Characterization of Orc6 function following pre-replicative complex assembly in *Saccharomyces cerevisiae*

by

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Abstract

Pre-replicative complex (pre-RC) components the origin recognition complex (ORC), Cdc6, and Cdt1, play key roles in the recruitment, and loading of the replicative helicase, the minichromosome maintenance complex (Mcm2-7), onto DNA to license origins for replication. Until recently, the prevailing model for pre-RC assembly predicted that once MCMs are loaded at origins, ORC, Cdc6, and Cdt1 are dispensible for replication. Contrary to this model, previous work has shown that Orc6 is required following origin licensing, for the continued association of the MCM complex in late G1 phase. In this study, a similar role in pre-RC maintenance has been demonstrated for Cdc6, and Cdt1. Chromatin immunoprecipitation (ChIP) analysis has shown that late G1 phase depletion of either Cdc6, or Cdt1 leads to the destabilization of MCMs from origins, although this destabilization is more pronounced for Cdc6 depletion than for Cdt1. Furthermore, the resynthesis of Cdc6 following its depletion, allows for the reassembly of pre-RCs in late G1 phase, and restores competence for DNA replication.

In this study, a potential role for Orc6 in mitosis/cytokinesis in budding yeast has also been characterized, as research with both *Drosophila* and human cell lines has pointed to a role for Orc6 in these processes. Deleting *HOF1* and *CYK3* (two proteins involved in cytokinesis in budding yeast) leads to a synthetic lethal phenotype, suggesting that the resulting gene products function in redundant cytokinetic pathways. Indeed, Hof1 has been shown to be primarily involved in actin ring contraction, while Cyk3 functions in septum formation, both pathways of which are important for budding yeast cytokinesis. Interestingly, previous work has identified an Orc6-Hof1 interaction in budding yeast. In this study, it has been demonstrated that following Orc6 depletion in a *GAL1-ORC6*/Δ*cyk3* strain, fluorescence activated cell sorting (FACS) analysis is consistent with a stronger cytokinetic defect phenotype than observed for Δ*cyk3* cells. Preliminary cell counts indicate that following Orc6 depletion, a higher percentage of *GAL1-ORC6*/Δ*cyk3* cells display misshapen mother bud necks than in

an isogenic Δ*cyk3* strain. Cell synchronization experiments have demonstrated that Orc6 depletion during a G2/M phase arrest, leads to a block in cell cycle progression following release.

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Abbreviations

ACS	ARS Consensus Sequence
APC/C	Anaphase Promoting Complex/Cyclosome
ARS	Autonomously Replicating Sequence
bp	base pair(s)
CDK	Cyclin Dependent Kinase
ChIP	Chromatin Immunoprecipitation
DDK	Dbf4-Dependent Kinase
FACS	Fluorescence Activated Cell Sorting
GAL	YPG/R (galactose-based medium)
GAL1	P_{GAL1} glucose-repressible promoter
GLU	YPD (glucose-based medium)
HA	Hemagglutinin epitope tag
HU	Hydroxurea
IN	Input DNA sample from chromatin immunoprecipitation assay
IP	Immunoprecipitated DNA sample from chromatin immunoprecipitation assay
MEN	Mitotic Exit Network
MCM	Minichromosome Maintenance proteins
ORC	Origin Recognition Complex (Orc1-6)
ORF	Open Reading Frame
P_{GAL1}	*GAL1* promoter
Pre-RC	Pre-Replicative Complex
WCE	Whole Cell Extract
WT	Wild Type

YPD	(1% Yeast extract, 2% Peptone, 2% D-glucose)
YPG/R	(1% Yeast extract, 2% Peptone, 2% Galactose, 1% Raffinose)

Chapter 1

General Introduction

Budding yeast as a model organism

Saccharomyces cerevisiae has been used extensively in baking and brewing processes because of its ability to ferment glucose to ethanol and carbon dioxide. More recently, however, budding yeast has become one of the most highly studied eukaryotes in the areas of cell cycle and molecular biology, genetics, and proteomics.

As a eukaryotic organism, *S. cerevisiae*'s cellular structure, as well as many of its biochemical processes, are similar to those in higher eukaryotes (reviewed in Bell & Dutta, 2002). As a unicellular organism, its short generation time, ability to grow on defined media, and ease of cultivation, make it an attractive organism for cell cycle studies. Budding yeast are also used in these types of studies because of the ease with which they respond to cell cycle arresting agents, which allow an entire population of cells to be synchronized at certain cell cycle stages. Following removal of the arresting agent, cells release and progress through one or more cycles synchronously. For example the mating pheromones **a**- and α-factor can be used to arrest haploid asynchronously growing populations of MATα and MAT**a** cells, respectively, in late G1 phase (reviewed in Bardwell, 2004). Hydroxyurea (HU) is a ribonucleotide reductase inhibitor, and arrests cells in mid-S phase by depleting dNTP pools, thus inhibiting DNA synthesis (Sanchez et al., 1996; de la Torre-Ruiz et al., 1998). Another type of arrest agent, nocodazole, is a microtubule depolymerizer, and arrests cells at the G2/M boundary since microtubules are required for spindle formation, and chromosome segregation during mitosis (Vasquez et al., 1997).

The complete sequence of the budding yeast genome was released in April of 1996, and was the result of six years of work and a world-wide collaboration of over 600 scientists (Goffeau et al., 1996). This body of data has provided yeast biologists with a powerful tool for the study of genetics and proteomics, since all genes, open reading frames (ORFs), and introns have been mapped to each of the sixteen chromosomes.

Most importantly, the manipulation of the budding yeast genome is accomplished using quick and simple protocols. Lab wild type (WT) strains are not generally true WT, but contain mutations in genes that code for enzymes involved in key biochemical pathways (Brachmann *et al*., 1998). An example is *URA3*, which encodes orotidine-5'-phosphate decarboxylase, an enzyme that is important for the biosynthesis of uracil. Without this gene, Δ*ura3* cells cannot synthesize uracil, and must be grown on medium supplemented with this pyrimidine (Boeke *et al*., 1987). A variety of these types of mutations have been generated in yeast strains. Transforming these strains with the DNA of interest attached to a selectable marker gene such as *URA3*, can allow for the selection of the modified yeast strains by plating transformants on media lacking uracil. In addition, yeast can exist in a haploid or diploid state, making it possible to study the direct phenotypic effect of gene deletion in haploids, while also being able to study the effect of deletion of an essential gene in a diploid cell, without loss of viability.

The development of yeast transformations, in combination with the ability of yeast to integrate DNA fragments into their genome via homologous recombination, has given scientists the capability of manipulating any section of the genome with ease. In particular, it is possible to delete a gene, replace it with a mutated/fragmented form, tag it with a sequence encoding an affinity, or fluorescence tag, or control gene expression, and importantly, observe the resulting phenotype *in vivo* (Longtine et al., 1998).

The control of gene expression is of particular importance with respect to this study, as it allows the experimenter to manipulate the abundance of a protein simply by changing the environment in which the yeast are grown. This can be accomplished via several mechanisms, including the creation of temperature-sensitive point mutations, degron strains, or the replacement of a gene's endogenous promoter with one that is capable of being regulated. Temperature-sensitive point mutants grow similarly to WT at the permissive temperature (usually 23°C, but can vary) because the protein product

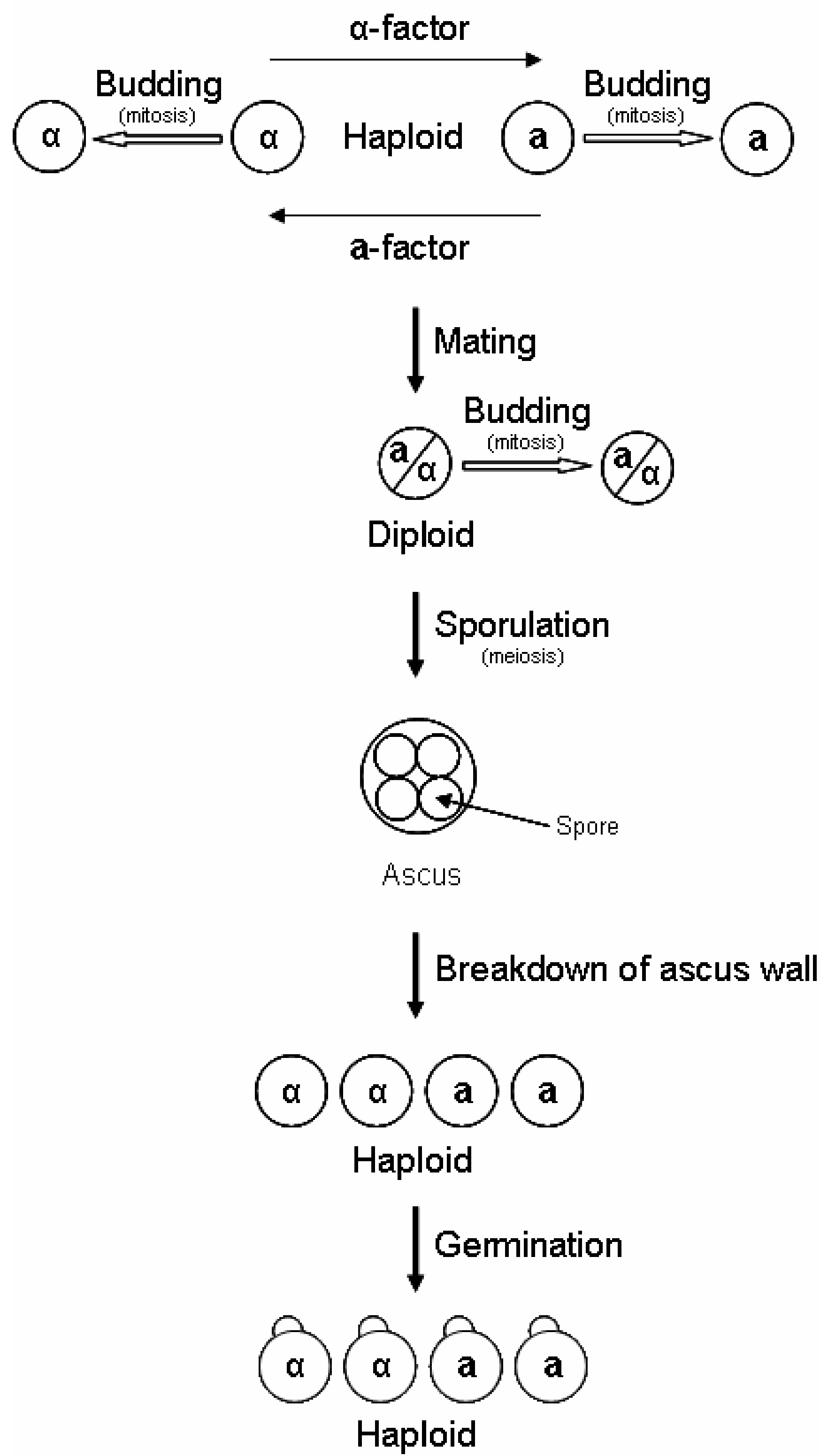
of the mutated gene is stable and fully functional. At the restrictive temperature (usually 37°C), the protein becomes unstable and does not function. The generation of temperature-sensitive point mutants can be time consuming, since they are created through random mutagenesis. For this reason, the degron mutant was designed. A heat-inducible degron cassette, consisting of the temperature-sensitive version of the mouse dihydrofolate reductase gene fused to a copper-inducible promoter, is integrated into the genome to replace the endogenous promoter of a gene of interest. Induction of the degron promoter causes the transcription of a fusion protein consisting of the degron cassette at the N-terminus of the gene of interest. Protein depletion can be accomplished by growing the strain at the restrictive temperature in the absence of copper (Dohmen et al., 1994; Kanemaki et al., 2003; Sanchez-Diaz et al., 2004). Another very simple and effective means of protein depletion is to replace an endogenous promoter with one that can be regulated, such as the P_{GAL1} promoter (abbreviated from now on as *GAL1*) used in this study. *GAL1* is turned on when strains are grown in the presence of galactose-based medium such as YPG/R (abbreviated from now on as GAL), but turned off when switched to glucose-based medium, such as YPD (abbreviated from now on as GLU).

Physiology and genetics of *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is a unicellular member of the fungi, which is a group of organisms that are heterotrophic, spore producing, and encased in a chitinous cell wall. The budding yeast genome is compact, and contains approximately 6000 genes on 16 chromosomes (the haploid or 1N number), 5885 of which are predicted to produce protein products. One explanation for this compactness is the scarcity of introns in comparison to other organisms. Only four percent of yeast genes are interrupted by an intron, compared to the forty percent of genes in *Schizosaccharomyces pombe* (Goffeau et al., 1996). Yeast are able to exist stably as either haploids or diploids, both of which can reproduce asexually via mitotic budding, to produce cells of identical genotype to the mother. Haploid cells exist

as one of two mating types, **a** or α, and in addition to budding, can reproduce sexually by mating with a haploid cell of the opposite mating type to produce a diploid **a**/α cell. Differences in mating type are controlled by the presence of one of the two alleles of the mating-type locus *MAT*, ***MATa***, or MATα. The *MAT* locus includes genes that direct the transcriptional program of the correct mating type, including those encoding the pheromones **a**- or α-factor, and specific cell surface receptors for detection of these pheromones. A ***MATa*** cell produces **a**-factor and has α cell surface receptors, while *MATα* cells produce α-factor and have **a** cell surface receptors. Two cells of the opposite mating type will mate if they are in close enough proximity to detect mating pheromones. Once detected, each haploid cell will arrest in late G1 phase and initiate mating, whereby cells will begin directional growth toward the mating partner. The resultant pear-shaped morphology is referred to as a shmoo, and is easily identified under the microscope. Transcription of genes required for mating allows cell fusion, followed by nuclear fusion to produce a diploid *MAT* **a**/α cell. Diploids are not able to mate, but in nutrient poor conditions, are capable of sporulating via meiosis to produce four haploid ascopores, two **a,** and two α, contained within an ascus. Sporulation facilitates cell survival because asci are capable of withstanding harsher environmental conditions than actively cycling cells. Once environmental conditions become favourable for growth and division, haploid ascopores will resume cycling and asexual budding (Figure 1) (Herskowitz, 1988).

Figure 1: The life cycle of *Saccharomyces cerevisiae*. Budding yeast can exist in both haploid and diploid states, and can replicate through mitosis in either of these states. This is known as budding, and produces an identical replicate of the mother cell in 90-100 min at 30ºC with WT laboratory strains. Haploids of opposite mating type (MAT**a** and MATα) can mate to produce a diploid (MAT**a**/α) cell, and are signaled to begin the mating process by sensing phermones (**a**- and α-factor) released by the opposite mating type through the binding of these pheromones to receptors located on the cell surface. Diploids can sporulate (meiosis) to produce four haploids, two MAT**a**, and two MATα, contained within an ascus. Breakdown of the ascus releases the haploid cells, and germination leads to the development of spores into newly budding cells. (Adapted from Herskowitz, 1988)


α-factor
Budding
(mitosis)
Budding
(mitosis)
α
α
Haploid
a
a
a-factor
Mating
Budding
(mitosis)
a/α
a/α
Diploid
Sporulation
(meiosis)
Spore
Ascus
Breakdown of ascus wall
α α a a
Haploid
Germination
α α a a
Haploid

The budding yeast cell cycle

Cell proliferation is accomplished through a series of steps that together constitute the eukaryotic cell cycle. This process involves the duplication of cellular components, the segregation of these components to different regions of the cell, and division to produce two daughter cells. For convenience, the cell cycle can be thought of as being comprised of a longer interphase, in which duplication of cellular contents occurs, and a shorter mitotic phase, in which the contents are divided between mother and daughter cells. Interphase can be further subdivided into S phase, during which DNA replication occurs, and the Gap phases G1 and G2 that precede and follow replication, respectively (Figure 2) (Alberts et al., 1994). As discussed in the previous sections *S. cerevisiae* can reproduce asexually through a process known as budding, and WT strains typically have a generation time of 90-100 min when grown at optimal temperature (Hartwell, 1974). Since bud emergence and growth can be correlated to certain cell cycle events, budding offers an approximate marker of cell cycle stage (Hartwell, 1974).

Gl phase can be thought of as the beginning of the cell cycle since during this phase the cell must integrate environmental inputs and decide on a particular pathway. This decision point is often referred to as START (Figure 2) (Pringle & Hartwell, 1981). In early G1 phase, or pre-start, cells are competent to enter the cell cycle if conditions are favourable, or, if conditions are not favourable cells can enter a state of quiescence known as G0. In contrast, cells in late G1 phase, or post-start, are committed to completing the cell cycle, starting first with the replication of the genome. Eukaryotic DNA replication is a tightly regulated process that ensures the accurate replication of the genome exactly once per cell cycle. The initiation of DNA replication involves an ordered set of steps that include the labelling of origins of replication, the assembly of several multiprotein complexes at these sites, the unwinding of origin DNA, and the recruitment of the replication machinery (Lee & Bell, 1997). The origin recognition complex (ORC) is the primary recognition protein for origins of

replication and is conserved throughout eukaryotes (Bell & Stillman, 1992; reviewed in Bell & Dutta, 2002). Following origin binding, ORC acts as a landing pad for the recruitment of a multiprotein complex (the pre-replicative complex, or pre-RC) that licenses origins for replication by loading the replicative helicase, the minichromosome maintenance complex (Mcm2-7) onto origin DNA during G1 phase. This process can only occur during a time of low cyclin abundance, since cyclins activate key kinases responsible for the inactivation or degradation of several pre-RC components. It is therefore extremely important that cyclins present in S and M phases of the previous cell cycle be degraded in late M phase prior to pre-RC assembly. This is accomplished by ubiquitin ligases active in late M and early G1 phases that target cyclins for degradation by the proteosome. Cells in G1 phase can be identified cytologically by the absence of a bud (Figure 2) (Hartwell, 1974), and through fluorescence activated cell sorting (FACS) analysis by the presence of a 1C (Copy) peak, indicating that cells have only one copy of the genome and have not yet replicated their DNA. This 1C peak is specific to haploids, as diploid cells at this stage would have a 2C peak.

