# Functional Characterization of the Origin Recognition Complex (ORC) in *S. cerevisiae*.

by

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**Abstract**

The origin recognition complex (Orc1-6) plays a fundamental role in the initiation of DNA replication by binding replication origins throughout the budding yeast cell cycle. ORC acts as a scaffold for the assembly of the pre-replicative complex (pre-RC) factors Cdc6, Cdt1 and a replicative helicase, the minichromosome maintenance (MCM2-7) complex in G1 phase. Upon assembly, origins are then said to be "licensed" for DNA replication. Previous models of pre-RC assembly and function have predicted that once MCMs have been loaded onto chromatin, ORC and Cdc6 are no longer required for DNA replication. In contrast, data from our lab strongly suggest a role for Orc6 in the maintenance of MCMs after pre-RC formation. Orc6 was found to be required for the chromatin maintenance of Mcm2 and more specifically, chromatin immunoprecipitation (ChIP) analysis demonstrated that Orc6 was necessary for the continued origin association of MCM proteins in late G1 at the early-firing origin ARS1, and the late-firing origin ARS609. It was also determined that after destabilization in the absence of Orc6, the pre-RC could be reassembled and facilitate DNA replication in late G1 after Orc6 expression had been turned back on. Interestingly, the clamp loading protein Cdc6 was also discovered to be essential to the maintenance of MCM proteins on bulk chromatin and at ARS1.

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## Abbreviations

| | |
|---|---|
| ACS | ARS Consensus Sequence; AT-rich 11bp element within the ARS |
| ARS | Autonomously Replicating Sequence; origin of DNA replication |
| CDK | Cyclin Dependent Kinase; Clb/Cdc28 |
| ChIP | Chromatin Immunoprecipitation |
| DDK | Dbf4-Dependent Kinase; Dbf4/Cdc7 |
| FACS | Fluorescence-Activated Cell Sorting |
| Gal/Raf | Synthetic Complete medium containing galactose and raffinose as carbon source; amino acids are typically "dropped out" for growth selection (ie. Gal/Raf –TRP) |
| GINS | Complex of Sld5-Psf1-Psf2-Psf3 (GINS: Go-Ichi-Ni-San or 5-1-2-3) |
| HA | Hemagglutinin epitope tag |
| HU | Hydroxurea; depletes dNTP pools by inhibiting ribonucleotide reductase |
| MCM | Mini Chromosome Maintenance; consists of subunits Mcm2 through 7 (Mcm2-7); eukaryotic replicative helicase |
| ORC | Origin Recognition Complex; consists of subunits Orc1 through 6 (Orc1-6) |
| pre-RC | Pre-Replicative Complex |
| RPA | Replication Protein A; single stranded DNA binding protein |
| RPC | Replisome Progression Complex |
| SC | Synthetic Complete medium containing glucose as carbon source; amino acids are typically "dropped out" for growth selection (ie. SC –TRP) |
| td | Temperature-sensitive Degron |
| WCE | Whole Cell Extract; yeast whole protein extract |
| YPD | 'GLU'; Yeast extract, Peptone, Dextrose (D-Glucose); non-selective yeast growth medium |
| YPG/R | 'GAL'; Yeast extract, Peptone, Galactose, Raffinose; non-selective yeast growth medium for inducing GAL1 promoter |

# GENERAL INTRODUCTION

## Physiology of *Saccharomyces cerevisiae*

The budding yeast *Saccharoymces cerevisiae* is a single-celled organism with approximately 6000 genes arranged on 16 chromosomes and exists in both haploid (1N) and diploid (2N) states. Polyploids, up to octaploid (Hartwell, 1974), also exist, but these are typically used in industrial applications. Yeast cells reproduce asexually by budding, or sexually by mating. Haploid yeast exist as one of two mating types termed **a** or α, encoded by the mating type locus, *MAT*. Asexual reproduction involves the development of a bud on the surface of a parent yeast cell. Interestingly, S and M phases are completed before the daughter cell grows as large as the mother cell; thus upon separation, produce a large mother cell and a smaller daughter cell. The daughter cell must then reach a critical size before it can form a bud of its own (Hartwell, 1974; Chen *et al*., 2000). Mating involves the fusion of two opposite mating types to form a diploid and is stimulated by the secretion of two small peptide pheromones, namely **a**-factor and α-factor from each of the respective mating types. The secretion of these pheromones causes yeast cells to arrest in the G1 (gap1) phase, and results in the transcription of genes required for cell adhesion, cell fusion and nuclear fusion (Gustin *et al*., 1998; Wu *et al*., 1999). Mating is characterized by a cell morphology known as a 'schmoo' directed towards the opposite mating type (Fig. 1A). In the absence of a suitable fermentable carbon source and typically in the absence of nitrogen, diploids will undergo meiosis and spore formation (Fig. 1A; Hartwell, 1974; Dickinson, 1999). Sporulation in diploids is only possible in heterozygous mating types (Mitchell, 1994) and involves the development of 4 haploid ascospores surrounded by an ascus (Xiao, 2006). These asci are more resistant to heat, solvents, dehydration and other environmental elements than actively dividing vegetative yeast cells. Once haploid spores are returned to nutrient-rich conditions they will re-enter the cell cycle and actively bud as haploids (Dickinson, 1999).

**Yeast as a Model Organism**

Budding yeast, more commonly called Baker's or Brewer's yeast, is a very significant model organism in molecular biology. Due to its importance in the baking of bread through the release of carbon dioxide and the production of ethanol for industry, and beer and wine production, much work has been done on the biochemistry and physiology of this yeast. Yeast is a model organism for the study of higher eukaryotic genetics and disease due to the conservation of several replication proteins and pathways (Bell and Dutta, 2002; Guthrie and Fink, 2002). Budding yeast is a relatively small single-celled organism as haploids are approximately 3-4μm in diameter, and they are readily cultured and typically complete their life cycle in approximately 90 minutes at 30°C. The full sequence of the *S. cerevisiae* genome, the first to be determined of any eukaryote, was completed in 1996 and combined the research of 600 scientists from Japan, Europe, and North America (Goffeau *et al*., 1996). Having elucidated the complete genome, budding yeast is well-suited for molecular biological research for it is easily manipulated by genetic techniques, such as transformation with plasmid DNA for exogenous protein expression (Gari *et al*., 1997), or homologous recombination. In an effort to study protein function, the most notable of these involves a rather simple transformation of linear DNA into the yeast cell that, via homologous recombination, has the advantage of either deleting or adding to gene sequences (Longtine *et al*., 1998; Gauss *et al*., 2005). Consequently, in both haploid and diploid yeast cells, gene deletion analysis, epitope tagging for protein detection, or expression control through the replacement of natural gene promoters with regulatable promoters (ie. $P_{GAL1}$) can be accomplished with ease, a technique unrivalled in other eukaryotes. In addition, most 'wild-type' yeast strains contain mutations in or complete deletions of vital metabolic genes (Brachmann *et al*., 1998). For example, a common mutation occurs in URA3 which encodes orotidine-5'-phosphate (OMP) decarboxylase necessary to catalyze the sixth enzymatic step in the *de novo* biosynthesis of uracil, converting OMP into uridine monophosphate (UMP) (Boeke *et al*., 1987). Exploiting this, the DNA of

interest to be transformed also contains URA3 and selection of modified yeast strains is accomplished by culturing on media lacking uracil.

In addition and due to the relative ease of genetic manipulation, an enormous number of *S. cerevisiae* temperature-sensitive mutants have been created in which essential genes have been modified by point mutations through random mutagenesis. The first of these involved the isolation of a number of temperature-sensitive cell division cycle (*cdc*) mutants (Hereford and Hartwell, 1974; Hartwell, 1976). These mutant gene products are typically fully functional at the permissive temperature of 23°C, but are unstable and usually non-functional at the higher restrictive temperature of 37°C. In some cases, these strains are affected even at the semi-permissive temperature of 30°C. To circumvent the arduous task of finding temperature-sensitive mutants, a recent development employs the use of temperature-sensitive degron fusions of essential proteins (Kanemaki *et al*., 2003; Sanchez-Diaz *et al*., 2004). Under the control of the copper-inducible promoter CUP1, a sequence encoding the degron consisting of a temperature-sensitive version of mouse dihydrofolate reductase (DHFRts) is integrated upstream of the open reading frame so as to replace the natural endogenous promoter of the gene of interest, and creates an N-terminal in-frame fusion between the degron and the gene (Kanemaki *et al*., 2003; Sanchez-Diaz *et al*., 2004).

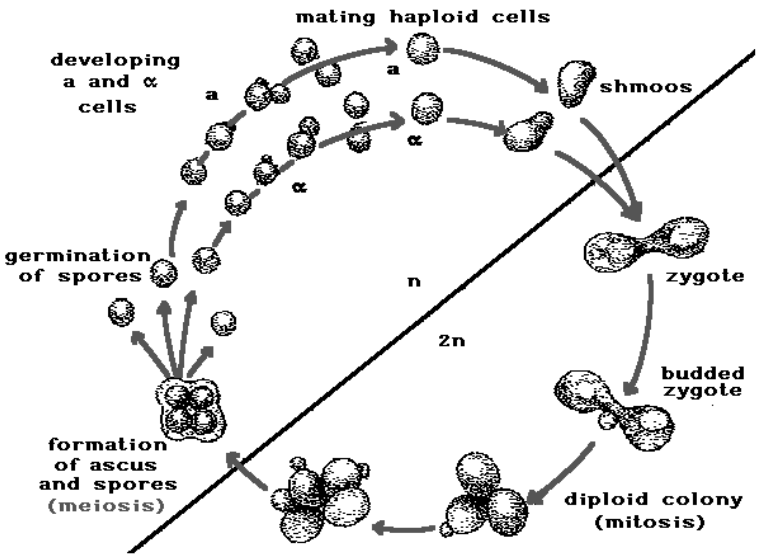
It should be noted that the elucidation of the key components involved in the various stages of the yeast cell cycle has been made possible in large part by the ease in which budding yeast respond to cell synchronizing agents. Briefly, asynchronously growing yeast cultures can be arrested at G1 phase using the small peptidyl mating pheromones **a**-factor, which acts on MATα cells, or α-factor which acts on MAT**a** cells. In this case, cells are tricked into thinking they are going to mate and arrest at G1, a process mediated by Far1 (Chang and Herskowitz, 1992; Remenyi *et al*., 2005). Long exposures to either pheromone will cause cells to form ‘schmoo’ (see above; Fig. 1A), and prolonged exposure will permanently prevent arrested cells from re-entering the cell cycle. Hydroxyurea (HU) is a ribonucleotide reductase inhibitor which depletes the cells of dNTP pools and inhibits DNA synthesis

(Sanchez *et al*., 1996; de la Torre-Ruiz *et al*., 1998). Approximately half of the replication origins fire and yeast cells arrest in mid-S phase (Semple *et al*., 2006). Yet another arresting agent, nocodazole, synchronizes cells at the G2/M boundary by inhibiting the polymerization of microtubules (Vasquez *et al*., 1997), vital as cells enter mitosis whereby spindle fiber networks facilitate chromosome segregation.

**The Yeast Cell Cycle**

The cell cycle of *S. cerevisiae* has been well studied by molecular biologists and represents just one of the major reasons for using this yeast as a model organism. Together with the ease of genetic manipulation and analysis, the various cell cycle stages of this organism serve as suitable landmarks for functional analysis (Fig. 1; Hartwell, 1974; Dickinson, 1999). Through microscopic observation it is easy to identify some of the stages of the cell cycle in which a given cell is. G1 phase is illustrated by the appearance of a developing bud (Fig. 1B) which increases in size as the mother cell replicates its genome (S phase). During G2 phase, the nucleus migrates toward the bud neck and the cell quickly enters mitosis (M phase), whereby the DNA is partitioned (nuclear division) between the mother and daughter cells. The two cells then separate in a stage known as cytokinesis, upon which time each cell begins the cycle from G1 phase. The most significant is bud emergence and simply involves distinguishing an unbudded from a budded cell (Fig. 1B; Hartwell, 1974; Dickinson, 1999). Other landmarks include the size of the emerging bud, cell separation (Dickinson, 1999) and mitosis, the latter of which requires staining the DNA of fixed cells and visualizing under a fluorescent microscope.

A




B

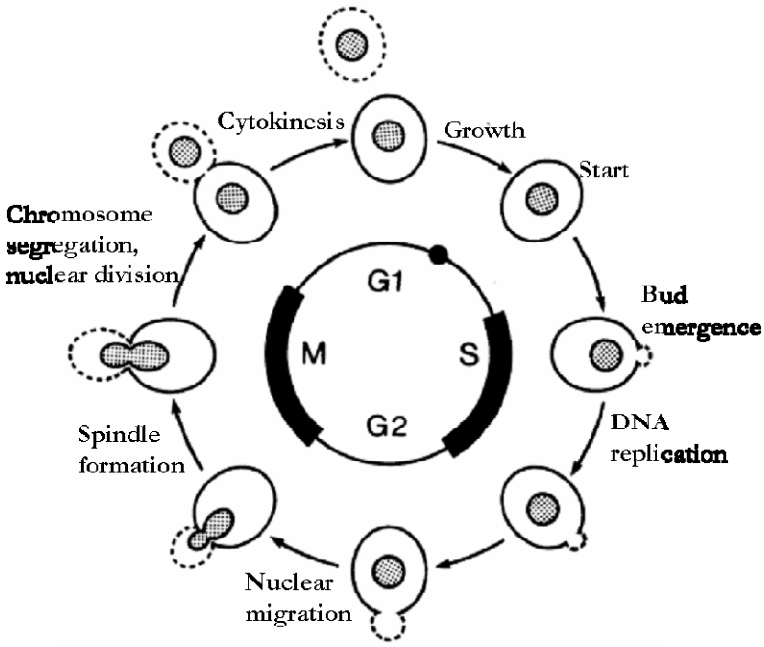



**Figure 1**. The life cycle of *Saccharomyces cerevisiae*. (**A**) Haploid cells, either Mat **a** or Mat α, enter the cell cycle, germinate and develop into actively budding cells. Diploid yeast cells are also shown, and via meiosis, transform into 4 haploid ascospores. Both the haploid and diploid cell cycle illustrates bud formation, mitosis and cytokinesis. (The GENE Project, Kansas State University, 2005). (**B**) Schematic outline of the cell cycle phases, drawn in approximate proportion to their length. The mother cell is illustrated with a solid line; the daughter bud and cell are drawn with a dotted line. The shaded area represents the nucleus. The solid circle at G1 phase depicts the position at which cells arrest following treatment with mating pheromones (ie. α-factor). Adapted from Herskowitz, 1988.

The yeast cell cycle begins with an unbudded cell at G1 phase, in which cells spend majority of their time (Dickinson, 1999). From this point, yeast cells will follow one of two paths: (i) they will either withdraw from the cell cycle and enter a resting phase known as G0, or (ii) they will commit themselves to initiating DNA replication and completing the cell cycle, beginning from a stage known as START (Fig. 1B). In G0 phase, also known as stationary phase, cells are viable and metabolically active, but cease to proliferate. For actively cycling cells, complete and accurate duplication of genetic information from one cell generation to the next is central to the genetic integrity of all organisms. Eukaryotes have developed intricate mechanisms to ensure this occurs once, and only once per cell cycle. The initiation of DNA replication is a complex and dynamic process involving the selection of sites for the initiation of DNA replication, the ordered assembly of multiple protein complexes at sites of DNA replication and the eventual unwinding of the DNA helix (Lee and Bell, 1997). Jacob, Brenner and Cuzin (1963) were the first to propose the replicon model for the initiation of DNA replication in bacteria. This model included two key components essential for the initiation of DNA replication: the initiator and the replicator. The initiator is a positively acting factor that recognizes a particular DNA sequence within the genetic domain required for initiation, called the replicator. Numerous studies over the past decade have demonstrated that a single conserved eukaryotic initiator called the origin recognition complex (ORC) facilitates these initiation events (Bell and Stillman, 1992; Bell, 2002).

S phase is marked by origin firing, a time in which the DNA duplex is unwound by a replicative helicase and DNA is replicated via bidirectional fork migration from origins of replication. Upon unwinding, single-stranded binding proteins attach to the individual strands to ensure they do not reanneal to one another. In order to relieve tension downstream of the fork, DNA topoisomerases, particularly topoisomerase II, nick double stranded DNA ahead of the replisome (Berger and Wang, 1996). The single stranded DNA acts as a template for newly synthesized DNA, facilitated by DNA polymerases $\alpha$, $\delta$ and $\varepsilon$ (Pol $\alpha$, -$\delta$, -$\varepsilon$) (Aparicio *et al*., 1997; Bell and Dutta, 2002). First, short RNA

primers, complementary to the template strand, are laid down by the DNA polymerase α-primase complex and are essential for DNA elongation. DNA Pol δ and Pol ε can only add nucleotides in the 5' – 3' direction, and as the replication fork progresses it exposes two complementary strands, one in the 3' – 5', and the other in the 5' – 3' direction (reviewed in Kornberg, 1988). Consequently, primers bind to the 3' – 5' strand and new DNA synthesis occurs in the 5' – 3' direction in which the fork is moving, and is called leading strand synthesis. On the other hand, to synthesis DNA from the 5' – 3' strand, RNA primers, synthesized by DNA primases, must wait to be synthesized and bind template DNA until the fork has travelled far enough away from the origin. In this case, primer extension is directed back towards the origin in a 5' – 3' direction in a semi-discontinuous manner in which Okazaki fragments approximately 100-200nt in length are synthesized and this is referred to as lagging strand synthesis (Maga *et al*., 2001). Due to the fact that eukaryotic chromosomes are linear, RNA primers are not efficiently converted to DNA at the ends of chromosomes, leading to the loss of genetic information if allowed to persist from one cell cycle to the next. To circumvent this, the terminal ends of chromosomes possess particular and highly repetitive DNA sequences called telomeres, the length of which is maintained by a specialized reverse transcriptase called telomerase (Grandin and Charbonneau, 2008).

The G2 phase of the yeast cell cycle involves the migration of the nucleus to the bud neck, mediated by cytoplasmic microtubules, where it will undergo nuclear division (reviewed in Hartwell, 1974). This stage is marked by an increase in the size of the daughter cell and the replicated DNA is monitored by surveillance mechanisms to ensure its integrity and will invoke a checkpoint to block the onset of anaphase if DNA damage is detected. In addition, there is a high rate of protein synthesis, particularly of factors involved in chromosome segregation during mitosis (see below). Chromosome duplication produces a pair of sister chromatids that are held together throughout G2 phase and also following their alignment on the mitotic spindle by a multisubunit complex called Cohesin (Guacci *et*

*al*., 1997; Michaelis *et al*., 1997; Alexandru *et al*., 1999). Some researchers, however, believe yeast do not have a true G2 phase because of the rapid transition from S phase to M phase (Nasmyth, 1995). Regardless, the end of G2 is marked by the movement of the nucleus to the bud neck.

Following G2 phase, the cell enters mitosis (M phase) and nuclear division commences. Unlike most eukaryotes, budding yeast do not show any noticeable condensation of chromosomes and complete DNA synthesis is not required for the early stages of M phase, but is necessary for the metaphase to anaphase transition (Nasmyth, 1995). To avoid erroneous chromosome segregation, anaphase must start only after sister chromatids have attached to opposite poles of the mitotic spindle. In this case, checkpoints ensure this has occurred before anaphase initiation (Hartwell and Weinert, 1989). The spindle checkpoint is dependent on Pds1, an anaphase inhibitor which prevents precocious dissociation of sister chromatids (Cohen-Fix *et al*., 1996; Yamamoto *et al*., 1996a; 1996b). Pds1 binds to and inhibits the separin Esp1, responsible for degrading the key subunit of Cohesin, Scc1 (Michaelis *et al*., 1997; Ciosk *et al*., 1998). During metaphase, the kinetochore of each sister chromatid is attached to an arrangement of mitotic microtubules termed the spindle pole body (SPB) which is responsible for aligning the chromatids at the nuclear equator (Hayden *et al*., 1990; Alexandru *et al*., 1999). Sister chromatid separation during anaphase depends on the proteolysis of Pds1 by a multisubunit ubiquitin protein ligase known as the anaphase-promoting complex (APC), specifically mediated by Cdc20 just prior to the onset of anaphase (Cohen-Fix *et al*., 1996; Visintin *et al*., 1997; Zachariae and Nasmyth, 1999). The activity of the APC increases in late mitosis and remains high throughout G1 (Amon *et al*., 1994; Zachariae and Nasmyth, 1996). The elongation of microtubules promotes the movement of chromosomes to the poles of the mother and daughter cells. Dissolution of the mitotic spindle then permits division of the nuclear envelope encasing the respective mother and daughter genomes, after which the phosphatase Cdc14 promotes mitotic exit and cytokinesis. Cdc14 is an indirect inhibitor of cyclin-dependent kinase (CDK; Clb/Cdc28) activity during late M phase. In this case, Cdc14 dephosphorylates Hct1, also known as Cdh1, a key activator of the APC and mediator of the

proteolysis of the cyclin Clb2 (Visintin *et al*., 1997). Cytokinesis marks the final stage of the cell cycle and is mediated by two seemingly redundant pathways, actomyosin and septin ring formation at the bud neck (Bi *et al*., 1998; Lippincott and Li, 1998a). It has, however, been established that cytokinesis in *S. cerevisiae* does not require the contractile actomyosin ring, but is instead mediated by septum formation (Bi *et al*., 1998). Moreover, Cdc42 has been shown to be a key component in the organization of the septin cytoskeleton and in the recruitment of other septins (Gladfelter *et al*., 2005; Iwase *et al*., 2005).

**Regulation of the Initiation of DNA Replication**

Key events in the budding yeast cell cycle are controlled by a single cyclin-dependent kinase (CDK; Cdc28) in combination with two families of cyclins: the G1 cyclins, Cln1-3; and the B-type cyclins, Clb1-6 (Nasmyth, 1993; Mendenhall and Hodge, 1998). Cln1,2/Cdc28 have essential roles in budding and spindle pole body duplication, and Cln3/Cdc28 mediates the size at which daughter cells execute start. Clb1,2/Cdc28 are essential for accurate completion of mitosis; Clb3,4/Cdc28 facilitate DNA replication and spindle formation; and Clb5,6/Cdc28 are vital for timely DNA replication (Chen *et al*., 2000). Cell cycle events are also mediated by another kinase complex known as the Dbf4-dependent kinase (DDK, Dbf4/Cdc7; Jackson *et al*., 1993; Varrin *et al*., 2005). The roles of CDK and DDK in DNA replication are discussed in more detail below.

In order to maintain the integrity of chromosomal DNA, *S. cerevisiae*, as well as other eukaryotes, have developed a highly conserved and temporally regulated pattern of assembly and disassembly of multiple protein complexes at sites of DNA replication. Genetic analysis has proven that a well-conserved eukaryotic initiator is responsible for the recruitment of these protein complexes. The origin recognition complex (ORC) plays a fundamental role in the initiation of DNA replication by binding specific chromosomal loci, called origins of replication, throughout the budding yeast cell cycle (Bell & Stillman, 1992; Bell & Dutta, 2002). ORC was initially isolated and characterized in

budding yeast (Bell and Stillman, 1992) and consists of six subunits (Orc1-6; decreasing in mass) that act as a scaffold for the controlled assembly of several protein factors in G1 phase (Bell, 2002; see also below). A role for ORC as an initiator protein is further solidified by its conservation in several higher eukaryotes (Dutta and Bell, 1997; DePamphilis, 2005).

