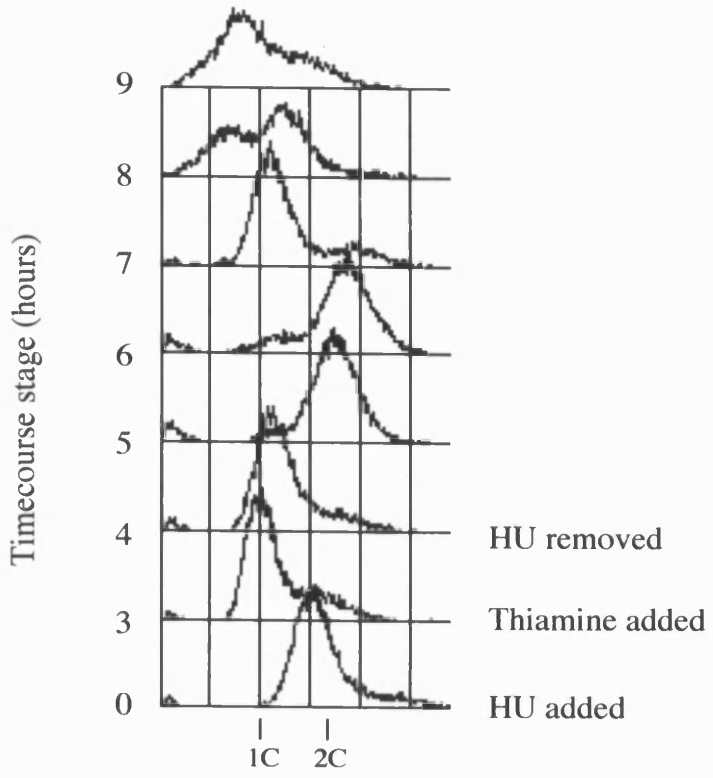


Figure 5.6 Cdc18-H2.3 is not present at the post-HU arrest point

Cdc18 levels in total extracts were analysed in the strains indicated during the sequencing experiment.

A



B

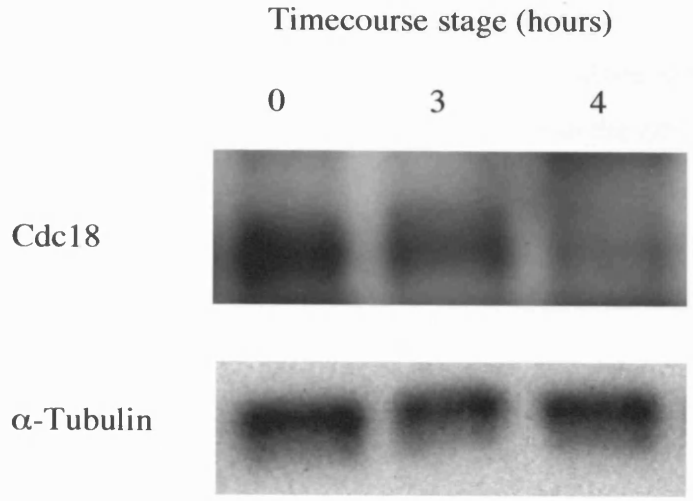


Figure 5.7 The sequencing experiment applied to the SO strain

A) FACS profile and B) Cdc18 levels of SO strain in the sequencing experiment.

Chapter 6. General discussion

The results described in this thesis suggest that Cdc18 has multiple roles in the cell cycle of fission yeast. A well-established role for Cdc18 is in the initiation of DNA replication. In addition, however, Cdc18 appears to be required in S phase for the S phase checkpoint (both for establishment and maintenance) and for an additional function required for cell cycle progression. The ability of Cdc18 to induce rereplication when overexpressed is well documented (Nishitani et al., 2000; Nishitani and Nurse, 1995; Yanow et al., 2001). Thus using Cdc18 for these additional roles provides the cell with the challenge of ensuring that Cdc18 does not induce rereplication from within S phase. As shown in section 4.2.4, expressing Cdc18 in G2 to levels normally seen in S phase (using the Cdc18-TA mutant) does not induce rereplication, demonstrating that this challenge can be met. In this respect it may be that fission yeast displays similarities with metazoan cells, where tight regulation of Cdt1 appears to be the primary means of preventing rereplication. The absence of Cdt1 in HU-arrested cells suggests that Cdt1 is degraded early in S phase, and phosphorylation of Orp2 may also contribute to this control system (Vas et al., 2001). The variety of roles for Cdc18 in the cell cycle poses questions about the timing and co-ordination of these roles, along with the mechanism by which each function is effected. As such I shall present a model for the behaviour of Cdc18 through the fission yeast mitotic cell cycle.

6.1 Cdc18 in M/G1 phases

Cdc18 appears in late mitosis/early G1, when Cdc2 kinase activity is minimal, and loads MCM proteins onto replication origins at this time (in concert with Cdt1) (Jallepalli et al., 1997; Kearsey et al., 2000; Kelly et al., 1993; Muzi Falconi et al., 1996; Nishitani et al., 2000; Nishitani and Nurse, 1995). From my results, there appears to be no nucleotide regulation of Cdc18 chromatin association, but binding and hydrolysis of ATP is essential for the MCM loading activity (if we assume that the WA and WB motifs of Cdc18 are required for ATP binding and hydrolysis respectively).

It is possible that, also during G1 (post start), Cdc18 initiates an S phase checkpoint signal that is sufficient to restrain mitosis. This would mean that the replication checkpoint could be active in G1, when replication is absent, as well as in S, when replication is incomplete. The notion of such a 'G1-M' checkpoint was first suggested after work with an Orp1 ts mutant, *orp1-4* (Synnes et al., 2002). At restrictive temperature S phase does not occur and replication intermediates appear to be absent (from 2D-gel analysis), yet a Rad3/Chk1-dependent inhibition of mitosis is present. In this thesis I have identified a replication-deficient Cdc18 ts mutant (*cdc18-3.9*) which also displays a Rad3/Chk1-dependent checkpoint in the apparent absence of replication intermediates. In both the *orp1-4* and *cdc18-3.9* mutants, Cdt1 and Cdc18 are bound to chromatin but Mcm4 is not. Collectively, these data suggest that these mutants arrest, in a Rad3-dependent manner, in late G1 due to an inability to load MCM proteins and hence to initiate DNA replication. In contrast, cells lacking Orp1 or Cdc18 (or indeed Cdt1) fail to initiate an S phase checkpoint and enter a lethal mitosis (Grallert and Nurse, 1996; Hofmann and Beach, 1994; Kelly et al., 1993). Whilst more work needs to be done to rule out the presence of a low level of replication intermediates in these ts arrests, it seems possible that Cdc18, perhaps in a complex with ORC and Cdt1, initiates an S phase checkpoint in late G1. From this work, Cdc18 needs to bind ATP (but not to hydrolyse it) to initiate this signal. Presumably wild-type Cdc18 is capable of initiating or maintaining this checkpoint signal following MCM loading (i.e. in the ADP-bound state).

