Characterization of the role of Orc6 in the cell cycle of the budding yeast

Saccharomyces cerevisiae

by

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required finalrevisions, as accepted by my examiners.

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Abstract

The heterohexameric origin recognition complex (ORC) acts as a scaffold for the G1 phase assembly of pre-replicative complexes. Only the Orc1-5 subunits are required for origin binding in budding yeast, yet Orc6 is an essential protein for cell proliferation. In comparison to other eukaryotic Orc6 proteins, budding yeast Orc6 appears to be quite divergent. Two-hybrid analysis revealed that Orc6 only weakly interacts with other ORC subunits. In this assay Orc6 showed a strong ability to self-associate, although the significance of this dimerization or multimerization remains unclear. Imaging of Orc6-eYFP revealed a punctate sub-nuclear localization pattern throughout the cell cycle, representing the first visualization of replication foci in live budding yeast cells. Orc6 was not detected at the site of division between mother and daughter cells, in contrast to observations from metazoans. An essential role for Orc6 in DNA replication was identified by depleting the protein before and during G1 phase. Surprisingly, Orc6 was required for entry into S phase after pre-replicative complex formation, in contrast to what has been observed for other ORC subunits. When Orc6 was depleted in late G1, Mcm2 and Mcm10 were displaced from chromatin, the efficiency of replication origin firing was severely compromised, and cells failed to progress through S phase. Depletion of Orc6 late in the cell cycle indicated that it was not required for mitosis or cytokinesis. However, Orc6 was shown to be associated with proteins involved in regulating these processes, suggesting that it may act as a signal to mark the completion of DNA replication and allow mitosis to commence.

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APPENDIX I

Acronyms

HU – Hydroxyurea

BrdU – Bromodeoxyuridine

eYFP – Enhanced Yellow Fluorescent Protein

eCFP – Enhanced Cyan Fluorescent Protein

DNA – Deoxyribonucleic Acid

PCR – Polymerase Chain Reaction

Co-IP – Co-Immunoprecipitation

WCE – Whole Cell Extract

ORF – Open Reading Frame

SC – Synthetic Complete

YPD – Non-selective, glucose-based medium for growing yeast (Yeast extract, Peptone, D-glucose).

YPG/R – Non-selective, galactose-based medium for growing yeast (Yeast extract, Peptone, Galactose/Raffinose).

SPB – Spindle Pole Body

HAT – Histone Acetyltransferase

NLS – Nuclear Localization Signal

ARS – Autonomously Replicating Sequence

ACS – ARS Consensus Sequence

Pre-RC – Pre-Replicative Complex

ORC – Origin Recognition Complex

MCM – Mini-Chromosomal Maintenance

CDK – Cyclin Dependent Kinase

DDK – Dbf4 Dependent Kinase

CHAPTER I: Literature Review and Research Objectives

<u>Working with Yeast</u>

A model organism

Throughout this project, the majority of experiments were performed on the budding yeast *Saccharomyces cerevisiae.* As with all fungi, *S. cerevisiae* is a spore-producing eukaryote that lacks the ability to undergo photosynthesis (reviewed in Sherman, 2002; Hong *et al.,* 2006). The term yeast describes a common group of fungi that are unicellular and do not use hyphal thalli to reproduce, but instead typically divide asexually (vegetatively) through budding or fission. Interestingly, yeast are able to live in either haploid or diploid states, and can maintain these states through mitosis. Under certain environmental conditions diploids can undergo meiosis to produce haploid spores (gametes). Most yeast, including budding yeast, are sac fungi or Ascomycetae, a division representing approximately 75% of described fungi. Following meiosis, these organisms house the resulting haploid spores in sacs known as asci. *S. cerevisiae,* is also a member of the family Saccharomycetaceae, which describes organisms that multiply through budding and are able to convert carbohydrates into alcohol and carbon dioxide. The most domesticated in the family, *S. cerevisiae* is commonly used in wine and beer production, as well as bread making. A relatively safe organism to work with, *S. cerevisiae* is not typically pathogenic to humans, except in a few isolated cases in which patients were immunocompromised (Henry *et al.,* 2004). Although it is acknowledged that *S. cerevisiae* is not the only "budding yeast", from this point on any mention of budding yeast refers specifically to *S. cerevisiae*.

Yeast are popular scientific model organisms as their metabolic machinery, including cell structure, macromolecule synthesis, DNA synthesis and cell division mechanisms

are all similar to those of plant and animal cells. It is their intrinsic advantages in the laboratory that make yeast ideal models. Among eukaryotes, the practical advantages of working with these species in a scientific laboratory are unparalleled (reviewed in Ostergaard *et al.,* 2000). *S. cerevisiae* is a very small organism, as haploid yeast cells are approximately 4 μm spheres. With such a small volume, these organisms divide rapidly under ideal conditions, with most yeast strains having a doubling time between 90-100 min while shaking in liquid culture at 30°C. Cell division in *S. cerevisiae* occurs through budding. Each new bud grows in size throughout the cell cycle, which presents a simple morphological marker of cell cycle stage. However, it should be noted that arrests in the cell cycle may not be represented by bud size, since they tend to continue to increase in size even if a checkpoint was activated and the cell cycle was blocked in S phase.

More specific to a molecular biology laboratory, working with *S. cerevisiae* offers several advantages compared to metazoans. Macromolecule isolation, particularly RNA, DNA and proteins are performed routinely with simple, short protocols. Budding yeast allow for easy, one-step transformation and recombination techniques (Longtine *et al.,* 1998), unmatched in eukaryotic organisms. Recombination gives researchers control over any aspect of the genome, with the ability to tag or control the expression of specific proteins. Since yeast can be propagated in both haploid and diploid states, knock-out mutations are easily identified and isolated. Most "wild-type" laboratory strains of *S. cerevisiae* contain several mutations to metabolic genes. For example, a common mutation occurs in *TRP1*, which encodes a phosphoribosylanthranilate isomerase necessary to catalyze the third step in tryptophan biosynthesis. Without a functional

copy of *TRP1*, tryptophan must be added to the culture medium for yeast to grow. Therefore, selection of yeast transformants can be facilitated by including the *TRP1* gene in the vector DNA and growing the cultures on medium lacking tryptophan. As well, research is facilitated by vast numbers of *S. cerevisiae* mutants which are commercially available. Particularly, numerous mutant strains of yeast express specific proteins that are temperature-sensitive. Typically, these proteins are stable at low *permissive* temperatures (i.e. 23°C), but gradually become unstable at higher *restrictive* temperatures (i.e. 37°C). This allows for a simple method to study the phenotype of yeast lacking a protein of interest. Temperature-sensitive mutants are usually rather serendipitously discovered, and researchers commonly rely on other methods to deplete proteins *in vivo*, such as promoter replacement (this will be discussed in more detail later).

Establishing synchronous cultures of yeast

While studying the cell cycle in yeast, it is sometimes necessary to obtain synchronous cultures, in which all the cells in a culture are at the same point of the cell cycle. Several techniques and arresting agents can be employed to arrest cells at a given point of the cycle. However, cell synchrony does not last long upon release from arrest, usually about two generations. Haploid mother cells bud much more rapidly than daughter cells (Hartwell, 1974). Therefore, after a few rounds of the cell cycle, the original parental cells are typically farther along in the cell cycle than the later generations, and cultures begin to appear asynchronous. The key to any arresting agent is that it is able to act uniformly and promptly in a culture, and that cells can be

released from the block quickly and synchronously. A few common methodologies used in this project are discussed below.

Alpha factor

Interestingly, although yeast are single-celled, they are indeed two "sexes", denoted by mating types, *MATa* and *MATα*. The *MAT* gene product controls the expression of several other mating type-specific genes. Both *MATa* and *MATα* produce signaling molecules (pheromones) and receptors that identify cells as a particular type or sex. For example, *MATα* cells produce a short peptide (13 amino acids) called α-factor that can be received by *MATa* cells, and subsequently initiates the mating process. Although haploid cells are able to undergo mitotic division to produce two daughter cells of identical DNA content, it is possible that two haploid cells of different type mate and produce a diploid cell. Mating results in nuclear fusion along with cell fusion; therefore, the resulting zygotes are not dikaryon. Although unable to mate themselves, diploid cells are able to undergo meiosis and sporulation under nutrient-limited circumstances. In budding yeast, sporulation occurs after 2-7 days in minimal medium, resulting in four haploid spores (tetrad) bound together in a single ascus.

When mating pheromone is supplied in a culture of the opposite cell sex, it is detected by the cells and a signal transduction pathway initiates the mating response pathway (reviewed in Bardwell, 2005). Although there are several cellular components affected by this signal, one consequence to the cell is an arrest in late G1 phase. Under a light microscope, the percentage of cells blocked in G1 can be determined by counting cells that are without a bud. As a consequence of binding the pheromone, cells also undergo

morphological changes to facilitate fusion. This involves an elongation of the cell into a distinct pear shape, called a "schmoo". This behaviour is of great use in the laboratory, as a population of *MATa* cells can be synchronized to G1 following exposure to the pheromone produced by a *MATα* strain (α-factor). Once given enough time to allow all cells to arrest, the pheromone can then be washed away, and the cells will synchronously enter back into the cell cycle. When exploiting yeast mating in the lab to produce synchronous cultures of yeast, it is important to use strains that are *bar1⁻*, as the end-product of this gene degrades α-factor. Outside the laboratory Bar1 is important to allow cells to release from cellular arrest if they did not find a mate.

Hydroxyurea (HU)

Other cell cycle arresting agents are commonly used when working with yeast to obtain synchronous cultures at various points of the cell cycle. Hydroxyurea is a ribonucleotide reductase inhibitor. Ribonucleotide reductase is a key enzyme in DNA precursor biosynthesis, specifically catalyzing dNDP formation (Lammers and Follmann, 1984). Therefore, in the presence of an inhibitor of this enzyme, cells enter S phase, but are not able to complete new strand synthesis as the pools of dNTPs rapidly become depleted. In this case, only early origins of replication fire and replication fork lengths are typically below 50 kb. Morphologically, yeast cultures arrested in HU can be monitored as cells usually have tiny buds at the start of S phase. However, it should be noted that bud size can only be used to observe the initial arrest, since buds increase in volume even though the cell cycle is blocked. Therefore, cells arrested in HU for several hours may have large buds and appear as though they have progressed

through the cell cycle.   Nevertheless, although it takes time for cells to synthesize dNTPs *de novo*, cells arrested in HU can be synchronously released to re-enter the cell cycle through washing the cell pellets with water and resuspending the cells in medium lacking HU.

Nocodazole

Nocodazole is another important chemical agent used in cell cycle research.   It acts on the cytoskeleton of the cell by depolymerizing tubulin subunits of microtubules.   This results in a change in the cytosketetal dynamics, essentially preventing microtubule turnover (Vasquez *et al.,* 1997). The integrity of microtubules is particularly important in cells entering mitosis, where fiber networks play a critical role in chromosome segregation.   Once added to cultures, nocodazole prevents cells from initiating mitosis, synchronizing cells at G2/M.   This block is dependent on a functional spindle assembly checkpoint, that monitors the integrity of microtubules during mitosis.   Interestingly, some human cell lines may attempt to complete mitosis in the absence of chromosome segregation, and prolonged exposure to nocodazole can result in polyploidy (Verdoodt *et al.,* 1999).   However, an apoptotic pathway is typically triggered under these conditions prior to chromosome segregation (reviewed in Rudner and Murray, 1996). Not surprisingly, yeast cultures do not behave well under long exposures to nocodazole. These cells tend to remain in G2/M long after release from the block, and do not always progress synchronously as expected.

As mentioned, observing bud size in cultures to determine cellular arrest is not only inconvenient, but may be inaccurate as well.   Therefore, monitoring cultures under a

microscope is usually done in parallel with measuring DNA content (as determined by FACS), in which case culture aliquots are taken and stained with a DNA-binding dye, which will emit light when excited by a laser. The amount of light emitted is then recorded, and is proportional to the DNA content. Clearly, cells in G2 (after DNA replication) will give a signal twice as strong as a cell in G1. With current instruments, the DNA content of thousands of cells in a culture can be rapidly measured and displayed graphically to monitor cell cycle progression. The only disadvantage to this type of measurement is that it is done *after* the experiment was performed, and can not be used to judge if the cells are completely arrested before the protocol is initiated.

S. cerevisiae genetics

In 1996, the entire genome sequence and physical map of *Saccharomyces cerevisiae* were completed (Cherry *et al.*, 1997) by an international effort of over 600 scientists in North America, Europe and Japan. At ~1.2 x 10^7 base pairs distributed between 16 chromosomes, the genome of yeast is relatively small compared to other eukaryotes. However, the *S. cerevisiae* genome is highly compact. Nearly 70% of its DNA encodes genes, that is one in every 2 kb on average. In contrast, typically 100+ kb of DNA sequence must be examined to uncover a protein-encoding gene in the human genome. Of the approximate 6,000 genes (estimates still range from 5,700-6,300), only 4% contain introns, which are usually small and near the 5'-end of the coding sequence (Goffeau *et al.*, 1996). This makes cloning of genes very straight-forward and they can typically be amplified through PCR of genomic DNA. Interestingly, the budding yeast

also has a 6.3 kb 2 μm closed circular plasmid, usually present at 50-100 copies per cell (1-5% of the total DNA of a yeast cell), that is inherited through non-Mendelian genetics. It is clear that knowledge of the DNA sequence of a gene does not decipher the function of the protein product. In fact, this information may prescribe little of the biological properties of the corresponding peptide. Due to the condensed and relatively small genome of *S. cerevisiae*, this species is on the forefront of proteomic research (Ho *et al.,* 2002; Gavin *et al.,* 2002). However, only around 70% of the ORFs of *S. cerevisiae* have homologs in other organisms (Cherry *et al.*, 1997; Goffeau *et al.*, 1996). This means that 30% of the yeast ORFs have little to no sequence similarity to any other known gene (Winzeler *et al.,* 1999). Along with trying to identify the function of these genes, focus has also been placed on discovering and describing the interactions of polypeptides as they form either transient or stable complexes. This project is designed to understand the role of Orc6 in DNA replication, concentrating on identifying and characterizing its cellular interactions.

Although consistent with most eukaryotic symbols, the nomenclature used in yeast genetics is very important, especially when dealing with mutant strains. Genes are designated by three italicized letters (e.g. *LEU*), where dominant alleles are in uppercase, and recessive alleles are in lowercase. Dominant and recessive nomenclature only applies when two or more copies of the gene are present in the cell. However, in the laboratory, many strains are haploid, in which case all mutations of a gene are in the lowercase. Unlike in *E. coli*, genetic loci in yeast are identified by numbers following the gene symbol (i.e. *leu2*). Various allele-specific mutations are designated by a hyphen (i.e. *leu2-3*). In the case of larger mutations in which part or all

of the gene is deleted a delta sign is used (i.e. *leu2-Δ1*). Finally, in the case of insertions or replacements two colons are used (i.e. *orc6::LEU2* in which the *ORC6* gene is mutated and a functional copy of *LEU2* is inserted at this locus).

The Budding Yeast Cell Cycle

S. cerevisiae undergoes cell division by forming a bud from the parental cell. A commitment to complete the cell cycle is observed when a bud emerges at the G1/S transition of the cell cycle (Herskowitz, 1988). The bud continues to grow in size throughout the cell cycle and can be used as a crude marker of the cell cycle stage of individual cells. As the bud grows, a channel connecting it to its parent remains open (designated the neck) for organelles, including eventually the nucleus, to pass through. The cell wall of the new bud, consisting of glucan, mannan, protein and some chitin, is not derived from the parent cell, but instead is synthesized from the bud (reviewed in Hartwell, 1974). Following cell separation at the end of the cell cycle, a permanent scar remains on the mother cell at the site of bud formation.

The cell cycle is a tightly controlled, coordinated process with the goal of cellular reproduction. To ensure two viable daughter cells, the order of cellular events must occur in the appropriate sequence. As in all eukaryotic organisms, the cell cycle of yeast consists of four main stages: a pre-synthetic gap/growth (G1 phase), DNA synthesis (S phase), a post-synthetic gap (G2 phase) and mitosis (M phase), as outlined in Figure 1 (Herskowitz, 1988). Once the cycle has begun, there are several cellular checkpoints that monitor appropriate progression as well as DNA stability. The

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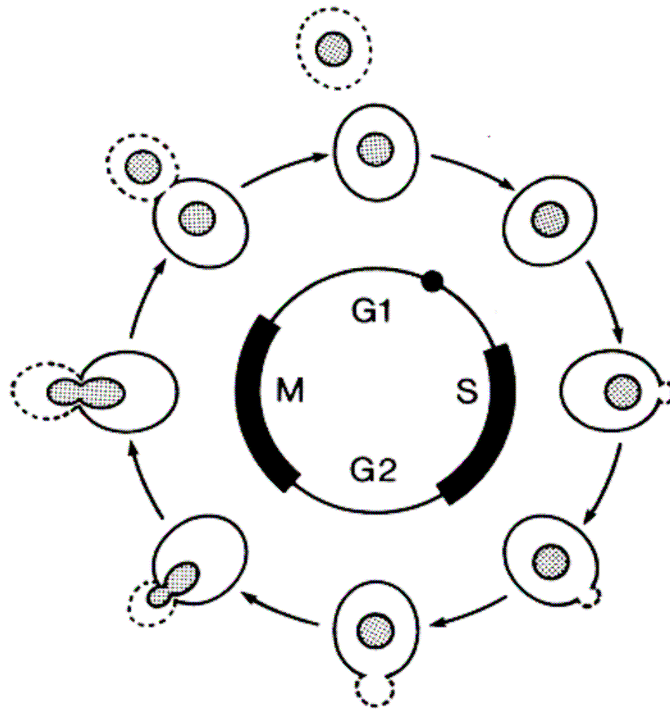
Figure 1. The yeast cell cycle. A schematic outline of each of the phases of the budding yeast cell cycle, drawn in proportional to their typical length. Dotted lines illustrate the daughter bud, solid lines represent the mother cell. The shaded area indicates the nucleus. The solid dot depicted in G1 designates the position at which cells arrest following treatment with pheromones (e.g. α-factor) (Herskowitz, 1988).

main checkpoints are situated between the G1-S and G2-M boundaries and will be discussed in more detail later on in this chapter.

G1 phase

Following cytokinesis, the daughter cells enter a control point late in G1, called START, to determine the fate of progression into the next cell cycle. The term START is a designation for the point in late G1 phase at which cells are no longer considered undifferentiated (a term typically used in multicellular organisms in which the function and morphology of a cell changes). Prior to START, cells can either enter another round of the cell cycle, begin meiosis or become quiescent entering into G0. Although there are many proteins that regulate the timing and speed of the cell cycle, once past START, cells are irreversibly committed to another cycle. Once mitosis is complete, the cell monitors several external stimuli, including a lack of sufficient nutrients (starvation) and mating pheromones, either of which would block passage through START. Yeast are able to survive well in this G0 phase arrest and resume growth after the stimuli or the environmental conditions have changed. In G1, budding yeast cells are small, unbudded and contain only one spindle plaque, a nuclear structure that gives rise to microtubules.

As this stage progresses, the parental cell increases in volume, as small cells are unable to enter S phase and undergo cell division. The amount of time needed to attain that volume usually dictates the length of G1 phase. As the cells increase their size, they are also synthesizing organelles in preparation for cell division. The metabolic rate of cells in this stage is high, as they are synthesizing numerous structural proteins and

enzymes to support the new growth. Since cells have committed to another round of the cell cycle by this point, several essential replicative proteins are actively transported into the nucleus from the cytoplasm, where they were originally quarantined to prevent any re-replication events. At the chromatin level, late G1 phase is marked by the loading of several factors at origins of replication in preparation for DNA replication (this will be discussed in more detail below).

S phase and DNA replication

The initiation of DNA replication marks the end of G1 and the commencement of S phase (reviewed in Hartwell, 1974; Herskowitz, 1988). Synthesis of the entire genome takes approximately 25 min, or one quarter of the cell cycle in wild-type yeast grown under optimal conditions. This process is strictly regulated, as it is essential that duplication occurs precisely. Clearly, any outcome other than complete and exact replication of the cell's DNA content can have serious consequences, not only to the immediate descendants, but to all successive generations. As this project focuses on a member of the origin recognition complex (ORC), the initiator protein in the process of DNA replication, S phase will be examined in more detail, with an emphasis on DNA replication. The events that unfold immediately prior to DNA replication will be discussed in the next section as they are more pertinent to my research.

S phase begins when origins of DNA replication are activated, and this process is outlined in Figure 2A. Once all the necessary cellular signals are sent, the process of DNA replication begins with bi-directional fork migration from origins (Figure 2B). The replication fork is derived from the unwinding of the double helix, resulting in a Y-shaped
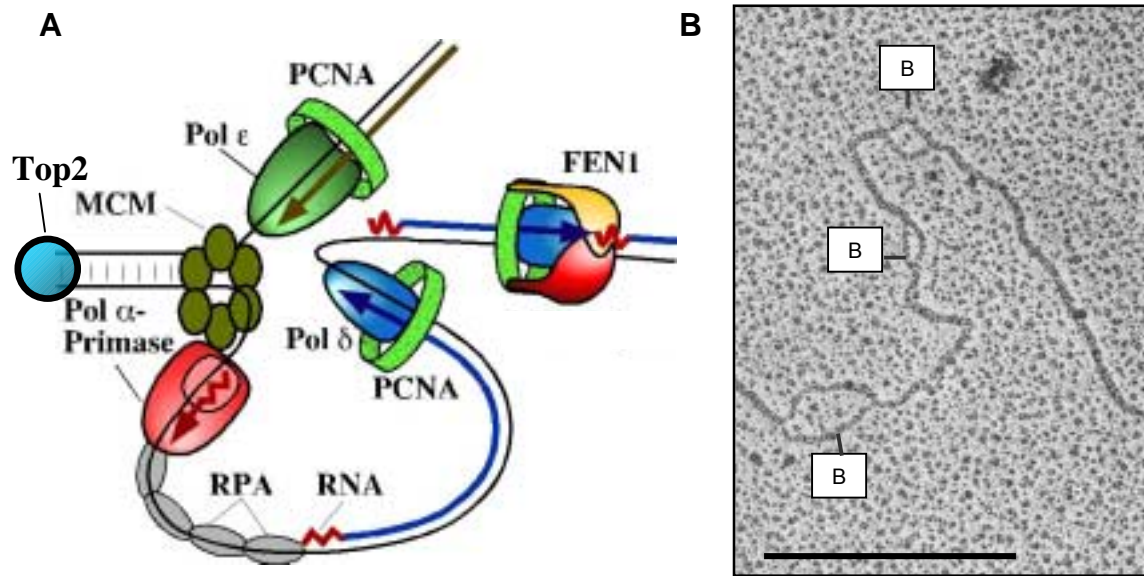
Figure 2. The eukaryotic replication fork. (A) A schematic diagram representing DNA replication at a progressing fork. Top2 is a topoisomerase II enzyme, MCM is the putative DNA helicase enzyme, RPA is the single-stranded DNA binding protein, PCNA is a replication processivity factor, and FEN1 is a 5'-3' exonuclease responsible for RNA primer degradation (modified from Garg and Burgers, 2005). (B) An electron micrograph of S phase DNA from the eukaryotic protist *Physarum polycephalum.* Bar represent 0.5 μm. B indicates maturing DNA replication forks (image obtained from Gillespie and Hardman, 1979).

structure as the two strands are divided. Separation of complementary DNA strands is carried out by helicases. These molecules use energy from ATP hydrolysis to break the hydrogen bonds that hold the two strands together. Immediately following DNA unwinding, single-stranded DNA binding (SSB, RPA in yeast) proteins attach to the individual strands (MacNeill, 2001). RPA proteins ensure that the single-stranded DNA remains in an extended state, so that separated regions do not fold back and adhere to each other. The unwinding process causes tension on the downstream helix as the fork progresses. DNA topoisomerases, more specifically topoisomerase II, relieves this strain by nicking the DNA ahead of the fork (Berger and Wang, 1996). This protein progresses along the double helix in front of the replication bubble to eliminate positive supercoils in the DNA as a result of the mechanical unwinding.

It is the resulting single strands that act as templates for the synthesis of new DNA. In eukaryotes, there are several DNA polymerases required for DNA replication (reviewed in Kawasaki and Sugino, 2001). Once individual strands are exposed, an enzyme complex called DNA polymerase α-primase binds to origins of replication (reviewed in Garg and Burgers, 2005). This four subunit complex is unique in eukaryotes for its ability to synthesize new strands of DNA, called primers, and is required for the initiation of DNA replication. Composed of short strands of RNA, primers are complementary to the exposed template strands and are necessary for subsequent DNA polymerases to elongate the DNA strands.

The elongation of each new strand is complicated by the fact that DNA polymerases (other than the polymerase α-primase complex) can only add nucleotides in the 5'-to-3' direction. As the replication fork progresses it exposes the two complementary strands,

one in the 3'-to-5' and the other in the 5'-to-3' direction. Therefore, primers can bind to the strand being exposed in the 3'-to-5' orientation, and synthesis of the new DNA molecule can progress in the direction that the fork is moving. This is known as the leading strand synthesis. To synthesize new DNA from the complementary template strand, RNA primers must wait until the fork has traveled sufficiently away from the origin before they bind. In this case new strand synthesis is directed back toward the origin, again in the 5'-to-3' direction. Since the progress of this strand is divided as the fork progresses, DNA replication is termed semi-discontinuous. Each segment of new strand synthesis is about 100-200 bases in length, and is known as an Okazaki fragment. Since this strand is discontinuous and must wait for the replication bubble to grow before beginning DNA synthesis, it is known as the lagging strand.

Once the primers are synthesized on the lagging strand, loading of the proliferating cell nuclear antigen, PCNA, causes the dissociation of polymerase α-primase complex. PCNA is then involved in the recruitment of DNA polymerase δ, which is responsible for the elongation of the Okazaki fragment. In contrast, the leading strand, which can synthesize DNA uninhibited behind the advancing replication fork is elongated by DNA polymerase ε. However, there appears to be some versatility in the polymerase enzymes, as the function of polymerase ε can be replaced by polymerase δ on the leading strand under conditions of dysfunction (Garg and Burger, 2005).

Behind the growing replication fork on the lagging strand, the RNA primers of each Okazaki fragment are digested by the FEN1 complex. The DNA polymerase δ and FEN1 complex work together to digest away the RNA primer and incorporate new nucleotides, causing nicks in the new DNA duplex. DNA ligase I then fills in the gaps

between the DNA fragments as the new strands are ligated together. Once one Okazaki fragment is properly incorporated into the new chromosome, the factors involved in lagging strand maturation are presumed to be recycled to new sites of RNA primers until DNA replication is complete.

Lagging strand DNA synthesis is further complicated by the fact that eukaryotic chromosomes are linear. As a result, the RNA primer can not be efficiently converted to DNA and there is a shortening of the chromosome at the extreme ends of lagging strands. If this shortening process were to continue indefinitely, chromosomes would continue to decrease in size from their ends and cause the loss of genes or other important DNA elements. However, the terminal ends of chromosomes contain specialized, highly repetitive DNA sequences called telomeres (reviewed in Chan and Blackburn, 2003). These regions do not encode genes, but act as a cap for the ends of chromosomes. Telomeres are very important as they allow the cell to distinguish natural ends of chromosomes from double-stranded chromosomal breaks. Clearly, the fusion of two telomeres or a telomere to a broken DNA strand would have catastrophic effects on the genomic stability of the cell. Replication of these regions is carried out by a ribonucleoprotein reverse transcriptase called telomerase. To ensure the maintenance of telomere lengths, this enzyme is able to bind to the extreme ends of chromosomes and extend the number of repeat sequences.

G2 phase

Once two complete copies of the genome are present, the cell then enters G2 phase (reviewed in Hartwell, 1974; Herskowitz, 1988). This is the final stage of interphase, in

which cells prepare for mitosis and cytokinesis. During this time the cell and the bud increase in size. Also, the DNA is monitored to ensure that no mutations or errors are present, since any genetic problem beyond this stage will be inherited by future generations. G2 phase also marks a period of high protein synthesis, typically of factors and enzymes responsible for chromosome segregation, including spindle formation. Some researchers suggest that budding yeast do not have a true G2 phase, due to a very quick transition from DNA synthesis to DNA segregation (reviewed in Nurse, 1997). Nevertheless, the end of this stage is evident by the movement of the nucleus to the bud neck.

Mitosis and cytokinesis

Following G2 phase, the cell enters mitosis and begins nuclear division. Clearly, the replicated chromosomes must be partitioned equally between the mother and daughter cells during this stage to ensure genomic stability (reviewed in Zachariae 1999). Typically, mitotic chromosomes are condensed to facilitate separation and segregation; however, in budding yeast, DNA is consistently in the form of chromatin and appears diffuse throughout all stages of the cell cycle (Hartwell, 1974). Interestingly, unlike vertebrate cells, the nuclear envelope of budding yeast remains intact throughout the cell cycle, including mitosis (Matile *et al.,* 1969; reviewed in Winey and O'Toole, 2001). Late in G2 phase, the nucleus migrates to the bud neck where it remains throughout M phase, and visualization of this localization is termed the "mitotic index". Prior to separation, chromosomes are physically aligned between the two cells during metaphase. Movement of both the nucleus to the bud neck and individual chromosomes

to the bud plane results from microtubule arrangements orchestrated by the spindle pole body (SPB; centrosome equivalent). This striated organelle is found within the nuclear envelope, with faces on both sides of the membrane. Microtubules on both sides of the nuclear envelope play important roles in mitosis. Cytoplasmic microtubules are responsible for positioning the nucleus at the bud neck after G2 phase. Nuclear (mitotic) microtubules attach directly to chromosomes at the kinetochore and facilitate their movement. SPBs are located at opposite poles during mitosis and are directly responsible for aligning chromosomes along the nuclear equator during metaphase.

Shortly before anaphase begins, a large complex of proteins is activated, termed the anaphase-promoting complex/cyclosome (APC/C) (Townsley and Ruderman, 1998). The APC/C is composed of at least 10 highly conserved subunits (reviewed in Zacharie and Nasmyth, 1999), and is responsible for the specific proteolysis of key regulating factors, including cyclins (discussed below). After APC/C activation, the dissolution of cohesion between sister chromatids triggers their separation and the transition to anaphase (reviewed in Miyazaki and Orr-Weaver, 1994). The mechanisms responsible for the movement of chromosomes are maintained between yeast and vertebrate cells (Bloom, 2002). The elongation of microtubule spindles facilitates the movement of each complete genome to the poles. As the yeast cell progresses through anaphase, the nuclear envelope forms a typical dumbbell shape encasing the separating chromosomes. Once all the chromosomes have migrated, the nucleus undergoes fission, establishing distinct nuclei within each cell.

Mitotic exit refers to sequential order of events that proceeds late anaphase and advances the cell into the next G1 phase (reviewed in Yeong, 2005). These events

typically include the disassembly of the mitotic spindle, the decondensation of chromosomes and cytokinesis. The final event of the yeast cell cycle is the complete separation of the new daughter cell from the mother cell. Cytokinesis involves removing all links between the two cells. In budding yeast, there are two main pathways for cytokinesis (reviewed in Lippincott and Li, 1998). Cell division is established through actinomysin or septin-based rings, which are distinctly localized to the bud neck. Although cells typically use the actinomysin structure, it is thought that the redundant septum structure is a means of protecting cells from degrading the links between buds before they are ready to separate. Many of the key factors involved in mitotic exit are regulated by the APC/C as well as the mitotic exit network (MEN). In conjunction with this role, the APC/C complex is also involved in a surveillance mechanism termed the mitotic checkpoint (reviewed in Zachariae, 1999). This system prevents cell-cycle progression until microtubules have attached to sister kinetochores from opposite ends. While this checkpoint is not essential for unperturbed cells, it is this mechanism that is responsible for arresting the cell cycle in the presence of microtubule depolymerizing drugs like nocodazole.

Regulation of the cell cycle

As mentioned above, there are several protein factors called cyclins that regulate the cell cycle (reviewed in Kelly and Brown, 2000; Zou and Stillman, 2000; Nguyen *et al.,* 2001). Cyclins are periodically expressed throughout the cell cycle, and different cyclins are involved in monitoring the progression of specific stages of the cycle (ie. S phase cyclins). Once present at a certain stage of the cell cycle, cyclins activate specific

kinases, known as cyclin-dependent kinases (Cdks). There are other kinases (Ddks) that regulate the cell cycle similar to Cdks, yet are dependent on Dbf4 as a regulatory subunit, not cyclins (Jackson *et al.,* 1993; Varrin *et al.,* 2005). Both Cdks and Ddks are inactive serine/threonine kinases found throughout the cell. Once bound to its specific regulatory factor, these kinases become activated and are able to regulate the cell cycle through site-specific phosphorylation events. Interestingly, the levels of most Cdks and Ddks are at high, constant levels throughout the cell cycle, and it is the level of their regulatory cyclins that fluctuates (Pines, 1991), and ensures that the Cdks perform their function at the appropriate time.

As the cell cycle is under strict control, there are several mechanisms involved in regulating Cdks and Ddks. Primarily, kinase activity is directly related to cyclin and Dbf4 concentrations. These regulatory genes are transcribed and up-regulated at specific times in the cell cycle and the resulting protein products are responsible for binding to and activating the kinases. Once activated, the Cdks and Ddks are then able to phosphorylate substrate proteins. Contrary to cyclins, there are also inhibitors of Cdks that negatively regulate their activity. For example, Sic1 is an inhibitor of Cdk activity during G1, and must be degraded prior to DNA synthesis (Lengronne and Schwob, 2002). Additionally, regulation of Cdks and Ddks is carried out by specific proteolysis after they are required, as well as compartmentalization of different regulators into various subcellular locations (typically nucleus and cytoplasm).

Initiation of DNA Replication

Origins of DNA replication

Precise replication of the genome is essential in maintaining the genetic integrity of an organism. Most eukaryotic cells contain several chromosomes and anywhere from millions to billions of nucleotides. Therefore, an efficient mechanism is required to initiate DNA replication in order to synthesize the entire genome in a reasonable amount of time during S phase. To manage the initiation event, eukaryotic cells employ hundreds or even thousands of chromosomal origins of replication. It is these DNA elements that are responsible for orchestrating the protein assemblages needed to begin DNA synthesis. Preliminary studies identifying and locating origins of replication stemmed from work on viral and prokaryotic organisms. In these systems, origins of replication and initiation events are well characterized (reviewed in Kelly *et al.,* 1988; Marians *et al.,* 1992). The first origins of replication discovered in yeast were identified as DNA elements that allow plasmids to be duplicated autonomously in the cell (reviewed in Fangman and Brewer, 1991). Originally given the name autonomous replicating sequences (ARSs), these DNA elements were later identified on yeast chromosomes, where they were shown to perform as they did on plasmids. Since the time of their discovery, hundreds of origins of replication have been identified in the budding yeast genome.

In *S. cerevisiae*, origins of replication share a great deal of sequence similarity. Each origin is composed of short regions of DNA (~100-200 bp) generated from multiple, conserved elements. Generally, budding yeast origins have a bipartite structure in yeast, consisting of both an A and one or more B domains (Marahrens and Stillman,

1992). The A domain contains a highly-conserved 11 bp ARS consensus sequence (ACS), found in nearly all ARS sequences and essential for origin function (Van Houten and Newlon, 1990). B domains are much larger, T-rich elements that exhibit less sequence similarity between origins than the A domain, and contain various enhancer sequences (Rao *et al.,* 1994). While there is only one A domain per origin, there can be a number of different variants of the B domain. Although the function of the B domains is still unclear, it is thought that at least one domain (B_1) is a second element of the recognition site of ORC (Rao and Stillman, 1995).

Translating origin structure and function from budding yeast to metazoans and other eukaryotes has been challenging. Mainly, identification and definition of specific c*is*-acting DNA sequences responsible for replication in these species has been rather difficult. There appears to be very little sequence conservation between origins, even within the same genome. As a result, only a few metazoan origins have been identified to date (reviewed in DePamphilis, 1999). Those origins that have been characterized are extremely AT-rich and mapped to relatively large regions of DNA (0.5-6 kb). Even these regions are not always associated with sites of initiation. Origins in *X. laevis* and *D. melanogaster* early embryos demonstrate very little or no sequence specificity (Blow, 2001). Virtually any DNA fragment can act as an origin of replication in these systems, although this is probably to facilitate a more rapid S phase.

To broaden the search of metazoan origins of DNA replication, microarray-based assays were performed (reviewed in MacAlpine and Bell, 2005). Although this method has discovered hundreds of putative ORC binding sites, very little sequence similarity was identified between the origins by this method. As such, there has yet to be a clear

consensus sequence that define metazoan origins. In *Drosophila*, characterization of the 491 origins identified by microarray analysis was unable to identify specific origin consensus sequences, although ORC binding was found to be limited to AT-rich regions. However, these studies show ORC association in *Drosophila* is clustered in discrete replication initiation zones spread throughout the genome, a pattern not observed in *S. cerevisiae*. However, even with this pattern of ORC association with DNA, it is clear that there is not a specific ACS consensus sequence located at metazoan origins.

There are several scenarios that could explain the lack of conservation among metazoan origins of replication. For one, it is known that ORC plays a role in more than one cellular process. This could interfere with genomic analyses in search for consensus sequences as ORC could be bound throughout the genome at sites not related to DNA replication. However, the most likely concept is that there simply aren't any metazoan origin consensus sequences. Initial metazoan studies focused on early frog and fly embryos, as the rapid DNA replication in these cells was shown to result from a dense network of origins spaced as little as 4-7 kb apart (Blumenthal et al. 1974). However, *in vitro* studies revealed that lowering the levels of ORC in *Xenopus* extracts applied to sperm DNA expands inter-ORC distances and reduces DNA replication rates (Blow *et al.,* 2001). It soon became apparent that ORC has little sequence specificity in these models and the increase in the number of origins and rates of DNA replication were a direct consequence of ORC levels. However, this relationship was only observed in developing embryo cells, as ORC levels tend to be constant in cells of an adult. Nevertheless, it appears as though the nature of eukaryotic origins is variable between

species and stages of development, making research into their structure and function complicated. Interestingly, although it is clear that there are significant differences between definitive origin sequences throughout eukaryotes, the proteins responsible for the recognition of these sites are highly conserved.

Timing of origin activation

Beyond the genomic location of origins of DNA replication, the timing of origin activation should also be addressed. It is clear from continued microarray-based studies on metazoans that the transcriptionally active regions of the genome replicate earlier in S phase than areas of few genes (reviewed in MacAlpine and Bell, 2005). This supports earlier theories derived from yeast and Chinese hamster ovary cells that suggest there are regions of the genome that consistently replicate early in S phase (Taljanidisz *et al.,* 1989; Friedman *et al.,* 1997). Similarly, areas have been identified that initiate replication in the middle or near the end of S phase. As a result, most eukaryotic origins can be roughly classified as early, middle or late firing, depending on its initiation in S phase. However, although this classification may apply to specific origins, an examination of global origin firing in yeast shows that origins of replication are continuously activated throughout S phase with most origins firing mid-S phase (Raghuraman *et al.,* 2001).