The beginning of S phase is marked by several key events including the unwinding of origin DNA, the recruitment of the replication machinery, and bud emergence (Figure 2). Ubiquitin ligases responsible for degrading cyclins in G1 phase are no longer active in S phase. This allows for a rise in S phase cyclin levels, and the activation of kinases required for origin unwinding and recruitment of the replication machinery. Once origins have been unwound, single stranded binding proteins stabilize unwound DNA, facilitating synthesis by the DNA polymerases (DNA Pol α -δ, and -ε) (reviewed in Bell & Dutta, 2002). In order for synthesis to occur, the DNA polymerases require a template strand to copy, as well as a primer, since they cannot initiate synthesis, but can only add nucleotides to the 3' end of a pre-existing strand. A short RNA primer is added to each of the single strands of DNA by a specialized RNA polymerase called primase. As the replication fork progresses, two single stranded templates are exposed, one in the 5'→3', and one in the 3'→5' direction. An additional difficulty arises

in DNA synthesis due to the inability of the DNA polymerases to synthesize in the 3'→5' direction. As the replication fork progresses, primase adds a primer to the exposed 3'→5' template, and DNA Pol δ can synthesize a new strand continuously chasing the replication fork. Because synthesis proceeds uninterrupted, this strand is called the leading strand. In contrast, the 5'→3' strand is synthesized discontinuously because synthesis must occur in a direction that moves away from the replication fork. This means that primase must wait for the helicase to move away from the origin before it can prime the template strand. DNA Pol δ then synthesizes in the 5'→3' direction in a discontinuous manner that produces Okazaki fragments that together make up the lagging strand. Finally a nuclease called FEN1 digests away the RNA primers, DNA Pol δ fills in the gaps, and DNA ligase joins the fragments together (reviewed in Kornberg, 1988). The completion of chromosome duplication in S phase produces two sister chromatids, which are then attached at the centromere by a cohesin protein complex.

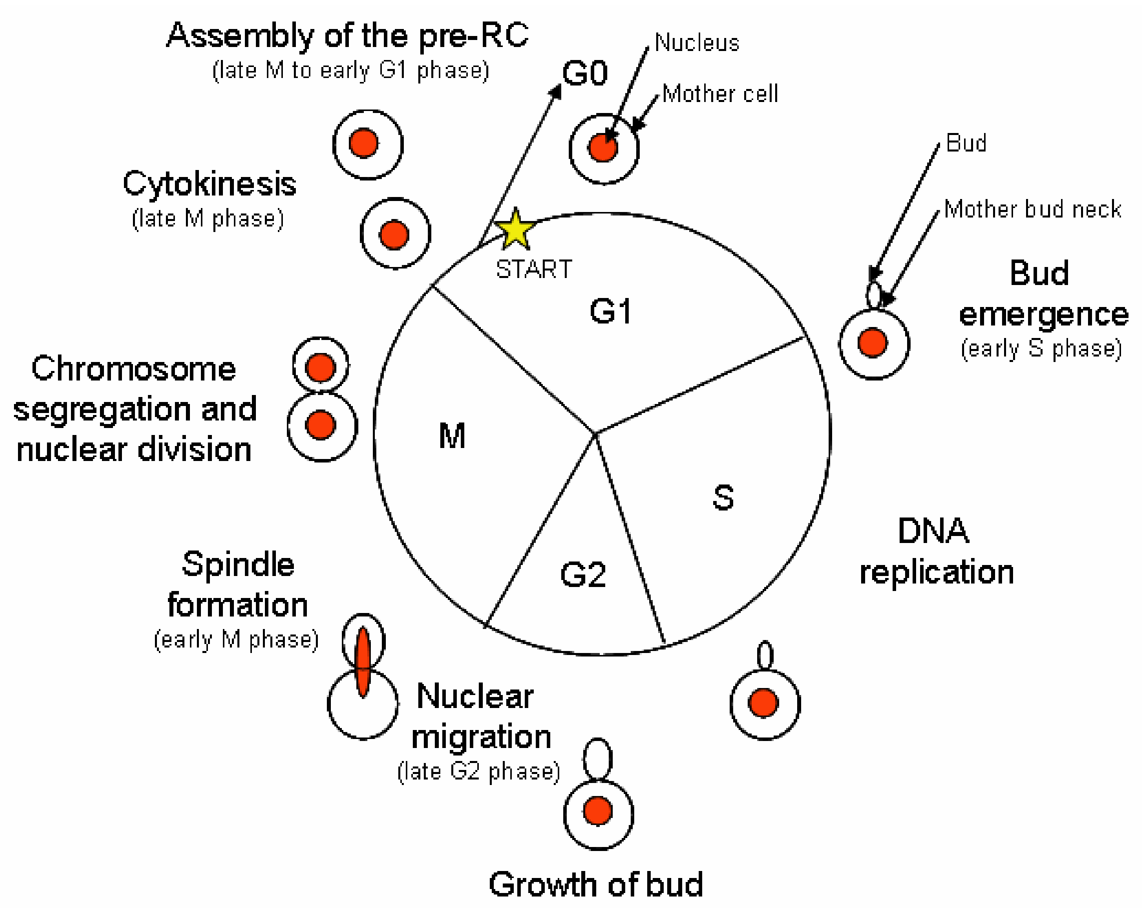
The budding yeast cell cycle includes a short G2 phase that is characterized by the migration of the nucleus to the neck of the cell, and the growth of the bud, which continues throughout M phase (Figure 2) (Hartwell, 1974). In addition, a high rate of protein synthesis ensures that components required for mitosis and cell separation are available when needed. G2 is represented as a 2C peak through FACS analysis with haploid cells, since cells now have two copies of the genome.

Mitosis is the final stage of cell division, and is characterized by nuclear division, which includes the segregation of sister chromatids generated during S phase to opposite poles of the nucleus (Figure 2). The nuclear spindle, which includes microtubules required for the movement of sister chromatids, is assembled throughout the cell cycle beginning at the G1/S phase boundary. In budding yeast, chromosome condensation is difficult to visualize under the microscope, and unlike in other eukaryotes, the nuclear envelope remains intact throughout M phase (Matile, 1969; reviewed in Pringle et al., 1997). Early in mitosis a protein complex called the kinetochore assembles on centromeric DNA

and mediates the proper connection of microtubules of the spindle (Hayden et al., 1990; Alexandru et al., 1999). Faithful distribution of genetic material between mother and daughter cells depends on the timely destruction of the cohesin complex holding sister chromatids together. During metaphase, the cohesion complex resists tension generated by microtubules, pulling sister chromatids to the center of the nucleus. High mitotic cyclin dependent kinase (CDK) levels are required for entry into mitosis, and activate the ubiquitin ligase called the anaphase promoting complex/cyclosome (APC/C). The APC/C initiates the separation of sister chromatids by mediating the degradation of the inhibitory protein securin, which normally inhibits separase. Separase is a protease which cleaves cohesin, allowing the separation of sister chromatids. Microtubule elongation moves sister chromatids to opposite poles of the nucleus, and spindle disassembly triggers nuclear envelope division. Finally, division of the cytoplasm through a process called cytokinesis, separates mother from daughter cell, and completes the cell cycle (Figure 2). Cyclin degradation by the APC/C results in low CDK activity required for cytokinesis, thus coordinating mitosis with the completion of the cell cycle (Miyazaki & Orr-Weaver, 1994; reviewed in Zachariae, 1999). In budding yeast, cytokinesis is mediated by two functionally overlapping processes, namely actomyosin ring formation/contraction, and septum formation (Bi et al., 1998; Lippincott & Li, 1998).

In order to ensure that a complete and accurate copy of the genome is distributed to each daughter, the cell is equipped with checkpoints set at various stages throughout the cell cycle that monitor key events such as DNA replication and sister chromatid segregation. If an error occurs in one of these processes, checkpoints act to stall cell cycle progress, allowing time for repair machinery to correct the error (reviewed in Nyberg et al., 2002; Lew & Burke, 2003).

Figure 2: The budding yeast cell cycle. The cell cycle can be divided into a longer interphase, consisting of G1, S, and G2 phases, and a shorter M phase. Before a point in G1 phase known as START (indicated by the star), a cell must integrate external signals and decide whether or not to enter the cell cycle. If conditions are favourable, a cell will continue actively dividing, but if conditions are not favourable, a cell will exit the cell cycle and enter a state of quiescence known as G0. Following this decision point, the cell is committed to completing the cell cycle. In early G1 phase pre-RCs are assembled, and are maintained at origins until the beginning of S phase, at which point a new bud emerges, and the mother cell begins replicating its genome. At the completion of S phase, each of the sixteen chromosomes has been replicated, and all chromosomes are now referred to as sister chromatids. S phase is followed by a short G2 phase during which the bud grows, the mother cell prepares for mitosis, and the nucleus migrates to the mother bud neck. At the beginning of M phase the mitotic spindle is formed, and is required for sister chromatid separation. During mitosis, nuclear division occurs and sister chromatids are evenly separated between mother and daughter cells. Cytokinesis marks the end of M phase, during which mother and daughter cells are separated from each other by the formation and constriction of a muscular ring at the site of division, followed closely by septum synthesis of cell wall material. (Adapted from Herskowitz, 1988)


Assembly of the pre-RC
(late M to early G1 phase)
G0
Nucleus
Mother cell
Bud
Mother bud neck
Cytokinesis
(late M phase)
START
G1
Bud
emergence
(early S phase)
Chromosome
segregation and
nuclear division
M
S
DNA
replication
Spindle
formation
(early M phase)
G2
Nuclear
migration
(late G2 phase)
Growth of bud

Formation of the pre-replicative complex

Eukaryotic DNA replication involves the assembly of several multiprotein complexes at sites of replication, beginning with the marking of origins by ORC. Replication initation occurs in two sequential stages: origin licensing in G1 phase of the cell cycle, and origin initation in S phase. During the first stage, pre-RC assembly at origins of replication results in the loading of the replicative helicase, onto origin DNA (Figure 3). Once the pre-RC is assembled, origins are said to be 'licensed' for DNA replication. In the second stage, cyclin-dependent kinases (CDKs) and Dbf4-dependent kinases (DDKs) are activated to phosphorylate key proteins that then activate the replicative helicase to unwind origin DNA, and recruit the replication machinery.

In order to replicate the entire genome in a timely manner, eukaryotic DNA replication initiates at many origins within the genome, however, despite the conservation of protein factors involved in replication, the origin sequences that bind them are highly divergent, and for most species, poorly characterized.

In budding yeast, replication starts from well-characterized origins known as autonomously replicating sequences (ARS) (Stinchcomb et al., 1979; Chan & Tye, 1980; Brewer & Fangman, 1987), named for their ability to allow for autonomous replication of plasmid DNA in yeast (Hsaio & Carbon, 1979). *S. cerevisiae* has approximately 332 functional origins (Raghuraman et al., 2001; Yabuki et al., 2002; Feng et al., 2006) spaced evenly across the genome (Patel et al., 2006). Most origins are found within intergenic regions lacking nucleosomes, and many are highly efficient since they are used in every cell cycle (Yuan et al., 2005; Nieduszynski et al., 2006). An ARS spans approximately 100 base pairs (bp), and contains a 17 bp A-element, which includes the highly conserved AT-rich 11bp ARS-consensus sequence (ACS), as well as several less conserved B-elements (Marahrens & Stillman, 1992; Bell, 1995). The A- and B1-elements are binding sites for ORC (Bell & Stillman, 1992; Rao &

Stillman, 1995; Rowley et al., 1995), while other B-elements act to enhance origin efficiency and contain DNA unwinding elements thought to aid in the recruitment of replication proteins (Umek & Kowalski, 1990; Newlon & Theis, 1993; Bell, 1995). The B2-element is thought to be a binding site for the Mcm2-7 complex and functions in pre-RC assembly (Zou & Stillman, 2000; Wilmes & Bell, 2002), while the B3-element binds Abf1, a transcription factor that limits nucleosome binding within origin sequences (Marahrens & Stillman, 1992). Many budding yeast origins, including the well characterized *ARS1*, contain A-, B1-, and B2-elements, however, some differ from this prototype. For example, some origins contain a B3-element, some contain multiple A-elements, while others contain a transcription factor-binding C-element (Walker et al., 1990; Marahrens & Stillman, 1992; Theis & Newlon, 2001).

In contrast to *S. cerevisiae*, the *cis*-acting elements required for replication in other eukaryotes are generally larger, more complex, and lacking of any comparable sequence speicificity, making these origins much more difficult to characterize. However, one common feature between these replicators and those of *S. cerevisiae*, is that they are all AT-rich, presumably to allow for efficient unwinding at the origin (Kelly & Brown, 2000; Kong et al., 2003; Vashee et al., 2003; Zhang & Tower, 2004). It is likely that in higher eukaryotes, origin selection and binding by ORC does not require sequence specific DNA binding regions, but relies on other, more complex mechanisms.

ORC, originally identified in *S. cerevisiae* as a complex that binds the ACS element of origins (Bell & Stillman, 1992), is composed of six subunits (Orc1-6), and is conserved in all eukaryotes studied to date. Although pre-RC assembly is restricted to G1 phase, budding yeast ORC remains bound to origins throughout the cell cycle (Diffley et al., 1994; Aparicio et al., 1997; Liang & Stillman, 1997; Tanaka et al., 1997). In contrast, studies of mammalian ORC revealed that while an Orc2-6 complex is constitutively bound to origins, Orc1 is removed during S phase and rebinds as cells enter G1 phase of the following cell cycle (Ritzi et al., 1998; Natale et al., 2000; Tatsumi et al., 2000; Kreitz et al., 2001). Protein-DNA cross-linking studies revealed that Orc1, Orc2, Orc4, and Orc5 are in close

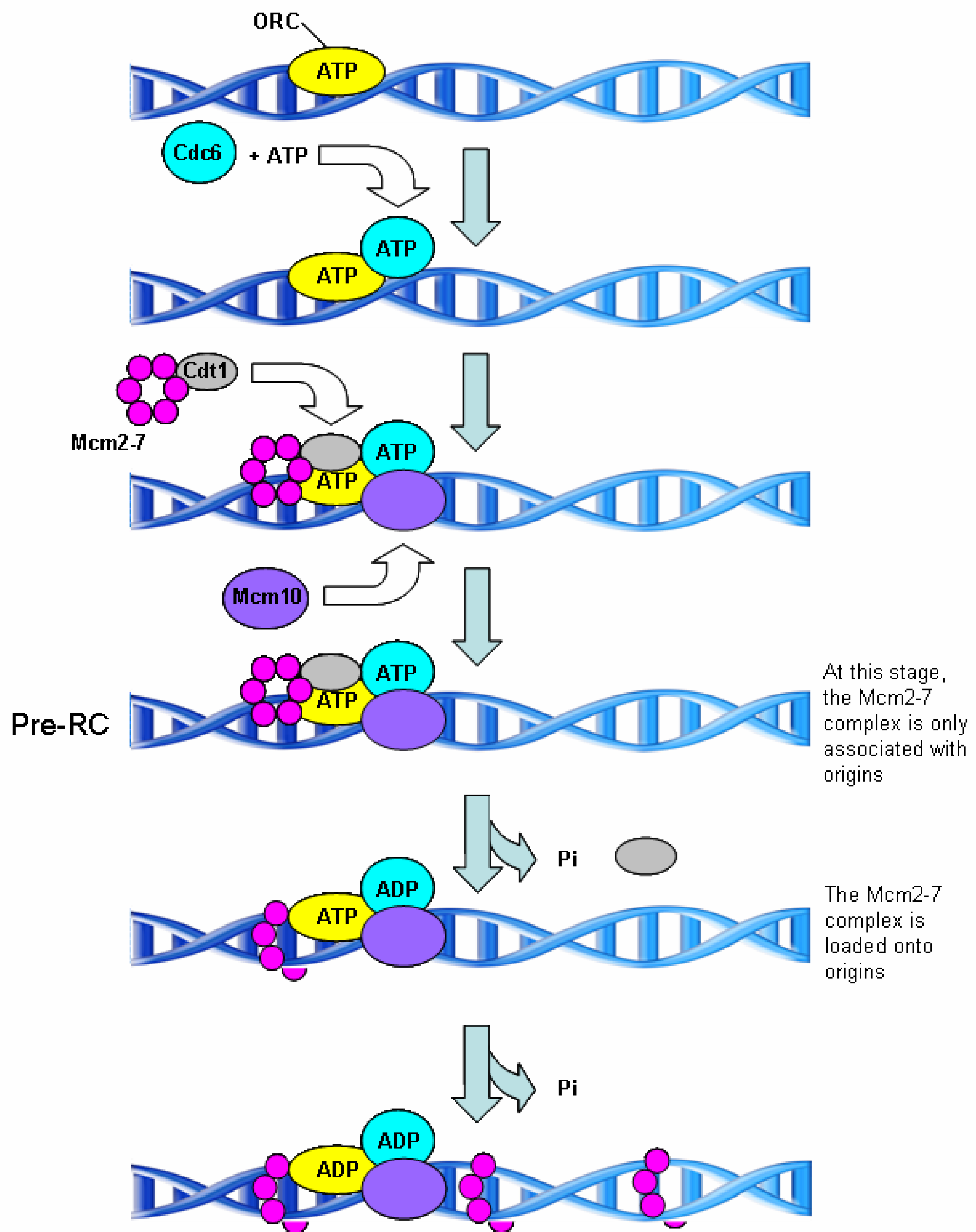
contact with origin DNA. Despite the lack of cross-linking between Orc3 and origins, this subunit remains essential for ORC-DNA binding (Lee & Bell, 1997; Harvey & Newport, 2003). Orc6 is the only subunit that is dispensable for binding to origins *in vitro*, but remains essential for viability and for ORC function *in vivo* (Li & Herskowitz, 1993). In contrast, studies with *Drosophila* ORC indicate that all six ORC subunits are required for both DNA binding, and *in vitro* DNA replication (Chesnokov et al., 2001). It is interesting to note that a clear DNA binding domain has not been identified within any of the ORC subunits, with the exception of the AT-hook at the N-terminus of Orc4 in *S. pombe*. This binding motif is not found in Orc4 homologs, but is essential for *S. pombe* viability (Chuang & Kelly, 1999). Another feature of ORC is its ability to bind and hydrolyze ATP. ORC must be in an ATP-bound state to bind origin DNA and although Orc1-5 each contain potential ATP binding sites, Orc1 and Orc5 are the only subunits that bind ATP (Bell & Stillman, 1992; Klemm et al., 1997; Austin et al., 1999; Chesnokov et al., 2001). Once ORC is bound, origin DNA inhibits ATP hydrolysis (Klemm et al., 1997).