6.2 Cdc18 in S phase

As cells proceed into S phase, and DNA replication begins, Cdc18 is apparently required for (at least) two further processes. Firstly, Cdc18 is required for maintaining the S phase checkpoint when DNA replication is perturbed. I have shown that Cdc18 is required to restrain mitosis when replication is disturbed by HU or by using ts mutants of DNA pol α or DNA ligase. Such perturbations are likely to represent a range of DNA insults (e.g. from stalled forks to damaged structures), and indeed involve both Cds1 (HU) and Chk1 (ts arrests) responses. Thus Cdc18 may be universally required for

the S phase checkpoint in much the same way as the Rad proteins. From my results it seems likely that Cdc18 is present throughout S phase and would thus be able to respond to insults to the integrity of the DNA during this time. In contrast, Cdt1 appears to be removed from the cell as it progresses into S phase, and Cdt1 is not required to restrain mitosis in an HU block (Damien Hermand, unpublished data). It will be interesting to illuminate the mechanism by which Cdc18 maintains the S phase checkpoint. My results reveal a correlation between checkpoint function and Cdc18 chromatin association, and this is consistent with findings that Cdc18 is required for Rad3 chromatin association in response to HU treatment (Damien Hermand, unpublished data). One approach to understanding further the behaviour of Cdc18 will be to locate the position of Cdc18 on the chromatin throughout S phase. One possibility is that Cdc18 (or a subpopulation of Cdc18), travels along the DNA with the replication fork. At the fork Cdc18 would be able to detect impediments to replication progression and hence signal to the Rad checkpoint system. Another possibility is that Cdc18 is recruited independently to sites of stalled forks or damage, perhaps in a Rad17-dependent manner. These possibilities could be explored by using ChIP to follow Cdc18 localisation in origin and non-origin areas, throughout S phase, and in relation to ORC, replication factors, and Rad proteins. Using ChIP on DNA microarrays (chip-ChIP) would enable this analysis on a genomic scale.

Investigation of the *cdc18-H2.3* ts mutant has revealed a putative third role for Cdc18 in the cell cycle, also within S phase. However, one caveat with the sequencing experiment used to identify this role is that HU was used to synchronise the cells in early S phase. Hence the loss of function displayed by *cdc18-H2.3* may reflect an inability to respond to HU at restrictive temperature, rather than an inability to effect a normal S phase function post initiation. However, even this possibility would reveal an interesting role for Cdc18; such a role in HU response would be separate from its checkpoint function and is unlikely to involve DNA metabolism. A novel, alternative method of arresting cells at early S would be required before this matter could be conclusively resolved.

For the remaining discussion I shall assume that the results from chapter 5 reveal a role for Cdc18 in a normal S phase as opposed to a non-checkpoint role in response to HU. The nature of this role is a matter for speculation at this stage, and more work needs to be carried out to confirm the process dependent upon this function. The lack of Cdc18 protein at the block point in the *cdc18-H2.3* sequencing experiment suggests that the cells arrest in G2 or mitosis, but further investigation is required to confirm this, and to locate the block more precisely. Two candidate arrest points would be at the G2/M transition and the metaphase/anaphase transition. From my results it seems that the putative third role does not relate to DNA metabolism, and hence I hypothesise that Cdc18 is required for some chromosome function which is subsequently needed for entry into or progress through mitosis. The proposals suggested in chapter 5 include the establishment of cohesion between sister chromatids, preparation of chromosomes for condensation in mitosis, or the localisation of the mitotic CDK complex to chromosomes in readiness for mitotic entry. RFC has been shown to have a least two specific functions in the cell, both of which involve loading proteins (PCNA and the 9-1-1 complex) onto DNA (Kim et al., 2005). Given the homology of Cdc18 to RFC it may be that the S phase roles of Cdc18, like its M/G1 role of recruiting MCM proteins to replication origins, involve loading of proteins or protein complexes onto DNA. For instance, Cdc18 may load Rad3 onto chromatin in response to stalled forks and may load cohesin subunits onto chromatin during S phase. If such functionality is identified, then it will be interesting to see if this activity is dependent upon ATP hydrolysis, and if so, whether these multiple catalytic roles require a cyclic replacement of ADP with fresh ATP.

As with the role for Cdc18 in the S phase checkpoint, knowing the localisation of Cdc18 during S phase is likely to help us model the behaviour of Cdc18 in this third, putative role. It will be interesting to know if Cdc18 effects its two S phase functions from the same location, or whether different subpopulations of Cdc18 effect different roles from different positions. For example, one

could imagine Cdc18 travelling with the replication fork during S phase, enabling the loading of cohesin subunits and also recruiting Rad3 if the fork is stalled. On the other hand, one subpopulation of Cdc18 may remain at the origin, perhaps to load condensins, whilst another subpopulation is recruited to sites of DNA damage for the S phase checkpoint. Many DNA replication factors have been shown to participate in cohesion, including DNA helicases, S-phase checkpoint factors (e.g. Mrc1), RFC, and DNA polymerases (reviewed in Skibbens, 2005). The process of DNA replication also appears to be closely linked to chromosome condensation (Christensen and Tye, 2003; Pflumm, 2002). Significantly, a number of DNA replication factors, including Cdc18, were recently shown to interact genetically with both cohesion and condensin components in fission yeast (Yuasa et al., 2004). Another possible role of Cdc18 at replication origins in S phase may be to recruit the mitotic CDK complex, Cdc2-Cdc13. This recruitment is dependent upon ORC and is required to prevent rereplication (Wuarin et al., 2002). It may be that Cdc18 is also required for this recruitment, and that Cdc2 localisation to origins both prevents rereplication and facilitates Cdc2 mitotic activity. If Cdc2-Cdc13 mediated repression of replication involves degradation of origin bound Cdc18 then we must additionally postulate that a separate, non-origin population of Cdc18 is responsible for its S phase checkpoint function.