The most well characterized eukaryotic replication origins are those found in *S. cerevisiae*. These *cis*-acting elements were initially identified by their ability to facilitate the stable transformation and the autonomous replication of plasmid DNA in yeast (Hsaio and Carbon, 1979). In light of this, these elements were subsequently called autonomous replicating sequence (ARS) elements (Stinchcomb *et al*., 1979; Chan and Tye, 1980). Yeast origins are non-coding regions of DNA, approximately 100-200bp in length and contain a highly conserved AT-rich 11bp element within the ARS, called the ARS consensus sequence (ACS or A element) that is essential but not sufficient for ORC-DNA binding and replication origin function *in vivo* (Bell and Dutta, 2002; Robinson and Bell, 2005). Multiple less conserved B elements also contribute to the function of origins of DNA replication (Bell, 2002; Robinson and Bell, 2005) and contain DNA unwinding elements (DUEs) that are thought to direct the recruitment of replication enzymes into the double helix (Umek and Kowalski, 1990; Bell, 1995). B2 elements are found in majority of, but not all, ARS elements and function in pre-replicative complex (pre-RC; discussed below) assembly (Wilmes and Bell, 2002). B3 elements function as a binding site for ABF1, an indispensable DNA binding protein involved in transcription, silencing and replication (Rowley *et al.*, 1995; Lipford and Bell, 2001). Some origins also contain C elements which also function as transcription factor binding sites (Walker *et al.*, 1990). Several studies on mutations in the ACS that abrogate ORC binding *in vitro* and *in vivo*, and ultimately ORC function, further support a role for ORC in the initiation of DNA replication (Bell and Stillman, 1992; Li and Herskowitz, 1993; Aparicio *et al.*, 1997). A puzzling fact to researchers is that there are over 10,000 matches to the ACS in *S. cerevisiae*, yet only a small number seem to be functional ORC binding sites. To differentiate functional ACS elements from non-functional ones, an extended ACS (EACS)

consisting of 17bp instead of 11 has been developed (Theis and Newlon, 1997). It is noteworthy that *S. cerevisiae* and its close relatives are presently the only eukaryotes known to employ specific sequence elements within its origins of replication (Robinson and Bell, 2005). However, the mechanism of origin selection is thought to differ in higher eukaryotes (reviewed in Gilbert, 2001). In mammals for example, DNA replication initiates from multiple, less well-defined sites along chromosomes, and only a handful of origin sequences have been found (Gilbert, 2001; Bell, 2002; Aladjem and Fanning, 2004; Bolon and Bielinsky, 2006).

The ORC complex is bound to origins throughout the yeast cell cycle and acts as a 'landing pad' to recruit several other replication factors including Mcm10, Cdc6, Cdt1, and the Mcm2-7 complex (MCM) to the origin (Fig. 2; Bell and Stillman, 1992; Bell, 2002; Bell and Dutta, 2002). Collectively these factors form the pre-replicative complex (pre-RC) which quickly changes to a pre-initiation complex (pre-IC) upon the activation of CDK (Liang and Stillman, 1997; Queralt and Igual, 2005), and DDK (Jackson *et al*., 1993; Varrin *et al*., 2005; Pacek *et al*., 2006), which help to trigger initiation and DNA unwinding by the replicative helicase Mcm2-7. Phosphorylation of Sld2 (Kamimura *et al*., 1998) by CDK is essential for DNA replication initiation, which in turn promotes the interaction with Dpb11 and the subsequent recruitment of replication proteins such as DNA polymerases, Cdc45 and GINS (Go, Ichi, Nii and San; five, one, two and three in Japanese) to origins (Masumoto *et al*., 2002; Takayama *et al*., 2003). Furthermore, CDK-dependent phosphorylation of Sld3, which associates with Cdc45 (Kamimura *et al*., 2001), is required and creates a binding site for Dpb11 (Tanaka *et al*., 2007). The helicase activity of Mcm2-7 is thought to be dependent on the phosphorylation of Mcm2 by DDK upon the initiation of S phase (reviewed in Bell and Dutta, 2002). DDK has also been shown *in vitro* to phosphorylate every MCM subunit except for Mcm5 (Lei *et al*., 1997; Weinreich and Stillman, 1999).

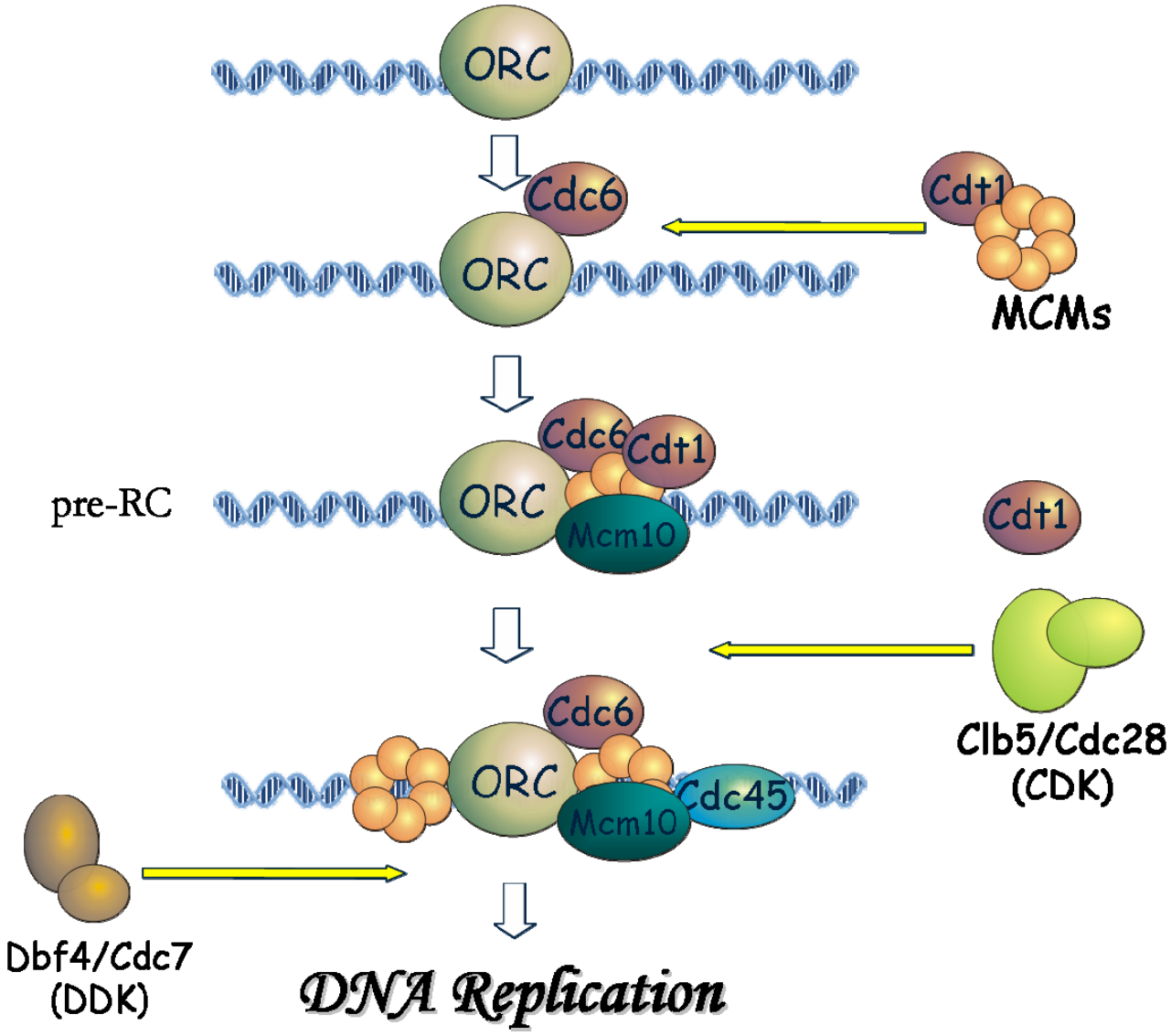



**Figure 2**. Model for pre-RC formation in budding yeast. During late M or early G1, Cdc6 binds origins after which Cdt1 promotes nuclear import of Mcm2-7. Cdc45 facilitates the loading of DNA polymerases to origins. Origin unwinding by the MCM replicative helicase is activated by CDK and DDK.

ORC specifically binds to both A and B1 elements of yeast origins throughout the cell cycle (Fig. 3; Liang and Stillman, 1997). *In vivo* DNaseI footprinting has shown this region of protection spans approximately 30-50bp (Bell and Stillman, 1992; Rowley *et al.*, 1995; Santocanale and Diffley, 1996). As stipulated earlier, ORC is conserved in higher eukaryotes and since its discovery in budding yeast, orthologs of each subunit were found in fission yeast (Moon *et al*. 1999), *Xenopus* (Rowles *et al*., 1996), *Drosophila* (Gossen *et al*., 1995), and humans (Vashee *et al*., 2001). Characterization of the ORC subunits has demonstrated a high degree of similarity between species for most. For instance, human Orc1 is 45% identical and 62% similar to *Drosophila*, and 29% identical and 46% similar to *S. cerevisiae* orthologs. In contrast, Orc6, the smallest of the ORC subunits, possesses little conservation among eukaryotes. Budding yeast Orc6 shares only 5% identity and 19% similarity to *Drosophila*, and 6% identity and 15% similarity to human orthologs (Dhar and Dutta, 2000). Unlike in budding yeast, not all human ORC (HsORC) subunits are constitutively bound to origins, but rather, a large fraction of HsOrc2 is chromatin-bound throughout the entire cell cycle (Ritzi *et al*., 1998; Kreitz *et al*., 2001).


**Figure 3**. Association of *Saccharomyces cerevisiae* Origin Recognition Complex (ORC). ORC binds specifically to the A element (ACS) and B1 element of an origin of DNA replication (Bell, 2002).

Interestingly, only five of the six budding yeast ORC subunits are required for origin recognition and binding *in vitro*, with Orc1, 2, and 4 specifically making contact with the ACS (Fig. 3; Lee and Bell, 1997; Harvey and Newport, 2003). Despite being an essential protein for viability in budding yeast, Orc6 appears to be dispensable for these functions (Li and Herskowitz, 1993). In contrast, *Drosophila* Orc6 is required for both origin-association of ORC and DNA replication (Chesnokov *et al*., 2001). Surprisingly, origins in the fission yeast *Schizosaccharomyces pombe* exhibit little similarity to those of budding yeast and lack detectable consensus sequences. A commonality with budding yeast is that origins of replication are AT rich as shown by *in situ* footprinting (Kong and DePamphilis, 2002), yet *S. pombe* ORC (SpORC) associates with origins via an AT-hook DNA binding domain on Orc4, and in addition, origin association of SpORC does not require ATP (Chuang and Kelly, 1999; Kong and DePamphilis, 2001; Lee *et al*., 2001; Robinson and Bell, 2005). Moreover, a recent study in *Drosophila* suggests that multiple ORC molecules distributed within large replication regions are required for efficient origin firing in organisms that require long chromosomal regions for DNA replication initiation, as is the case in metazoans (Takahashi *et al*., 2003).

Orc1-5 are members of the AAA+ ATPase family (Speck *et al.*, 2005), an extension of the AAA (ATPases associated with a variety of cellular activities) family (Neuwald *et al.*, 1999). ATP binding by Orc1, mediated through Orc4 is required for ORC-origin binding (Bowers *et al.*, 2004) and this event inhibits ATPase activity by Orc1 (Speck *et al.*, 2005). It is interesting to note that studies have shown this also holds true in *Drosophila*, and that ATP hydrolysis is not required for DNA binding in either budding yeast or fruit flies (Klemm *et al.*, 1997; Austin *et al.*, 1999; Chesnokov *et al.*, 2001).

In addition to its essential role in DNA replication, ORC has been implicated in other cellular processes. ORC is involved in chromatin reorganization such that it renders regions of chromosomes inactive, a process termed transcriptional silencing and reminiscent of the function of heterochromatin in higher eukaryotes (Foss *et al*., 1993; Fox *et al*., 1995). Silencing involves the assembly of certain

chromosomal regions into an inactive chromosome structure which blocks transcription (Dillin and Rine, 1997). Mutations in ORC genes that derepress the HML and HMR mating type loci suggest a link between DNA replication and silencing (Foss *et al*., 1993). In addition, the silencing function of ORC requires that Orc1 physically interact with Sir1, a protein involved in chromatin remodeling (Triolo and Sternglanz, 1996). The silencing function also holds true in metazoans whereby fruit fly Orc1 interacts with heterochromatin protein 1 (HP-1), and mutations in *Drosophila* Orc2 result in the loss of heterochromatin-regulated silencing in this organism (Pak *et al*., 1997). Further, research in human and fruit fly cell lines implicate a mitotic and/or cytokinetic role for Orc6 in addition to its role in the initiation of DNA replication (Prasanth *et al*., 2002; Chesnokov *et al*., 2003), yet to date, a role for budding yeast Orc6 in this capacity has not been discovered.

**Formation of the Pre-Replicative Complex**

In early G1 phase, ORC promotes the origin association of the clamp loading protein Cdc6 (Fig. 3; Bell and Dutta, 2002). Cdc6 is a member of the AAA+ ATPase family, which includes Orc1-5 and Mcm2-7 (Speck *et al*., 2005), and as with ORC, this association is dependent on ATP. Studies by Mizushima and colleagues (2000) and Harvey and Newport (2003) have shown that Cdc6 increased the sequence specificity of ORC by restricting ORC binding to functional origins, and induced a conformational change in ORC *in vitro* (Mizushima *et al*., 2000). Cdc6 possesses strong sequence similarity with Orc1 (Bell *et al*., 1995; Muzi-Falconi and Kelly, 1995) and based on their distinct, yet coordinated roles, a recent report showed that ATP binding and hydrolysis are essential to the function of Cdc6, and to the downstream loading of the replicative helicase, the Mcm2-7 complex (Randell *et al*., 2006). Further evidence in *Xenopus* helps substantiate a role for Cdc6 in pre-RC formation. In *Xenopus* egg extracts, origin association of XCdc6 was dependent on XOrc2, and was essential for the subsequent loading of XMcm3 (Coleman *et al*., 1996). Moreover, there are three modes of regulation that help to inhibit origin association of Cdc6 following the initiation of DNA replication. First, both

transcript and protein levels of Cdc6 are cell cycle regulated such that they are high in late M and early G1, but very low in S, G2 or early M phases (Piatti *et al*., 1995). Second, phosphorylation of Cdc6 by CDK targets the protein for ubiquitin-mediated proteolysis by the Cdc4-Cdc34-Cdc53-Skp1 ($SCF^{CDC4}$) pathway (Drury *et al*., 1997). Finally, phosphorylation of Cdc6 near its N-terminal nuclear localization signal is thought to inhibit nuclear import (Jong *et al*., 1996). It is also of note that overexpression of Cdc6 leads to inhibition of CDK activity marked by elongated yeast cell and bud morphology (Elsasser *et al*., 1996; Honey and Futcher, 2007), and can inhibit mitosis and slow yeast growth (Bueno and Russell, 1992). Further, *cdc6-3* temperature-sensitive mutants display wanton initiation of DNA replication, increase in nuclear DNA content and persistent Mcm2-7 chromatin association throughout the cell cycle despite the presence of high mitotic CDK levels (Liang and Stillman, 1997). In addition, overexpression of the fission yeast homolog, Cdc18, leads to re-replication of DNA in the absence of mitosis (Nishitani and Nurse, 1995).

Cdt1 (Devault *et al*., 2002; Tanaka and Diffley, 2002), previously called Tah11 and Sid2 and isolated in two independent studies (Fiorani and Bjornsti, 2000; Jacobson *et al*., 2001) maintains a constant level throughout the yeast cell cycle and facilitates the nuclear import of the Mcm2-7 complex (see below). It is thought that Mcm2-7 and Cdt1 form a complex and subsequently associate with origin-bound ORC and Cdc6 (Fig. 3; Tanaka and Diffley, 2002; Mendez and Stillman, 2003; Randell *et al*., 2006). Hydrolysis of ATP by Cdc6 is thought to result in Cdt1 dissociation and tighter MCM binding at origins (Fig. 4). Subsequent ATP hydrolysis by ORC facilitates reiterative MCM loading onto origin sequences. The biological significance of this remains elusive, yet a mutation in ORC4 that prevents this is lethal (Bowers *et al*., 2004; Kawasaki *et al*., 2006; Randell *et al*., 2006). After Mcm2-7 loading, Cdc6 dissociates from origins and will only reassociate late in M phase when CDK levels are low (Honey and Futcher, 2007). Interestingly, inhibiting ATP hydrolysis by Cdc6 during pre-RC formation causes Cdt1 stabilization at origins and consequently results in an unstable association between Mcm2-7 and origins (Randell *et al*., 2006).

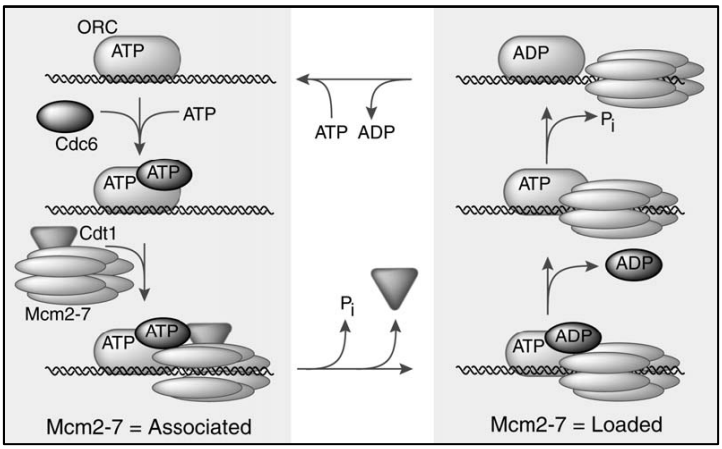



**Figure 4**. A model for loading Mcm2-7 by ORC and Cdc6. ATP-bound ORC binds to origins, followed by the binding of ATP-bound Cdc6. Cdt1 and Mcm2-7 form a complex and bind to ORC and Cdc6. Cdc6 hydrolyzes its ATP, releasing Cdt1 and stably loading Mcm2-7. Reiterative loading is mediated by ATP hydrolysis by ORC. (Randell *et al*., 2006).

The Mcm2-7 complex is essential for the initiation and elongation of DNA replication in yeast and is highly conserved from yeast to mammals (Tye, 1999; Forsburg, 2004). It is also thought to be the replicative helicase involved in origin unwinding upon CDK and DDK activation (reviewed in Bell and Dutta, 2002). The MCM proteins were initially identified in *S. cerevisiae* from screens for mutants defective in minichromosome maintenance and cell cycle control (Maine *et al*., 1984; Takahashi *et al*., 1994), and are very similar to each other, particularly within a 200-amino-acid core domain. MCMs are members of the AAA+ superfamily of proteins and as such, contain an ATPase motif within this core, including Walker A and B motifs, which are highly conserved in DNA helicases (Koonin, 1993). Based on *in vivo* studies all six MCM subunits associate with each other with equal stoichiometry (Forsburg, 2004). Interestingly, only complexes containing Mcm4,6,7 exhibit helicase activity *in vitro* (Kaplan *et al*., 2003). While there is no evidence yet for the formation of a functional Mcm4,6,7 complex *in vivo*, *S. cerevisiae* temperature-sensitive degron mutants of MCM proteins illustrate that all six subunits are required for both initiation and elongation, demonstrating that the entire heterohexameric complex is maintained at replication forks (Labib *et al*., 2000). It should also be noted that MCM subunits have been shown to bind chromatin in *Xenopus* egg extracts (Chong *et al*., 1995; Coleman *et al*., 1996) and mammalian cells (Kimura *et al*., 1995). As stated above, Mcm2-7 loading requires the coordinated function of ORC, Cdc6 and Cdt1, and indeed, physical interactions between MCMs and origin-associated components have been documented, including ORC (Semple *et al*., 2006), Cdt1 (Tanaka and Diffley, 2002), Cdc6 (Jang *et al*., 2001), Mcm10 (Homesley *et al*., 2000), Cdc45 (Zou *et al*., 1997) and Dbf4/Cdc7 (Varrin *et al*., 2005). Importantly, the MCM complex is inactive as a replicative DNA helicase until the action of CDK and DDK, and most likely involves post-translational modification and association with other factors (see below; Zou and Stillman, 1998; Labib *et al*, 2000; Pacek and Walter, 2004).

Once the MCM complex is loaded, the continued association of at least Mcm2 with chromatin in G1 is dependent upon the presence of another ORC associated protein, Mcm10 (Homesley *et al*.,

2000). There is also evidence linking Mcm10 and Mcm7, and together with the above, suggest that Mcm10 plays an important role in maintaining Mcm2-7 at origins during G1 (Homesley *et al*., 2000). It was recently shown in budding yeast that Mcm10 forms a complex with and recruits DNA polymerase α (Pol α) to replication origins, independent of Cdc45. Noteworthy is the fact that Mcm10 regulates the stability of the catalytic subunit of Pol α, Cdc17 (Ricke and Bielinsky, 2004). Mcm10 was also reported to be recruited to origins in a cell cycle mediated fashion, dependent on pre-RC assembly (Ricke and Bielinsky, 2004), in contrast to an earlier study stipulating that Mcm10 was constitutively chromatin-bound in budding yeast (Homesley *et al*., 2000; Kawasaki *et al*., 2000). ORC, Cdc6, Cdt1, Mcm2-7 and Mcm10 collectively form the pre-replicative complex (pre-RC) and consequently, origins are said to be licensed for DNA replication.

In addition to pre-RC formation, activation of pre-RCs at the G1-S transition requires the recruitment of Cdc45 and Sld3, which in turn, are dependent on the two protein kinase complexes, Clb5/Cdc28 (CDK) and Dbf4/Cdc7 (DDK) (Zou and Stillman, 2000; Takeda and Dutta, 2005). It has also been proposed that Cdc45 and replication protein A (RPA) are targets for the activation of origins by these kinases (Tanaka and Nasmyth, 1998; Zou and Stillman, 1998). Mcm2-7, but not RPA, is present at origins during G1, but no origin firing takes place until S phase CDK (S-CDK) and DDK are activated at the G1-S transition. Both the MCM complex and Cdc45 are central to DNA initiation and elongation for the progression of replication forks and for unwinding the DNA duplex (Aparicio *et al*., 1997). In this capacity, Cdc45 binds to Mcm2-7 and chromatin upon activation of S-CDK (Mimura and Takisawa, 1998; Zou and Stillman, 1998), and association of RPA with origins depends on both S-CDK and DDK activity (Tanaka and Nasmyth, 1998). Moreover, Cdc45 is required for chromatin association of Pol α in *Xenopus* (Mimura and Takisawa, 1998), and RPA is integral in the recruitment of primase to origin sequences in yeast (Tanaka and Nasmyth, 1998). During the G1-S transition, Cdc45, RPA and Mcm2 start to associate with each other, an interaction dependent on S-CDK activity.

Specifically, chromatin immunoprecipitation (ChIP) experiments have shown that Cdc45 and RPA associate with early-firing origins at the G1-S boundary, concomitant with the disappearance of Cdc6 from chromatin and origins. This origin-association is also dependent on S-CDK and DDK activity (Zou and Stillman, 2000). Association of Cdc45 with chromatin requires Mcm2, and origin association of RPA requires Mcm5 (Tanaka and Nasmyth, 1998; Zou and Stillman, 1998). In addition, origin association of Cdc45 and Sld3 during G1 and S phase is mutually dependent (Kamimura *et al*., 2001). Also noteworthy is the fact that the Cdc45-Sld3 complex associates specifically with origins via Mcm2-7, and in *sld3-5* mutant cells, which exhibit a reduced Cdc45-Sld3 interaction, the interaction between Cdc45 and Mcm2 is also reduced, and origin association of RPA is abrogated. These findings suggest an essential role for the Cdc45-Sld3 complex in origin unwinding and the ensuing initiation of DNA replication (Kamimura *et al*., 2001).

Budding yeast Sld3 is also essential for loading the tetrameric protein complex, GINS (Go, Ichi, Nii and San; five, one, two, and three in Japanese), to origins. GINS consists of Psf1 (partner of Sld five 1), Psf2, Psf3, and Sld5, all of which are highly conserved in eukaryotes and initially associates with replication origins, then moves with the replication fork (Takayama *et al*., 2003; Calzada *et al*., 2005; Gambus *et al*., 2006). GINS is present throughout the yeast cell cycle, but is only recruited to origins around the time of their initiation (Takayama *et al*., 2003). CDK facilitates Sld3 to recruit Dpb11-Sld2 and GINS to origins, possibly in association with Pol ε. DDK is also essential and Mcm2-7 is thought to be its key target (Sheu and Stillman, 2006), yet the effects of phosphorylation of MCM are unclear. Furthermore, a yeast two-hybrid assay showed an interaction between Sld3 and Psf1 (Takayama *et al*., 2003). In fact, ChIP analysis has shown that GINS, much like Pol2, the catalytic subunit of Pol ε, associates with both stalled replication forks after cells have been arrested in early S phase with hydroxyurea (HU), and paused forks from a replication fork block after cells have been arrested and released from late G1 with the mating pheromone, α-factor (Calzada *et al*., 2005).

Kanemaki and Labib (2006) recently showed in ChIP experiments that GINS is necessary for the recruitment of RPA, and thus DNA unwinding by the replicative helicase, Mcm2-7. Moreover, GINS is essential for normal progression of replication forks, and is required after DNA replication initiation to maintain association between Mcm2-7 and Cdc45 (Gambus *et al*., 2006). Interestingly, in the absence of GINS, MCM-Cdc45 does not progress from origins and genomic footprinting demonstrates that the origin remains in the pre-RC state (Kanemaki and Labib, 2006).

