Coordination of Origin Licensing and Cell-cycle Entry

by

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Submitted to the Department of Biology in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy in Biology

at the

Massachusetts Institute of Technology

October 17, 2007

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Summary

The faithful duplication of the genome is a fundamental requirement for cellular propagation. To ensure successful transmission of genetic material to progeny, the preparation for, initiation, and completion of DNA replication are closely coordinated with cell growth. Central to this coordination is the regulated assembly and activation of the pre-Repli cative Complex (pre-RC) at specific sites of future initiation, or origins of replication. In cycling cells, pre-RC formation can only occur in G1 when cyclin-dependent kinase activity is low, whereas activation of assembles pre-RCs can only occur as cells exit G1 and enter S-phase. The focus of my research has been investigating the parameters that regulate the selection of potential origins and their subsequent activation.

My initial insights into the coordination between licensing and cell cycle progression came through the examination of origin usage during the first cell cycle as cells return to growth from quiescence. Surprisingly, I found that yeast retain a subset of pre-RCs at origins in G0. Although these origins are sufficient to duplicate the genome, additional pre-RCs are normally assembled as cells exit G0 and use of these additional licensed origins increases viability. Finally, this additional licensing is monitored by a checkpoint that is dependent on the Rad53 checkpoint kinase, but is distinct from other cellular surveillance mechanisms that Rad53 is involved in.

I have gained further understanding of the control of origin usage through a genetic approach that identified novel factors that enhance the replication of weak origins. One factor that I investigated in more detail, acts by redistributing pre-RC formation amongst origins of replication. This perturbation of origin licensing appears to particularly affect replication when cells re-enter the cell cycle from quiescence, by perturbing the re-formation of pre-RCs.

Thesis Supervisor: Stephen P. Bell
Professor of Biology
aan mijn ouders en grootouders
Acknowledgments

“In the long history of humankind (and animal kind, too) those who learned to collaborate and improvise most effectively have prevailed”

-Charles Darwin

Although the goal of science may be to better explain the world we live in, the practical pursuit of science entails far more posing of questions than it ever does collecting of answers. To that end, I owe a great debt, not only to the many people that supplied answers during my time here, but also to those colleagues whose conversations left me with new, more challenging questions.

I am grateful for the practical support of the many labs in the department whose reagents and expertise I've benefited from, the building and support staffs whose foundation work I've depended on, and the members of the lab, whose assistance was as immediate as it was constant; I am indebted, as well, to the moral support from those colleagues who most believed in the potential of this work and in my ability to complete it; and I am appreciative, in particular, of the intellectual support from diverse members of this department – in corridors and in coffee shops and at all hours.

I would especially like to thank the members of my dissertation committee – Angelika Amon, Phil Sharp, Terry Orr-Weaver, Nick Rhind, and Frank Solomon – for their dedication and critical feedback. It is also a pleasure to acknowledge my debt to my advisor, Steve Bell, whose commitment to scientific excellence has seen much of this work through to its completed form and from whom I've learned more about science than any other person I've interacted with in my time here.

Finally I would like to humbly recognize the friends, and most especially the family, whose succor made this dissertation possible and whose love make its completion meaningful.
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Chapter I

Introduction
Introduction

The emergence of DNA as the primary hereditary vehicle of life stands as a landmark event in evolution. As the central transducer of genetic events in the cell, DNA must balance opposing molecular demands. The molecule must be accessible enough to direct the production of cellular building blocks yet be stable enough to preserve the integrity of genetic information. The ability to transmit this stored genetic information to progeny is a defining facet of living things. The opposing functional demands on this molecule present a particular challenge during cellular propagation. The duplication of the genetic molecule must occur very rapidly, to allow continued use of the DNA to support growth, but also accurately, to prevent loss of genomic integrity. The regulatory mechanisms that have evolved to ensure efficient and precise DNA replication are therefore of central importance to the development of life on earth.