The first step in assembling the pre-RC at origins is the recruitment of Cdc6 by ORC in early G1 phase (Figure 3). Like Orc1-5, Cdc6 is also a member of the AAA+ (ATPases associated with various cellular activities) ATPase family and possesses strong amino acid similarity to Orc1, suggesting that ATP binding and hydrolysis are key to its function (Neuwald et al., 1999). Consistent with this, Randell et al., (2006) demonstrated that not only is ATP binding and hydrolysis required for the functioning of Cdc6, but it is also required for loading of the Mcm2-7 replicative helicase onto origin DNA. Interestingly, Cdc6 has been shown to regulate ORC selection of origin DNA, allowing for a higher degree of sequence specificity. Following ORC-dependent Cdc6 recruitment to origins, ATP-bound Cdc6 binds ORC, altering its shape so that ORC and Cdc6 form a ring-like structure that can then cooperatively bind origin DNA with a higher specificity. ORC activates the ATPase activity of Cdc6, while origin DNA suppresses it. On DNA sequences lacking origin activity, Cdc6 ATPase

activity promotes the dissociation of Cdc6 from the ORC-Cdc6-DNA complex, whereas on origin DNA, Cdc6 ATPase activity is suppressed, stabilizing the ORC-Cdc6-DNA complex (Speck & Stillman, 2007). Consistent with this model, mutations in the A-element of origins induces Cdc6 ATPase activity, while Cdc6 ATPase mutants promote binding of the ORC-Cdc6 complex to non-origin specific DNA. Once stably associated with origin DNA, Cdc6 and ORC can then recruit, and in conjunction with Cdt1, load the Mcm2-7 complex onto origin DNA. Cdt1 forms a complex with the Mcm2-7 helicase (Tanaka & Diffley, 2002), and together are recruited to origins, binding onto origin DNA in a Cdc6 ATPase-dependent manner (Randell et al., 2006). Cdt1 associates with the C-terminus of Cdc6 (Nishitani et al., 2000) and Cdc6 ATPase activity acts to load the Mcm2-7 helicase from the Cdt1-Mcm2-7 complex (Figure 3) (Randell et al., 2006). The Mcm2-7 complex, is composed of six proteins that associate in equal stoichiometry (Forsburg, 2004) to form a ring-shaped structure (Adachi et al., 1997), and functions as the replicative helicase to unwind origin DNA (Bochman & Schwacha, 2008, reviewed in Bell & Dutta, 2002). MCM (minichromosome maintenance) proteins share a high degree of sequence similarity, and all are members of the AAA+ ATPase family (Koonin, 1993). The Mcm2-7 complex is inactive as a helicase until CDK and DDK activation at the beginning of S phase (Zou & Stillman, 1998; Labib et al., 2000; Pacek & Walter, 2004). It has been proposed that Cdc6 acts as a clamp loader to open the ring shaped MCM complex using the energy from ATP hydrolysis (Perkins & Diffley, 1998; Weinreich & Stillman, 1999). Once the Mcm2-7 complex has been loaded onto origin DNA, Cdt1 dissociates and ATP hydrolysis by ORC allows for reiterative loading of Mcm2-7 complexes onto origins (Randell et al., 2006). Thus Cdc6 ATPase directs a single round of Mcm2-7 complex loading, whereas ATP hydrolysis by ORC is required for reiterative Mcm2-7 complex loading at origins of replications (Figure 3) (Bowers et al., 2004). The function of reiterative Mcm2-7 loading is unclear, however, it has been proposed that additional helicase complexes move

away from the origin and act to unwind DNA at various points between replication origins (Randell et al., 2006).

Figure 3: Current model for pre-RC formation in budding yeast. Assembly of the pre-RC occurs in late M and early G1 phases. ORC binds origins of DNA replication throughout the budding yeast cell cycle, and acts as a scaffold for the assembly of the pre-RC. ORC first binds origins in an ATP-bound state, and recruits Cdc6, which binds ORC, and ATP. Both Cdc6, and ORC then act to recruit Cdt1, and Mcm2-7 (the replicative helicase), possibly as a complex. Finally Mcm10, a protein believed to help stabilize Mcm2-7 association with origin DNA, is recruited to form the pre-RC. Mcm2-7 associates with origin sequences following recruitment, however, ATP hydrolysis by Cdc6 is required to load the helicase onto DNA, and simultaneously release Cdt1. Finally, ATP hydrolysis by ORC directs the reiterative loading of Mcm2-7 complexes onto origin DNA. (Adapted from Randell et al., 2006)


ORC
ATP
Cdc6
+ ATP
ATP
ATP
Cdt1
Mcm2-7
ATP
ATP
Mcm10
ATP
ATP
Pre-RC
At this stage, the Mcm2-7 complex is only associated with origins
Pi
ADP
ATP
The Mcm2-7 complex is loaded onto origins
Pi
ADP
ADP

It is clear that pre-RC components are required for the loading of Mcm2-7 complexes at origins of replication, however, whether these components have any additional functions following pre-RC assembly has been the subject of debate. The proper functioning of pre-RC components is important for the prevention of over- or under-replication, and thus for the maintenance of genome integrity. It is of interest to investigate any additional roles for these proteins in DNA replication, since a loss of genomic integrity can ultimately lead to cancer in higher eukaryotes. Previous models of pre-RC assembly had predicted that following MCM loading at origins, both ORC and Cdc6 were dispensable for DNA replication. MCM proteins were shown to exhibit ORC- and Cdc6-dependent association with chromatin during G1 phase, but were then associated with non-origin sequences as cells progressed through S phase (Aparicio et al., 1997; Donovan et al., 1997). Similarly, investigating the origin association of Cdc6 indicated that MCM proteins remained bound to origin DNA longer than Cdc6, whereas origin association of Cdc6 declined before entry into S phase (Tanaka & Nasmyth, 1998). *In vitro* studies showed that high salt treatment of pre-RCs assembled on origin DNA bound to magnetic beads removed ORC, while MCMs remained (Bowers et al., 2004). It is important to note, however, that while MCMs remained at origins, Bowers et al. (2004) did observe an almost fifty percent reduction in MCM association with pre-RCs following ORC removal. Studies using *Xenopus* egg extracts showed that treatment of late G1 chromatin with cyclin A or high salt to remove ORC following MCM loading, did not interfere with initiation or DNA replication, as measured by radioactive dATP incorporation. Cdc6 was also shown to be rapidly removed from chromatin following MCM loading (Hua & Newport, 1998; Rowles et al., 1999). Using *Xenopus* as a model organism, Edwards et al., (2002) similarly concluded that Mcm2-7 maintenance at origins, whether *in vitro* or *in vivo*, did not require ORC, Cdc6, or Cdt1.

In contrast, a number of studies in *S. cerevisiae* have shown that ORC and Cdc6 are not dispensable following pre-RC assembly. *In vivo* studies have shown that depletion of Orc2 in late G1

phase using an *orc2-1* temperature sensitive strain resulted in the rapid loss of at least Mcm2 from chromatin (Zhang et al., 2002). FACS analysis of cells depleted of Orc2 in late G1 phase using an *orc2-1* temperature sensitive strain showed no impairment of the initiation or the elongation stages of DNA replication (Shimada et al., 2002). It should be noted, however, that results from numerous papers disagreed with this model, and found that depletion of Orc2 in late G1 phase resulted in a significant reduction in DNA replication efficiency (Weinberger et al., 2005). Differences between the results of these studies may have been due to the requirement of an extended period of time for pre-RC disassembly, as previously noted using a *cdc6-1*strain (Detweiler & Li, 1997). Weinberger et al., (2005) also noted a loss of MCM maintenance, as well as a decrease in replication efficiency, following Cdc6 depetion in G1 phase arrested cells. A study investigating the function of ORC during the cell cycle observed defective initiation and incomplete replication in cells containing conditional mutations in either *ORC1*, *ORC2,* or *ORC5*, after cells had been arrested in late G1 phase, shifted to the restrictive temperature, and released at this temperature (Gibson et al., 2006).

Research in our lab supports a role for Orc6 in the maintenance of MCM proteins at origins of replication following pre-RC assembly (Semple et al., 2006). Orc6 depletion in late G1 phase was shown to result in an accumulation of cells at the G1/S phase boundary, indicating that Orc6 is required for S phase progression. Depletion was also shown to destabilize Mcm2 chromatin association, as measured by chromatin binding assays, and DNA combing analysis indicated a significant reduction in origin firing efficiency (Semple et al., 2006). Additionally, Orc6 depletion was found to activate the DNA damage checkpoint protein Rad53, consistent with incomplete replication (Da-Silva & Duncker, 2007).

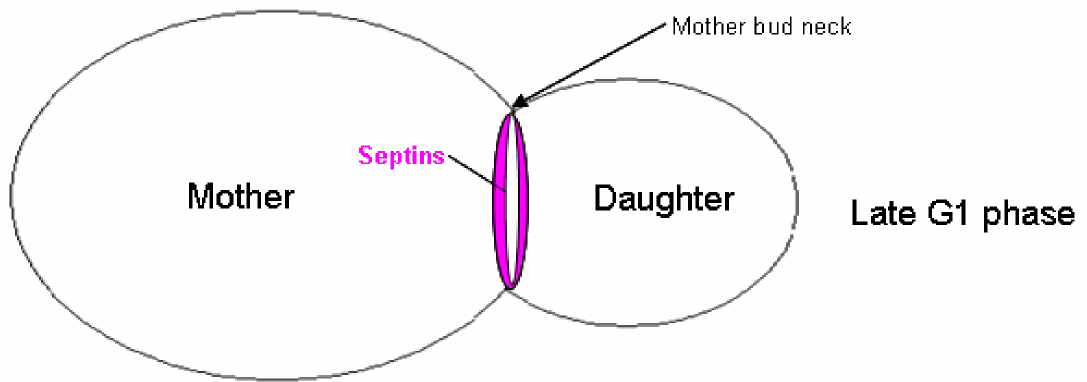
Cytokinesis

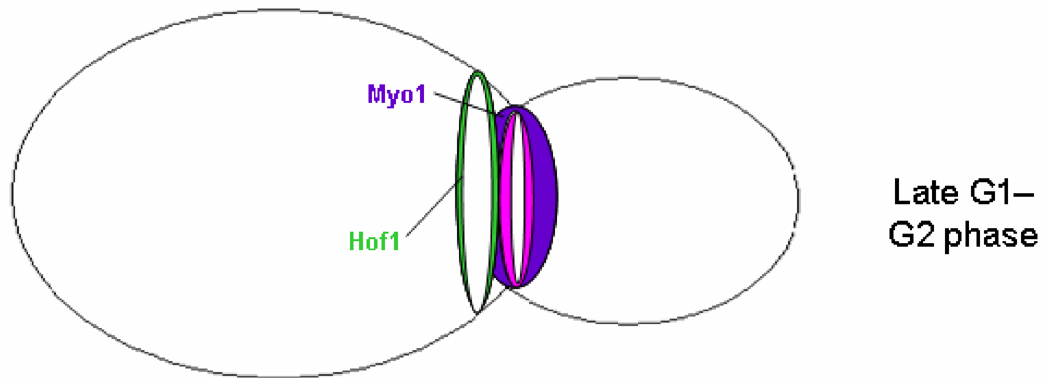
Cytokinesis is the final stage in the cell division cycle, and is the process by which two cells physically separate following the duplication and segregation of genetic material during S and M phases. In all eukaryotes studied to date, cytokinesis involves the assembly and contraction of an actomyosin ring at the site of cell division. Cortical cues at the future division site facilitate ring assembly through the recruitment of actin and myosin, along with accessory proteins, to form the mature ring. Ring contraction acts to physically separate the cytoplasm of two dividing cells, and this process is tightly coordinated with the completion of chromosome segregation to ensure that each new cell receives exactly one copy of the genome. Finally, membrane deposition at the division site closes the small gap between dividing cells that remains following contraction. In yeast, which have cell walls, septum synthesis to generate new cell wall material, is coordinated with these other processes. Important differences also exist between yeast and animal cells, in regards to both how and when the site of cell division is determined. In budding yeast, the division site is determined in G1 phase, and is dependent upon the previous bud site (Chant, 1999; Casamayor & Snyder, 2002). In contrast, selection of a division site in fission yeast, and animal cells occurs prior to anaphase, and depends on the position of the interphase nucleus (Chang & Nurse, 1996), or the position of the mitotic spindle, respectively (Glotzer, 1997). Irrespective of the mechanism, the division site must be positioned between the separated sister chromatids to ensure equal chromosome segregation. The molecules that serve as spatial cues in actomyosin ring assembly also differ between organisms, but all function to recruit components to assemble a functional contractile ring.

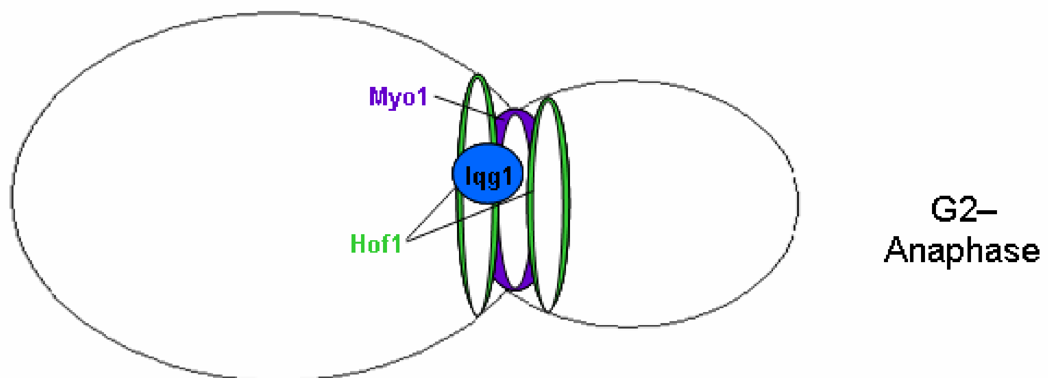
The components that make up the contractile ring are conserved across eukaryotes, but are assembled at different times in accordance with division site selection. In budding yeast, a group of bud-site selection proteins are important in determining the site of budding in G1 phase, but are also important for contractile ring assembly. These proteins recruit and activate the Cdc42 GTPase, which

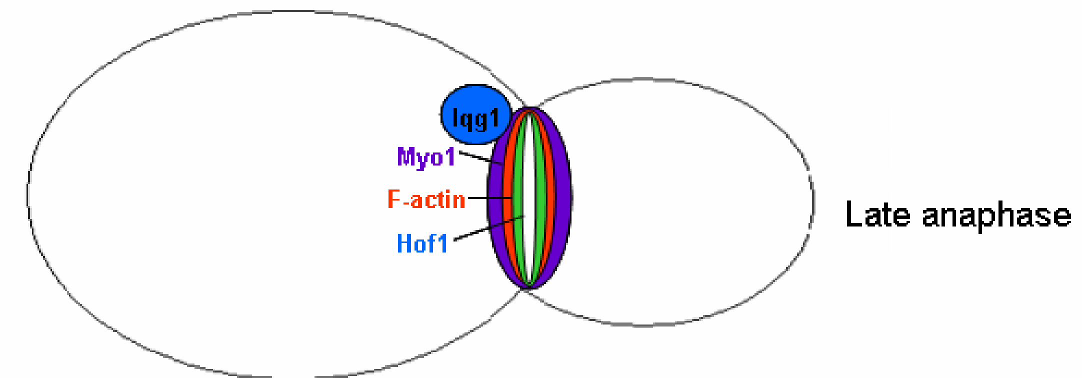
helps to direct budding, as well as actomyosin ring formation, by recruiting the septins in late G1 phase. The septins are a family of structurally related GTP-binding proteins that associate to form filaments *in vitro*, and are generally found to be involved in cytokinesis (Field et al., 1996; Longtine et al., 1996; Frazier et al.; 1998). At the bud neck, the septins associate to form a ring structure that acts as a scaffold in the recruitment and assembly of many of the components that make up the contractile ring (Figure 4). The septin ring is also required for the recruitment of cell wall synthesizing enzymes during cytokinesis, and so iniates both processes required for budding yeast cytokinesis (DeMarini et al., 1997). There are seven septins in budding yeast; inactivation of any one prevents the septin ring from forming, and results in a complete block in cytokinesis (Bi, 2001).

Figure 4: Current model for actomyosin ring formation in budding yeast. Cytokinesis in budding yeast involves two functionally overlapping processes: actomyosin ring assembly and constriction at the mother bud neck, and the synthesis of a septum of cell wall material between dividing cells. Contractile ring formation begins in late G1 phase, when septin proteins are recruited to the mother bud neck. The type II myosin, Myo1, is recruited in a septin-dependent manner, also in late G1 phase. Maintenance of Myo1 association with the mother bud neck until the completion of contractile ring assembly in late anaphase, also depends on the septin ring. It is thought that Myo1 acts as a motor to drive ring contraction by sliding actin filaments. During G2 phase Hof1, a protein involved in actomyosin ring contraction as well as septum synthesis, is recruited in a Myo1-independent, septin-dependent manner, and forms a single ring on the mother side of the neck region. Shortly after, a second Hof1 ring appears on the bud side of the neck region. During anaphase Iqg1 is recruited, and is responsible for the recruitment and polymerization of the final ring component, actin, into filamentous actin (F-actin). Following ring contraction at the end of mitosis, a septum of cell wall material is synthesized between mother and daughter cells.