Previous models of pre-RC assembly and function have predicted that once the MCMs have been loaded onto chromatin (origin licensing), ORC is no longer required for DNA replication (Aparicio *et al*., 1997). *In vitro* studies showed that high salt treatment of either budding yeast late G1 chromatin (Donovan *et al*., 1997) or pre-RCs assembled on origin-coated magnetic beads (Bowers *et al*., 2004) removed ORC and Cdc6, but Mcm2-7 remained. It should however be noted that Bowers and colleagues (2004) reported a significant reduction in MCM dissociation following salt extraction, yet DNA replication could not be evaluated due to the constraints of the pre-RC assembly assay. Furthermore, research with *Xenopus* egg extracts suggest that once MCM proteins are assembled on chromatin, ORC and Cdc6 are dispensable for its helicase activity (Hua and Newport, 1998; Rowles *et al*., 1999).

In a recent study through mutational analysis of three ORC subunits, Gibson and colleagues (2006) showed that *orc1-161*, *orc2-1* and *orc5-1* temperature-sensitive mutants displayed slower S phase progression than a wild-type control, after cells had been arrested in late G1 with α-factor, shifted to the restrictive temperature, and released from the block at the restrictive temperature. Another study showed a significant defect in S phase progression in *orc2-1* cells following a shift to the restrictive temperature for 6h in late G1. Also noteworthy was a displacement of Mcm2 from chromatin after a shift to the restrictive temperature (Zhang *et al*., 2002), however, the effect of Mcm2 displacement on DNA replication was not evaluated. In contrast, Shimada and colleagues (2002) did not find a defect in S phase progression following a 2h depletion of Orc2 in late G1 in GAL1-*orc2-1*

cells at the permissive temperature. Interestingly, another study using the same strain found that a 2h depletion of Orc2 at the restrictive temperature resulted in less efficient DNA replication (Weinberger *et al*., 2005).

Data from our lab strongly suggest a role for Orc6 in the maintenance of MCMs after pre-RC formation (Semple *et al*., 2006). Orc6 depletion in late G1 significantly reduced the number of origins that initiated DNA replication following release from alpha factor, and inhibited S phase progression. Moreover, yeast two-hybrid studies showed a physical interaction between Orc6 and Mcm10. In addition, Orc6 was found to be required for the chromatin maintenance of Mcm10. In this study, Orc6 depletion in late G1 resulted in a 50% reduction of Mcm10 from chromatin compared to the wild-type control. Mcm10 is essential for Mcm2 maintenance at origins during G1 (Homesley *et al*., 2000). Orc6 was also found to be necessary for the continued origin association of Mcm2 in late G1 at both the well-characterized early-firing origin, ARS1 and the late-firing origin ARS609 (Semple *et al*., 2006; Da-Silva and Duncker, 2007). Orc6 is thought to be the stability factor for MCMs, via an interaction between Mcm2 and Mcm10. These processes, in turn, are thought to maintain MCMs at origins where they function as the DNA replicative helicase to unwind origin DNA, and facilitate the initiation of DNA replication.

The aims of the present study conducted in *S. cerevisiae* were four-fold. First, it was of importance to determine if the above-mentioned MCM maintenance role is unique to Orc6, or if in fact other ORC subunits function in this capacity. Second, equipped with the knowledge that MCMs are displaced from chromatin and origins in the absence of Orc6 following pre-RC formation (Semple *et al*., 2006; Da-Silva and Duncker, 2007), it was of great interest to determine if this was reversible such that the pre-RC could reassemble and initiate DNA replication. Third, it was also of significance to establish whether or not other pre-RC components facilitate MCM maintenance since it was previously reported that along with ORC, Cdc6 is dispensable once MCM proteins have been loaded onto

chromatin (Donovan *et al*., 1997; Bowers *et al*., 2004). Finally, it was of interest to determine if the role of Orc6 in late G1 extends into S phase for MCM maintenance at late-firing origins.

## MATERIALS AND METHODS

### Yeast Strains

The genotypes of the strains used in this study are listed in Table I. Unless otherwise indicated, DY-26 was used as the standard wt strain.

**Table 1.** Yeast Strains used in this study

| Strain | Genotype | Source |
|---|---|---|
| DY-26 | MATa, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0 | ATCC BY4733 |
| DY-36 | MATa, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, orc6::Pgal-3HA-ORC6/TRP1 | Semple *et al*., 2006 |
| DY-39 | MATa, ade2-1, can1-100, trp1-1, his3-11, his3-15, ura3-1, leu2-3, leu2-112, pep4::LEU2, orc6::ORC6-13Myc/KanMX6 | J. Semple |
| DY-93 | MATa, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, orc6::ORC6-3HA/TRP1 | Semple *et al*., 2006 |
| DY-103 | MATa, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, orc4::Pgal-3HA-ORC4/TRP1 | L. Kummer |
| DY-109 | MATa, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, orc4::ORC4-3HA/TRP1 | L. Kummer |
| DY-115 | MATa ade2-1, ura3-1, his3-11,15, trp1-1, leu2-3,112, can1-100 UBR1::GAL-HA-UBR1/HIS3 | Euroscarf YKL200 |
| DY-118 | MATa, ade2-1 ura3-1 his3-11,15, trp1-1, leu2-3,112, can1-100, UBR1::GAL-HA-UBR1/HIS3; orc1::myc-ORC1td/KanMX6 | This study |
| DY-119 | MATa, ade2-1 ura3-1 his3-11,15, trp1-1, leu2-3,112, can1-100, UBR1::GAL-HA-UBR1/HIS3; orc2::myc-ORC2td/KanMX6 | This study |
| DY-120 | MATa, ade2-1 ura3-1 his3-11,15, trp1-1, leu2-3,112, can1-100, UBR1::GAL-HA-UBR1/HIS3; orc4::myc-ORC4td/KanMX6 | This study |
| DY-121 | MATa, ade2-1 ura3-1 his3-11,15, trp1-1 leu2-3,112, can1-100, UBR1::GAL-HA-UBR1/HIS3; orc5::myc-ORC5td/KanMX6 | This study |
| DY-122 | MATa, ade2-1 ura3-1 his3-11,15, trp1-1, leu2-3,112, can1-100, UBR1::GAL-HA-UBR1/HIS3; orc6::myc-ORC6td/KanMX6 | This study |
| DY-125 | MATa, ade2-1 ura3-1 his3-11,15, trp1-1, leu2-3,112, can1-100, UBR1::GAL-HA-UBR1/HIS3; orc3::myc-ORC3td/KanMX6 | This study |
| DY-139 | MATa, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, cdc6::Pgal-3HA-CDC6/TRP1 | A. Broom |
| DY-140 | MATa, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, cdt1::Pgal-3HA-CDT1/TRP1 | A. Broom |
| DY-142 | MATa, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, cdc6::CDC6-3HA/TRP1 | This study |
| DY-143 | MATa, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, cdt1::CDT1-3HA/TRP1 | This study |

**Genomic Integration**

Genomic tagging of ORFs was performed by homologous recombination with PCR fragments containing gene-specific sequences including the promoter and/or the eptitope to be added, and selectable marker genes amplified from plasmid templates as described by Longtine *et al.* (1998) for GAL1 and/or HA tagging, and Sanchez-Diaz *et al*. (2000) for degron fusion. PCR fragments were amplified, purified and transformed into yeast using general molecular biology techniques (Burke *et al*., 2000). Correct integration was confirmed by PCR analysis of yeast genomic DNA using primers flanking the gene of interest. Table 2 lists primers used for both genomic modification and PCR confirmation. Confirmation of expression of tagged genes was determined by western blot.

**Yeast Genomic DNA Isolation**

Yeast genomic DNA was isolated according to Burke *et al.* (2000). 10ml saturated yeast culture was harvested, washed with 0.5ml dd$H_2O$, spun down and resuspended in 0.2ml DNA isolation mix (10mM Tris-HCl, pH 8; 100mM NaCl; 1mM EDTA, 2% Triton X-100, 1% SDS), 0.2ml phenol:chloroform:isoamylalcohol (25:24:1) and 0.5g glass beads. Samples were then vortexed 3-4min, and 0.2ml TE (pH 8) was added and samples were mixed by inversion. Samples were spun down 5min at 16,000 x g and the top layer was transferred to a new tube. To each sample, 1ml 100% EtOH (RT) was added, mixed by inversion, and then spun 2min at 16,000 x g. The DNA pellet was resuspended in 0.4ml TE (pH 8) containing 100µg RNaseA, and incubated 10-15min at 37°C. Ammonium acetate was then added to 0.1M, followed by 1ml 100% EtOH (RT), mixed by inversion, and spun 2min at 16,000 x g. The DNA pellet was then air-dried and resuspended in 50µl TE (pH 8).

**Protein Isolation and Western Blotting**

Yeast whole-cell extracts (WCE) were prepared according to Burke *et al.* (2000). Briefly, cells were pelleted and resuspended in 400µl ice-cold lysis buffer (10mM Tris-HCl pH 8; 140mM NaCl; 1mM EDTA, 1% Triton X-100; protease inhibitors). Cell lysis was performed at 4°C with 0.5g glass beads

using a Bead Beater (Biospec Products, Inc.) with 8 cycles of 30s on/30s incubation on ice. Lysates were centrifuged at 4°C for 1min at 16,000 x g to remove cell debris. Protein concentration was quantified by Bradford assay (Bradford, 1976) using a protein assay solution (BioRad) and protein expression was determined by western blot analysis. Unless otherwise specified nitrocellulose blots were analyzed on a Typhoon 9400 (GE Healthcare).

**Chromatin Binding Assay**

Approximately 5 x $10^8$ cells were harvested at 1000 x g according to Conradt *et al.* (1992) and Pasero *et al.* (1999) with modifications. Cells were washed once with $ddH_2O$ and incubated in 5 ml pre-spheroplasting buffer (0.1M PIPES-KOH, pH 9.4; 10mM DTT) for 10 min at 30°C. Cells were then incubated in 5 ml spheroplasting buffer (1X YPD; 1.1M Sorbitol) containing 0.5 mg/ml Zymolyase 20T (Saikagaku Corp., Japan) and 0.2mg/ml Lyticase (Sigma, L4025) for 30min at 30°C with gentle mixing. Cells were washed once with 20ml spheroplasting buffer containing 0.5mM PMSF, followed by resuspension in 1ml ice-cold wash buffer (20mM Tris-HCl, pH 7.4; 20mM KCl; 2mM EDTA-KOH pH 7.4; 125µM spermidine; 50µM spermine; 1M sorbitol; 1% thiodiglycol; protease inhibitors [0.1mM benzamidine HCl; 1µg/ml Pepstatin A; 2µg/ml antipain; 2µg/ml leupeptin; 0.5mM PMSF]). Cells were pelleted at 1000 x g for 1min at 4°C and washed twice with 1 ml ice-cold wash buffer, followed by resuspension in 0.4 ml ice-cold breakage buffer (20mM Tris-HCl, pH 7.4; 20mM KCl; 2mM EDTA-KOH pH 7.4; 125µM spermidine; 50µM spermine; 1M sorbitol; 1% thiodiglycol; protease inhibitors). Cells were then lysed with 0.5ml ice-cold breakage buffer containing 4% Triton X-100 and incubated on ice for 2 min with occasional gentle mixing. The lysed cells were pelleted at 16,000 x g for 10min at 4°C. The soluble fraction was transferred to a new tube and the insoluble chromatin pellet was digested on ice in 0.1ml ice-cold breakage buffer containing 5mM $MgCl_2$ and 5µg DNaseI. Digestion was stopped with 10mM EDTA-KOH, pH 7.4.

**Chromatin Immunoprecipitation Assay**

Chromatin immunoprecipitation (ChIP) was performed as previously described (Tanaka *et al.*, 1997) with modifications. Approximately 2.5 x $10^8$ cells were cross-linked with 1% formaldehyde for 20min at 30°C with gentle shaking and the reaction quenched with 125mM glycine for 5min at 30°C. Cells were harvested, washed once with ice-cold PBS and the pellet was snap frozen in liquid nitrogen and stored at -80°C. Cell lysis was performed at 4°C with 0.5g glass beads in 500µl lysis buffer (50mM HEPES-KOH pH 7.5; 140mM NaCl; 1mM EDTA-KOH, pH 7.5; 1% Triton X-100, 0.1% sodium deoxycholate) containing protease inhibitors using a Mini-Beadbeater-8 (Biospec Products, Inc) with 8 cycles of 30s lysis/30s incubation on ice. Samples were spun down into a 2ml tube 30s at 1000 x g so as to separate the glass beads from the cell lysate. This slurry was spun down 5min at 16,000 x g at 4°C and the soluble fraction of the whole-cell extract (WCE) was discarded and the insoluble pellet was resuspended in 500µl lysis buffer containing protease inhibitors. This extract was sonicated five times for 20s at 5–6W to achieve an average DNA fragment size of 500bp using a Microson$^{TM}$ XL 2000 (Misonix). Samples were spun down twice for 2min at 4500g to remove cell debris. Approximately 1/50 of the WCE was saved as "input" (sonicated only). Immunoprecipitation was carried out with 30µl anti-goat IgG agarose beads (Sigma, A9294), which were preincubated with 2 mg each of anti-Mcm2 (Santa Cruz, sc-6680) and anti-Mcm5 (Santa Cruz, sc-6686) antibodies. Samples were incubated overnight at 4°C on a rotator. Samples were then washed and processed as described in the above reference. DNA samples were resuspended in a final volume of 60µl and PCR was carried out in a 20µl volume with 0.5µl input or 2µl immunoprecipitated (IP) sample using two pairs of primers (Table 2) in each reaction as follows: 2µM dNTP, 5µM upstream ARS primers and 30µM ARS-specific primers. PCR products were separated on a 2% agarose gel.

**Flow Cytometry**

Approximately $10^7$ cells were harvested and resuspended in 1ml 70% ice-cold EtOH to fix cells and stored at 4°C. Cells were then spun down and washed once with $ddH_2O$ and incubated in 0.5ml 50mM Tris-HCl (pH 8) containing 0.2mg/ml RNase A for 2-4h at 37°C. Cells were then incubated in 50mM Tris-HCl (pH 7.5) containing 2mg/ml proteinase K for 30-60min at 50°C. Cells were then harvested and resuspended in 100µl FACS buffer (200mM Tris-HCl, pH 7.5; 200mM NaCl; 78mM $MgCl_2$) and the entire volume was transferred to 0.5ml Sytox solution (50mM Tris-HCl, pH 7.5; Sytox [1:5000; Invitrogen]). Samples were then analysed for DNA content on a Becton Dickinson FACSVantage SE.

**Synchronizing Yeast Cultures**

***Alpha factor arrest and protein depletion in GAL1 strains***

Late G1 arrest and protein depletion during late G1 was performed as previously described (Semple *et al.*, 2006). Briefly, strains were diluted to 0.5-1 x $10^7$ cells/ml in fresh media and arrested in late G1 with α-factor (10µg/ml) for 3h at 30°C in YPG/R (1% yeast extract; 2% peptone; 2% galactose; 1% raffinose), replenishing the α-factor (10µg/ml) at 1.5h. The cells were then harvested, washed once with $ddH_2O$ and resuspended in YPD (1% yeast extract; 2% peptone; 2% glucose) containing α-factor (10µg/ml) for 3h (*GAL1-CDC6*) or 5h (*GAL1-ORC4* or *GAL1-ORC6*) to achieve complete protein depletion, replenishing the α-factor (10µg/ml) every hour. Cells were subsequently washed once with $ddH_2O$ and once with either YPG/R or YPD and released in either YPG/R or YPD containing 100µg/ml pronase E (Sigma, P6911).

***Hydroxyurea (HU) arrest***

Hydroxyurea was used to synchronize yeast cultures in S phase. Cells were diluted to 0.5-1 x $10^7$ cells/ml in fresh YPG/R and initially arrested with α-factor as above. Cells were then harvested, washed once in $ddH_2O$ and resuspended in fresh YPG/R containing 0.4M HU (Sigma, H8627) and

incubated for 45min. Cultures were then spun down, washed once with dd$H_2O$ and resuspended in YPD and incubated for 6h, replenishing HU (0.4M) at 3h.

**Construction of Heat-Sensitive Degron Strains**

Degron strains were created by a one-step PCR approach using a yeast strain in which the sole copy of the *UBR1* gene is expressed under the control of the glucose-repressible GAL1 promoter (Kanamaki *et al.*, 2003; Sanchez-Diaz *et al.*, 2004). For each ORC subunit, a PCR product was generated using two 70-base oligonucleotides, the forward primer beginning with 50 nucleotide homology to the promoter region of the ORC subunit, chosen ~50bp upstream of the ATG start codon, and ending with 20 nucleotides identical to the beginning of the degron cassette in pKL187. The reverse primer contained 20 nucleotides identical to the end of the degron cassette in pKL187 and the first 50 nucleotides from the open reading frame of the target ORC subunit including the ATG. The PCR products were transformed into the haploid *GAL-UBR1* strain YKL200, and transformants grown at 24°C on YPD containing 0.1mM $CuSO_4$ to induce the CUP1 promoter, under which the degron fusion protein is expressed, and 200µg/ml G418. Colonies were then replica-plated and re-streaked onto fresh YPD containing 0.1mM $CuSO_4$ and 200µg/ml G418. These colonies represented putative integrants and confirmatory PCR analysis of yeast genomic DNA using primers that flank the ORC subunit of interest was performed.

**Growth of Degron Strains**

Degron strains were grown according to Kanamaki *et al.* (2003) and Sanchez-Diaz *et al.* (2004), with modifications. Briefly, asynchronous cultures were grown at 24°C in YPD supplemented with 0.1mM $CuSO_4$. The cells were then pelleted, washed with dd$H_2O$ and transferred to YPG/R containing 0.1mM $CuSO_4$ for 35-60min at 24°C to induce overexpression of ubiquitin (Ubr1). Cells were then harvested and resuspended in YPG/R pre-warmed to 37°C and incubated for 45-60min to promote proteolysis of the ORC degron fusion proteins.

**Table 2.** PCR primers created/used in this study.

| Gene/Region | Name | Sequence (5' ➔ 3') | Description |
|---|---|---|---|
| ARS1 | ARS1 Up Fwd | TAC CTC GCT CCT GAA AAA TCC CG | Forward primer to amplify 9kb upstream of ARS1 |
| | ARS1 Up Rev | TTC CGC GTA CTT CCC GCT GG | Reverse primer to amplify 9kb upstream of ARS1 |
| | ARS1 Fwd | CGG AGG TGT GGA GAC AAA TGG TG | Forward primer to amplify ARS1 |
| | ARS1 Rev | GGT AAA AGT CAA CCC CCT GCG ATG | Reverse primer to amplify ARS1 |
| ARS603 | ARS603 -9kb Fwd | CTC CAG CTT TTG CGC ATG GTT ATC | Forward primer to amplify 9kb upstream of ARS603 |
| | ARS603 -9kb Rev | CCA CCT GAT CTC CAA TTA ACC CAG GG | Reverse primer to amplify 9kb upstream of ARS603 |
| | ARS603 Fwd | CTC TTT CCC AGA TGA TAT CTA GAT GG | Forward primer to amplify ARS603 |
| | ARS603 Rev | CGA GGC TAA ATT AGA ATT TTT GAA GTC | Reverse primer to amplify ARS603 |
| ARS609 | ARS609 Up Fwd | TGA TCA ATC CCC TGC CTC CAA G | Forward primer to amplify 9kb upstream of ARS609 |
| | ARS609 Up Rev | ATG CGG TGA TTC TTG CCA TGG | Reverse primer to amplify 9kb upstream of ARS609 |
| | ARS609 Fwd | GAA CGC CAT GTG TAA GGT GCA ATG | Forward primer to amplify ARS609 |
| | ARS609 Rev | CCA TTG AAT TAT GCC GAG AGC TGA C | Reverse primer to amplify ARS609 |
| ORC1 | Orc1 td For | GAA AAC TGC ATA GGC GGC AAA TTC AGC CTA AAA GTT TCC AGA AGC AGG AAC TC*A TTA AGG CGC GCC AGA TCT G* [a] | Forward primer to amplify degron cassette for ORC1-degron fusion |
| | Orc1 td Rev | TCA GTT GTT ATT ATC TCC CAA CCC TGT AAA TCC TTC AAC GTT TTT GCC AT*G GCA CCC GCT CCA GCG CCT G* [a] | Reverse primer to amplify degron cassette for ORC1-degron fusion |
| | Orc1 Fwd Flank | TGC ACC AAC GAA CAC CAC TAC ATA TG | Forward primer to amplify region flanking ORC1 |
| | Orc1 Rev Flank | GAA AAC AAC TAT ACC CGC CTG CTA CC | Reverse primer to amplify region flanking ORC1 |
| ORC2 | Orc2 td For | CAA AGT AGA ATA TAT TAG CTG AAA TTG TAT TTG ATA ATT GAT CAT TGA TCT TA*A TTA AGG CGC GCC AGA TCT G* [a] | Forward primer to amplify degron cassette for ORC2-degron fusion |
| | Orc2 td Rev | GGA GAC GAT AGG ATA TCA TTA TGC TCT ACA AAG TCT TCC CCA TTT AGC AT*G GCA CCC GCT CCA GCG CCT G* [a] | Reverse primer to amplify degron cassette for ORC2-degron fusion |
| | Orc2 Fwd Flank | CAC CGC ATC ATT GAG GTG GAA ATG AC | Forward primer to amplify region flanking ORC2 |
| | Orc2 Rev Flank | GAG CTC ATC AGA CGT TTT TCA GTA GGG | Reverse primer to amplify region flanking ORC2 |
| ORC3 | Orc3 td For | GAC GTA TTT TTC CGC ACC GTA ATT TGA AGA AAA AGA AAA GTG ACA AAA GA*A TTA AGG CGC GCC AGA TCT G* [a] | Forward primer to amplify degron cassette for ORC3-degron fusion |

| | | | |
|---|---|---|---|
| | Orc3 td Rev | GTC AGC AAA CTC GCT GAC GTT CAT CTT TTT GGA TTG GTT AAG GTC GCT CAT *GGC ACC CGC TCC AGC GCC TG* [a] | Reverse primer to amplify degron cassette for ORC3-degron fusion |
| | Orc3 Fwd Flank | GGT TGC GTT ACG AGA CAG GCT TGA C | Forward primer to amplify region flanking ORC3 |
| | Orc3 Rev Flank | AAT CTT CGC GCA TCA TGG CAC TAT C | Reverse primer to amplify region flanking ORC3 |
| ORC4 | Orc4 td Fwd | GAA AAA GCA TTA ACA ATT AAA AAA AAA AAA AAA ATC TAA ATA ATA CTG ATA *ATT AAG GCG CGC CAG ATC TG* [a] | Forward primer to amplify degron cassette for ORC4-degron fusion |
| | Orc4 td Rev | CTT TAT TGG GAG AAG ATT GAC TTG CGG TGA TAG ACG AGC TTC GCT TAT AGT CAT *GGC ACC CGC TCC AGC GCC TG* [a] | Reverse primer to amplify degron cassette for ORC4-degron fusion |
| | Orc4 Fwd Flank | AAG CAA AAT GGA GAC GCA AAA GAA AAC | Forward primer to amplify region flanking ORC3 |
| | Orc4 Rev Flank | TCG CTG GAT GGC TGG TGT ACT CTT C | Reverse primer to amplify region flanking ORC3 |
| ORC5 | Orc5 td Fwd | CCG AAA AAT TAG CCC TTG AAC ATA ATT AAC ACT CTT CTT TGA TAT TTA *ATT AAG GCG CGC CAG ATC TG* [a] | Forward primer to amplify degron cassette for ORC5-degron fusion |
| | Orc5 td Rev | CAG TTG GTT TGA TAT TCC CTA AAA GCA ACT TCC GGA GTG GTC ACA TTC AT*G GCA CCC GCT CCA GCG CCT G* [a] | Reverse primer to amplify degron cassette for ORC5-degron fusion |
| | Orc5 Fwd Flank | AAC TGC TGA AGA TAT TTG AAT TGC GTG AG | Forward primer to amplify region flanking ORC5 |
| | Orc5 Rev Flank | CGC CGT TCA GTC TCC TCA TTC ATT G | Reverse primer to amplify region flanking ORC5 |
| ORC6 | Orc6 td Fwd | GTG TAT TTC TTT GTT CTT TGC CGT TGT TTA CGT TAG TAA GAA ATC GGC AT*A TTA AGG CGC GCC AGA TCT G* [a] | Forward primer to amplify degron cassette for ORC6-degron fusion |
| | Orc6 td Rev | TCT AGT CGA AGT ACT TCT GCG ACA CAA TGT TGG ACT TGT TGC ATG GAC AT*G GCA CCC GCT CCA GCG CCT G* [a] | Reverse primer to amplify degron cassette for ORC6-degron fusion |
| | Orc6 Fwd Flank | ATG CAA GGC TTT CGA ATA CAC TCC | Forward primer to amplify region flanking ORC6 |
| | Orc6 Rev Flank | GCG GAA GCT GCA AAG ATA CAA AAG | Reverse primer to amplify region flanking ORC6 |
| CDC6 | Cdc6 F2 | GAT GAG ATG ACC AAA ATT TCA ATT TTG AAA CCT TTC CTT CAC *CGG ATC CCC GGG TTA ATT AA* [b] | Forward primer to amplify 3HA-TRP1 cassette |
| | Cdc6 R1 | TGG AAC TGT AAA AAA AAA AGA AAA AAT AAA TCT ATT AGC TGA *GAA TTC GAG CTC GTT TAA AC* [b] | Reverse primer to amplify 3HA-TRP1 cassette |
| | Cdc6 Fwd Flank | TGA GTT GGC TGG CAG AAA AGA TAT A | Forward primer to amplify region flanking CDC6 |
| | Cdc6 Rev Flank | TCA GCC AAA CGG TTT AAC CGT TTC C | Reverse primer to amplify region flanking CDC6 |
| CDT1 | Cdt1 F2 | TTT TCT AAG CTG TTG CAA ATC CAC AAA TCA AAA CAA CAA GAT *CGG ATC CCC GGG TTA ATT AA* [b] | Forward primer to amplify 3HA-TRP1 cassette |

| | | | |
|---|---|---|---|
| | Cdt1 R1 | ACC GTA AAT GTA CGT ATT CTA CCT TAT ATC TAA AAA TAT TCT *GAA TTC GAG CTC GTT TAA AC* [b] | Reverse primer to amplify 3HA-TRP1 cassette |
| | Cdt1 Fwd Flank | CTC TGC CGT TGT GGG TGA TAT AC | Forward primer to amplify region flanking CDT1 |
| | Cdt1 Rev Flank | CGC AGG TTC GAG TCC TGC AGT TGT C | Reverse primer to amplify region flanking CDT1 |

[a] For PCR-based degron modification (Sanchez-Diaz *et al.,* 2004), the gene-specific sequences included in the primers were chosen so as to delete ~50 nucleotides upstream of the target gene start codon (underlined). The plasmid sequences are italicized.