The mechanism behind origin activation timing in yeast or any other eukaryote is not completely understood. It is not specifically the replicator sequence that dictates this temporal arrangement, as an early origin sequence moved to a region that typically initiates later in S phase, will be activated at the later timepoint (Friedman *et al.,* 1996).

It is thought that the structure of adjacent chromatin contributes to the timing of origin activation (Aparicio *et al.,* 2004). In yeast, transcriptionally active euchromatin typically replicates early in S phase, whereas silent heterochromatin and telomeric regions were found to consistently replicate late in S phase (reviewed in Gilbert, 2002; McCarroll and Fangman, 1988).

The pre-replicative complex

Due to the complex nature of DNA replication, the initiation of this process is under very tight control. During G1 phase, a number of protein factors and complexes sequentially bind to origins of replication. The formation of the pre-replicative complex (pre-RC) is required to initiate DNA replication (reviewed by Kelly and Brown, 2000; Bell and Dutta, 2002). At the heart of the pre-RC lies the origin recognition complex (ORC), which is responsible for locating and binding origins. Although ORC is bound to origins throughout the cell cycle (Liang and Stillman, 1997), loading of other replicative factors begins in early G1, when cyclin-dependent kinase (Cdk) activity is low. It is thought that ORC acts as a platform onto which other protein factors bind and initiate DNA synthesis. To commence the initiation process, two important loading factors bind to origin-associated ORC. The first protein to bind to ORC is the nucleotide-dependent loading factor Cdc6 (Perkins and Diffley, 1998). Once Cdc6 is bound to the initiation complex, Cdt1 recruits the mini-chromosome maintenance (MCM; MCM_{2-7}) complex to the origin (Randell *et al.,* 2006). Part, or all of the MCM complex acts as a helicase (Labib *et al.,* 2000), assisting in the unwinding of the double helix. The assemblage of ORC, Cdc6, Cdt1 and the six MCM proteins constitutes a pre-RC. An overview of the
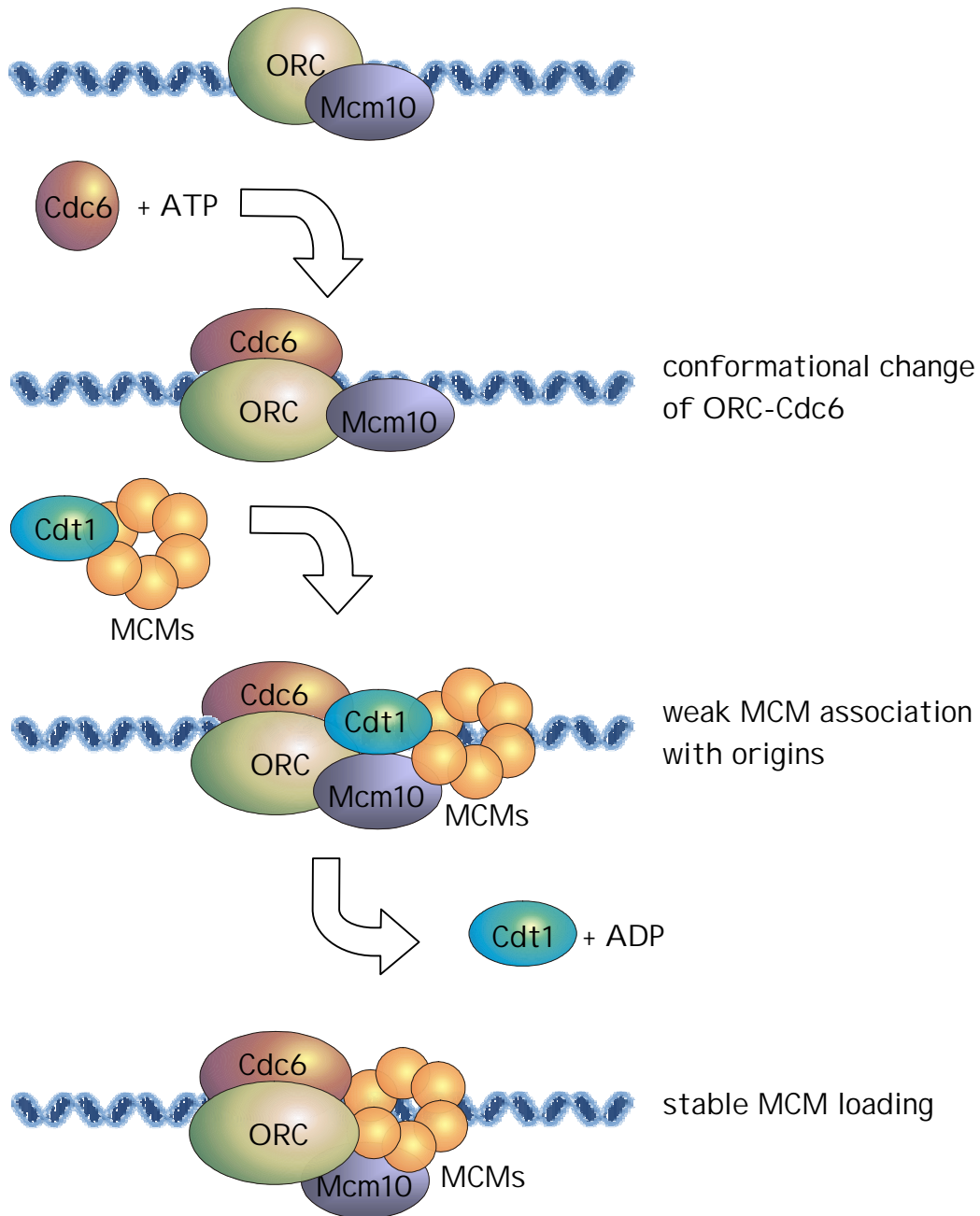
Figure 3. Pre-RC formation. Outlined are the events leading to the stable loading of the MCM complex at origins of replication in budding yeast. Both ORC and Mcm10 are found at origins throughout the cell cycle. During G1 phase Cdc6 binds in an ATP dependent manner. Once Cdc6 is bound to origins, Cdt1 stabilized following the ATP hydrolysis of Cdc6 and the dissociation of Cdt1 (adapted from Randell *et al.,* 2006).

events involved in the formation of the pre-RC is described in Figure 3. Each member of the pre-RC will be discussed in the section below.

Once the pre-RC has formed, the origin is considered to be "licensed", and thus competent for the initiation DNA replication. As the cell cycle nears the end of G1, the levels of activated cyclin- and Dbf4-dependent kinases rise, and these two protein kinase complexes (Clb/Cdc28 and Dbf4/cdc7) activate the pre-RC. These enzymes are involved in the recruitment of Cdc45 and the subsequent activation of the replicative helicases. As the MCMs begin unwinding the DNA, single-stranded DNA binding proteins (RPAs) stabilize the melted strands and the polymerase-α/primase complex joins the assembly at origins to synthesize RNA primers. Finally, once the DNA is replicated, pre-RCs are disassembled anddisassociated from origins. To prevent re-replication of part or all of the genome within the same cell cycle, there are multiple, partially redundant mechanisms in place resulting from high Cdk activity. In *S. cerevisiae*, Cdk activity directs at least three events that inhibit re-replication. Phosphorylation of Cdc6, inducing its proteolysis (Drury *et al.,* 2000; Nguyen *et al.,* 2001), phosphorylation of the MCM complex, leading to its export from the nucleus (Labib *et al.,* 1999; Nguyen *et al.,* 2000), and the phosphorylation of two ORC complex subunits (Orc2 and Orc6), prevent another round of DNA replication until Cdk levels drop at the end of M phase (Nguyen *et al.,* 2001).

The origin recognition complex (ORC)

The ORC complex was originally discovered in budding yeast through glycerol gradient sedimentation of nuclear extracts (Bell and Stillman, 1992). Aliquots of the extract were

incorporated in DNase protection assays using ACS sequences of *ARS1*. These footprinting reactions determined that a six protein complex, given the name origin recognition complex (ORC), specifically bound *ARS1* in an ATP-dependent manner. In all eukaryotes studied to date, ORC is a heteromeric protein composed of six different subunits (reviewed in Bell and Dutta, 2002). Each protein subunit is numbered according to decreasing size (Orc1-6), with Orc1 being the largest subunit at 104 kDa and Orc6 the smallest at 50 kDa. The proposed structure of ORC determined by UV-crosslinking studies is illustrated in Figure 4, with the Orc1, 2, 4 and 5 subunits all having close associations with origin DNA (Lee and Bell, 1997).

It is evident that the ORC complex is conserved throughout eukaryotes. Soon after its discovery in *S. cerevisiae*, orthologs of each ORC subunit were identified in various other eukaryotic organisms, including *S. pombe* (Moon *et al.*, 1999), *X. laevis* (Rowles *et al.*, 1996), *D. melanogaster* (Gossen *et al.*, 1995), and *H. sapiens* (Vashee *et al.*, 2001; Dhar and Dutta, 2000). Identification of ORC was more difficult in other species as it was with budding yeast since individual ORC subunits are not as tightly associated in *S. pombe* and mammals. As a result, extraction of ORC in these species does not typically yield the full complex. Following characterization of different ORC complexes, it is clear that individual subunits share a great deal of similarity between species. The human homologs of Orc1, Orc2, Orc4, and Orc5 show considerable similarity to the yeast and metazoan equivalents. For example, human Orc1 is 45% identical/62% similar and 29% identical/46% similar to the *Drosophila* and *S. cerevisiae* orthologs respectively (Dhar and Dutta, 2000). However, the same can not be said for all ORC
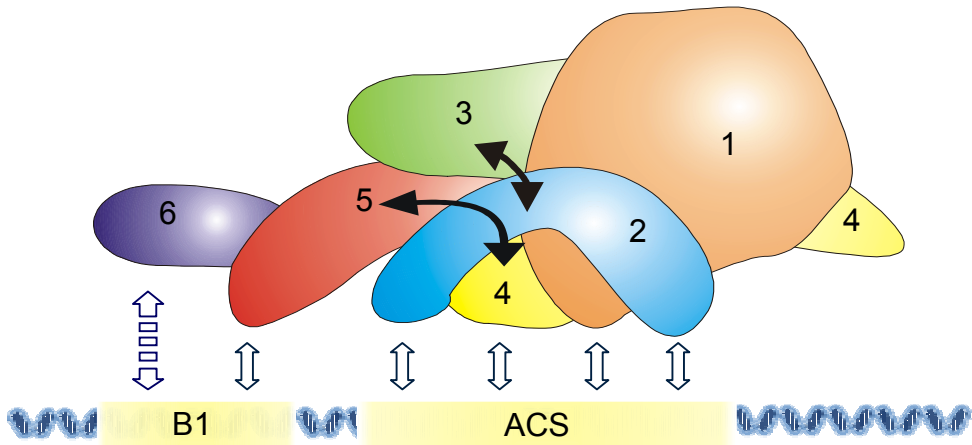
Figure 4. Assembly of the origin recognition complex (ORC). This model of budding yeast ORC was derived from UV-crosslinking studies (adapted from Lee and Bell, 1997). Black arrows indicate putative subunit interactions. White arrows indicate putative subunit-DNA interactions.

subunits identified to date. There are some ORC subunits, particularly budding yeast Orc6, that are quite divergent and share very little conservation among different species (Dhar and Dutta, 2000). Although ORC is found constitutively associated with origins of replication in yeast, the same is not true for all species. In humans, it appears that not all of the ORC subunits remain chromatin-bound throughout the cell cycle. Human Orc1 is only found on chromatin during G1 phase and gets removed during DNA synthesis (Kreitz *et al.,* 2001). This translocation of Orc1 is thought to be a mechanism by which reformation of the pre-RC and re-replication of the genome is prevented within the same cell cycle. It is possible that in humans, Orc1 is required for the initial binding of ORC, but not for its maintenance at origins, thus it is dispensable for ORC function after it is loaded on to chromatin. In budding yeast, it is the five largest ORC subunits (ORC_{1-5}) that are required to recognize and bind to DNA (Lee and Bell, 1997). The smallest protein, Orc6, is not required in this action, but still remains an essential protein to the cell. In contrast, fruit fly Orc6 is required for both ORC association with origins, and DNA replication (Chesnokov *et al.,* 2001). Thus, there appears to be variability among eukaryotes regarding the mechanisms that load and maintain ORC at origins.

Since origins of replication are well defined in budding yeast, characterization of ORC association with DNA has been studied in more detail in this species than in any other. Budding yeast ORC specifically binds to both the A and B1 elements of origins of replication in an ATP-dependent manner. Single base-pair mutations within either the A or B1 domains causes a significant decrease in ORC association and in levels of DNA replication (Bell and Stillman, 1992; Rowley *et al.,* 1995). Throughout the cell cycle, budding yeast ORC is localized to ~460 chromosomal sites (Pak *et al.,* 1997), spaced

approximately every 20-30 kb. Interestingly, this number is significantly lower than the total number of ACS sequences identified throughout the *S. cerevisiae* genome. This suggests that there is some selectivity over origin sequences. In contrast to budding yeast, fission yeast exhibits a significantly different method of origin selection. Origins of replication contain similar AT-rich domains to budding yeast, yet ORC association with origins is dependent upon a AT-hook located on Orc4. It is this DNA binding motif found at the N terminus of the protein that conveys the DNA sequence specificity of ORC in fission yeast (Chuang and Kelly, 1999). Although not found in any other Orc4 orthologs, the AT-hook in *S. pombe* Orc4 is essential for cell viability.

In metazoans, origins of replication and the nature of ORC binding are much less defined. As mentioned earlier, there does not appear to be a consensus sequence linked to metazoan origins, and several factors are involved in ORC association with origin DNA. It has been difficult to study ORC association with origins, as it is clear that metazoan ORC possesses little selectivity over DNA sequences. It is possible that loading factors involved in pre-RC formation, such as Cdc6, may affect ORC binding. Cdc6 was shown to increase the stability of ORC on chromatin (Speck *et al.,* 2005), which may ultimately dictate the final location of pre-RC formation. Alternatively, it is known that ORC sites typically co-localize with transcription promoters. It is possible that ORC is recruited to chromatin by various transcription factors (Bosco *et al.,* 2001; Danis *et al.,* 2004). Thus, it appears as though chromatin association of ORC in metazoans is governed by *trans* factors outside of the ORC complex itself.

It is clear that in all organisms examined thus far ORC is essential for DNA replication (reviewed in Kelly and Brown, 2000; Bell and Dutta, 2002). As stated earlier, the

primary function for ORC is the recognition of origins and subsequent loading of the pre-RC components. In *S. cerevisiae* and *D. melanogaster*, ORC association with chromatin requires ATP binding by Orc1. An ATP-bound complex is also a prerequisite for recruiting Cdc6, the first member of the pre-RC to load onto ORC. However, in both of these species, hydrolysis of the bound ATP occurs later in initiation but this is not a requirement for ORC binding. Instead, it is the ATP bound state of ORC that is needed to assemble the pre-RC.

In addition to its role in DNA replication, ORC has been found to function in several other cellular processes. For example, ORC is involved in the reorganization of chromatin. As part of this role, it appears as though ORC is involved in transcriptional silencing, which renders regions of chromosomes inactive, similar to heterochromatin in higher eukaryotes. The best characterized example of ORC participation in gene silencing is in *S. cerevisiae* (Foss *et al.,* 1993)*.* It is known that ORC binds to regions adjacent to the silent mating type loci and recruits silent information regulator (Sir) proteins (Triolo and Sternglanz, 1996). The loading of Sir proteins results in a position effect, in which the genes around these loci are repressed. As well, ORC is typically found in intergenic regions of DNA, as most ORC in *Drosophila* is observed in areas of heterochromatin. It is possible that ORC alters the conformation of local chromatin structures. In humans, ORC has been found to interact with histone acetyltransferases (HATs, Iizuka and Stillman, 1999). Acetylation of histones typically results in a loosening of the nucleosome, which generally allows genes to be transcribed. Although the significance of ORC interaction with HATs is unclear, it is possible that ORC could regulate the acetylation of adjacent areas ensuring that gene silencing is maintained.

Several ORC subunits have been found to be required in M phase. In budding yeast, Orc5 was shown be required in early M phase (Dillin and Rine, 1998). Although it function later in the cell cycle is unclear, asynchronous cultures depleted of Orc5 arrest primarily in late G2/M phase. It is possible that ORC, along with other pre-RC members are involved in a G1/M checkpoint to ensure the proper sequence of cellular events. It has been shown that checkpoint signals from pre-RC components during G1 and S phase inhibit premature entry into mitosis (Kelly *et al.*, 1993; Maiorano *et al.*, 1996; Piatti *et al.*, 1995; van Brabant *et al.*, 2001), thus ensuring that chromosome segregation is only initiated after DNA replication is complete. Outside of the checkpoint control of mitosis, Orc6 has been found to be directly associated with M phase and cytokinesis in both humans and flies (to be discussed in more detail below; Prasanth *et al.,* 2002; Chesnokov *et al.,* 2003).

Cdc6/18

Found in all eukaryotes studied to date, the Cdc6 protein (Cdc18 in *S. pombe*) is a member of the AAA$^+$ ATPase family, as are several of the ORC subunits and the MCM proteins. It is thought that members of this family bind and hydrolyze ATP, and in doing so cause conformational changes to the protein. It is likely that this is the mechanism by which a controlled, sequential assembly of pre-RC components is performed (Neuwald *et al.,* 1999). Orthologs of Cdc6 share a great deal of sequence and structural similarity (Crevel *et al.,* 2005). The budding yeast protein sequence is approximately 25% identical/45% similar to the *Xenopus, Drosophila* and human

orthologs. As well, Cdc6 is similar to members of the ORC complex, particularly Orc1, in which it is 22% identical/41% similar (Bell *et al.,* 1995).

Both mRNA transcript and protein levels for this protein peak at the M/G1 transition of the cell cycle. Upon completion of M phase Cdc6 initiates pre-RC assembly by binding ORC. Repression of *CDC6,* at the mRNA or protein level prevents pre-RC formation in G1 and subsequent initiation of DNA replication. In budding yeast, Cdc6 is targeted for degradation following S phase (Piatti *et al.,* 1995). In higher eukaryotes, Cdc6 appears to be stable throughout the cell cycle, but is actively transported from the nucleus after the start of S phase (Saha *et al.,* 1998). However, there may be a small population of Cdc6 that remains localized to the nucleus throughout the cell cycle in mammalian cells (Alexandrow and Hamlin, 2004). The remaining Cdc6 found in the nucleus is thought to be involved in checkpoint surveillance.

Cdc6 appears to have several roles leading up to DNA replication. Primarily, it is significantly involved in licensing origins of replication. Cdc6 is targeted to origins and the ORC complex during early G1 phase. In budding yeast, the ORC-Cdc6 complex has an enhanced DNA-binding specificity relative to ORC alone (Speck *et al.,* 2005). As mentioned earlier, it is thought that this increased specificity may be the key to metazoan origin identification. More recently, human cells depleted of Cdc6 in G1 phase resulted in a G1 phase arrest, consistent with its role in pre-RC assembly (Lau *et al.,* 2006). However, this study also showed that removing Cdc6 during S phase causes a reduction in the rate of DNA replication, followed by mitotic lethality. Chromosomal combing of these cells indicated that forks that fired prior to the Cdc6 depletion were able to continue synthesizing new strands, but no new origins were able to fire.

In budding yeast, Cdc6 binding to ORC causes ATP hydrolysis and a conformational change in ORC leading to the formation of an ORC-Cdc6 complex (Speck *et al.,* 2005). This ORC-Cdc6 structure is thought to contain six highly conserved AAA+ ATPase proteins, consisting of Cdc6 and five $ORC_{(1-5)}$ subunits. Reconstruction of electron microscopic images revealed that the ORC-Cdc6 complex forms a ring around the double helix (Speck *et al.,* 2005). Interestingly, along with several ORC subunits, Cdc6 contains regions conserved in clamp loaders, which function to load ring-shaped protein factors onto DNA. Therefore, it is not surprising that Cdc6 is a key factor during pre-RC formation in early G1 phase (Piatti *et al.,* 1995; Cocker *et al.,* 1996), as it functions to load the MCM complex, which has been shown to form a ring structure with a central cavity the size of double-stranded DNA (Takahashi *et al.,* 2005). Interestingly, inhibiting ATP hydrolysis by Cdc6 during pre-RC formation causes a stabilization of Cdt1 at origins (Randell *et al.,* 2006), and as a result, MCM association with origins remains unstable. It is not until ATP hydrolysis of Cdc6 and the dissociation of Cdt1 that the MCM complex is tightly loaded at origins.

Cdc6 is also involved in cell cycle checkpoints, although this function is much less understood as it appears to be species specific. In *S. cerevisiae*, Cdc6 plays a role in mitotic exit (Bueno and Russell, 1992), in *S. pombe* it is needed for the intra-S phase checkpoint (Murakami *et al.,* 2002), whereas the *X. laevis* ortholog may play a role in monitoring both S and M phase progression (Clay-Farrace *et al.,* 2003). As mentioned earlier, signals from yeast Cdc6 during G1 and S phase inhibit premature entry into mitosis (Kelly *et al.*, 1993; Piatti *et al.*, 1995;). However, when Cdc6 is depleted during G1 phase the cells later enter into a reductional division in which they undergo partial

mitosis without completing DNA replication. This lethal "cut" phenotype, along with other cell cycle abnormalities has been observed in both budding and fission yeast (Piatti *et al.,* 1995; Kelly *et al.,* 1993), as well as *Drosophila* (Crevel *et al.,* 2005), and is the result of a global inhibition of DNA replication. Without Cdc6 in these systems, DNA replication fails to initiate. Since DNA synthesis does not begin, there are no signals (i.e. stalled replication forks) to notify the cell that replication is incomplete. Therefore, cell cycle regulators attempt to continue the cell cycle even though DNA replication did not occur, causing partial mitosis. Interestingly, simple over-expression Cdc6/18 is sufficient to cause re-replication of the genome in the absence of cell division in *S. pombe (*Muzi-Falconi *et al.,* 1996; Nishitani *et al.,* 1995*)*, although this phenotype is not observed when Cdc6 is over-expressed in either humans or budding yeast. As mentioned earlier, re-replication in *S. cerevisiae* results from a lack of phosphorylation of key components of the pre-RC, including ORC, Cdc6, and the MCM complex (Nguyen *et al.,* 2001). As well, over-expression of Cdc6 leads to a mild delay in initiation of M phase (Elsasser *et al.,* 1996), with little effect on the rate of cell cycle progression. Similarly, microinjection of high levels of Cdc6 into higher eukaryote cells during G2 phase blocks progression into M phase (Clay-Farrace *et al.,* 2003). These results suggest that Cdc6 is involved in a number of crucial cell cycle pathways, including DNA licensing and replication, as well as cellular checkpoint control.

Cdt1

Although only transiently associated with the pre-RC, Cdt1 is key element in pre-RC development. Originally identified in *S. pombe,* Cdt1 is conserved among all eukaryotes

studied to date, including yeast, frogs, flies and humans (reviewed in Bell and Dutta, 2002). Initially it was thought that there was no Cdt1 ortholog in budding yeast, since it exhibits low conservation being 10% and 12% identical to the fission yeast and *Xenopus* proteins respectively (Tanaka and Diffley, 2002). Although it is quite divergent at the amino acid level, Cdt1 function appears to be quite similar among species. Along with Cdc6, Cdt1 is required to load the MCM complex onto origins during G1 phase. Mutations in *CDT1* inhibit the initiation of DNA synthesis and also results in a "cut" phenotype similar to null Cdc6 mutations (Hofmann and Beach, 1994). When only Cdt1 is loaded onto chromatin in a *Xenopus in vitro* assay, it is insufficient to load the MCMs, even if Cdc6 is subsequently added (Tsuyama *et al.,* 2005). This suggests that there is a defined order in which pre-RC components must be loaded. Failure in this sequential order of events renders origins incompetent. Therefore, after Cdc6 is loaded onto origins, it works with Cdt1 to promote loading of the MCM complex. A current model suggests that the MCM complex may enter the pre-RC via Cdt1 and subsequent ATP hydrolysis of Cdc6 stimulates the disassociation of the Cdt1 molecule (Randell *et al.,* 2006; reviewed in Cvetic and Walter, 2006). It is thought that the conformational changes resulting from Cdt1 disassociation may be required for a stable interaction between the MCM complex and origin DNA.

As with Cdc6, the levels of Cdt1 are strictly monitored in the nucleus. In *S. pombe* and most metazoans, global levels of Cdt1 are regulated by cyclins and Cdks, with protein levels peaking in early G1, and then decaying at the start of S phase (Nishitani *et al.,* 2000). In budding yeast, the cellular levels of Cdt1 remain constant throughout the cell cycle, yet it is only found in the nucleus during G1 (Tanaka and Diffley, 2002). In this

system, the transport of Cdt1 into the nucleus appears to be linked with the similar translocation of the MCM complex.

As an added mechanism of control over the initiation of DNA replication, metazoans produce a specific inhibitor of Cdt1, called geminin (McGarry and Kirschner, 1998). When present, geminin specifically binds to and inactivates Cdt1. Although ORC and Cdc6 are still able to associate with origins in the presence of geminin, MCM loading is impaired. The levels of the geminin peak at S phase, thus preventing the reformation of pre-RCs at early origins. Most of the cellular population of geminin is degraded during M phase, although some pools are maintained in an inactive state (Li and Blow, 2004). If Cdt1 is added to *Xenopus* extracts *in vitro* following DNA synthesis, it induces re-replication, particularly in the absence of geminin (Arias and Walter, 2005). This suggests that targeted proteolysis of Cdt1 and geminin work in a redundant pathway to inhibit rereplication in these organisms.

Minichromosome maintenance proteins

The MCM $_{(2-7)}$ complex

The MCM complex is the final component of the pre-RC necessary to license origins of replication in preparation for DNA synthesis. It consists of six proteins, Mcm2 to Mcm7, with equal stoichiometry (Forsburg, 2004). Each MCM protein was identified in *S. cerevisiae* in a screen to isolate proteins required to efficiently replicate plasmids (minichromosomes) (Maine *et al.,* 1984). Mutations in any of these proteins prevents DNA replication. This complex of proteins are highly conserved among eukaryotes, and even *Archaea* (reviewed in Maiorano *et al.,* 2006). Even among the complex, individual

MCM proteins are very similar, as the N-terminus of each protein contains highly conserved Walker A and B motifs. Each MCM protein is required for cell viability, as they are not functionally redundant.

As mentioned above, ORC, Cdc6 and Cdt1 are all required to load the MCM complex. The MCM complex does not possess any intrinsic affinity for DNA (Mendez and Stillman, 2003), and thus relies on being loaded by pre-RC components onto origins. In budding yeast, direct interactions have been observed between the MCM complex and several pre-RC proteins (reviewed in Kelly and Brown, 2000), including ORC (this study), Cdt1 (Tanaka and Diffley 2002), Cdc6 (Jang *et al.,* 2001), Mcm10 (Homesley *et al.,* 2000), Cdc45 (Zou *et al.,* 1997) and Cdc7/Dbf4 (Varrin *et al.,* 2005). However, there are also very strong interactions among the MCM proteins, suggesting that they function as a complex (reviewed in Bell and Dutta, 2002). Indeed, a 560 kDa complex was isolated from *S. pombe*, comprising stoichiometric amounts of each protein. Once bound to chromatin, it is thought that some of the initial loading factors (ORC, Cdc6) are not necessary to maintain the MCM complex at origins (Donovan *et al.,* 1997).

Recent evidence has altered the original understanding of the pre-RC, as it was shown that up to 20 molecules of the MCM complex are loaded onto individual origins prior to initiation (Bowers *et al.,* 2004). This successive loading occurs after, and through a different mechanism, than the initial MCM complex. As mentioned earlier, this first MCM complex is stably loaded at origins following the ATP hydrolysis of Cdc6 and dissociation of Cdt1. Subsequent MCM complexes are loaded through a process that requires the continued ATP hydrolysis of Orc1, and is dependent on the Orc1 and Orc4 proteins. The functional significance of this reiterative loading is unclear; however,

mutations that abolish Orc1 hydrolysis are lethal. Even with the greater number of MCMs present, it is still thought that only two are activated per origin at the onset of S phase, and establish the bi-directional fork.

Recent studies indicate that the phosphorylation state of MCM proteins may affect their DNA binding ability. Different phosphorylation states of Mcm4 can enhance its chromatin association and alter its localization pattern throughout the cell cycle (Komamura-Kohno *et al.,* 2006). Phosphorylation of Mcm4 inhibits DNA helicase activity, and phosphorylated Mcm4 does not co-localize to sites of DNA replication (Ishimi and Komamura-Kohno, 2001). Unexpectedly, populations of phosphorylated Mcm4 were found localized to the nucleolus, although its function at this location is unclear (Komamura-Kohno *et al.,* 2006). At this point, very little is known about the function of MCM proteins outside of DNA replication, but evidence from phosphoylation events and localization studies suggests that some MCMs may have roles beyond DNA synthesis.

During DNA replication the MCM complex is thought to be involved in the unwinding of the helix. Structural analysis of the MCM complex revealed a ring shape with a large central opening sufficient for double-stranded DNA (Takahashi *et al.,* 2005). As expected of helicases, members of the MCM complex are observed travelling along DNA with the replication fork (Aparicio *et al.,* 1997). As well, ATP hydrolysis by the MCM complex is required for its enzymatic activity (Ying and Gautier, 2005). When the ATPase activity is inhibited, the MCM complex is able to bind to origins and participate in the formation of the pre-RC, but these cells are unable unwind origin DNA. However, there is some resistance in the literature to labeling the MCM complex as *the* eukaryotic

helicase enzyme (reviewed in Takahashi *et al.,* 2005). For one reason, the entire MCM complex is devoid of helicase activity *in vitro*. Biochemical evidence indicates that only a subset of the complex (Mcm4/6/7) exhibits helicase activity (Ishimi, 1997). Even the helicase activity shown by this complex is minimal when compared to other helicases. Although smaller complexes have been purified *in vitro*, only the full $MCM_{(2-7)}$ complex has been observed *in vivo*. Interestingly, biochemical analysis indicates that some of the other MCM subunits (Mcm2, Mcm3/5) may in fact inhibit the enzymatic action of the Mcm4/67 complex. Therefore, the full MCM complex may represent the catalytic helicase along with its regulatory subunits.

In budding yeast, the MCM complex is translocated into the nucleus at the onset of G1 phase, and rapidly exported again to the cytoplasm after S phase is complete (reviewed in Kelly and Brown, 2000). However, this extra measure to prevent re-replication is not observed in metazoans, as MCMs are found in the nucleus throughout the cell cycle in these organisms. Once loaded onto DNA, the MCM complex associates directly with the origin and proximal DNA sequences. In *S. cerevisiae*, mutations in the B1 domain hinder MCM association with origins. Once bound in G1 phase, the MCM complex remains on the DNA until the completion of S phase, and this displacement is consistent with the completion of DNA synthesis.

<u>Mcm10/Cdc23</u>

Mcm10 is not a member of the MCM$_{(2-7)}$ complex mentioned above, and has not been shown to possess helicase activity. As well, this protein does not share any sequence or structural similarities of the other MCM proteins. However, this protein was originally identified in the same screen that isolated members of the MCM complex above (Maine *et al.,* 1984), and has been shown to be involved in the initiation of DNA replication. It is a highly conserved protein, found in various eukaryotes including yeast and humans (reviewed in Lei and Tye, 2001). Preliminary characterization of *MCM10* and its protein product were carried out in yeast (Merchant *et al.,* 1997), which showed that Mcm10 remains localized to the nucleus throughout the cell cycle. Later studies confirmed that Mcm10 is chromatin-associated at all stages, and is found at known origins of replication (Homesley *et al.,* 2000). This differs from observations from humans, where Mcm10 levels fluctuate throughout the cell cycle and is only bound to chromatin during S phase (Izumi *et al.,* 2001). In fact, more recent evidence indicates that the localization of human Mcm10 varies even within S phase (Izumi *et al.,* 2004). Prior to initiation in humans, Mcm10 is recruited to origins, but dissociates after origin activation and localizes at the nuclear periphery and nucleolar regions.

Mcm10 has been shown to interact with several replicative and pre-replicative proteins. Primarily, Mcm10/Cdc23 associates with several members of the MCM complex in both fission and budding yeast, as well as humans (Hart *et al.,* 2002; Merchant *et al.,* 1997; Izumi *et al.,* 2000). However, removal of Mcm10/Cdc23 in *S. pombe* did not affect MCM$_{(2-7)}$ association with chromatin nor their integration into pre-RCs (Gregan *et al.,* 2003). An interesting discovery came after using budding yeast strains containing the

double *mcm10-1/mcm7-1* mutations.   Each single mutation results in the loss of association between the two proteins; however, genetic analysis shows that when combined the *mcm10-1/mcm7-1* mutants regain cell viability even at restrictive temperatures (Homesley *et al.,* 2000). Mcm10 has also been shown to self-interact by co-immunoprecipitation and two-hybrid analysis, assembling into large homocomplexes (~800 kDa) consisting of approximately 12 Mcm10 molecules (Cook *et al.,* 2003). The region of Mcm10 required for this interaction is essential for cell viability, suggesting that this is a physiologically significant complex.

Outside of MCM proteins, preliminary studies indicate that Mcm10 interacts with ORC subunits. Immunoprecipitation of budding yeast Orc1 also pulled down Mcm10, although the same reverse experiment did not precipitate Orc1 (Kawasaki *et al.,* 2000). Also, weak genetic interactions were observed between *mcm10-1* and both *orc2-1* and *orc5-1*, in which the double mutants exhibited lower permissive temperatures than the single mutants. A Mcm10 and Orc2 interaction was later confirmed physically in yeast (Izumi *et al.,* 2000), *Drosophila* (Christensen and Tye, 2003), and humans (Izumi *et al.,* 2000). In fission yeast, Mcm10/Cdc23 was shown to interact with several ORC subunits (Orc1/2/5/6), as observed through two-hybrid analysis (Hart *et al.,* 2002).

Interestingly, eliminating Mcm10 activity from the cell through growth of a temperature-sensitive strain *(mcm10-1)* at non-permissive temperatures revealed that it is an essential protein (Merchant *et al.,* 1997; Kawasaki *et al.,* 2000). Once depleted of Mcm10, cells arrest with a 2C DNA content, a large bud and a single nucleus. As well, these cells have significantly reduced initiation of DNA replication at origins. Even at semi-permissive temperatures the *mcm10-1* mutant exhibited replication defects, with a

prolonged S phase. This replication flaw was also observed in *Xenopus*, and can be

rescued if recombinant Mcm10 is added (Wohlschlegel *et al.,* 2002). Surprisingly, the

stalling of activated replication forks was mapped in yeast to *ARS* regions that have yet

to fire (Merchant *et al.,* 1997). It is possible that Mcm10 functions to remove large pre-

RC complexes and other protein blocks in front of progressing replication forks, which

would therefore stall if Mcm10 were not present. It has been shown that Mcm10 travels

with the replication fork and might therefore play a more direct role in new strand

synthesis. Chromatin association studies in budding yeast indicate that Mcm10 is

required to maintain Mcm2 bound to DNA (Homesley *et al.,* 2000). Even at low levels of

Mcm10, the MCM complex is displaced from chromatin, without affecting ORC binding.

This defect in pre-RC formation following Mcm10 depletion was not observed in

Xenopus or human.   In *Xenopus*, Mcm10 was found to be essential after pre-RC

assembly in order to load Cdc45 (Wohlschlegel *et al.,* 2002). This was supported by

Drosophila data that showed Mcm10 could associate directly with Cdc45 (Christensen

and Tye, 2003). Cdc45 is important in initiation as it binds to pre-RCs to recruit the

polymeraseα-primase complex (Mendez and Stillman, 2003), which is one of the last

steps before DNA synthesis begins.   In budding yeast, it was shown that Mcm10

regulates the chromatin association of polymerase-α through an interaction with Cdc45

(Ricke and Bielinsky, 2004). As well, Mcm10 travels with replication forks during DNA

synthesis to ensure the stability of the polymerase-α complex. Recent evidence

suggests that the fission yeast Mcm10 possesses primase activity, and a point mutation

in the region required for this function is lethal (Fien and Hurwitz, 2006). Therefore, in

budding yeast, Mcm10 appears to be involved in both the initiation and elongation stages of DNA replication.

Finally, recent results have implicated Mcm10 in transcriptional silencing. As mentioned above, ORC and other replication factors, including MCM proteins and Cdc45, have been connected with gene silencing (Bell *et al.,* 1993; Ehrenhofer-Murray *et al.,* 1999; Dziak *et al.,* 2003). These proteins recruit various Sir proteins and other factors associated with silencing. Once bound, Sir complexes spread along the adjacent DNA forming heterochromatin-like structures. Reporter genes can be integrated into areas known to be silenced and act as markers in biochemical assays. Mutations in budding yeast *MCM10* significantly decrease silencing at these loci (Liachko and Tye, 2005). Two-hybrid analysis indicates that Mcm10 associates with members of the Sir complex, and that this interaction is abolished in *MCM10* mutants (Douglas *et al.,* 2005). Through specific mutations, this novel role for Mcm10 can be isolated from its role in DNA replication. Taken together with earlier studies, these results suggest that Mcm10 may play a role in several cell cycle mechanisms along with DNA replication.

Research Objectives

The budding yeast Orc6 protein is essential for cell viability, however its function in the cell cycle is unknown. Although it was initially isolated along with the other ORC subunits, UV-crosslinking studies did not find Orc6 present at all ORC-bound origins (Lee and Bell, 1997), and there was no evidence that directly linked this protein to DNA replication. Amino acid comparisons indicated that *S. cerevisiae* Orc6 possesses very

little similarity to metazoan Orc6 proteins (Dhar and Dutta, 2000), so it is unclear if budding yeast Orc6 is a functional ortholog of these proteins. Therefore, this project was based on the hypothesis that budding yeast Orc6 plays an essential role in DNA replication. To sufficiently test this proposition, the goal of this project was to characterize the function(s) of Orc6 in the budding yeast cell cycle. There were three main approaches taken to assess the role(s) of Orc6 within the cell. The first was to determine the stage(s) at which Orc6 executes its function in the cell cycle. The second objective was to visualize the cellular localization of Orc6 throughout the cell cycle. The final aim of this project was to identify the proteins that associate with Orc6. The knowledge gained from each of these objectives advanced our understanding of Orc6 and offers insight into its function(s) throughout the cell cycle. Each of these objectives are outlined in more detail below.