Mother bud neck
Septins
Mother
Daughter
Late G1 phase


Myo1
Hof1
Late G1–
G2 phase


Myo1
Iqg1
Hof1
G2–
Anaphase


Iqg1
Myo1
F-actin
Hof1
Late anaphase

The first ring components to be recruited are the type II myosin Myo1 (Figure 4) along with its essential light chain Mlc1, and this occurs in a septin-dependent manner in late G1 phase (Bi et al., 1998; Lippincott & Li, 1998; Luo et al., 2004). Mlc1 binds to an IQ motif of Myo1 and so is recruited to the septin ring in conjunction with Myo1 (Boyne et al., 2000; Shannon & Li, 2000; Luo et al., 2004). Since ring formation is not completed until late anaphase, these two proteins are maintained at the bud neck for most of the cell cycle, an association that is also dependent on the septins. Myo1 is required for the assembly of a functional contractile ring, and is thought to act as a motor for actin sliding during ring contraction (Bi et al., 1998; Lippincott & Li, 1998). Although Myo1 is required for efficient cytokinesis and cell separation (Tolliday et al., 2003), in some strain backgrounds cells are able to close the small gap at the bud neck by synthesizing cell wall material that forces the membranes together without requiring Myo1 function (Bi et al., 1998; Vallen et al., 2000; Tolliday et al., 2003). This is only possible because the gap at the bud neck is so narrow, however, it has led to an ongoing debate as to whether or not the contractile ring is required for cytokinesis. Another protein required for ring formation is Iqg1, a member of the IQGAP family characterized by an N-terminal calponin-homology domain (CHD) required for interaction with actin (Epp & Chant, 1997; Shannon & Li, 1999), middle IQ motifs for interaction with calponin and binding of myosin light chain Mlc1 (Cheney & Mooseker, 1992; Xie et al., 1994), and a C-terminal GAP-related domain (GRD) thought to be involved in a signaling function (Lippincott & Li, 1998; Shannon & Li, 1999). Iqg1 is recruited to the bud neck during anaphase in an Mlc1-dependent manner (Figure 4) (Boyne et al., 2000; Korinek et al., 2000; Shannon & Li, 2000). Following association with the septin ring, Iqg1 is responsible for the recruitment and assembly of the final component of the actomyosin ring, actin, into filamentous actin (F-actin) (Figure 4) (Boyne et al., 2000; Korinek et al., 2000; Shannon & Li, 2000). F-actin in turn, is required for actomyosin ring contraction. In the absence of a functional actomyosin ring, as in Δ*myo1* cells, cells form abnormally thick, irregular septa, and arrest in chains indicative of cytokinetic and/or

cell separation defects (Watts et al., 1987). In addition, cells that can form an actomyosin ring, but that cannot contract, usually form a septum that is misaligned at the bud neck. Thus, the proper formation of a septum is dependent on the actomyosin ring system to guide septum formation to the correct location, allowing it to occur more efficiently (Vallen et al., 2000).

Several publications point to a function for Hof1 in actomyosin ring contraction. Hof1 contains an N-terminal FCH domain flanked by coiled coil domains, PEST sequences, and a C-terminal SH3 domain, and is a member of the PCH family of proteins (Lippincott & Li, 2000). In a recent study, it was shown that Hof1 is degraded following actomyosin ring assembly, and that degradation was necessary for efficient ring contraction (Blondel et al., 2005). Following activation of the mitotic exit network (MEN), a protein kinase signaling cascade important for exit from mitosis, Hof1 is phosphorylated (Vallen et al., 2000) and targeted for degradation by the E3 ubiquitin ligase SCF^{GRR1} (Blondel et al., 2005). The SCF regulatory protein, Grr1, is seen to localize to the mother bud neck late in mitosis following MEN activation, and to interact with Hof1 through its PEST domain (Blondel et al., 2005). This interaction requires the prior phosphorylation of Hof1, by an as yet unidentified protein. It has been suggested, however, that this factor is a member of the MEN (Vallen et al., 2000). Hof1 accumulates at the mother bud neck in a septin-dependent manner early in the cell cycle, but disappears late in mitosis concurrently with the localization of Grr1 (Blondel et al., 2005). There is growing evidence that MEN components play an important role in the regulation of cytokinesis (Jaspersen et al., 1998). Several MEN components localize the actin ring following ring formation, including the downstream effector kinase Dbf2, whose localization correlates with Hof1 phosphorylation (Corbett et al., 2006). These results strongly suggest that Hof1 is in fact involved in the negative regulation of the actomyosin ring, and that its MEN- and SCF^{GRR1}-dependent degradation is required for efficient actomyosin ring contraction and subsequent disassembly. Thus, Hof1 may act to link the completion of

mitosis with the onset of cytokinesis, in addition to its role in septum synthesis, which is described below (Vallen et al., 2000; Corbett et al., 2006).

Interestingly, it has been proposed that Hof1 and Cyk3 function in coupling the actomyosin ring system with septum formation, thereby providing a mechanism by which these processes are coordinately regulated. This hypothesis was based on localization patterns of both proteins, which were seen to localize to the actomyosin ring just before contraction, despite their apparent roles in septum formation (Lippincott & Li, 1998; Vallen et al., 2000). *HOF1* deletion results in temperature sensitive growth with cells arrested in chains at the non-permissive temperature, but does not affect actomyosin ring assembly or contraction at the permissive temperature (Vallen et al., 2000). Deletion of *HOF1* prevents actomyosin ring contraction at the non-permissive temperature, but has no affect on its assembly. This is similar to the phenotype observed in cells depleted of one of the two chitin synthases, proteins required for the synthesis of cell wall material during septum synthesis (Shaw et al., 1991). In addition, Hof1 is targeted to the septin ring in a Myo1-independent manner, while deletion of both *HOF1* and *MYO1* is synthetic lethal (Vallen et al., 2000). Taken together, these results point to a role for Hof1 in septum formation, independent of the actomyosin ring. Localization of Hof1 changes throughout the cell cycle, initially localizing as a double ring structure at the bud neck. Interestingly, fluorescence microscopy has revealed that during cytokinesis Hof1 rings coalesce and become coincident with the actomyosin ring, in a Myo1-dependent manner (Figure 4) (Lippincott & Li, 1998; Vallen et al., 2000). This localization pattern prompted the suggestion that Hof1, while mainly involved in septum formation, may act to coordinate this event with the actomyosin ring system.

Cyk3 has an SH3 domain at its N-terminus, and was initially identified as a high-copy suppressor of Δ*iqg1* lethality that restores viability without restoring the actomyoisn ring (Korinek et al., 2000). Since deletion of both *MYO1* and *CYK3* results in synthetic lethality, it is likely that Cyk3 is involved in septum formation. Deletion of *CYK3* results in misshapen mother bud necks, a phenotype

that is believed to be a result of defects in secretory targeting or septum synthesis (Korinek et al., 2000). Like Cyk3, Hof1 is also a high-copy suppressor of *Δiqg1*, indicating that Cyk3 and Hof1 perform similar roles in cytokinesis. Deletion of both *HOF1* and *CYK3* is synthetic lethal, raising the possibility that these proteins act in redundant cytokinetic pathways (Korinek et al., 2000). Consistent with these observations, Cyk3 localizes to the neck region just after actomyosin ring assembly, and becomes coincident with the actomyosin ring upon contraction (Korinek et al., 2000). Also in support of a role for Cyk3 in septum formation, is the fact that Δ*myo1* slow growth phenotype can be suppressed by mutations in non-essential components of the APC/C. Iqg1 has been shown to be a direct target of this complex, and mutations in certain APC/C components results in the accumulation of Iqg1, which then interacts with Cyk3 to promote an actomyosin-independent cytokinetic pathway. In addition, Δ*myo1* suppression can be achieved through the overexpression of either Iqg1 or Cyk3 (Ko et al., 2007).

Septum formation occurs concurrently with actomyosin ring contraction, and involves cell wall synthesis to close the gap between mother and daughter cells to complete cell separation. This process is also accompanied by membrane deposition, which is required to close the small opening still present in the plasma membrane following actomyosin ring contraction. Chitin synthases CSII and CSIII function to synthesize cell wall material and localize to the mother bud neck in a septin-dependent manner during telophase (Ziman et al., 1996; DeMarini et al., 1997). A primary septum is laid down between mother and daughter cells (Cabib et al., 2001; Schmidt et al., 2002), followed by synthesis of a secondary septum around the primary one. Cell separation is achieved through the degradation of the primary septum by the chitinase Cts1 (Kuranda & Robbins, 1991). Depletion of both chitin synthases is lethal, and causes defects in both cytokinesis, and cell wall formation, pointing to a role for septum formation in actomyosin ring function (Shaw et al., 1991). Consistent with this, compromising septum formation by depletion of CSII, prevents actomyosin ring contraction (Bi, 2001). Thus the correct and

efficient functioning of both septum formation and the actomyosin ring requires the close coordination of these two processes.

Recent work has shown that Orc6 is required in G1 phase following origin licensing, for the continued association of the MCM complex with origin sequences (Semple et al., 2006; Chen et al., 2007). However, these results conflict with previous models of pre-RC assembly, which predicted that following MCM loading at origins, the remaining pre-RC components were dispensable for replication. A recently proposed model that would reconcile this role for Orc6 with previous research, is one in which an MCM unloading activity is invoked throughout G1 phase in WT cells, that is overcome by the normal functioning of the pre-RC. This hypothesis would predict that any protein required for pre-RC assembly, would also be required for its maintenance throughout G1 phase (Chen et al., 2007). Thus, the late G1 depletion of any one of these proteins should destabilize pre-RCs from origins, while its resynthesis should lead to pre-RC reassembly. This is true for Orc6, and preliminary results have shown that Cdc6 may also function in pre-RC maintenance in late G1 phase (Semple et al., 2006; Da-Silva, 2007). The characterization of roles for pre-RC proteins in MCM maintenance at origins is of key importance since changes in the levels, or activity of these proteins can result in either over- or under-replication of DNA. This in turn can often have deleterious consequences for genome integrity (Piatti et al., 1995, Nguyen et al., 2001). A greater understanding of pre-RC protein functioning is therefore important, since in higher eukaryotes such perturbations may ultimately result in cancer (Shima et al., 2007).

The objectives for Chapter 3 of the present study were to confirm preliminary results indicating that Cdc6 is involved in MCM maintenance at origins in late G1 phase (Da-Silva, 2007), and to determine whether this pre-RC maintenance function is also shared by Cdt1. To answer these questions, either Cdc6 or Cdt1 was depleted from cells in late G1 phase, and the effects of depletion on

cell cycle progression, and MCM association with origins, were monitored. It was expected that if Cdc6 and Cdt1 are involved in MCM maintenance at origins of replication, the late G1 phase depletion of either of these pre-RC proteins would result in the destabilization of MCMs from origin sequences. As a confirmation of the role for Cdc6 in pre-RC maintenance, depleted cells were allowed to resynthesize Cdc6 in late G1 phase, and the effects of this resynthesis on cell cycle progression and MCM association with origins, were again monitored. It was expected that if Cdc6 functions in pre-RC maintenance, that resythesis of Cdc6 following depletion in late G1 phase, would result in a retargeting of MCMs to origin sequences, and allow cells to replicate their DNA.

In addition to its role in DNA replication, Orc6 has been shown to be important for other cell cycle regulated events such as chromosome segregation, and cytokinesis in metazoans. *Drosophila* Orc6 has an important role in cytokinesis, a function mediated by an Orc6 C-terminal cytokinetic domain (Chesnokov et al., 2003). Additionally, Orc6 in human cells localizes to structures important for chromosome segregation. Consistent with this, silencing of Orc6 using siRNA causes an increase in polyploidy, as well as the accumulation of cells with multi-polar spindles, aberrant mitosis, multiple nuclei, and defects in DNA replication (Prasanth et al., 2002). As a result of these findings, it has been suggested that Orc6 may function as a signaling molecule, to coordinate important cell cycle stages such as DNA replication, chromosome segregation, and cytokinesis, ensuring that these processes are each completed correctly, and in the right order (Prasanth et al., 2002). To date, Orc6 has not been shown to be involved in cytokinesis in budding yeast, however, ORC is known to have multiple functions, including a role in silencing of the HML and HMR mating type loci in yeast (Foss et al., 1993; Fox et al., 1995), and heterochromatin formation in metazoans (Pak et al., 1997). Work in our lab by former Ph.D. student Jeff Semple, has identified a physical interaction between budding yeast Orc6 and Hof1, a protein known to be involved in cytokinesis (Semple, 2006). This interaction was

identified through a two-hybrid screen of the entire genome, followed by additional yeast-two-hybrid analysis and co-immunoprecipitation to confirm the interaction. A role for Orc6 in cytokinesis would be consistent with the previously hypothesized function for this protein in the coordination of chromatin dynamics and cell cycle stages (Prasanth et al., 2002), and would therefore implicate Orc6 in the maintenance of genomic integrity throughout the cell cycle.

The objective of Chapter 4 of the present study was to investigate whether Orc6 has a role in cytokinesis in budding yeast. To help answer this question, the effects of Orc6 depletion in asynchronous cells containing a *CYK3* deletion, were monitored. Both FACS analysis of cell cycle progression and cell scoring were used to detect any increases in cytokinetic defects as a result of Orc6 depletion. Finally, cell synchronization experiments were used to deplete cells of Orc6 at a time after their DNA had been replicated, in order to monitor the effects of depletion on later cell cycle stages, such as cytokinesis, without interfering with DNA replication. In all cases, it was hypothesized that if Orc6 does function in cytokinesis, depletion of Orc6 would result in an increase in cytokinetic defects.

Chapter 2

Materials and Method

The genotypes of the yeast strains used in this study are described in Table l. For all strains used, DY 26 is the isogenic WT from which they were constructed. Since *ORC6*, *CDC6*, and *CDT1* are essential for viability, *GAL1* strains were used to deplete Orc6, Cdc6, and Cdt1, rather than deleting the genes entirely. The *GAL1* promoter has been integrated into the ORF of the genes whose protein products were depleted throughout this study, to replace the endogenous promoter. *GAL1* directs high levels of transcription in GAL medium, but is strongly repressed in GLU medium. Therefore, protein depletion is achieved by simply growing these strains in GLU medium. As part of the construction of all strains used, a sequence encoding three copies of the HA epitope was fused to the ORF of the genes of interest, in order to facilitate the monitoring of protein levels by immunoblot. For simplicity, the term 'WT' will be used to describe the HA-tagged isogenic parental strain, although it is acknowledged that this strain is not truly WT. All strains used are haploid, MAT**a** strains. MAT**a** strains can be synchronized in late G1 phase with α-factor.

Table 1: Genotypes of yeast strains used in this study

Strain	Genotype	Source
DY 26	MAT**a**, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0	ATCC BY4733
DY 36	MAT**a**, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, orc6::Pgal-3HA-ORC6/TRP1	Semple et al., 2006
DY 93	MAT**a**, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, orc6::ORC6-3HA/TRP1	Semple et al., 2006
DY 135	MAT**a**, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, orc6::ORC6-3HA/TRP1, cyk3::HIS3	This study
DY 136	MAT**a**, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, orc6::Pgal-3HA-ORC6/TRP1, cyk3::HIS3	This study
DY 139	MAT**a**, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, cdc6::Pgal-3HA-CDC6/TRP1	A. Broom
DY 140	MAT**a**, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, cdt1::Pgal-3HA-CDT1/TRP1	A. Broom
DY 142	MAT**a**, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, cdc6::CDC6-3HA/TRP1	L. DaSilva
DY 143	MATa, his3Δ200, leu2Δ0, met15Δ0, trp1Δ63, ura3Δ0, cdt1::CDT1-3HA/TRP1	L. DaSilva

Genomic integration and gene deletion

Genomic *CYK3* deletion was accomplished through homologous recombination of a transformed *HIS* selectable marker gene with *CYK3* flanking sequences. Initially, *HIS* was amplified from pFA6a-HIS3MX6 plasmid template as described by Longtine et al. (1998), using a primer set (primer sequences are described in Table 2) that anneals to promoter and terminator regions of *HIS*, and which also contain 5' regions homologous to flanking regions of *CYK3*. Thus, *HIS* amplification using these PCR primers allowed for the incorporation of regions homologous to *CYK3* flanking sequences into the ends of the *HIS* gene. PCR amplification of the selectable marker, as well as subsequent purification and transformation into DY 93, and DY 36 yeast strains (refer to Table 1 for genotypes), were performed using basic molecular biology techniques (Burke et al., 2000). Transformants were plated on media lacking histidine, and two rounds of replica plating were performed. Following replica plating, individual colonies were used to inoculate 10 ml of medium lacking histidine, grown to saturation, and genomic DNA was isolated (see below) for each. *CYK3* deletion was confirmed through PCR analysis of genomic DNA using primers specific for regions flanking the *CYK3* gene (Table 2).

Yeast genomic DNA isolation

Yeast genomic DNA was isolated as previously described in Burke et al. (2000). Specifically, 10 ml of saturated culture was washed with ddH_2O, and resuspended in 0.2 ml DNA isolation mix (10 mM Tris-HCl pH=8, 100 mM NaCl, 1 mM EDTA, 2% Triton X-100, 1% SDS), and placed in a 2 ml screw cap tube. In order to lyse cells, 0.5 g glass beads were added and samples were vortexed for 3-4 min. Following lysis, 0.2 ml TE pH=8 was added, and samples was gently mixed by inversion. Samples were then centrifuged for 5 min at 16 000 x g, and the supernatant was saved. To this, 1 ml of room temperature 100% EtOH was added, mixed gently by inversion, and centrifuged again for 2 min

at 16 000 x g. The DNA pellet was resuspended in 0.4 ml TE pH=8 containing 100 µg/ml RNAse A, and incubated at 37°C for 15 min. Ammonium acetate was then added to a final concentration of 0.1 M, followed by the addition of 1 ml of room temperature 100% EtOH, and the entire solution was mixed by inversion. Following a final 2 min centrifugation at 16 000 x g, the DNA pellet was air dried, and resuspended in 50 µl TE pH=8.