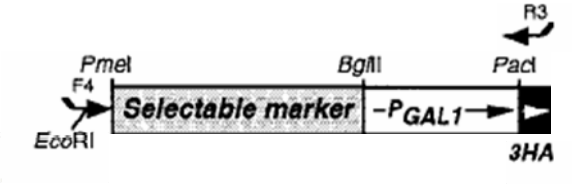
[b] For PCR-based tagging (Longtine *et al.,* 1998), the gene-specific sequences of the forward primer were chosen to end just upstream of the stop codon, preserving the reading frame, whereas those of the reverse primer were chosen to end just downstream of the stop codon (underlined). The plasmid sequences are italicized.

# RESULTS

## Role of Orc4 in cell cycle progression

An effective way to study the biological function of a protein both *in vitro* and *in vivo* is simply to delete it and monitor the effect. This approach, however, is unsuitable *in vivo* for essential proteins such as the ORC subunits. To circumvent this and in collaboration with Lutz Kummer from our lab, the role of ORC subunits in cell cycle progression was investigated by replacing the natural promoter of each of the ORC genes (*ORC*1-6) in a haploid yeast strain (DY-26) with the glucose-repressible *GAL1* promoter (Fig. 5A), in much the same manner as was done previously for Orc6 (Semple *et al*., 2006). As part of each strain construction, a sequence encoding three copies of the HA epitope was fused to the start of each respective ORF in order to facilitate observation of protein levels. In an effort to compare overexpressed protein levels to those of endogenous wild-type (wt) levels, using the same isogenic DY-26 strain a separate strain was created in which 3 copies of the HA epitope was fused to the extreme 3' end of each respective ORF (Fig. 5B), just upstream of the stop codon. For simplicity, the term wt will refer to the isogenic HA-tagged ORC counterpart of the *GAL1-ORC1-6* strain being discussed. Cultures of *GAL1-ORC1-6* and the wt strains were grown in YPG/R medium overnight, washed in dd$H_2O$ and resuspended in YPD medium to deplete the ORC subunit in the respective *GAL1-ORC* strain. Results showed that both growth and protein depletion kinetics were strikingly similar between Orc4 (Fig. 6 and data not shown) and Orc6 (Semple *et al*., 2006) and as such, the role of Orc4 in cell cycle progression was examined more closely.

A




B

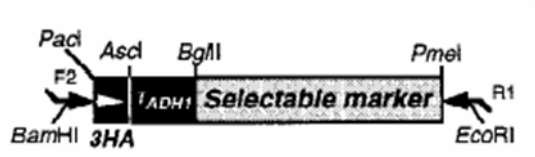



**Figure 5**. Modules for use as PCR templates to generate DNA fragments for genomic manipulation. (**A**) Cassette for $P_{GAL1}$-HA replacement of natural ORC promoters. (**B**) Cassette for C-terminal 3xHA-tagging of ORC subunits. Grey boxes represent selectable markers including the *S. cerevisiae* TRP1 gene. Black boxes represent protein-tagging modules consisting of 3HA together with the *S. cerevisiae* ADH1 transcriptional terminator. Arrows within the boxes indicate direction of transcription. Arrows outside the boxes indicate forward (F) and reverse (R) PCR primers; the bent portion of each primer represents the regions homologous to the yeast target sequence. (Longtine *et al*., 1998)

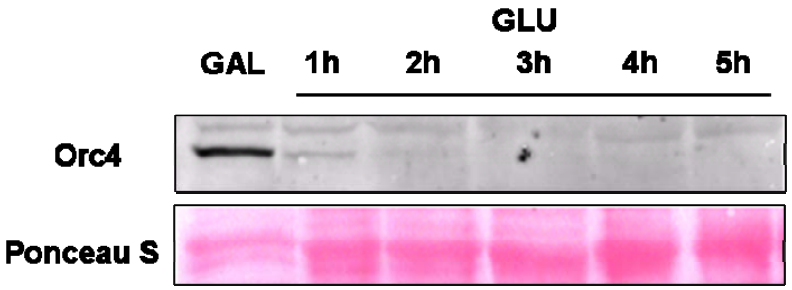



**Figure 6**. Orc4 is rapidly depleted in yeast following a shift of *GAL1-ORC4* cells to glucose medium. An asynchronous *GAL1-ORC4* (DY-103) strain was grown in YPG/R (2% galactose/1% raffinose) medium to $10^6$ cells/ml, washed and resuspended in YPD (2% glucose) medium. Whole-cell extracts were prepared from culture aliquots taken prior to (GAL) and at the indicated time points following a shift to YPD (GLU). Eighty micrograms of protein was used for immunoblot analysis. HA-tagged Orc4 was detected using an anti-HA antibody (Sigma) and a fluorescent secondary antibody (Invitrogen). Ponceau S staining of the region detected by the blot verifies equal loading of whole-cell extracts.

High salt treatment of either budding yeast late G1 chromatin, or pre-RCs assembled on origin-coated magnetic beads has been shown to remove ORC and Cdc6 while MCM proteins remain (Donovan *et al*., 1997; Bowers *et al*., 2004). Similar results were shown in *Xenopus* egg extracts which demonstrate that once MCM proteins are assembled on chromatin, ORC and Cdc6 can be removed without affecting DNA replication (Hua and Newport, 1998; Rowles *et al*., 1999). As such and armed with the knowledge that Orc6 is required for efficient initiation of DNA replication after MCM proteins have been loaded onto chromatin (Fig. 7B; Semple *et al*., 2006), it was of interest to determine if this also held true for Orc4. This was accomplished by the G1 phase synchronization of *GAL1-ORC4* and isogenic wt cultures growing in YPG/R through addition of the mating pheromone α-factor, as outlined in Materials and methods. After the initial arrest, cells were transferred to YPD in the presence of α-factor to deplete Orc4 in the *GAL1-ORC4* strain. Cells were then transferred to fresh YPD medium in the absence of the pheromone. If indeed Orc4 is required for initiation of DNA replication following origin licensing, cells would be unable to undergo DNA synthesis and replicate their genome. By fluorescently labeling the DNA of haploid yeast cells and analyzing them via fluorescence-activated cell sorting (FACS), cells can be monitored for either the absence of genome duplication, as depicted by a 1C DNA content, or the presence of a replicated genome, as illustrated by a 2C DNA content. FACS analysis showed that following release from depletion in late G1, and in contrast to Orc6 depletion, *GAL1-ORC4* cells were able to replicate their DNA, albeit at a slower rate than the wt control (Fig. 7A).

It was then of interest to determine if destabilization of the MCM complex at origins was the cause for this slower rate of DNA replication. To answer this, chromatin association of Mcm2 prior to and following Orc4 depletion was monitored by way of a chromatin binding assay. Briefly, whole cell extracts of yeast cultures were isolated by spheroplasting cells, which removed the cell wall, and were then lysed in buffer containing TX-100. Low speed centrifugation then separated the chromatin-

enriched nuclear fraction (pellet) and the soluble fraction (supernatant). The nuclear fraction was then further treated with DNaseI to degrade chromatin DNA, thereby releasing chromatin-bound proteins. Fractionation efficiency is determined by the percentage of protein in the chromatin pellet relative to the supernatant fraction and an efficient fractionation is represented by approximately 5-10% and 95-90% in the chromatin pellet and supernatant fractions, respectively. Protein samples were then run on SDS-PAGE gels and immunoblotted using antibodies specific to Mcm2, Orc2 and HA-tagged Orc4. There was no displacement of Mcm2 from chromatin following Orc4 depletion, yet there was an enrichment of chromatin-bound Mcm2 after overexpression of Orc4 (Fig.7B, GAL). The level of chromatin-bound Orc2 was unaltered following Orc4 depletion. Taken together, these results supported the view that Orc6, but not all ORC subunits, are required for initiation of DNA replication subsequent to the loading of MCM proteins on chromatin.

**Mechanism of cell cycle arrest from Orc6 depletion**

In an effort to elucidate the mechanism by which Orc6 depletion caused a cell cycle arrest, it was of value to first determine if a checkpoint was triggered. There are three main checkpoints in the cell cycle, namely G1/S, intra-S and G2/M and they exist to monitor the integrity of the DNA being replicated. If an error is detected, proteins involved in these checkpoint mechanisms will arrest cell cycle progression until such time as the DNA damage can been repaired (Kolodner *et al*., 2002). Thus, DNA damage due to the lack of Orc6 and/or MCM proteins on chromatin could potentially invoke a G1/S checkpoint. In order to investigate whether this might be the case, we examined the status of the checkpoint kinase Rad53, which is typically upregulated or hyperphosphorylated when a checkpoint is triggered. No upregulation or higher mobility states on immunoblots were observed for Rad53 with samples taken either directly following Orc6 depletion in late G1 or an hour after release from the pheromone (Fig. 8). As a control, wt cells were treated with the ribonucleotide reductase inhibitor

hydroxurea (HU), which depletes dNTP pools and arrests cells in mid-S phase, triggering the intra-S phase checkpoint, and illustrates the characteristic hyperphosphorylation of Rad53 (Fig. 8).

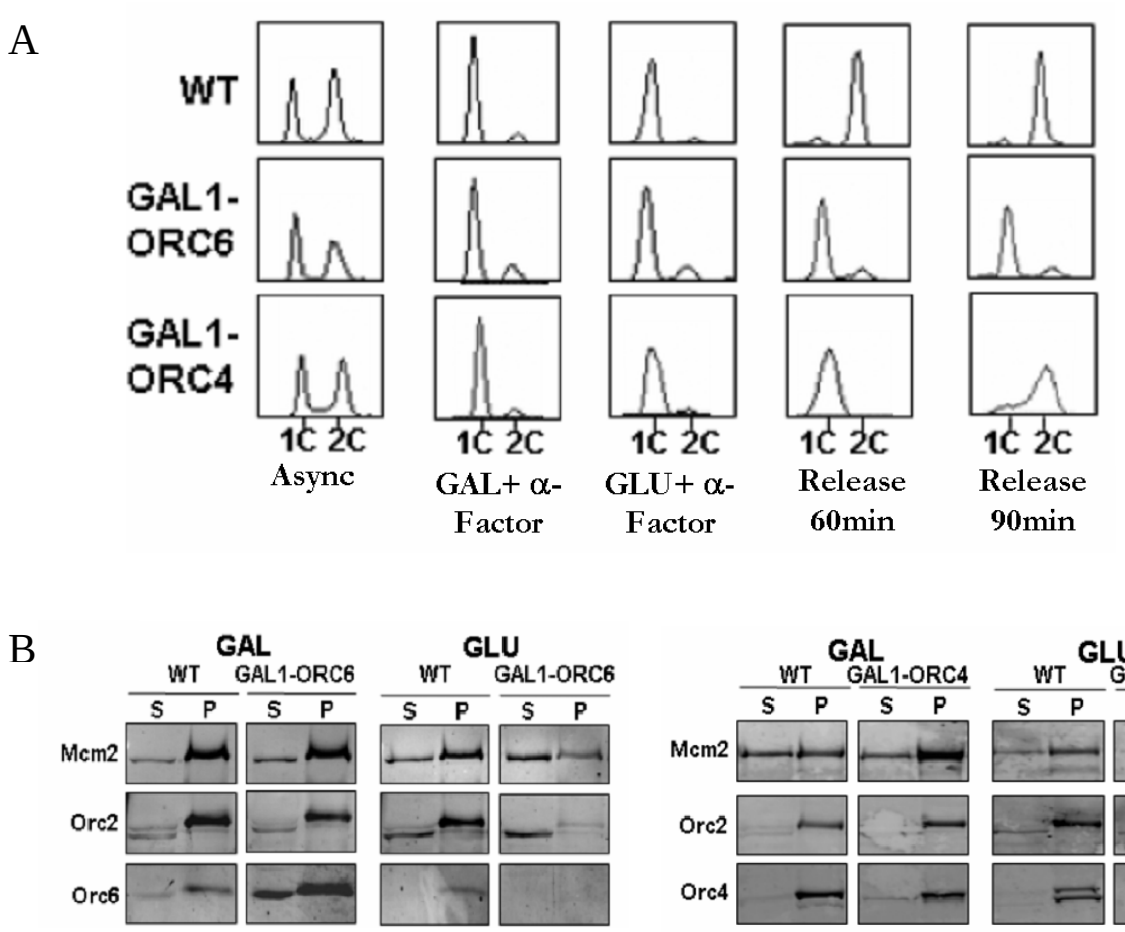



**Figure 7**. Depletion of Orc4 slows S phase progression, but does not destabilize MCM association with chromatin. (**A**) wt (DY-26), *GAL1-ORC6* (DY-36) and *GAL1-ORC4* (DY-103) cells were grown to 5 x $10^6$ cells/ml (Exp) and arrested in late G1 with α-factor (50µg/ml) for 2-3h in YPG/R. The cells were then pelleted, washed with $ddH_2O$ and transferred to YPD containing α-factor (50µg/ml) for 4h. Cells were subsequently washed and released into YPD without α-factor. Culture aliquots were taken at the indicated times for FACS analysis. (**B**) Isogenic wt, *GAL1-ORC6* and *GAL1-ORC4* cells were treated as described in (A). Samples were removed following the initial arrest (GAL) and following Orc4 depletion in late G1 (GLU), chromatin binding assays were conducted. Thirty microlitres of the chromatin-associated pellet (P) and 15µl of the soluble fraction (S) were analyzed by immunoblot. Proteins were detected as follows: HA-tagged Orc4 was detected using an anti-HA antibody (1:1000; Sigma); Mcm2 was detected by anti-Mcm2 (1:500; Santa Cruz) and Orc2 was detected by anti-Orc2 (1:1000) (Semple *et al*., 2006).

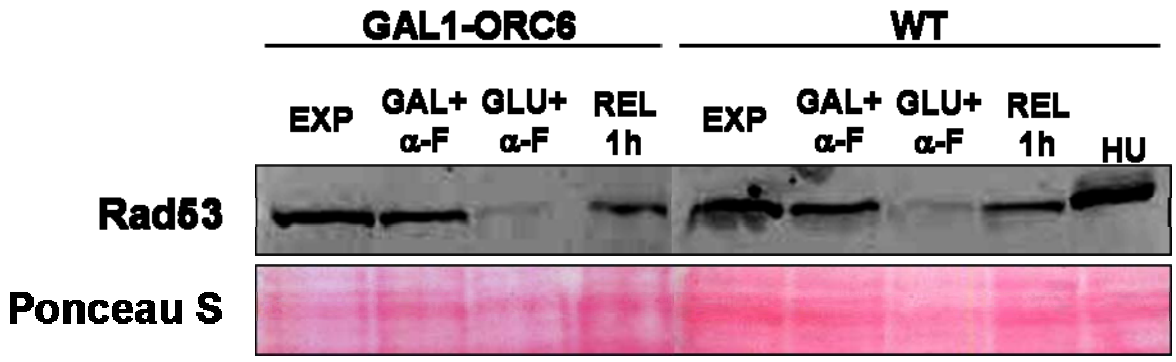



**Figure 8**. No checkpoint is triggered following Orc6 depletion in late G1, as indicated by a lack of Rad53 phosphorylation. Isogenic wt (DY-26) and *GAL1-ORC6* (DY-36) cells were grown to 5 x $10^6$ cells/ml (Exp) and arrested in late G1 with α-factor (50μg/ml) for 2-3h in YPG/R (GAL+α-F). The cells were then pelleted, washed with $ddH_2O$ and transferred to YPD containing α-factor (50μg/ml) for 4h (GLU+α-F). Cells were subsequently washed and released into YPD without α-factor for 1h (REL 1h). Samples were removed at the indicated time points and whole-cell extracts were prepared as described in Materials and methods. Rad53 was detected by using anti-yeast Rad53 antibody (1:200; Santa Cruz). Ponceau S staining of the region detected by the blot verifies equal loading of whole-cell extracts (Semple *et al*., 2006).

After ruling out a checkpoint mechanism, the most likely scenario for a lack of DNA replication subsequent to Orc6 depletion in late G1 was the specific loss of the MCM complex at replication origins, which was investigated by ChIP analysis (described in Materials and methods). Wt and *GAL1-ORC6* strains were initially arrested in late G1 with α-factor in YPG/R and then shifted to YPD containing α-factor as described above. Briefly, cells were then fixed with formaldehyde to crosslink proteins to DNA and whole-cell extracts were sonicated to shear chromatin to approximately 500bp fragments. Sample aliquots were removed for whole genome controls (INPUT) while the remainder was co-immunoprecipitated (IP) with a mixture of Mcm2 and Mcm5 antibodies. PCR of INPUT and IP templates was carried out by amplifying the early-firing ARS1 origin sequence and a non-origin region 9kb upstream of ARS1 (ARS1 -9kb); and the late-firing ARS609 origin sequence and a non-origin region 9kb upstream of ARS609 (ARS609 -9kb). The non-origin sequences were used to gauge the enrichment, or lack thereof, of MCM association at origin sequences, and demonstrated a significant drop in the level of both ARS1 and ARS609 DNA that had co-immunoprecipitated with MCMs in the absence of Orc6 (Fig. 9).

**Depletion of temperature-sensitive Orc6**

As an alternative approach and to further substantiate the glucose-repressible *GAL1-ORC6* results, conditional inactivation of temperature-sensitive alleles of ORC1-6 was carried out by use of a heat-sensitive degron tag. In this case, the degron cassette, under the control of the copper-inducible CUP1 promoter, was integrated upstream of each ORC subunit open reading frame (ORF) so as to replace the natural endogenous promoter in a MAT **a** haploid yeast strain. This created an N-terminal in-frame fusion between the degron and each ORC subunit (Dohmen *et al*., 1994; Sanchez-Diaz *et al*., 2004). As part of the strain construction, a sequence encoding a single MYC epitope was fused to the start of each ORF to monitor protein levels of each ORC-degron fusion protein. For simplicity, these

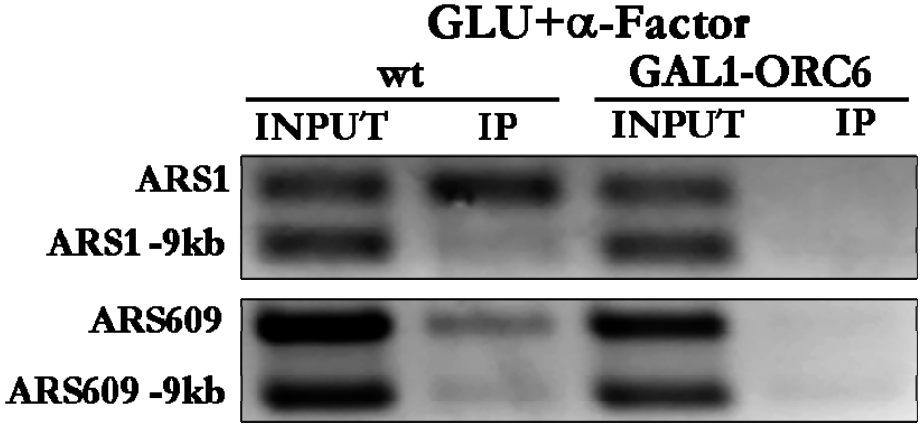



**Figure 9**. MCM proteins are displaced from replication origins following Orc6 depletion in late G1 phase. Isogenic wt (DY-93) and *GAL1-ORC6* (DY-36) cells were grown in YPG/R to 5 x $10^6$ cells/ml and arrested in late G1 with α-factor (15µg/ml) for 2.5h. The cells were then pelleted, washed with $ddH_2O$ and transferred to YPD containing α-factor (15µg/ml) for 4h. ChIP was performed with a mixture of Mcm2 and Mcm5 antibodies as described in Materials and methods and as described previously (Semple *et al*., 2006). PCR using sample aliquots taken after sonication (INPUT) and following purification of co-immunoprecipitated DNA (IP) was carried out using primers specific for the early-firing ARS1 origin sequence, and a region 9kb upstream of ARS1 (ARS1 -9kb); and the late-firing ARS609 origin sequence, and a region 9kb upstream of ARS609 (ARS609 -9kb). (Da-Silva and Duncker, 2007)

strains will be referred to as *ORC1-6td*. Degron fusion proteins are rapidly targeted for degradation by the N-end rule pathway (Varshavsky, 1997) in which multiple ubiquitin molecules bind exposed lysine residues of the temperature-sensitive version of mouse protein dihydrofolate reductase (DHFRts; the "degron") when cells are shifted from the permissive temperature of 23°C to the non-permissive, or restrictive temperature of 37°C. In addition, efficient proteolysis is achieved by the continued expression of ubiquitin (Ubr1) and as such, degron fusions were created in a haploid yeast strain in which the sole copy of UBR1 was under the control of the glucose-repressible GAL1 promoter (Labib *et al*., 2000). To first characterize these strains, asynchronous cultures of *ORC1-6td* and the isogenic wt (DY-115) were grown at 23°C in YPD supplemented with 0.1mM $CuSO_4$. The cells were then harvested and washed with $ddH_2O$ and transferred to YPG/R containing 0.1mM $CuSO_4$ for 35-60min at 24°C to overexpress Ubr1. Cells were subsequently pelleted and resuspended in YPG/R pre-warmed to 37°C and incubated for 45-60min to inactivate each ORC degron fusion protein (Orc1-6td). At all time points following the shift to the restrictive temperature, the wt strain exhibited prominent 1C and 2C peaks characteristic of asynchronous cultures (Figure 10). Unlike that observed previously with Orc6 depletion in the *GAL1-ORC6* strain (Semple *et al*., 2006), Orc6td did not show defects in S phase progression after 4h, yet by 16h, cells indeed demonstrated a defect with an accumulation of cells with a DNA content between 1C and 2C.