Timing of the essential function(s) of Orc6

Orc6 is known to be an essential protein in budding yeast; however, it is not known when Orc6 is precisely needed in the cell cycle. Therefore, by synchronizing cultures to specific stages of the cell cycle and then depleting Orc6 while the cells are blocked, the point(s) at which Orc6 is required can be determined by then releasing the cultures from their arrest. Cell cycle progression after release from the arresting agent can be monitored by FACS analysis as well as microscopy. Our laboratory recently developed a strain of yeast in which the endogenous promoter of *ORC6* was replaced by an inducible *GAL1* promoter. Therefore, in this strain *ORC6* is over-expressed when grown in galactose-based medium, whereas the gene is shut-off when cultures are shifted to glucose medium. By first determining the rate of protein turnover through whole cell

extracts and immunoblot analysis, cultures can be grown in glucose for a sufficient amount of time to deplete Orc6. This requires a comparable wild-type strain in which the levels of Orc6 can be monitored as a control to ensure the protein concentration in the *GAL1-ORC6* strain is below that of endogenous levels. Knowing precisely when Orc6 carries out its essential function contributed to its characterization and established points for further investigation.

Localization of Orc6 throughout the cell cycle

The second objective of this project was to determine to the localization of Orc6 at different stages of the cell cycle. The endogenous copy of *ORC6* was tagged with a DNA sequence encoding a fluorescent protein and the resultant fusion protein was visualized through epifluorescent microscopy. It is known that DNA synthesis occurs at 15-20 discrete nuclear foci, consisting of ~400 replication forks (Pasero *et al.,* 1997). It was therefore of interest to observe whether Orc6 is found in these discrete foci or in a more diffuse pattern in the nucleus. Another objective was to observe whether the nuclear localization of Orc6 changes throughout the cell cycle to determine if Orc6 is only present at origins during G1 phase to assist in the initiation of DNA replication. Similarly, this experiment would determine if budding yeast Orc6 localizes to the cell membrane and the bud site during cytokinesis, as it was observed in metazoans (Prasanth *et al.,* 2002; Chesnokov *et al.,* 2003). Finally, comparing the localization of Orc6 to other ORC subunits could lead to a better understanding of the specific function(s) of Orc6, particularly if other ORC subunits were found contained within the nucleus throughout the cell cycle while Orc6 was not, or vice versa.

Protein interactions of Orc6

An important means by which to help determine the role of a protein is to identify its ligands. It was expected that Orc6 would be found to associate with other ORC subunits, as it was initially isolated with this complex (Bell and Stillman, 1992). As well, since ORC is involved in DNA replication, it seemed likely that Orc6 also plays a role in this process, in which case it could associate with different initiation factors. In other species, Orc6 was shown to be directly involved in additional cellular events, including mitosis and cytokinesis (Prasanth et al, 2002; Chesnokov et al, 2003). If the budding yeast protein plays a role in several cell cycle stages, it would be expected to interact with proteins involved in separate cellular mechanisms. Furthermore, the identification of novel proteins which bind to Orc6 could lead to the characterization of additional functions. Therefore, by identifying all the ligands of Orc6 it will be interesting to discover what other functions Orc6 may play in the cell. Two assays commonly used in parallel to evaluate protein interactions are two-hybrid analysis (reviewed in Brent and Finley, 1997) and co-immunoprecipitation (Gavin *et al.,* 2002). A liquid two-hybrid assay could be used to determine Orc6 associations with other ORC subunits and known DNA replication factors. To identify interactions between unknown proteins, a *S. cerevisiae* cDNA library screen could be used. Alternatively, immunoprecipitation of Orc6 could be used to isolate associated proteins as well, which could then be identified through mass spectrometry. Identification of ligands of budding yeast Orc6 was expected to implicate the protein in alternate cellular pathways and offer insight into new mechanisms to be characterized.

CHAPTER II: Materials and Methods

<u>Cloning</u>

All cloning was performed using a directional approach, using different restriction enzyme sites incorporated into the 5'-ends of PCR primers. Construction details for specific plasmids are provided in the proceeding section. In general, genes were amplified from wild-type, haploid yeast DNA (DY1), that was isolated as described previously (Isolation of genomic DNA for Southern Blot analysis; Burke *et al.,* 2000). Once DNA was harvested, amplification of yeast ORFs was performed using Expand High Fidelity PCR System (Roche), according to manufacture's instructions. PCR products were run on a 0.8% agarose gel and purified using a Gel Extraction Kit (Qiagen). The amplified DNA and the desired vector DNA were digested using the appropriate restriction enzymes, and subsequently purified using a PCR Purification Kit (Qiagen). These products were used in ligation reactions along with T4 DNA ligase (Promega) and incubated overnight at $16^{o}C$. Each ligation reaction was transformed into competent DH5α *E. coli* cells. Plasmid DNA was then isolated using a GenElute Plasmid MiniPrep Kit (Sigma) according to manufacturer's instructions. Once purified, the new plasmids were digested to confirm inserts of the appropriate size with the original enzymes used in their construction. The plasmids were then transformed into yeast and plated on the proper selective medium (Schiestl and Gietz, 1989).

Although most plasmid constructs were established with the above method, some were the result of alterations to pre-existing vectors. To create an eYFP tagging vector, the DNA sequence encoding the GST tag was removed from pFA6a-GST-KanMX6 (Longtine *et al.,*1998) through digestion with PacI and AscI. Following the double digest, the plasmid backbone was purified using a Gel Extraction Kit (Qiagen). The

gene encoding eYFP was amplified from pEYFP (Promega) using an Expand High Fidelity PCR System (Roche). The primers used in the PCR were designed to amplify the entire coding region of the gene, with PacI and AscI sites in the 5'-end of the forward and reverse primers respectively. Following digestion and purification of the PCR product, *EYFP* was ligated into the pFA6a-KanMX6 vector backbone to create pFA6a-eYFP-KanMX6. Similarly, the pFA6a-GST-TRP1 plasmid was digested with PacI and AscI to release the GST coding region. *ECFP* was amplified from pECFP (Promega) and cloned into pFA6a-TRP1 as above, creating pFA6a-eCFP-TRP1.

Similarly, pCM190-ORC6-MYC was digested with PacI and AscI to pop out the *ORC6* gene, creating a linear pCM190-MYC vector backbone. Both *HOF1* and *RLF2* genes were amplified with primers that have PacI and AscI restriction sites incorporated into their 5'-ends, as described above. Once the PCR products were digested and purified, they were used in a ligation reaction with the pCM190-MYC backbone established earlier, creating pCM190-HOF1-MYC and pCM190-RLF2-MYC.

All other constructs were developed by amplifying the entire coding sequence (unless specified) of each gene from DY1 using a Expand High Fidelity PCR System (Roche). Each PCR primer had a restriction enzyme site incorporated into its 5' end to facilitate the ligation reaction. Once amplified, the products were cloned in-frame with any fusion cassettes of the vector.

Protein Tagging

Genomic tagging of ORFs was performed by homologous recombination with linear PCR fragments amplified using plasmid templates, as described by Longtine *et al.,*

(1998). Epitope tags were amplified from a set of plasmids, along with selectable marker genes to select for integrants. Specifically, pFA61-13Myc-kanMX6 and pFA6a-13Myc-TRP1 were used to create the ORC6myc13 and ORC2myc13 strains respectively. The pFA6a-TRP1-PGAL1-3HA was used to create the GAL1-ORC6-TK+ and the GAL1-orc2-1-TK+ strains. The pFA6a-eCFP-TRP1 and pFA6a-eYFP-KanMX6 were used to create the ORC6-eCFP, ORC6-eYFP, ORC2-eYFP, NUP49-eYFP, ORC6-eCFP/ORC2-eYFP, ORC6-eCFP/NUP49-eYFP and ORC2-eCFP/NUP49-eYFP strains. The amplification process involved using primers that were able to recombine in the yeast genome. The forward PCR primer recombined immediately upstream of the stop codon, while the reverse primer recombined downstream of the ORF. Therefore, the tag was incorporated in-frame immediately after the last amino acid-encoding codon, thus resulting in a C-terminal extension to the protein. Confirmation of the appropriate integration was obtained through PCR using primers that flank the region of recombination. The strains that contain the tag in the desired location would produce a PCR fragment larger than observed from wild-type yeast DNA. When possible, whole-cells extracts were analyzed by a western blot to confirm appropriate protein expression. A list of tagged strains created in the project are included in Table 1.

Table 1. Yeast strains used/created in this project.

Strain	Common Name	Genotype	Source
DY-1	GA-1020	MATa, ade2-1, can1-100, trp1-1, his3-11, his3-15, ura3-1, leu2-3, leu2-112, pep4::LEU2	S. Gasser
DY-26	BY4733	MATa, his3D200, leu2D0, met15D0, trp1D63, ura3D0	ATCC
DY-36	GAL1-ORC6	MATa, his3D200, leu2D0, met15D0, trp1D63, ura3D0, orc6::Pgal-3HA ORC6 TRP1	L. DaSilva
DY-39	ORC6myc13	MATa, ade2-1, can1-100, trp1-1, his3-11, his3-15, ura3-1, leu2-3, leu2-112, pep4::LEU2, orc6::ORC6-Myc13	J. Semple
DY-40	ORC6-eCFP	MATa, ade2-1, can1-100, trp1-1, his3-11, his3-15, ura3-1, leu2-3, leu2-112, pep4::LEU2, orc6::ORC6-ECFP	J. Semple
DY-41	ORC6-eYFP	MATa, ade2-1, can1-100, trp1-1, his3-11, his3-15, ura3-1, leu2-3, leu2-112, pep4::LEU2, orc6::ORC6-EYFP	J. Semple
DY-43	ORC2-eYFP	MATa, ade2-1, can1-100, trp1-1, his3-11, his3-15, ura3-1, leu2-3, leu2-112, pep4::LEU2, orc2::ORC2-EYFP	J. Semple
DY-45	NUP49-eYFP	MATa, ade2-1, can1-100, trp1-1, his3-11, his3-15, ura3-1, leu2-3, leu2-112, pep4::LEU2, nup49::NUP49-EYFP	J. Semple
DY-64	ORC6-eCFP ORC2-eYFP	MATa, ade2-1, can1-100, trp1-1, his3-11, his3-15, ura3-1, leu2-3, leu2-112, pep4::LEU2, orc6::ORC6-eCFP (kanMX6), orc2::ORC2-eYFP (TRP1)	J. Semple
DY-65	ORC6-eCFP NUP49-eYFP	MATa, ade2-1, can1-100, trp1-1, his3-11, his3-15, ura3-1, leu2-3, leu2-112, pep4::LEU2, orc6::ORC6-ECFP (TRP1), nup49::NUP49-eYFP (kanMX6)	J. Semple
DY-66	ORC2-eCFP NUP49-eYFP	MATa, ade2-1, can1-100, trp1-1, his3-11, his3-15, ura3-1, leu2-3, leu2-112, pep4::LEU2, orc6::ORC2-ECFP (kanMX6), nup49::NUP49-eYFP (TRP1)	J. Semple
DY-67	E1000	MATa ura3::URA3[GPD-TK(7x)] ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 GAL psi+	G. Brown
DY-70	mcm10-1	MATa, ade2-1, can1-100, his3-11,15, leu2-3,112, trp1-1, ura3-52, mcm10-1	A. Bielinsky
DY-79	GAL1-ORC6 TK+	MATa ura3::URA3[GPD-TK(7x)] ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 GAL psi+, GAL1-3HA-ORC6 (TRP)	J. Semple
DY-80	orc2-1 TK+	Mata, ura3::URA3/GPD-TK, orc2-1, trp, ade, his, leu	G. Brown
DY-81	ORC2myc13	MATa, ade2-1, can1-100, trp1-1, his3-11, his3-15, ura3-1, leu2-3, leu2-112, pep4::LEU2, ORC2::ORC2myc13 TRP1	J. Semple
DY-83	CDT1myc13	MATa, his3D200, leu2D0, met15D0, trp1D63, ura3D0, CDT1::CDT1myc13 HIS3	L. DaSilva
DY-84	MCM10myc13	MATa, his3D200, leu2D0, met15D0, trp1D63, ura3D0, MCM10::MCM10myc13 HIS3	L. DaSilva
DY-91	GAL1-ORC6 CDT1myc13	MATa, his3D200, leu2D0, met15D0, trp1D63, ura3D0, orc6::Pgal-3HA ORC6 TRP1, CDT1::CDT1myc13 HIS3	L. DaSilva
DY-92	GAL1-ORC6 MCM10myc13	MATa, his3D200, leu2D0, met15D0, trp1D63, ura3D0, orc6::Pgal-3HA ORC6 TRP1, mcm10::MCM10myc13 HIS3	L. DaSilva
DY-93	ORC6 3HA	MATa, his3D200, leu2D0, met15D0, trp1D63, ura3D0, orc6::ORC6-3HA-TRP1	L. DaSilva
DY-103	GAL1-ORC4	MATa, his3D200, leu2D0, met15D0, trp1D63, ura3D0, orc4::Pgal-3HA ORC4 TRP1	L. Kummer
DY-109	ORC4 3HA	MATa, his3D200, leu2D0, met15D0, trp1D63, ura3D0, orc4::ORC4-3HA-TRP1	L. Kummer
DY-126	GAL1-orc2-1 TK+	MATa, ura3::URA3/GPD-TK, orc2-1, trp, ade, his, leu, orc2-1::PGAL-3HA-ORC2-1 TRP1	J. Semple

Yeast DNA Isolation

Genomic yeast DNA was isolated as described previously (Burke *et al.,* 2000). Briefly, 10 ml of saturated culture was pelleted at 4,000 RPM for 5 min. Cells were resuspended in 0.2 ml of DNA isolation mix (2% Triton X-100, 1% SDS, 100 ml NaCl, 10 mM Tris-HCl (pH=8.0), 1 mM EDTA), along with 0.2 ml phenol:chloroform:isoamyl alcohol (25:24:1) and 0.3 g of acid-washed glass beads. Samples were vortexed for 3-4 min, 0.2 ml of 1X TE was added and subsequently hand mixed. Each sample was then centrifuged at maximum speed for 5 min and the top phase was transferred to a new tube. To precipitate the DNA, 1 ml of 100% ethanol was added to the samples. After 2 min of centrifugation the supernatant was discarded and the pellet was resuspended in 0.4 ml 1X TE. RNase A (125 μl/ml) was added to each sample and incubated for 15 min at 37°C. Then, ammonium acetate (0.1 M) was added, followed by 1 ml of 100% ethanol was added to each tube. The DNA was pelleted by centrifugation for 2 min and the supernatant was discarded. Finally, the pellets were air-dried and resuspended in 50 μl of 1X TE.

To isolate plasmid DNA from yeasts, 2 ml of saturated culture was spun down to collect the cells. Once the supernatant was removed, the cell pellets were resuspended in 0.2 ml of DNA isolation mix (as above), 0.2 ml of phenol:chloroform and 0.3 g of glass beads. After 3-4 min of vortexing, the samples were centrifuged for 5 min at maximum speed. 10 μl of the top aqueous layer was added directly to competent *E. coli* cells for transformation.

Protein Extracts and Western Blotting

Unless otherwise stated, yeast protein isolation was performed using a whole-cell extraction method described in (Burke *et al.,* 2000). Briefly, cells were pelleted and resuspended in 400 μl of ice-cold lysis buffer (10mM Tris-Cl pH 8.0/140 mM NaCl/1 mM EDTA/1% Triton X-100, with protease inhibitors). While kept at 4°C, 0.3 g of 0.5 mm glass beads were added to each sample and lysis occurred through 12 cycles (20 sec on/20 sec off) of bead beating (BioSpec). The lysates were centrifuged (10, 000 x *g,* 30 s) and the supernatant (whole-cell extract; WCE) was removed. Immediately following the isolation protocol, protein concentrations were quantified using a Bio-Rad Protein Assay (Bio-Rad) and expression of specific proteins was determined by western blot.

Detections were carried out following an initial incubation with Blocking Buffer (1X TEN + 5% skim milk powder) overnight at 4°C. The blot was then incubated with primary antibody for 1-2 h while gently shaking at RT (see Table 2 for details on specific antibodies, including dilutions), and then washed with 3 times with 1X TEN. Secondary antibody incubations were carried out similarly; however, each secondary antibody was conjugated to a fluorophore to facilitate the visualization process, so all incubations were carried out in the dark. The blots were then washed 2X with 1X TEN, 2X with ddH$_2$O and then detected on a Typhoon 9400. Densitometry of bands was carried out using ImageQuant v3.3 (Molecular Dynamics).

Table 2. Antibodies used in this project

Antibody	Company	Dilution
AlexaFluor 488 donkey anti-goat	Invitrogen	1:3000
AlexaFluor 488 goat anti-mouse	Invitrogen	1:3000
AlexaFluor 647 goat anti-rabbit	Invitrogen	1:3000
anti-GST (rabbit polyclonal IgG)	Sigma	1:3000
anti-HA (mouse monoclonal)	Sigma	1:5000
anti-LexA (rabbit polyclonal)	Invitrogen	1:2000
anti-Mcm2 (yN-19) (goat polyclonal IgG)	Santa Cruz Biotech	1:500
anti-MYC (mouse monoclonal)	Sigma	1:5000
anti-Orc2 (rabbit polyclonal)	S. Gasser	1:1000

Silver Staining of Protein Gels

After PAGE the gel was stained with silver according the manufacture's instructions (Sigma). Briefly, the protein gel was initially fixed in solution (50% ethanol, 10% acetic acid) overnight. The gel was then washed (30% ethanol) for 10 min, followed by a water wash for another 10 min. To prepare for the stain the gel was incubated in a 1% ProteoSilver Sensitizer solution for 10 min. After two 10 min water washes, the gel was equilibrated in Silver solution for 10 min. Carefully, the solution was decanted, the gel was immediately washed with water for 1.5 min and developed in Developer solution until the desired intensity. The developing reaction was stopped with 5 ml of ProteoSilver Stop solution incubated for 5 min. Gels were then stored in a sealed plastic bag with water.

Chromatin Binding Assay

Approximately 2.5×10^7 cells were harvested at 1000 x g and spheroplasted as performed previously (Pasero *et al.,*1999), with modifications. Cells were washed once with ddH$_2$O and incubated at 30°C for 10 min with gentle mixing in 10 ml/g prespheroplasting buffer [100 mM EDTA-KOH (pH 8), 10mM DTT], followed by incubation in 10 ml/g spheroplasting buffer [0.5XYPD, 1.1M Sorbitol] containing 0.5 mg/ml Zymolyase 20T (Seikagaku Corp., Japan) at 30°C for 10-15 min with gentle mixing. Cells were washed once with 20 ml spheroplasting buffer containing 0.5 mM PMSF followed by resuspension in 1 ml ice-cold wash buffer [5 mM Tris-HCl (pH 7.4), 20 mM KCl, 2 mM EDTA-KOH (pH 7.4), 1 M Sorbitol, 1% Thiodiglycol, 125 μM spermidine, 50 μM spermine] and protease inhibitors [0.1 mM benzamidine HCl, 1 μg/ml

pepstatin, 2 μg/ml antipain, 2 μg/ml leupeptin, 0.5 mM PMSF]. Cells were pelleted at

400 x g for 2 min in a microcentrifuge at 4°C and washed twice with 1 ml ice-cold wash

buffer, followed by resuspension in 0.4 ml ice-cold breakage buffer [5 mM Tris-HCl (pH

7.4), 20 mM KCl, 2 mM EDTA-KOH (pH 7.4), 0.4 M Sorbitol, 1% Thiodiglycol, 125 μM

spermidine, 50 μM spermine] and protease inhibitors as above. Cells were then lysed

with 0.5 ml ice-cold breakage buffer containing 2% Triton X-100 and incubated on ice

for 5 min with occasional mixing. The lysed cells were then spun at 16,000 x g for 5 min

in a microcentrifuge at 4°C. The chromatin pellet was digested on ice for 10 min in 100

μl ice-cold breakage buffer containing 5 mM $MgCl_2$ and 5 μl DNaseI (1 mg/ml).

Digestion was stopped by adding EDTA-KOH (pH 7.4) to 10 mM.

Fluorescent Microscopy

All images were taken with live cells. Prior to imaging cells were grown to ~5 x 10^6

cells/ml in SC medium (2% glucose, 0.02% adenine), washed in ddH_2O and

resuspended in fresh medium. Cultures were diluted with fresh medium to ~1 x 10^6

cells/ml, with 500 μl added to growth chambers for imaging. Chambers were created

using 0.5" I.D. glass tubing cut to 0.5" and glued to a standard glass (22 x 66 mm)

coverslip with medical grade silicon adhesive. Cells were imaged on a Zeiss Axiovert

100 with a 63X, 1.4 N/A objective lens. To image eCFP and eYFP we used filter sets

consisting of exciter D436/20x; dichroic 455DCLP; emitter D480/40m and exciter

HQ500/20x; dichroic Q515LP; emitter HQ520LP respectively (Chroma Technology,

Rockingham, VT). Images were collected with a Sony SX700 CCD (1024 x 768) and

processed in ImageJ 1.30v (NIH, Bethesda). The maximum exposure time with this

digital camera is only 2 sec and as a result imaging of some FP-tagged proteins under their endogenous promoters resulted in rather weak signals. To obtain a higher signal, 20 fluorescent images were taken in succession and subsequently summed using ImageJ software. Camera gain was adjusted to maximize signal to noise ratios in individual frames.

DNA staining was performed using Hoescht 33342 stain (Molecular Probes, USA). *GAL1-ORC6* cells were transferred to glucose for 6 h to deplete Orc6. As a control, wild-type (DY26) cells were also grown under the same conditions.   After the 6 hours, cultures were supplemented with Hoescht stain (5 μg/ml) and allowed to incubate for another 30 min. Cells were then allowed to settle in a growth chamber and imaged as above using an exciter 380; emitter 450 filter set.

FACS Analysis

Preparation of cells for FACS analysis was carried out as described in Davierwala *et al.,* (2005). Briefly, 10^7 cells were pelleted and resuspended in 70% ethanol to fix the cells. The cells were then treated with RNase A (200 μg/ml in 50 mM Tris HCl pH 8) for 2-4 h at 37°C. Subsequently, the cells were incubated in proteinase K (2 mg/ml in 50 mM Tris HCl pH 8) for 30-60 min at 50°C. Finally, the cells were pelleted and washed in FACS buffer (200mM Tris-HCl pH 7.5, 200mM NaCl, 78mM $MgCl_2$) before being transferred to Sytox solution (50mM Tris-HCl pH 7.5, 1:5000 dilution Sytox [Molecular Probes; 5mM Sytox in DMSO]) to stain the DNA. Cells were kept at 4°C in the dark until they were analyzed. The analysis was performed with a FACscalibur (Becton Dickinson) flow cytometer in the Immunology Department at the University of Toronto.

Synchronizing Yeast Cultures (α-Factor, Hydroxyurea, Nocodazole)

Alpha factor arrest

A synthetic version of the *S. cerevisiae* mating pheromone (α-factor; Louisiana State University Health Sciences Centre) was used to synchronize cultures to G1 phase. For a complete arrest, α-factor was added to each culture ($\sim 5 \times 10^6$ cells/ml) and incubated for 2.3 h. The actual amount of α-factor used in each experiment was determined by pre-testing each strain, although there were a few variations, typically 10 μg/ml was used for most strains. For longer arrests (ie. during Orc6 depletion experiments), after the initial 2.3 h block, a fresh aliquot of α-factor was added every hour. For example, if 20 μl of α-factor was needed to arrest the cells, then another 20 μl was added every hour beyond the original 2.3 h. Bud size and cell morphology, as determined by light microscopy, was used to evaluate the efficiency of the arrest before progressing with the experiment. After the arrest, cells were washed with water and released into fresh media containing 50 μg/ml Pronase E (Sigma).

Nocodazole arrest

To synchronize liquid cultures at G2/M, cultures were grown to log phase and diluted to $\sim 5 \times 10^6$ cells/ml in fresh media containing 15 μg/ml of nocodazole (Sigma). Cultures were then incubated for 2.3 h for a complete arrest. For longer arrests (ie. during Orc6 depletion experiments), the concentration of nocodazole was adjusted. If the culture was arrested up to another 3 h *after* the initial arrest, 12 μg/ml of nocodazole was used (instead of 15 μg/ml). The volume of nocodazole used to maintain the arrest in this extended block was added again a half-way through the duration to ensure cells remain

arrested. Cells usually had a difficult time coming out of longer arrests in nocodazole, therefore, upon release from a nocodazole block cells were washed in water, followed by the media that they were to released into, then water and media again. After a total of 5.5 h in nocodazole, cells had a very difficult time releasing from the block, and those cultures that did release, typically did not do so completely synchronously.

Hydroxyurea (HU) arrest

Hydroxyrurea was used to synchronize cultures in S phase. Cells were initially diluted to ~5×10^6 cells/ml in fresh media with 0.2 M HU (Sigma). Cultures were then incubated for 2.3 h to initially arrest the cells. For longer block durations (ie. during Orc6 depletion experiments), a higher concentration of HU (0.36 M) was used *after* the initial arrest.   In these cases, the amount of HU used to maintain the extended arrest was also added every 1.3 h to ensure the cells remain synchronized.   After the arrest, cells were washed with water and released into fresh media.

Mating Yeast Strains

Mating of haploid yeast strains was carried out as described previously (Burke *et al.,* 2000). Opposite mating-type strains were grown up in the appropriate medium until log phase. Approximately 10^7 cells of each type was pelleted by centrifugation. Each cell pellet was resuspended in 25 ml of enriched (2X YPD) medium, after which the cultures were combined and mixed.   The resulting culture was added to a 500 ml flask and incubated for 24 h at 50 RPM and 30°C. The cells were then collected by gentle centrifugation at 1,000 RPM for 5 min. Pellets were resuspended in 50 ml of selective

medium, and 100 μl of the final culture was spread on a plate of the same selective medium. Typically, mating was performed using opposite mating-type strains containing plasmids with different selectable markers; therefore, after mating the cultures were plated on drop-out medium that requires both plasmids to be present in the diploid cell.

<u>Yeast Two-Hybrid Assay</u>

Liquid two-hybrid assays were performed as described previously (Ausubel *et al.*, 1995). The LacZ-reporter plasmid (pSH18-34), along with bait (pEG202) and prey (pJG4-6) plasmids were serially transformed into yeast strain DY-1. Exponential cultures were grown in SC (2% glucose) medium lacking uracil, histidine and tryptophan to a concentration of 5 x 10^6 cells/ml, washed with ddH$_2$0 and then resuspended in SC (2% galactose/1% raffinose) lacking the same three components for 6 h to induce prey expression. Following induction, 5 x 10^6 cells were harvested and permeabilized. The relative strength of interaction was quantified through a β-galactosidase assay utilizing the substrate o-nitrophenyl-β-D-galactopyranoside (ONPG) (Wang *et al.,* 2001) and the formula: β-galactosidase activity = 1000 x A_{420nm}/(t x v x A_{600nm}), where t= time of reaction (min) and v = volume of culture used in the assay (ml).

Plate two-hybrid assays were also performed to screen for Orc6 ligands. Following yeast transformation cultures were plated on SC medium (2% galactose/1% raffinose, pH = 7.0) lacking uracil, tryptophan, and histidine, and supplemented with X-gal (80 μg/ml) to select for strains with the appropriate plasmids and indicate colonies with a successful interaction. Similarly, after mating cultures were plated on SC medium (2%

galactose/1% raffinose) lacking uracil, tryptophan, histidine and leucine to select for colonies with the correct plasmids as well as a interaction between fusion proteins.

Co-Immunoprecipitation

Genes of interest were cloned into pCM190 (with a MYC-encoding epitope) and pJG4-6 expression vectors and transformed into DY26 wild-type cells. Cultures were grown in glucose medium lacking uracil and tryptophan and supplemented with 5 μg/ml doxycycline (Dox), since the expression from pCM190 plasmids is repressed by Dox. Cells were transferred to culture containing 2% galactose/1% raffinose medium (BD Bioscience) lacking uracil, tryptophan and Dox for 6 h to induce expression from the pJG4-6 and pCM190 constructs. All subsequent steps were performed as described previously (Duncker *et al.*, 2002). Briefly, WCEs were prepared from each culture and added to 40 μl of magnetic Dynabeads (Invitrogen) conjugated with an anti-MYC antibody. Extracts were incubated with the beads for 1 h on a rotator at 4°C. The beads were washed 2X with lysis buffer and 2X in wash buffer before being stored at – 20°C. WCEs and bead samples were run on a western blot and detected with both anti-MYC and anti-HA.

GST Pull-Down

The experiment was performed as described previously (Holland *et al.*, 2002). Briefly, the gene of interest was cloned in the pGEX-KG vector, which contains DNA encoding a GST-tag, and transformed into competent BL21 cells. Plasmid-based protein expression was induced through the addition of IPTG followed by a 20 h incubation at 25°C. The

cells were then resuspended in TEN buffer with protease inhibitors and sonicated. Supernatants were conjugated to glutathione beads. Beads were stored at -20°C in glycerol. At the same time, the other gene of interest was cloned into pJG4-6 and transformed into DY26 wild-type cells. Exponential cultures were transferred to 2% galactose/1% raffinose medium (BD Bioscience) lacking tryptophan for 6 h to induce expression. WCEs were prepared from these samples and the supernatants were incubated with 40 μl of the GST beads for 1-2 h at 4°C. Samples were then washed 4X with wash buffer (20 mM Tris HCl ph 7.5, 100 mM NaCl, 0.2% NP-40, 0.3 mM EDTA, 0.3 mM EGTA and protease inhibitors), after which the beads were collected by centrifugation at 2000 rpm for 2 min.

DNA Combing

DNA combing was performed as in Versini *et al.,* (2003). In brief, wild-type and GAL1-ORC6 cultures were released synchronously from G1 following an initial arrest with α-factor and subsequent 4 h incubation in YPD and the pheromone to deplete Orc6. The cells were released into YPD containing both hydroxyurea (0.2 M) to stop S phase progression, and BrdU (0.4 mg/ml) to mark nascent DNA synthesis. After 90 min, genomic DNA plugs (800 ng DNA/plug) were made and stored at 4°C until combing. The plugs were initially stained with YOYO-1 (Molecular Probes), and then digested with agarase (Roche) and resuspended at 150 ng/ml in 50 mM MES pH 5.7. Isolated DNA was combed on silanized coverslips. Appropriate antibodies were used to detect both the DNA molecules (Argene) and BrdU incorporation (Sera Labs). Images were captured through a Leica DMRA microscope with a CCD camera. Analysis of track

lengths was performed using MetaMorph (Universal Imaging Corp.). Adenovirus DNA was used as a standard to convert distances to base pairs.

CHAPTER III: Initial Characterization of Orc6 in Budding Yeast

<u>Introduction</u>

ORC was discovered in budding yeast as a complex of proteins that bound ARS sequences and protected them from DNase treatment (Bell and Stillman, 1992). At the same time, a one-hybrid screen was performed in *S. cerevisiae* using a reporter gene (*GAL4*) with four ACS repeats imbedded in its promoter region (Li and Herskowitz, 1993). To determine which proteins bind to the ACS region, various protein fragments from a set of three complementary yeast expression libraries were fused to a transcriptional activator and used in the one-hybrid. Surprisingly, only one protein exhibited a strong interaction with the ACS region, which was later sequenced and determined to be Orc6 (Li and Herskowitz, 1993). Although this suggests that Orc6 recognizes yeast replication origins *in vivo*, it is possible that this is an indirect interaction mediated through ORC or another factor. However, if indeed this was an indirect interaction one would expect to also isolate the intermediate proteins (ie. other ORC subunits) from this screen. Four independent *ORC6* constructs were isolated using this assay, while no other protein convincingly associated with the ACS sequences.

It is thought that, at least in *S. cerevisiae*, Orc6 associates with ORC at origins of DNA replication. However, in both *S. cerevisiae* and *Xenopus*, Orc6 is the only subunit not required for chromatin binding of the recognition complex, nor was it found in larger complexes containing the other five ORC subunits (Lee and Bell, 1997; Gillespie *et al.,* 2001). Similar purifications of endogenous ORC from HeLa cells do not contain Orc6 (Vashee *et al.,* 2001). However, transfection of human cells with viruses expressing all six subunits resulted in the isolation of ORC complexes with stoichiometric levels of

Orc1-Orc5 and low amounts of Orc6 (Giordano-Coltart *et al.,* 2005). Thus, the Orc6 association with ORC in budding yeast and humans may be fairly weak, or require other cellular signals in obtain the appropriate binding conditions. Interestingly, the only species in which Orc6 has been shown to be required for chromatin association of ORC is *Drosophila* (Chesnokov *et al.,* 2001). This suggests that orthologs of Orc6 may exhibit species-specific functional variations.

A comparison of Orc6 amino acid sequences between species confirmed the differences observed in its function (Figure 5). Most of the ORC subunits share a great deal of similarity between species, particularly Orc1, Orc2, Orc4 and Orc5; however, amino acid sequences for both Orc3 and Orc6 vary dramatically (Dhar and Dutta, 2000). The similarities between human and budding yeast amino acid sequences of each ORC subunit are illustrated in Figure 6. It appears as though Orc6 is the most divergent of the group. Budding yeast Orc6 is only 5% identical/19% similar and 6% identical/15% similar to the *Drosophila* and human Orc6 proteins respectively (Dhar and Dutta, 2000), although the peptide sequence similarity between the latter two species is much higher (28% identical/49% similar). As well, the size of the budding yeast protein is much larger (~48 kDa) than the other Orc6 subunits identified to date (~28 kDa). Thus, not only is Orc6 the least conserved of all the ORC subunits, Orc6 in *S. cerevisiae* appears to be the most divergent ortholog characterized to date.

Although there is little conservation of Orc6 sequence, similarities in structure may point to similar functions among the orthologs. Preliminary characterization of the budding yeast Orc6 sequence did not yield any DNA binding or helicase motifs (Li and Herskowitz, 1993), which would support its role in DNA replication. Two potential

Figure 5. An alignment of Orc6 sequences from human (hsORC6), *Drosophila* (dmORC6), *S. pombe* (spORC6) and *S. cerevisiae* (scORC6) using GeneDoc program. Dark shading indicates identical amino acid residues, light shading represents similar residues. Numbers on the right designate the Orc6 amino acid residue for that organism (Dhar and Dutta, 2000).

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A   Human vs. *Drosophila*   **B**   Human vs. Budding Yeast
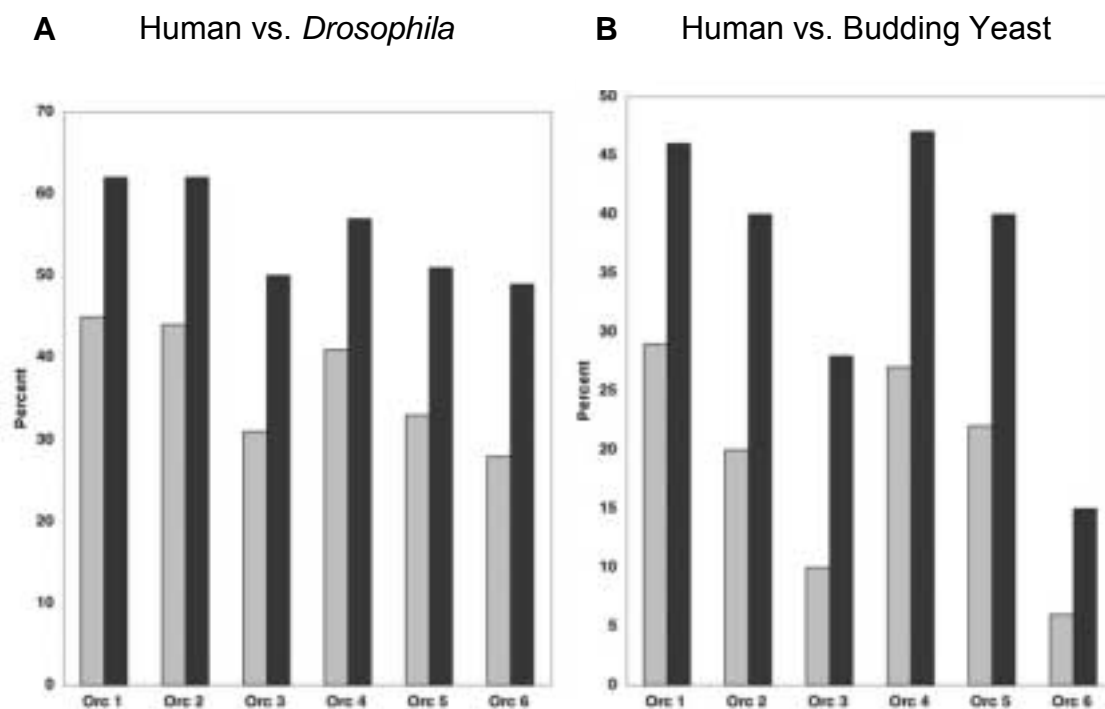
Figure 6. Comparison of ORC protein sequences between human, *Drosophila* and budding yeast. (A) A bar diagram illustrating the percent identity (light bars) and percent similarity (dark bars) between human and *Drosophila* ORC subunits. (B) A similar bar diagram showing the percent identity and similarity between human and budding yeast ORC subunits (Dhar and Dutta, 2000).

nuclear localization signals (NLSs) were identified, although it is not known if these motifs are functional. As well, several putative phosphorylation sites were identified in the first half of the amino acid sequence.

It appears as though Orc6 may be regulated by cyclin-dependent protein kinases, which are known to govern much of the machinery involved in cell cycle progression. Studies indicate that Orc6 is phosphorylated *in vivo*, and four possible phosphorylation sites have been identified in the N-terminus region of Orc6 (Li and Herskowitz, 1993). As mentioned earlier, it is known that Orc6 phosphorylation after initiation of DNA replication is part of a mechanism to prevent re-replication of DNA (Wilmes *et al.,* 2004; Archambault *et al.,* 2005). However, it is unclear if this is the only point in the cell cycle in which Orc6 is phosphorylated. In humans, Orc6 appears to be only weakly phosphorylated, and the level of phosphorylation remains constant throughout the cell cycle (Dhar and Dutta, 2000). This suggests that phosphorylation of human Orc6 is not a key event in a transition through the cell cycle.

Localization of Orc6 throughout the cell cycle can offer insight into its function, and can also be compared among different species. In both humans and budding yeasts, the levels of Orc6 remain constant throughout the cell cycle, similar to the other ORC subunits (Dhar and Dutta, 2000). However, very little is known about the location of endogenous Orc6 at various stages of the cell cycle. Over-expression of Orc6 in *Drosophila* shows the protein localized to both the nucleus and the cytoplasmic membrane throughout the cell cycle (Chesnokov *et al.,* 2001). This is similar to human cells, where Orc6 localized to the nucleus, as well as the cell periphery (Prasanth *et al.,* 2002). However, human cells also showed other interesting localization patterns for

Orc6. During mitosis, Orc6 was observed in reticular patterns adjacent to chromatin as well as in a punctate pattern on chromosomes proximal to the centromeres. Toward the end of mitosis human Orc6 is redistributed, and a subset is localized to the midbody between the separating cells. Judging from these patterns, it is possible that human Orc6 plays a role in DNA replication, chromosome segregation and cytokinesis.