Cell imaging and scoring

In order to image yeast cells for scoring purposes during protein depletion time courses, 3 µl of culture was removed and placed on a slide, covered with a cover slip, and sealed around all edges with nail polish. Cells were visualized using a Zeiss Axiovert 200 inverted microscope equipped with a LudI motorized stage and Qimaging retiga 1494 digital camera. Multiple pictures were taken for each strain at each time point to allow for the scoring of a large number of cells. Cell scoring was completed by one person, in a blind experiment using the guidelines that follow. Each cell was categorized as either budded or unbudded. Budding cells were further categorized as budding normally or aberrantly. An aberrantly budding cell is one in which the bud neck appears large, bent, or misshapen, and is described further in Korinek et al., as the characteristic morphology of fifty percent of *Δcyk3* cells (Korinek et al., 2000). In order to ascertain what percentage of an entire population was budding aberrantly, it was important to determine the total number of cells counted. In order to remain consistent, a cell that was budding but whose bud was almost equal in size to the mother cell was counted as two cells, since these cells were near separation. Those cells whose bud was significantly smaller than the mother cell was counted as only one cell. The total number of cells counted for each strain at each time point are as follows: nGAL = 480, nGLU = 384 for the WT strain; nGAL = 378, nGLU = 588 for the *GAL1-ORC6* strain; nGAL = 558, nGLU = 330 for the *Δcyk3* strain; and nGAL = 504, nGLU = 384 for the *GAL1-ORC6/ Δcyk3* strain.

Protein isolation and Western blotting

Yeast whole-cell extracts (WCE) were prepared according to Burke et al. (2000). Specifically, 100-200 ml of culture (1-2x10^7 cells/ml) was centrifuged, the supernatant discarded, and the pellet resuspended in 400 µl ice-cold lysis buffer (10 mM Tris-HCl pH=8, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, protease inhibitors). 0.5 g glass beads were added to samples, and cells were lysed at 4°C, using a Biospec Minibead beater 8, with 8 rounds of 30 s lysis/30 s incubation on ice. Cell lysates were then centrifuged, again at 4°C for 1 min at 16,000 x g to remove cell debris. Protein concentration was quantified by Bradford assay (Bradford, 1976) using BioRad, a protein assay solution. Protein expression was then determined by Western blot analysis. Blots were analyzed on a Typhoon 9400.

Fluorescence activated cell sorting (FACS) analysis

Approximately 10^7 cells were centrifuged, resuspended in 1 ml 70% ice-cold EtOH to fix cells, and stored at 4 °C. Cells were then centrifuged, washed once with 0.5 ml ddH_2O, resuspended in 0.5 ml 50 mM Tris-HCl pH=8 containing 0.2 mg/ml RNase A, and incubated for 2-4 h at 37°C. Cells were then centrifuged, resuspended in 0.5 ml 50 mM Tri-HCl pH=7.5 containing 2 mg/ml proteinase K, and incubated for 30-60 min at 50°C. Cells were then centrifuged, resuspended in 0.1 ml FACS buffer (200 mM Tris-HCl pH=7.5, 200 mM NaCl, 78 mM $MgCl_2$), and added to 0.5 ml Sytox solution (50 mM Tris-HCl pH=7.5, 1:5000 dilution Sytox green) to stain DNA. Samples were then analyzed on a Beckton Dickinson FACSVantage SE flow cytometer.

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation (ChIP) was performed as previously described in Tanaka et al. (1997), but with the following modifications. A final concentration of 1% formaldehyde was added to approximately 5x10^8 cells (50 ml; 0.5-2x10^7cells/ml) for 20 min at 30°C, with gentle shaking, to cross-

link protein to DNA. The reaction was quenched with a final concentration of 125 mM glycine for 5 min at 30°C, with gentle shaking. Cells were then centrifuged, washed once with 40 ml ice-cold PBS, snap frozen in liquid nitrogen, and stored at -80°C. Cell lysis was performed at 4°C with 0.5 g glass beads in 0.5 ml lysis buffer (50 mM HEPES-KOH pH=7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton-X100. 0.1% sodium deoxycholate, protease inhibitors), with 8 rounds of 30 s lysis/30 s incubation on ice, using a Biospec Minibead beater 8. Sample was separated from glass beads by centrifuging for 30 s at 1000 x g into a 2 ml tube. Samples were then centrifuged 5 min at 16000 x g to separate soluble from insoluble fractions of the WCE; the soluble fraction was discarded, and the insoluble fraction was resuspended in 0.5 ml lysis buffer. Sonication of samples was performed at 4°C, with 5 rounds of 20 s sonication at 5-6W/2 min incubation on ice, using a Microsonix Microson XL 2000 sonicator, to achieve an average DNA fragment size of 500 bpIN. Samples were then centrifuged for 2 min at 4500 x g, to remove cell debris; the supernantant was removed and saved. 25 µl of the sonicated WCE was saved as input. Immunoprecipitation was performed with 30 µl anti-goat IgG agarose beads (Sigma A9294), which were pre-incubated with 2 mg of each of anti-Mcm2 (Santa Cruz sc-6680) and anti-Mcm5 (Santa Cruz sc-6686) antibodies; samples were incubated at 4°C, overnight, on a rotator. Samples were then washed, and processed as described in Tanaka et al., (1997). DNA samples were resuspended in a final volume of 60 µl and PCR was carried out in a total volume of 20 µl. PCR was carried out with 0.5 µl input (IN), and 2 µl immunoprecipitated (IP) samples using two sets of primers in each reaction as follows: 0.5 mM dNTP, 0.4 ρM upstream *ARS1* primers, and 1.1 ρM *ARS1*-specific primers (Table 2). The PCR program used included an initial 5 min at 95°C, followed by 34 rounds of (30 s at 95°C, 30 s at 50°C, 1 min at 72°C), followed by a final 7 min at 72°C. In all cases, PCR products were separated on a two percent agarose gel.

Synchronizing yeast cultures

Alpha factor arrest and protein depletion in GAL1 strains

Late G1 phase arrest with α-factor, and subsequent protein depletion were performed as previously described in Semple et al. (2006). Cells were grown to 1 x 10^7 cells/ml in YPG/R (1% yeast extract, 2% peptone, 2% galactose, 1% raffinose), centrifuged, washed once with ddH_2O, resuspended in fresh YPG/R containing 10 μg/ml α-factor, and grown for 3 h at 30°C with shaking (α-factor was replenished after 1.5 h with 10 μg/ml), to arrest cells in late G1 phase. Cells were then centrifuged, washed once with ddH_2O, once with YPD (1% yeast extract, 2% peptone, 2% glucose), and resuspended in YPD containing 10 μg/ml α-factor for protein depletion. Cdc6 (*GAL1-CDC6*) was depleted for 3 h, and Cdt1 (*GAL1-CDT1*) for 8 h, both growing at 30°C with shaking. For protein depletion, α-factor was replenished after every hour with 10 μg/ml. For resynthesis of Cdc6, cells were then centrifuged, and each culture was split into two, washed once with ddH2O, once with either YPG/R or YPD, and resuspended in either YPG/R (for protein resynthesis) or YPD (to continue protein depletion), respectively, both containing 100 μg/ml pronase E. Cells were then grown for 2 h at 30°C with shaking.

Nocodazole arrest and protein depletion in GAL1 strains

Nocodazole was used to arrest cells at the G2/M boundary prior to protein depletion. Cells were grown in YPG/R to1x10^7 cells/ml, and arrested in late G1 phase using α-factor as previously described in Semple et al. (2006). Cells were then centrifuged, washed with TBS (20 mM Tris, 150 mM NaCl), resuspended in YPG/R containing 15 μg/ml nocodazole, and grown for 1.5 h at 30°C with shaking, to arrest cells. Cells were then centrifuged, washed with TBS, resuspended in YPD containing 15 μg/ml nocodazole, and incubated for 4 h at 30°C with shaking, to deplete Orc6 in the *GAL1* strains.

Nocodazole was replenished at 1, 2, and 3 h of depletion in YPD with 15 μg/ml each time. Finally, cells were centrifuged, washed twice with TBS, once with YPD, and released in YPD (to maintain protein depletion) containing 10μg/ml α-factor. Cells were then grown for 1 h at 30°C with shaking.

Table 2: PCR primers used in this study

Gene/Region	Name	Sequence (5' ➔ 3')	Description
CYK3	*Cyk3* del fwd 1	ATTTAATTCCTGAATTTACCGTATTACATTTAAATTTGCATACGGA	Forward primer to amplify *HIS* selectable marker gene
CYK3	*Cyk3* del rev 1	TGATACAGATTATAGCGCTGTAAAAATTTGTGAAAAACGTGAA	Reverse primer to amplify *HIS* selectable marker gene
CYK3	*Cyk3* fwd flank	CCTTAATATTTTAACACATTAACTGTTTGC	Forward primer to confirm *HIS3* replacement of *CYK3* in genome
CYK3	*Cyk3* rev flank	ACATCACTAAATACGAAAAAGTTGGACCAG	Reverse primer to confirm *HIS3* replacement of *CYK3* in genome
ARS1 -2kb	*ARS1* up fwd A	TAAACGGCAAACTAAAATACAAACAAT	Forward primer to amplify a region 2 kb upstream from the *ARS1* origin of replication
ARS1 -2kb	*ARS1* up rev A	ACGAAAATGTTACCGCTGGC	Reverse primer to amplify a region 2 kb upstream from the *ARS1* origin of replication
ARS1	*ARS1* fwd	CGGAGGTGTGGAGACAAATGGTG	Forward primer to amplify the *ARS1* origin of replication
ARS1	*ARS1* rev	GGTAAAAGTCAACCCCCTGCGATG	Reverse primer to amplify the *ARS1* origin of replication

Chapter 3

Multiple Pre-replicative Complex Components are Required in Late G1 Phase to Maintain MCM Association with Origins of DNA Replication

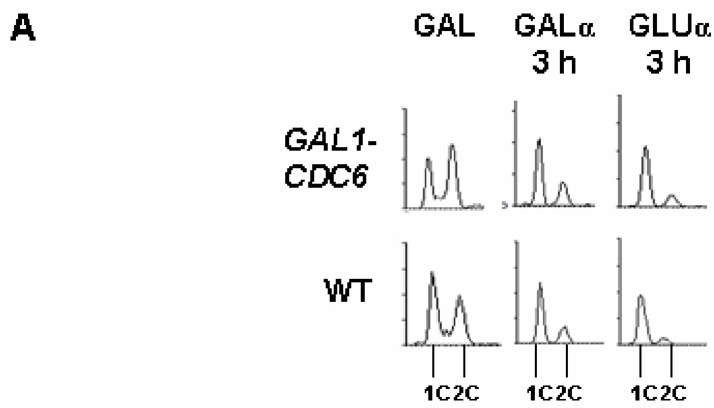
Results

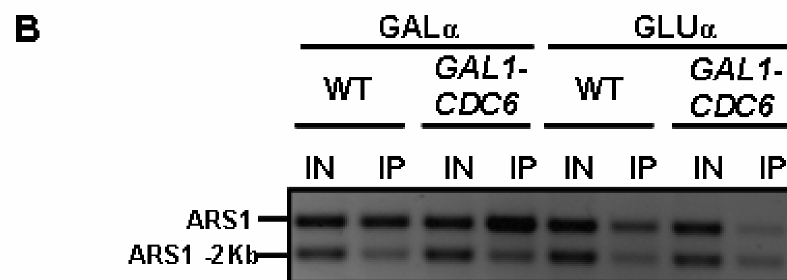
In order to confirm a previously reported role for Cdc6 in pre-RC maintenance following origin licensing in G1 phase (Aparicio et al., 1997; Da-Silva, 2007), and to determine whether Cdt1 has a similar role, transcriptional shut-off strains, *GAL1-CDC6* (DY 139), and *GAL1-CDT1* (DY 140), along with isogenic WT strains (DY 142, and DY 143, respectively), were used in cell synchronization experiments to monitor the effects of pre-RC component depletion in late G1 phase, on cell cycle progression, and MCM-origin association. A former lab member, Lance DaSilva, performed the initial characterization of these strains, and showed that both *GAL1-CDC6,* and *GAL1-CDT1* strains demonstrated a rapid turnover of Cdc6 and Cdt1 proteins, respectively, following a shift from GAL to GLU medium. In particular, Cdc6 was shown to be depleted to below endogenous levels by 1 h growth in GLU medium, while Cdt1 was similarly depleted by 2 h. In the case of the *GAL1-CDC6* strain, a rapid G1 phase arrest was also observed, as judged by an accumulation of cells with a 1C DNA content only 1 h after the medium switch. In contrast, the *GAL1-CDT1* strain did not show defects in S phase progression until 8 h growth in GLU medium (Da-Silva, 2007).

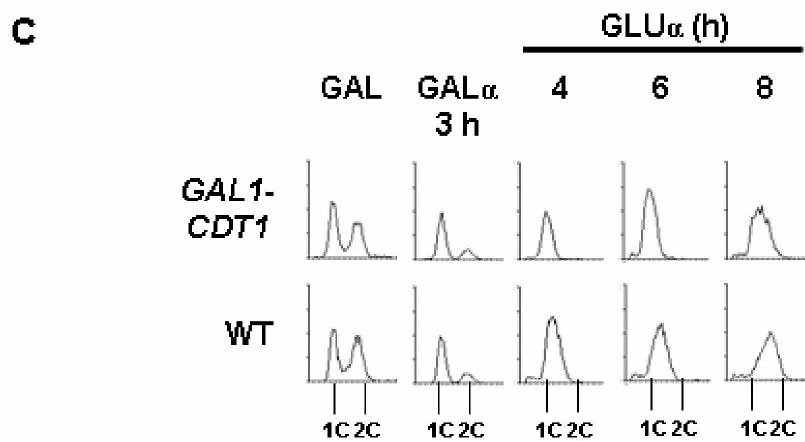
To assess whether Cdc6 and Cdt1 are required in late G1 phase, *GAL1-CDC6*, and *GAL1-CDT1* strains, along with their WT counterparts, were grown on GAL medium, arrested with α-factor (GALα), and switched to GLU medium containing α-factor (GLUα) for 3 h (*GAL1-CDC6*), and 8 h (*GAL1-CDT1*), to deplete Cdc6 and Cdt1, respectively. FACS analysis was used to ensure that late G1 phase cell cycle arrest was maintained throughout the depletion, as evinced by the presence of a 1C peak in FACS samples taken following arrest in α-factor. ChIP analysis was used to monitor MCM association with *ARS1* origin sequences. Enrichment/destabilization of MCMs at origin sequences can be assessed by comparing IN to IP DNA between origin (*ARS1*) and non-origin (*ARS1* -2kb) sequences. As can be seen from Figure 5, depletion of Cdc6 and Cdt1 leads to a loss of MCMs from *ARS1* sequences. Specifically, from Figure 5B it can be seen that the late G1 phase depletion of Cdc6 (GLUα)

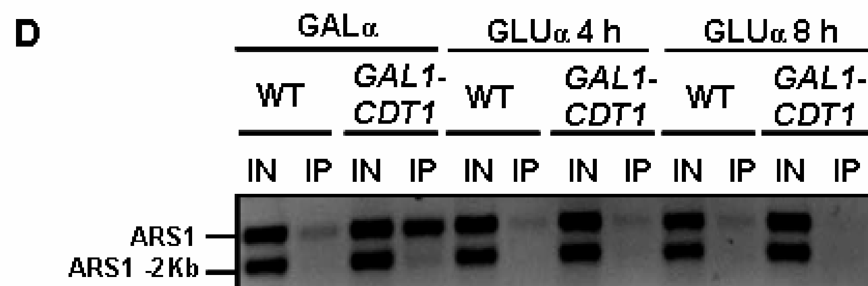
leads to a destabilized of MCMs from *ARS1* as compared to MCM-*ARS1* association in the WT strain (compare *GAL1-CDC6* strain IN to IP with WT strain for GLUα). FACS analysis confirms that late G1 phase α-factor arrest held throughout the time course, since cells from both *GAL1-CDC6* and WT strains remain arrested with a 1C DNA content (Figure 5A). In contrast, FACS analysis shows that *GAL1-CDT1* and WT strains did not hold in the α-factor arrest following 6 h and 8 h growth in GLUα medium respectively, as can be seen by progression of these cells to mid S phase (Figure 5C). Nevertheless, it can be seen that depletion of Cdt1 also leads to the destabilization of MCMs from *ARS1*, however, this destabilization is much more subtle than in the case of Cdc6 depletion (Figure 5D, compare *GAL1-CDT1* strain IN to IP with WT strain for GLUα 8 h).