In an effort to determine the rate of protein depletion, wt and *ORC1-6td* strains were grown as described above and protein extracts were run on SDS-PAGE gels and immunoblotted. It should be noted that the single MYC epitope as part of the fusion protein (see above) was insufficient to allow Orc1-6td protein levels to be determined after immunoblotting with anti-MYC monoclonal antibodies (data not shown). This was most likely due to the fact that the single MYC epitope does not provide enough antibody binding sites for efficient detection, unlike use of the 3HA tag described above. Sanchez-Diaz and colleagues (2004) have found that the best way to circumvent this is to use an

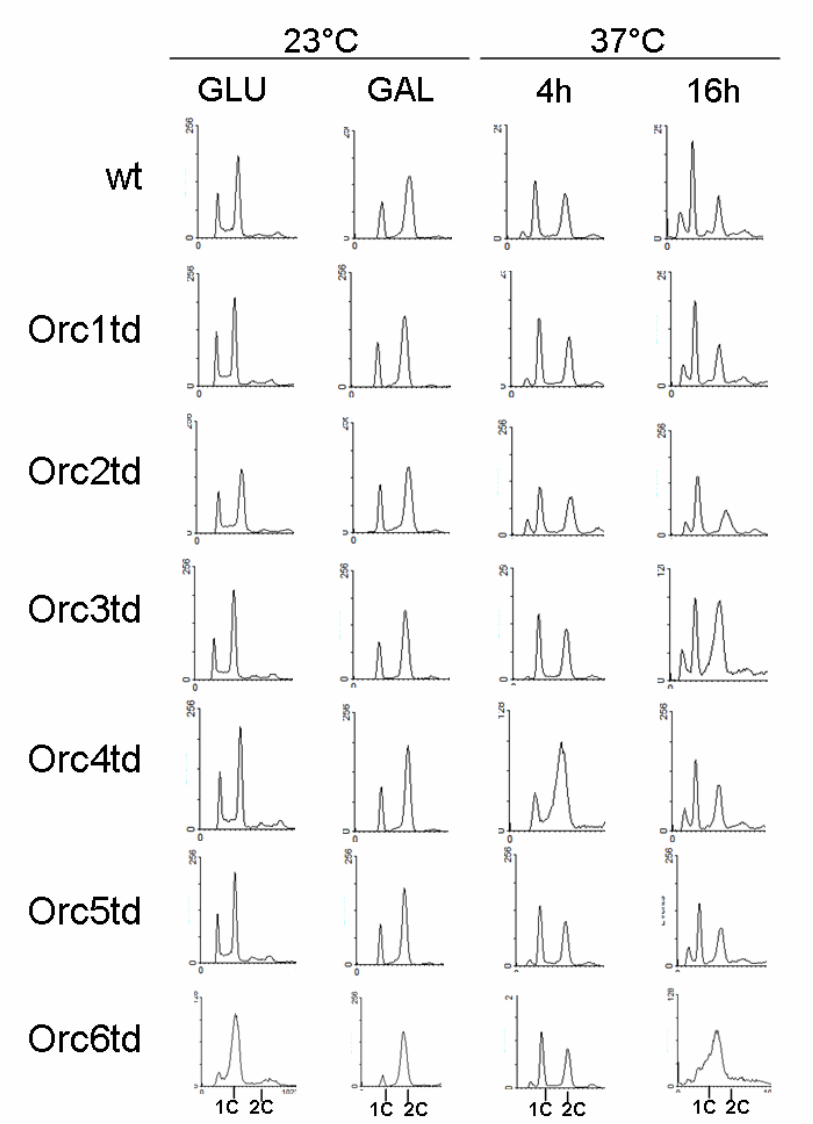



**Figure 10**. Depletion of temperature-sensitive ORC subunit degron strains. Asynchronous cultures were grown to 1 x $10^6$ at 23°C in YPD supplemented with 0.1mM $CuSO_4$ (GLU). To promote proteolysis of an ORC subunit degron fusion protein, cells were harvested, washed with $ddH_2O$ and to induce expression of Ubr1, cells were resuspended in YPG/R containing 0.1mM $CuSO_4$ for 60min at 23°C (GAL). The cells were then pelleted and resuspended in YPG/R pre-warmed to 37°C and incubated for 4-16h. Samples were removed at the indicated time points for FACS analysis.

affinity-purified polyclonal antibody to the DHFR region of the degron as this provides multiple epitopes and in turn, produces a stronger signal on immunoblots. After using such an affinity-purified rabbit polyclonal anti-DHFR antibody (kindly donated by Karim Labib) the depletion status of each Orc1-6td fusion protein was visualized (Figure 11). A dramatic drop in protein levels was seen for all ORC subunits, except Orc3td, by at least 2h at the non-permissive temperature of 37°C. Unfortunately, normal endogenous protein levels could not be examined because of the lack of available antibodies specific to the ORC subunits. It should also be stipulated that several attempts to synchronize these ORC degron strains in late G1 with α-factor proved unsuccessful (data not shown) and consequently, all future work was conducted using the *GAL1-ORC6* strain.

**Pre-RC reconstitution following Orc6 depletion and resynthesis**

Now that it was established that Orc6, as opposed to the entire ORC complex, plays an essential function in the maintenance of MCM proteins at replication origins (see above), it was intriguing to determine if the effect of its depletion was reversible, in so far as the ability of the pre-RC to reassemble once being dismantled or destabilized, and the ability for initiation of DNA replication to be restored subsequent to resynthesis of Orc6. This was investigated by initially synchronizing cultures of *GAL1-ORC6* and the isogenic wt counterpart growing in YPG/R in late G1 phase by adding α-factor. Following this arrest, cells were transferred to YPD containing α-factor to deplete Orc6 in the *GAL1-ORC6* strain. Subsequently, cells were released from the pheromone block into either YPD to continue depletion or YPG/R to resynthesize Orc6 in the *GAL1-ORC6* strain. Persistence of 1C DNA content was observed for the Orc6-depleted *GAL1-ORC6* strain maintained in YPD for a further 60min following release indicating Orc6 was required for DNA replication initiation. In contrast, DNA content between 1C and 2C was demonstrated after resynthesizing Orc6 for 60min following its depletion in late G1 (Fig. 12, compare GLU and GAL 60min). It was also observed that the wt strain

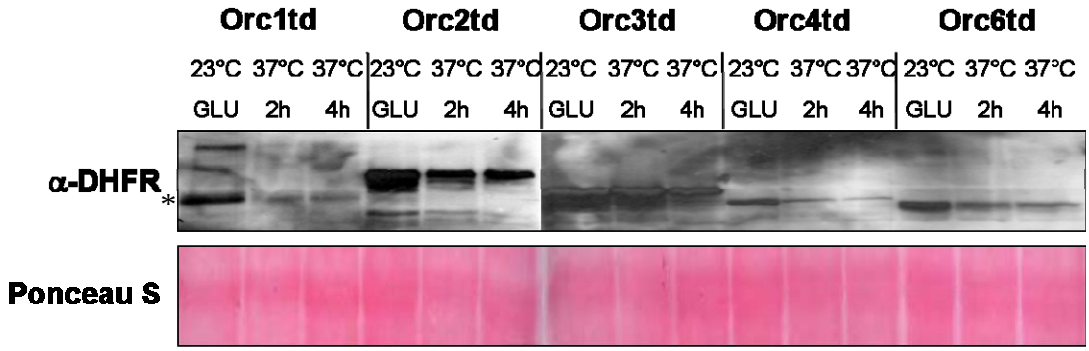



**Figure 11**. ORC subunit degron fusion protein depletion. Asynchronous cultures were treated as described in Figure 10. Sample aliquots were removed prior to (23°C GLU) and following a shift to the restrictive temperature in YPG/R after 2h (37°C 2h) and 4h (37°C 4h). Eighty micrograms of protein was used for immunoblot analysis. Each ORC subunit was detected using an anti-DHFR antibody (1:1000; K. Labib) and an HRP-conjugated rabbit secondary antibody (1:1000; Promega). X-ray film was developed on an AGFA CP1000 developer. The degradation product of Orc1td is indicated by an asterix. Ponceau S staining of the region detected by the blot verifies equal loading of whole-cell extracts.

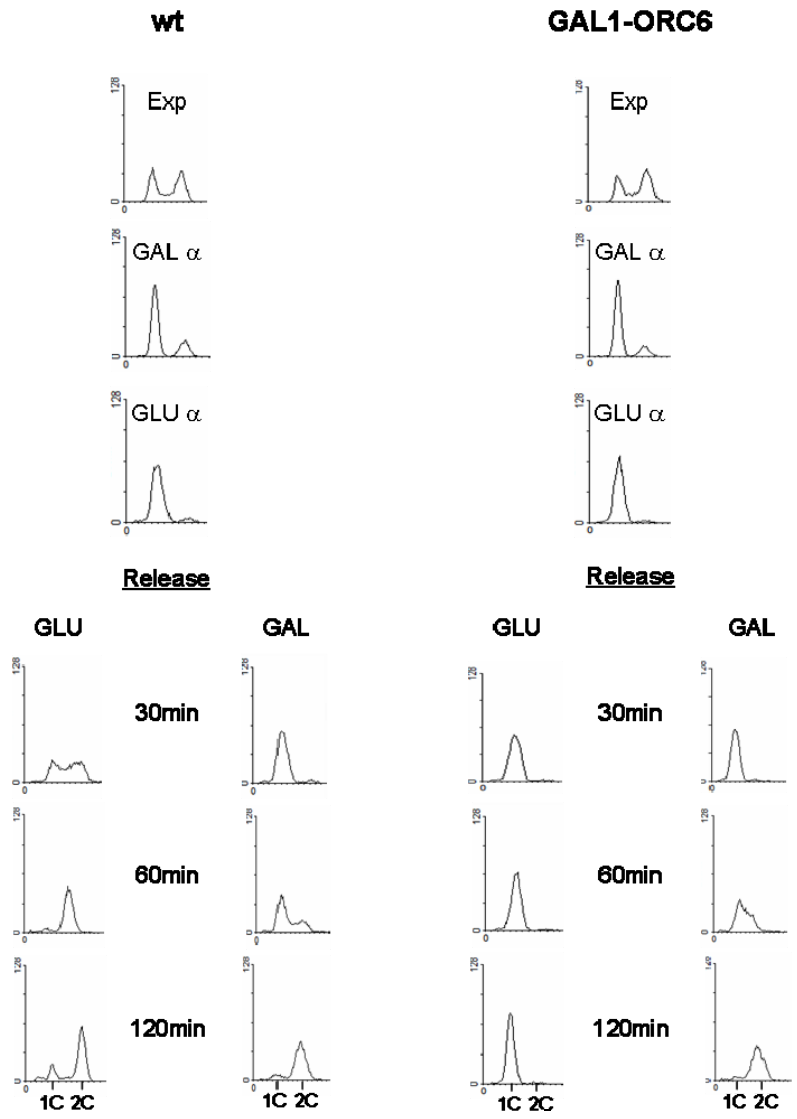



**Figure 12**. MCM destabilization from Orc6 depletion in late G1 is reversible after Orc6 resynthesis. Isogenic wt (DY-93) and *GAL1-ORC6* strains were grown to 5 x $10^6$ cells/ml (Exp) and arrested in late G1 with α-factor (10µg/ml) for 3h in YPG/R (GALα). The cells were then harvested, washed with $ddH_2O$ and resuspended in YPD containing α-factor (10µg/ml) for 5h (GLUα). Cells were subsequently washed and released into either YPD (GLU) or YPG/R (GAL) without α-factor. Samples were taken at the indicated times for FACS analysis.

progressed through the cell cycle at a faster rate in YPD than in YPG/R, but this was most likely a consequence of *S. cerevisiae* having a preference for glucose as its carbon source (Geladé *et al*., 2003). Nevertheless, the resumption of the cell cycle upon the resynthesis of Orc6 suggested that pre-RC components, subsequent to being destabilized, could in fact reassemble and initiate DNA replication.

Since Mcm2-7 are thought to act as the replicative helicase necessary to unwind the DNA duplex, it was important to investigate the dynamics of chromatin association of this complex subsequent to Orc6 resynthesis. Isogenic wt and *GAL1-ORC6* strains were grown as described above during Orc6 resynthesis and sample aliquots were taken for a chromatin binding assay as outlined in the Materials and methods. It was clear that depletion of Orc6 in the *GAL1-ORC6* strain resulted in a destabilization of Mcm2 and Orc2 chromatin association (Fig. 13, compare wt and *GAL1-ORC6*, GLUα), consistent with previous observations (Semple *et al*., 2006). Interestingly, after only 15min of resynthesis, a robust accumulation of chromatin-associated Orc6 was observed and indeed by 30min, this high level of Orc6 coincided with an appreciable increase in the amount of chromatin-associated Mcm2 (Fig. 13, compare *GAL1-ORC6*, GLU and GAL 30min). Finally, it was of value to determine if MCM proteins reassociate specifically with replication origins following Orc6 resynthesis by carrying out ChIP analysis (Fig. 14). Again, wt and *GAL1-ORC6* strains were grown as described above and samples were taken following the initial arrest (GALα), at the end of the YPD/α-factor incubation (GLUα) and during the subsequent release in either YPD (GLU) or YPG/R (GAL) for 1h and 2h. PCR using sample aliquots taken after sonication (INPUT) and following purification of co-immunoprecipitated DNA (IP) was done using primers specific for ARS1 origin sequence, and a non-origin region 9kb upstream of ARS1 (ARS1 -9kb). While there was a clear destabilization of MCM proteins following Orc6 depletion in late G1 (Fig. 14, GLUα), a clear reassociation of MCMs with ARS1 was observed after 1h of Orc6 resynthesis (Fig. 14). While the chromatin fractionation was performed several times, it should be noted that the ChIP data are preliminary. Nevertheless, these

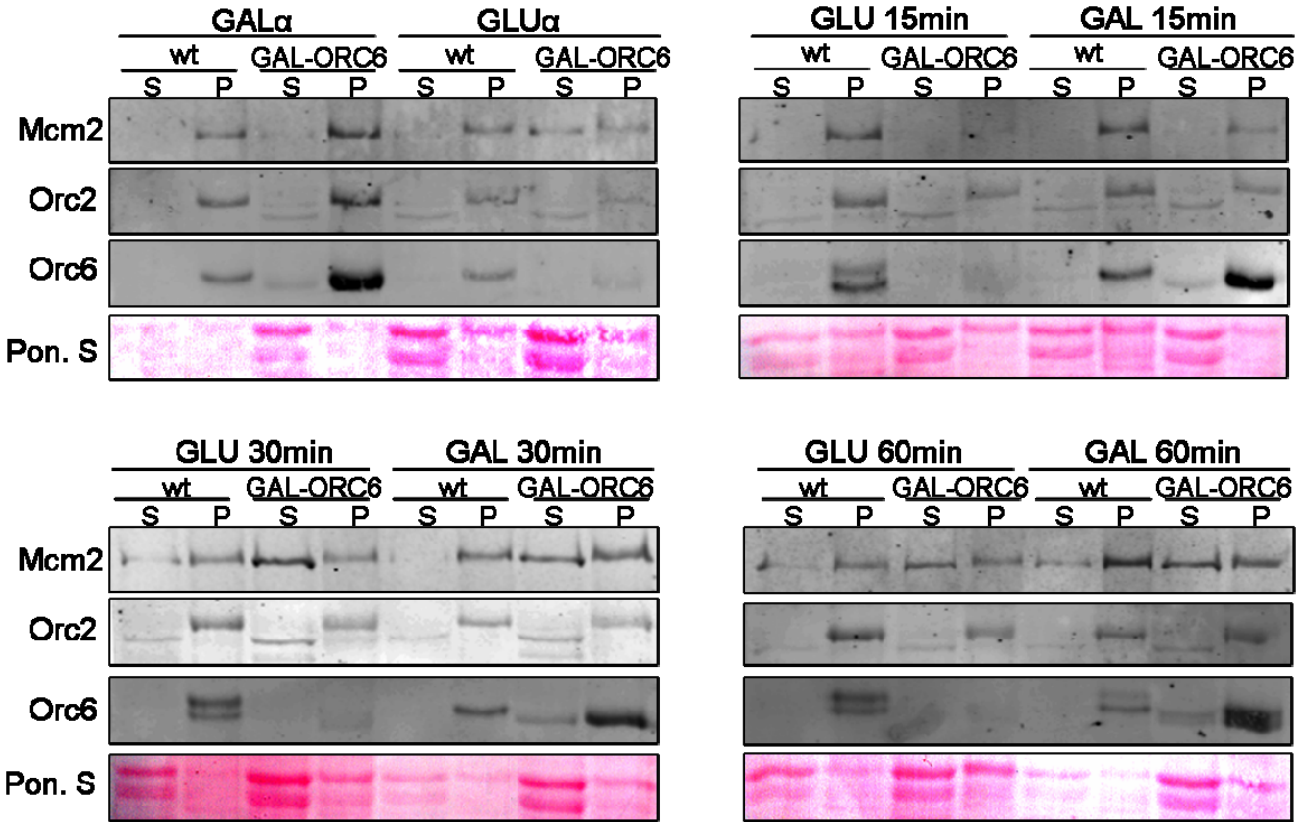



**Figure 13**. The pre-RC can reassemble following Orc6 resynthesis. Isogenic wt (DY-93) and *GAL1-ORC6* (DY-36) strains were grown as described in Figure 12. Samples were removed following the initial arrest (GALα), after Orc6 depletion in late G1 (GLUα) and following the release into either YPD (GLU) or YPG/R (GAL) at the indicated time points and chromatin binding assays were performed as outlined in Figure 7.

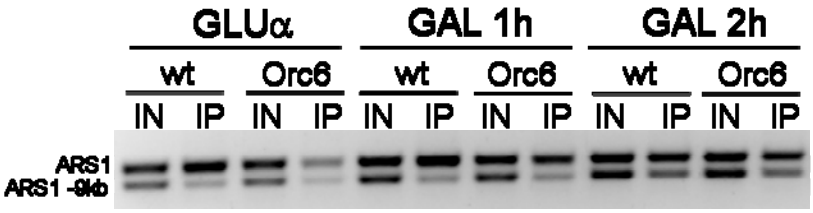



**Figure 14**. Association of MCM proteins at an early-firing replication origin following Orc6 depletion in late G1 and subsequent resynthesis. Isogenic wt (DY-93) and *GAL1-ORC6* (DY-36) strains were grown as described in Figure 12. ChIP was performed on the culture aliquots indicated as described in Materials and methods. PCR analysis of ARS1 was carried out as described in Figure 9.

results bolster the notion that MCM proteins, subsequent to their destabilization in late G1, are able to reassociate at origins and initiate DNA replication.

**Role of other pre-RC components in cell cycle progression**

Since it was previously reported that in *Xenopus* egg extracts (Hua and Newport, 1998; Rowles *et al*., 1999) and through *in vitro* studies of budding yeast late G1 chromatin (Donovan *et al*., 1997) and pre-RCs assembled on origin-coated beads (Bowers *et al*., 2004) that MCM proteins remain while ORC proteins and Cdc6 are removed after high salt treatment, it was of interest to determine if Cdc6 is indeed dispensable for DNA replication once MCMs have been loaded onto chromatin. In addition, Cdt1 was examined in this capacity since it is also a member of the pre-RC and is responsible for the nuclear import of the MCM complex (Tanaka and Diffley, 2002; Mendez and Stillman, 2003; Randell *et al*., 2006). Due to the fact that both Cdc6 and Cdt1 are essential proteins, the same approach taken to conditionally deplete the ORC subunits was employed. In collaboration with Aron Broom from our lab, glucose-repressible *GAL1-CDC6*, *GAL1-CDT1* strains, and their isogenic HA-tagged wt counterparts were created. Initially, cultures of *GAL1-CDC6*, *GAL1-CDT1* and wt strains were grown in YPG/R medium overnight, washed with $ddH_2O$ and resuspended in YPD medium. Following growth in YPD, the levels of Cdc6 and Cdt1 had both fallen below normal endogenous levels by at least 1h and 2h, respectively, as judged by immunodetection of whole-cell extracts (Fig. 15A). The *GAL1-CDC6* cells clearly showed defects in S phase progression, with an accumulation of a prominent 1C DNA content after only 1h in YPD, relative to the wt control (Fig. 15B). In contrast, the *GAL1-CDT1* cells did not show any defect in S phase progression until 8h subsequent to a shift to YPD, as compared to the wt control (Fig. 15B).

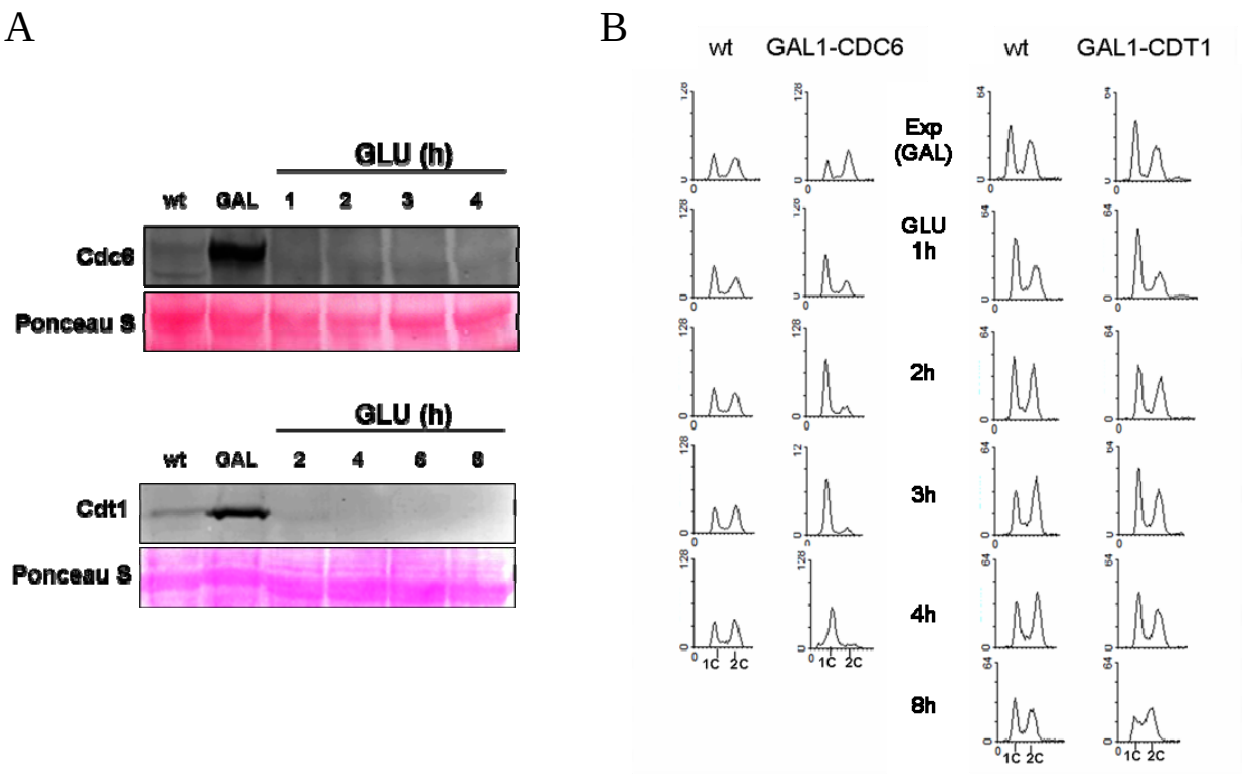



**Figure 15**. Depletion of pre-RC components in yeast cultures. (**A**) Asynchronous cultures of *GAL1-CDC6* (DY-139), *GAL1-CDT1* (DY-140) and their wt counterparts DY-142 and DY-143, respectively, were grown to $10^6$ cells/ml, washed and resuspended in YPD. Whole-cell extracts were prepared from culture aliquots taken prior to (GAL) and at the indicated time points following resuspension in YPD (GLU). Eighty micrograms of protein was used for immunoblot analysis. HA-tagged Cdc6 and HA-tagged Cdt1 were detected using an anti-HA antibody (Sigma) and a fluorescent secondary antibody (Invitrogen). Ponceau S staining of the region detected by the blot to judge loading of whole-cell extracts is also shown. (**B**) FACS analysis of culture aliquots as described in (A).

To further determine whether or not Cdc6 and/or Cdt1 are dispensable following MCM loading, cultures of *GAL1-CDC6*, *GAL1-CDT1* and wt strains growing in YPG/R were synchronized in late G1 with α-factor. The cells were then transferred to YPD containing α-factor to deplete Cdc6 and Cdt1 in the *GAL1-CDC6* and *GAL1-CDT1* strains, respectively. Reminiscent of what was seen when asynchronous cultures were shifted to YPD, an accumulation of 1C cells was observed for the Cdc6-depleted *GAL1-CDC6* culture by 60min, while the isogenic wt strain displayed a prominent 2C peak at the same time point, consistent with a role for Cdc6 in S phase progression (Fig. 16). In contrast, the depletion of Cdt1 in late G1 did not affect S phase progression as illustrated by a prominent 2C peak 60min following release from the pheromone block (Fig. 16). In addition, chromatin fractionation demonstrated a clear displacement of Mcm2 from the pellet to the supernatant fraction following Cdc6 depletion in the *GAL1-CDC6* strain (Fig. 17A, GLU). Densitometry analysis indicated there was close to a 70% reduction in the Mcm2 pellet:supernatant ratio in the *GAL1-CDC6* strain relative to the isogenic wt strain (Fig. 17B). Intriguingly, no effect in Orc2 levels was observed after Cdc6 depletion. Consistent with these findings, preliminary data shows that MCM proteins were indeed displaced specifically from replication origins following late G1 depletion of Cdc6 (Fig. 18). Also of note was a 50% increase in the enrichment of MCMs at ARS1 from the overexpression of Cdc6 relative to the wt control (Fig. 18B, GAL).