Although the protein sequence of budding yeast Orc6 is unlike its orthologs, the question remains as to whether it functions similar to the other Orc6 molecules. Since it was initially discovered in a complex with the other members of ORC, it is assumed that Orc6 plays a role in DNA replication. Marked deletions of the *ORC6* gene arrest the cell cycle, indicating that this protein is essential for cell viability (Li and Herskowitz, 1993). When diploid strains heterozygous for this mutation are induced to undergo meiosis, the deletion co-segregated with cell cycle arrest following tetrad dissection. Microscopic analysis illustrated that germinating mutant spores completed one or two rounds of cell division and arrested at a large bud state, consistent with a defect in DNA replication or nuclear division. An intriguing result from human studies indicates that Orc6 is involved in more than just DNA replication, as treatment of cells with Orc6 siRNA revealed a number of phenotypes (Prasanth *et al.,* 2002). Cells were unable to align chromosomes at the metaphase plate, which caused an increase in multinucleation and polyploidy, verifying roles for Orc6 in mitosis and cytokinesis. Treated cells were also unable to efficiently incorporate BrdU, confirming a defect in DNA replication. Prolonged siRNA treatment resulted in a decrease in cell division and ultimately a loss of cell viability. Although a similar depletion of Orc2 revealed some of the same phenotypes and eventual M-phase arrest, many of the mitotic defects

observed after the Orc6 siRNA treatment were not observed in these cells. In a comparable study on *Drosophila* using RNAi, Orc6 depletion also resulted immediately in multinucleation and a loss of cytokinesis (Chesnokov *et al.,* 2003). As well, a marked decrease in DNA replication and eventual cell death followed prolonged treatment with Orc6 dsRNA, supporting the results observed in human cells. Therefore, it is possible that metazoan Orc6 may be a link between cytokinesis and DNA replication. Clearly, Orc6 functions in several key mechanisms governing the cell cycle in other species, although little characterization of the protein has been done in *S. cerevisiae.*

Indirect evidence indicates that there may be differences between the structure and function of Orc6 between species. It may be useful to return to the budding yeast model to obtain a complete and accurate definition of the role of Orc6 in the cell cycle, and determine if it has roles common to all eukaryotes. To begin, this chapter focuses on obtaining a broad description of budding yeast Orc6 by examining a few fundamental questions. What is known about the structure of the budding yeast Orc6 molecule? Can the function of *S. cerevisiae* Orc6 be complemented by a metazoan ortholog? The tools needed to begin preliminary work on the function of yeast Orc6 are acquired through work in this chapter. In particular, it will be necessary to be able to monitor Orc6 *in vivo* throughout the yeast cell cycle. Therefore, a tagged version of *S. cerevisiae* Orc6 is needed. If functional, this fusion protein can be used to determine the general localization of Orc6 in the cell and compare its levels to those of other ORC subunits. It is intended that this will provide the groundwork for future studies in examining the functional significance of budding yeast Orc6.

Examination of budding yeast Orc6 in comparison to the metazoan proteins

Orc6 is an essential protein in *S. cerevisiae,* originally identified as a member of the origin recognition complex. Since ORC is known to play a pivotal role in DNA replication, it is assumed that Orc6 would also function during this process. However, this had yet to be confirmed as very little is known about the function of Orc6 in budding yeast. It is possible that a closer look at its principle domains/motifs and secondary structure could uncover a clue as to its function. A few significant domains and motifs have been reported in the literature (summarized in Figure 7), including several putative NLS domains and phosphorylation sites, as well as a Cy motif. To check for more putative domains, the sequence of *S. cerevisiae* Orc6 was examined with psi-Blast (NCBI database), but no additional domains were identified. Only one motif was recognized in budding yeast Orc6 after entering its amino acid sequence into the Interpro database (through the *Saccharomyces* Genome Database), which integrates domain and motif information from several comprehensive databases. That motif is a inhibitor of apoptosis (IAP) repeat, found near the C-terminal end of the protein, and is only found in one other protein in *S. cerevisiae,* Bir1 (Huang *et al.,* 2001; Nevill-Manning *et al.,* 1998).

As mentioned earlier, there is little conservation between Orc6 in *S. cerevisiae* compared to the other Orc6 proteins. A Basic Local Alignment Search Tool (BLAST) search for proteins with a similar sequence to the budding yeast protein failed to identify any human proteins or putative ORFs. In fact, *S. cerevisiae* Orc6 was not similar to any protein sequence in the NCBI database using a standard low complexity search, except