Figure 5: Depletion of either Cdc6 or Cdt1 in late G1 phase leads to the destabilization of MCMs from origins of DNA replication. In order to determine whether the previously characterized role for Orc6 in MCM maintenance at origins, is shared by other pre-RC components, *GAL1-CDC6* (DY 139), and *GAL1-CDT1* (DY 140) strains, along with WT counterparts (DY 142 and DY 143, respectively), were grown in GAL medium, arrested in late G1 phase with α-factor (GALα), then switched to GLU medium containing α-factor (GLUα) for protein depletion in the *GAL1* strains. Cdc6 was depleted for 3 h, while Cdt1 was depleted for 8 h. FACS analysis was used to monitor maintenance of the α-factor arrest throughout the time course, while ChIP analysis was used to monitor MCM association with origin sequences (*ARS1*). As a negative control for association of MCMs with origins, MCM association with non-origin sequences (*ARS1* -2 kb) was also monitored. As described in the materials and methods, the IN DNA was sonicated only, while the IP DNA was immunoprecipitated with a combination of anti-Mcm2 and anti-Mcm5 antibodies, based on the binding of MCM proteins to DNA sequences. PCR was performed using IN or IP DNA as a template, and primer sets specific to *ARS1* and *ARS1* -2 kb DNA sequences. When interpreting ChIP results, it is important to compare the ratio of intensity of origin specific to non-origin specific bands between IP and IN samples of the same time point, or between IPs of different time points. If MCMs are associated with origin sequences, a DNA gel should show an enrichment for origin specific over non-origin specific sequences in the IP sample when compared to the IN sample at the same time point. A) Culture aliquots for FACS analysis were taken at the indicated time points for the Cdc6 time course. Following arrest in GALα medium, it can be seen that the majority of cells have a 1C DNA content, indicating that the late G1 phase arrest held throughout the depletion B) ChIP analysis reveals that MCMs are destabilized from *ARS1* following Cdc6 depletion (GLUα) in late G1 phase, since the *ARS1* -2kb band appears more enriched than the *ARS1*-specific band for the *GAL1-CDC6* strain when compared to the IN, as well as to the IP for the WT at the same time point. Comparison with the *GAL1-CDC6* strain GALα IP is consistent with this result since this sample shows an enrichment for origin specific sequences compared to the IN, and has not yet been depleted of Cdc6. C) Following arrest in GALα medium, FACS analysis for the Cdt1 time course showed that the late G1 arrest did not hold throughout the depletion, since samples from *GAL1-CDT1* strain at 8 h, and WT strain at 6 h, and 8 h, indicate that the majority of cells are in S phase. D) Nevertheless, ChIP analysis of Cdt1 depletion is consistent with MCMs being destabilized from origin sequences following 8 h depletion, although the destabilization is more subtle than for Cdc6 depletion. **NOTE: GAL = transcription ON; GLU = transcription OFF for the *GAL1* strains**


A
GAL
GALα 3 h
GLUα 3 h
GAL1-CDC6
WT
1C 2C
1C 2C
1C 2C

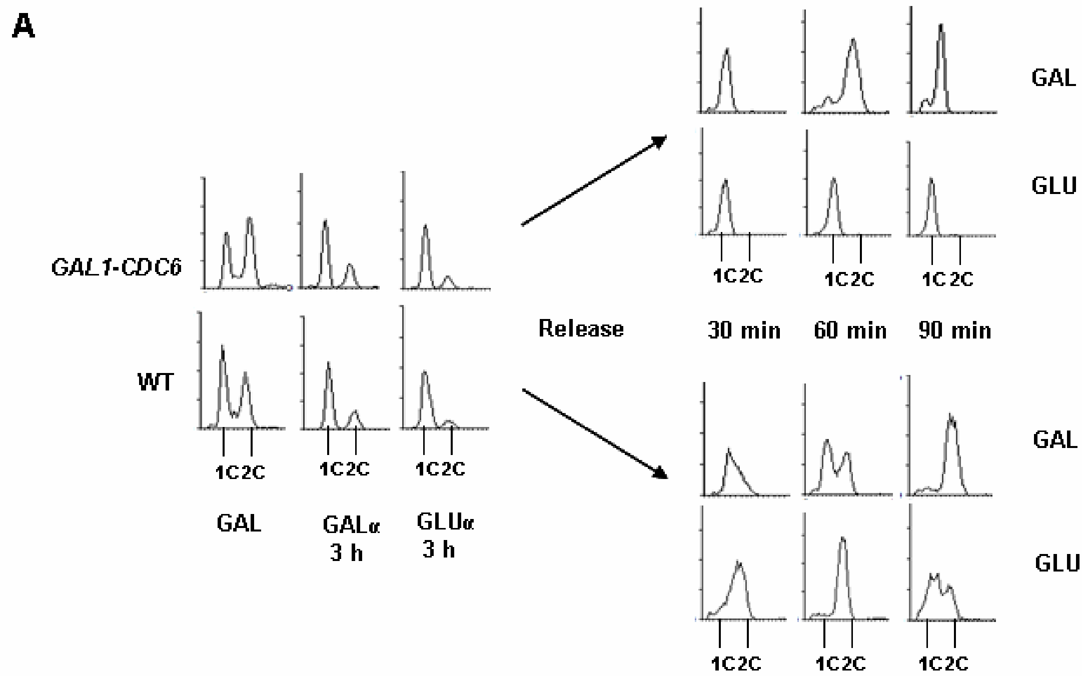

B
GALα
GLUα
WT
GAL1-CDC6
WT
GAL1-CDC6
IN IP IN IP IN IP IN IP
ARS1
ARS1 -2Kb

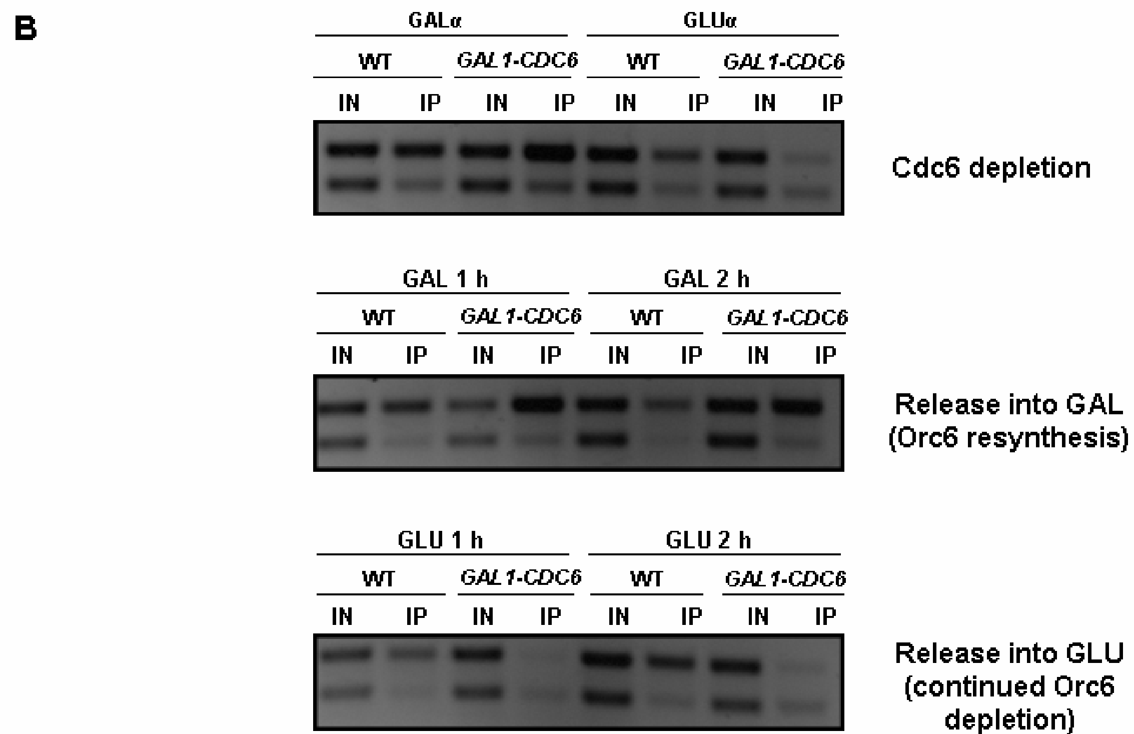

C
GLUα (h)
GAL
GALα 3 h
4
6
8
GAL1-CDT1
WT
1C 2C
1C 2C
1C 2C
1C 2C
1C 2C


D
GALα
GLUα 4 h
GLUα 8 h
WT
GAL1-CDT1
WT
GAL1-CDT1
WT
GAL1-CDT1
IN IP IN IP IN IP IN IP IN IP IN IP
ARS1
ARS1 -2Kb

In order to determine if Cdc6 resynthesis in late G1 phase retargets MCMs to origin sequences, *GAL1-CDC6*, and WT strains were arrested in late G1 phase in GAL medium, then switched to growth in GLUα medium for protein depletion. Following this, cultures were split, and half was released into GLU medium to continue protein depletion, while the other half was released into GAL medium to resynthesize Cdc6. As can be seen from Figure 6A, *GAL1-CDC6* cells that were released into GAL medium to resynthesize Cdc6 following protein depletion, are fully competent to complete DNA replication, as evidenced by the FACS sample progression from a 1C to 2C DNA content with a rate comparable to WT strain. In contrast, *GAL1-CDC6* cells released into GLU medium to maintain protein depletion remain arrested with a 1C DNA content, indicating that these cells are unable to enter S phase. As expected, the WT strain is able to complete DNA replication in both cases. Consistent with these results, ChIP analysis in Figure 6B indicates that following Cdc6 depletion, MCMs are retargeted to *ARS1* by 1 h of resynthesis in GAL medium (compare *GAL1-CDC6* strain IN to IP with WT strain at GAL 1 h time point), and remain at origin sequences following 2 h of resynthesis. As a further control, ChIP analysis was also performed on strains released into GLU medium to maintain Cdc6 depletion. As can be seen in Figure 6B, when Cdc6 resynthesis is not allowed in the *GAL1-CDC6* strain, MCMs are not retargeted to *ARS1*, consistent with the notion that it is Cdc6 resynthesis that retargets pre-RC to origin sequences (compare *GAL1-CDC6* strain IN to IP with WT strain at GLU 1 h and GLU 2 h time points).

Figure 6: Resynthesis of Cdc6 in late G1 phase retargets MCMs to origins and restores competence for DNA replication. In order to determine whether the resynthesis of Cdc6 following its depletion, will lead to the retargeting of MCMs to origins in late G1 phase, *GAL1-CDC6* strain (DY 139), along with WT counterpart (DY 142) were arrested in late G1 phase in GALα medium, and switched to GLUα medium to deplete Cdc6. Strains were then split in two, and half was released into GAL medium to resynthesize Cdc6, while the other half was released into GLU medium to continue protein depletion. FACS analysis was used to monitor arrest throughout the deplete, as well as progression through S phase following release, while ChIP was again used to monitor MCM association with origin sequences (for an explanation on how to interpret IN and IP ChIP samples, refer to Figure 5. A) FACS analysis for the Cdc6 time course shows that the majority of cells remained arrested in late G1 phase throughout the 3 h deplete. Following release, it can be seen that *GAL1-CDC6* cells released into GAL medium (to resynthesize Cdc6) are able to progress through S phase, as evidenced by the progression of cells from a 1C to a 2C DNA content. In contrast, *GAL1-CDC6* cells released into GLU medium (to continue Cdc6 depletion), are not competent for DNA replication, since cells remain arrested in late G1 phase with a 1C DNA content. As expected, WT cells are fully able to replicate their genome following release from the late G1 phase block, into either GAL or GLU medium. B) ChIP analysis is consistent with the retargeting of MCMs to origins of replication following resynthesis of Cdc6. Specifically, it can be seen that even following 1 h resynthesis (*GAL1-CDC6* GAL 1 h sample), there is an enrichment of MCMs at origin sequences compared to non-origin sequences. This enrichment is comparable to MCM enrichment at origins in the *GAL1-CDC6* strain GALα medium sample that had not yet been depleted of Cdc6. Also shown is a control ChIP for samples from each strain released into GLU medium. It can be seen that when the *GAL1* strain is not allowed to resynthesize Cdc6, MCMs are not retargeted to origin sequences. **NOTE: GAL = transcription ON; GLU = transcription OFF for the *GAL1-CDC6* strain**


A
GAL1-CDC6
WT
1C2C
GAL
GALα
3 h
GLUα
3 h
Release
GAL
GLU
30 min
60 min
90 min
GAL
GLU


B
GALα
GLUα
WT
GAL1-CDC6
IN
IP
Cdc6 depletion
GAL 1 h
GAL 2 h
Release into GAL
(Orc6 resynthesis)
GLU 1 h
GLU 2 h
Release into GLU
(continued Orc6
depletion)

Discussion

The association of MCM proteins with origins of DNA replication is a key step in promoting cell cycle progression, as this is a requirement for DNA replication (Chong et al., 1995). Since pre-RCs are assembled in early G1 phase (Tanaka et al., 1997), it is essential that the integrity of this complex is preserved until origin firing at the G1/S phase transition. To date, the way in which this association is maintained has not been well characterized. Apart from the ORC-associated protein Mcm10, the extent to which other origin-associated factors play a role in safeguarding licensing for DNA replication is unclear. Mcm10 has been implicated in pre-RC stability, however, the mechanism behind this function is unclear (Kawasaki et al., 2000). Although previously thought to be dispensable after pre-RC assembly, a number of recent studies have indicated that the continued association of ORC subunits with origin sequences in late G1 phase is important to ensure subsequent DNA replication (Weinberger et al., 2005; Gibson et al., 2006; Semple et al., 2006; Chen et al., 2007; Makise et al., 2008). Studies of Cdc6 function using temperature-sensitive *cdc6*-1 mutants have found that shifting these cells to the restrictive temperature in late G1 phase converted the DNaseI protection footprint of the 2μm plasmid origin of replication from a pre-RC to a post-RC pattern (Cocker et al., 1996), and displaced Mcm4 from origin sequences (Aparicio et al., 1997). However, high salt extraction of ORC and Cdc6 from late G1 budding yeast chromatin did not result in an equivalent reduction of MCM proteins (Donovan et al., 1997). These results have contributed to a model proposing that the other pre-RC components were no longer required for Mcm2-7 maintenance at origins following licensing (Randell et al., 2006). Data from this study argues against this, as depletion of Cdc6 and to a lesser extent Cdt1 in late G1 phase leads to a destabilization of MCMs from origin sequences (Figure 5B, D). This study is the first in which the depletion of Cdt1 has been correlated to a loss of MCMs from origins, however, the finding for Cdc6 is consistent with results of a previous study, in which a *cdc6*-1 strain was shifted to restrictive temperature for three hours while being held in late G1 phase. Following release, initiation

of DNA replication was not inhibited to the same extent as seen in this study (Figure 6A), nevertheless, progression through S phase was severely impaired (Detweiler and Li, 1997). Differences in the observed effects may indicate that depletion, rather than conformational change of a mutant, is a more efficient means of reducing Cdc6 activity in G1 phase.

Interestingly, it was also shown in this study that pre-RC disassembly due to depletion of Cdc6 can be efficiently reversed by its resynthesis (Figure 6). These results complement previously reported results for late G1 phase Orc6 depletion and resynthesis (Da-Silva, 2007), and are consistent with the idea that an MCM unloading activity exists in G1 that can be overcome by the pre-RC assembly machinery (Chen et al., 2007). The fact that the reconstituted pre-RCs are fully competent to promote DNA replication indicates that the ability to faithfully rebuild these complexes remains robust even in late G1 phase. This may be an important consideration as human pre-RC components are evaluated as potential anti-cancer drug targets (Gonzalez et al., 2005). Another observation relevant to such applications is that depletion of individual replication factors can vary widely in terms of how quickly, and to what extent DNA replication is inhibited. When comparing the effects of Cdc6 and Cdt1 depletion, it is striking to note that, despite a similar rate of depletion, inhibition of S phase progression occurs much sooner in the case of Cdc6 (Da-Silva, 2007). Furthermore, this slower inhibition of S phase progression correlates with results from Figure 5D, which are consistent with the notion that 8 h of Cdt1 depletion are required before a destabilization of MCMs from origin sequences is observed. These results suggest that levels of Cdc6 are more limiting for pre-RC integrity, and may reflect the fact that it is a core component of this complex as opposed to the more transient nature of origin association reported for Cdt1 (Randell et al., 2006).

Results from this study point to a role for multiple pre-RC components in the maintenance of MCMs at origin sequences following licensing in early G1 phase. These results, along with similar previously reported results for Orc6 (Da-Silva, 2007), help solidify the hypothesis that an MCM

unloading activity exists in G1 phase that is overcome by the normal functioning of the pre-RC components (Chen et al., 2007). The fact that disruption of the regular functioning of Orc6 (Semple et al., 2006), Cdc6, and Cdt1 through late G1 phase protein depletion, leads to a destabilization of MCMs from origins, suggests that when these pre-RC components are compromised, the MCM unloading activity predominates. In contrast, when Orc6 and Cdc6 are resynthesized following depletion in late G1 phase, thereby restoring full functioning of these pre-RC components, MCMs are once again retargeted to origin sequences, since pre-RC assembly can once again overcome the hypothesized G1 phase MCM unloading activity. It will now be of interest to see if resynthesis of Cdt1 also leads to pre-RC reassembly in late G1 phase.