In this introduction I will discuss these regulatory mechanisms with particular attention to how DNA replication is coordinated within the eukaryotic cell cycle. I will first describe the molecular machines that assemble onto chromatin to promote replication initiation. These complexes assemble at specific sequences called origins, at which replication begins. The number and identity of origins varies depending on the developmental state of the cell as well as external conditions. Next I will describe the basic network of regulators that drives cell-cycle progression. I will then put the selection and activation of origins into the context of this cell-cycle framework both in normally
cycling cells as well as in a special out-of-cycle state known as cellular quiescence. Finally I will discuss the cellular surveillance mechanisms, or checkpoints, that monitor the fidelity of various aspects of cellular duplication before allowing progression through specific cell-cycle phases.

The replicon model of DNA replication

The central architecture of DNA replication was first proposed by Francois Jacob and Sydney Brenner (Jacob and Brenner, 1963). The replicon model postulates that the replication of a single unit of DNA, or replicon, is controlled by two factors - a soluble gene product, encoded on the DNA, called an initiator and a regulatory sequence on the DNA called a replicator. The model proposed that the initiator binds to the replicator and acts as a positive stimulant of replication initiation. This model was validated in bacteria by the discovery of an *E. coli* sequence, OriC, that fulfills the criteria of a replicator and a cognate initiator protein, DnaA (Fuller and Kornberg, 1983). Genetic mapping experiments and autoradiography revealed the *E. coli* genome to be encoded by a circular chromosome which is duplicated bidirectionally from a single start point, called an origin of replication (Marsh and Worcel, 1977). The OriC replicator sequence maps to near the origin of replication (von Meyenburg et al., 1977) consistent with the stimulatory DnaA-OriC interaction promoting replication initiation locally. About ten DnaA molecules bind sequence-specifically to OriC. This binding cooperatively induces a distortion of the DNA that results in unwinding of nearby AT-rich DNA. This unwinding is sufficient to
allow the helicase loader, DnaC, to recruit and assemble the replicative helicase, DnaB around the ssDNA region (Bramhill and Kornberg, 1988). This allows a bidirectional replication fork to be established which then initiates DNA synthesis.

The paradigm, established by the bacterial replicon model, provided a useful framework for early studies of eukaryotic DNA replication, although key differences between the regulation of replication in these two systems have since emerged. Eukaryotic replicators were first identified in the yeast *Saccharomyces cerevisiae* as sequences able to promote the autonomous replication of episomal elements (Struhl et al., 1979). These autonomous replicating sequence (ARS) elements were subsequently demonstrated to also direct chromosomal replication (Stinchcomb et al., 1979) and, like in bacteria, to coincide with sites of replication initiation, or origins (Brewer and Fangman, 1987; Huberman et al., 1987). Yeast origins are 100-150 bp helically-unstable, AT-rich sequences containing an essential match to the 11 bp ARS Consensus Sequence (ACS). The ACS, however, is not sufficient to promote initiation. Each replicator also contains one or more B-elements, which are not individually required, but are together necessary for origin activity (Marahrens and Stillman, 1992). These elements are conserved in function, but divergent in sequence between origins.

In contrast to yeast, replicators are ill-defined in multicellular eukaryotes. The difficulty in identifying replicators in these organisms may reflect the difficulty in
determining specific sites of initiation. In fact, it is unclear to what extent replication initiates from defined origins in these systems. Both Xenopus and Drosophila embryos are capable of duplicating DNA without any apparent sequence preference (Harland and Laskey, 1980; Sasaki et al., 1999). During development, initiation eventually becomes focused to more specific sites (Hyrien et al., 1995) in a transition that may depend on the induction of transcription at the mid-blastula stage (Danis et al., 2004). Although some instances of highly localized sites of initiation have been described (Abdurashidova et al., 2000; Carminati et al., 1992; Toledo et al., 1998), replication in other regions has been found to initiate less specifically from within broad zones (Phi-van et al., 1998; Shinomiya and Ina, 1994; Vaughn et al., 1990). The specificity of initiation site selection may be influenced either by the level of nucleotide pools (Anglana et al., 2003) or by changes in transcription in those regions during development (Norio et al., 2005). In both origins with specific initiation sites and those with broad initiation zones, attempts to genetically define replicator sequences have identified only very few sequences contributing to replication and it is possible that other factors, such as chromatin structure and other chromosomal processes, contribute to specifying sites of replication (Gilbert, 2004).