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  1    MSMQQVQHCVAEVLRLDPQEKPDWSSGYLKKLTNATSILYNTSLNKVMLK

 51    QDEEVARCHICAYIASQKMNEKHMPDLCYYIDSIPLEPKKAKHLMNLFRQ

101    SLSNS**S**PMKQFAWTP**SPKKNKRS**PVKNGGRFTSSDPKELRNQLFG**T**PTKV

151    RKSQNNDSFVIPELPPMQTNESPSITR**RKL**AFEEDEDEDEEEPGNDGLSL

201    KSHSNKSITGTRNVDSDEYENHESDPTSEEEPLGVQESRSGRTKQNKAVG

251    KPQSELKTAKAL**RKRGRIPNSLLVKKYCK**MTTEEIIRLCNDFELPREVAY

301    KIVDEYNINASRLVCPWQLVCGLVLN**CTFIVFNERRRKDPRIDHFIVSKM**

351    **CSLMLTSKVDDVI**ECVKLVKELIIGEKWFRDLQIRYDDFDGIRYDEIIFR

401    KLGSMLQTTNILVTDDQYNIWKKRIEMDLALTEPL
```

Blue - Potential NLS sites (aa. 117-122; 263-279)

Red – Potential Phosphorylation sites (aa. S106, S116, S123 and T146)

⬜ - **"Cy motif" Clb5 binding site** (aa. 178-180)

Underlined - Inhibitor of apoptosis (IAP) repeat (aa 327-363)

Figure 7. Common motifs found in budding yeast Orc6. The amino acid sequence of Orc6 displaying several known/putative motifs in the protein. Potential NLS sequences and putative phosphorylation sites are identified in blue and red respectively (Li and Herskowitz, 1991; Nguyen *et al.,* 2001). The "Cy" motif, or cyclin B5 binding site, is boxed in green (Wilmes *et al.,* 2004). The inhibitor of apoptosis (IAP) repeat in underlined (Huang *et al.*, 2001; Nevill-Manning *et al.,* 1998).

a few unnamed and hypothetical proteins, the vast majority being from *Saccharomyces sp.* As reported earlier, an alignment of the amino acid sequences of various Orc6 proteins does not show any obvious areas of conservation (Figure 5). However, the primary structure of proteins do not always reveal functionally important domains. Therefore, the secondary structures of budding yeast, fly and human Orc6 proteins were predicted using the Protein Structure Prediction Server (PSIPRED) (Figure 8A). It is clear that the secondary structures of both human and fly are strikingly similar with parallel patterns of alpha helices and random coils. This indicates that although these two proteins have only a modest sequence similarity, the secondary structure may be very much alike. However, yeast Orc6 showed very little similarity to the predicted secondary structures of the other two Orc6 proteins. To get a better idea of the structure of each Orc6 molecule, the globular structure of the proteins were predicted from the amino acid sequence using Prediction of Intrinsically Unstructured Proteins (IUPred) (Figure 8B; Dosztányi *et al.,* 2005). This program outlines functional globular domains on the basis of estimated pair-wise energy content. The assumption for this analysis is that the functional component of a protein consisting of a well-defined, globular structure is composed of amino acids that have a higher potential to interact with other proteins. Areas where the amino acid composition is less likely to associate with other proteins adopt an intrinsically unstable structure. The predicted structure of the yeast Orc6 indicates that it is made up of two globular domains, one at the N-terminus (a.a. 1-95) and the other at the C-terminus (a.a. 262-435). Alternatively, the structures of both the human and fly Orc6 proteins are mostly all globular (a.a. 1-182), with an unstable C-terminal end. However, it should noted that yeast Orc6 is much larger than the other
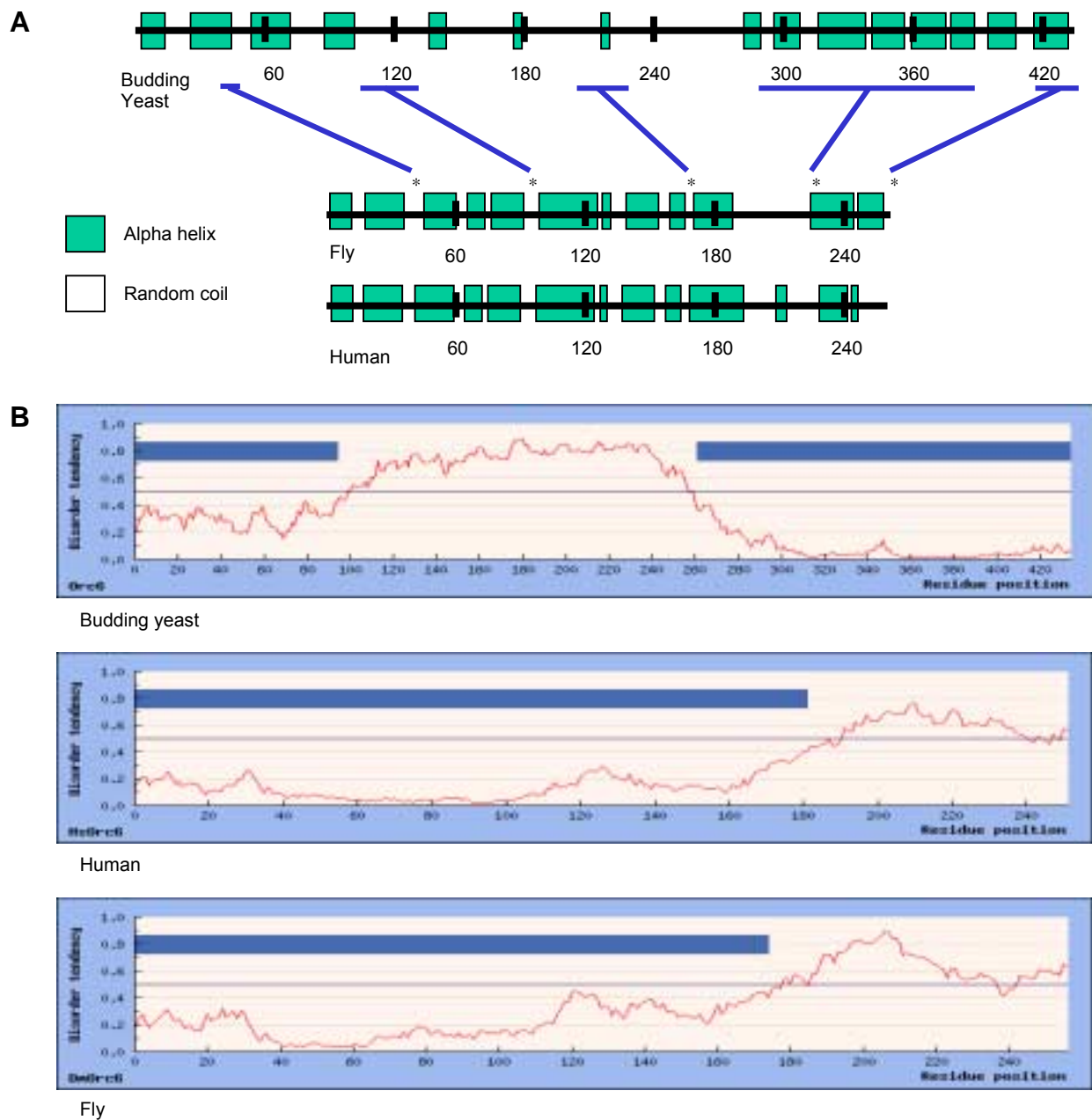
Figure 8. Structure predictions for Orc6. (A) The secondary structure of budding yeast Orc6 as determined by the PSIPRED Protein Structure Prediction Server (Bryson *et al.,* 2005; McGuffin *et al.,* 2003; Jones, 1999), is displayed in comparison to the fly (DmOrc6) and human (HsOrc6). This image is to scale. Green boxes represent alpha helices, the gaps in between are areas of random coils. * indicate points in the human and fly sequences that have yeast insertions, blue lines represent the size and region of the insert (refer to Figure 5). (B) The amino acid sequences of budding yeast, human and fly Orc6 proteins were analyzed using IUPred, which identifies intrinsically unstructured regions in proteins (Dosztányi *et al.,* 2005). High disorder tendencies are plotted and the solid blue line appearing over the histogram indicates globular domains.

two proteins, and it is possible that the C-terminal globular structure is simply an addition to the yeast protein.

Although little similarity has been observed at any level of the protein structure between budding yeast and human Orc6 molecules, it is still possible that they carry out comparable molecular functions. Therefore, a plate-based growth assay was performed to determine if the essential function(s) of budding yeast Orc6 can be complemented by human Orc6. The human *ORC6* gene was amplified from cDNA created from RKO cells (human colon carcinoma; ATCC CRL-2577) and cloned into a yeast expression vector (pEG202). The pEG202 vector contains a poly-linker region under the control of a strong promoter (alcohol dehydrogenase) and a 2 μm origin of replication to maintain a high (20-100) copy number in the cell. It also has a *HIS3* marker to allow for selection of transformants. pEG202 is typically used in yeast two-hybrid assays as it fuses a LexA DNA binding domain to the insert upon expression. In this experiment, two independent plasmid constructs with a human *ORC6* gene insert were obtained and confirmed through restriction enzyme digestion. Both constructs were then transformed separately into *GAL1-ORC6* yeast strains, along with pEG202-*ORC6(yeast)* and pEG202 as positive and negative controls, respectively. Transformation reactions were plated on galactose media lacking histidine to allow for expression of *ORC6* and selection of transformants. Individual colonies were then patched and subsequently streaked out on plates containing galactose or glucose selective media (Figure 9). Growth on glucose media suppresses the expression of *GAL1-ORC6*, essentially depleting endogenous yeast Orc6. The results show that human Orc6 is not able to complement the essential function(s) of the budding yeast protein, as neither strain

pEG202 HsOrc6-2	pEG202 HsOrc6-1
pEG202 ScOrc6	Vector alone

Figure 9. Growth assay to determine whether a reduction in *S. cerevisiae* Orc6 can be complemented by *H. sapiens* Orc6. *GAL1-ORC6* was transformed with an expression vector either carrying yeast *ORC6* (pEG202-Sc*ORC6*) or one of two independently-amplified human *ORC6* genes (pEG202-Hs*ORC6*-1/2). As a negative control, *GAL1-ORC6* was also transformed with an empty expression vector (pEG202). From a master GAL plate, individual colonies were streaked on a fresh galactose (GAL) plate or on a glucose (GLU) plate. When grown on glucose endogenous Orc6 is shut-off in the *GAL1-ORC6* strain, thus the only source of Orc6 in these cells originates from the expression vectors.

containing a human construct grew on the glucose medium. However, it should be noted that although the plasmid was sequenced to confirm the correct frame of the human *ORC6* insert, the expression of human Orc6 was not confirmed in this assay, and a lack of expression could explain the absence of growth.

Characterization of budding yeast Orc6 in comparison to Orc2

Comparing the endogenous levels of Orc6 to other ORC proteins may offer insight to their cellular activities. If the ORC complex is composed of stoichiometric amounts of each subunit, then higher levels of one protein would suggest it is involved in mechanisms outside of ORC. To monitor proteins levels *in vivo*, most molecular techniques require an effective antibody; unfortunately there are no antibodies commercially available that efficiently recognize budding yeast Orc6. Instead of developing an antibody, which could take months to acquire, it is possible to tag the endogenous copy of genes with an epitope that already has available antibodies. Directed recombination events for this purpose are quite common when using *S. cerevisiae,* and the experimental procedure is outlined in the Materials and Methods. Essentially, a plasmid carrying the desired epitope (i.e. *MYC*) tag along with a selectable marker is amplified using special PCR primers. These primers have extended 5'-ends that recombine specifically into the host genome at the 3'-end of *ORC6.* If the integration is successful, Orc6-Myc will remain under the regulation of the endogenous *ORC6* promoter, and Orc6-Myc levels can be monitored through western blotting and other biochemical techniques. Using this method, the C-terminus of Orc6 was tagged with 13 copies of a Myc epitope (Figure 10). Proper integration of the Myc

Figure 10. Tagging ORFs in budding yeast with a one-step PCR method. Recombination-based tagging of genes as a result of linear DNA transformation was performed as described in the Materials and Methods. (A) Using various plasmid cassettes (Longtine *et al.,* 1998), DNA encoding a desired protein tag along with a selectable marker was amplified. In this example, DNA encoding 13 consecutive *MYC* epitopes was amplified along with the *KANMX* gene, which infers resistance to G418. The primers used in this PCR reaction have 3'-ends (~25 bp) complementary to the plasmid cassette. However, the 5'-ends are greatly extended (42 bp), with the forward primer complementary to the extreme 3'-end of the ORF (in this case *ORC6*) without the stop codon, while the reverse primer has a 5'-end complementary to a region downstream of the ORF. (B) Following amplification, the PCR product was purified and transformed into the desired yeast strain, which was subsequently plated on selective media. Colonies were then harvested and genomic DNA extracted. (C) Using a set of primers that flank the original ORF, PCR was performed to determine if there is an increase in size from wild-type yeast product, confirming integration of the PCR product. (D). Once the PCR products indicated a recombination event at that locus, whole-cell protein extracts were isolated from the transformants and the fusion protein (Orc6-Myc) was detected using an anti-Myc antibody as described in the Materials and Methods.
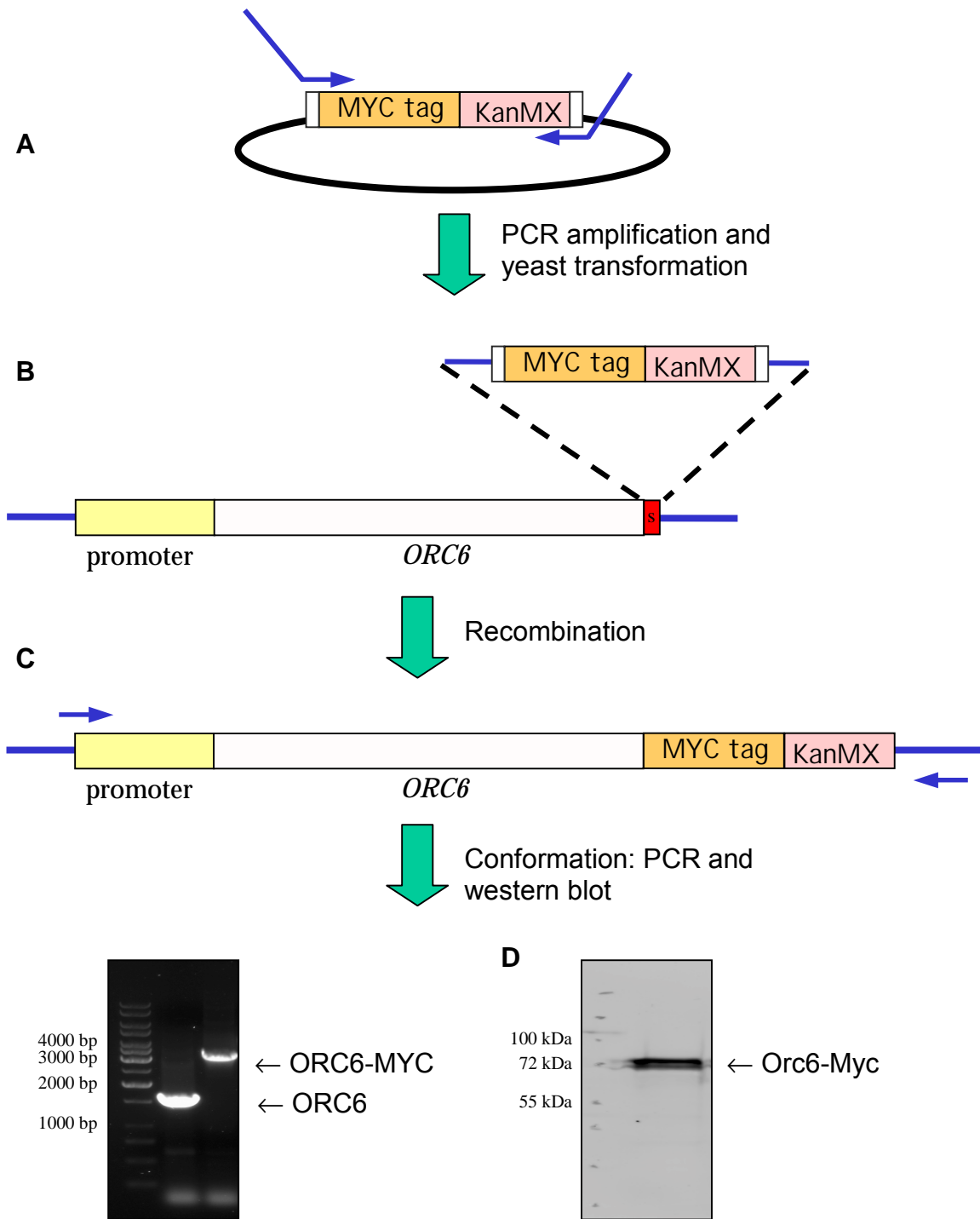
tag was confirmed on several independent clones through both PCR and western blotting. As well, growth rates were monitored to ensure cell viability and that no partial growth defects are observed relative to the untagged parental strain. Epitope tagging of genes was used throughout this project to allow observation of several proteins. The newly developed mutants are among those listed among the strains used in this project (Chapter II, Table 1).

Using the new Orc6-Myc strains, the global levels of the protein were determined by western blotting. As a control, several Orc2-Myc strains were developed by the same approach as above. Since both tagged proteins have the same number of Myc epitopes a direct comparison of protein content is permissible. Whole cell extracts were prepared from asynchronous cultures of three transformants of each strain (Figure 11). Densitometry was performed following detection of the blots with an anti-Myc antibody and the ratio of average Orc6-Myc to Orc2-Myc intensity was observed to be 1.03. This indicates that the level of Orc6 in the cell is relative equal to the level of Orc2.

It is possible that both Orc2 and Orc6 function in pathways independent of the ORC complex, outside of the nucleus. For example, a portion of human and *Drosophila* Orc6 appears to localize to the cytosolic side of the cell membrane (Prasanth *et al.,* 2002; Chesnokov *et al.,* 2003). A chromatin binding assay was performed on asynchronous cultures to compare the general localization of both Orc6 and Orc2 proteins as described in the Materials and Methods, and fractions were analyzed on western blots. The pellet to supernatant (pel/sup) ratios indicate that both Orc6-Myc and Orc2-Myc have similar concentrations of protein bound to chromatin, with Orc6-Myc having a slightly higher fraction associated with DNA.

Orc6-Myc/Orc2-Myc = 1.03
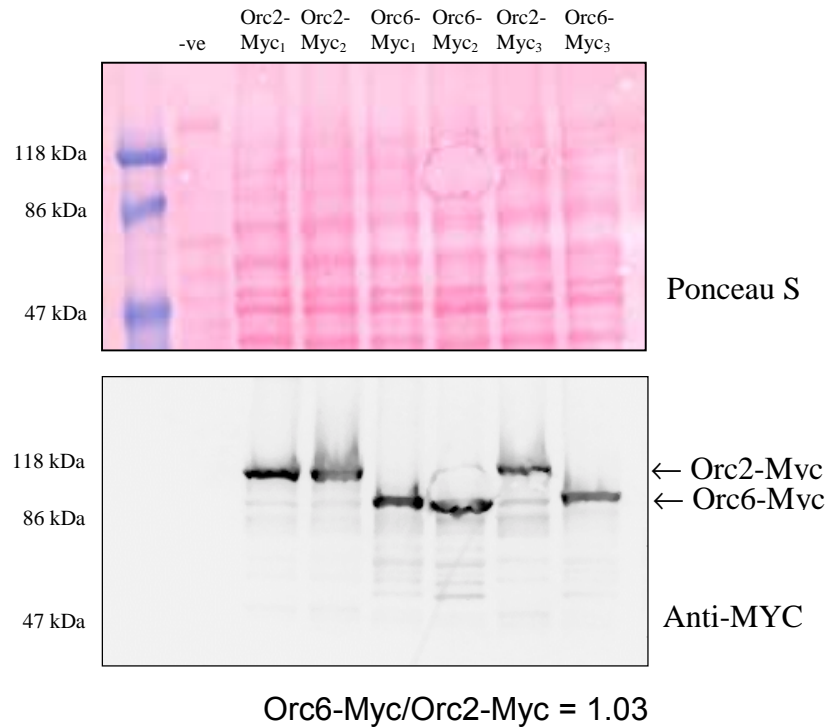
Figure 11. Comparison of Orc6 and Orc2 levels in whole-cell extracts. Using the method described in Figure 10, Orc6 was tagged with a Myc_{13} epitope creating the DY39 strain. Orc2 was also MYC_{13}-tagged using a similar method (DY81). In each case, three independent colonies were isolated from the transformation plate and used to establish separate liquid cultures (Orc6-Myc_{1-3}, and Orc2-Myc_{1-3}). Whole-cell extracts were prepared from each culture and run on a western blot. The blot was then detected using an anti-Myc antibody as in Figure 10, and densitometry of the corresponding bands was performed. –ve corresponds to a WCE from the parental strain. The results from the densitometry are displayed as Orc6-Myc/Orc2-Myc levels shown in the bottom right.
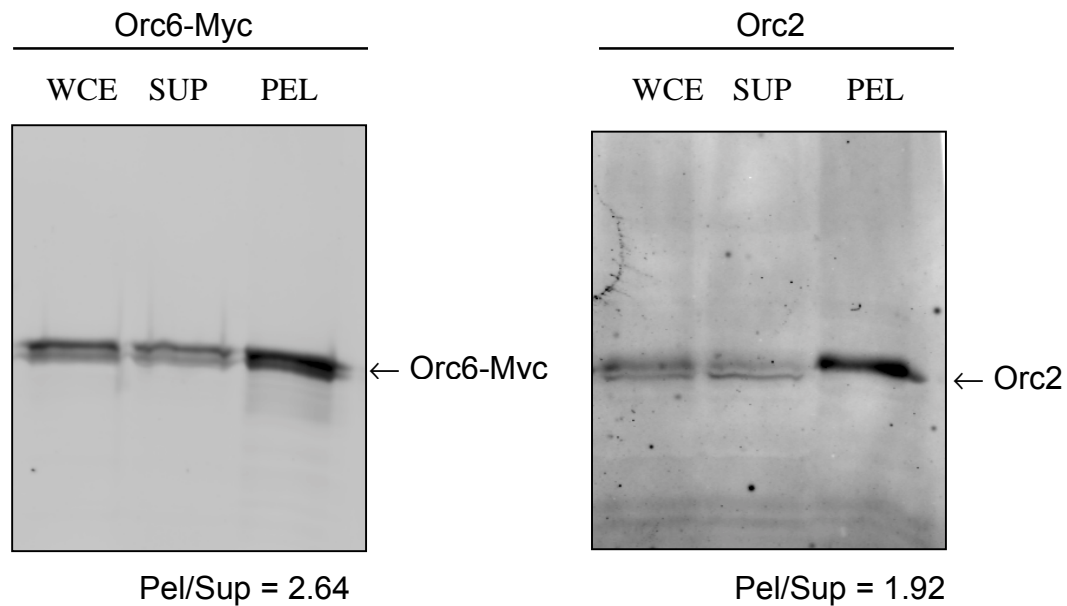
Figure 12. Comparison of Orc6 and Orc2 localization as determined by chromatin association. A chromatin binding assay was performed as described in the Materials and Methods using the Orc6-Myc stain (DY39). 15 μl of both the whole-cell extract (WCE) and supernatant (SUP), and 30 μl of the insoluble pellet fraction (PEL) were then analyzed by immunoblotting. The blot was originally detected with an anti-Myc antibody as in Figure 4, followed by an anti-Orc2 antibody. Densitometry depicting the PEL/SUP ratio for each protein is given below each representative blot.

<u>Discussion</u>

Orc6 structure

Biochemical evidence suggests that budding yeast Orc6 is a member of the ORC complex and essential for cell cycle progression (Bell and Stillman, 1992, Li and Herskowitz, 1993). However, the lack of similarity between this protein and Orc6 in other species draws into question whether or not budding yeast Orc6 is a true ortholog of the these other proteins. Based on the amino acid sequence, yeast Orc6 is much larger and only 6% identical/15% similar to human Orc6 (Dhar and Dutta, 2000). An alignment of these proteins along with other Orc6 molecules did not reveal any short sequences of conservation (Figure 5). The results of a BLAST search indicated that no other human protein sequences are similar to yeast Orc6, nor are there any proteins in the yeast proteome similar to the human Orc6 sequence. Since the human and yeast Orc6 proteins both bind to ORC and do not have related proteins in the others proteome, they may still in fact be functional orthologs.

To clarify the structural dissimilarity between various Orc6 proteins, the secondary structures of both human and *S. cerevisiae* proteins were predicted (Figure 8). Examination of predicted secondary structures did little to suggest that the *S. cerevisiae* Orc6 molecule is related to its metazoan counterpart. There is no clear similarity between patterns of alpha helices and random coils, whereas the two metazoan Orc6 proteins are quite alike. As well, putative globular structures are also significantly different between budding yeast and human Orc6. Therefore, it is difficult to suggest that these two proteins are related following analysis of amino acid structure and predicted higher structures.

Orc6 function

While the function of Orc6 in budding yeast is still uncertain, it is clear that on the structural level it bears little resemblance to human Orc6. The question still remains as to whether or not these two proteins still have similar functions. A complementation assay was performed to address this issue. This is not the first attempt at cross-species complementation of DNA replication proteins as *Xenopus* extracts depleted of all ORC subunits were supplemented with ORC from either humans (Giordano-Coltart *et al.,* 2005) or *Drosophila* (Chesnokov *et al.,* 1999). Results from that study showed that human ORC$_{(1-5)}$ was able to bind chromatin and facilitate binding of *Xenopus* Cdc6 and MCM complex. As well, both human and *Drosophila* ORC were able to support DNA replication in the *Xenopus* ORC-depleted extracts, however, not as efficient as if supplemented back with *Xenopus* ORC. Nevertheless, it was still able to perform this essential function. A similar experiment in which *Drosophila ORC2* was incorporated into the genome of a budding yeast *orc2-1* mutant revealed interesting results. The silencing function of budding yeast Orc2 was complemented by the *Drosophila* gene, but the essential DNA replication function was not (Ehrenhofer-Murray *et al.,* 1995). Therefore, although it is interesting that some functions of ORC can be complemented with proteins from another species, clearly this is not always the case.

Pre-RC components outside of the ORC complex are also able to undergo cross-species complementation. As mentioned earlier, Mcm10 is required for pre-RC formation and DNA replication in *S. cerevisiae.* Initial attempts at complementing either *S. cerevisiae* or *S. pombe* Mcm10 with the human protein failed (Izumi *et al.,* 2000). Considering that human Mcm10 is only 21% and 23% similar to the *S. cerevisiae* and *S.*

pombe versions respectively, this result was not unexpected. However, when a plasmid containing *Drosophila MCM10* was transformed into a budding yeast strain null for the chromosomal copy, it was able to complement the essential function of this gene (Christensen and Tye, 2003). This was surprising, given that the sequence similarity between these two proteins is quite modest (24.1%). Therefore, even in cases where the similarity between two versions of the protein is low, cross-species complementation is still possible.

If complementation was a success, a re-evaluation of the amino acid sequence alignment would be necessary. Although only a few amino acids are identical between the two proteins, they would offer reasonable starting points for determining the active sites of budding yeast Orc6. Specific site-directed mutagenesis of the amino acids identical to the human protein could be used to try to knock out the essential function of Orc6. Once other proteins are identified that interact with Orc6, it would be possible to perform two-hybrid assays to determine which protein association is lost in the mutant. This loss of interaction would point to an association essential to cell viability. Unfortunately, although it is possible that the human Orc6 constructs were not expressing the protein, it appears as though this protein was unable to complement the essential function of budding yeast Orc6. However, given the sequence and structural dissimilarity, this observation was not a unexpected. Overall, with little evidence to suggest that the *S cerevisiae* Orc6 protein is physically or functionally similar to human Orc6, it is possible that these proteins may not be the functional orthologs.

Although clearly distinct from the human protein, the amino acid sequence of budding yeast Orc6 could still be useful in determining functional aspects of the protein by

looking for functional domains and motifs. These conserved sites could offer insight into the cellular mechanisms and pathways that employ Orc6. Two putative NLS sequences were identified in budding yeast Orc6 (Li and Herskowitz, 1993), although it is unknown if they are indeed functional. In humans, two NLS sequences on Orc2 are required for the transport of $ORC_{(1-5)}$ into the nucleus (Radichev *et al.,* 2006), suggesting that Orc6 does not enter the nucleus with the rest of the ORC complex. Therefore, it is possible that budding yeast Orc6 also enters the nucleus independent of the ORC complex, and may be able to cross into the nucleus on its own.

Several putative phosphorylation sites were also identified in budding yeast Orc6, and at least one of these sites is functional as *in vivo* phosphorylation of Orc6 was observed (Figure 10 and 12, Li and Heskowitz, 1993; Wilmes *et al.,* 2004). Closer examination has indicated that phosphorylation of Orc6 is mediated by S phase cyclin Clb5 and may be part of a mechanism that prevents re-replication of the genome during and immediately following S phase (Wilmes *et al.,* 2004; Archambault *et al.,* 2005). The Cy motif identified in Orc6 is essential for Clb5 association, which is maintained from S phase through to M phase. With a total of four putative phoshorylation sites identified in the N-terminus of Orc6, it is possible that more than one phosphorylation site is functional and necessary for Orc6 action.

A rare inhibitor of apoptosis (IAP) repeat was identified in the budding yeast Orc6 sequence. As the name implies, the biological significance of the IAP motif (if functional) is that proteins containing these repeats are involved in inhibiting apoptosis through interacting with caspases (reviewed in Shi, 2004). It has been suggested that there are low levels of active caspases in the cell, and that IAP proteins minimize their effects,

preventing the caspase cascade that leads to apoptosis in healthy cells (Muro *et al.,* 2002). If the IAP motif in Orc6 is functional, a mutation or deletion of this region may lead to an increased sensitivity to death signals. IAP proteins have been shown to be important in the cell cycle. Intriguingly, IAP proteins are involved in regulating cytokinesis (reviewed in Verhagen *et al.,* 2001), although the mechanism of action is not yet known. Recently, IAP-containing proteins have become targets for cancer therapeutics (reviewed in Nachmias *et al.,* 2004; Schimmer, 2004). The only other *S. cerevisiae* protein containing IAP is Bir1. Although the specific molecular function of Bir1 is unknown, it is a chromosomal protein that is involved in coordinating major events of the cell cycle, including chromosome segregation (Yoon and Carbon, 1999; Li *et al.,* 2000). It is thought that Bir1 acts similarly to metazoan IAP-containing proteins, for example human Survivin, which not only functions in spindle formation during mitosis, but also inhibits the caspase cascade by interacting directly with caspase 3 and 7 (Uren *et al.,* 1999). Although Bir1 has yet to be directly linked to the cell death pathway, it would be still be interesting to investigate the similarities between Bir1 and Orc6 and examine the significance of the IAP in Orc6.

Orc6 regulation

Key cellular pathways including DNA replication are tightly regulated, and proteins involved in these processes are strictly monitored in the cell. Most cells adopt one of several main methods of controlling these essential proteins. One possibility is to transport proteins away from the site of action during phases of the cell cycle that they are not required. As well, the level of protein is controlled so that concentrations peak

when they are needed and quickly degraded after that point. Alternatively, cell cycle regulated post-translational modifications (i.e. phosphorylation events) (un)activate proteins at various phases of the cell cycle. Since ORC proteins are known to be present at constant levels throughout the cell cycle, it is possible that Orc6 is relocated after it performs its essential function. Several lines of evidence indicate that budding yeast Orc6 is a nuclear protein, as it has been shown to associate with chromatin, analysis of its amino acid sequence revealed putative NLS sequences, and when over-expressed Orc6 is localized to the nucleus (Li and Herskowitz, 1993). Biochemical analysis showed Orc6 interacts with both ACS sequences and with the chromatin-associating ORC complex (Li and Herskowitz, 1993; Bell and Stillman, 1992). However, there is also evidence from UV-crosslinking studies to suggest that Orc6 is not always bound to ORC, nor is it needed for the DNA binding of ORC (Lee and Bell, 1997; Gillespie *et al.,* 2001). Therefore, if Orc6 functions outside of ORC, where does it perform this role? A chromatin association study of Orc6 was performed to determine the ratio of cellular Orc6 bound to DNA. Any Orc6 found in the soluble (supernatant) fraction may indicate that it is transported from the nucleus at specific stages of the cell cycle, or that has a function outside of the nucleus. Populations of both human and *Drosophila* Orc6 have been observed in the cytosol, localized to the cell membrane and cleavage furrow (Chesnokov *et al.,* 2003; Prasanth *et al.,* 2002), where it is thought that they are involved in cytokinesis. Results from the chromatin association assay found the vast majority of budding yeast Orc6 bound to chromatin. In fact, it appears as though a slightly higher ratio of Orc6 is bound to chromatin when compared to Orc2. This suggests that the main function(s) of Orc6 is localized to chromatin, although it is

possible that small, cell phase-specific cytosolic populations are masked by the abundant nuclear fraction when examining asynchronous cultures. Therefore, to obtain a more accurate description of budding yeast Orc6 localization, a more sensitive technique is needed to monitor cells synchronized at various stages of the cell cycle.

Although the levels of ORC in budding yeast appear to be constant throughout the cell cycle (review in Kelly and Brown, 2000), it is still important to examine relative amounts of the Orc6 protein. By comparing to another ORC subunit, it is possible to make insinuations as to whether or not Orc6 functions outside the role of ORC, since ORC is made up of stoichiometric amounts of each subunit. Immunoblot data showed that Orc6 levels are quite similar to Orc2 (Figure 11), and does little to suggest that Orc6 functions independently of ORC. However, it is possible that both proteins function together with ORC as well as independently in other cellular pathways. Therefore, it may be useful to compare the levels of Orc6 to more than one other ORC subunit, particularly in cells arrested at different stages of the cell cycle to see if there are minor fluctuations in the concentration of Orc6 not mirrored by the other ORC subunits. If the levels of all the ORC subunits are equivalent, it would strongly indicate that they function solely as a complex.

CHAPTER IV: The Essential Role of Orc6 in the Cell Cycle

* This version includes several supplemental Figures that do not appear in the original manuscript.

Figure 18 was contributed by L. Da-Silva.
Figure 24 was contributed by L. Da-Silva and H. Al-Attar.
Figure 26 was assisted by L. Da-Silva.
Figure 27 was assisted by J. Ah-Kee and P. Pasero.

Introduction

The origin recognition complex (ORC) plays an essential role in the initiation of DNA replication by binding to origin sequences throughout the cell cycle and acting as a scaffold for the association of additional protein factors in G_1 phase (reviewed in Bell, 2002). Originally isolated and characterized in the budding yeast *Saccharomyces cerevisiae* (Bell *et al.*, 1992), ORC is composed of six distinct subunits, and orthologs of each have now been found in a wide range of eukaryotic species, including *Schizosaccharomyces pombe* (Moon *et al.*, 1999), *Drosophila melanogaster* (Chesnokov *et al.*, 1999), *Arabidopsis thaliana* (Masuda *et al.*, 2004), *Xenopus laevis* (Carpenter *et al.*, 1996; Rowles *et al.*, 1996; Tugal *et al.*, 1998), *Mus musculus* (Kneissl *et al.*, 2003), and *Homo sapiens* (Gavin *et al.*, 1995; Takahara *et al.*, 1996; Quintana *et al.*, 1997; Quintana *et al.*, 1998; Tugal *et al.*, 1998; Dhar and Dutta, 2000). In early G_1 phase, ORC promotes the origin-association of the clamp loading protein Cdc6 in an ATP-dependent manner (Speck *et al*, 2005). Another factor, Cdt1 (Devault *et al.*, 2002; Tanaka and Diffley, 2002), directs the nuclear import of the MCM (minichromosome maintenance) family of proteins, Mcm2-7, which act as replication fork helicases (reviewed in Bell and Dutta, 2002).The transient association of Cdt1 with origin DNA, and its subsequent release, allows the reiterative loading of multiple Mcm2-7 complexes (Randell *et al.,* 2006). Once tightly bound, the continued association of at least Mcm2 with chromatin in G_1 phase requires the presence of another ORC-associated protein, Mcm10 (Homesley *et al.*, 2000). Collectively, this assemblage of proteins is known as the pre-replicative complex (pre-RC). In addition to pre-RC formation, the initiation of DNA replication requires the activation of two kinase complexes, Clb5/Cd28 and

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Dbf4/Cdc7, which promote the origin-association of Cdc45 (Nougarede *et al.*, 2000, Zou and Stillman, 2000). Cdc45 in turn recruits DNA polymerases to origins (Mimura and Takisawa, 1998; Aparicio *et al.*, 1999; Zou and Stillman, 2000).

Curiously, only five of the six ORC subunits are required for origin recognition and binding (Lee and Bell, 1997). Even though Orc6 is an essential protein in budding yeast (Li and Herskowitz, 1993), it is dispensable for these functions and its role in cell cycle progression has yet to be determined. Clearly, Orc6 association with the other budding yeast ORC subunits suggests a function in DNA replication. Li and Herskowitz disrupted one copy of *ORC6* in a diploid yeast strain and, following sporulation, were able to observe up to two of rounds of cell division from spores inheriting the *ORC6* knock-out. Arrested cells had a large budded phenotype often observed for DNA replication mutants, but the stage of cell cycle arrest could not be determined by FACS analysis due to an insufficient number of cells. Studies involving the replication of *Xenopus* sperm DNA in *Drosophila* egg extracts indicate that Orc6 can promote DNA replication in this *in vitro* system (Chesnokov *et al.*, 2001). With human cancer cells, depletion of Orc6 by transfection with siRNA duplexes results in a significant reduction in the number of positive cells in BrdU incorporation assays, consistent with a replicative function (Prasanth *et al.*, 2002). As well, research with both human and fruit fly cells point to mitotic and/or cytokinetic functions in addition to a role for Orc6 in DNA replication (Chesnokov *et al.*, 2003).

Here, we demonstrate that Orc6 is required for the initiation of DNA replication in normal cycling cells and is dispensable for progression through mitosis and cytokinesis. Localization of Orc6-YFP to subnuclear foci, provides evidence for the existence of

discrete 'replication factories' in living yeast cells. We further show that Orc6 is required for the maintenance of MCM protein association with chromatin, and that depletion of Orc6 after pre-RC formation inhibits replication origin firing.

Results

Orc6 localizes to the nucleus throughout the cell cycle

As a first step to establishing the cell cycle role of Orc6, we examined its cellular localization. This was accomplished by tagging genomic *ORC6* with a sequence encoding the GFP variant eYFP in a haploid yeast strain (DY1), resulting in expression of full-length Orc6 with a C-terminal eYFP tag, under the control of the natural *ORC6* promoter (DY41). Studies with human cancer cells indicate that, in addition to the expected nuclear localization during interphase, cytoplasmic pools of Orc6 exist with localization observed at both the cell periphery during mitosis and at the midbody during cytokinesis (Prasanth *et al.*, 2002). Similar results have been reported for *Drosophila* cells (Chesnokov *et al.*, 2003). When we followed single cells as they progressed through the cell cycle, only nuclear Orc6 localization was observed (Figure 13). To confirm that *S. cerevisiae* Orc6 is confined to the nucleus, we constructed a double-tagged haploid strain expressing Orc6 with a C-terminal eCFP fusion as well as the nuclear pore protein Nup49 with a C-terminal eYFP fusion (DY65). Irrespective of cell cycle stage, Orc6 was consistently bounded by the nuclear membrane, as designated by the signal for Nup49 (Figure 14). Although we cannot rule out additional pools of Orc6 below our threshold of detection, we conclude that there was no significant localization of budding yeast Orc6 at either the cell periphery or at the mother-bud neck.

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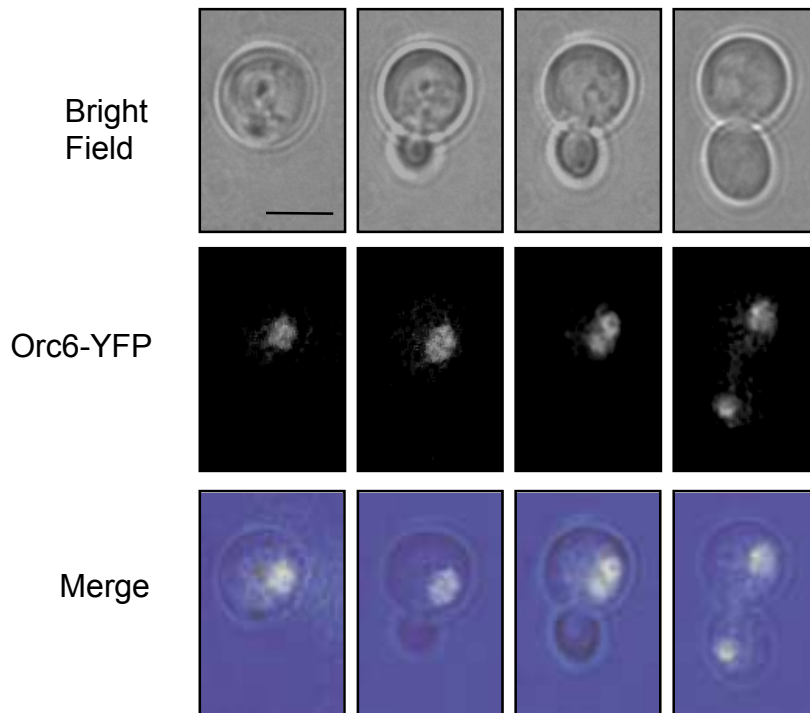
Figure 13. Localization of Orc6 within an individual cell at several stages of the cell cycle. A single Orc6-YFP cell was imaged over a 3 h time period, as described in the Materials and Methods. Both bright field and fluorescent images were taken every 10 min and stacked using imageJ 1.30v. Scale bar, 5 μm.
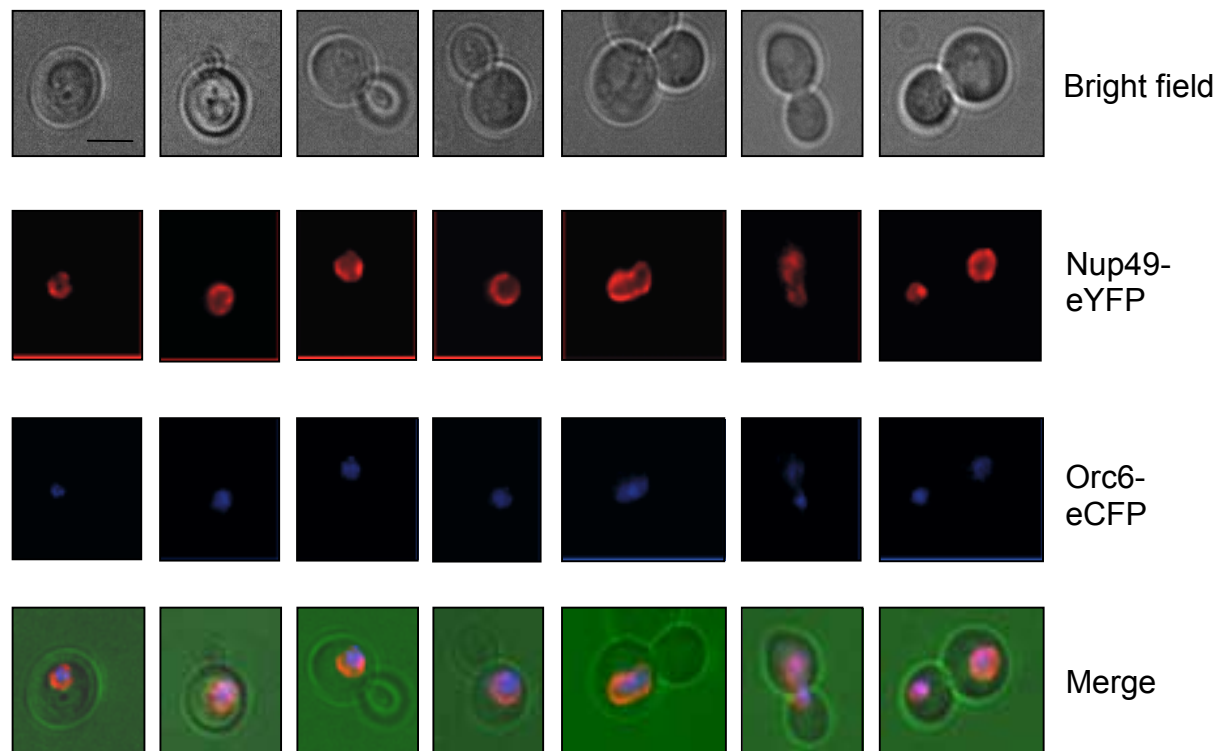
Figure 14. Orc6 is confined to the nucleus throughout the cell cycle. Cells were prepared as described in the Materials and Methods. Co-localization of Orc6 and the nuclear membrane protein Nup49 in a series of Orc6-eCFP/Nup49-eYFP cells showed that Orc6 is found exclusively within the nucleus at all stages of the cell cycle observed, as judged by bud morphology. At each time point, 20 fluorescent images were taken with the eCFP filters and summed as in Figure 8. Red and blue signals correspond to Nup49-eYFP and Orc6-eCFP respectively. Scale bar corresponds to 5 μm.

To further investigate the subnuclear localization of Orc6, 20 images taken in rapid succession were stacked to increase the signal as described in the Materials and Methods. The resultant higher resolution images revealed a pattern of punctate Orc6 foci (Figure 15), consistent with its presence at discrete 'replication factories' within the nucleus. As a control, images of an Orc2-eYFP strain were also taken in rapid succession as above. The Orc2 signal also revealed punctate foci as observed with the Orc6-eYFP strain. Finally, a double-tagged haploid strain was developed which expresses Orc6 and Orc2 with C-terminal eCFP and eYFP fusions respectively (Figure 16). Images from this strain confirms the co-localization of the two ORC subunits, and confirms that both proteins are localized to the nucleus throughout the cell cycle.

Depletion of Orc6 leads to an S phase arrest

Given that the cellular localization of Orc6 was indicative of a role in DNA replication, and provided no evidence for additional cellular function(s), we wanted to determine the point in the cell cycle at which cells arrest when depleted of Orc6. A haploid yeast strain (DY36) was generated replacing the natural *ORC6* promoter with a repressible *GAL1* promoter. As part of this strain construction, a sequence encoding 3 copies of the HA epitope was fused to the start of the *ORC6* ORF, to facilitate the monitoring of Orc6 levels. The *GAL1* promoter is active when cells are grown on medium containing galactose, but is tightly repressed on glucose medium. Cultures of DY36 and isogenic wild-type (wt) strain DY26, were grown on 2% galactose/1% raffinose (YPG/R) medium overnight, washed in ddH$_2$O, and resuspended in 2% glucose (YPD) medium. Following