Chapter 4

Characterization of a Potential Role for Orc6 in Cytokinesis

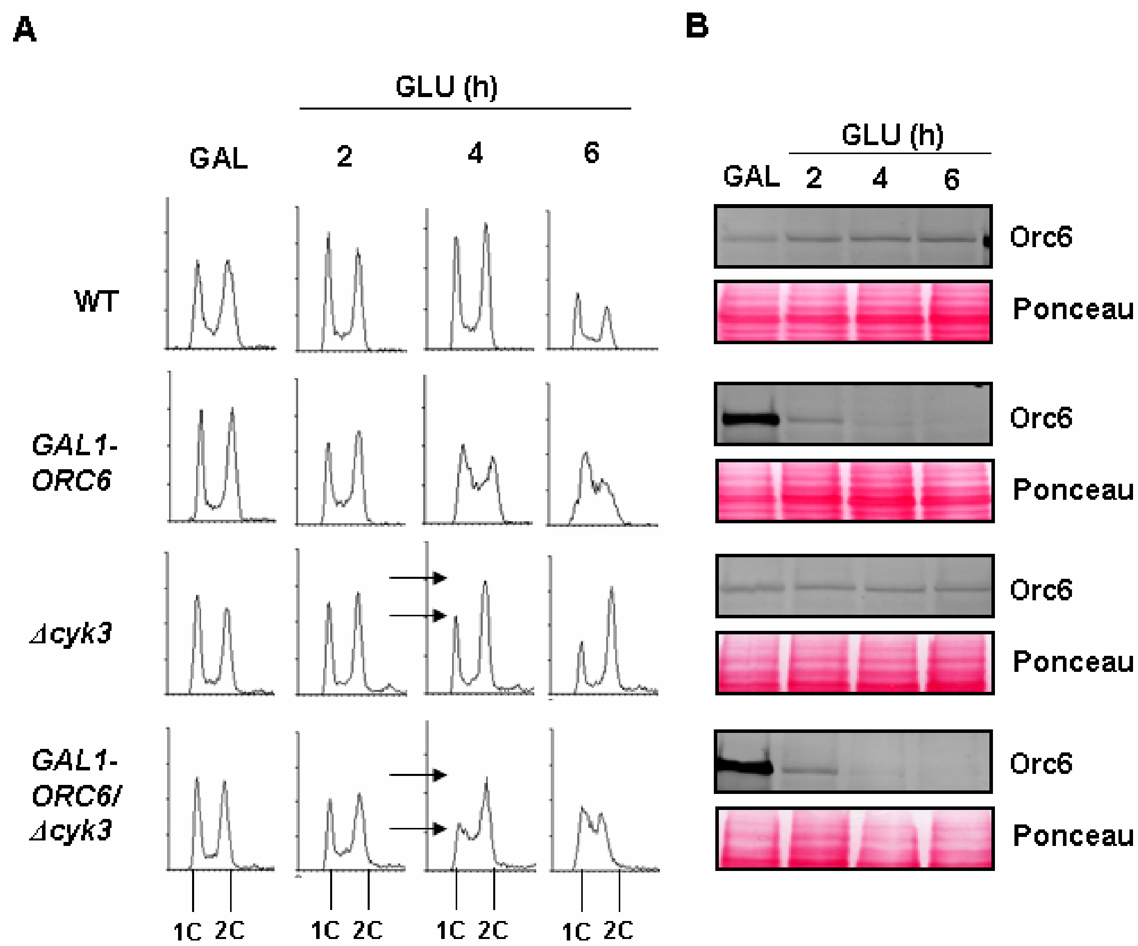
Results

Given that Hof1 and Orc6 physically interact (Semple, 2006), and that a *Δhof1* and *Δcyk3* double mutant produces a synthetic lethal phenotype (Korinek et al., 2000), suggesting redundant roles in cytokinesis, it was hypothesized that Hof1 and Orc6 may function in one cytokinetic pathway, and that Cyk3 functions in a second redundant cytokinetic pathway. This is true at least for Hof1 and Cyk3, since Hof1 functions in actomyosin ring assembly and contraction (Blondel et al., 2005), while Cyk3 functions in a parallel redundant pathway involved in septum formation (Korinek et al., 2000). If this hypothesis is correct, depletion of Orc6 in a strain containing a *CYK3* deletion could lead to a more severe cytokinetic phenotype than deletion of *CYK3* or depletion of Orc6 would alone. This hypothesis was initially tested by performing an asynchronous shift from GAL to GLU medium to deplete Orc6, using WT (DY 93), *GAL1-ORC6* (DY 36), *Δcyk3* (DY 135), and *GAL1-ORC6 Δcyk3* (DY 136) strains, and monitoring cell cycle progression through FACS analysis (Figure 7A). Since Orc6 has an essential role in DNA replication (Li & Herskowizt, 1993), FACS analysis from this type of experiment will show defects in replication in strains depleted of Orc6, in addition to any potential defects in cytokinesis. Given the difficulty in interpreting results from this type of experiment, an asynchronous shift was performed only as a preliminary characterization of a potential role for Orc6 in cytokinesis that did not require the use of cell cycle arresting agents, many of which can be difficult to work with. Cell synchronization experiments are essential for differentiating a role for Orc6 in replication from a potential role in cytokinesis, and are described below. For the asynchronous shift, WT, *GAL1-ORC6*, *Δcyk3*, and *GAL1-ORC6/ Δcyk3* strains were grown in GAL medium, then switched to growth in GLU medium to deplete Orc6. Culture aliquots of each strain were taken every 2 h for a total of 6 h for FACS and Western blot analysis, to monitor cell cycle progression, and Orc6 depletion in the *GAL1* strains, respectively. As previously reported, Orc6 is depleted to below endogenous levels in the *GAL1-ORC6* strain following 4 h growth in GLU medium (Semple et al., 2006). Consistent with this, it can be

seen in Figure 7B that Orc6 is depleted to below endogenous levels in the *GAL1* strains by 4 h growth in GLU medium, when compared to endogenous WT and *Δcyk3* Orc6 levels. It is important to note that WCE samples for WT and *GAL1-ORC6* strains were run on the same immunoblot, as were *Δcyk3* and *GAL1-ORC6/ Δcyk3* (Figure 7B), but were separated in Figure 7 to match up with FACS analysis of the corresponding strains in Figure 7A. Therefore, it is possible to directly compare endogenous Orc6 levels in WT and *Δcyk3* strains to Orc6 levels in *GAL1-ORC6* and *GAL1-ORC6/Δcyk3* strains throughout the deplete. FACS analysis for this asynchronous shift, following depletion of Orc6 to below endogenous levels, is consistent with a role for Orc6 in cytokinesis (Figure 7A). When comparing FACS profiles at the 4 h time point, it can be seen that the difference between the 1C and 2C peak heights in the *GAL1-ORC6/ Δcyk3* FACS profile is greater than for FACS profiles of the *GAL1-ORC6* and *Δcyk3* strains (Figure 7A, arrows indicate differences between 1C and 2C peak heights in the *Δcyk3* and *GAL1-ORC6/ Δcyk3* strains). This suggests that following Orc6 depletion, *GAL1-ORC6*/Δ*cyk3* cells have more difficulty completing the cell cycle, resulting in a slower progression. Consistent with previously reported results using a non-isogenic Δ*cyk3* strain, deletion of *CYK3* produces a mild cytokinetic defect (Korinek et al., 2000) as evidenced by the 4 h FACS profile for this strain, which also has a more prominent 2C than 1C peak. However, deletion of *CYK3* cannot fully explain the defects in cell cycle progression observed in the *GAL1-ORC6*/Δ*cyk3* strain, since the difference in peak heights is not as large in the Δ*cyk3* strain 4 h FACS profile, indicating that this strain has less difficulty completing the cell cycle. Therefore, these results are consistent with a role for Orc6 in cytokinesis, since depletion of Orc6 in the *GAL1-ORC6*/Δ*cyk3* strain results in a more pronounced defect in cytokinesis than can be explained by *CYK3* deletion, or Orc6 depletion alone. As can be seen in the 4 h FACS sample for the *GAL1-ORC6* strain*,* depletion of Orc6 has consequences for DNA replication, resulting in cells accumulating with a 1C DNA content due to an inability to replicate their DNA. Replication defects are also seen in the 4 h FACS profile for the *GAL1-ORC6/ Δcyk3* strain,

making it more difficult to identify potential cytokinetic defects through FACS analysis. For this reason we were interested to know what effect a G2/M phase Orc6 depletion and subsequent release would have on *GAL1-ORC6*/Δ*cyk3* when compared to *GAL1-ORC6* and *Δcyk3* strains. This cell synchronization experiment is described in detail below.

Figure 7: FACS analysis of a *GAL1-ORC6/Δcyk3* strain depleted of Orc6 is consistent with a role for Orc6 in cytokinesis. Since Orc6 and Hof1 physically interact, and *Δhof1* and *Δcyk3* are synthetic lethal, it was hypothesized that Hof1 and Orc6 are involved in a cytokinetic pathway that acts in parallel to a redundant cytokinetic pathway involving Cyk3. If correct, this would predict that depletion of Orc6 in a strain containing a *CYK3* deletion would cause a more severe defect in cytokinesis than Orc6 depletion or *CYK3* deletion would alone. Therefore, in order to characterize a potential role for Orc6 in cytokinesis, an asynchronous shift from GAL to GLU medium was performed with a *GAL1-ORC6*/*Δcyk3* (DY 136) strain and the effects of Orc6 depletion on cell cycle progression were monitored through FACS analysis. Specifically, a *GAL1-ORC6*/*Δcyk3* strain, along with control strains WT (DY 93), *GAL1-ORC6* (DY 36), and *Δcyk3* (DY 135) were grown in GAL medium, then switched to growth in GLU medium to deplete Orc6. Culture aliquots for each strain were removed at the indicated time points for FACS analysis, and for WCE samples to monitor Orc6 levels. A) FACS analysis of the *GAL1-ORC6*/*Δcyk3* strain, following depletion of Orc6 to below endogenous levels, is consistent with a role for Orc6 in cytokinesis. When comparing FACS profiles between strains at the 4 h time point (at this point, Orc6 has been depleted to below endogenous levels in the *GAL1* strains; see Figure 7B), it can be seen that the difference between 1C and 2C peak heights (arrows show difference between 1C and 2C peak heights) for the *GAL1-ORC6*/*Δcyk3* strain FACS profile is greater than for FACS profiles of either *GAL1-ORC6* or *Δcyk3* strains. This is consistent with the idea that following Orc6 depletion, *GAL1-ORC6*/Δ*cyk3* cells have more difficulty completing the cell cycle, resulting in a slower cell cycle progression. B) Western blots showing Orc6 levels for each strain at the indicated time points are shown to confirm Orc6 depletion in the *GAL1* strains. WCE samples for WT and *GAL1-ORC6* strains were run on the same blot, as were *Δcyk3* and *GAL1-ORC6/ Δcyk3*, but were separated for this figure, to align with FACS profiles from the corresponding strain. It can be seen that following 4 h growth in GLU medium, Orc6 is depleted to below endogenous levels in the *GAL1* strains when compared to WT and Δ*cyk3* strains. As part of the construction of each of the four strains used in this asynchronous shift, three copies of the HA epitope were fused to the ORF of *ORC6* to facilitate the comparison of endogenous Orc6 levels with Orc6 levels in the *GAL1* strains. Orc6 was detected using an anti-HA antibody (1:5000 mouse monoclonal) and fluorescent secondary antibody (1:3000, Alexafluor 488 goat anti-mouse). Ponceau stains are shown beneath each blot to confirm equal protein loading. **NOTE: GAL = transcription ON; GLU = transcription OFF for the *GAL1* strains**



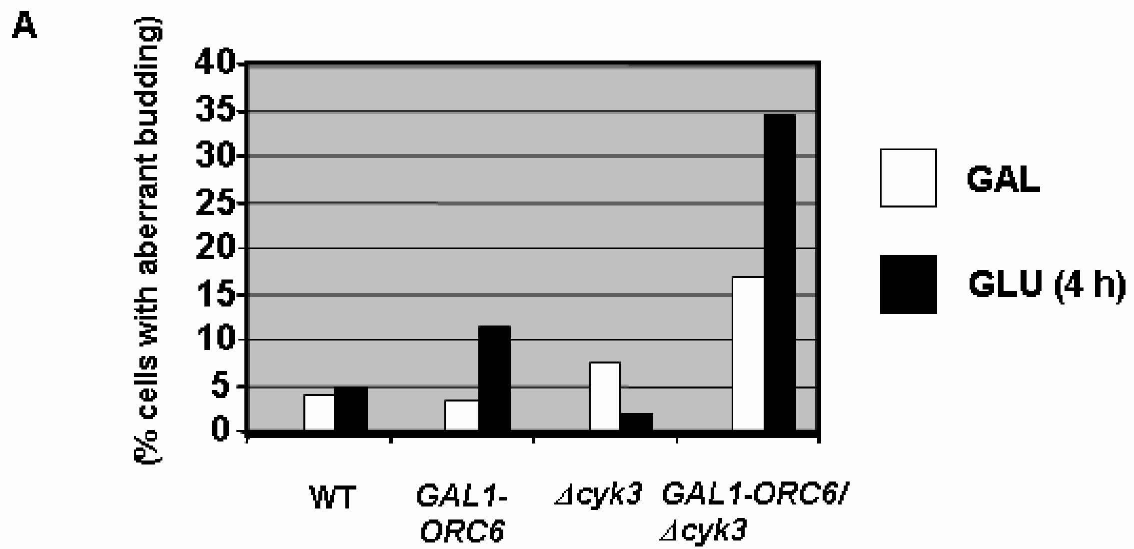
A
GLU (h)
GAL
2
4
6
WT
GAL1-
ORC6
Δcyk3
GAL1-
ORC6/
Δcyk3
1C 2C
1C 2C
1C 2C
1C 2C
B
GLU (h)
GAL 2 4 6
Orc6
Ponceau
Orc6
Ponceau
Orc6
Ponceau
Orc6
Ponceau

In order to confirm results obtained through the asynchronous shift, scoring of aberrantly budding cells was performed by taking high resolution microscope pictures of cells from each of the four strains following 4 h growth in GLU medium. As controls for each strain growing in GLU medium, scoring was also performed on each strain after 4 h growth in GAL medium. Microscopic examination of the Δ*cyk3* strain revealed that some cells displayed misshapen mother bud necks that appeared to be elongated, or bent, consistent with previous observations reported for a non-isogenic Δ*cyk3* strain (Korinek et al., 2000). Since FACS analysis of the *GAL1-ORC6*/Δ*cyk3* strain following Orc6 depletion to below endogenous levels is consistent with a more severe cytokinetic defect than for the Δ*cyk3* strain, it was predicted that a larger proportion of cells in the *GAL1-ORC6*/Δ*cyk3* strain would also display misshapen mother bud necks. Initially, cells from each strain were scored as being either budded or unbudded. From the population of budding cells, the proportion that displayed 'aberrant' budding, defined by a misshapen mother bud neck, was determined. A graphical representation of data obtained from these cell counts is shown in Figure 8A, and representative microscopic pictures showing WT and *GAL1-ORC6*/Δ*cyk3* strains after 4 h growth in GLU medium are shown in Figure 8B. The arrows in Figure 8B point to cells budding 'normally' in the WT strain picture, and 'aberrantly' in the *GAL1-ORC6*/Δ*cyk3* strain. In support of a role for Orc6 in cytokinesis, cell scoring confirmed that following 4 h growth in GLU medium, the *GAL1-ORC6*/Δ*cyk3* strain had a much higher number of cells displaying aberrant budding than any of the other strains. In addition, the *GAL1-ORC6*/Δ*cyk3* strain had a much higher proportion of cells budding aberrantly after 4 h of Orc6 depletion than after 4 h growth in GAL medium, indicating that depletion of Orc6 significantly increased cytokinetic defects in this strain. These results are consistent with those obtained from the asynchronous shift, however, some surprising observations from the cell counts of the control strains make it more difficult to draw definitive conclusions. For example, the Δ*cyk3* strain seems to have more cells budding aberrantly when grown in GAL medium than when grown in GLU medium.

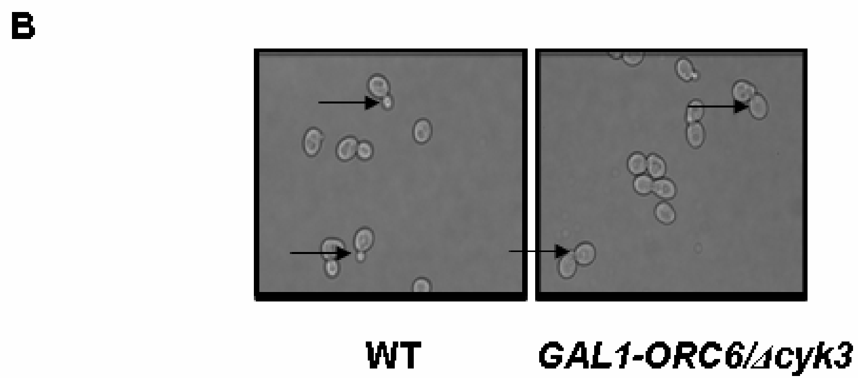
Additionally, the *GAL1-ORC6*/Δ*cyk3* strain growing in GAL medium should behave similarly to the Δ*cyk3* strain, however, cell counts indicated that the *GAL1-ORC6*/Δ*cyk3* strain had more cells budding aberrantly in GAL medium than the Δ*cyk3* growing in either type of media. Interestingly, the *GAL1-ORC6* strain shows an increase in aberrantly budding cells when grown in GLU medium, suggesting that Orc6 depletion on its own may be sufficient to cause a significant defect in cytokinesis.

Figure 8: Scoring for aberrantly budding cells in a *GAL1-ORC6/Δcyk3* strain depleted of Orc6 is consistent with a role for Orc6 in cytokinesis. In order to confirm results obtained from the asynchronous shift from GAL to GLU medium, which were consistent with a role for Orc6 in cytokinesis, the number of *GAL1-ORC6/Δcyk3* (DY 136) cells budding aberrantly following a 4 h depletion of Orc6, was scored and compared to control strain scoring at the same time point. Specifically, the *GAL1-ORC6/Δcyk3* strain, along with control strains WT (DY 93), *GAL1-ORC6* (DY 36), and *Δcyk3* (DY 135) were grown in GAL medium, then switched to growth in GLU medium as described in Figure 7. Culture aliquots for microscopic examination of each strain were taken following growth in GAL medium, and then following 4 h growth in GLU medium. Microscopic pictures at 63x magnification were taken for each strain at each time point, and used to score for aberrantly budding cells. Since previously reported results indicated that a subset of *Δcyk3* cells bud aberrantly (defined by budding cells displaying misshapen mother bud necks that appeared elongated or bent), it was hypothesized that a *GAL1-ORC6/*Δcyk3 strain grown in GLU medium for 4 h to deplete Orc6 would have a higher percentage of cells budding aberrantly than either *Δcyk3* or *GAL1-ORC6* strains after 4 h growth in GLU medium. A) The total numbers of cells scored for each strain at each time point are as follows: nGAL = 480, nGLU = 384 for the WT strain; nGAL = 378, nGLU = 588 for the *GAL1-ORC6* strain; nGAL = 558, nGLU = 330 for the *Δcyk3* strain; and nGAL = 504, nGLU = 384 for the *GAL1-ORC6/ Δcyk3* strain. From this scoring data, the proportion of cells budding aberrantly was determined for each strain growing in GLU medium for 4 h. As a control for scoring of GLU medium samples, scoring was also performed on microscopic pictures taken following growth of each strain in GAL medium. Scoring data is consistent with a role for Orc6 in cytokinesis, and confirms results obtained from the asynchronous shift from GAL to GLU medium shown in Figure 7. Following 4 h growth in GLU medium, the *GAL1-ORC6/*Δcyk3 strain had a much higher percentage of cells budding aberrantly than any other strain growing in either type of media, or than when grown in GAL medium. Interestingly, the *GAL1-ORC6* strain has a higher percentage of cells budding aberrantly when grown in GLU medium than when grown in GAL medium. B) A representative microscopic picture for both WT and *GAL1-ORC6/Δcyk3* strains following 4 h growth in GLU medium is shown, with arrows pointing to ‘normal’ and ‘aberrantly’ budding cells, respectively. **NOTE: GAL = transcription ON; GLU = transcription OFF for the *GAL1* strains**