6.3 The Cdc18/Cdc2 switch

One important issue raised by the newly discovered functions of Cdc18 in S phase is the relationship between Cdc18 and Cdc2. A traditional view describes Cdc18 simply as a target for negative regulation by Cdc2. Hence at late mitosis and G1, a period of low Cdc2 kinase activity, Cdc18 can accumulate and functions in pre-RC formation; increased Cdc2 activity at the G1/S transition triggers DNA replication and the removal of Cdc18 from the cell. Whilst Cdc2 kinase activity acts to repress *cdc18* transcription (Ayte et al., 2001; Baum et al., 1998) and targets the Cdc18 protein for ubiquitin-mediated proteolysis (Jallepalli et al., 1997; Jallepalli et al., 1998; Kominami et al., 1998; Kominami and Toda, 1997; Wolf et al., 1999), other work has shown that Cdc18 can in turn inhibit Cdc2 kinase activity via the Rad

checkpoint system (Greenwood et al., 1998; Zarzov et al., 2002). Hence Cdc18 and Cdc2 have an antagonistic relationship, and the cell cycle sees a switch from a Cdc18 dominant state (M/G1) to a Cdc2 dominant state (G2). What is unclear is how this switch is coordinated during S phase, and a model of the Cdc18/Cdc2 switch in this part of the cell cycle must take into account the role of Cdc18 in maintaining an S-phase checkpoint and its putative role in preparing the cell for mitosis. In both cases, the specific localisation of Cdc18 (or a subpopulation of Cdc18) on the chromatin may influence its susceptibility to Cdc2-mediated phosphorylation or/and degradation. For example, a subpopulation of Cdc18 travelling with the replication fork may be immune to proteolysis, whilst other subpopulations are sensitive. From the replication fork Cdc18 may load cohesin proteins and recruit Rad3 at times of replication perturbation. When forks meet and resolve at the end of S phase Cdc18 would be released and degraded. Another factor to consider is the possibility that Cdc18 is modified or/and stabilised in response to perturbation of DNA replication. When transcription of *cdc18* is repressed in an HU block the Cdc18 protein level rapidly decreases (figure 4.2). Thus the Cdc18 pool in an HU block is subject to constant degradation, and the level of Cdc18 in the cell is maintained by a supply of newly synthesised Cdc18. Closer investigation on a timescale of minutes rather than hours is required, however, to determine if the rate at which Cdc18 is degraded in an HU block is reduced compared to the rate in other circumstances. This will help to determine if subtle changes in Cdc18 stabilisation are a feature of the Cdc18/Cdc2 switch in S phase.

6.4 Conservation of Cdc18 function amongst eukaryotes

The role of Cdc18/Cdc6 proteins (herein referred to as Cdc6) in pre-RC formation has been well studied and is a highly conserved feature of eukaryotic DNA replication (reviewed in Bell and Dutta, 2002). Cdc6 binds to replication origins via the ORC complex and loads the MCM complex onto DNA in concert with Cdt1. This MCM loading activity of Cdc6 is dependent upon ATP hydrolysis and is required for DNA replication. The requirement for ATP binding for Cdc6 origin association in *Xenopus* may reflect an

additional control step for pre-RC formation in this system (Frolova et al., 2002).

Other functions for Cdc6 have been less well studied. Deletion of Cdc6 in budding yeast, fission yeast or *Drosophila* gives rise to inappropriate entry into mitosis, implicating Cdc6 in the S-phase checkpoint (Crevel et al., 2005; Kelly et al., 1993; Piatti et al., 1995). Recent work in fission yeast, described and presented in this thesis, has demonstrated that Cdc6 works intimately with the Rad checkpoint system as part of the S phase checkpoint after initiation of DNA replication. A role for Cdc6 in S phase checkpoint function is also likely to be detailed in higher eukaryotes; Cdc6 is required for Chk1 activation in response to aphidicolin in *Xenopus* (Oehlmann et al., 2004) whilst overexpression of Cdc6 in human cells leads to a Chkl-dependent inhibition of mitosis (Clay-Farrace et al., 2003).

The putative involvement of Cdc6 in chromosome dynamics during S phase in fission yeast, identified in this thesis and supported by genetic interactions between Cdc6 and separase/condensin subunits (Yuasa et al., 2004), has not been reported in any other system to date. Interestingly, a separate, additional function for Cdc6, other than its role in DNA replication and the S phase checkpoint, has been suggested from work with mouse Cdc6. Mouse Cdc6 expressed in hamster cell lines associates with the spindle apparatus (Illenye and Heintz, 2004), and Cdc6 is essential for spindle formation during maturation of mouse oocytes (Anger et al., 2005). It will be interesting to see if mammalian Cdc6, which is present throughout the mitotic cell cycle, is required for all of the emerging functions for Cdc6 in these systems. The interaction between DNA replication and other cellular processes, such as chromosome cohesion, is an expanding area of research. As such, factors with a well-defined role in DNA replication are becoming implicated in other processes, and a good example is the ORC complex. Components of budding yeast ORC have been known to be involved in transcriptional silencing for some time (Bell et al., 1993; Loo et al., 1995), and increasingly ORC has been implicated in the regulation of heterochromatin and chromosome structure in a

range of systems (Leatherwood and Vas, 2003; Prasanth et al., 2004). A similar picture may emerge for Cdc6-a replication and checkpoint factor with additional roles in chromosome metabolism.