Bright Field	Fluorescence	Merge
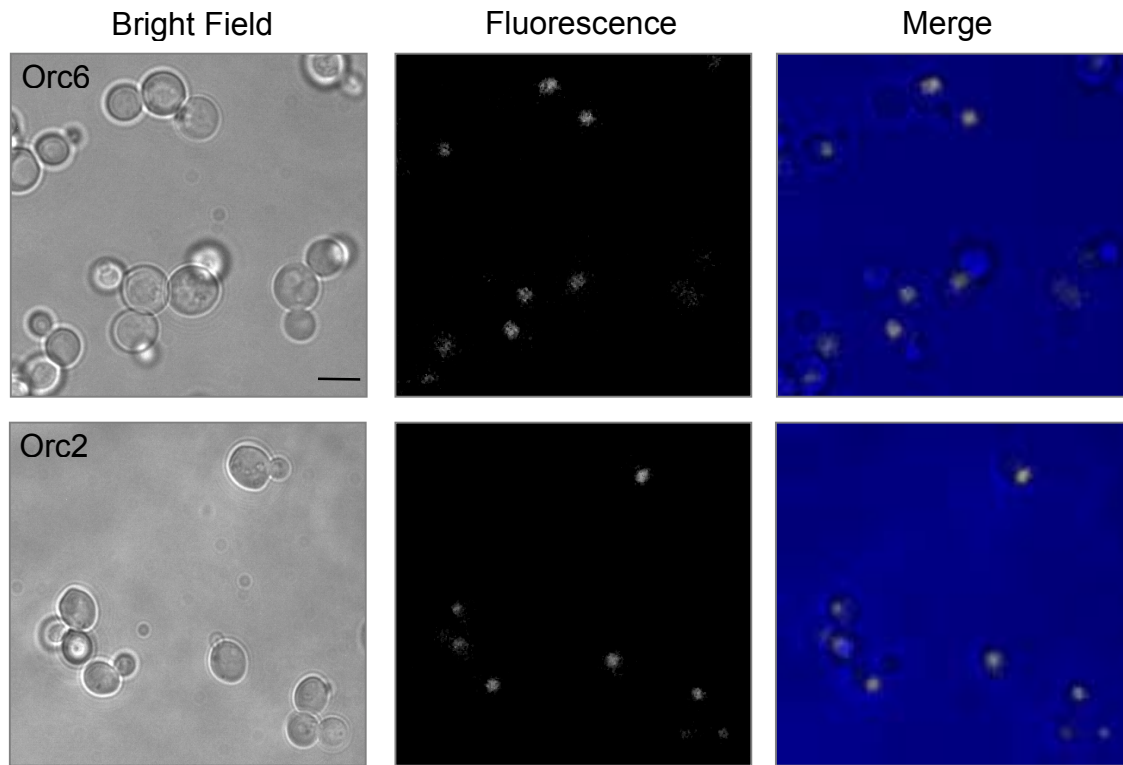
Figure 15. Localization of Orc6 and Orc2 in asynchronous cultures. Orc6-eYFP and Orc2-eYFP cell preparations and live imaging was performed as described in the Materials and Methods. A representative field of view showing cells in various stages of the cell cycle is shown. Since expression of each fusion protein is low under its endogenous promoter, a stronger signal with reduced background was obtained by taking 20 fluorescent images under low gain in rapid succession and stacking them using ImageJ 1.30v. Scale bar corresponds to 5 μm.
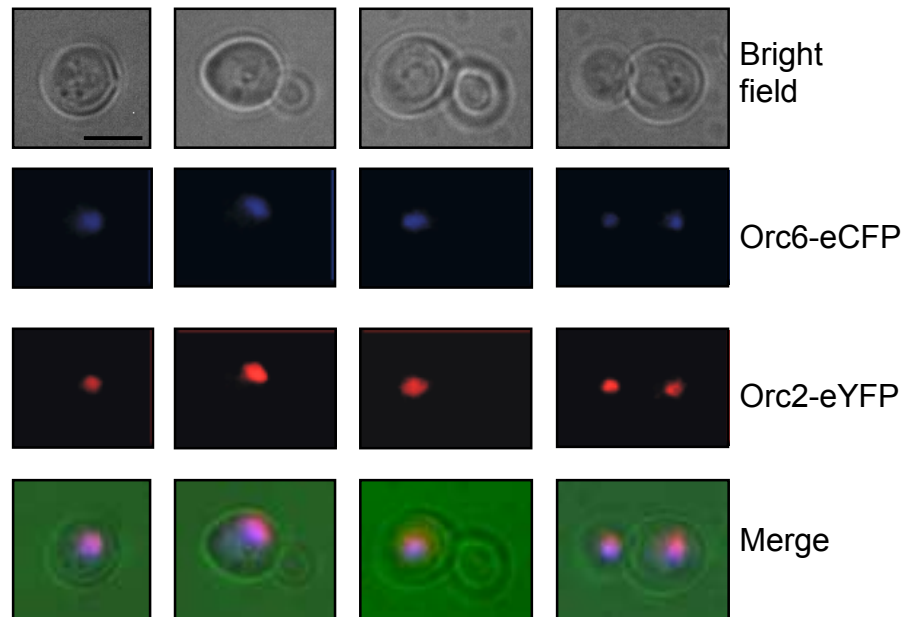
Figure 16. Orc6 and Orc2 co-localize to the nucleus throughout the cell cycle. A series of Orc6-eCFP/Orc2-eYFP cells were imaged as in Figure 9, showing specific co-localization of the two ORC subunits throughout the cell cycle. A stronger Orc2-eYFP signal with less background was obtained by summing 5 images taken from each time point using ImageJ 1.30v. Red and blue signals correspond to Orc2-eYFP and Orc6-eCFP respectively. Scale bar, 5 μm.

four hours of growth in YPD, the amount of Orc6 in the *GAL1-ORC6* cells had fallen below normal endogenous levels as judged by immunodetection of whole cell extracts (Figure 17), and these cells clearly showed growth defects at subsequent time points, relative to the wild-type controls (Figure 18). We further compared *GAL1-ORC6* and wt strain growth in YPD by removing aliquots at three hour intervals for FACS analysis. At all time points following the shift to YPD, the wt strain exhibited prominent 1C and 2C peaks characteristic of asynchronous cultures (Figure 19). In contrast, by three hours the *GAL1-ORC6* cells were already showing defects in S phase progression, with an accumulation of cells with a DNA content between 1C and 2C. No significant accumulation of cells with 2C or greater DNA content was observed, as would be expected for mitotic or cytokinetic defects. Indeed, by six hours, the size of the 2C peak was markedly reduced compared to earlier time points, and the wt control. DNA content and localization was also determined through imaging following depletion of Orc6 (Figure 20). *GAL1-ORC6* was initially grown in Gal/Raf medium (+0.02% adenine) until log phase and split into either similar Gal/Raf or glucose medium for 6 h to deplete Orc6. DNA was stained for 30 min with Hoescht stain (Molecular Probes) and the cells were imaged as described in the Materials and Methods. Cells depleted of Orc6 were typically observed in a large bud state, with the size of each cell being much larger than those observed in Gal/Raf. The DNA staining revealed that the Gal/Raf culture had 4.2% of its cells in G2/M, whereas 53% of cells in the Orc6-depleted culture were observed at that stage. However, FACS data following a 6 h depletion of Orc6 indicated that most cells at this point have not completely replicated their DNA (Figure 19). This contradiction suggests that the increased number of G2/M cells observed from
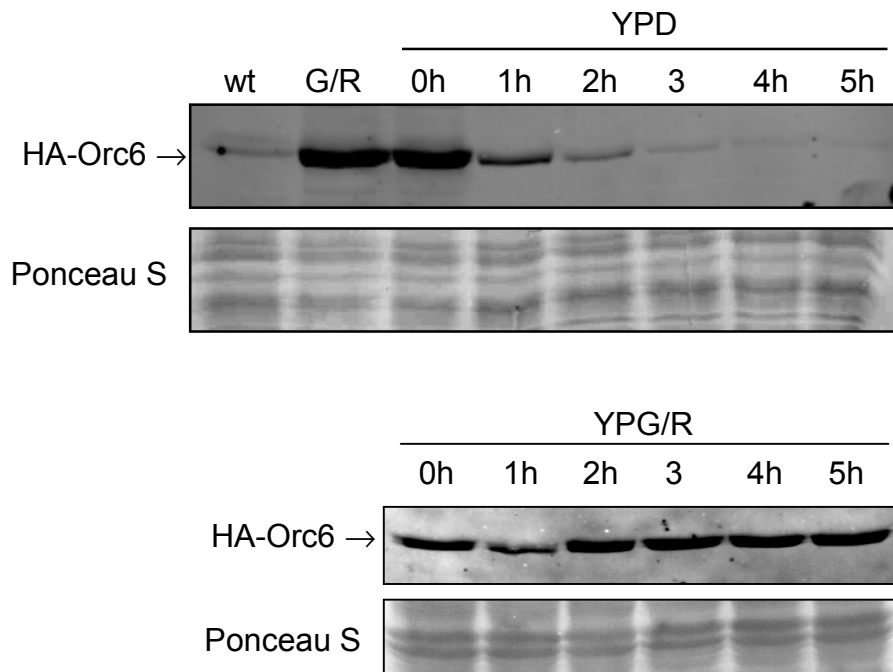
Figure 17. Time course of Orc6 depletion. Whole-cell extracts from both *GAL1-ORC6* (DY36) and wild-type cells (DY26) were taken every hour after the transfer to YPD (glucose) or fresh YPG/R (galactose). 3HA-Orc6 by significantly reduced by 2 h of growth in YPD, and below wild-type control levels by 4 h. 75 µg of each extract was used for immunoblot analysis. 3HA-Orc6 was detected using an anti-HA antibody (1:5000; mouse monoclonal, Roche) and fluorescent secondary antibody (1:3000; AlexaFluor 488 goat anti-mouse, Invitrogen). Ponceau S staining of region detected is shown to verify equal loading of whole-cell extracts.
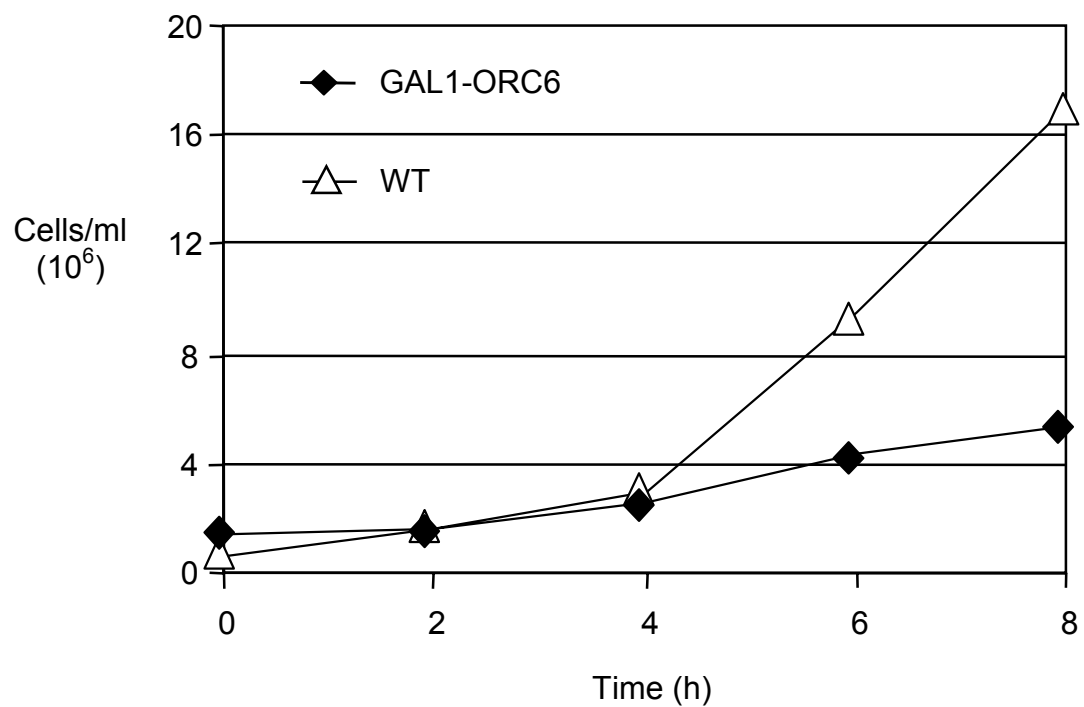
Figure 18. Growth of *GAL1-ORC6* cells is compromised following Orc6 depletion. The growth rate of *GAL1-ORC6* (DY36) and its parental strain (DY26) was determined following transfer to glucose medium. Asynchronous cultures of both strains were grown in YPG/R to 10^6 cells/ml, washed in ddH$_2$O and resuspended in fresh YPD (glucose). Cell counts of both cultures were then taken every 2 h for eight hours.
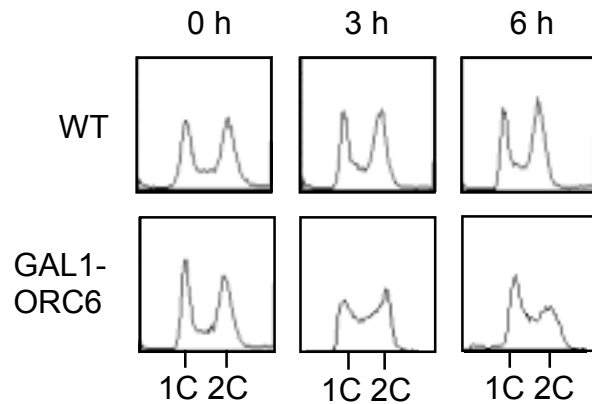
Figure 19. Cell cycle arrest of *GAL1-ORC6* following shift to glucose medium. FACS analysis of *GAL1-ORC6* (DY36) and its parental strain (DY26) was determined after they were transferred to glucose medium as described in the Materials and Methods. Exponential cultures of both *GAL1-ORC6* and wild-type cells were grown to 5×10^6 cells/ml in YPG/R, washed in ddH$_2$O and resuspended in YPD. Following the transfer, culture aliquots were removed at the indicated time points for FACS analysis.

GAL/RAF

7/168 – 4.2%

GLU
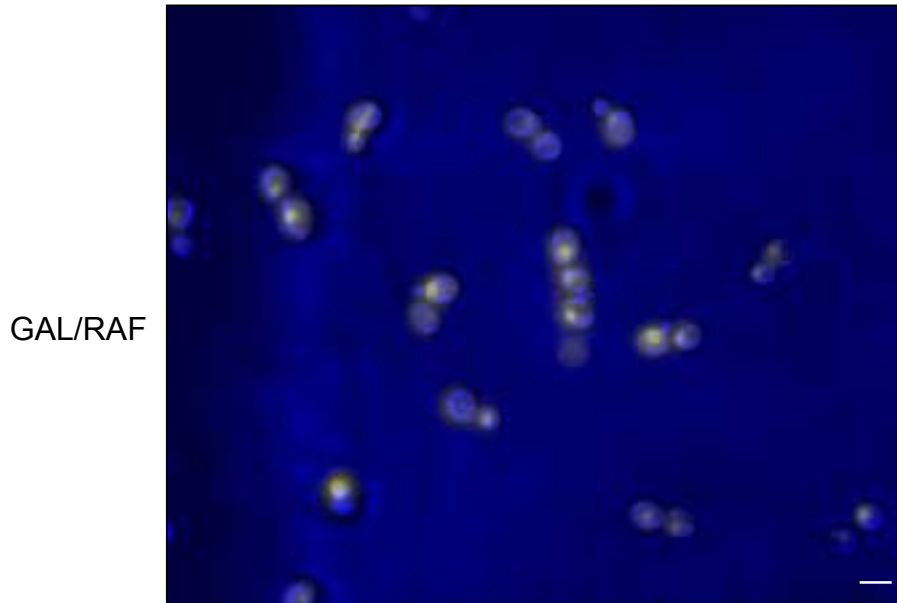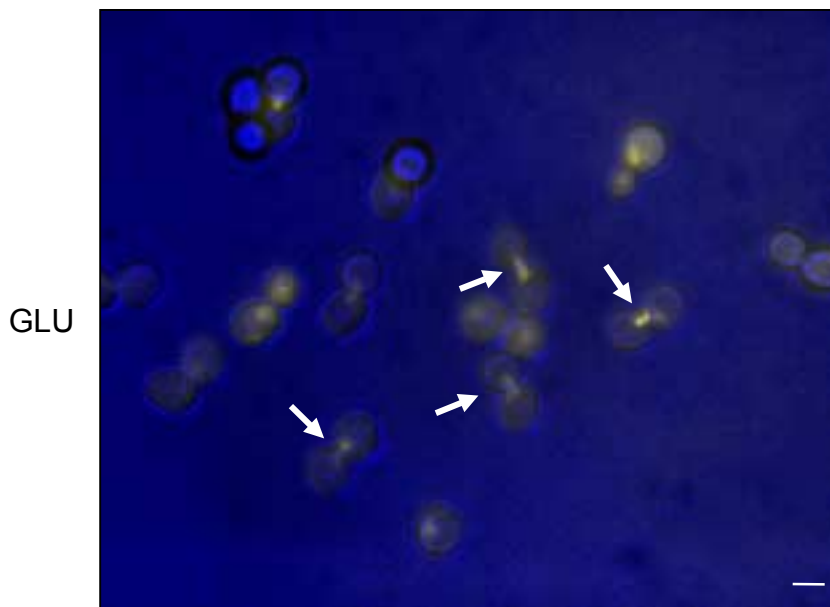
110/206 – 53%

Figure 20. DNA staining and cell morphology of cells depleted of Orc6. Asynchronous cultures of *GAL1-ORC6* were grown exponentially in YPG/R and then shifted to either glucose (GLU) or galactose/raffinose (GAL/RAF) media for 6 h to deplete Orc6. DNA staining and cell imaging was performed as described in the Materials and Methods. White arrows indicate cells in which DNA is localized to the bud neck. Scale bar corresponds to 5 μm.

microscopy may be the result of a checkpoint response. Cells that have not completed DNA replication are prevented from entering mitosis through a Rad53-dependent checkpoint pathway (Allen *et al.,* 1994). When protein extracts from these cells were examined by immunodetection, cells depleted of Orc6 exhibited Rad53 phosphorylation, confirming the checkpoint activation (data not shown).

Orc6 is required for entry into S phase

In order to determine whether Orc6 is required for entry into and/or progression through S phase, Orc6 was depleted in *GAL1-ORC6* cells synchronized at G2/M by initially arresting cells in YPG/R supplemented with nocodazole, washing the culture and resuspending the cells in YPD/nocodazole. An schematic diagram outlining arrest/deplete/release experiments is outlined in Figure 21. Since origins are rendered competent for DNA replication during G1 phase, if Orc6 is essential in this process one would expect to see the formation of a 1C peak as Orc6-depleted cells fail to efficiently fire origins following release from the block. Such a 1C peak was indeed observed with the *GAL1-ORC6* strain following both partial and rigorous Orc6 depletion (Figure 22).

A central function of the ORC complex in promoting DNA replication is to facilitate the loading of the MCM proteins at origins (reviewed in Bell and Dutta, 2002). To further explore the role of Orc6 in rendering origins competent, we monitored Mcm2 loading by means of a chromatin fractionation assay (Donovan *et al.,* 1997; Liang and Stillman, 1997) using *GAL1-ORC6* cells that had been depleted of Orc6 during a G2/M arrest. Mcm2 association with the chromatin pellet was reduced, in Orc6-depleted cells relative to wild-type cells, following release into a G1 block with α-factor (Figure 23). To confirm

Figure 21. A schematic diagram of cell cycle stage-specific depletion of Orc6. In several experiments, Orc6 was depleted at precise stages of the cell cycle, as described in the Materials and Methods. In brief, asynchronous cultures were diluted to the appropriate cell concentration using fresh YPG/R, and an arresting agent was added to each culture to block cell cycle progression at a given phase. Cultures were then incubated for 2.3 h to ensure the cells are synchronized. During which time HA-*ORC6* was expressed as shown by the green arrow. The cells were then washed and resuspended in YPD containing the same arresting agent. At which point, the cells in the culture no longer expressed HA-*ORC6*, and any protein present was eventually degraded. Essentially, all the cells were depleted of Orc6 at the same point in the cell cycle. After Orc6 depletion, the cells were washed thoroughly and resuspended again in YPD, this time without the original arresting agent. Therefore, cells should re-enter the cell cycle from that point and proceed until Orc6 is required. Cell cycle progression was monitored by FACS analysis to determine when the cell cycle is blocked. As well, chromatin binding assays or DNA combing experiments could be performed on the cells to examine DNA-association and initiation of DNA replication respectively.

Asynchronous culture

Dilute cells in
fresh medium

Galactose — GAL | HA | *ORC6* —
- Over-expression of HA-Orc6
- Arrest cell cycle

+ arresting agent
(Nocodazole, α-factor or HU)

Wash cells

STOP

Glucose — GAL | HA | *ORC6* —
- Depletion of HA-Orc6
- Maintain cellular arrest

+ arresting agent
(Nocodazole, α-factor or HU)

Wash cells

STOP

Glucose — GAL | HA | *ORC6* —
- Release cells from arrest

DNA
Combing

FACS

Chromatin
Binding
Assay

Depletion of Orc6

Figure 22. Cells depleted of Orc6 in G2/M arrest in the G1 phase of the following cell cycle. Asynchronous cultures of *GAL1-ORC6* cells (DY36) and its parental strain (DY26) were grown in YPG/R to 5×10^6 cells/ml and then arrested at G2/M with nocodazole (15 μg/ml) as described in the Materials and Methods. Once blocked, the cells were washed and transferred to YPD again with nocodazole (10 μg/ml) for 3 h to deplete HA-Orc6 from the *GAL1-ORC6* strain while maintaining the cell cycle arrest. The cells were then washed and released into fresh YPD without any arrest agents and samples were removed at the indicated intervals for FACS analysis.
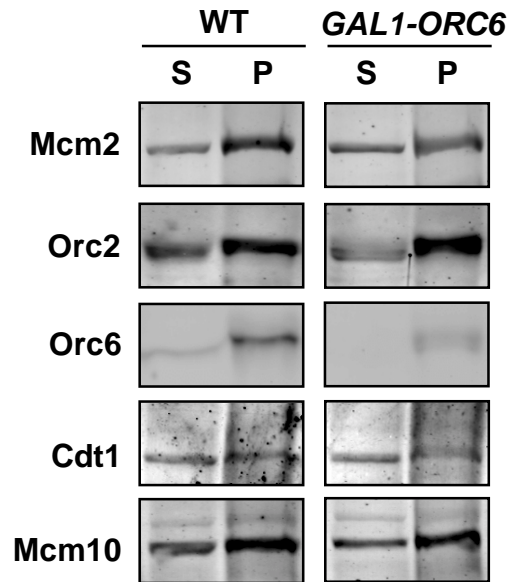
Figure 23. Orc6 mediates the level of chromatin-associated Mcm2 in G1 phase. Exponential cultures of *GAL1-ORC6* and its parental wild-type strain were grown to 5 x 10^6 cells/ml and arrested at G2/M with nocodazole (15 μg/ml) for 2.3 h in YPG/R as in Figure 16. The cultures were then washed in ddH$_2$O and shifted to YPD containing nocodazole (10 μg/ml) for 3 h. Cells were released into fresh YPD containing α-factor (50 μg/ml) and incubated for 2 h to arrest the cells in G1. Samples of the culture were taken following the arrest in α-factor for chromatin binding assays, which were carried out as described in the Materials and Methods. 15 μl of supernatant (S) and 30 μl of the chromatin-associated pellet (P) were analyzed by immunoblot. An anti-Mcm2 antibody was used to determine the extent of which the MCM proteins are loaded to chromatin, and an anti-Orc2 antibody was used to detect the status of Orc2 association. An anti-HA antibody was used to determine the level of Orc6 depletion and the levels of Cdt1 and Mcm10 were monitored using an anti-Myc antibody.

that the decline of Mcm2 loading following Orc6 depletion in G2/M was not the result of ORC destabilization, we also monitored the level of Orc2 to examine the overall state of the ORC complex. *GAL1-ORC6* cells depleted of Orc6 in G2/M and then released into the G1 block had levels of chromatin-bound Orc2 comparable to wild-type cells. It therefore appears that the reduction in Mcm2 loading was specific to the absence of Orc6.

Orc6 interacts with members of the pre-RC involved in MCM loading and maintenance
To determine whether Orc6 physically interacts with Mcm2 or factors that play a role in MCM chromatin association, the full-length *MCM2, CDC6, CDT1* and *MCM10* coding sequences were cloned into the two-hybrid bait vector pEG-202 (Ausubel *et al.*, 1995). Each of these constructs were separately transformed into the wild-type yeast strain DY1, already harboring the lacZ reporter-plasmid pSH18-34, and one of six prey plasmids expressing Orc1-6. When Mcm2 was tested as bait, the strongest β-galactosidase signal was obtained with Orc2, indicating that this ORC subunit and not Orc6, is most likely to associate directly with Mcm2 (Figure 24). We next evaluated ORC subunit interactions with Cdc6 and Cdt1, which are required for MCM loading onto chromatin (reviewed in Bell and Dutta, 2002). The strongest signal for Cdc6 was obtained with the Orc1 subunit, consistent with the previously reported Orc1-Cdc6 interaction in both yeast (Wang *et al.*, 1999) and human cells (Saha *et al.*, 1998). However, Orc2, Orc5 and Orc6 preys produced positive two-hybrid signals when co-expressed with the Cdt1 bait, with Orc6 the highest of the three. Finally, we tested the ORC subunits against Mcm10, which is required to preserve the interaction of Mcm2

Figure 24. Orc6 interacts with members of the pre-RC. Liquid two-hybrid assays were performed using pEG-Mcm2 (*A*), pEG-Cdc6 (*B*), pEG-Cdt1 (*C*), and pEG-Mcm10 (*D*) as baits in combination with each ORC subunit as preys (pJG-ORC1-6), as described in the Materials and Methods. Immunoblots of whole-cell extracts prepared from culture aliquots removed just prior to β-galactosidase activity determination are shown. Detection of the bait construct was performed using an anti-LexA antibody and prey expression was determined using an anti-HA antibody. Ponceau S stains are shown to illustrate protein loading. All two-hybrid results are representative of two or more assays and error bars represent standard deviation.
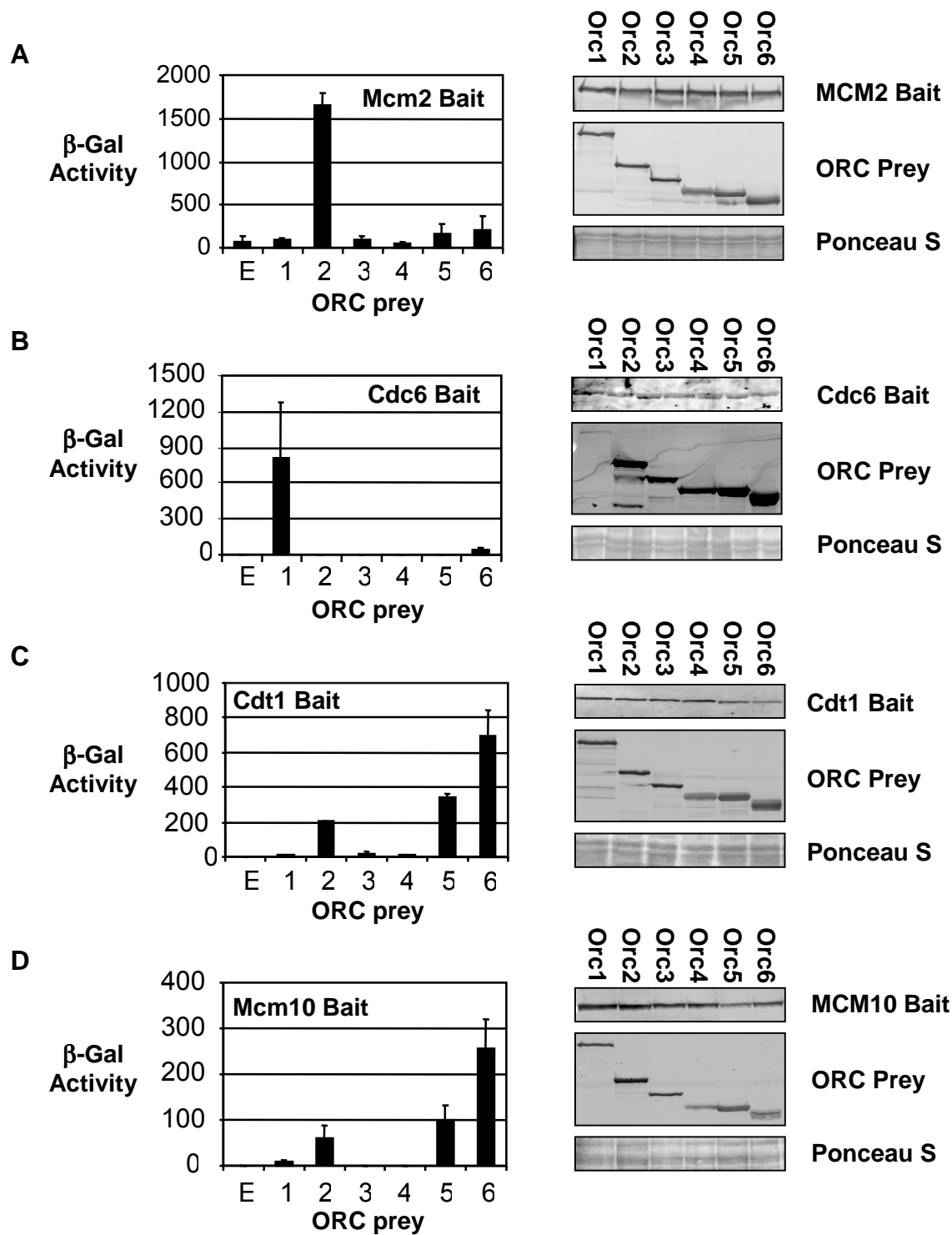
with chromatin in G1 phase (Homesley *et al.,* 2000). Here, we noticed a very similar pattern to that observed for Cdt1, with Orc6 again exhibiting the highest two-hybrid signal, and several attempts were made to confirm this interaction through different molecular assays (See Appendix I). Since both Cdt1 and Mcm10 interacted with Orc6, we evaluated the effect of G2/M Orc6 depletion on their chromatin association in G1. There was no appreciable effect on the degree chromatin binding of either protein (Figure 23).

Orc6 is required to maintain MCM chromatin association following pre-RC formation

Studies with *Xenopus* egg extracts indicate that once the MCM proteins are assembled onto chromatin, ORC and Cdc6 can be removed without affecting their putative DNA helicase function (Hua and Newport, 1998; Rowles *et al.,* 1999). Therefore, we were interested to determine whether Orc6 is still required for the efficient initiation of DNA replication after MCM proteins have been loaded onto chromatin. This was carried out by synchronizing cultures of *GAL1-ORC6* and its parental strain growing in YPG/R in late G1 phase by adding α-factor. Following the initial arrest, cells were transferred to YPD again with α-factor to deplete Orc6 in the *GAL1-ORC6* strain. After release from the block, an accumulation of 1C cells was observed for the Orc6-depleted *GAL1-ORC6* culture by 30 min, while the isogenic wt strain displayed a prominent 2C peak at the same time point, consistent with a role for Orc6 in S phase progression (Figure 25). As an additional control, a *GAL1-ORC4* strain was similarly assessed. Orc4 depletion in this strain occurs with similar kinetics to what is observed for Orc6 depletion (data not shown), but in this case cells were able to replicate their DNA, albeit at a slower rate

Depletion of Orc6
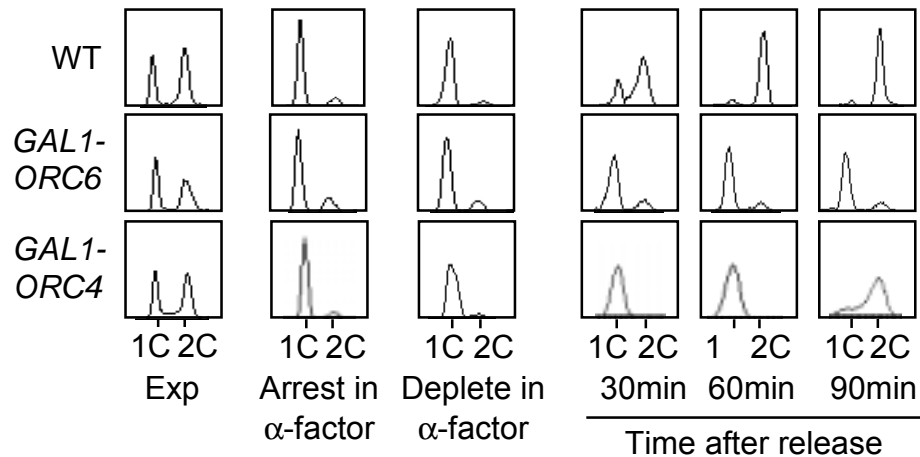
Figure 25. Cells depleted of Orc6 during late G1 do not progress into S phase. Asynchronous cultures of *GAL1-ORC6* cells (DY36) and its parental strain (DY26) were grown to 5 x 10^6 cells/ml and then arrested at G1 with α-factor (50 μg/ml) for 2.3 h in YPG/R as described in the Materials and Methods. Once synchronized, the cells were washed and transferred to YPD again with α-factor (50 μg/ml) for the indicated 4 h while maintaining the cell cycle block to deplete Orc6 from the *GAL1-ORC6* strain. The cells were then washed and released into fresh YPD without α-factor and samples were removed at the indicated intervals for FACS analysis.

than the wt control. Therefore, it appears that Orc6, as opposed to the entire ORC complex, is required in late G1 phase for subsequent DNA replication. This is consistent with previous work showing that depletion of budding yeast Orc2 from late G1 cells does not impede S phase progression (Shimada *et al*, 2002).

Since the most likely explanation for a lack of DNA replication following Orc6 depletion in late G1 phase was again a destabilization of the MCM complex at origins, we monitored the chromatin association of Mcm2 prior to and following Orc6 depletion. While equivalent chromatin pellet to supernatant Mcm2 ratios were observed during the initial α-factor arrest between the wt and *GAL1-ORC6* strains, there was a clear displacement of Mcm2 from the pellet to the supernatant fraction following Orc6 depletion in the *GAL1-ORC6* strain (GLU, Figure 26). Interestingly, we also observed a dramatic reduction in the level of chromatin-associated Orc2 following Orc6 depletion. Although we cannot rule out the possibility that an overall destabilization of ORC is responsible for the displacement of Mcm2, the observation that a robust Mcm2 chromatin association is maintained in cells depleted of Orc4 argues against this. In the case of Cdt1, little difference was observed between cells with normal Orc6 levels or those that had been depleted, although in each case, as expected, very little Cdt1 was detected in the chromatin fraction (Randell *et al.,* 2006). In contrast, the high levels of chromatin-bound Mcm10 initially detected were markedly reduced following Orc6 shut-off. These results are consistent with a mechanism whereby Orc6 promotes Mcm10 chromatin association, and Mcm10 in turn stabilizes the MCM complex (Homesley *et al.*, 2000).
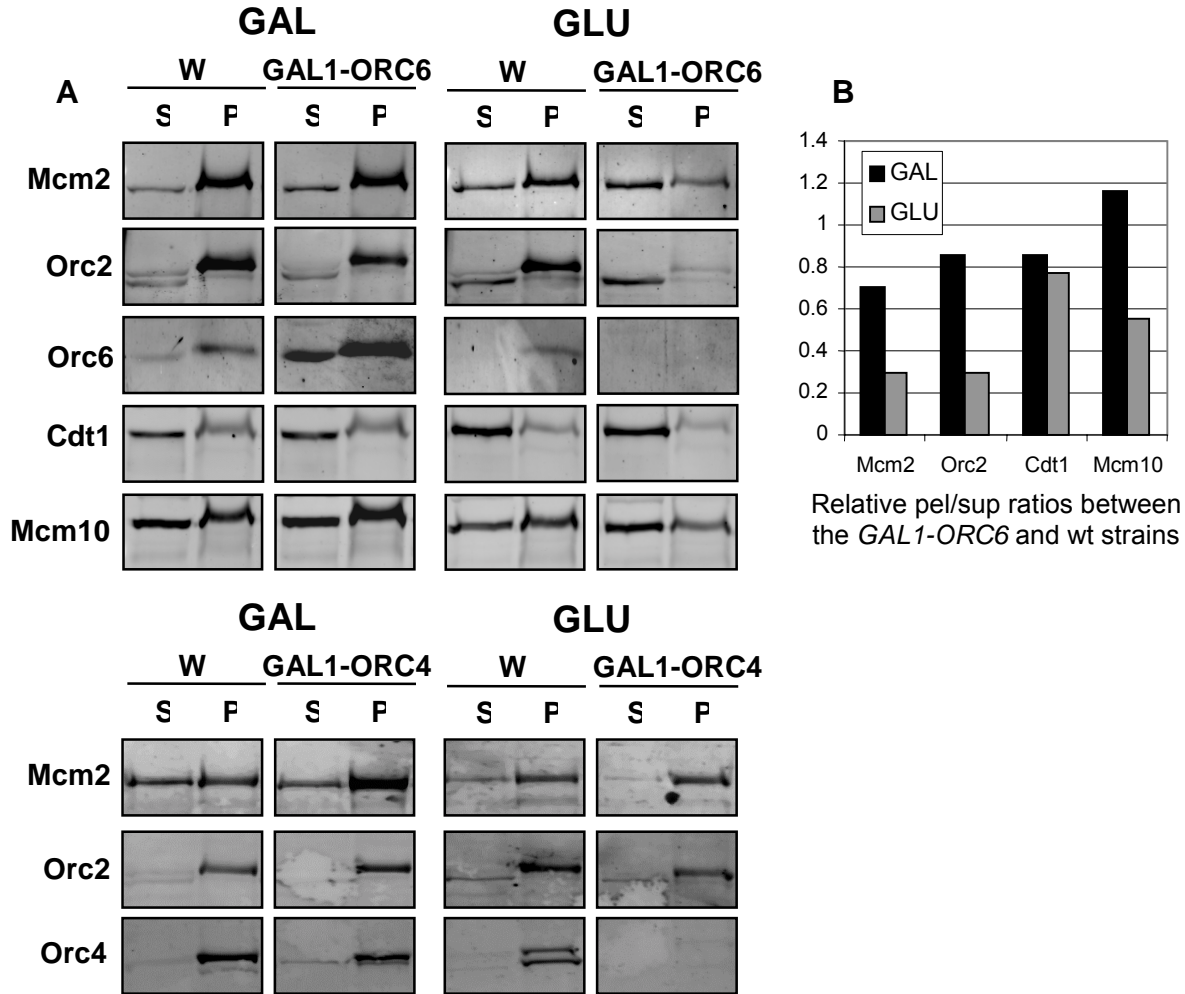
Figure 26. Orc6, but not Orc4, is required for pre-RC stability. (A) *GAL1-ORC6, GAL1-ORC4* and wt strains were grown up as outlined in Figure 22. Following the arrests in both YPG/R and YPD, culture aliquots were removed for CBAs as described in the Materials and Methods. 15 μl and 30 μl of the supernatant (S) and pellet (P) fractions respectively were analyzed by immunoblotting. Detection of each protein was carried out as performed in Figure 23. (B) Densitometry from the *GAL1-ORC6* immunoblots. The pellet to supernatant (pel/sup) ratios were obtained for each blot. Presented are the *GAL1-ORC6* ratios directly compared to the wt ratios, in both galactose/raffinose (GAL) and glucose (GLU) medium.

Depletion of Orc6 in late G1 reduces the efficiency of DNA replication initiation

The inability of cells lacking Orc6 to progress through S phase may have been due to a reduction of origin firing, inhibition of elongation, or a combination of both. To investigate the effect of Orc6 depletion on initiation events, we constructed a TK+ *GAL1-ORC6* strain (DY79), to allow the incorporation of BrdUinto newly synthesized DNA. Cultures of DY79 and its parental TK+ strain (DY67) were initially synchronized in late G1 with α-factor in YPG/R, then shifted to YPD with α-factor, to deplete Orc6 in the DY79 cells. The cells were subsequently washed and resuspended in YPD supplemented with BrdU (0.4 mg/ml) and the ribonucleotide reductase inhibitor hydroxyurea (HU; 0.2 M), which normally results in an early-S phase arrest, after about half the replication origins have fired. Following 90 min in HU, cells were harvested, genomic DNA was isolated and single molecule DNA combing was carried out, as previously described (Versini *et al.*, 2003). Comparison of inter-origin distances from *GAL1-ORC6* and wt cells revealed that approximately half the number of origins fired in cells depleted of Orc6 (Figure 27), consistent with a role for Orc6 in promoting initiation events. Following administration of HU and the reduction of nucleotide pools, the length of individual BrdU tracks were more than double those observed with the wt controls. Clearly cells that have fewer origins firing have more nucleotides available for the migration of individual replication forks.

Whether Orc6 might play a role in the elongation stage as well as the initiation stage of DNA replication was also investigated. *GAL1-ORC6* and isogenic wt cells were arrested in YPG/R supplemented with HU, then washed and resuspended in YPD/HU for 4 h. Following release from the HU block in YPD, the two cultures progressed through S
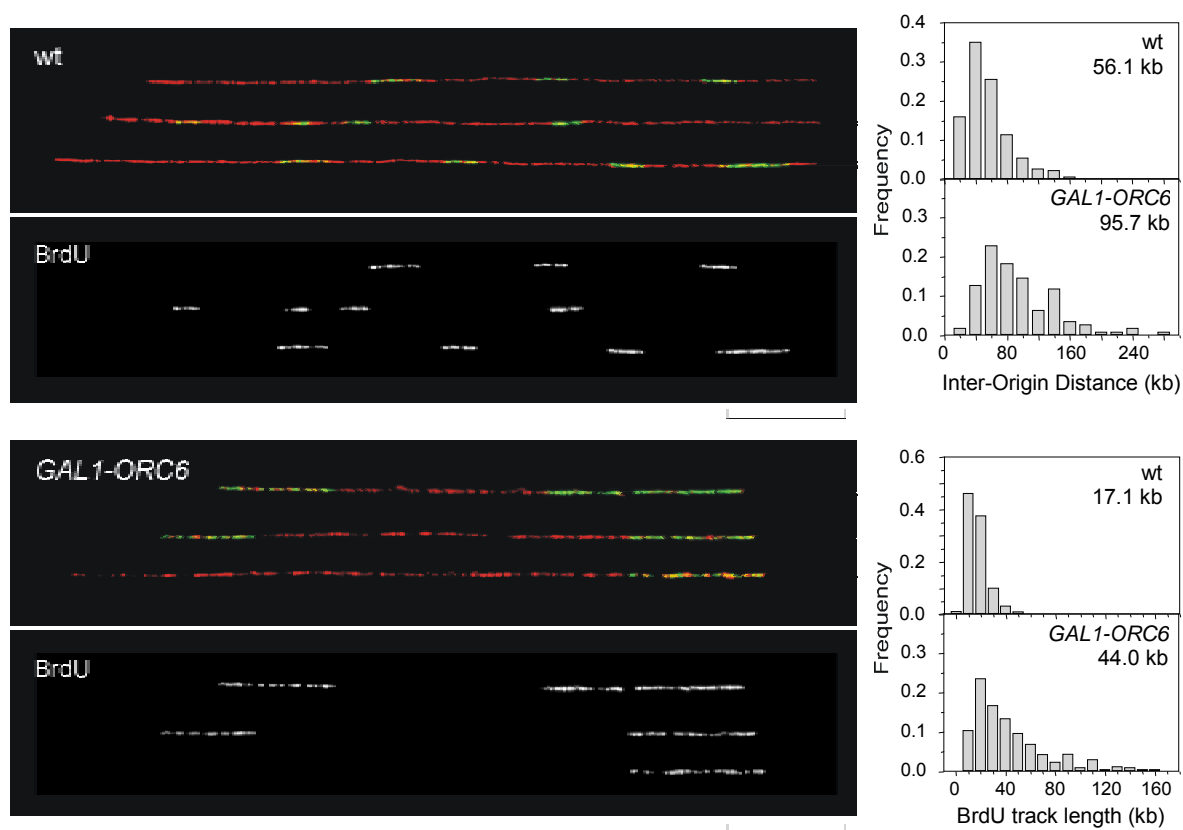
Figure 27. Orc6 is needed for efficient firing of origins after pre-RC formation. DNA combing of cells arrested with HU, following depletion of Orc6 while in late G1. Initiation frequency was monitored in both wild-type (DY67) and *GAL1-ORC6* (DY -79) TK+ cells. Cells were synchronized in G1 using α-factor and then transferred to YPD medium for 4 h to deplete Orc6 while still arrested with the pheromone. Cells were then released into YPD containing HU (0.2 M) to block elongation, and BrdU to track new strand synthesis, DNA plugs were prepared 90 after the transfer as described in the Materials and Methods. DNA combing was performed on silanized coverslips as described in Materials and Methods. Representative DNA fibers from both the wild-type and *GAL1-ORC6* strains are presented. BrdU incorporation appears green, DNA red. Scale bar represents 50 kb. Inter-origin distances as well as BrdU tract lengths following analysis of over 200 origins are shown in the histograms.

phase at comparable rates (Figure 28), suggesting that Orc6 was not required for efficient replication fork progression. Although we cannot exclude the possibility that residual Orc6 following our shut-off regime may have played a role in replication fork progression, we can rule it out as the cause of the S-phase arrest we see for this length of depletion (Figure 25).

Discussion

The essential cell cycle role of Orc6 is specific to S phase

Since its original biochemical purification from budding yeast (Bell and Stillman, 1992), ORC has been shown to play a central role in the initiation of DNA replication (reviewed in Bell, 2003). ORC lacking Orc6 is fully competent to bind origin sequences, but omission of any other subunit abrogates this capability (Lee and Bell, 1997). Orc6 is nevertheless an essential protein for cell proliferation (Li and Herskowitz, 1993), and previous studies suggest that some budding yeast ORC subunits may have important functions at other cell cycle stages (Bell *et al.*, 1993; Dillin and Rine, 1998). Recent attention has focused on additional roles for the Orc6 subunit in mitosis and cytokinesis in metazoans (Prasanth *et al.*, 2002; Chesnokov *et al.,* 2003). It is therefore reasonable to ask whether budding yeast Orc6 is required for DNA replication and/or other cell cycle events. Our localization of Orc6-eYFP/eCFP to sub-nuclear foci in living cells provides compelling evidence for a replicative function, since it is similar to the punctate pattern previously shown for immunolocalization of Orc2 in fixed cells (Pasero *et al.,* 1999), and with the detection of discrete zones of new DNA synthesis in TK+ yeast cells following a BrdU pulse (Lengronne *et al.,* 2001). To our knowledge, the visualization of

Depletion of Orc6
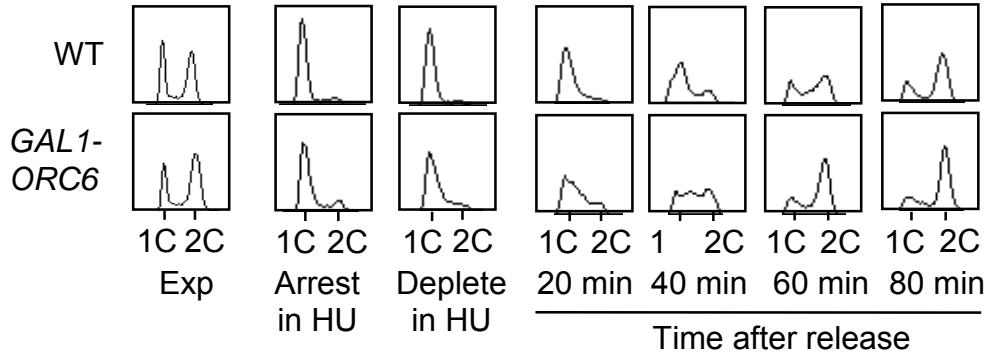
Figure 28. FACS analysis of cells depleted of Orc6 while in S phase and then released. Asynchronous cultures of *GAL1-ORC6* cells (DY36) and its parental strain (DY26) were grown in YPG/R to 5 x 10^6 cells/ml and then arrested at early S phase with HU (0.2 M) as outlined in the Materials and Methods. Once blocked, the cells were washed and transferred to YPD, again with HU (0.36 M) for 4 h deplete Orc6 while maintaining the cell cycle arrest. The cells were then washed and released into fresh YPD without HU and samples were removed at the indicated intervals for FACS analysis.

Orc6 foci represents the first detection of a protein constituent of what are thought to be replication factories involving clusters of replication forks (reviewed in Laskey and Madine, 2003) in living *S. cerevisiae* cells. This pattern of localization was also observed *in vivo* in the Orc2-eYFP strain (Figure 15). Intriguingly, we have also found that among ORC subunits, both Orc5 and Orc6 are able to homodimerize and thus may conceivably contribute to the formation of replication compartments by bridging different origin sequences (unpublished data – Chapter V, Figure 30).

In contrast to what has been seen in both human and fruit fly cells, we observed no accumulation of Orc6 at the midplane of division between mother and daughter cells (Figure 13, 14 and 15). Given that the nuclear envelope in budding yeast remains intact throughout the cell cycle, it is possible that Orc6 could play a karyokinetic role that is analogous to the cytokinetic function of its metazoan orthologs. Our results argue against this, however, as we do not observe a population of Orc6 at the site of nuclear division in any of the cells we observed (Figure 13 and 14, rightmost set of figures, and results not shown). As well, it appears as though Orc6 is limited to areas of general co-localization with Orc2 (Figure 16). FACS analysis following depletion of Orc6 from an asynchronous culture shows no accumulation of cells with $\geq$ 2C DNA content (Figure 19). The fact that no significant 2C DNA peak was observed even six hours after Orc6 was depleted suggests that chromosome segregation was not compromised as reported for human cells treated with Orc6 siRNA (Prasanth *et al.,* 2002). Although microscopy following DNA staining of Orc6-depleted cells indicated an increase in G2/M cells (Figure 20), it is thought that this is due to incomplete DNA replication that resulted in S-phase checkpoint activation. We show that upon depletion of Orc6, the reduced

origin firing clearly limits the rate of DNA replication (Figure 28), and Rad53 is activated (data not shown). Rad53 dependent checkpoint activation is known to occur when the rate of DNA replication is drastically reduced (Paulovich and Hartwell, 1995), thus preventing cells from dividing prior to a complete round of DNA synthesis. Therefore, although the defect occurs during G1/S, the Rad53-mediated S-phase checkpoint arrests cells in G2/M, as it prevents entry into mitosis (Allen *et al.,* 1994). Indeed, cells depleted for Orc6 during a G2/M block were fully capable of completing mitosis and proceeding into G1 phase following release (Figure 22). Although we cannot rule out that residual levels of Orc6 capable of carrying out mitotic and/or cytokinetic functions remain following our depletion protocol, it is clear from the marked accumulation of cells with DNA content between 1C and 2C following Orc6 depletion in an asynchronous culture, that the cell cycle arrest we observed under these conditions was primarily due to S phase defects.

Why would metazoan roles for Orc6 in cytokinesis and mitosis not be present in budding yeast? An important consideration in answering this question is that *S. cerevisiae* Orc6 shares only low sequence conservation with its *Drosophila* (5% identity, 19% similarity) and human (6% identity, 15% similarity) orthologs. Furthermore, sequence comparison of Orc1-Orc6 from these species suggests that Orc6 is more divergent than any of the other ORC subunits (Dhar and Dutta, 2000). Thus, it appears that metazoan Orc6 has conserved its original role in DNA replication, while acquiring cytokinetic and, at least in the case of human Orc6, mitotic functions. It was proposed that Orc6 helps to coordinate the processes of DNA replication and chromosome segregation with cytokinesis in metazoans (Prasanth *et al.*, 2002), which might be more

critical for multicellular than for unicellular organisms. The fact that these additional functions are not present in *S. cerevisiae* makes it an advantageous system to further characterize the role of Orc6 in DNA replication, without the complication of mitotic and cytokinetic phenotypes.

Orc6 mediates the G$_1$ phase chromatin association of Mcm2

To our knowledge, the most substantial evidence of an essential role for *S. cerevisiae* Orc6 in DNA replication comes from the original work of Li and Herskowitz (1993) in which they found that following sporulation and tetrad dissection of *ORC6*+/- heterozygotes, haploid *ORC6-* spores undergo up to two rounds of cell division, arresting with the type of large bud morphology often associated with mutations affecting DNA replication. More recently, the hyperphosphorylation of both Orc6 and Orc2 following START was shown to be part of a series of overlapping mechanisms that prevent DNA re-replication, which also include Cdc6 degradation and export of Mcm2-7 from the nucleus (Nguyen *et al.*, 2001). All three processes are Clb/Cdc28 (CDK) mediated, and a direct interaction between Clb5 and Orc6 has been reported in budding yeast (Wilmes *et al.*, 2004). Either mutation of both the Orc2 and Orc6 consensus CDK phosphorylation sites or abrogation of the Clb5/Orc6 interaction predisposes cells to re-replication in combination with stabilized Cdc6 and/or constitutively nuclear Mcm2-7 (Nguyen *et al.*, 2001; Tanny *et al.*, 2006). However, neither change has any effect on cell cycle progression in the presence of wild-type Cdc6 and MCM proteins. Thus, prevention of re-replication is not the essential function of Orc6.

In the present report, we provide strong evidence that Orc6 is required for efficient initiation of DNA replication. Cells that were released from a G2/M block during which Orc6 was depleted, arrested in the same cycle with a 1C DNA content (Figure 22). How is the lack of Orc6 inhibiting DNA replication? One possibility is that the absence of Orc6 prevents proper pre-RC assembly, which normally culminates in the chromatin loading of the MCM complex. There was a clear drop in the level of chromatin-associated Mcm2 after cells depleted of Orc6 in nocodazole were released and subsequently blocked with α-factor (Figure 23). More striking, however, was the strong displacement of previously loaded Mcm2 by depleting Orc6 during an α-factor arrest (Figure 26). This indicated that the role of Orc6 is primarily to maintain the chromatin association of MCM complexes after they have associated with the pre-RC. Possible mechanisms for how Orc6 facilitates MCM chromatin association were suggested by our observations that Orc6 interacts with both Cdt1 and Mcm10, two factors required for stable association of Mcm2 with chromatin (Homesley *et al.*, 2000; Devault *et al.*, 2002; Tanaka and Diffley, 2003). While there appeared to be no significant effect on the chromatin levels of either Cdt1 or Mcm10 following the G2/M Orc6 depletion, the chromatin pellet to supernatant ratio of Mcm10 dropped by approximately 50% when Orc6 expression was shut-off in late G1 phase, again suggesting a maintenance role. Interestingly, Mcm10 has also been shown to regulate the chromatin association of DNA polymerase-α (Ricke and Bielinsky, 2004), providing another way in which its displacement may inhibit DNA replication. In contrast to what was seen with Orc6 depletion at the earlier time point Orc2 was displaced from chromatin (Figure 26). Thus, a general destabilization of ORC may be responsible for the MCM displacement, and

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subsequent replication defects; however, the fact that Orc4 depletion neither displaces Mcm2 from chromatin (Figure 26), nor prevents progression through S phase (Figure 25) argues against this and points to a role for Orc6 that is distinct from other ORC subunits.

Orc6 plays a role in the initiation of DNA replication after pre-RC formation

Previous models of pre-RC assembly and function have proposed that once MCM proteins have been loaded onto chromatin, ORC is dispensable for DNA replication. Treatment of either budding yeast late G1 chromatin or pre-RCs assembled on magnetic beads with high salt has been reported to remove ORC, while MCM proteins remain present (Donovan *et al.*, 1997; Bowers *et al.*, 2004). In each case, however, there was a significant reduction in the level of associated MCM proteins and the consequences of the ORC removal on DNA replication was not evaluated. Subsequent DNA replication was determined to be unaffected by salt extraction of ORC after pre-RC formation in a *Xenopus* cell free system, although the successful removal of only the Orc1 and Orc2 subunits was monitored (Rowles *et al.*, 1999). In contrast to these results, our data clearly points to a role for Orc6 in promoting initiation events after the pre-RC has formed, since its depletion in late G1 severely reduced the number of origins that fired (Figure 27) and clearly inhibited S phase progression (Figure 25). One explanation for varying observations with budding yeast and *Xenopus* is that there are simply differences between the two species with respect to the cell cycle window during which ORC is required to ensure origin firing. Another possibility is that some ORC subunits are required for initiation after pre-RC formation, while others are not. We favor

this idea, since the late G1 depletion of neither Orc2 nor Orc4 prevents cells from traversing S phase (Shimada *et al.,* 2002 and Figure 28 of the present study).

Clearly, a better understanding of the functions of individual ORC components is required to fully characterize the multiple roles of this complex in cell cycle progression. For the first time, we have demonstrated a unique function for the Orc6 subunit in promoting DNA replication by mediating the stability of pre-RCs after their formation. It will now be of considerable interest to determine whether this function is conserved among eukaryotes, and to investigate the precise cell cycle roles of other ORC subunits.

CHAPTER V: Orc6 Interactions

Introduction

DNA replication is initiated by a series of protein interactions that culminate in the formation of the pre-RC and the eventual recruitment of the polymerase α/primase complex to origins of replication. In *S. cerevisiae*, numerous protein components involved in the initiation process have been well characterized (reviewed by Kelly and Brown, 2000; Bell and Dutta, 2002). At the heart of the pre-RC lies ORC, which is considered to be the foundation onto which other factors assemble. ORC is typically described as a functional complex with little consideration for the responsibilities of each independent subunit and the distinct roles that they may play in during DNA replication. It is known that Orc6 is the only subunit not required for chromatin association of ORC, and it has been shown to be absent from some ORC complexes bound to origins (Lee and Bell, 1997). Since it does not facilitate DNA binding of ORC, it is possible that Orc6 is involved in the recruitment of other factors during pre-RC development. In the previous chapter, it was shown that Orc6 is involved in MCM loading and maintenance, as well as Mcm10 and Orc2 stability on chromatin in late G1 phase. However, only a limited number of physical interactions with Orc6 were examined in that study, and the details of this mechanism need to be clarified.