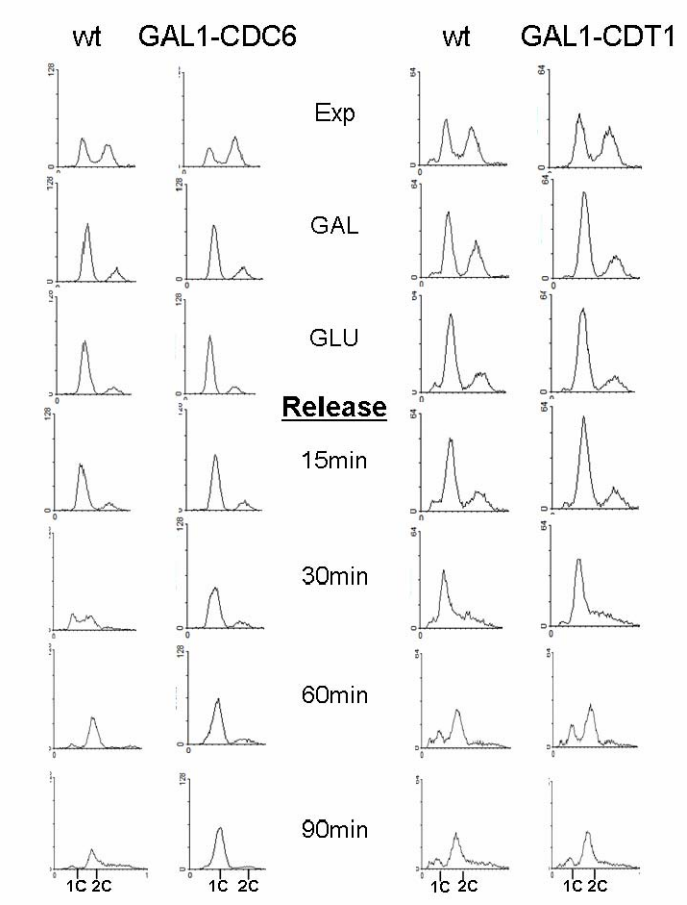



**Figure 16**. Cdc6 is required for entry into S phase. Cultures of *GAL1-CDC6* (DY-139), *GAL1-CDT1* (DY-140) and their wt counterparts DY-142 and DY-143, respectively, were grown to 5 x $10^6$ cells/ml (Exp) and arrested in late G1 with α-factor (5µg/ml) for 2.5h in YPG/R (GAL). The cells were then pelleted, washed with $ddH_2O$ and transferred to YPD containing α-factor (5µg/ml) for 2h (GLU). Cells were subsequently washed and released into YPD without α-factor. Culture aliquots were taken at the indicated time points for FACS analysis.

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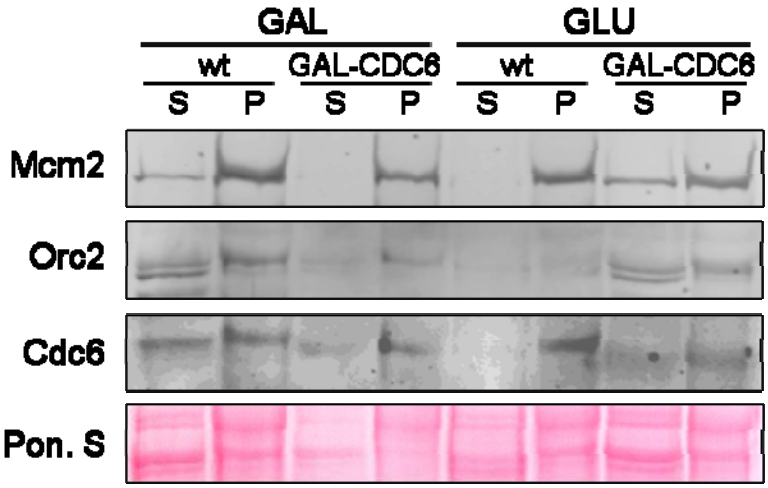


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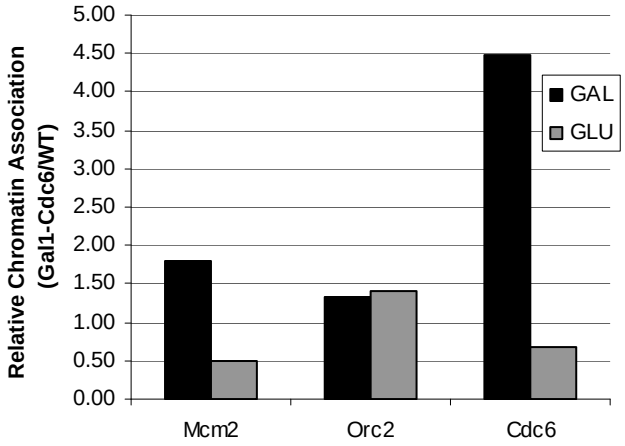


**Figure 17**. Depletion of Cdc6 in late G1 destabilizes MCM association with chromatin. (**A**) Isogenic wt (DY-142) and *GAL1-CDC6* (DY-139) cultures were grown to 1 x $10^7$ cells/ml and arrested in late G1 with α-factor (5μg/ml) for 2.5h in YPG/R (GAL); representative of all trials. The cells were then harvested, washed with $ddH_2O$ and transferred to YPD containing α-factor (5μg/ml) for 2h (GLU). Culture aliquots were taken at the indicated times and chromatin fractionation was performed as outlined in Figure 7. (**B**) Relative chromatin association of pre-RC components following the depletion of Cdc6 as determined by densitometry analysis of (A); average of three trials.

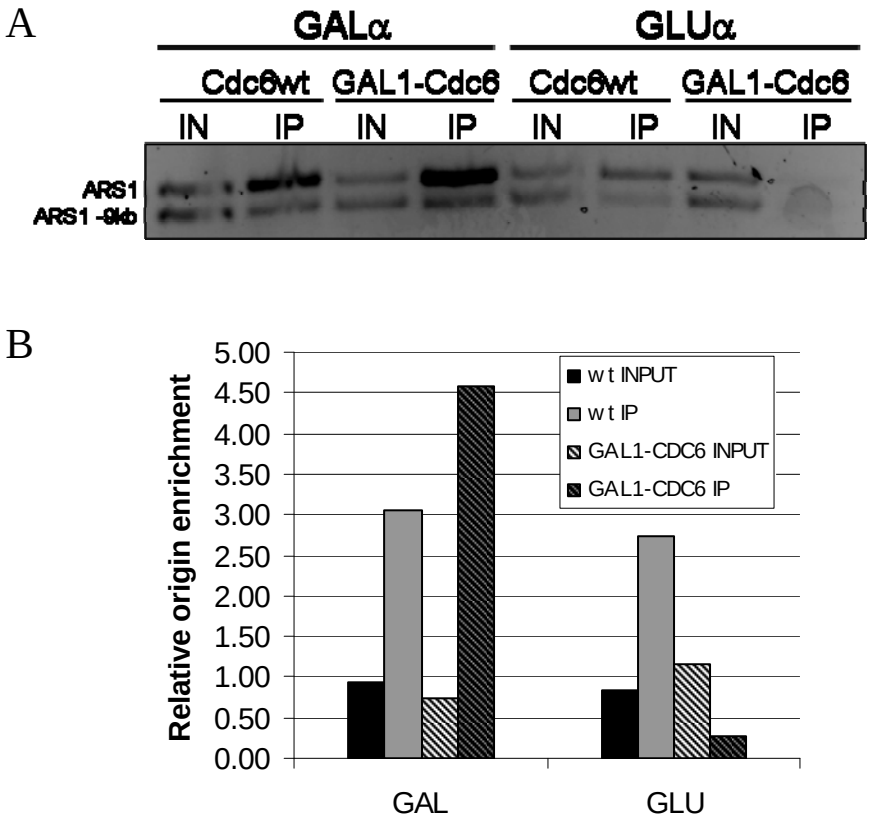


**Figure 18**. MCM proteins are displaced from replication origins following Cdc6 depletion in late G1. (**A**) Isogenic wt (DY-142) and *GAL1-CDC6* (DY-139) strains were grown in YPG/R to 5 x $10^6$ cells/ml and arrested in late G1 with α-factor (10μg/ml) for 2.5h (GAL). The cells were then pelleted, washed with dd$H_2O$ and transferred to YPD containing α-factor (10μg/ml) for 3h (GLU). ChIP was performed with a mixture of Mcm2 and Mcm5 antibodies as described in Materials and methods. PCR using sample aliquots taken after sonication (INPUT) and following purification of co-immunoprecipitated DNA (IP) was carried out using primers specific for the early-firing ARS1 origin sequence, and a region 9kb upstream of ARS1 (ARS1 -9kb). (**B**) Relative ARS1 origin enrichment over the non-origin region (ARS1 -9kb) following Cdc6 depletion in late G1 as determined by densitometry analysis of (A).

**Role of Orc6 in MCM maintenance during S phase**

Data in our lab has shown that when Orc6 is depleted in the presence of the ribonucleotide reductase inhibitor hydroxurea (HU), which arrests cells in mid-S phase by depleting dNTP pools, and subsequently released from this block, *GAL1-ORC6* cells are unable to progress beyond a 2C peak, even after 3h, relative to an isogenic wt control (S. Cutting, pers. comm.). This could suggest a mitotic and/or cytokinetic defect such that cells are unable to partition the replicated DNA and/or split into individual mother and daughter cells. However, it is unlikely that Orc6 has an essential role in mitosis or cytokinesis since cells progress through these stages after Orc6 depletion at the G2/M boundary with the microtubule polymerization inhibitor nocodazole (Semple *et al*., 2006). On the other hand, despite the presence of a 2C peak, defects in late-origin firing may prevent complete genome duplication, consequently triggering a checkpoint that prevents progression through mitosis. It is proposed that the phosphorylation of chromatin-bound Mcm2-7 permits the simultaneous association of Cdc45 and GINS to origins just prior to the point of replication fork migration (Kanemaki *et al*., 2003; Takayama *et al*., 2003). Once DNA replication initiates, MCM proteins, Cdc45 and GINS travel with the replication fork (Aparicio *et al*., 1997; 1999; Kanemaki *et al*., 2003), and this process is thought to be mediated by Mcm10 (Homesley *et al*., 2000; Wohlschlegel *et al*., 2002). Indeed, depletion of Orc6 in late G1 resulted in a marked reduction in chromatin-bound Mcm10 and is consistent with a mechanism whereby Orc6 mediates Mcm10 chromatin association (Semple *et al*., 2006), and Mcm10 in turn stabilizes the MCM complex (Homesley *et al*., 2000). Since it was recently established that Mcm10 is recruited to replication origins in a cell cycle regulated manner (Ricke and Bielinsky, 2004), it is conceivable that via Mcm10, Orc6 may function to stabilize MCM proteins beyond late G1 and into S phase at late-firing origins. In an effort to elucidate this, the chromatin association of Mcm2 following Orc6 depletion in S phase was examined. This was performed by first synchronizing wt and *GAL1-ORC6* strains growing in YPG/R in late G1 by adding α-factor, and then releasing these cultures into YPG/R containing HU to arrest cultures in S phase. The cells were then transferred to YPD again with

HU to deplete Orc6 in the *GAL1-ORC6* strain and this resulted in a modest reduction in the levels of chromatin-associated Mcm2, relative to the wt (Fig. 19). It is noteworthy that while Orc6 levels dropped dramatically, they did not however, drop below normal endogenous levels, even after 6h in YPD/HU. It is conceivable that Orc6 depletion may have a more profound effect on MCM complex association specifically with origins than on bulk chromatin. To investigate if this modest reduction in MCM association coincided with the specific displacement from replication origins, a ChIP assay was performed. Preliminary results showed that indeed wt and *GAL1-ORC6* strains were arrested efficiently prior to (GAL+HU) and following Orc6 depletion (GLU+HU) in S phase, as illustrated by DNA content analysis (Fig. 20A). It should be noted that cultures were initially arrested with HU for a total of 90min to ensure an efficient S phase arrest prior to incubation in YPD/HU. ChIP analysis of wt samples demonstrated a gradual reduction in early-firing ARS1-specific association, consistent with early-origin firing in the presence of HU. In contrast, the *GAL1-ORC6* strain showed a robust ARS1-specific MCM association in the initial arrest, with a greater loss in the enrichment of MCM association at ARS1 after Orc6 had been depleted (Fig. 20B). An appreciable MCM association at the late-firing origin ARS603 remained prior to and following Orc6 depletion. MCM association at another late-firing origin, ARS609, was inconclusive and collectively, interpretation of these results is difficult, particularly in light of the inability to deplete Orc6 below endogenous levels during S phase.

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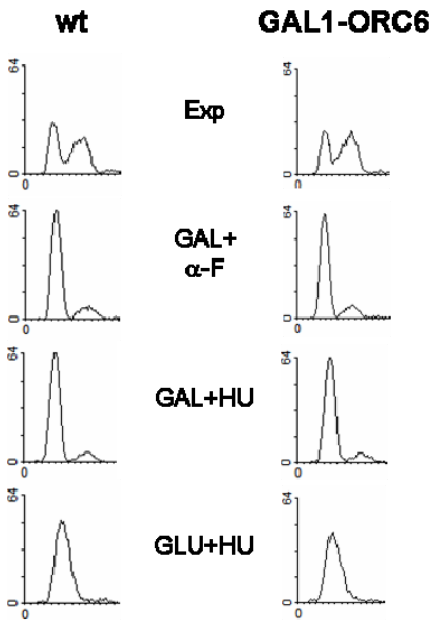


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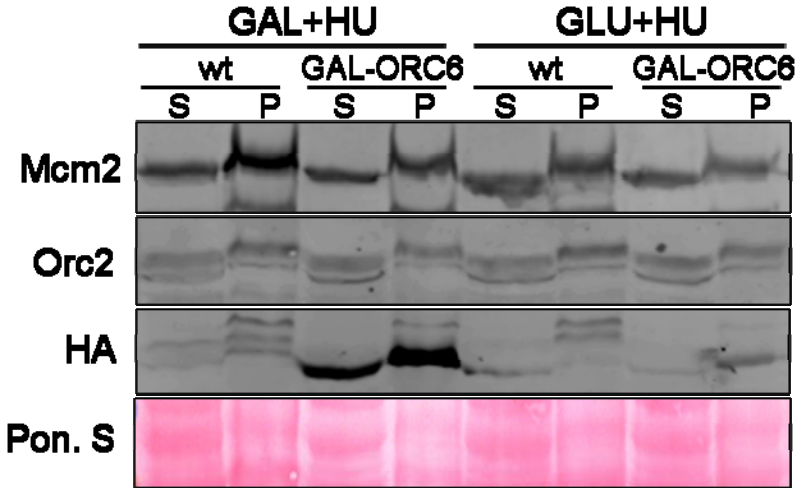


**Figure 19**. Chromatin association of pre-RC components following Orc6 depletion in S phase. (**A**) Isogenic wt (DY-93) and *GAL1-ORC6* (DY-36) strains were grown to 1 x $10^7$ cells/ml and arrested in late G1 with α-factor (20μg/ml) for 3h in YPG/R. The cells were then harvested, washed with $ddH_2O$ and then arrested in S phase with HU (0.4M) for 45min in YPG/R. Cells were subsequently washed and transferred to YPD with HU (0.4M) for 6h. Samples were collected for FACS analysis at the indicated time points. (**B**) Cultures were grown as described for (A). Chromatin fractionation and immunoblotting was performed as described in Materials and methods, with samples taken following the initial arrest in S phase (GAL+HU) and at the end of the YPD/HU incubation (GLU+HU).

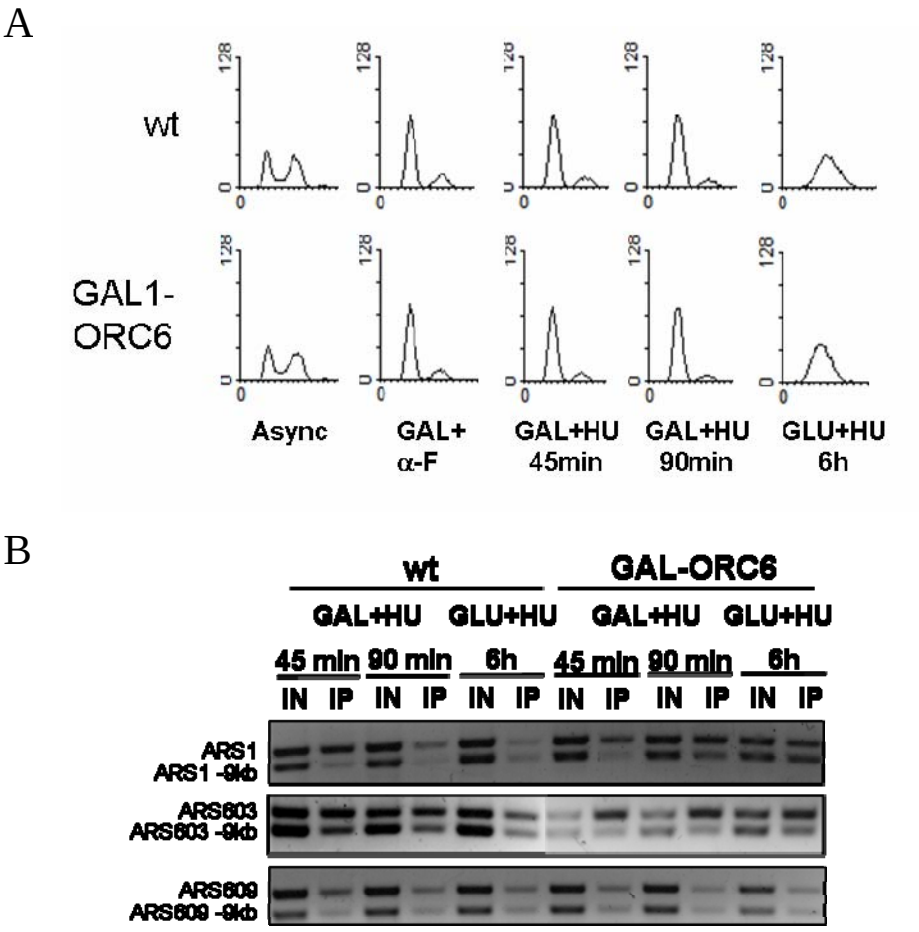


**Figure 20**. Origin association of MCM proteins following Orc6 depletion in S phase. (**A**) wt (DY-93) and *GAL1-ORC6* (DY-36) strains were grown to 5 x $10^6$ cells/ml and arrested in late G1 with α-factor (10μg/ml) for 3h in YPG/R. The cells were then harvested, washed with dd$H_2O$ and then arrested in S phase with HU (0.4M) for 45min and 90min in YPG/R. Cells were subsequently washed and transferred to YPD with HU (0.4M) for 6h. (**B**) Samples were collected at the indicated time points and ChIP was performed with a mixture of Mcm2 and Mcm5 antibodies as described in Materials and methods. PCR using sample aliquots taken after sonication (INPUT) and following purification of co-immunoprecipitated DNA (IP) was carried out using primers specific for the early-firing ARS1 origin sequence, and a non-origin region 9kb upstream of ARS1 (ARS1 -9kb); and two late-firing origin sequences: ARS603, and a non-origin region 9kb upstream of ARS603 (ARS603 -9kb); and ARS609, and a non-origin region 9kb upstream of ARS609 (ARS609 -9kb).

## DISCUSSION

It is well established that ORC plays a vital role in the initiation of DNA replication (reviewed in Bell, 2002) and ORC complexes lacking Orc6 are fully capable of binding replication origins, but the absence of any other subunit abrogates this ability (Lee and Bell, 1997). Nevertheless, all six ORC subunits are essential for cell viability (Li and Herskowitz, 1993). Interestingly, work presented in this thesis suggests an essential role for Orc6 in the efficient initiation of DNA replication. In this case, Orc6 is required for the maintenance of MCM proteins in late G1, which in turn, are necessary for origin unwinding. In contrast, cells depleted of Orc4 following MCM loading were proficient in DNA replication (Fig. 7). This is corroborated by the original work of Li and Herskowitz (1993) who found that subsequent to sporulation and tetrad dissection of *ORC6* +/- heterozygotes, haploid *ORC6*- spores undergo up to two rounds of cell division, and arrest with the large bud morphology typical of mutations affecting DNA replication. This raises the question as to the mechanism by which Orc6 depletion inhibits DNA replication. One possibility is that the absence of Orc6 invokes a G1/S checkpoint by way of stalled replication forks. The lack of checkpoint kinase Rad53 hyperphosphorylation following Orc6 depletion, however, argues against this (Fig. 8). The most likely scenario was that Orc6 depletion inhibits proper pre-RC formation, as evidenced by the displacement of MCM proteins from chromatin (Semple *et al*., 2006). The previous dogma of pre-RC assembly and function stipulated that once the MCM complex has been loaded onto chromatin, ORC is dispensable for DNA replication. This originated from studies whereby the high salt treatment of either budding yeast late G1 chromatin or pre-RCs assembled on origin-coated magnetic beads removed ORC, yet MCM proteins remained (Donovan *et al*., 1997; Bowers *et al*., 2004). Interestingly in each case, there was a reduction in the level of associated MCM proteins, but the effect of ORC removal on DNA replication was not monitored. Similarly, there was a marked displacement of previously loaded MCM proteins (Mcm2/5) from replication origins by depleting Orc6 during late G1 (Fig. 9; Da-Silva and Duncker, 2007). Further data from our lab showed that Orc6 depletion in late G1 significantly reduced

the number of origins that fired (Semple *et al*., 2006). Together, these data suggest a role for Orc6 in maintaining origin association of Mcm2-7 after they have associated with the pre-RC, a stage at which origins are poised, or "licensed", for DNA replication (Bell and Dutta, 2002). In support of this, Chen and colleagues have recently shown that Orc6 function is required for pre-RC assembly and maintenance at all origins (Chen *et al*., 2007), forcing a re-evaluation of their previous model (Bowers *et al*., 2004). In this study, late G1 depletion of Orc6 hindered progression through S phase. Interestingly, Orc6 depletion did not affect the levels of other ORC subunits or Mcm2-7 proteins (Chen *et al*., 2007). In addition, Orc6 was shown to be required for origin association of MCM proteins prior to pre-RC assembly. Importantly, Orc6 depletion after pre-RC assembly in late G1 caused a loss of Mcm2-7 association from all sites across the genome (Chen *et al*., 2007).