Despite the difficulty in defining replicators, the eukaryotic initiator appears to be conserved in all organisms studied to date. Like DnaA in bacteria, the Origin Recognition Complex (ORC) binds to DNA and stimulates replication initiation locally.
In yeast, origin-binding of the six-member ORC depends on the ACS (Bell and Stillman, 1992). Association of ORC with origin DNA is essential for origin function as mutations of the ACS that eliminate ORC binding abolish origin function (Bell and Stillman, 1992). ATP-binding by the largest ORC subunit, Orc1, stimulates the origin association of the complex and ORC-origin binding inhibits the ATP-hydrolysis activity of Orc1 (Klemm et al., 1997). In contrast, ssDNA stimulates ORC-ATPase activity, suggesting that unwinding of origin DNA may promote some energy-driven ORC function, although no such event has as yet been clearly defined (Lee et al., 2000). ORC-binding to origins of replication is conserved in higher eukaryotes (Abdurashidova et al., 2003; Austin et al., 1999; MacAlpine et al., 2004) although no sequence specificity has been found in these organisms (Remus et al., 2004; Vashee et al., 2003). This non-sequence specific origin-binding could be mediated by DNA topology, interactions with other DNA-binding proteins, such as transcription factors, or chromatin state (reviewed in Gilbert, 2005). Indeed, the DNA binding-specificity implied by the ARS-consensus sequence is insufficient to explain the relatively limited number of ORC binding sites in the yeast genome. Rather, it is likely that additional, less-conserved sequences or sequence-independent chromatin features contribute to determining sites of ORC binding even in yeast.

Unlike in bacteria, binding of the initiator to an origin region is not sufficient to trigger initiation of replication in eukaryotic cells. Studies in *Xenopus* revealed mitotic
nuclei must undergo nuclear-envelope breakdown to become competent to replicate their DNA in a cell-free system (Blow and Laskey, 1988). This finding was interpreted to mean that a positively active “licensing factor” must interact with chromatin after the end of mitosis before cells can initiate replication. Subsequently, it was found that yeast origins also became “licensed” to replicate only after mitosis despite the fact that ORC is bound to origins throughout the cell-cycle (Diffley et al., 1994). The molecular manifestation of this origin licensing is the formation of an ORC-dependent, multi-protein complex called the pre-Replicative Complex (pre-RC). Subsequent activation of replication at an origin occurs through the assembly of a bidirectional replication fork following recruitment of additional initiation proteins to the pre-RC (see below). Thus ORC-binding determines the sites of origins by directing the formation of the pre-RC at those sequences.

Although the exact subunit architecture of the pre-RC remains unknown, it is clear that bound ORC recruits additional factors to the origin that together load multiple copies of the Mcm2-7 presumptive helicase complex onto origin DNA. ORC interacts directly with Cdc6, an essential pre-RC component related to Orc1 (Liang et al., 1995). Like Orc1, Cdc6 is a member of the AAA+ family of ATPases and ATP binding and hydrolysis by Cdc6 is necessary for pre-RC formation to occur (Cocker et al., 1996; Perkins and Diffley, 1998). Cdc6 is a low-abundance protein that may act catalytically at origins during pre-RC formation rather than being a stable component of the pre-RC (Donovan et
ORC and Cdc6 sequentially hydrolyze ATP during the loading of the Mcm2-7 complex. ORC directly recruits ATP-bound Cdc6 to the origin (Wang et al., 1999). This interaction is sufficient to recruit Cdt1 and the Mcm2-7 complex to the origin. ATP-hydrolysis by Cdc6 promotes loading of Mcm2-7 onto DNA and release of both Cdc6 and Cdt1, whose association with origins is only transient (Randell et al., 2006; Weinreich et al., 1999). Structural studies reveal the Mcm2-7 complex to be a hetero-hexameric ring-shaped molecule suggesting that loaded Mcm2-7 may encircle origin DNA. ATP-hydrolysis by ORC then completes a round of Mcm2-7 loading. This may be accomplished by releasing Mcm2-7 from other pre-RC components or by resetting the conformation of ORC to allow for a new round of Mcm2-7 loading (Bowers et al., 2004).
Eukaryotic genomes are replicated using many origins