A



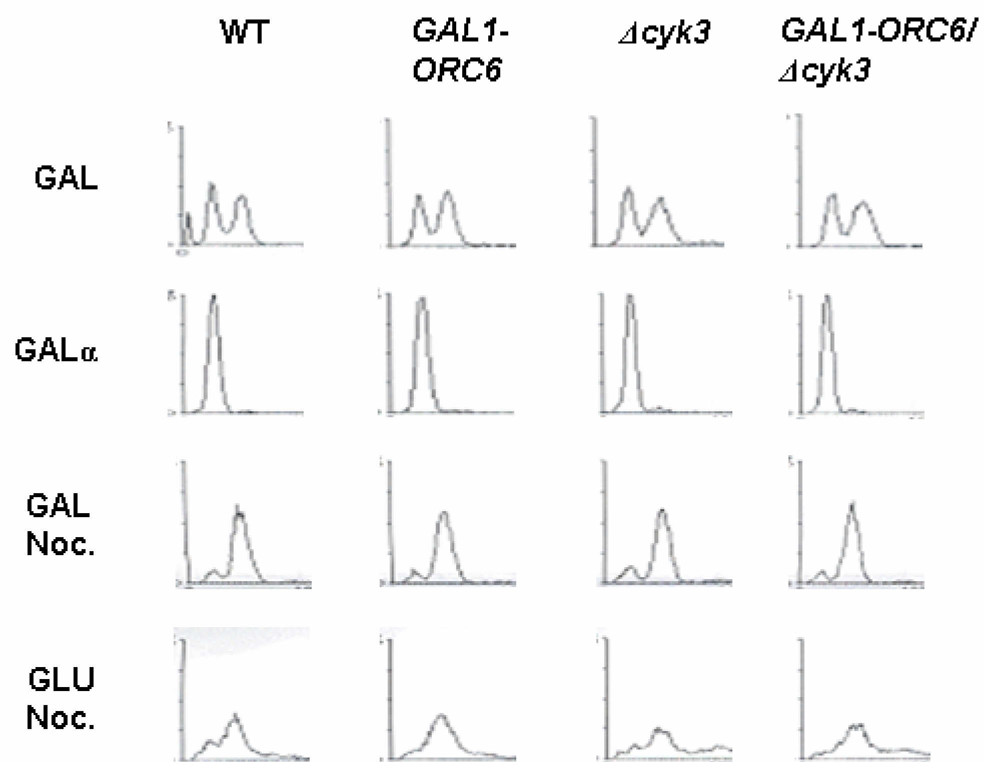
B

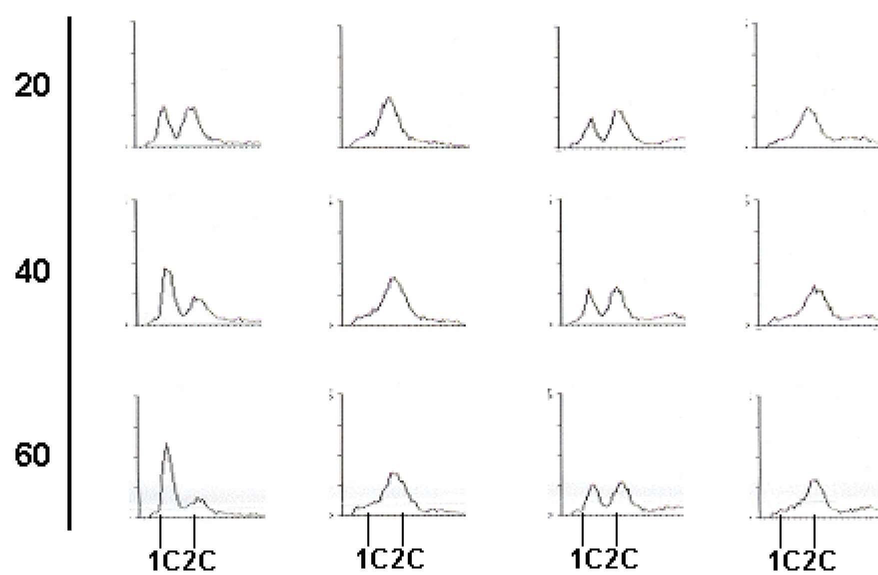


Confounding the interpretation of results obtained from the asynchronous shift, is the fact that Orc6 has an essential role in DNA replication through the recruitment and maintenance of Mcm2-7 at origins of replication. Because of this, it is more difficult to interpret FACS analysis from asynchronous cultures of the *GAL1-ORC6/ Δcyk3* strain, since depletion of Orc6 will cause DNA replication defects in addition to potential cytokinetic defects. For this reason, it is desirable to synchronize the cell cycle of *GAL1-ORC6/ Δcyk3* and control strains at a time after their DNA has been replicated. Nocodazole, a microtubule depolymerizer, was used in cell synchronization experiments, to arrest cells at the G2/M phase boundary. The *GAL1-ORC6/ Δcyk3* strain, along with control strains WT, *GAL1-ORC6*, and *Δcyk3* were initially grown in GAL medium containing α-factor (GALα) to arrest cells in late G1 phase, since synchronizing cultures initially with α-factor leads to a tighter subsequent nocodazole arrest. Strains were then transferred into GAL medium containing nocodazole (GAL Noc.) to arrest cells at the G2/M phase boundary, and then into GLU medium containing nocodazole (GLU Noc.) for 4 h to deplete Orc6. Strains were then released from the nocodazole block into GLU medium containing α-factor (GLUα), and culture aliquots for FACS analysis were taken every 20 min for 60 min to monitor progression through the cell cycle (Figure 9). Since the efficiency of nocodazole releases can vary between experiments, cells were released into medium containing α-factor to arrest cells in G1 phase of the following cell cycle. This allows for an easily identifiable marker indicating that cells had successfully completed cytokinesis following release from the nocodazole block. As can be seen from the FACS analysis of this cell synchronization experiment shown in Figure 9, the nocodazole arrest held reasonably well through the 4 h depletion in GLU medium. Interestingly, following release from the nocodazole arrest into GLUα medium, both *GAL1-ORC6* and *GAL1-ORC6*/Δ*cyk3* strains remain arrested with a 2C DNA content throughout the 60 min of FACS analysis, suggesting that depletion of Orc6 on its own is enough to cause a significant defect in cytokinesis. In comparison, the WT strain is able to release and complete the cell cycle, arresting

with a 1C DNA content by 60 min. The release for the Δ*cyk3* strain is more difficult to interpret. The FACS samples look similar to an asynchronous culture, but there seems to be no change between time points following release. It is possible that some cells are arrested with a 2C DNA content that are unable to complete cytokinesis, while some cells have made it through to G1 phase. Nevertheless, the fact that Orc6 depletion can cause an arrest late in the cell cycle is a novel phenotype, and points to a role for Orc6 in later cell cycle stages not previously characterized in budding yeast.

Figure 9: Orc6 depletion at the G2/M phase boundary leads to a block in cell cycle progression following release, suggesting that Orc6 depletion is sufficient to cause a significant defect in cytokinesis. It is difficult to interpret FACS analysis from an asynchronously growing culture of the *GAL1-ORC6/ Δcyk3* (DY 136) strain depleted of Orc6, since depletion will cause DNA replication defects in addition to potential defects in cytokinesis. For this reason, *GAL1-ORC6/Δcyk3* cells were synchronized at the G2/M phase boundary to deplete Orc6 at a time point after cells had fully replicated their DNA. Following release, it is possible to monitor cell cycle progression through FACS analysis, and determine whether Orc6 depletion has consequences for cytokinesis independent of its essential role in DNA replication. Specifically, the *GAL1-ORC6/Δcyk3* strain, along with control strains WT (DY 93), *GAL1-ORC6* (DY 36), and *Δcyk3* (DY 135) were grown in GAL medium, and arrested in late G1 phase with α-factor (GALα), since an initial α-factor arrest leads to a tighter subsequent nocodazole arrest. Strains were then switched to growth in GAL medium containing nocodazole (GAL Noc.) to arrest cells at the G2/M phase boundary. Following this, strains were then switched to growth in GLU medium containing nocodazole (GLU Noc.) and grown for 4 h to deplete Orc6. Finally, strains were released into GLU medium containing α-factor (GLUα). Since the efficiency of nocodazole releases are variable, α-factor was added to the release medium to arrest cells in G1 phase of the following cell cycle, allowing for an easily identifiable marker to indicate that strains had successfully completed cytokinesis following release. Culture aliquots were taken for each strain at the indicated time points for FACS analysis to monitor cell cycle progression. It can be seen from this cell synchronization experiment, that FACS analysis of the release of the *GAL1-ORC6/Δcyk3* strain into GLUα medium following Orc6 depletion at the G2/M phase boundary, is consistent with a role for Orc6 in cytokinesis. Following depletion of Orc6 at the G2/M phase boundary, it is interesting to note that cells from both the *GAL1-ORC6/Δcyk3* strain and the *GAL1-ORC6* strains remain arrested with a 2C DNA content after being released into GLUα medium, indicating that Orc6 depletion on its own, and independent of a *CYK3* deletion, is enough to cause a significant defect in cytokinesis.
NOTE: GAL = transcription ON; GLU = transcription OFF for the *GAL1* strains


WT
GAL1-ORC6
Δcyk3
GAL1-ORC6/Δcyk3
GAL
GALα
GAL Noc.
GLU Noc.


Release into GLUα (min)
20
40
60
1C2C
1C2C
1C2C
1C2C

Discussion

A role for Orc6 in DNA replication has been well documented in multiple model organisms, however, these roles are not the same in each organism. For example, Orc6 is required for ORC-DNA binding in *Drosophila*, but is dispensable for this role in budding yeast (Li & Herskowitz, 1993; Chesnokov et al., 2001). Similarly, roles for Orc6 outside of DNA replication vary from one organism to the next. Orc6 has an important role in chromosome segregation in human cells (Prasanth et al., 2002), but a similar role in *Drosophila* or budding yeast has not been identified to date. Even in budding yeast, ORC has been shown to be involved in both DNA replication and silencing, making it possible that this complex is involved in the coordination or coupling of important cell cycle events (Foss et al., 1993; Fox et al., 1995).

Results presented in this chapter are preliminary, but are nevertheless consistent with a role for Orc6 at a later cell cycle stage than DNA replication. It is reasonable to assume that this role is specific to cytokinesis, since an Orc6-Hof1 interaction has been identified (Semple, 2006), and Hof1 is known to be involved in cytokinesis both as a negative regulator of actomyosin ring constriction, and in septum formation (Vallen et al., 2000; Blondel et al., 2005). Results obtained from an initial asynchronous shift from GAL to GLU medium using the *GAL1-ORC6/Δcyk3* strain, and control strains WT, *GAL1-ORC6*, and Δ*cyk3*, revealed that Orc6 depletion, in combination with a *CYK3* deletion produced a more severe defect in cell cycle progression than for Orc6 depletion, or *CYK3* deletion alone (Figure 7). This is consistent with a role for Orc6 in cytokinesis, and was confirmed with cell scoring, which revealed that the *GAL1-ORC6/Δcyk3* strain had a higher proportion of cells budding aberrantly than any other strain (Figure 8). Further, when depleted of Orc6 in GLU medium, the *GAL1-ORC6*/Δ*cyk3* strain showed a large increase in the number of cells aberrantly budding as compared to growth in GAL medium (Figure 8). Since Orc6 interacts with Hof1 (Semple, 2006), and deletion of *HOF1* and *CYK3* is known to cause a synthetic lethal phenotype (Korinek et al., 2000), it is possible

that Orc6 functions in a similar pathway to Hof1, and in a parallel pathway to Cyk3. In this case, Orc6 may be involved in septum formation, in conjunction with Hof1s role in this process. It seems more likely, however, that Orc6 would be involved in Hof1's other role, as an inhibitor of actomyosin ring contraction, since all other organisms in which a role for Orc6 in later cell cycle stages has been established, do not have cell walls, and so do not produce a septum.

If Orc6 had a crucial role in cytokinesis, you would expect that its depletion alone would lead to a severe cytokinetic phenotype. *Δhof1* and *Δcyk3* are only synthetic lethal in combination, and each individually only produces a mild cytokinetic defect, indicating that these proteins play more minor roles in cell division (Korinek et al., 2000). Orc6 is present outside of the nucleus and accumulates to high levels at the cell periphery and at the mid-body during mitosis in both *Drosophila* and human cells, and is easily detected when tagged for fluorescence microscopy (Prasanth et al., 2002; Chesnokov et al., 2003). Conversely, published work from our lab has shown that Orc6 is consistently confined to within the nucleus throughout the cell cycle (Semple et al., 2006). It is possible, however, that small amounts of Orc6 are present outside of the nucleus that went undetected under microscopic examination. Interestingly, Orc6 depletion in the *GAL1-ORC6* strain is sufficient to increase the number of cells budding aberrantly when compared to a non-depleted control (Figure 8). This result was confirmed through cell synchronization studies, which showed that Orc6 depletion at the G2/M phase boundary, was sufficient to arrest the *GAL1-ORC6* strain with a 2C DNA content, so that it could not complete the cell cycle (Figure 9). In a recently published paper (Shimada & Gasser, 2007), it was shown that the late G1 phase depletion of Orc2 resulted in a significant delay in progression through M phase, with a large proportion of cells arresting completely. Upon further investigation, it was found that this delay was caused by the activation of both the DNA damage and spindle checkpoints at the G2/M transition, and that Orc2 depleted cells showed signs of impaired sister chromatid cohesion. It is possible then, that the arrest seen in the *GAL1-ORC6* strain following release from a nocodazole

arrest/Orc6 deplete, could reflect defects in mitosis rather than defects in cytokinesis. Since it is impossible to differentiate between defects in mitosis or cytokinesis from FACS analysis, it is possible that rather than being involved in cytokinesis, Orc6 is involved earlier in mitosis, through a role in sister chromatid cohesion, or in chromosome segregation as in human cells (Prasanth et al., 2002). An attractive model would be one in which Orc6 monitored sister chromatid cohesion or chromosome segregation, and interacted with Hof1 once mitosis had been completed, thereby linking the end of mitosis to the intiation of cytokinesis by promoting the constriction of the actin ring. Indeed Hof1 has been proposed to link mitotic exit with the initiation of cytokinesis through interaction with the MEN (Blondel et al., 2005; Corbett et al., 2006).

Chapter 5

General Conclusions

Results in chapter 3 of this study demonstrate for the first time that late G1 phase depletion of the pre-RC component Cdt1 results in a displacement of MCMs from origin sequences (Figure 5). It was also shown that late G1 depletion of Cdc6 leads to a similar displacement of MCMs (Figure 5), confirming previously reported data from Aparicio et al., (1997). These results complement published work from our lab which identified a similar role for Orc6 (Semple et al., 2006), and together show that multiple pre-RC components are required following assembly in early G1 phase, for the maintenance of MCMs at origins. Since *in vitro* experiments and studies involving isolated late G1 chromatin have shown that ORC and Cdc6 are dispensable following pre-RC assembly (Hua & Newport, 1998; Rowles et al., 1999), a recent model was proposed by Chen et al., (2007) that would reconcile these findings with results from this study is one in which an MCM unloading mechanism, not present in the *in vitro* system, is invoked throughout G1 phase in WT cells, but is overcome by the normal functioning of pre-RC components. This model predicts that any protein required for the initial establishing of pre-RCs in early G1 phase, would also be required for its maintenance. Thus results from the present study, in conjunction with published Orc6 data (Semple et al., 2006; Da-Silva, 2007) support this model by showing that all proteins required for the loading of MCMs thus far investigated, are also involved in their maintenance at origins throughout G1 phase. Further confirmation is derived from the fact that while Cdc6 and ORC depletion result in a rapid inhibition of S phase progression, a much longer depletion of Cdt1 is required to observe a similar effect (Semple et al., 2006; Da-Silva, 2007). This makes sense considering that both ORC and Cdc6 are core components of the pre-RC, while Cdt1 association with this complex is more transient in nature.

This study is also the first to demonstrate the ability of pre-RCs to reform following the depletion and subsequent resynthesis of Cdc6 in late G1 phase (Figure 6). In addition, these reconstituted pre-RCs were fully capable of initiating and completing DNA replication (Figure 6).

Similar results for Orc6 were obtained by a previous M.Sc. student in our lab, and together with my results indicate that cells retain the ability to form pre-RCs throughout G1 phase (Da-Silva, 2007).

Since accurate DNA replication is necessary for the faithful transmission of genetic material from one generation to the next, the proper functioning of the pre-RC is intimately linked to the maintenance of genomic integrity. The impairment of pre-RC components has deleterious effects on DNA replication, and can thus result in inaccurate or uneven distribution of genetic material to daughter cells, which in turn can lead to cancer through the loss of genomic integrity. This implicates the improper functioning of pre-RC components in cancer mechanisms, since these components control if and when DNA replication begins. In support of this, the deregulation of several pre-RC components, by over-expression or deficiency, results in the loss of genomic integrity that could potentially result in tumorigenesis. Pre-RC proteins have thus been suggested as having potential for both diagnostic and therapeutic value (reviewed in Lau et al., 2007). Since the development of these therapeutic agents requires a full understanding of pre-RC functioning, results from this study have important implications in the choice of pre-RC component targeted, as well as the constraints under which a therapeutic agent must function.

Interestingly, results from chapter 4 have similar implications. In this study, a role for the DNA replication protein Orc6 in cytokinesis in budding yeast has been implicated. A prominent hypothesis on the mechanism of tumorigenesis states that the failure of a cell to properly divide results in the formation of tetraploid cells, which are genetically unstable (Fujiwara et al., 2005). Consistent with this, mutations in some tumor suppressors lead to an increase in cytokinetic failure. In addition, many of the proteins required for cytokinesis are upregulated in tumor-derived cell lines, and some of the genes encoding these proteins lie in regions shown to be deleted or amplified in these cancerous cells (Caldwell et al., 2007). Importantly, several mammalian homologs of MEN components are known tumor-suppressors and have been implicated in cytokinesis (Nishiyama et al., 1999; Tao et al., 1999;

Yang et al., 2004). One of these is the homolog of yeast Dbf2, which has been implicated in the phosphorylation of Hof1, leading to its degradation and subsequent activation of actin ring contraction. Support for this is seen in mammalian cells where Dbf2 promotes contractile ring disassembly, consistent with the known role of Hof1 as a negative regulator of cytokinesis (Yang et al., 2004). Thus the potential involvement of Orc6 in cytokinetic processes would be consistent with a role for this protein in the maintenance of genomic integrity during several cell cycle stages.

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