Possible mechanisms for how Orc6 mediates Mcm2-7 chromatin association emerge from our previous yeast two-hybrid data showing an interaction between Orc6 and Mcm10 (Semple *et al*., 2006). Interestingly, the late G1 depletion of Orc6 not only resulted in a dramatic drop in Mcm2 chromatin association, but also resulted in a 50% reduction in the chromatin pellet to supernatant ratio of Mcm10 (Semple *et al*., 2006). Consistent with this, Mcm10 is required for the maintenance of at least Mcm2 with chromatin (Homesley *et al*., 2000). This is the favoured mechanism based on experimental results and is depicted in Figure 21. Under this model, depletion of Orc6 also abrogates the chromatin-association of Orc2 (Semple *et al*., 2006). However, alternative mechanisms are possible. Mcm10 has also been shown to mediate the chromatin association of DNA polymerase $\alpha$ (Ricke and Bielinsky, 2004), providing the possibility that Orc6, via Mcm10, mediates the proper assembly of the polymerase machinery. Third, a general destabilization of ORC may be the cause for MCM displacement as Orc6 depletion in late G1 also coincided with a marked reduction in the chromatin association of Orc2 (Semple *et al*., 2006). This seems unlikely in light of the fact that depletion of an Orc6 degron fusion protein did not affect other ORC subunits (Chen *et al*., 2007). A fourth possibility is that the combined displacement of Orc2 and Mcm10 contributes to Mcm2 dissociation from chromatin. However, data

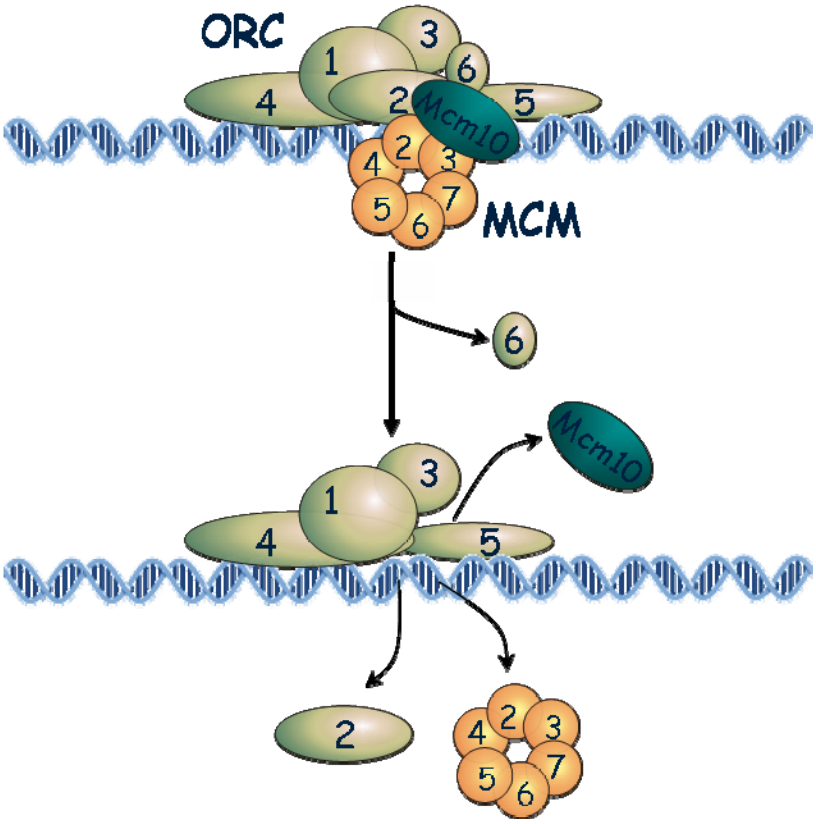


**Figure 21**. Model of pre-RC destabilization following Orc6 depletion in late G1. During unperturbed conditions, Orc6 is required for the chromatin-association of Mcm10, which in turn, is required for the maintenance of at least Mcm2 on chromatin. The absence of Orc6 during late G1 causes the release of Mcm10 from chromatin, which in turn, leads to the dissociation of the MCM complex from chromatin. Orc6 depletion also abrogates the chromatin association of Orc2.

on the depletion of Orc2 in late G1 have been inconsistent. S phase was inhibited when an *orc2-1* strain was shifted to restrictive temperature in late G1 (Zhang *et al*., 2002). Shimada and colleagues (2002) reported that the late G1 depletion of Orc2 at permissive temperature using a *GAL1-orc2-1* strain had little effect on S phase progression, yet another study using the same strain found that Orc2 depletion resulted in less efficient DNA replication (Weinberger *et al*., 2005). However, we were the first to report an interaction between Orc6 and Cdt1 (Semple *et al*., 2006), the protein required for nuclear import of the MCM complex. In concert with this, i*n vitro* studies showed that Orc6 depletion inhibited the initial association of both Cdt1 and Mcm2-7 (Chen *et al*., 2007). In addition, Orc6 contains at least two domains capable of binding Cdt1 and both of these domains are essential for Orc6 function *in vivo*. Recruitment of Cdt1 during pre-RC formation is not solely accomplished by Orc6, as it was established that Cdc6 is also required (Chen *et al*., 2007). This is consistent with previous results demonstrating that stable Cdt1 association with origins is dependent upon the origin association of ATP-bound Cdc6 (Randell *et al*., 2006).

The maintenance role of Orc6 provoked an intriguing question: after being destabilized by Orc6 depletion, is the pre-RC capable of re-forming and initiating DNA replication following Orc6 resynthesis? Upon resuming Orc6 expression, the 2C DNA content of cells grown in GAL for 2h after Orc6 had been depleted demonstrated that pre-RC components could in fact reassemble and initiate DNA replication (Fig. 12). More striking was the robust accumulation of chromatin-associated Orc6, concomitant with a marked increase in the level of chromatin-associated Mcm2 after 30min of Orc6 resynthesis (Fig. 13). Further, preliminary data suggest that MCM proteins specifically reassociate with the early-firing replication origin ARS1 (Fig. 14). These data illustrate that upon being displaced, MCM proteins are fully capable of reassembling and initiating DNA replication following the resynthesis of Orc6. More importantly, however, MCM protein loading can occur beyond the normal late M or early G1 cell cycle window in which pre-RC assembly occurs. In *S. cerevisiae*, there are three ways in which CDK activity inhibits replication. Both the transcript and protein levels of Cdc6

are high in late M and early G1, but low in S, G2 and early M phases (Piatti *et al*., 1995; Drury *et al*., 2000). Secondly, phosphorylation of MCM proteins leads to their exclusion from the nucleus (Labib *et al*., 1999; Nguyen *et al*., 2000). Finally, the phosphorylation of Orc2 and Orc6 also helps to prevent promiscuous replication (Nguyen *et al*., 2001; Weinreich *et al*., 2001). Interestingly, a doublet was observed in the chromatin pellet fraction for wt Orc6 after being released from late G1 into the preferred YPD medium (Fig. 13; wt, GLU 30 and 60), a time in which cells showed an accumulation between 1C and 2C DNA content, indicating some DNA synthesis had occurred (Fig. 12; wt, GLU 30). This doublet, however, was not evident during the late G1 block with α-factor (Fig. 12; wt, GALα and GLUα). Further, the *GAL1-ORC6* strain clearly showed an accumulation of cells between 1C and 2C DNA content after 60min of Orc6 resynthesis (Fig. 12; *GAL1-ORC6*, GAL 60), concomitant with the appearance of an Orc6 doublet at this time in the chromatin pellet (Fig. 13; *GAL1-ORC6*, GAL 60). Indeed, a recent report demonstrated an interaction between Clb5 and Orc6 at origins of replication, only after initiation of DNA replication, and that this interaction is maintained during S phase and into M phase (Wilmes *et al*., 2004). It is therefore proposed that the higher mobility state observed during S phase (ie. during release from α-factor) represents the phosphorylated form of Orc6. This is consistent with a recent study demonstrating that Orc6 alternated between a phosphorylated state during S and G2/M, and a non-phosphorylated form during G1 (Chen *et al*., 2007).

The intriguing observation that Cdc6 functions in the maintenance of Mcm2-7 at origins demonstrates the mutual, yet essential role both Orc6 and Cdc6 play in the initiation of DNA replication. This too is in contrast to previous models suggesting that once MCM proteins have been loaded onto chromatin, ORC and Cdc6 are dispensable for DNA replication (Donovan *et al*., 1997; Bowers *et al*., 2004). Similar to what is seen with Orc6 turnover, the depletion of Cdc6 in late G1 led to a marked reduction in chromatin-associated Mcm2 (Fig. 17) and the displacement of MCMs from the early-firing origin ARS1 (Fig. 18). However, in contrast to the dramatic drop in Orc2 chromatin

association as a result of Orc6 depletion in late G1, the chromatin association of Orc2 was not affected by Cdc6 turnover (Fig. 17). ORC promotes the origin association of Cdc6, and in turn, Cdc6 mediates the formation of pre-RCs (Cocker *et al*., 1996; Seki and Diffley, 2000) and is essential for the initiation of DNA replication (Bueno and Russell, 1992; Liang *et al*., 1995; Piatti *et al*., 1995), particularly facilitating the chromatin association of Mcm2-7 (reviewed in Bell and Dutta, 2002). More recently, it was reported that ATP hydrolysis by Cdc6 facilitates the loading of MCM proteins (Randell *et al*., 2006). These and the present data raise the possibility that Cdc6 is the limiting factor for MCM maintenance, such that the observed displacement of MCMs from chromatin in the absence of Orc6 may have been mediated by the displacement of Cdc6 from chromatin. Previous data from our lab, however, did not show a two-hybrid interaction between Cdc6 and Orc6, but rather we observed a strong interaction between Cdc6 and Orc1 (Semple *et al*., 2006). It is also feasible that since Cdc6 interacts with MCM proteins (Tanaka *et al*., 1997; Jang *et al*., 2001) and is required for their efficient association with chromatin (reviewed in Bell and Dutta, 2002), Cdc6 is directly involved in MCM maintenance, distinct from the mechanism by which Orc6 regulates MCM chromatin association. However, it would be interesting to determine if depletion of Orc6 is also coincident with a reduction in the chromatin association of Cdc6 in late G1, and vice versa. It would, however, seem more likely that Orc6 depletion would affect Cdc6 association, rather than the reciprocal effect, since the association of Cdc6 with chromatin is downstream to that of Orc6. Interestingly, Orc6 was shown to be required for recruiting Cdt1 to origins, which is in turn, required for the nuclear import of the MCM complex. This was also found to be dependent on Cdc6 function (Chen *et al*., 2007).

The accumulation of cells with 2C DNA content after Orc6 depletion in S phase and subsequent release (S. Cutting, unpublished data) raised the possibility that in addition to a requirement during late G1, Orc6 is also required for pre-RC assembly during S phase at late-firing origins. The modest reduction in the chromatin association of Mcm2 from Orc6 depletion relative to the wt control (Fig. 19) suggests that Orc6 may be required during S phase. However, the appreciable association of MCM

proteins at the late-firing origin ARS603 after Orc6 depletion (Fig. 20B) suggests Orc6 is not required for late-origin firing. It must be noted that this was a preliminary result and should be repeated several more times. It is possible that an intra-S phase checkpoint was triggered as a result of stalled replication forks from Orc6 depletion and somehow a tighter Orc6 association with chromatin is maintained to stabilize pre-RCs at late-firing origins. This seems unlikely, however, given the fact that cells depleted of Orc6 progress through S phase at a comparable rate to the isogenic wt. Regardless, it is evident that a 6h Orc6 depletion regime is insufficient to drop below normal endogenous levels on bulk chromatin (Fig. 19), and therefore to make any conclusions as to the role of Orc6 during S phase, levels must indeed be reduced below those of the wt at origins.

## GENERAL CONCLUSIONS

In concert with our previous published results, the present study is the first to demonstrate an essential role for Orc6, and indeed of any ORC subunit, in facilitating DNA replication by regulating the stability of pre-RCs after they have been assembled at origins of replication. The fact that late G1 phase Orc4 depletion had only a modest effect on the initiation of DNA replication or progression through S phase suggests a scenario whereby different ORC subunits are required to varying degrees for initiation. In support of this, distinct roles for ORC subunits have been documented. For example, only Orc1-5 are required for origin recognition and binding *in vitro,* yet all six subunits are essential for cell viability (Li and Herskowitz, 1993; Lee and Bell, 1997). Evidently, a more comprehensive understanding of the roles of the different ORC subunits in cell cycle progression is necessary. Moreover, this study reveals that not only can MCM proteins reassociate and initiate DNA replication subsequent to their displacement, but more significantly, pre-RC assembly can occur outside of the typical late M or early G1 phases. This observation also extends our knowledge of the cell cycle window, proposed to be late G1 (Piatti *et al.*, 1996), in which CDK levels remain low enough to promote pre-RC assembly. It was previously shown that Mcm4 was displaced from origins in late G1

in a *cdc6-1* temperature sensitive mutant at the restrictive temperature (Aparicio *et al*., 1997). This is consistent with the findings in the present study, yet this is the first to show that MCM displacement, as a consequence of late G1 Cdc6 depletion, inhibits subsequent DNA replication.

It will now be of considerable interest to investigate the role, if any, of Mcm10 in Mcm2-7 maintenance specifically at origins of replication, in the context of the separate depletion of Orc6 and Cdc6. It will also be beneficial to determine if Orc6 depletion during late G1 displaces Cdc6 from chromatin, and vice versa. Finally, the role of Orc6 in the maintenance of MCMs at late-firing origins should be investigated further, such as by ChIP analysis and in particular, late-origin firing as demonstrated by 2D DNA gel analysis.

Additionally, it is of importance to consider the role of pre-RC stability for genome integrity. DNA replication is tightly regulated to ensure the genome is replicated once per cell cycle; however, impairment of pre-RC components can deregulate DNA replication initiation such that it can have deleterious effects on the genomic material transferred to daughter cells, ultimately leading to cancer (Karakaidos *et al*., 2004). Replication proteins, such as Cdc6 and Mcm5, are significantly upregulated in cancerous tissues (Williams *et al*., 1998), illustrating the importance of monitoring their expression levels in cancer diagnosis and therapy (reviewed in Lau and Jiang, 2006). Furthermore, overexpression of Cdt1 leads to chromosomal damage in the absence of re-replication, and its deregulation causes genomic instability in normal human cells. In addition, re-replication induced by Cdt1 overexpression is enhanced by the simultaneous overexpression of Cdc6 (Yanow *et al*., 2001). Interestingly, Cdt1 is overexpressed in human cancer cells (Karakaidos *et al*., 2004; Xouri *et al*., 2004), suggesting a new mechanism leading to carcinogenesis (Fujita, 2006). In light of these findings, would pre-RC destabilization and subsequent re-formation affect genome integrity? The absence of >2C DNA content 2h after turning back on Orc6 expression in *GAL1-ORC6* cells following pre-RC destabilization (Fig. 12) demonstrates the genome was replicated efficiently and implies there were no deleterious effects on DNA replication.

In conclusion, and consistent with recent findings (Semple *et al*., 2006; Chen *et al*., 2007), this study has shown strong evidence for a role for Orc6 in MCM maintenance after origin licensing. Furthermore, pre-RC destabilization is reversible such that the pre-RC can be reassembled and initiate DNA replication in late G1 after Orc6 resynthesis. Interestingly, and similar to Orc6, Cdc6 is essential for the maintenance of the MCM complex at origins in late G1.

## REFERENCES


Aladjem MI, Fanning E (2004) The replicon revisited: an old model learns new tricks in metazoan chromosomes. *EMBO Rep* **5:** 686-691.

Alexandru G, Zachariae W, Schleiffer A, Nasmyth K (1999) Sister chromatid separation and chromosome re-duplication are regulated by different mechanisms in response to spindle damage. *EMBO J* **18:** 2707-2721.

Amon A, Irniger S, Nasmyth K (1994) Closing the cell cycle circle in yeast: G2 cyclin proteolysis initiated at mitosis persists until the activation of G1 cyclins in the next cycle. *Cell* **77:** 1037-1050.

Aparicio OM, Weinstein DM, Bell SP (1997) Components and dynamics of DNA replication complexes in *S. cerevisiae*: redistribution of MCM proteins and Cdc45p during S phase. *Cell* **91:** 59-69.

Austin RJ, Orr-Weaver TL, Bell SP (1999) *Drosophila* ORC specifically binds to ACE3, an origin of DNA replication control element. *Genes Dev* **13:** 2639-2649.

Bell SP, Stillman B (1992) ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. *Nature* **357:** 128-134.

Bell SP (1995) Eukaryotic replicators and associated protein complexes. *Curr Opin Genet Dev* **5:** 162-167.

Bell SP (2002) The origin recognition complex: from simple origins to complex functions. *Genes Dev* **16:** 659-672.

Bell SP, Dutta A (2002) DNA replication in eukaryotic cells. *Annu Rev Biochem* **71:** 333-374.

Berger JM, Wang JC (1996) Recent developments in DNA topoisomerase II structure and mechanism. *Curr Opin Struct Biol* **6:** 84-90.

Bi E, Maddox P, Lew DJ, Salmon ED, McMillan JN, Yeh E, Pringle JR (1998) Involvement of an actomyosin contractile ring in *Saccharomyces cerevisiae* cytokinesis. *J Cell Biol* **142:** 1301-1312.

Boeke JD, Trueheart J, Natsoulis G, Fink GR (1987) 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol* **154:** 164-175.

Bolon YT, Bielinsky AK (2006) The spatial arrangement of ORC binding modules determines the functionality of replication origins in budding yeast. *Nucleic Acids Res.*

Bowers JL, Randell JC, Chen S, Bell SP (2004) ATP hydrolysis by ORC catalyzes reiterative Mcm2-7 assembly at a defined origin of replication. *Mol Cell* **16:** 967-978.

Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD (1998) Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14:** 115-132.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248-254.

Bueno A, Russell P (1992) Dual functions of CDC6: a yeast protein required for DNA replication also inhibits nuclear division. *EMBO J* **11:** 2167-2176.

Burke D, Dawson D, and Stearns T (2000) *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual*. New York: Cold Spring Harbor Laboratory Press.

Calzada A, Hodgson B, Kanemaki M, Bueno A, Labib K (2005) Molecular anatomy and regulation of a stable replisome at a paused eukaryotic DNA replication fork. *Genes Dev* **19:** 1905-1919.

Chan CS, Tye BK (1980) Autonomously replicating sequences in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* **77:** 6329-6333.

Chang F, Herskowitz I (1992) Phosphorylation of FAR1 in response to alpha-factor: a possible requirement for cell-cycle arrest. *Mol Biol Cell* **3:** 445-450.

Chen KC, Csikasz-Nagy A, Gyorffy B, Val J, Novak B, Tyson JJ (2000) Kinetic analysis of a molecular model of the budding yeast cell cycle. *Mol Biol Cell* **11:** 369-391.

Chen S, de Vries MA, Bell SP (2007) Orc6 is required for dynamic recruitment of Cdt1 during repeated Mcm2 7 loading. *Genes Dev* **21:** 2897-2907.

Chesnokov I, Remus D, Botchan M (2001) Functional analysis of mutant and wild-type *Drosophila* origin recognition complex. *Proc Natl Acad Sci U S A* **98:** 11997-12002.

Chesnokov IN, Chesnokova ON, Botchan M (2003) A cytokinetic function of *Drosophila* ORC6 protein resides in a domain distinct from its replication activity. *Proc Natl Acad Sci U S A* **100:** 9150-9155.

Chong JP, Mahbubani HM, Khoo CY, Blow JJ (1995) Purification of an MCM-containing complex as a component of the DNA replication licensing system. *Nature* **375:** 418-421.

Chuang RY, Kelly TJ (1999) The fission yeast homologue of Orc4p binds to replication origin DNA via multiple AT-hooks. *Proc Natl Acad Sci U S A* **96:** 2656-2661.

Ciosk R, Zachariae W, Michaelis C, Shevchenko A, Mann M, Nasmyth K (1998) An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast. *Cell* **93:** 1067-1076.

Cocker JH, Piatti S, Santocanale C, Nasmyth K, Diffley JF (1996) An essential role for the Cdc6 protein in forming the pre-replicative complexes of budding yeast. *Nature* **379:** 180-182.

Cohen-Fix O, Peters JM, Kirschner MW, Koshland D (1996) Anaphase initiation in *Saccharomyces cerevisiae* is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p. *Genes Dev* **10:** 3081-3093.

Coleman TR, Carpenter PB, Dunphy WG (1996) The *Xenopus* Cdc6 protein is essential for the initiation of a single round of DNA replication in cell-free extracts. *Cell* **87:** 53-63.

Conradt B, Shaw J, Vida T, Emr S, Wickner W (1992) In vitro reactions of vacuole inheritance in *Saccharomyces cerevisiae*. *J Cell Biol* **119:** 1469-1479.

Da-Silva LF, Duncker BP (2007) ORC function in late G1: maintaining the license for DNA replication. *Cell Cycle* **6:** 128-130.

de la Torre-Ruiz MA, Green CM, Lowndes NF (1998) RAD9 and RAD24 define two additive, interacting branches of the DNA damage checkpoint pathway in budding yeast normally required for Rad53 modification and activation. *EMBO J* **17:** 2687-2698.

DePamphilis ML (2005) Cell cycle dependent regulation of the origin recognition complex. *Cell Cycle* **4:** 70-79.

Devault A, Vallen EA, Yuan T, Green S, Bensimon A, Schwob E (2002) Identification of Tah11/Sid2 as the ortholog of the replication licensing factor Cdt1 in *Saccharomyces cerevisiae*. *Curr Biol* **12:** 689-694.

Dhar SK, Dutta A (2000) Identification and characterization of the human ORC6 homolog. *J Biol Chem* **275:** 34983-34988.

Dickinson JR (1999). The Metabolism and Molecular Physiology of *Saccharomyces cerevisiae*. In Dickinson, JR and Schweizer, M (Eds.), Taylor & Francis Ltd., pp. 1-22.

Dillin A, Rine J (1997) Separable functions of ORC5 in replication initiation and silencing in *Saccharomyces cerevisiae*. *Genetics* **147:** 1053-1062.

Dohmen RJ, Wu P, Varshavsky A (1994) Heat-inducible degron: a method for constructing temperature-sensitive mutants. *Science* **263:** 1273-1276.

Donovan S, Harwood J, Drury LS, Diffley JF (1997) Cdc6p-dependent loading of Mcm proteins onto pre-replicative chromatin in budding yeast. *Proc Natl Acad Sci U S A* **94:** 5611-5616.

Drury LS, Perkins G, Diffley JF (1997) The Cdc4/34/53 pathway targets Cdc6p for proteolysis in budding yeast. *EMBO J* **16:** 5966-5976.

Drury LS, Perkins G, Diffley JF (2000) The cyclin-dependent kinase Cdc28p regulates distinct modes of Cdc6p proteolysis during the budding yeast cell cycle. *Curr Biol* **10:** 231-240.

Dutta A, Bell SP (1997) Initiation of DNA replication in eukaryotic cells. *Annu Rev Cell Dev Biol* **13:** 293-332.

Elsasser S, Lou F, Wang B, Campbell JL, Jong A (1996) Interaction between yeast Cdc6 protein and B-type cyclin/Cdc28 kinases. *Mol Biol Cell* **7:** 1723-1735.

Fiorani P, Bjornsti MA (2000) Mechanisms of DNA topoisomerase I-induced cell killing in the yeast *Saccharomyces cerevisiae*. *Ann N Y Acad Sci* **922:** 65-75.

Forsburg SL (2004) Eukaryotic MCM proteins: beyond replication initiation. *Microbiol Mol Biol Rev* **68:** 109-131.

Foss M, McNally FJ, Laurenson P, Rine J (1993) Origin recognition complex (ORC) in transcriptional silencing and DNA replication in *S. cerevisiae*. *Science* **262:** 1838-1844.

Fox CA, Loo S, Dillin A, Rine J (1995) The origin recognition complex has essential functions in transcriptional silencing and chromosomal replication. *Genes Dev* **9:** 911-924.

Fujita M (2006) Cdt1 revisited: complex and tight regulation during the cell cycle and consequences of deregulation in mammalian cells. *Cell Div* **1:** 22.

Gambus A, Jones RC, Sanchez-Diaz A, Kanemaki M, van DF, Edmondson RD, Labib K (2006) GINS maintains association of Cdc45 with MCM in replisome progression complexes at eukaryotic DNA replication forks. *Nat Cell Biol* **8:** 358-366.

Gari E, Piedrafita L, Aldea M, Herrero E (1997) A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in *Saccharomyces cerevisiae*. *Yeast* **13:** 837-848.

Gauss R, Trautwein M, Sommer T, Spang A (2005) New modules for the repeated internal and N-terminal epitope tagging of genes in *Saccharomyces cerevisiae*. *Yeast* **22:** 1-12.

Gelade R, Van d, V, Van DP, Thevelein JM (2003) Multi-level response of the yeast genome to glucose. *Genome Biol* **4:** 233.

Gibson DG, Bell SP, Aparicio OM (2006) Cell cycle execution point analysis of ORC function and characterization of the checkpoint response to ORC inactivation in *Saccharomyces cerevisiae*. *Genes Cells* **11:** 557-573.

Gilbert DM (2001) Making sense of eukaryotic DNA replication origins. *Science* **294:** 96-100.

Gladfelter AS, Kozubowski L, Zyla TR, Lew DJ (2005) Interplay between septin organization, cell cycle and cell shape in yeast. *J Cell Sci* **118:** 1617-1628.

Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M, Louis EJ, Mewes HW, Murakami Y, Philippsen P, Tettelin H, Oliver SG (1996) Life with 6000 genes. *Science* **274:** 546, 563-546, 567.

Gossen M, Pak DT, Hansen SK, Acharya JK, Botchan MR (1995) A *Drosophila* homolog of the yeast origin recognition complex. *Science* **270:** 1674-1677.

Grandin N, Charbonneau M (2008) Protection against chromosome degradation at the telomeres. *Biochimie* **90:** 41-59.