A key difference between bacterial and eukaryotic replication is that, unlike the single replicon of bacteria, eukaryotic genomes are duplicated using many origins of replication in parallel. Eukaryotic genomes are generally larger and structurally more complex than their bacterial counterparts. The increased number of origins ensures that, despite a polymerization rate that is about twenty times slower than in bacteria and a genome that is ten to a thousand times larger, the time required to duplicate the genome only varies within a single order of magnitude. Indeed, the activation of multiple origins ensures that eukaryotic replicons range in size from ~10kb, in rapidly dividing embryonic cells, to 30-100kb in differentiated cells.

The replicon model originally envisioned that the duplication of every replicon in the cell might be regulated by a unique replicator and a unique initiator (Jacob and Brenner, 1963). However, the parallel replicons that combine to duplicate a eukaryotic genome all use the same initiator (ORC) and licensing system (pre-RC). Despite this,
individual origins differ in their usage. Every origin has both a characteristic efficiency and a characteristic time of replication in S-phase. Origin efficiency is defined as the frequency of replication cycles in which replication initiates from a given origin. Yeast origins range from highly efficient (>90%) to those that rarely, if ever, initiate under normal conditions. Higher eukaryotic origins characteristically show even lower frequency of activation than yeast origins, with very few origins exceeding 50% efficient. Despite this low efficiency and despite the fact that origin activation appears to be independent of the activation of nearby origins (Patel et al., 2006), cells appear to possess an as yet unelucidated mechanism to prevent excessively large gaps in origin activation which would lead to very long replicon sizes.

Origin timing refers to the defined temporal pattern of origin initiation within S-phase. Not all origins initiate replication at the same time. Some origins initiate characteristically early while others initiate later. It is not known what factors govern the initiation time of an origin, although studies in yeast suggest that chromosomal context and not origin sequence plays a primary role (Ferguson and Fangman, 1992; Friedman et al., 1996). Consistent with a role for chromatin in determining replication timing, deletion of the histone deacetylase $RPD3$ advances the replication of late-initiating origins (Aparicio et al., 2004). Additionally, artificially tethering a histone acetylase to a late-replicating region advances the time of origin initiation in the region (Vogelauer et al., 2002) whereas tethering a histone deacetylase to an early-replicating region delays origin
Origin timing and efficiency combine to determine the kinetics of DNA replication of different regions of the genome (Rhind, 2006). Those regions of the genome nearest early, efficient origins will replicate earlier than those near later, efficient origins. In addition, sequences near inefficient origins are more likely to be passively duplicated by replication forks stemming from adjacent, more efficient origins. In higher eukaryotes, there is a general correlation between late duplicating regions and heterochromatin, although it is not known whether this effect is primarily the result of late-initiating origins, or the lack of efficient origins in those regions.

**Redundancy of origin licensing**

In all eukaryotic cell cycles it appears that more origins of replication are licensed than are required to efficiently duplicate the genome (Okuno et al., 2001; Walter and Newport, 1997). Indeed, deletion of individual origins in yeast has no apparent deleterious consequences to the cell. This is likely due, in part, to replication forks being more processive than the average distance between origins (Dershowitz and Newlon, 1993). Additionally, the failure of one origin to initiate can allow nearby dormant origins that are normally passively replicated to initiate (Santocanale et al., 1999; Vujcic et al., 1999).
Although individual origin function is both variable and redundant, several lines of evidence suggest that reducing the overall number of activated origins reduces cellular fitness. Reducing the number of pre-RCs formed decreases the number of replication forks established, increasing the average replicon size, and therefore increasing the length of time required to duplicate the genome (Devault et al., 2002; Dimitrova and Gilbert, 1998; Walter and Newport, 1997). Additionally, many mutations that partially impair pre-RC formation also decrease viability, although it is has not been generally elucidated whether the reduced viability is a direct consequence of a reduced number of active origins. For instance, mutations in both Orc3 and Orc5 result in increased levels of gross chromosomal rearrangements on a yeast artificial chromosome (YAC). However, the Orc5-mutant isolated in this study does not have a defect in pre-RC formation and the increased rate of rearrangements is suppressed by the deletion of origins on the YAC, suggesting that ORC may have an additional, pre-RC formation-independent role in maintaining genomic stability (Huang and Koshland, 2003). Additionally, reducing the number of activated origins may impair the ability of cells to elicit a checkpoint response as a result of exogenous damage (Shimada et al., 2002), thereby complicating such studies. A more direct approach is to look at the consequences of deleting multiple origins in a single region. Deletion of a subset of origins on an arm of a yeast chromosome does not cause any discernible phenotype, consistent with the origin-redundancy discussed above (Dershowitz and Newlon, 1993). However, deletion of multiple efficient origins of replication on the same arm extends the size of the replicon
duplicating that arm and increases loss of the chromosome (Dershowitz et al., 2007; Newlon et al., 1993).