Chapter 7. Materials and methods

7.1 Fission yeast physiology and genetics

7.1.1 Nomenclature for genes and proteins

The following nomenclature has been used for the thesis. For fission yeast genes, the name of the gene is in lower case and italicised (e.g. *cdc18*). The wild-type allele is designated by a + sign (e.g. *cdc18+*). The mutant form of the gene is in lower case and italicised, with an allele number or abbreviated description when specified (e.g. *cdc18-K46* or *cdc18-WA*). Tag abbreviations are then added to the genotype description were necessary (e.g. *cdc18-WA-HA*). The protein encoded by the gene has the first letter in upper case and the rest of the gene name in lower case (e.g. Cdc18 or Cdc18-WA). For budding yeast genes, the name of the gene and the wild-type allele are in upper case letters and italicised (e.g. *CDC6*). The mutant form of the gene is in lower case letters and italicised, with an allele number when specified (e.g. *cdc6-1*). The protein encoded by that gene has the first letter in upper case and the rest of the gene name in lower case (e.g. Cdc6). When referring to homologous genes or proteins in fission yeast and budding yeast, the two names may be divided by a forward slash (e.g. *cdc18/CDC6*). For genes and proteins in metazoa, the nomenclature used for fission yeast has been applied. Genes present within a cell but located on a plasmid are preceded by the character '*p*'. Where *nmt81-cdc18* (SO) has been crossed into a mutant strain, or where *pnmt41/81-cdc18* has been transformed into a mutant strain, individual genes are separated by a semi-colon (;).

Table 7.1 Strains used in this study

Genotype	Shorthand used in text
h- ade6-M210	
h- ade6-M210 leu1-32	
h- ade6-M216 leu1-32 ura4-D18	
h+ ade6-M210 leu1-32 ura4-D18	
h- ade6-M210 nmt81-cdc18+	SO
h- ade6-M210 nmt81-cdc18+ cdc18-WA-HA	*cdc18-WA-HA;* SO
h- ade6-M210 nmt81-cdc18+ cdc18-WB-HA	*cdc18-E287G-HA;* SO
h- ade6-M210 ura4-D18 his3-D1 Δcdc18::ura4+ leu1+::cdc18-DE286AA-midT with *pDAD112* (containing *nmt81-cdc18 his3+*)	*cdc18-DEAD-midT;* SO
h- leu1-32 ura4-D18 cdc18-TAP-Kan^R	*cdc18-TAP*
h- ade6-M210 leu1-32 rad3-136	*rad3Δ*
h- ade6-M210 leu1-32 rad3ts	*rad3ts*
h- ade6-M216 leu1-32 cdc17-K42	*cdc17-K42*
h+ ade6-M210 leu1-32 pol1-1	*pol1-1*
h- leu1-32 ura4-D18 rad3::ura4 cdc25-22 cdc18-P1-6-TAP-Kan^R	*cdc18-TA* in *rad3Δ* and *cdc25-22* background
h- leu1-32 ura4-D18 rad3::ura4 cdc25-22 cdc18-TAP-Kan^R	WT *cdc18* in *rad3Δ* and *cdc25-22* background
h-/h- ade6-M210/ade6-M216 leu1-32/leu1-32	Homozygous wild-type diploid
h- ade6-M210 leu1-32 cdc25-22 orp1-HA	
h+ leu1-32 ura4-D18 cdc25-22 cdc18-TAP	

7.1.2 Strain growth and maintenance

All strains were derived from the wild-type *972h-* and *975h+* strains. Medium and growth conditions are as previously described (Moreno et al., 1991). Techniques used to grow and maintain fission yeast strains, to store and revive frozen cultures, to check phenotypes, to perform and analyse genetic crosses

by tetrad analysis or random spore analysis were performed as previously described (MacNeill and Fantes, 1993; Moreno and Nurse, 1994). All experiments were performed using cells in exponential growth in minimal medium at 32°C. For ts strains, 25°C was used as the permissive temperature and 36.5°C was used as the restrictive temperature (unless otherwise stated).

7.1.3 Strain construction

For construction of ts mutants by random mutagenesis, a total of 56 mutagenic PCR reactions were performed, and pairs were pooled. Pooled mixtures (28) were then purified using a Qiagen QIAquick kit and DNA eluted in 30μl water. 2μl of this prep was run on a 0.8% agarose gel (to check for the presence of the required PCR product), whilst the remainder was used for transformation of wild-type cells. For construction of ts mutants by site-directed mutagenesis, a total of 6 high-fidelity PCR reactions were performed, and pairs pooled. These pooled mixtures were then treated as above.

All other haploid strains were constructed by genetic crosses. Where each desired genotype is individually traceable (e.g. by antibiotic resistance or temperature sensitivity) strains were isolated following random spore analysis. For other cases (e.g. double ts mutants) tetrad analysis was employed. To construct the COT strain, ten isolates were obtained demonstrating temperature sensitivity (*cdc25-22*) and resistance to Kanamycin (*cdc18-TAP*). Protein extracts were then prepared from these isolates which were probed for HA staining, identifying strains with the desired *orp1-HA* genotype.

Diploid strains were constructed by crossing *h+ ade6-M210* strains with *h- ade6-M216* strains on low nitrogen plates, and transferring the cross to minus adenine plates after 16 hours. Diploids were then maintained on YE medium, or sporulated on low nitrogen plates. Non-sporulating diploids were obtained by plating out approximately 10^3 cells and screening for stable isolates on low nitrogen. These stable diploid versions were used for experiments in liquid culture. Sporulation of these stable diploid strains (to check for heterozygosity) was achieved by plating out approximately 10^3 cells and screening for sporulating isolates on low nitrogen medium.