Loading and maintaining initiation factors at origins may not be the only function Orc6 plays during the G1/S transition. As well, Orc6 could be involved in several redundant mechanisms throughout the cell cycle. To help characterize the functions of Orc6 in budding yeast, it is important to identify the proteins that associate with Orc6, as protein-protein interactions are typically observed between factors involved in the same biological pathway. Once protein interactions are identified, they can be examined to

determine their phenotypic significance. This chapter deals with the identification of Orc6 ligands to further investigate its role in the budding yeast cell cycle.

Since Orc6 was originally discovered in *S. cerevisiae* as part of the ORC complex, it is expected that it interacts with one or more of the ORC subunits. However, this association may be coincidental as UV cross-linking studies have shown that Orc6 is not always associated with ORC at budding yeast origins (Lee and Bell, 1997). When bound to ORC at origins, Orc6 appears to associate with a segment of DNA overlapping the Orc5 subunit (see Chapter 1, Figure 4), indicating that these two subunits may interact. However, this is not conclusive evidence of a physical association, and the mechanism by which Orc6 interacts with ORC in budding yeast is unclear.

To date, the only species in which Orc6 has been shown to be required for both chromatin association of ORC and DNA replication is *Drosophila* (Chesnokov *et al.,* 2001). However, gel filtration assays using *Drosophila* extracts revealed only a small fraction of Orc6 present in the cell is found in large protein complexes with the other ORC subunits (Chesnokov *et al.,* 2001). Even in *Xenopus*, Orc6 was the only ORC subunit that did not co-purify with ORC (Gillespie *et al.,* 2001). Similar gel filtration assays using recombinant human ORC subunits revealed only low levels of Orc6 migrating with the other ORC subunits in a large complex, as the majority of Orc6 was present in a much smaller complex (just over 50 kDa), possibly the result of homodimerization (Vashee *et al.,* 2001). In HeLa cells, isolation of endogenous ORC through immunoprecipitation with an anti-Orc2 antibody does not contain Orc6, whereas all the other subunits were co-purified (Vashee *et al.,* 2001). In a similar experiment using human 293T cell lysates, immunoprecipitation of ORC using an anti-Orc2

antibody pulled out Orc2-Orc5, whereas both Orc1 and Orc6 were not found in the precipitate (Dhar and Dutta, 2000). When an anti-Orc6 antibody was used in a similar co-IP experiment under the same conditions no ORC subunit other than Orc6 was detected in the precipitate, supporting the claim that Orc6 only weakly binds the ORC complex. In a study mapping the binary interactions between recombinant human ORC subunits, Orc6 was shown to co-immunoprecipitate with Orc3 and to a lesser extent Orc2 (Vashee *et al.,* 2001). In murine cells, Orc6 appears to interact well with Orc4 and weakly with Orc3 and Orc5 by two-hybrid analysis (Kneissl *et al.,* 2003). Clearly, these results indicate that Orc6 does not have defined place within the ORC complex across all eukaryotes as there is little consistency in how it associates with ORC.

Why is Orc6 occasionally absent from the ORC complex? It is possible that the weak associations observed between Orc6 with other ORC subunits are additive, and a stable complex is only formed after Orc6 has bound several subunits. However, UV cross-linking experiments put budding yeast Orc6 at the end of the complex proximal only to Orc5 and not in direct contact with any other ORC subunit (Lee and Bell, 1997). An alternate hypothesis is that a conformational change is required of Orc6 or another ORC subunit, possibly Orc5, before it binds to ORC. This change could be the consequence of earlier associations among the ORC subunits or from an external stimulus. With so many species showing a weak interaction between Orc6 and ORC, it is possible that this association is only transient. If Orc6 is not required for chromatin association of ORC, it may be involved in targeting other replication factors to origins, in which case an extended association is not needed. Nevertheless, little insight into the connection between Orc6 and ORC has been gained from other species. Therefore, it would be of

interest to re-examine the physical interactions between Orc6 and the other ORC subunits in more detail to clearly define its associations within ORC in yeast.

Outside of ORC, several interactions have been identified between Orc6 and various DNA replication factors, including members of the pre-RC. In budding yeast, genetic interactions were observed between *ORC6* and both *cdc6* and *mcm5* (Li and Herskowitz, 1993; Kroll *et al.,* 1996). In each case, over-expression of Orc6 resulted in a decrease in the restrictive temperature in these temperature-sensitive mutants. Other Orc6 interactions were identified from synthetic dosage lethality screens, in which Orc6 over-expression in mutants harboring non-essential mutations caused cell death. This method of screening identified an interaction between *ORC6* and the catalytic subunit of DNA polymerase delta (*CDC2*), which is required for both DNA replication and mitosis (Kroll *et al.,* 1996). All the genetic interactions mentioned above point to a role for budding yeast Orc6 in DNA replication.

Physical interactions between Orc6 and proteins involved in DNA replication have also been observed in several species, supporting the genetic findings. Yeast two-hybrid data from a previous chapter of this project (Chapter 4, Figure 24) indicated that budding yeast Orc6 associates with both Mcm10 and Cdt1. A similar interaction between Orc6 and Mcm10 (Cdc23) was observed in fission yeast (Hart *et al.,* 2002). In mice, Orc6 appears to interact in a two-hybrid assay with a suite of initiation factors including Mcm5, Cdc45 and the single-stranded DNA binding protein Rpa70 (Kneissl *et al.,* 2003). Therefore, it appears as though Orc6 is directly involved in the assembly of the pre-RC components in several species.

Beyond DNA replication, Orc6 may have other roles throughout the cell cycle. In budding yeast, several genetic interactions have implicated Orc6 in mitosis and cytokinesis. Interactions between *ORC6* and both *CDC14* and *CDC16* were identified from synthetic dosage lethality screens (Kroll *et al.,* 1996). Cdc14 is a important anaphase-promoting protein that is involved in separating sister chromatids, particularly at sites of rDNA and telomeres (Torres-Rosell *et al.,* 2005). As well, Cdc14 is needed to inactivate mitotic Cdks, which is necessary for the appropriate timing of mitotic exit (Visintin *et al.,* 1998). Cdc16 is a member of the anaphase-promoting complex, which is responsible for the specific ubiquitination and subsequent degradation of cell cycle regulators, including several Cdks (Passmore *et al.,* 2005). Lowering of Cdk activity establishes a window for the pre-RC to form at origins of replication during G1 phase (Mailand and Diffley, 2005). In budding yeast, Cdc16 is involved in preventing re-replication of the chromosomal DNA, as cells defective in Cdc16 undergo re-replication even when arrested in G2 with high Cdk activity (Heichman and Roberts, 1998). Taken together, these genetic interactions indicate that budding yeast Orc6 may be involved in coordinating mitotic events.

In metazoans, several studies have shown Orc6 to be associated with factors involved in mitosis and cytokinesis. Yeast two-hybrid screens performed using *Drosophila* ovary and embryo cDNA libraries and an *ORC6* construct (Chesnokov *et al.,* 2003) identified 16 different cDNA clones. Currently, only one ligand isolated from this screen has been characterized, and that was with Pnut, a septin protein important in cell division (Chesnokov *et al.,* 2003). Septins are highly conserved proteins involved in filament assembly and cytosolic re-organization, particularly during cytokinesis (reviewed in

Versele and Thorner, 2005). They are essential proteins in both yeast and *Drosophila*
that typically localize to the site of bud formation and cleavage furrow respectively.
Orc6 and Pnut have been found to not only interact through two-hybrid analysis and co-
immunprecipitation, but they were also found to co-localize in *Drosophila* cells
(Chesnokov *et al.,* 2003). Over-expression of an Orc6 mutant unable to interact with
Pnut resulted in both a loss of Orc6 localization to the cytoplasmic membrane and an
increase in cells with multiple nuclei, implicating *Drosophila* Orc6 in cytokinesis. A
similar mitotic/cytokinetic role for Orc6 was observed in humans, although no ligand for
Orc6 has yet been identified in this species (Prasanth *et al.,* 2002). In both species, the
mitotic phenotypes were observed independent of a defect in DNA replication, indicating
that they are two separable functions of metazoan Orc6.

In yeast, Orc6 may be involved in the coordination of other cellular events, as it has
been shown to interact with several important cell cycle regulators. As mentioned
earlier, cyclin-dependent and Dbf4-dependent kinases are key regulators of the cell
cycle (reviewed in Kelly and Brown, 2000; Zou and Stillman, 2000, Nguyen *et al.,* 2001,
Varrin *et al.,* 2005). High Cdk/Ddk activity facilitates the G1/S transition (Zou and
Stillman, 2000). Continued high levels of Cdks prevent re-replication of the genome
post-S-phase (Nguyen *et al.,* 2001), and low Cdk activity is needed for cells to prepare
for a new round of DNA replication following the metaphase/anaphase transition. Yeast-
two hybrid data indicates that budding yeast Orc6 interacts with the regulatory subunits
of both Cdks (Wilmes *et al.,* 2004) and Ddks (Duncker *et al.,* 2002), although it should
be noted that stronger Dbf4 interactions were observed between both Orc2 and Orc3.
While it is unclear whether or not the Orc6-Dbf4 interaction is significant in budding

yeast, this interaction appears to conserved in mice where Orc6 was found to associate with both Dbf4 and Cdc7 (Kneissl *et al.,* 2003). It is possible that the Dbf4/Cdc7 interaction with Orc6 is important in regulating the initiation of DNA replication. Along with promoting DNA replication at the G1/S transition, Cdks are also involved in inhibiting pre-RC formation post-S-phase. There are three mechanisms regulated by Cdks that inhibit re-replication, including phosphorylation of ORC (Orc2 and Orc6, Nguyen *et al.,* 2001), Cdc6 (Drury *et al.,* 2000; Nguyen *et al.,* 2001) and the MCM proteins (Labib *et al.,* 1999; Nguyen *et al.,* 2000). Interestingly, these control mechanisms are not completely redundant, as alterations in any one of them can cause slight re-replication (Archambault *et al.,* 2005). A closer examination of this process revealed a conserved hydrophobic patch on the Clb5 molecule common to several cyclins that is essential for substrate association (Brown *et al.,* 1999; Loog and Morgan, 2005; Wohlschlegel *et al.,* 2001; Cross and Jacobson, 2000). Clb5 is one of many cyclins involved in S phase regulation, and targets several proteins including Orc6 (Loog and Morgan, 2005; Wilmes *et al.,* 2004). After initiation of DNA replication, Clb5 interacts with Orc6 through the hydrophobic patch and RXL or "Cy" motif, respectively (Wilmes *et al.,* 2004, see Chapter 3, Figure. 7). This interaction requires initiation of DNA replication and is maintained throughout S phase and early M phase. Although mutations that prevent this interaction have no effect on DNA replication, they increase cell vulnerability to re-replication (Archambault *et al.,* 2005). Although the interaction between Clb5 and Orc6 is not essential for cell viability under normal conditions, this interaction shows the importance of Orc6 in regulating key events in the cell cycle.

Orc6 interactions with ORC subunits

Very little is known about how Orc6 actually associates with the rest of the ORC complex. To clarify this interaction, yeast two-hybrid assays were performed using Orc6 as a bait, and each ORC subunit as prey. The intent of these experiments was to identify which ORC subunits strongly interact with Orc6. Most of the plasmids required for this assay were already available in the laboratory (Duncker *et al.,* 2002; pEG-*ORC6* was prepared by Nayan Modi), so they were immediately transformed into a wt yeast strain (DY1) containing the β-galactosidase reporter plasmid, pSH18-34. All cultures were grown up in selective media to maintain each plasmid prior to the yeast assay. To ensure that negative results were the consequence of a lack of protein-protein interaction, bait and prey expression was confirmed in each culture by western blotting. Each assay was performed twice to verify reproducibility of results as described in the Materials and Methods. A representative histogram showing the relative strength of the interactions between Orc6 and each ORC subunit is shown in Figure 29. Surprisingly, the most robust interaction observed in this assay was between Orc6 and Orc6, suggesting that Orc6 may form homodimers or mulitmers. Much weaker interactions were detected between Orc6 and Orc2, Orc3 and Orc4. Therefore, the key to a stable interaction between Orc6 and ORC may be through one or more of these subunits.

It was not expected that Orc6 would interact so strongly with itself. To determine the significance of this interaction, it was important to determine if Orc6 is the only ORC subunit that exhibits a self-association. Since our laboratory already had prey constructs containing each ORC subunit and pEG-*ORC2* (made by Nayan Modi), only the
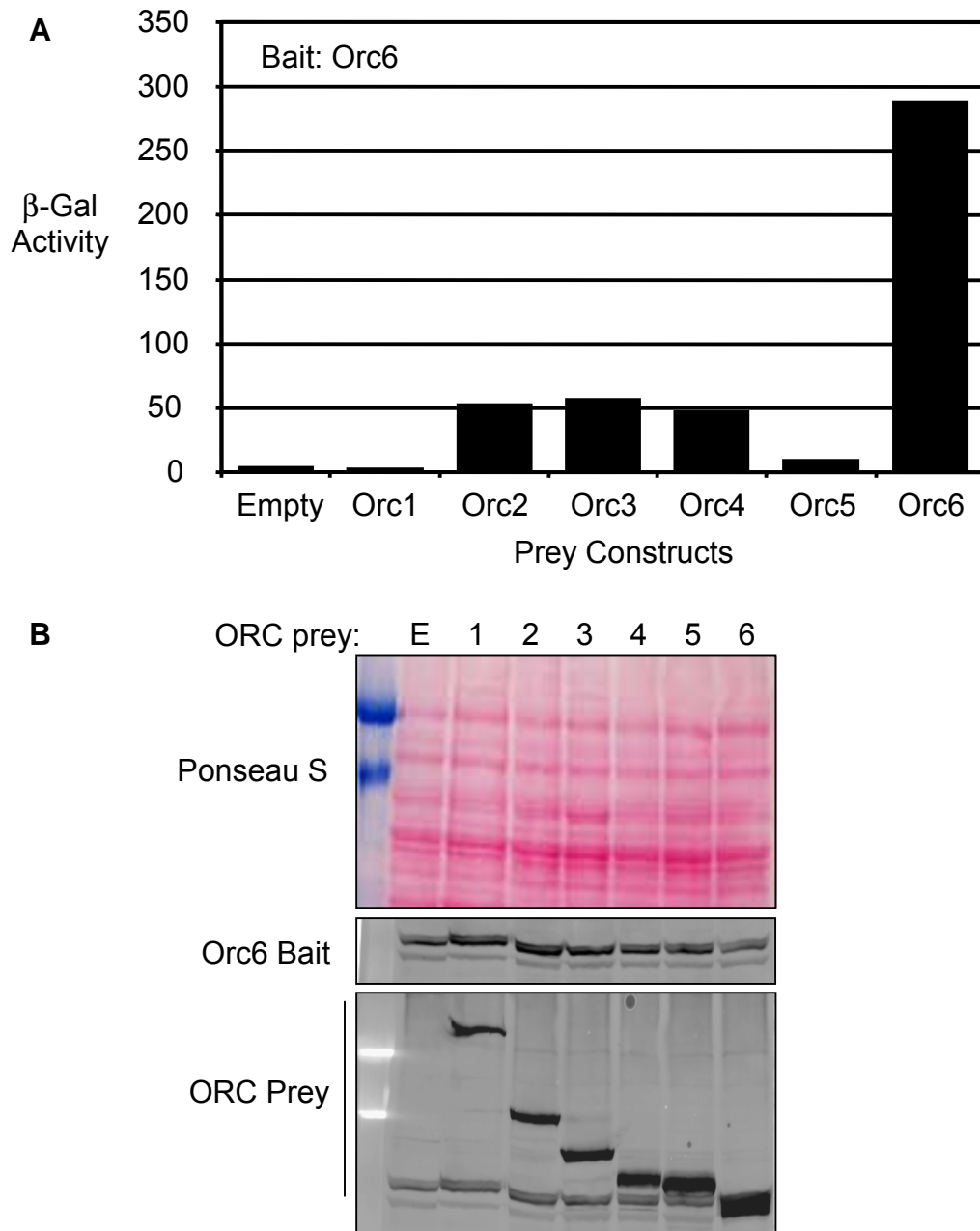
Figure 29. Examination of Orc6 associations within the ORC complex. (A) A liquid two-hybrid assay was performed using pEG-*ORC6* as a bait and individual ORC subunits as preys (in pJG4-6). The assay was carried out described in the Materials and Methods. All two-hybrids were performed at least twice, and a representative histogram is shown. E represents an empty prey construct. (B) Immunoblots of whole-cell extracts prepared from culture aliquots removed just prior to β-galactosidase activity determination are shown. Detection of the bait and prey constructs was performed as in Figure 18. A Ponceau S stain is shown to illustrate equal protein loading.

additional bait plasmids were required. Therefore, the full-length coding sequences of *ORC1, ORC3, ORC4* and *ORC5* were amplified and cloned into pEG202 as outlined in the Materials and Methods. Bait and prey constructs containing the same ORC subunit were transformed into a wt (DY1) strain already possessing the reporter plasmid (pSH18-34). Yeast two-hybrid assays and western blots were performed using each culture as described earlier to confirm appropriate expression of the bait and prey constructs, and duplicate assays were performed to verify reproducibility of observed results. A representative histogram and series of western blot detections are shown in Figure 30. Clearly, the only other ORC subunit that self-associates is Orc5. In the example shown, Orc6 bait expression was fairly weak, resulting in a lower β-galactosidase activity. However, when this assay was repeated on two more occasions (without western blots), the strength of the Orc5-Orc5 and Orc6-Orc6 interactions were relatively equal (data not shown). Therefore, both Orc5 and Orc6 are able to self-associate, possibly forming homodimers or multimers.

In an effort to determine the significance of the Orc6 self-association, the region of Orc6 necessary for this interaction was mapped. PCR primers were designed to amplify discrete fragments of the *ORC6* gene with restriction enzyme sites incorporated into each 5'-end to facilitate cloning into the bait vector. Various bait plasmids were constructed containing fragments of the *ORC6* gene as outlined in the Materials and Methods, and are illustrated in Figure 31. These new bait plasmids were transformed along with an *ORC6* prey plasmid into a wt (DY1) strain containing the reporter plasmid. Two-hybrid analysis and western blotting was performed as described in the Materials and Methods. This assay was performed twice, and a representative histogram
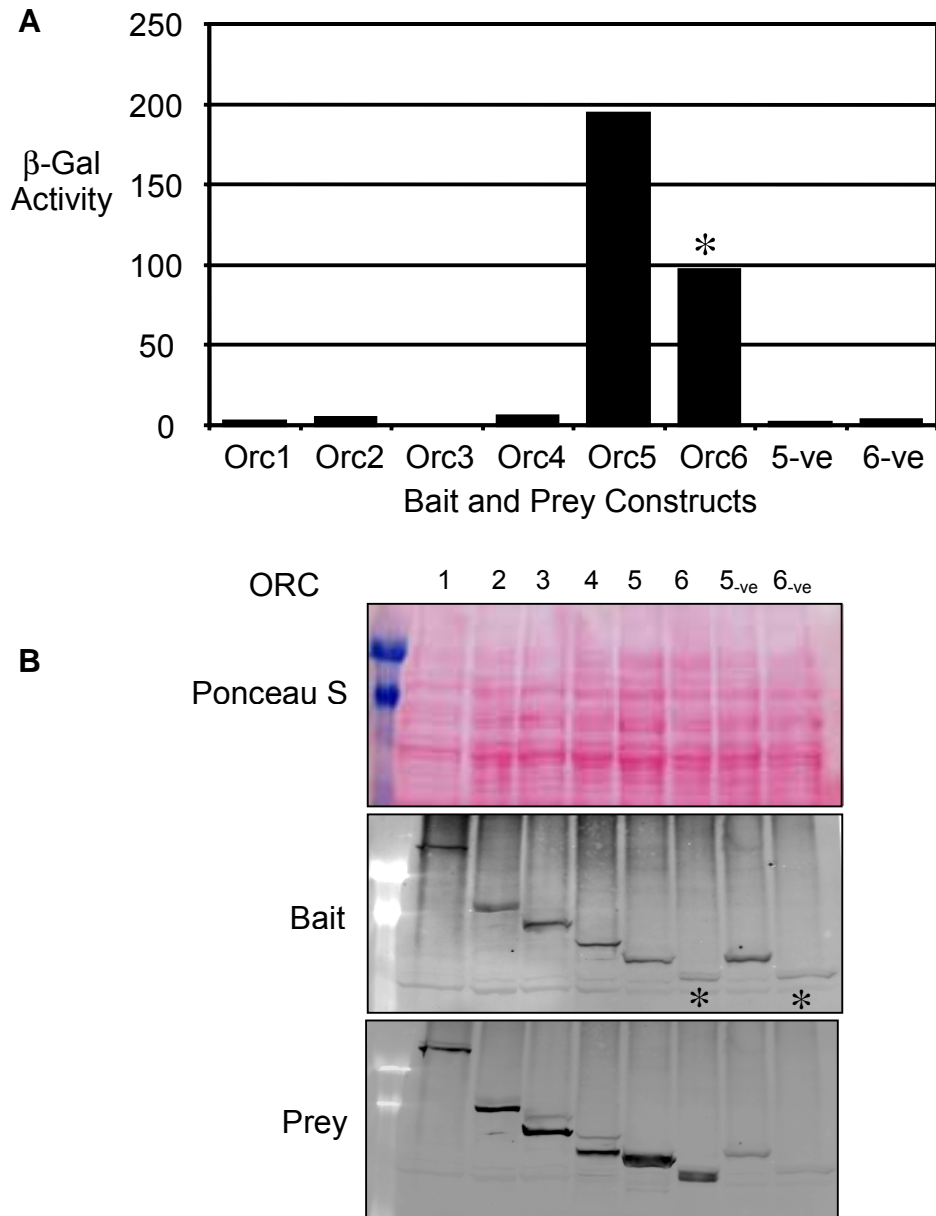
Figure 30. Identification of ORC subunits that are able to form multimers. (A) A liquid two-hybrid assay was performed using combinations of the same ORC subunit as both bait (pEG202) and prey (pJG4-6), as described in the Materials and Methods. Subunits that showed the highest interactions were also analyzed for auto-activation of baits, using the same bait construct along with an empty prey vector. –ve represents two-hybrid assays performed with an empty prey vector. All two-hybrid assays were performed at least twice, and a representative graph is shown. (B) Immunoblots of whole-cell extracts prepared from culture aliquots removed just prior to β-galactosidase activity determination. Detection of the bait and prey constructs was performed as in Figure 18. A Ponceau S stain is shown to illustrate equal protein loading. An asterisk denotes bait proteins that were expressed only at low levels.
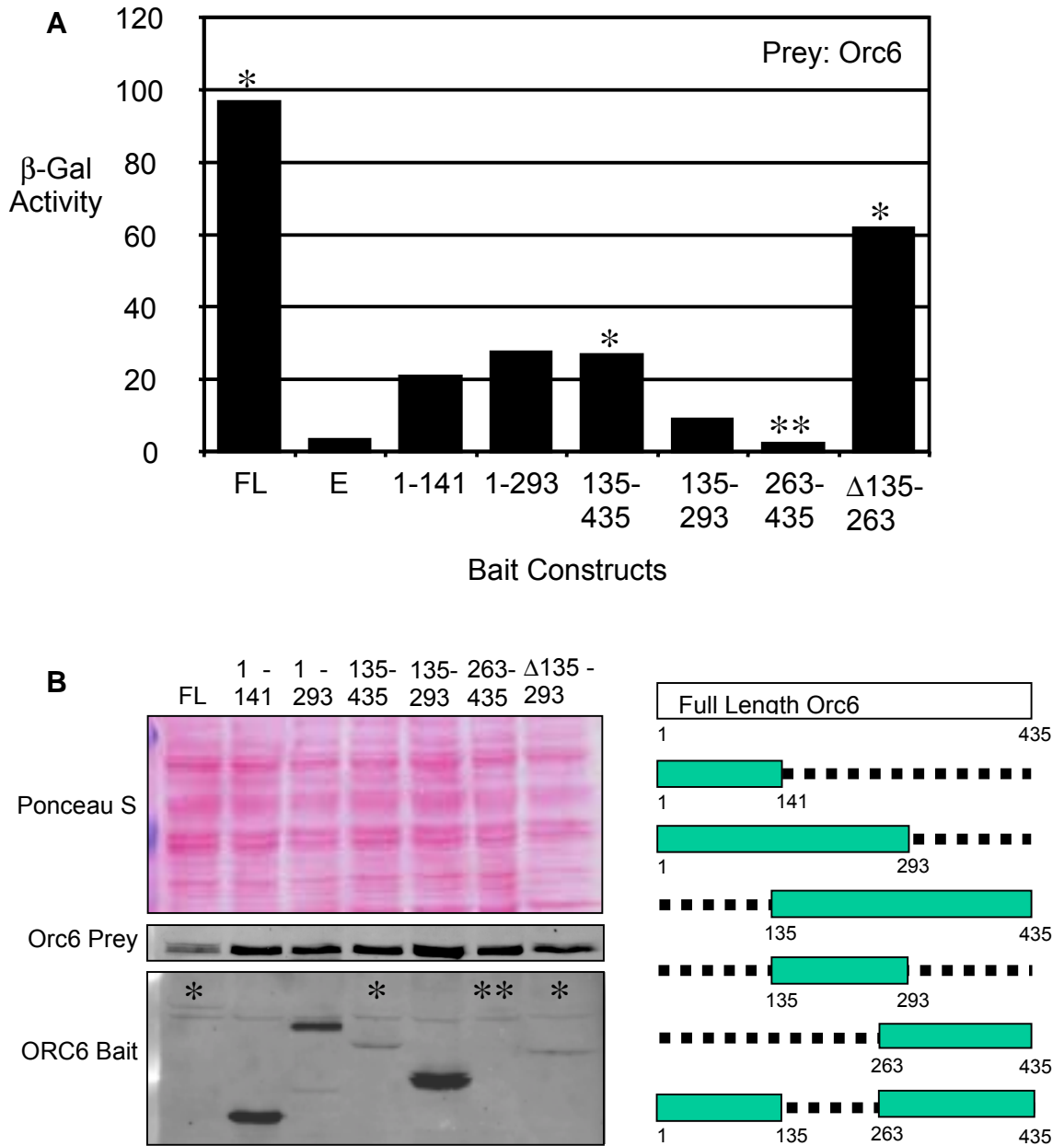
Figure 31. Identification of the region responsible for Orc6 dimerization. (A) A liquid two-hybrid assay was performed using O*RC6* as a prey and fragments of the *ORC6* gene as baits, as described in the Materials and Methods. All two-hybrids were performed at least twice, and a representative graph is shown. FL represents the full-length Orc6 protein, E represents an empty bait vector. (B) Immunoblots of whole-cell extracts prepared from culture aliquots removed just prior to β-galactosidase activity determination are shown. Detection of the bait and prey constructs was performed as in Figure 18. A Ponceau S stain is shown to illustrate equal protein loading. * indicate low expression of bait constructs, ** indicates no expression of bait constructs.

illustrating the relative β-galactosidase activity is given in Figure 31. The immuno-detections indicated that there was a problem with expression of several bait constructs, particularly $Orc6_{263-435}$, for which no protein was detected. Similar expression patterns were obtained when the assay was repeated, suggesting that some of the smaller polypeptides may be unstable. Nevertheless, the most robust interaction was observed between full-length Orc6 and $Orc6_{\Delta135-263}$, even though expression of this construct was weak. Although this interaction is not as strong as the full-length protein, this result indicates that the region(s) responsible for the Orc6-Orc6 interaction lies in either the N- or C-terminus, or both. The N-terminal fragment alone ($ORC6_{1-141}$) revealed a significantly decreased signal. As well, a larger fragment containing both the middle and C-terminal regions ($ORC6_{135-435}$) did not display a strong interaction with the full-length protein, although again the expression of this construct was fairly weak. Unfortunately, the extreme C-terminus peptide alone appeared to be unstable and could not be successfully expressed on it own to confirm this prospect. Overall, although the results were complicated by expression problems, it is likely that the Orc6-Orc6 interaction requires both N- and C-terminal motifs.

Confirmation of Orc6 interactions in budding yeast

Prior to the commencement of this project, very little was known about the function of Orc6 in budding yeast. Originally isolated as a subunit of ORC, it was assumed to be involved in DNA replication. However, since it is not required for ORC to bind to origins of replication, it is thought that Orc6 may facilitate the recruitment of other initiation factors. Earlier studies suggest a link between Orc6 and proteins known to be involved in DNA replication as genetic interactions between *ORC6* and *CDC6, MCM5* and *CDC2*

have been identified (Li and Herskowitz, 1993; Kroll *et al.,* 1996). In mice, Orc6 was shown to physically interact with Mcm5, Cdc45, Cdc7, Dbf4 and the single-stranded DNA binding protein Rpa70 (Rfa1 in *S. cerevisiae*) (Kneissl *et al.,* 2003). However, conclusive evidence of a physical interaction between Orc6 and DNA replication factors in budding yeast is still lacking. As for a role at other cell cycle stages, genetic analyses identified a link between budding yeast *ORC6* and two mitotic regulators *CDC14* and *CDC16* (Kroll *et al.,* 1996). To investigate whether these genetic interactions and mouse associations were indicative of physical interactions in *S. cerevisiae*, each yeast ORF was cloned into a yeast two-hybrid bait construct. As well, *NOC3* was also cloned into the bait vector and included in this study as it was recently identified as a replication factor that associates with ORC (Zhang *et al.,* 2002). Similarly, little was known about *MCM10* at the time of these initial experiments, other than it was involved in DNA replication and it genetically interacted with members of ORC (Kawasaki *et al.,* 2000). Therefore, it was also included in this study and cloned into a bait vector. Each newly developed bait construct was transformed into yeast possessing Orc6 prey and reporter plasmids. An Orc6 bait construct was also transformed into similar yeast cultures to act as a positive control.

Two-hybrid analysis performed on each transformant revealed very little interaction between Orc6 and the other proteins (Figure 32). However, immunoblot detection showed that the bait (pEG202-*ORC6*) construct was not expressing very well in these strains. A clear doublet is observed in the Orc6 lane, which is typical of Orc6 as it is a phosphoprotein; however, this is not observed in the other lanes. Although this experiment was repeated on several occasions, each trial showed the same outcome.

Clearly, the assay failed to work efficiently, possibly as a result of minimal bait expression, as even the strength of the Orc6-Orc6 interaction observed in this experiment was much weaker than in previous experiments (Figures 29-31). As a result, the sensitivity of this assay was drastically reduced. Therefore, the only conclusion that can be made is that the previous genetic analyses and mouse model studies were not indicative of strong physical interactions with budding yeast Orc6. It is possible that some of the proteins examined do indeed interact with Orc6, but at a level lower than what could be detected in this assay.

Using a liquid two-hybrid assay to identify novel associations with any protein is labour intensive. Groundwork is require to amplify, clone and transform the yeast strains prior to each experiment. As well, it usually requires prior knowledge of the putative proteins in question, which clearly limits the scope of the search. As a result, a direct two-hybrid approach is not the best way to identify novel ligands. Therefore, several alternative techniques were used to broaden the search for proteins that interact with Orc6.

Identification of Orc6 ligands through co-immunoprecipitation

Co-immunoprecipitation was initially used as an approach to find Orc6 ligands. It is assumed that careful immunoprecipitation will not only isolate Orc6, but also preserve and co-precipitate protein complexes to which it belongs. The precipitates can then be subjected to SDS-PAGE, followed by silver staining to identify proteins in the precipitate, which can then be excised and purified for mass spectrometry. In this experiment, whole cell extracts were isolated from an Orc6-Myc strain and immunoprecipitated using anti-Myc antibodies conjugated to magnetic beads as

145
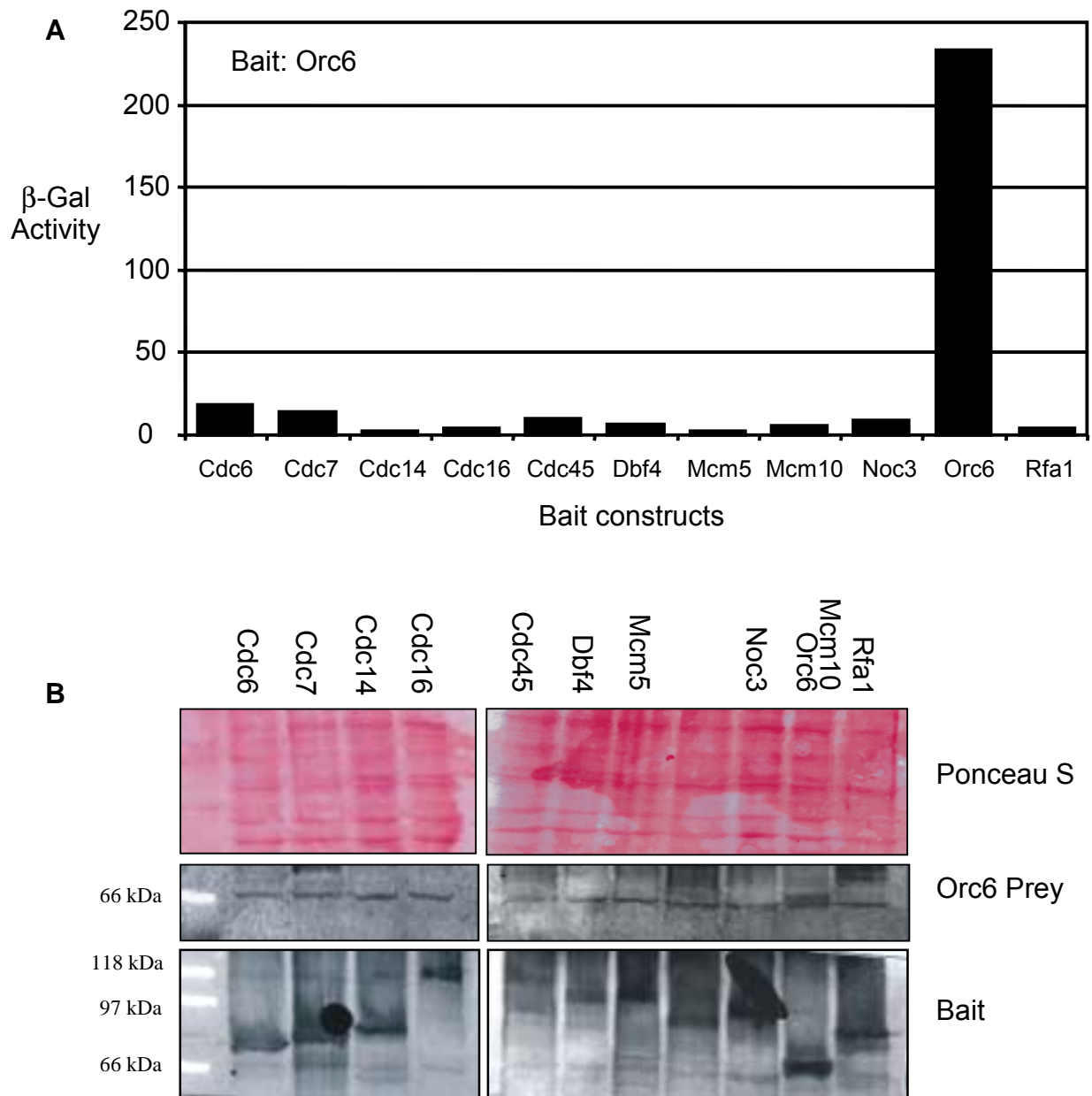
Figure 32. Analysis of potential interactions between Orc6 and candidate ligands. (A) A liquid two-hybrid assay was performed using ORC6 as a bait and several proteins as preys, as described in the Materials and Methods. All two-hybrids were performed at least twice, and a representative graph is shown. (B) Immunoblots of whole-cell extracts (50 μg) were prepared from culture aliquots. Detection of the bait and prey constructs was performed as in Figure 32. Ponceau S stain is shown to illustrate equal protein loading. Sizes of the protein ladder bands are given on the left of the detections.

described in the Materials and Methods. A control sample using an Orc2-Myc strain was obtained to distinguish any background proteins or ligands that associate indirectly through ORC, as opposed to a direct interaction with Orc6. After elution, the samples were subjected to a 10% SDS PAGE gel and silver stained. Unfortunately, no difference was observed between the banding patterns of the Orc2-Myc and Orc6-Myc extracts (Figure 33). Even increasing the salt concentration had little effect on the represented bands. Therefore, it was thought that contamination from proteins associating with the beads was too high to isolate Orc6 associated proteins. As a result, no bands were cut out and isolated for mass spectrometry.

An increase in stringency of the immunoprecipitation assay can be achieved by performing a tandem affinity purification, or TAP purification (reviewed in Puig *et al.,* 2001). In brief, a protein of interest is fused to a TAP tag, consisting of a *Staphylococcus aureus* protein A (ProtA) domain and a calmodulin binding peptide (CBP), separated by a TEV protease cleavage site. The CBP domain is usually incorporated immediately adjacent to the protein of interest, whereas the ProtA domain lies exterior to the CBP. When expressed, the ProtA domain binds tightly to an IgG matrix, which can be used to isolate the initial complex. The TEV protease can be used to elute the protein complex, which is then added to calmodulin beads. The final purified complex can be released under mild conditions containing EGTA. This technique allows two separate washing steps of the precipitate to remove contaminating proteins, and is currently underway in our laboratory. Once the precipitate is isolated, it can be run on a two-dimension protein gel to increase the protein resolution and assist in ligand purification. TAP-tagged Orc6 and Orc2 strains are currently available in our
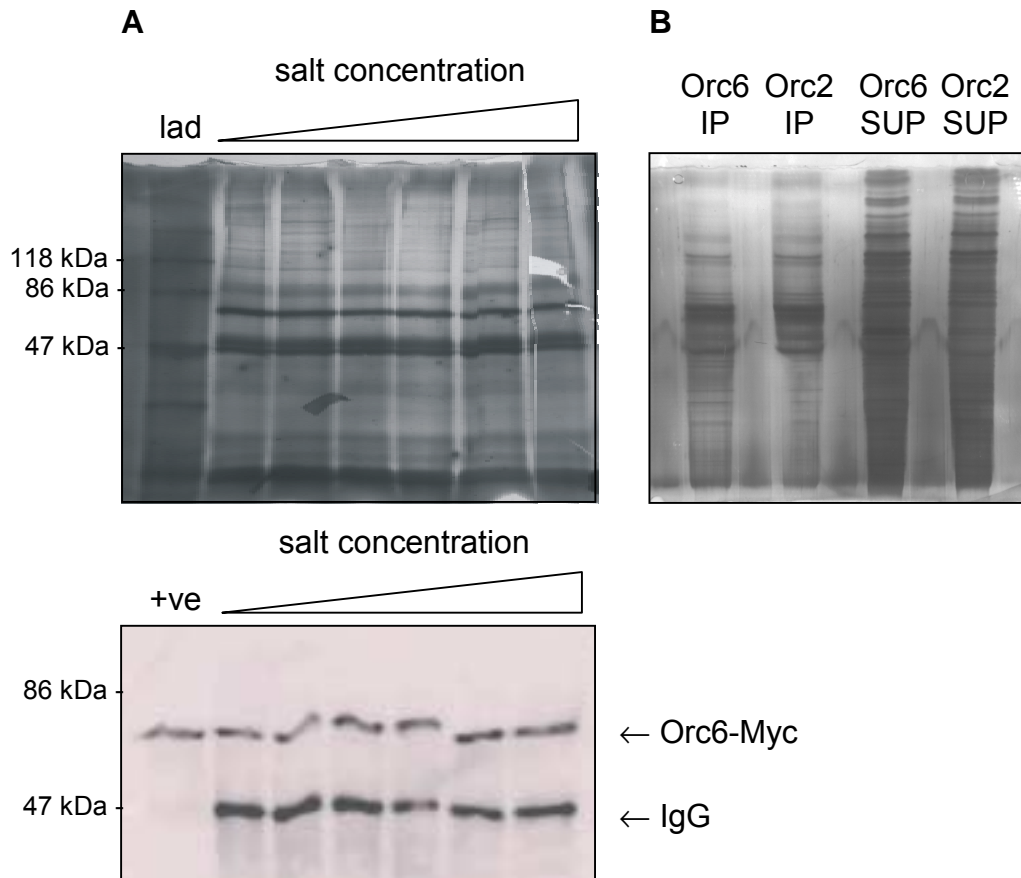
Figure 33. Identification of Orc6 ligands through co-immunoprecipitation. (A) Co-IPs were performed using Orc6-Myc with varying salt concentrations (250 mM – 500 mM) in the wash buffer to increase stringency. 10 µl of each precipitate was run on a 10% SDS gel and stained with silver. Below the silver-stained gel is a corresponding western blot in which 10 µl of each precipitate was run and detected with an anti-Myc antibody as in Figure 10. (B) A comparison of Orc2 and Orc6 immunoprecipitations. Both Orc2-Myc and Orc6-Myc strains were used in a co-IP experiment using 400 mM salt concentrations. 10 µl of precipitate (IP) and 20 µl of supernatants (SUP) were run and detected as in A. +ve corresponds to 20 µl the whole-cell extract of the 250 mM Orc6-Myc fraction prior to the immunoprecipitation. lad refers to a protein standard ladder.

laboratory, and work is underway to use them to identify Orc6 ligands. Although beyond the scope of this project, TAP purification and 2D gel analysis will no doubt help to identify proteins that associate with budding yeast Orc6.

Yeast two-hybrid library screens for Orc6 ligands

As an alternate approach in the search for Orc6 ligands, a two-hybrid screen was performed using a *S. cerevisiae* cDNA library obtained from the Yeast Resource Center (YRC). This library was already cloned into a two-hybrid prey construct (pOAD), however, this vector was not compatible with the system available in our laboratory so *ORC6* had to be cloned into a new bait vector (pOBD2) donated by YRC. In this assay, no reporter plasmid was needed and bait and prey constructs were cloned into identical haploid strains of the opposite sex. Upon successful mating of these strains, the diploid clones should contain both bait and prey plasmids. If the fusion proteins created by the two-hybrid constructs interact, they facilitate the expression of *ADE2* and *HIS3*. Therefore, only clones with successful interactions are able to grow on medium lacking both adenine and histidine. Initial attempts at screening for an Orc6 interaction failed to produce any positive colonies even though mating efficiency indicated ~60,000 diploids were examined. Therefore, the haploid culture containing the bait *ORC6* construct was sent to the YRC to be analyzed using their array (Uetz *et al.,* 2000). The array consists of 6,144 yeast colonies containing different proteins fused to specific activation domains. The strain containing the pOBD2-*ORC6* construct was then mated with the entire array, and resulting diploids were pinned onto a selective plate, as mentioned above. Results from the screen identified several putative Orc6 ligands (see Table 3).

Table 3. List of genes isolated from the two-hybrid library screen. All functions were ascertained from the *Saccharomyces* Genome Database (Hong *et al.,* 2006). All genes were identified only once in this screen unless marked with a "*", which designates genes identified more than once.

Gene	Function
YCP4	Function unknown, localizes to the cytoplasm
RIB1	Riboflavin biosynthesis
ALR2	Probable ion transporter
VHR1	Uncharacterized transcription factor
YML084W	Function unknown
PAM18	Mitochondrial import protein
YLR076C	Function unknown
ICY1	Function unknown, interacts with the cytoskeleton
MMS4	DNA recombination and repair
PPH21	Catalytic subunit of protein phosphatase, involved in regulating mitosis
YDR018C	Function unknown
YEF3(B)	Translational elongation factor
SSN2	Transcription factor, subunit of the RNA polymerase II mediator complex
PAK1	Regulatory kinase of the SNF1 complex, regulates transcription
*HOF1**	Cytokinesis and cytoskeleton organization
*RLF2**	Histone organization and transcriptional silencing

Two ORFs, *HOF1* and *RLF2*, were detected more than once in this screen and fourteen other proteins were identified once.

To confirm the putative interactions observed in the two-hybrid screen, DNA encoding Hof1 and Rlf2 were individually cloned into two-hybrid bait vectors (pEG202). These constructs were then transformed along with *ORC6* prey plasmids into wt yeast strains (DY1) containing the reporter plasmid. Two-hybrid assays were performed as described in the Materials and Methods. Immunoblots were performed using an aliquot of each culture to confirm expression of the fusion proteins. Interactions between Orc6 and both Hof1 (Figure 34) and Rlf2 (Figure 35) were observed.

To verify the Orc6 interactions observed between Hof1 and Rlf2 by two-hybrid analysis, a co-immunoprecipitation assay was performed. *HOF1* and *RLF2* were amplified and cloned into an expression vector (pCM190) that incorporates a Myc tag on the C-terminal end of the protein, as described in the Materials and Methods. These constructs were transformed along with pJG4-6-*ORC6* or pJG4-6-*ORC2* into wt (DY26) yeast strains. After induction of protein expression, WCEs were prepared and incubated with magnetic beads conjugated to anti-Myc antibodies. An aliquot of each WCE was incubated with beads lacking the anti-Myc antibody to confirm that immunoprecipitation is the result of the interaction between this antibody and the Myc fusion protein. The precipitates were analyzed by western blotting and detected using anti-Myc and anti-HA antibodies. Clearly, Orc6 was pulled-down with Hof1-Myc in this assay, confirming a physical interaction between these proteins. Similarly, Orc6 was co-immunoprecipitated with the Rlf2-Myc protein. However, a stronger signal was observed for the Orc2 protein suggesting that the direct interaction may lie with Orc2.

Figure 34. Confirmation of an Orc6-Hof1 interaction. (A) A liquid two-hybrid assay was performed using Orc6 as a prey and Hof1 as a bait, as described in the Materials and Methods. As a control, the Hof1 bait was also analyzed with an empty prey vector (-ve). Two-hybrids were performed at least twice, and a representative graph is shown. Immunoblots of whole-cell extracts prepared from culture aliquots removed just prior to β-galactosidase activity determination are shown. Detection of the bait and prey constructs was performed as in Figure 18. A Ponceau S stain (Pon. S) is shown to illustrate equal protein loading. (B) To confirm the two-hybrid results, a co-IP was performed as described in the Materials and Methods, using Hof1-Myc expressed from pCM190, and Orc6-HA expressed from pJG4-6. As a control, the Hof1 co-IP was also performed with pJG-Orc2. WCEs were added to magnetic beads with (myc) and without (-ve) anti-Myc antibodies conjugated to them. Immunoblots of the WCEs added to the beads is shown along with the IPs. MYC and HA detections were performed as in Figure 4 and Figure 11 respectively.
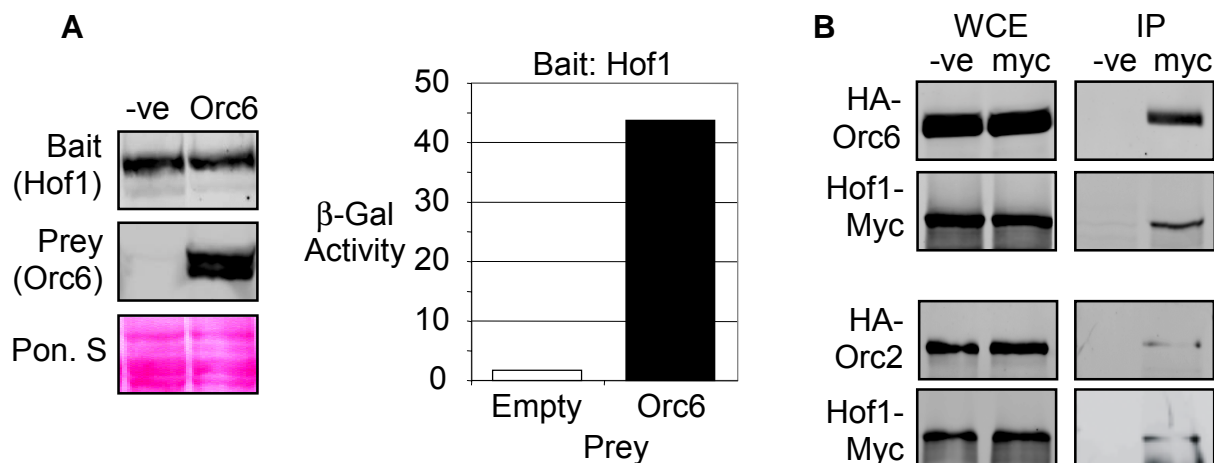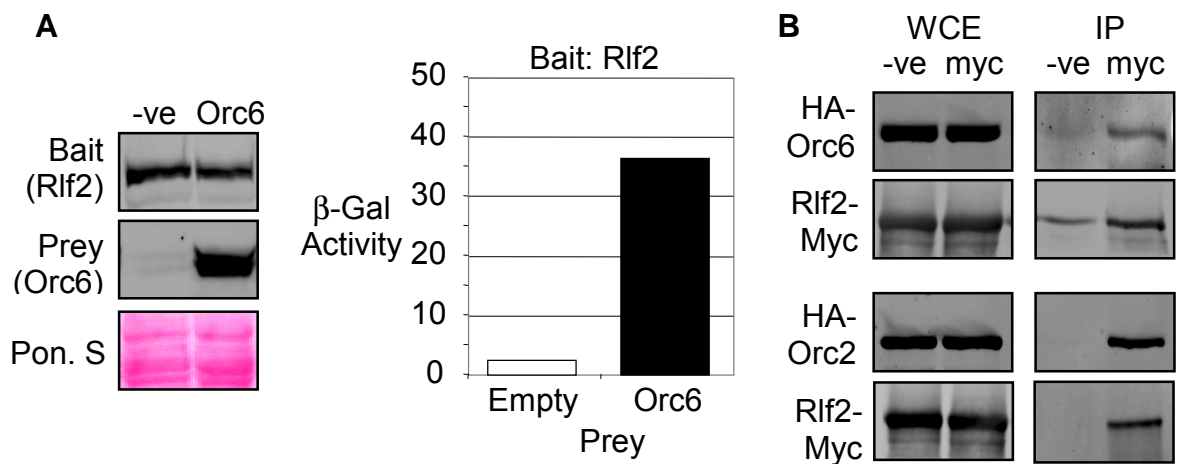
Figure 35. Confirmation of an Orc6-Rlf2 interaction. (A) A liquid two-hybrid assay was performed using Orc6 as a prey and Rlf2 as a bait, as described in the Materials and Methods. As a control, the Rlf2 bait was also analyzed with an empty prey vector (-ve). Two-hybrids were performed at least twice, and a representative graph is shown. Immunoblots of whole-cell extracts prepared from culture aliquots removed just prior to β-galactosidase activity determination are shown. Detection of the bait and prey constructs was performed as in Figure 18. A Ponceau S stain (Pon. S) is shown to illustrate equal protein loading. (B) To confirm the two-hybrid results, a co-IP was performed as described in the Materials and Methods, using Rlf2-Myc expressed from pCM190, and Orc6-HA expressed from pJG4-6. As a control, the Hof1 co-IP was also performed with pJG-Orc2. WCEs were added to magnetic beads with (myc) and without (-ve) anti-Myc antibodies conjugated to them. Immunoblots of the WCEs added to the beads is shown along with the IPs. MYC and HA detections were performed as in Figure 4 and Figure 11 respectively.