**Origin licensing and activation is integrated with cell-cycle progression**

The cell-cycle divides the DNA replication and chromosome segregation steps of cell-division into four distinct steps. This ordered regulation is promoted, in yeast, by the activity of a single Cyclin-Dependent Kinase (CDK), Cdc28. Cdc28 activity is modulated by its association with different cyclins at different times in the cell-cycle (Miller and Cross, 2001).

In mitosis Cdc28 combines with the B-type cyclins Clb1 and Clb2 to promote chromosome segregation and cytokinesis (Surana et al., 1991). As cells complete a round of division and exit mitosis, B-type cyclins are degraded by ubiquitin-dependent proteolysis (Glotzer et al., 1991). CDK activity is further reduced due to accumulation of the CDK-inhibitor Sic1 (Schwob et al., 1994). As cells progress through G1, Cdc28 is reactivated by the accumulation of the G1-specific cyclins Cln1, Cln2, and Cln3 (Richardson et al., 1989). This accumulation is nucleated by Cln3, which is constitutively expressed at low levels throughout the cell-cycle (Tyers et al., 1993). Exit from G1 depends on cells attaining a critical size. The regulated retention of Cln3 in the endoplasmic reticulum may play a role in monitoring cell-growth by coupling entry of active Cln3 into the nucleus in late G1 with sufficient biosynthetic activity (Verges et al.,
Nuclear Cln3-Cdc28 counteracts the Whi5 inhibition of two hetero-dimeric transcription factors, SBF (Swi6 and Swi4) and MBF (Swi6 and Mbp1) that activate the transcription of both Cln1 and Cln2 and of the B-type cyclins Clb5 and Clb6 (de Bruin et al., 2004). Clb5 and Clb6 direct Cdc28 activity towards S-phase targets but are kept inactive in G1 by the CDK inhibitor, Sic1 (Schwob and Nasmyth, 1993).

Cln-CDK activity promotes the G1/S transition in three ways. First, phosphorylation of SBF and MBF stimulates further cyclin transcription in a feed-forward loop (Cross and Tinkelenberg, 1991; Dirick and Nasmyth, 1991). Second, Cln-CDKs promote bud formation, which initiates daughter-cell formation (Lew and Reed, 1993). Third, phosphorylation of Sic1 promotes its proteosome-dependent destruction thereby activating the S-phase cyclins Clb5 and Clb6 (Schneider et al., 1996). The switch from a CDK-low state to a CDK-high state in late G1 constitutes an important transition step in the cell-cycle, called START. Passage through START commits a cell to completion of a round of replication and cell-division. Activated Clb5- and Clb6-Cdc28 both promote initiation from licensed origins and spindle elongation as well as promoting the degradation of Cln-type cyclins, thus ensuring the unidirectional progression of the cell-cycle and preventing cells from returning to G1 until B-type cyclins are again degraded at the end of mitosis.
Figure 2: Control of cell-cycle transitions in S. cerevisiae. The cyclin-dependent kinase (blue circle) pairs with different cyclins to promote different cell-cycle events. As cells exit mitosis, mitotic cyclins (violet triangles) are degraded and further inhibited by Sic1 (red arch). Entry of Cln3 (dark green triangle) into the nucleus in G1 simulates Cln1,2 (light green) as well as Clb5,6 (orange) production. Accumulated Cln-Cdc28 activity promotes budding and frees Clb5,6-Cdc28 from Sic1 inhibition to promote the G1/S transition.