Table 7.2 Strains constructed in this study

Genotype	Shorthand used in text
h+ ade6-M210 leu1-32 ura4-D18 cdc18-3.2-ura4+	*cdc18-3.2*
h+ ade6-M210 leu1-32 ura4-D18 cdc18-3.6-ura4+	*cdc18-3.6*
h+ ade6-M210 leu1-32 ura4-D18 cdc18-3.9-ura4+	*cdc18-3.9*
h+ ade6-M210 leu1-32 ura4-D18 cdc18-D8-ura4+	*cdc18-D8*
h- ade6-M216 leu1-32 ura4-D18 cdc18-B2.1-ura4+	*cdc18-B2.1*
h- ade6-M216 leu1-32 ura4-D18 cdc18-B3.1-ura4+	*cdc18-B3.1*
h- ade6-M216 leu1-32 ura4-D18 cdc18-F2.1-ura4+	*cdc18-F2.1*
h- ade6-M216 leu1-32 ura4-D18 cdc18-H1.2-ura4+	*cdc18-H1.2*
h- ade6-M216 leu1-32 ura4-D18 cdc18-H2.3-ura4+	*cdc18-H2.3*
h- ade6-M216 leu1-32 ura4-D18 cdc18-I1.2-ura4+	*cdc18-I1.2*
h- ade6-M216 leu1-32 ura4-D18 cdc18-K3.2-ura4+	*cdc18-K3.2*
h- ade6-M216 leu1-32 ura4-D18 cdc18-L2.1-ura4+	*cdc18-L2.1*
h- ade6-M216 leu1-32 ura4-D18 cdc18-G317D-ura4+	*cdc18-G317D*
h+ ade6-M210 leu1-32 ura4-D18 cdc18-3.2-ura4+ rad3-136	*cdc18-3.2 rad3Δ*
h+ ade6-M210 leu1-32 ura4-D18 cdc18-3.6-ura4+ rad3-136	*cdc18-3.6 rad3Δ*
h+ ade6-M210 leu1-32 ura4-D18 cdc18-3.9-ura4+ rad3-136	*cdc18-3.9 rad3Δ*
h+ ade6-M210 leu1-32 ura4-D18 cdc18-D8-ura4+ rad3-136	*cdc18-D8 rad3Δ*
h- ade6-M210 leu1-32 ura4-D18 cdc18-B2.1-ura4+ rad3-136	*cdc18-B2.1 rad3Δ*
h- ade6-M210 leu1-32 ura4-D18 cdc18-B3.1-ura4+ rad3-136	*cdc18-B3.1 rad3Δ*
h- ade6-M210 leu1-32 ura4-D18 cdc18-F2.1-ura4+ rad3-136	*cdc18-F2.1 rad3Δ*

h- ade6-M210 leu1-32 ura4-D18 cdc18-H1.2-ura4+ rad3-136	*cdc18-H1.2 rad3Δ*
h- ade6-M210 leu1-32 ura4-D18 cdc18-H2.3-ura4+ rad3-136	*cdc18-H2.3 rad3Δ*
h- ade6-M210 leu1-32 ura4-D18 cdc18-I1.2-ura4+ rad3-136	*cdc18-I1.2 rad3Δ*
h- ade6-M210 leu1-32 ura4-D18 cdc18-K3.2-ura4+ rad3-136	*cdc18-K3.2 rad3Δ*
h- ade6-M210 leu1-32 ura4-D18 cdc18-L2.1-ura4+ rad3-136	*cdc18-L2.1 rad3Δ*
h- ade6-M210 leu1-32 ura4-D18 cdc18-G317D-ura4+ rad3-136	*cdc18-G317D rad3Δ*
h- ade6-M210 leu1-32 ura4-D18 cdc18-H2.3-ura4+ rad3ts	*cdc18-H2.3 rad3ts*
h+leu1-32 his3-D1 cdc25-22 orp1-HA cdc18-TAP-Kan^R	COT
h+ ade6-M210 leu1-32 ura4-D18 his3-D1 cdc25-22 cdc18-TAP-Kan^R	*cdc25-22 cdc18-TAP*
h+ ade6-M210 leu1-32 ura4-D18 his3-D1 cdc10-v50 cdc18-TAP-Kan^R	*cdc10-v50 cdc18-TAP*
h+ ade6-M210 leu1-32 ura4-D18 his3-D1 orp1-4 cdc18-TAP-Kan^R	*orp1-4 cdc18-TAP*
h+ ade6-M210 leu1-32 ura4-D18 his3-D1 cdc23-M36 cdc18-TAP-Kan^R	*cdc23-M36 cdc18-TAP*
h+ ade6-M210 leu1-32 ura4-D18 his3-D1 pol1-1 cdc18-TAP-Kan^R	*pol1-1 cdc18-TAP*
h+ ade6-M210 leu1-32 ura4-D18 his3-D1 cdc17-K42 cdc18-TAP-Kan^R	*cdc17-K42 cdc18-TAP*
h+ ade6-M210 leu1-32 ura4-D18 his3-D1 cdc27-P11 cdc18-TAP-Kan^R	*cdc27-P11 cdc18-TAP*
h- ade6-M210 leu1-32 cdc17-K42 nmt81-cdc18+	*cdc17-K42*; SO
h- ade6-M210 leu1-32 pol1-1 nmt81-cdc18+	*pol1-1*; SO
h-/h- ade6-M210/ade6-M216 leu1-32/leu1-32 ura4-D18/ura4-D18 cdc18+/cdc18-H2.3-ura4+	heterozygous mutant diploid

7.1.4 Transformation of fission yeast

Approximately 10^8 exponentially growing cells were used per transformation. Cells were washed once in 1ml water, then resuspended in 200μl water. Herring sperm DNA (100μg) and transforming DNA were added, along with 0.5ml PLATE (40% PEG 4000, 100mM lithium acetate, 10mM Tris-HCl (pH 7.5), 1mM EDTA). This mixture was vortexed and incubated at room temperature overnight. Cells were washed twice in 1ml water before resuspension in water. Cells were then plated out onto selective medium at 32°C (or 25°C for ts strains).

7.1.5 Use of Hydroxyurea (HU)

Hydroxyurea (HU) was used at 12mM in minimal medium. For timecourses in solution, a 6mM top-up was added every generation time (e.g. every 4hrs at 25°C). To release cells from an HU block, cells were filtered, washed and resuspended in minimal medium lacking HU.

7.1.6 Use of *nmt* expression system

The *nmt* promoter is derived from the *nmt1* gene, which is required for thiamine biosynthesis (Maundrell, 1990). Proteins lying behind the *nmt* promoter in expression systems are expressed *in vivo*. There are three versions of the nmt promoter; the strongest is *nmt1*, *nmt41* is weaker and *nmt81* is the weakest. All are repressed in the presence of thiamine, although with the wild-type promoter, *nmt1*, there is a significant background expression level even in the presence of thiamine. The relative, approximate expression strengths of *nmt* promoters are given in the table below, along with the laboratory nomenclature for the plasmids containing these promoters.