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<u>Discussion</u>

Orc6 association with ORC

Two-hybrid analysis showed that Orc6 does not interact well with any of the other ORC subunits in budding yeast (Figure 29). Although it is intriguing as to how this protein was first purified with ORC, this would explain observations in which Orc6 was not present in all ORC complexes bound to origins (Lee and Bell, 1997). It is possible that Orc6 is only transiently or weakly bound to the larger complex, or that an ATP hydrolysis or phosphorylation event is needed to stably load Orc6 onto ORC. Alternatively, Orc6 association with other ORC subunits may be cell cycle-specific, requiring conformational changes that were not achieved through the assay. It is possible to perform liquid two-hybrid assays while cells are under cell cycle arrest, which would allow cell cultures to be arrested at various cell stages (e.g. late G1 using α-factor). Studying cells at specific points may enrich a transient two-hybrid signal that was masked by the other stages of the cell cycle. The results indicate that Orc6 weakly associates with Orc2, Orc3 and Orc4 in budding yeast (Figure 29). These are similar to interactions observed in humans, in which Orc6 co-immunoprecipitates with Orc3 and to a lesser extent Orc2 (Vashee *et al.,* 2001). As well, two-hybrid results in murine cells indicated that Orc6 associates with Orc4 and weakly with Orc3 (Kneissl *et al.,* 2003). In most cases, the Orc6 interactions detected in these other species were quite weak. One possibility is that the stability gained by each interaction may be additive, each increasing the strength of the association of Orc6 with ORC. Nevertheless, results from these two-hybrid assays as well as those from other species indicate that Orc6 does not strongly associate with the ORC complex.

Orc6 dimerization

Unexpectedly, budding yeast Orc6 was determined to strongly associate with itself (Figure 29), and is only one of two ORC proteins that exhibit this property (Figure 30). Although this is the first direct evidence to show that an ORC subunit in any species is able to dimerize, it is possible that it also occurs in other species. In humans, glycerol sedimentation showed some Orc6 was present in large complexes with the other ORC subunits; however, the majority of Orc6 was present in a much smaller complex just over 50 kDa (Vashee *et al.,* 2001). From the size of this complex and the intensity of the signal, it was proposed that most of human Orc6 may exist as dimers. Similar small complexes were observed with the human Orc4 and Orc5 subunits after sedimentation; however, in each case only a small fraction of the protein was found in these lower complexes.

The question remains as to why Orc6 self-associates. One intriguing possibility centers on replication foci. In budding yeast and metazoan systems, ORC has been shown to be present in discrete sub-nuclear foci (Pasero *et al.,* 1997; Nakamura *et al.,* 1986; Cox and Laskey 1991; reviewed in Newport and Yan, 1996). Indeed, results from Chapter 3 are the first to show budding yeast Orc6 present in a similar punctate pattern *in vivo.* During S phase, these sites have been linked to regions of new DNA synthesis, and exhibit variations in the timing of replication initiation (Pasero *et al.,* 1997; Nakamura *et al.,* 1986). In yeast, there are 15-20 of these new DNA synthesis sites, however, there are ~400 active origins of replication. Therefore, it is thought that origins congregate to these foci, which possibly represent regions with high local concentrations of replication factors. Currently, it is unknown how origins of replication aggregate to form foci. It is

possible since ORC is a key component of pre-RCs present at origins, that the Orc6 and/or Orc5 self-associations facilitate the clustering of origins.

Current results indicate that the Orc6-Orc6 interaction is dependent on both an N- and C-terminal domain, although protein expression was a concern in this assay (Figure 31). The presence of two binding regions will make it more difficult to map the specific amino acid residues of Orc6 necessary for dimerization. However, determining the regions of Orc6 involved in this interaction is still possible by continually removing smaller regions from either end, as well as the middle of the protein and performing two-hybrid analyses. From there, it will be interesting to learn the phenotype of mutant strains with these regions knocked out. Confocal microscopy could determine if replication foci are dismantled in a fluorescently-tagged mutant strain. This could offer insight into the significance of replication foci, as it is possible that Orc6 dimerization is necessary for the congregation of replication origins, and its disruption could cause an increase in the length of S phase or even inhibit DNA replication altogether.

Orc6 in DNA replication

Clearly, it is thought that budding yeast Orc6 is involved in DNA replication, although only indirect evidence is currently available in the literature (Li and Herskowitz, 1993). One of the goals of this project was to confirm if this protein is indeed required for the initiation of DNA replication. Results from Chapter 4 indicate that Orc6 plays an essential role in G1 phase (Figure 25), and a loss of this protein decreases the number of active origins (Figure 27). The mechanism of Orc6 action appears to be through the loading and stability of the MCM complex on chromatin, while also affecting Mcm10 and

Orc2 stability (Figures 23 and 24). A physical interaction between Orc6 was observed with Mcm10 and Cdt1 (Figure 24). Although not all initiation factors were examined, several other proteins that have illustrated a link to ORC were tested in the current two-hybrid assay. Unfortunately, there were problems with this experiment, as it appears likely that the expression of the Orc6 bait vector was low during the two-hybrid analysis with several important cell cycle proteins (Figure 32). Although the positive control revealed a positive interaction, it did not exhibit the same binding strengths observed previously (Figure 29). With the decrease in sensitivity of the assay, only robust interactions would be identified by this experiment. Therefore, it is possible that key interactions were missed due to expression difficulties, although it is likely that many of the proteins do not physically interact with Orc6. Genetic analyses only infer that two proteins function in related pathways and do not always indicate physical associations. Also, earlier work on this project revealed that budding yeast Orc6 is not closely related structurally to other eukaryotic Orc6 proteins examined in the literature. Therefore, it is not surprising that some physical associations observed in other species are not conserved in the budding yeast protein.

Orc6 in chromatin assembly

In addition to its role in DNA replication, ORC has a genetically separable role in chromatin reorganization and gene silencing (Dillin and Rine, 1997; Bell *et al.,* 1995; Fox *et al.,* 1995). Disassembly of nucleosomes and the removal of histones is necessary to gain access to DNA. In eukaryotes, this process is mediated by the chromatin assembly complex (CAF-1) (Smith and Stillman, 1989). CAF-1 is a conserved

group of proteins that is involved in loading histones (H3 and H4) onto newly replicated DNA and during DNA repair (Verreault *et al.,* 1996; Gaillard *et al.,* 1996; Linger and Tyler, 2005). It is thought that CAF-1 is targeted to replication forks through its interaction with PCNA (Shibahara and Stillman, 1999). Interestingly, the CAF-1 has also been shown to be specifically involved in the formation of centromeric chromatin and kinetochores (Sharp *et al.,* 2002). The function of the CAF-1 at kinetochores appears to be redundant of the histone regulatory (Hir) proteins, although deletions in both complexes results in mitotic defects including chromosome missegregation. As well, CAF-1 is particularly important in gene silencing, not only at telomeres (Enomoto *et al.,* 1997; Kaufman *et al.,* 1997; Monson *et al.,* 1997), but also at mating loci (Enomoto and Berman 1998; Kaufman et al. 1998) and ribosomal DNA (Smith *et al.,* 1999).

Rlf2 is the largest subunit of the CAF-1 complex. Although strains with *RLF2* deletions are viable (Giaever *et al.,* 2002), knocking out this gene causes reduced heterochromatin and telomere silencing as well as global defects in transcription (Game and Kaufman, 1999; Krawitz *et al.,* 2002; Zabaronick and Tyler 2005). Rap1 is a significant component of telomeres, as it binds to telomere DNA repeats and regulates telomere length and gene silencing. Rlf2 (Rap1 localization factor) is responsible for the appropriate localization of Rap1 on telomeres (Enomoto *et al.,* 1997). Mutations in *RLF2* result in alternations in Rap1 loading and a reduction in telomere lengths. The localization patterns of both Rap1 and Rlf2 are different, suggesting that Rlf2 is not a constituent of telomeric chromatin. Rlf2 is also responsible for nucleosome assembly (Enomoto *et al.,* 1997).

What is the significance of the Orc6-Rlf2 interaction observed in Figure 35? Prior to this project, there was no evidence to a indicate a direct link between CAF-1 and ORC, although both were shown to be involved in gene silencing. ORC, Rap1 and Abf1 bind to silencers at mating loci and recruit the silent information regulator (Sir) proteins (Rusche *et al.,* 2003), which establish yeast heterochromatin at mating type loci (Gasser and Cockell, 2001). Therefore, both ORC and Rap1 are required to co-localize to silencer sequences prior to the loading Sir proteins. It is possible that the association between Orc6 and Rlf2 is responsible for targeting Rap1 to ORC-bound silencers. Eliminating the region required for the Orc6-Rlf2 interaction may abrogate the localization of Rap1. As well, it would be intriguing to examine heterochromatin formation following Orc6 depletion.

Interestingly, the *Xenopus* homolog of Rlf2 has been shown to dimerize, and this self-association is essential for CAF-1 function (Quivy *et al.,* 2001). However, the 36 amino acid region responsible for this interaction is only found in the human and *Xenopus* proteins. Earlier two-hybrid results indicated that Orc6 is able to dimerize; therefore, it is possible that Orc6 may mediate the assembly of Rlf2 multimers in budding yeast. Currently, our laboratory is working on establishing Orc6 mutants with the alterations in the minimal dimerization domain(s). It will be interesting to study the effects of this mutation on Rlf2 function, particularly with regard to Rap1 targeting and subsequent gene silencing.

Orc6 in cytokinesis

In several metazoan species, Orc6 was shown to be involved in mitosis and cytokinesis (Prasanth *et al.,* 2002; Chesnokov *et al.,* 2003). Preliminary studies depleting Orc6 at the G2/M in budding yeast indicate that this protein is not essential for cell division (Chapter 4, Figure 22). However, budding yeast Orc6 was shown to interact with Hof1 (Figure 34), which is a involved in cytokinesis. Although Hof1 is not an essential protein (Giaever *et al.,* 2002), its expression is cell cycle-regulated, commencing during anaphase and continuing until cytokinesis. As well, disruption of Hof1 leads to an increase in cytokinetic defects (Lippincott and Li, 1998). Hof1 co-localizes with septin proteins at two rings adjacent to the bud neck during cell division (Kamei *et al.,* 1998). This is very interesting as Orc6 was shown to interact with septins in *Drosophila* (Chesnokov *et al.,* 2003), and this interaction is essential for proper cytokinesis.

There are two redundant pathways for cytokinesis in budding yeast (Lippincott and Li, 1998). One involves the establishment of a functional actinomysin ring around the bud neck. The other pathway incorporates several proteins, centering around a structurally distinct ring composed of septin proteins. It is thought that Hof1 acts as a negative regulator of cytokinesis through this ring, as over-expression of Hof1 in budding yeast appears to lead to a defect in septin localization and interfere with cell division (Ren *et al.,* 2005; Lippincott and Li, 1998). Hof1 association with Vrp1 is needed to prevent the inhibitory effects of Hof1 and allow cytokinesis to progress (Ren *et al.,* 2005). As well, Hof1 is specifically targeted for degradation late in mitosis (Blondel *et al.,* 2005). Degradation of Hof1 prior to cytokinesis is required for proper contraction of the actinomyosin ring and efficient cell separation.

What could the non-essential link be between Orc6, Hof1 and cytokinesis? It is possible that Orc6 acts as a cellular signal to ensure that proper progression through the cell cycle is maintained. It has already been shown that interaction of Orc6 with Clb5 prevents re-replication within the same cell cycle (Wilmes *et al.,* 2004; Archambault *et al.,* 2005). An interesting possibility is that the association between Orc6 and Clb5 facilitates the interaction between Orc6 and Hof1. Similar to the role of Vrp1, Orc6 association could eliminate the negative effects of Hof1 and allow progression into M phase. In the case of a replication defect, Orc6 would not associate with Clb5, and Hof1 would remain at the site of septin localization, preventing mitotic entry. Although an intriguing possibility, this is only speculative and needs to be substantiated through experimentation.

Other functions of Orc6

Several proteins were isolated from the two-hybrid screen using Orc6 as a bait (see Table 3). To date, only the proteins that were isolated more than once from this screen were confirmed to interact with Orc6 through other methods. It may be of interest to examine the remaining proteins as they may hint toward other roles for Orc6 in budding yeast. Particularly, seven of the proteins isolated from this screen have unknown functions. If their association with Orc6 is confirmed, they could offer new insight and direction in the characterization of Orc6 function. Alternatively, these putative interactions could reinforce other roles for Orc6 already under investigation. Through descriptions for other species and its confirmed interaction with Hof1, it is possible that Orc6 plays a non-essential role in cytokinesis. *PPH21* is a protein phosphatase

implicated in mitosis (Evans and Stark, 1997), and was isolated as a putative ligand of Orc6 from the two-hybrid screen. As well, there is a clear link between transcriptionally active regions of the genome and early replication in eukaryotes (reviewed by MacAlpine and Bell, 2005). The mechanism behind this observation is unclear. Two genes implicated in gene transcription, *SSN2* and *PAK1*, were isolated from the Orc6 screen and may offer potential insight into the targeting of origin activation to transcriptionally dynamic regions. As well, there is a putative interaction between Mms4 and Orc6. Mms4 is an endonuclease responsible for cleaving branched DNA structures and is involved in DNA recombination and repair, particularly at sites of stalled replication forks (Kaliraman *et al.,* 2001). Interestingly, the Mms4 protein is also a transcriptional activator (Xiao *et al.,* 1998), again linking Orc6 to areas of transcription. This also supports evidence in the literature to suggest that ORC is involved in DNA repair mechanisms (Suter *et al.,* 2000). Although these interactions are speculative at the moment, it would be interesting to confirm them through liquid two-hybrid and co-immunoprecipitation assays, and later determine their significance through functional assays.

**CHAPTER VI: General Conclusions
and Future Directions**

DNA replication is a highly coordinated event that is required for the successful inheritance of genetic information every time a cell divides. This process is governed by various proteins that assemble on chromosomes to prepare for initiation and monitor cell cycle progression to ensure that this process occurs only once per cycle. Cellular checkpoints guarantee that replication is restricted to S phase of the cell cycle, and that no section of any chromosome is left unreplicated. Disturbances in DNA replication or its regulation can trigger chromosome instability and mutations, which can be inherited and can even be lethal. In multicellular organisms, genetic mistakes can lead to cancer. In fact, several proteins involved in the initiation of DNA replication are effective markers for cancer progression (reviewed in Semple and Duncker, 2004), since they indicate cells actively advancing through the cell cycle. With an estimated 153,000 new cases each year, and over 70,000 deaths as a result of cancer in Canada in 2006 (National Cancer Institute of Canada, 2006), it is no wonder that research on cell cycle regulation is of high interest. As such, there is a need for understanding the components involved in DNA replication and its regulation.

Due to the very large size of eukaryotic genomes, numerous sites of initiation of DNA replication are distributed along each chromosome. Pre-replicative complexes form at each origin that when activated lead to bi-directional replication forks. At the heart of each pre-RC lies ORC. In all eukaryotes studied to date, this essential complex is responsible for initiating pre-RC formation. In various species, members of ORC are involved in more than just the assembly of pre-RC components in early G1 phase. Over the past few years, a great deal of attention has focused on the smallest of the ORC subunits, Orc6. In humans, Orc6 was shown to be required for DNA replication, as well

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as mitosis and cytokinesis (Prasanth *et al.,* 2002), and similar results were obtained

from *Drosophila* (Chesnokov *et al.,* 2003). In *S. cerevisiae*, Orc6 is required for cell

viability, yet little is known about cellular role(s) of this protein in yeast. Recently, a non-

essential function was discovered for Orc6, as it was shown to be involved in a complex

pathway to prevent re-replication of the genome within the same cell cycle (Wilmes *et

al.*, 2004). The main goal of this project was to confirm the hypothesis that budding

yeast Orc6 is involved in DNA replication. In doing so, several objectives were

established in order to characterize Orc6 in the cell cycle, with emphasis on determining

its essential function.

As described in the first chapter, there were three main tasks outlined for this project in

order to examine the function(s) of Orc6, which focus on determining its cellular

localization, observing the timing of its essential function and identifying its ligands.

Initial characterization of Orc6 in budding yeast made it apparent that this protein is

significantly different than the metazoan proteins bearing the same name. At the level

of its amino acid sequence to its predicted structure, *S. cerevisiae* Orc6 appears to be

quite divergent from other Orc6 molecules, emphasized by the fact the essential

function of budding yeast Orc6 was not complemented by the human protein. It would

be interesting to compare the amino acid sequences of Orc6 from a diverse range of

species, including other yeast and fungi, and even plants. It may be possible to trace

back to the point of divergence in this gene, which may offer some insight into the

conservation of particular domains/regions. Characterization of the known motifs of

budding yeast Orc6 identified an inhibitor of apoptosis (IAP) repeat. Future studies

could determine if the IAP motif in Orc6 is functional, as a mutation or deletion of this

region may lead to an increased sensitivity to apoptotic signals. IAP proteins have been shown to be important in the cell cycle, particularly in regulating cytokinesis (reviewed in Verhagen *et al.,* 2001), although the mechanism of action is not yet known. These findings could offer insight into a putative role of budding yeast Orc6 in mitosis.

Localization of *S. cerevisiae* Orc6 is nearly exclusively nuclear and bound to chromatin as judged by live-cell fluorescent imaging and chromatin association assays, contrasting its metazoan counterparts. No population of Orc6 was found localized to the cell membrane or bud neck region during cytokinesis. It would be beneficial to repeat Orc6-eYFP imaging using a confocal microscope, as an increase in resolution and sensitivity may recognize a weak but significant signal previously masked by the background intensity. Close examination of Orc6 localization in yeast identified a punctate sub-nuclear pattern, presumably at site of DNA replication foci. This is very exciting as this is the first time that replication factors were observed localized to nuclear foci in living cells. Future experiments should confirm that these sites are actual sites of DNA replication through co-localization analysis of BrdU incorporation. As well, both Orc2 and Orc6 were localized exclusively to the nucleus and exhibited similar protein levels in the cell. Although clearly not a definitive analysis, this suggests that these two proteins function together in cellular pathways. To examine whether of not Orc6 plays a role in the cell cycle separate of that of ORC, it would be useful to compare the levels of each subunit (as in Chapter III, Figure 11) at different stages of the cell cycle to determine if the concentration of Orc6 fluctuates independently of the other subunits.

The timing of the essential function of Orc6 in budding yeast was determined through synchronizing cells at various points in the cell cycle, depleting Orc6, then releasing the

cells from the block and monitoring their progression. Cells depleted of Orc6 in G2/M were able to progress to the following G1 phase, further confirming that the protein is not required for mitosis and cytokinesis as in metazoans. However, these cells arrested at the G1/S transition, typical of initiation factors required for DNA replication. A closer examination of the role of Orc6 at this time point revealed a very interesting discovery: Orc6 is required for the efficient maintenance of the MCM complex on chromatin in early G1. Although it is unknown whether this is the result of a reduction of MCM loading or simply a decrease in the stability of the MCM complex once loaded. A direct interaction between Mcm2 and Orc6 was not observed through two-hybrid analysis, although only one of the MCM proteins were monitored. It would be worth looking for Orc6 associations with the other MCM proteins in the future, as a direct interaction between Orc6 and a MCM protein would be of interest. To explain the lack of chromatin-bound Mcm2 following Orc6 depletion, interactions were examined between Orc6 and pre-RC factors responsible for MCM recruitment. This revealed interactions between Orc6 and both Mcm10 and Cdt1, consistent with the idea that these proteins may need to associate with Orc6 to efficiently load the MCM complex. As well, this could explain the lack of MCM loading following the G2/M depletion of Orc6 in the previous cell cycle. Future work could determine how Orc6 is involved in mechanism of MCM loading, with the emphasis on the timing of each interaction identified in this project. Also, the minimal regions required for the Orc6-Mcm10 and Orc6-Cdt1 can be identified, and the significance of each interaction could be examined using specific mutant proteins.

Surprisingly, depletion of Orc6 in late G1 phase after MCM loading and pre-RC formation revealed that these cells still could not progress through S phase. The

accepted model at that time was that ORC is dispensable for DNA replication following pre-RC formation (Hua and Newport, 1998; Rowles *et al.,* 1999; Shimada *et al.*, 2002). However, it was unclear as to whether this was still an initiation defect, or an elongation defect, or both. It is possible that DNA replication was initiated at early origins, but immediately stalled due to a problem in replication fork progression. However, when Orc6 was depleted after the initiation of S phase it appears that cells could still complete DNA synthesis, ruling out an elongation defect. DNA combing following Orc6 depletion in late G1 phase revealed that it was indeed an initiation defect, as a significant decrease in origin firing was observed. Chromatin association analysis of these cells revealed that Orc6 is needed to maintain the stability of the pre-RC on chromatin, particularly Mcm10, Mcm2 and Orc2 prior to activation. These results could be specific to Orc6, as depleting Orc4 under the same conditions does not affect the stability of other initiation factors nor does it prevent S phase progression. However, preliminary results from this project indicated that depleting Orc2 in late G1 phase decreases origin firing as did Orc6 and prevents S phase progression (data not shown), contrary to published results (Shimada *et al.*, 2002). Therefore, it would be interesting to examine each ORC subunit under these conditions to understand the role of ORC after pre-RC formation.

A main objective of this project was to identify the proteins that associate with budding yeast Orc6. Prior to the commencement of this work, UV cross-linking studies revealed that *S. cerevisiae* Orc6 does not always associate with ORC at origins (Lee and Bell, 1997). To clarify the interaction between Orc6 and ORC, several two-hybrid analyses were performed. Results from this experiment indicate that Orc6 does not strongly

associate with any other ORC subunit. Therefore it is possible that Orc6 is only transiently associated with ORC, or that a stable association requires an interaction with more than one subunit. It would be interesting to examine the binary interactions again, but at specific stages of the cell cycle to see if the Orc6 interaction with ORC is temporally regulated, perhaps at early or late G1 phase, and fails to associate at other time points. Interestingly, Orc6 showed an ability to self-associate, although the function of this interaction is unknown. Preliminary mapping of the protein regions responsible for this interaction revealed both N- and C-terminal domains are required. It would be interesting to continue to narrow these regions down to their essential amino acids. From there, site-directed mutations of these regions could uncover the significance of this interaction. As well, it would be worth examining Orc6 proteins in other species to determine if they also self-associate.

To examine Orc6 ligands outside of the ORC complex and components of the pre-RC, a cDNA library screen was performed. This experiment identified Rlf2 and Hof1 as ligands for budding yeast Orc6, and both interactions were confirmed through liquid two-hybrid analysis and co-immunoprecipitation. Rlf2 is the largest subunit of the chromatin assembly complex. It was shown that *RLF2* deletions are viable (Giaever *et al.*, 2002), but defects in this protein cause reduced heterochromatin and telomere silencing as well as decreased transcription levels (Game and Kaufman, 1999; Krawitz *et al.*, 2002; Zabaronick and Tyler, 2005). Both ORC and Rlf2 are involved in recruitment of Sir proteins, and chromatin localization of these complexes could be mediated through Orc6. Future studies should focus on studying the effects of this interaction, particularly on heterochromatin formation. Interestingly, Rlf2 is able to form dimers in metazoans

(Quivy *et al.*, 2001), but not in budding yeast. The impact of Orc6 dimerization could be investigated in this context, as it may be involved in forming Rlf2 multimers. If the dimerization domain(s) of Orc6 could be knocked out without disrupting cell viability, it would be interesting to monitor the subsequent effects on heterochromatin and telomere silencing.

Hof1 is a non-essential protein involved in cytokinesis, that associates with septins and localizes at site of bud formation. The observation that Hof1 interacts with Orc6 is very exciting as it could explain observations in metazoans that show a role for Orc6 in cytokinesis, possibly through associating with septins, and localizing to the cleavage furrow (Prasanth *et al.*, 2002; Chesnokov *et al.*, 2003). It is possible that the Orc6 association with the septin Pnut observed in flies, requires the presence of Hof1 in budding yeast. Although an intriguing hypothesis, more work is needed to determine the significance of this interaction. As a negative regulator of mitosis, over-expression of Hof1 interferes with cell division (Lippincott and Li, 1998). Therefore, it is possible that following DNA replication, Orc6 associates with Clb5 (Wilmes *et al.,* 2004), and that this interaction promotes an association between Orc6 and Hof1, which in turn alleviates the negative regulation of Hof1. Interestingly, knocking out the interaction between Orc6 and Clb5 appears to cause cells to arrest at G2 (Archambault *et al.,* 2005), and prevents entry into mitosis, which is consistent with the proposed model. It would be interesting to knock-out the Orc6-Hof1 interaction and observe the cytokinetic phenotypes. As well, two-hybrid analysis can be performed on cells synchronized to G2/M to see if the level of interaction is increased at this time point.

Overall, this project has added significant new knowledge to the function of Orc6 in budding yeast. Clearly, Orc6 is involved in the initiation of DNA replication, where it serves its essential cellular function. It is possible that Orc6 is also involved in gene silencing and cytokinesis. More work is required to adequately define each of its functions, but work here has identified new avenues for investigation and set a solid groundwork for future studies. Although it is unlikely that Orc6 will act as a marker for tumor progression as other replicative proteins, since the levels of this protein are not known to increase in dividing cells, this research can still offer medical significance. As a key regulator of the cell cycle, it is possible that Orc6 or factors that associate with this protein are implicated in some forms of cancer. For example, if a change in protein level of one of these factors or a mutation in one of the genes is found to be specific to some forms of cancer, a method of screening for these alterations could offer a more efficient and accurate method of diagnostic testing.

APPENDIX I: Revaluation of the Orc6-Mcm10 interaction

An interaction between Mcm10 and Orc6 was observed by two-hybrid analysis (Chapter IV, Figure 24). Due to the potential significance of this interaction, particularly in reference to the lack of MCM stability on chromatin following Orc6 depletion (Chapter IV, Figure 26), alternate methods were used to confirm the two-hybrid results. First, co-immunoprecipitation of Mcm10 was attempted. DNA encoding Mcm10-Myc was cloned into a expression vector (pCM190) and the resulting construct was transformed into a wt yeast strain (DY1) along with the expression vectors containing the ORC subunits (pJG-ORC$_{(1-6)}$). Co-immunopreciptation was performed as described in the Materials and Methods. No detectable signal was identified for Orc6 when Mcm10-Myc was precipitated (Figure 25). The strongest signals were observed in the Orc2 and Orc3 lanes; however, the Mcm10-Myc was not properly expressed in the Orc3 lane.

A preparation of bacterially expressed Mcm10-GST was used in an alternate precipitation assay described in the Materials and Methods. Protein extracts from yeast containing the same ORC constructs (pJG-ORC$_{(1-6)}$) used in the previous experiment were incubated with the Mcm10-GST beads. Precipitation of Mcm10-GST with glutathione beads failed to pull-down Orc6 (Figure 36). Again, Orc2 exhibited the strongest signal in this assay.

Unfortunately, neither experiment was able to confirm the early interaction observed between Orc6 and Mcm10. So the question remains, which results are to be believed? The two-hybrid assay was the only experiment to take place *in vivo* at physiological temperatures, thus it is thought to be the more reliable of the methods. In addition, later results revealed a distinct correlation between the depletion of Orc6 and the reduced loading of Mcm10 on chromatin during a late G1 phase arrest (Chapter IV, Figure 26).

Although this result on its own does not indicate an interaction between the two proteins, it acknowledges that these two proteins are functionally related, supporting the two-hybrid results.
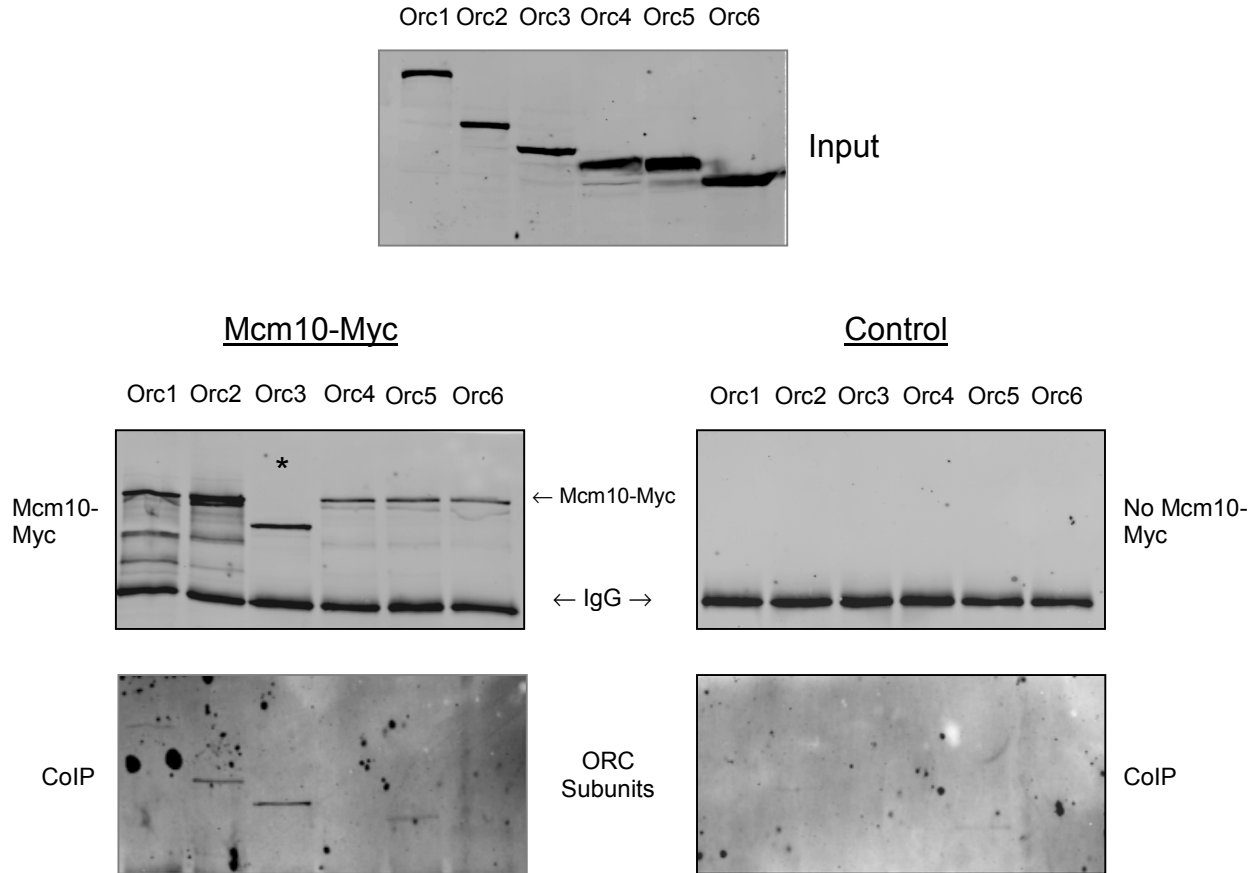
Figure 36. Co-immunoprecipitation of Mcm10-MYC with each ORC subunit. A co-immunoprecipitation assay was performed as described in the Materials and Methods. Mcm10-Myc was expressed from a pCM190 vector and precipitated by anti-Myc antibodies conjugated to magnetic beads. A strain without the pCM190-*MCM10*-Myc plasmid was used as a control. In each culture an ORC subunit was over-expressed using the pJG4-6 vector, which also adds three HA tags to the protein so it can be monitored by western blot. 10 μl of the WCEs and 20 μl of the IPs were run on a western blot. An anti-HA antibody was used to monitor the initial expression of each ORC subunit in the input and coIP fractions. An anti-Myc antibody was used to check expression of Mcm10-Myc. * indicates samples in which Mcm10-Myc was not properly expressed.
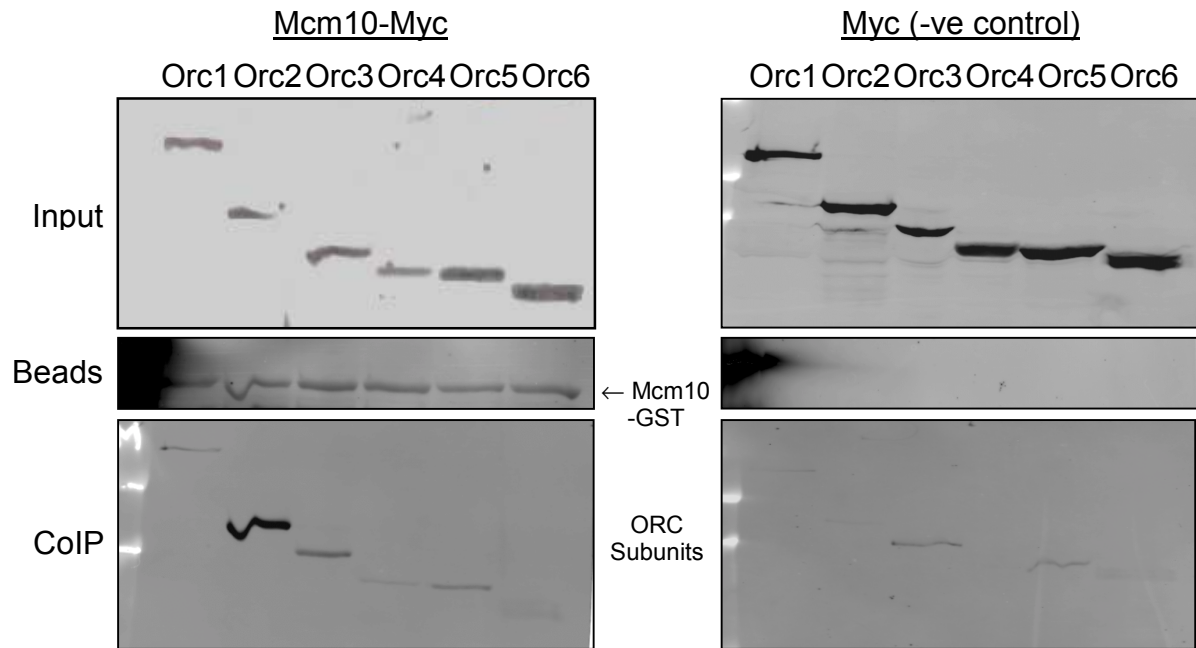
Figure 37. Pull-down assay of ORC subunits using Mcm10-GST. A GST pull-down assay was performed as described in the Materials and Methods. Initially, Mcm10-GST was expressed in bacteria using a pGEX-Mcm10 plasmid and subsequently used to create Mcm10-GST glutathione beads. An empty pGEX vector was used to express GST alone in the production of the control GST beads. 200 μl of WCEs of cells expressing ORC subunits in pJG4-6 was added to each bead type. 20 μl of each precipitate was run on a western blot and incubated with anti-GST and anti-HA antibodies, to detect Mcm10 and the ORC subunits respectively.

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