Guacci V, Koshland D, Strunnikov A (1997) A direct link between sister chromatid cohesion and chromosome condensation revealed through the analysis of MCD1 in S. cerevisiae. *Cell* **91:** 47-57.

Gustin MC, Albertyn J, Alexander M, and and Davenport K (1998) MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. *Microbiology and Molecular Biology Reviews* **62:** 1264-1300.

Guthrie C, Fink, GR Guide to Yeast Genetics and Molecular Biology, *Methods in Enzymology*, Vol. 169, Academic Press, San Diego, 1991.

Hartwell LH. (1974) *Saccharomyces cerevisiae* cell cycle. *Bacteriol.Rev.* **38:** 164-198.

Hartwell LH (1976) Sequential function of gene products relative to DNA synthesis in the yeast cell cycle. *J Mol Biol* **104:** 803-817.

Hartwell LH, Weinert TA (1989) Checkpoints: controls that ensure the order of cell cycle events. *Science* **246:** 629-634.

Harvey KJ, Newport J (2003) Metazoan origin selection: origin recognition complex chromatin binding is regulated by CDC6 recruitment and ATP hydrolysis. *J Biol Chem* **278:** 48524-48528.

Hayden JH, Bowser SS, Rieder CL (1990) Kinetochores capture astral microtubules during chromosome attachment to the mitotic spindle: direct visualization in live newt lung cells. *J Cell Biol* **111:** 1039-1045.

Hereford LM, Hartwell LH (1974) Sequential gene function in the initiation of *Saccharomyces cerevisiae* DNA synthesis. *J Mol Biol* **84:** 445-461.

Herskowitz I (1988) Life cycle of the budding yeast *Saccharomyces cerevisiae*. *Microbiol Rev* **52:** 536-553.

Homesley L, Lei M, Kawasaki Y, Sawyer S, Christensen T, Tye BK (2000) Mcm10 and the MCM2-7 complex interact to initiate DNA synthesis and to release replication factors from origins. *Genes Dev* **14:** 913-926.

Honey S, Futcher B (2007) Roles of the CDK phosphorylation sites of yeast Cdc6 in chromatin binding and rereplication. *Mol Biol Cell* **18:** 1324-1336.

Hsiao CL, Carbon J (1979) High-frequency transformation of yeast by plasmids containing the cloned yeast ARG4 gene. *Proc Natl Acad Sci U S A* **76:** 3829-3833.

Hua XH, Newport J (1998) Identification of a preinitiation step in DNA replication that is independent of origin recognition complex and cdc6, but dependent on cdk2. *J Cell Biol* **140:** 271-281.

Iwase M, Luo J, Nagaraj S, Longtine M, Kim HB, Haarer BK, Caruso C, Tong Z, Pringle JR, Bi E (2006) Role of a Cdc42p Effector Pathway in Recruitment of the Yeast Septins to the Presumptive Bud Site. *Mol Biol Cell*. **17:** 1110-1125

Jackson AL, Pahl PM, Harrison K, Rosamond J, Sclafani RA (1993) Cell cycle regulation of the yeast Cdc7 protein kinase by association with the Dbf4 protein. *Mol Cell Biol* **13:** 2899-2908.

Jacob F, Brenner S, Cuzin F (1963) On the regulation of DNA replication in bacteria. *Cold Spring Harbor Symp Quant Biol* **28:** 329–348.

Jacobson MD, Munoz CX, Knox KS, Williams BE, Lu LL, Cross FR, Vallen EA (2001) Mutations in SID2, a novel gene in *Saccharomyces cerevisiae*, cause synthetic lethality with sic1 deletion and may cause a defect during S phase. *Genetics* **159:** 17-33.

Jang SW, Elsasser S, Campbell JL, Kim J (2001) Identification of Cdc6 protein domains involved in interaction with Mcm2 protein and Cdc4 protein in budding yeast cells. *Biochem J* **354:** 655-661.

Jong A, Young M, Chen GC, Zhang SQ, Chan C (1996) Intracellular location of the *Saccharomyces cerevisiae* CDC6 gene product. *DNA Cell Biol* **15:** 883-895.

Kamimura Y, Masumoto H, Sugino A, Araki H (1998) Sld2, which interacts with Dpb11 in *Saccharomyces cerevisiae*, is required for chromosomal DNA replication. *Mol Cell Biol* **18:** 6102-6109.

Kamimura Y, Tak YS, Sugino A, Araki H (2001) Sld3, which interacts with Cdc45 (Sld4), functions for chromosomal DNA replication in *Saccharomyces cerevisiae*. *EMBO J* **20:** 2097-2107.

Kanemaki M, Sanchez-Diaz A, Gambus A, Labib K (2003) Functional proteomic identification of DNA replication proteins by induced proteolysis in vivo. *Nature* **423:** 720-724.

Kanemaki M, Labib K (2006) Distinct roles for Sld3 and GINS during establishment and progression of eukaryotic DNA replication forks. *EMBO J* **25:** 1753-1763.

Kaplan DL, Davey MJ, O'Donnell M (2003) Mcm4,6,7 uses a "pump in ring" mechanism to unwind DNA by steric exclusion and actively translocate along a duplex. *J Biol Chem* **278:** 49171-49182.

Karakaidos P, Taraviras S, Vassiliou LV, Zacharatos P, Kastrinakis NG, Kougiou D, Kouloukoussa M, Nishitani H, Papavassiliou AG, Lygerou Z, Gorgoulis VG (2004) Overexpression of the replication licensing regulators hCdt1 and hCdc6 characterizes a subset of non-small-cell lung carcinomas: synergistic effect with mutant p53 on tumor growth and chromosomal instability--evidence of E2F-1 transcriptional control over hCdt1. *Am J Pathol* **165:** 1351-1365.

Kawasaki Y, Hiraga S, Sugino A (2000) Interactions between Mcm10p and other replication factors are required for proper initiation and elongation of chromosomal DNA replication in *Saccharomyces cerevisiae*. *Genes Cells* **5:** 975-989.

Kawasaki Y, Kim HD, Kojima A, Seki T, Sugino A (2006) Reconstitution of *Saccharomyces cerevisiae* prereplicative complex assembly in vitro. *Genes Cells* **11:** 745-756.

Kimura H, Takizawa N, Nozaki N, Sugimoto K (1995) Molecular cloning of cDNA encoding mouse Cdc21 and CDC46 homologs and characterization of the products: physical interaction between P1(MCM3) and CDC46 proteins. *Nucleic Acids Res* **23:** 2097-2104.

Klemm RD, Austin RJ, Bell SP (1997) Coordinate binding of ATP and origin DNA regulates the ATPase activity of the origin recognition complex. *Cell* **88:** 493-502.

Kolodner RD, Putnam CD, Myung K (2002) Maintenance of genome stability in *Saccharomyces cerevisiae*. *Science* **297:** 552-557.

Kong D, DePamphilis ML (2002) Site-specific ORC binding, pre-replication complex assembly and DNA synthesis at *Schizosaccharomyces pombe* replication origins. *EMBO J* **21:** 5567-5576.

Koonin EV (1993) A common set of conserved motifs in a vast variety of putative nucleic acid-dependent ATPases including MCM proteins involved in the initiation of eukaryotic DNA replication. *Nucleic Acids Res* **21:** 2541-2547.

Kreitz S, Ritzi M, Baack M, Knippers R (2001) The human origin recognition complex protein 1 dissociates from chromatin during S phase in HeLa cells. *J Biol Chem* **276:** 6337-6342.

Labib K, Diffley JF, Kearsey SE (1999) G1-phase and B-type cyclins exclude the DNA-replication factor Mcm4 from the nucleus. *Nat Cell Biol* **1:** 415-422.

Labib K, Tercero JA, Diffley JF (2000) Uninterrupted MCM2-7 function required for DNA replication fork progression. *Science* **288:** 1643-1647.

Lau E, Jiang W (2006) Is there a pre-RC checkpoint that cancer cells lack? *Cell Cycle* **5:** 1602-1606.

Lee DG, Bell SP (1997) Architecture of the yeast origin recognition complex bound to origins of DNA replication. *Mol Cell Biol* **17:** 7159-7168.

Lee JK, Moon KY, Jiang Y, Hurwitz J (2001) The *Schizosaccharomyces pombe* origin recognition complex interacts with multiple AT-rich regions of the replication origin DNA by means of the AT-hook domains of the spOrc4 protein. *Proc Natl Acad Sci U S A* **98:** 13589-13594.

Lei M, Kawasaki Y, Young MR, Kihara M, Sugino A, Tye BK (1997) Mcm2 is a target of regulation by Cdc7-Dbf4 during the initiation of DNA synthesis. *Genes Dev* **11:** 3365-3374.

Li JJ, Herskowitz I (1993) Isolation of ORC6, a component of the yeast origin recognition complex by a one-hybrid system. *Science* **262:** 1870-1874.

Liang C, Stillman B (1997) Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle in cdc6 mutants. *Genes Dev* **11:** 3375-3386.

Lipford JR, Bell SP (2001) Nucleosomes positioned by ORC facilitate the initiation of DNA replication. *Mol Cell* **7:** 21-30.

Lippincott J , Li R (1998a) Sequential assembly of myosin II, an IQGAP-like protein, and filamentous actin to a ring structure involved in budding yeast cytokinesis. *J Cell Biol* **140:** 355-366.

Lippincott J, Li R (1998b) Dual function of Cyk2, a cdc15/PSTPIP family protein, in regulating actomyosin ring dynamics and septin distribution. *J Cell Biol* **143:** 1947-1960.

Longtine MS, McKenzie A, III, Demarini DJ, Shah NG, Wach A, Brachat A, Philippsen P, Pringle JR (1998) Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast* **14:** 953-961.

Maga G, Villani G, Tillement V, Stucki M, Locatelli GA, Frouin I, Spadari S, Hubscher U (2001) Okazaki fragment processing: modulation of the strand displacement activity of DNA polymerase delta by the concerted action of replication protein A, proliferating cell nuclear antigen, and flap endonuclease-1. *Proc Natl Acad Sci U S A* **98:** 14298-14303.

Maine GT, Sinha P, Tye BK (1984) Mutants of *S. cerevisiae* defective in the maintenance of minichromosomes. *Genetics* **106:** 365-385.

Masumoto H, Muramatsu S, Kamimura Y, Araki H (2002) S-Cdk-dependent phosphorylation of Sld2 essential for chromosomal DNA replication in budding yeast. *Nature* **415:** 651-655.

Mendenhall MD, Hodge AE (1998) Regulation of Cdc28 cyclin-dependent protein kinase activity during the cell cycle of the yeast *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev* **62:** 1191-1243.

Mendez J, Stillman B (2003) Perpetuating the double helix: molecular machines at eukaryotic DNA replication origins. *Bioessays* **25:** 1158-1167.

Michaelis C, Ciosk R, Nasmyth K (1997) Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. *Cell* **91:** 35-45.

Mimura S, Takisawa H (1998) Xenopus Cdc45-dependent loading of DNA polymerase alpha onto chromatin under the control of S-phase Cdk. *EMBO J* **17:** 5699-5707.

Mitchell AP. Control of Meiotic Gene Expression in *Saccharomyces cerevisiae*. Microbiological Reviews , 56-70. 1994.

Mizushima T, Takahashi N, Stillman B (2000) Cdc6p modulates the structure and DNA binding activity of the origin recognition complex in vitro. *Genes Dev* **14:** 1631-1641.

Moon KY, Kong D, Lee JK, Raychaudhuri S, Hurwitz J (1999) Identification and reconstitution of the origin recognition complex from *Schizosaccharomyces pombe*. *Proc Natl Acad Sci U S A* **96:** 12367-12372.

Muzi-Falconi M , Kelly TJ (1995) Orp1, a member of the Cdc18/Cdc6 family of S-phase regulators, is homologous to a component of the origin recognition complex. *Proc Natl Acad Sci U S A* **92:** 12475-12479.

Nasmyth K (1993) Control of the yeast cell cycle by the Cdc28 protein kinase. *Curr Opin Cell Biol* **5:** 166-179.

Nasmyth K (1995) Evolution of the cell cycle. *Philos Trans R Soc Lond B Biol Sci* **349:** 271-281.

Neuwald AF, Aravind L, Spouge JL, Koonin EV (1999) AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res* **9:** 27-43.

Nguyen VQ, Co C, Irie K, Li JJ (2000) Clb/Cdc28 kinases promote nuclear export of the replication initiator proteins Mcm2-7. *Curr Biol* **10:** 195-205.

Nguyen VQ, Co C, Li JJ (2001) Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. *Nature* **411:** 1068-1073.

Nishitani H, Nurse P (1995) p65cdc18 plays a major role controlling the initiation of DNA replication in fission yeast. *Cell* **83:** 397-405.

Pacek M, Walter JC (2004) A requirement for MCM7 and Cdc45 in chromosome unwinding during eukaryotic DNA replication. *EMBO J* **23:** 3667-3676.

Pak DT, Pflumm M, Chesnokov I, Huang DW, Kellum R, Marr J, Romanowski P, Botchan MR (1997) Association of the origin recognition complex with heterochromatin and HP1 in higher eukaryotes. *Cell* **91:** 311-323.

Pasero P, Duncker BP, Gasser SM (1999) *In vitro* DNA replication in yeast nuclear extracts. *Methods* **18:** 368-76, 323.

Piatti S, Lengauer C, Nasmyth K (1995) Cdc6 is an unstable protein whose de novo synthesis in G1 is important for the onset of S phase and for preventing a 'reductional' anaphase in the budding yeast *Saccharomyces cerevisiae*. *EMBO J* **14:** 3788-3799.

Prasanth SG, Prasanth KV, Stillman B (2002) Orc6 involved in DNA replication, chromosome segregation, and cytokinesis. *Science* **297:** 1026-1031.

Queralt E, Igual JC (2005) Functional connection between the Clb5 cyclin, the protein kinase C pathway and the Swi4 transcription factor in *Saccharomyces cerevisiae*. *Genetics* **171:** 1485-1498.

Randell JC, Bowers JL, Rodriguez HK, Bell SP (2006) Sequential ATP hydrolysis by Cdc6 and ORC directs loading of the Mcm2-7 helicase. *Mol Cell* **21:** 29-39.

Remenyi A, Good MC, Bhattacharyya RP, Lim WA (2005) The role of docking interactions in mediating signaling input, output, and discrimination in the yeast MAPK network. *Mol Cell* **20:** 951-962.

Ricke RM, Bielinsky AK (2004) Mcm10 regulates the stability and chromatin association of DNA polymerase-alpha. *Mol Cell* **16:** 173-185.

Ritzi M, Baack M, Musahl C, Romanowski P, Laskey RA, Knippers R (1998) Human minichromosome maintenance proteins and human origin recognition complex 2 protein on chromatin. *J Biol Chem* **273:** 24543-24549.

Robinson NP, Bell SD (2005) Origins of DNA replication in the three domains of life. *FEBS J* **272:** 3757-3766.

Rowles A, Chong JP, Brown L, Howell M, Evan GI, Blow JJ (1996) Interaction between the origin recognition complex and the replication licensing system in *Xenopus*. *Cell* **87:** 287-296.

Rowles A, Tada S, Blow JJ (1999) Changes in association of the *Xenopus* origin recognition complex with chromatin on licensing of replication origins. *J Cell Sci* **112:** 2011-2018.

Rowley A, Cocker JH, Harwood J, Diffley JF (1995) Initiation complex assembly at budding yeast replication origins begins with the recognition of a bipartite sequence by limiting amounts of the initiator, ORC. *EMBO J* **14:** 2631-2641.

Sanchez-Diaz A, Kanemaki M, Marchesi V, Labib K (2004) Rapid depletion of budding yeast proteins by fusion to a heat-inducible degron. *Sci STKE* **2004:** L8.

Sanchez Y, Desany BA, Jones WJ, Liu Q, Wang B, Elledge SJ (1996) Regulation of RAD53 by the ATM-like kinases MEC1 and TEL1 in yeast cell cycle checkpoint pathways. *Science* **271:** 357-360.

Santocanale C, Diffley JF (1996) ORC- and Cdc6-dependent complexes at active and inactive chromosomal replication origins in *Saccharomyces cerevisiae*. *EMBO J* **15:** 6671-6679.

Seki T, Diffley JF (2000) Stepwise assembly of initiation proteins at budding yeast replication origins in vitro. *Proc Natl Acad Sci U S A* **97:** 14115-14120.

Semple JW, Da-Silva LF, Jervis EJ, Ah-Kee J, Al-Attar H, Kummer L, Heikkila JJ, Pasero P, Duncker BP (2006) An essential role for Orc6 in DNA replication through maintenance of pre-replicative complexes. *EMBO J* **25:** 5150-5158.

Sheu YJ, Stillman B (2006) Cdc7-Dbf4 phosphorylates MCM proteins via a docking site-mediated mechanism to promote S phase progression. *Mol Cell* **24:** 101-113.

Shimada K, Pasero P, Gasser SM (2002) ORC and the intra-S-phase checkpoint: a threshold regulates Rad53p activation in S phase. *Genes Dev* **16:** 3236-3252.

Speck C, Chen Z, Li H, Stillman B (2005) ATPase-dependent cooperative binding of ORC and Cdc6 to origin DNA. *Nat Struct Mol Biol* **12:** 965-971.

Stinchcomb DT, Struhl K, Davis RW (1979) Isolation and characterisation of a yeast chromosomal replicator. *Nature* **282:** 39-43.

Takahashi K, Yamada H, Yanagida M (1994) Fission yeast minichromosome loss mutants cause lethal aneuploidy and replication abnormality. *Mol Biol Cell* **5:** 1145-1158.

Takahashi T, Ohara E, Nishitani H, Masukata H (2003) Multiple ORC-binding sites are required for efficient MCM loading and origin firing in fission yeast. *EMBO J* **22:** 964-974.

Takayama Y, Kamimura Y, Okawa M, Muramatsu S, Sugino A, Araki H (2003) GINS, a novel multiprotein complex required for chromosomal DNA replication in budding yeast. *Genes Dev* **17:** 1153-1165.

Takeda DY, Dutta A (2005) DNA replication and progression through S phase. *Oncogene* **24:** 2827-2843.

Tanaka S, Diffley JF (2002) Interdependent nuclear accumulation of budding yeast Cdt1 and Mcm2-7 during G1 phase. *Nat Cell Biol* **4:** 198-207.

Tanaka S, Umemori T, Hirai K, Muramatsu S, Kamimura Y, Araki H (2007) CDK-dependent phosphorylation of Sld2 and Sld3 initiates DNA replication in budding yeast. *Nature* **445:** 328-332.

Tanaka T, Knapp D, Nasmyth K (1997) Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. *Cell* **90:** 649-660.

Tanaka T, Nasmyth K (1998) Association of RPA with chromosomal replication origins requires an Mcm protein, and is regulated by Rad53, and cyclin- and Dbf4-dependent kinases. *EMBO J* **17:** 5182-5191.

Theis JF, Newlon CS (1997) The ARS309 chromosomal replicator of *Saccharomyces cerevisiae* depends on an exceptional ARS consensus sequence. *Proc Natl Acad Sci U S A* **94:** 10786-10791.

Triolo T, Sternglanz R (1996) Role of interactions between the origin recognition complex and SIR1 in transcriptional silencing. *Nature* **381:** 251-253.

Tye BK (1999) MCM proteins in DNA replication. *Annu Rev Biochem* **68:** 649-686.

Umek RM, Kowalski D (1990) The DNA unwinding element in a yeast replication origin functions independently of easily unwound sequences present elsewhere on a plasmid. *Nucleic Acids Res* **18:** 6601-6605.

Varrin AE, Prasad AA, Scholz RP, Ramer MD, Duncker BP (2005) A mutation in Dbf4 motif M impairs interactions with DNA replication factors and confers increased resistance to genotoxic agents. *Mol Cell Biol* **25:** 7494-7504.

Varshavsky A (1997) The N-end rule pathway of protein degradation. *Genes Cells* **2:** 13-28.

Vashee S, Simancek P, Challberg MD, Kelly TJ (2001) Assembly of the human origin recognition complex. *J Biol Chem* **276:** 26666-26673.

Vasquez RJ, Howell B, Yvon AM, Wadsworth P, Cassimeris L (1997) Nanomolar concentrations of nocodazole alter microtubule dynamic instability in vivo and in vitro. *Mol Biol Cell* **8:** 973-985.

Visintin R, Prinz S, Amon A (1997) CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis. *Science* **278:** 460-463.

Walker SS, Francesconi SC, Eisenberg S (1990) A DNA replication enhancer in *Saccharomyces cerevisiae. Proc Natl Acad Sci U S A* **87:** 4665-4669.

Weinberger M, Ramachandran L, Feng L, Sharma K, Sun X, Marchetti M, Huberman JA, Burhans WC (2005) Apoptosis in budding yeast caused by defects in initiation of DNA replication. *J Cell Sci* **118:** 3543-3553.

Weinreich M, Stillman B (1999) Cdc7p-Dbf4p kinase binds to chromatin during S phase and is regulated by both the APC and the RAD53 checkpoint pathway. *EMBO J* **18:** 5334-5346.

Weinreich M, Liang C, Chen HH, Stillman B (2001) Binding of cyclin-dependent kinases to ORC and Cdc6p regulates the chromosome replication cycle. *Proc Natl Acad Sci U S A* **98:** 11211-11217.

Williams GH, Romanowski P, Morris L, Madine M, Mills AD, Stoeber K, Marr J, Laskey RA, Coleman N (1998) Improved cervical smear assessment using antibodies against proteins that regulate DNA replication. *Proc Natl Acad Sci U S A* **95:** 14932-14937.

Wilmes GM, Bell SP (2002) The B2 element of the *Saccharomyces cerevisiae* ARS1 origin of replication requires specific sequences to facilitate pre-RC formation. *Proc Natl Acad Sci U S A* **99:** 101-106.

Wilmes GM, Archambault V, Austin RJ, Jacobson MD, Bell SP, Cross FR (2004) Interaction of the S-phase cyclin Clb5 with an "RXL" docking sequence in the initiator protein Orc6 provides an origin-localized replication control switch. *Genes Dev* **18:** 981-991.

Wohlschlegel JA, Dhar SK, Prokhorova TA, Dutta A, Walter JC (2002) *Xenopus* Mcm10 binds to origins of DNA replication after Mcm2-7 and stimulates origin binding of Cdc45. *Mol Cell* **9:** 233-240.

Wu C, Leberer E, Thomas DY, Whiteway M (1999) Functional characterization of the interaction of Ste50p with Ste11p MAPKKK in *Saccharomyces cerevisiae*. *Mol Biol Cell* **10:** 2425-2440.

Xiao W (2006). *Yeast Protocols.* Humana Press, Totowa, New Jersey.

Xouri G, Lygerou Z, Nishitani H, Pachnis V, Nurse P, Taraviras S (2004) Cdt1 and geminin are down-regulated upon cell cycle exit and are over-expressed in cancer-derived cell lines. *Eur J Biochem* **271:** 3368-3378.

Yamamoto A, Guacci V, Koshland D (1996a) Pds1p, an inhibitor of anaphase in budding yeast, plays a critical role in the APC and checkpoint pathway(s). *J Cell Biol* **133:** 99-110.

Yamamoto A, Guacci V, Koshland D (1996b) Pds1p is required for faithful execution of anaphase in the yeast, Saccharomyces cerevisiae. *J Cell Biol* **133:** 85-97.

Yanow SK, Lygerou Z, Nurse P (2001) Expression of Cdc18/Cdc6 and Cdt1 during G2 phase induces initiation of DNA replication. *EMBO J* **20:** 4648-4656.

Zachariae W, Nasmyth K (1996) TPR proteins required for anaphase progression mediate ubiquitination of mitotic B-type cyclins in yeast. *Mol Biol Cell* **7:** 791-801.

Zachariae W, Nasmyth K (1999) Whose end is destruction: cell division and the anaphase-promoting complex. *Genes Dev* **13:** 2039-2058.

Zhang Y, Yu Z, Fu X, Liang C (2002) Noc3p, a bHLH protein, plays an integral role in the initiation of DNA replication in budding yeast. *Cell* **109:** 849-860.

Zou L, Mitchell J, Stillman B (1997) CDC45, a novel yeast gene that functions with the origin recognition complex and Mcm proteins in initiation of DNA replication. *Mol Cell Biol* **17:** 553-563.

Zou L, Stillman B (1998) Formation of a preinitiation complex by S-phase cyclin CDK-dependent loading of Cdc45p onto chromatin. *Science* **280:** 593-596.

Zou L, Stillman B (2000) Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase. *Mol Cell Biol* **20:** 3086-3096.