Table 7.3 Comparison of the expression levels of *nmt* promoters

nmt promoter	Repressed conditions	Induced conditions	*ura4+* marker	LEU2+ marker
nmt1	20	6160	pREP4	pREP1
nmt41	4	100	pREP42	pREP41
nmt81	1	6	pREP82	pREP81

To repress the *nmt* promoter, thiamine was added to a minimal medium at a final concentration of 30μM. To induce expression from an *nmt* promoter, cells cultured in the presence of thiamine were filtered, washed and resuspended in minimal medium lacking thiamine. Induction typically occurs approximately 16 hours later.

7.1.7 Population mass and cell number

Population mass was followed by measuring the optical density (OD) of the cell culture at 595nm using an Amersham Ultraspec 2100 pro spectrophotometer. All experiments were carried out in logarithmic phase for growth, between OD of 0.1 and 0.9. Cell number was determined using a Coulter counter. Cells were fixed by adding 1.6mls of formal saline (0.9% saline, 3.7% formaldehyde) to 0.4ml of cell culture and stored at 4°C. Before counting, 18mls of filtered ISOTON solution was added and cells were sonicated for 30 seconds on level 6 of Soniprep 150 sonicator (MSE). The number of cells in a 0.5ml aliquot of each sample was taken in triplicate and averaged.

7.1.8 Flow cytometric analysis

The DNA content of cells was assessed using a Becton Dickinson fluorescence activated cell sorter (FACS). Cells were fixed by suspension in 1ml 70% ethanol and stored at 4°C. Cells were then rehydrated by spinning through 3ml 50mM sodium citrate, and treated with RNase (0.2mg/ml final in 0.5ml 50mM sodium citrate) at 37°C for at least 3 hours. Cells were then stained with propidium iodide (2μg/ml final in 0.5ml 50mM sodium citrate) and sonicated for 30 seconds as (described above) prior to analysis.

7.1.9 Visualisation of nuclei by DAPI staining

Cells fixed in 70% ethanol were rehydrated in water, heat fixed to a slide and mounted in 1μg/ml of DAPI (4', 6-diamidino-2-phenylindole) in 50% glycerol. Cells were visualised using a Zeiss Axioplan microscope and photographed with a Hamamatsu C4742-95 digital camera.

7.2 Molecular biology techniques

7.2.1 General techniques

The following techniques were performed essentially as described (Sambrook et al., 1989): preparation of competent bacteria, transformation of bacteria with DNA, restriction enzyme digests of DNA, and gel electrophoresis of DNA. Both small (miniprep) and large scale (maxiprep) preparation of DNA from bacteria was done using Qiagen QIAprep kits. PCR products were purified with a Qiagen QIAquick kit.

7.2.2 PCR reactions

All PCR reactions were carried out in a Biometra TGradient PCR cycler. The mutagenic PCR reactions were carried out in a final volume of 100μl containing 500ng template DNA (pDH17), 50pmol each of primers 247 and 256, 1X Taq buffer, 1.76mM $MgCl_2$, 0.5mM $MnCl_2$, 0.56mM dATP, 0.9mM dCTP, 0.2mM dGTP, 0.14mM dTTP and 8U of Taq DNA polymerase. 25 cycles were used in the PCR, with a melting temperature of 94°C (30s), annealing temperature of 42°C (30s) and extension temperature of 72°C (4 minutes).

High fidelity PCR of the pOH1 plasmid was carried out in a final volume of 50μl containing 50ng plasmid, 50pmol each of primers 247 and 256, 0.1mM dNTP mix, 3.5U of Expand High Fidelity enzyme mix (Roche) and 1X High Fidelity reaction buffer. 35 cycles were used in the PCR, and cycle details were as above.

For the Colony PCR reactions, a small loop of the appropriate colony was innoculated into the reaction mix before addition of the polymerase. The mix was then boiled for 10 minutes before cooling on ice and adding of 3.5U of Expand High Fidelity enzyme mix. The reaction contained 50pmol of each primer, 0.1mM dNTP mix and 1X High Fidelity reaction buffer. 35 cycles were used in the PCR, and cycle details were as above, with the following exceptions. For colony PCR of the new ts *cdc18* mutants (with primers 275 and R6) an annealing temperature of 45°C was used. For colony PCR of isolates rescued by a cDNA plasmid (with primers CO8 and R5) an annealing temperature of 50°C was used, with an extension time of 3 minutes.

Site directed mutagenesis was carried out using a Stratagene QuickChange Site-Directed Mutagenesis Kit, according to the kit instructions, using 50-500ng of DNA template and 18 PCR cycles. Mutation of pDH17 plasmid (to yield pOH1) was effected using primers G317DF and G317DR with an annealing temperature of 55°C and an extension time of 24 minutes. Mutation of *pnmt1-cdc18-C* (ARC 746) to yield various mutant forms of the Cdc18 C-terminus was effected using primers K205AF, K205AR, D286GF, D286GR, E287GF, E287GR, D286AF, D286AR, E287AF and E287AR with an annealing temperature of 55°C and an extension time of 12 minutes.

Table 7.4 Primers used in this study

Primer Name	Primer Sequence (5' to 3')
G317DF	CCGACTTCGAGATTAATTTTGGTAGACATTGCAAATGCC
G317DR	GGCATTTGCAATGTCTACCAAAATTAATCTCGAAGTCGG
247	CGATATGTGTGAAACTCC
256	CAATCATATTCAGTTTTGTGT
275	GAAGTTAATATTACTTAGTAGC
R6	GCATACATATAGCCAGTGGG
CO8	GCTACTGGATGGTTCAGTCAC
R5	CATCGGCAACACTCAATGAGG
K205AF	GCCCCTGGCACAGGAGAGACCGTTCTGCTTCACAACG
K205AR	CGTTGTGAAGCAGAACGGTCTCTCCTGTGCCAGGGGC
D286GF	CCCAGTCATTATTGTTTTAGGTGAAATGGATCACTTGATTGC
D286GR	GCAATCAAGTGATCCATTTCACCTAAAACAATAATGACTGGG
E287GF	CCCAGTCATTATTGTTTTAGATGGAATGGATCACTTGATTGC
E287GR	GCAATCAAGTGATCCATTCCATCTAAAACAATAATGACTGG
D286AF	CCCAGTCATTATTGTTTTAGCTGAAATGGATCACTTGATTGC
D286AR	GCAATCAAGTGATCCATTTCAGCTAAAACAATAATGACTGGG
E287AF	CCCAGTCATTATTGTTTTAGATGCAATGGATCACTTGATTGC
E287AR	GCAATCAAGTGATCCATTGCATCTAAAACAATAATGACTGGG
236	TAATTCTACTGCCAAATTG
237	GAACAAAGCATATTACTCCC
238	AACAAGTTCGGAATTTTGCG
R1	GGTCCTTTTGGGGGTTTTAGGAG
284	CAGAACGGTCTTTCCTGTGCC
R3	CGAGAGGGCCATTCAAAAAGCG
R4	CCGGGCACATAATTCAATTGCAGC

7.2.3 DNA sequencing

Sequencing was carried out using the dideoxynucleotide method (Sanger et al., 1977). DNA sequencing PCR was effected in a final volume of 20μl containing 50-500ng template DNA, 1μM sequencing primer and 9μl of CRUK termination ready reaction mix (A-dye terminator, C-dye terminator, G-dye terminator, T-dye terminator, dITP, dATP, dCTP, dTTP, Tris-HCl (pH 9), $MgCl_2$, thermal stable pyrophosphatase, and AmpliTaq DNA polymerase, FS). Samples were run on a 4.8% acrylamide gel and detected using an ABI Prism 377 DNA sequencer. The Cdc18 ORF was sequenced in the forward direction using primers 275, 247, 236, 237 and 238, and in the reverse direction using R1, 284, R3, R4, R5 and 256 (or R6 when *ura4+* lies 3' to the *cdc18* ORF).

7.2.4 Fission yeast plasmid extraction

Fission yeast cultures (10ml) were grown to exponential phase in selective medium. Cells were then collected by centrifugation, resuspended in 1ml water, and transferred to an Eppendorf. Cells were collected using a 5 second spin in a microfuge, the supernatant was decanted away and cells resuspended in 200μl lysis solution (2% Triton X-100, 10mM Tris-HCl (pH 8), 1% SDS, 1mM Na_2EDTA, 100mM NaCl). 200μl phenol:chloroform:IAA (25:24:1) and 0.3 g acid washed glass beads were added before vortexing for 2 minutes. The tube was then centrifuged for 5 minutes and the upper, aqueous layer removed and added to 200μl phenol:chloroform:IAA in a fresh eppendorf. Vortexing and centrifugation was repeated as above and the upper layer removed. DNA was precipitated from this layer, washed in 70% ethanol and resuspended in 10μl TE. This DNA preparation was further purified using the Q-Biogene Geneclean II kit, with an elution volume of 15μl water. 5 μl of this purified prep was transformed into competent *E.coli* cells.

7.2.5 Pulsed-field gel electrophoresis

Approximately $2x10^8$ cells were stopped with sodium azide (1mM) and collected by centrifugation. Cells were washed and resuspended in SP1 buffer (40mM EDTA, 1.2M sorbitol, 50mM sodium citrate, 50mM disodium

phosphate, pH 5.6). Cell density was estimated using a haemocytometer before Zymolyase-100T was added to 0.6mg/ml. Cells were incubated at 37°C and spheroplasting monitored by mixing 10μl samples with 1μl 20% SDS. When 95% lysis was achieved spheroplasts were collected by centrifugation and resuspended in 1% low melting point agarose in TSE (0.9M sorbitol, 45mM EDTA, 10mM Tris-HCl, pH 7.5) at a concentration of 8 x 10^7 cells per 100μl. Cells were dispensed into 100μl plug moulds and allowed to solidify at 4°C. Plugs were removed and incubated in 0.25M EDTA, 1% SDS, 50mM Tris-HCl, pH 7.5, at 55°C for 90 minutes. Plugs were then incubated in 0.5M EDTA, 1% lauryl sarcosine, 1mg/ml proteinase K, 10mM Tris-HCl, pH 9.5, at 55°C for 24 hours. This proteinase K step was repeated before washing twice in T10xE (10mM EDTA, 10mM Tris-HCl, pH 7.5) at 25°C for 30 minutes, once in T10xE containing 0.04mg/ml PMSF (50°C, one hour), and twice in T10xE (25°C, 30 minutes). Plugs were stored in 0.5M EDTA, 10mM Tris-HCl, pH 9.5

For whole chromosome gels, plugs were loaded into a 0.8%, 1 x TAE gel and electrophoresed at 14°C in 1 x TAE using a BioRad CHEF-DR III Variable Angle System with the following conditions: 24 hours with switch time of 1200 seconds, angle of 96° at 2V/cm, 24 hours with switch time of 1500 seconds, angle of 100° at 2V/cm, and 24 hours with switch time of 1800 seconds, angle of 106° at 2V/cm. Gels were stained in water containing 5μg/ml ethidium bromide and destained in water for one hour and overnight prior to photographing on a UV box.

For NotI digested chromosome gels, plugs were washed twice in T10xE at 25°C for 30 minutes, once in 2x NotI buffer (37°C, one hour,) and once in 1x NotI buffer (37°C, one hour). Chromosomes were then digested in 1x NotI buffer and 50 units of NotI at 37°C overnight. Plugs were washed in T10xE (25°C, 30 minutes), loaded into a 1%, 0.5 x TBE gel and electrophoresed at 14°C in 0.5 x TBE for 24 hours (switch time of 60-120 seconds, angle of 120° at 6V/cm).

7.2.6 Protein extract preparation

Total boiled cell extracts were prepared from $2x10^8$ OD units as described (Nishitani and Nurse, 1995). Cells were washed once in STOP buffer (150mM NaCl, 50mM sodium fluoride, 1mM sodium azide, 10mM EDTA, pH 8.0), resuspended in 200μl of HB buffer (25mM MOPS, pH 7.2, 15mM $MgCl_2$, 15mM EGTA, 1mM DTT, 1% Triton X-100, 60mM ß-glycerophosphate, 0.1mM sodium vanadate, 1mM PMSF, 20μg/ml leupeptin and 10μg/ml aprotinin) and boiled for 6 minutes. Cells were then broken by adding glass beads and vortexing in a Bio 101 FastPrep 120. Extracts were recovered by piercing the bottom of the tube with a needle and centrifuging into an Eppendorf tube at 2000 rpm for 1 minute. Protein concentrations were determined with a BCA/Copper (II) sulfate assay (Sigma B9643). Approximately 50μg of protein were run on gels for Western blotting.

7.2.7 Chromatin extract preparation

Approximately $2x10^8$ OD units were stopped with sodium azide (1mM) and collected by centrifugation. Cells were washed once with water, once with 1.2M sorbitol, and resuspended in 0.9ml of SP2 (1.2M sorbitol, 50mM sodium citrate, 50mM disodium phosphate, pH 5.6). Lysing enzymes (Sigma) were added at 1mg/ml (L2265) or 5mg/ml (L1412) and cells were incubated at 30°C. Spheroplasting was followed by mixing 2μl samples with 2μl 2% Triton X-100, and analysing the extent of lysis. When two thirds of the cells lysed the reaction was terminated with 10mM Tris-HCl, pH7.5. Spheroplasts were then spun through a sucrose gradient (15% sucrose, 1.2M sorbitol, 10mM Tris-Cl, pH7.5) at 2000rpm for 4 minutes, and washed twice in 1.2M sorbitol, 10mM Tris-HCl, pH7.5. Spheroplasts were lysed in BE10 buffer (20mM Hepes, pH7.9, 1.5mM magnesium acetate, 50mM potassium acetate, 10% glycerol, 0.5mM DTT, 1mM PMSF, 20μg/ml leupeptin, 40μg/ml aprotinin, 1.5μg/ml pepstatin and 2mM benzamidine) containing 1% Triton X-100, 0.1mM sodium vanadate and 5mM ATP, and incubated on ice for 10 minutes. A proportion of the lysate was kept for the total cell extract, and a proportion used to estimate total protein concentration by BCA method (see previous). The remaining fraction was centrifuged through a 30% sucrose cushion, and

the supernatant removed (corresponding to the Triton-extractable fraction). The pellet was washed once in BE10 buffer containing 5mM ATP and 0.1mM sodium vanadate. Chromatin associated proteins were released from the pellet by resuspending in BE10 buffer containing 0.5M NaCl and incubating with 550 units of DNase I (Sigma D7291) for 30 minutes at 25°C. NP40 was optionally added at 1% for the final 15 minutes of this incubation. Spinning through 30% sucrose gave a final pellet and a supernatant containing the chromatin-associated proteins.

7.2.8 Western blotting

Protein extracts were mixed with 4X LDS sample buffer and loaded onto BioRad pre-cast SDS polyacrylamide gels of varying percentages (15X 15μl wells). Unless otherwise stated, gels were run at 150V for 8minutes, then at 200V for 42 minutes. Proteins were transferred to PVDF Immobilon membrane (Millipore) in 10% methanol, 10mM CAPS (pH 11) at 46V for one hour. Membranes were blocked in PBSA containing 5% milk and 0.05% Tween 20 for one hour. Primary antibodies were incubated in 5% milk, 0.05% Tween 20 for one hour or overnight, and the membrane washed three times in PBSA containing 0.05% Tween 20 for 10 minutes. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies were used as secondary antibodies and were detected using an ECL kit (Amersham). Proteins tagged with the TAP tag (Protein A and Calmodulin Binding Protein) were detected with a peroxidase-anti-peroxidase antibody (PAP) which can be detected directly with the ECL kit. Quantitative analysis of the resultant films (densitometry) was perfomed using Image J 1.33u (http://rsb.info.nih.gov/ij/).

Table 7.5 Antibodies used for Western blotting

Antibody	Incubation conditions
anti-Cdc18 polyclonal (This study)	1:1000 dilution of affinity purified Ab, overnight.
anti-Cdt1 polyclonal (H. Nishitani)	1:500 dilution of affinity purified Ab, overnight.
anti-Cdc21 polyclonal (Z. Lygerou)	1:500 dilution of affinity purified Ab, overnight
anti-Orp2 polyclonal (P. Russell)	1:2500 dilution of serum, one hour.
anti-midT polyclonal (Bethyl A190-211A)	1:1000 dilution of affinity purified Ab, two hours
anti-HA monoclonal (CRUK, 12CA5)	1:500 dilution, one hour
anti-α-Tubulin monoclonal (Sigma T5168)	1:10000 dilution, 30 minutes
PAP (Sigma P1291)	1:500 dilution, one hour.
anti-rabbit HRP (Amersham NA934V)	1:1000 dilution, one hour
anti-mouse HRP (Amersham NA931V)	1:1000 dilution, one hour

7.2.9 Antibody affinity purification

Polyclonal antibodies were affinity-purified from crude rabbit serum using a Western blot procedure. Purified antigen protein (250-500μg) was loaded onto a single, large well in a polyacrylamide preparation gel. The percentage gel and run time depended on the size of the antigen. After running the gel, protein was transferred to nitrocellulose membrane (Amersham) in10% methanol, 10mM CAPS (pH 11) at 46V for one hour. The antigen band (located by Ponceau staining) was excised and blocked in PBSA containing 5%milk for one hour. The membrane was then washed three times in PBSA for 10 minutes before an overnight incubation with crude serum. The membrane was washed three times in PBSA for 10 minutes prior to elution.

To elute antibody from the blot the membrane was incubated in 300μl elution buffer (0.2M glycine, 1mM EGTA, pH 2.5) for 10 minutes. Elution buffer was removed and neutralised with 0.1M Tris-HCl, pH 8.8. The elution step was then repeated.

7.2.10 Isolation of anti-Cdc18 polyclonal antibody

Purified Cdc18-6His protein, produced in our lab, was used as antigen by Harlan Sera-Lab for rabbit immunisation (400μg primary immunisation and five 200μg boosters). Terminal bleed serum was affinity purified and used in test Western blots for Cdc18 specificity (data not shown). This antibody was designated as OH1 anti-Cdc18 polyclonal.

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