The importance of this cell-cycle commitment point in regulating DNA replication is underscored by the inability of pre-RCs to form after cells pass through START. Pre-RCs can only form in G1, when CDK activity is low (Dahmann et al., 1995; Piatti et al., 1996). When cells enter S-phase, the activity of two kinases, Cdc28 paired with Clb5 or Clb6 and the S-phase specific Dbf4-dependent kinase (Cdc7/Dbf4 or DDK)
combine to promote initiation from licensed origins. Importantly, Clb-CDK activity also prevents pre-RCs from forming outside of G1 through phosphorylation of multiple pre-RC components (Blow and Dutta, 2005; Hayles et al., 1994). Phosphorylation of Mcm2-7 subunits promotes export of the complex from the nucleus together with Cdt1 (Labib et al., 1999; Nguyen et al., 2000; Tanaka and Diffley, 2002b). Phosphorylation of Cdc6 stimulates its proteosome-dependent degradation as well as an inhibitory interaction with Clb2-Cdc28 (Drury et al., 2000; Mimura et al., 2004). Phosphorylation of Orc2 and Orc6 inhibits pre-RC formation through unknown mechanisms (Nguyen et al., 2001). Additionally phosphorylated Orc6 interacts directly with Clb5 and this interaction contributes to preventing pre-RCs from being formed (Wilmes et al., 2004). Bypassing these mechanisms, by disrupting the interactions between Clb-CDK and pre-RC components, allows pre-RCs to form outside of G1 (Nguyen et al., 2001). This inappropriate origin licensing allows origins to re-initiate within the same cell-cycle which causes over-duplication and results in lesions that cells cannot correct (Archambault et al., 2005; Green and Li, 2005). Multicellular eukaryotes similarly prevent over-replication by coupling entry into S-phase to the inhibition of pre-RC formation, although the exact molecular consequences of CDK phosphorylation of initiation factors differs in different organisms (Arias and Walter, 2007). Additionally, these organisms contain an inhibitor of Cdt1, called Geminin, not present in yeast, that prevents pre-RC formation until its destruction at the metaphase to anaphase transition (McGarry and Kirschner, 1998).
The importance of licensing a sufficient number of origins, discussed above, is further highlighted by mutations that decrease the length of the CDK-low phase of the cell-cycle. Pre-RC formation is restricted to G1 and mutations that shorten this phase of the cell-cycle results in similar defects to those observed when pre-RC formation is impaired. Over-production of the G1-cyclin Cln2 reduces pre-RC formation and causes increased genomic instability (Tanaka and Diffley, 2002a). Similarly, constitutively high levels of Cyclin E expression in human cells, result in decreased pre-RC formation and are often associated with chromosome instability (Ekholm-Reed et al., 2004). Additionally, deletion of the G1/S inhibitor Sic1 shortens G1 by allowing earlier activation of Clb5,6-Cdc28. This mutation also leads to an increased average replicon size, increased DNA damage and an increase in gross chromosomal rearrangements (Lengronne and Schwob, 2002).
Exit from the cell-cycle

Completion of mitosis is not always followed directly by progression through G1. At the end of mitosis cells can either continue cycling or else exit the cell-cycle to a metabolically inactive state called quiescence, or G0. In yeast, the decision to continue cycling or cease growth depends on the integration of three signaling pathways that sense the availability of nutrients and level of cellular stress (Gray et al., 2004). In response to unfavorable growth conditions cells complete division and then, rather than proceeding through G1, arrest growth. Quiescent cells are physiologically distinct from cycling cells.
due to a number of characteristic changes to cellular composition and metabolism that allow them to maintain viability for extended periods of time in the absence of proliferation. In yeast, these changes include decreased transcription and translation, increased accumulation of storage carbohydrates, thickened cell walls, and increased thermotolerance (Werner-Washburne et al., 1993).

With respect to the cell cycle, cells in G0 most closely resemble pre-START G1-cells (Johnston et al., 1977). Quiescent cells have an unreplicated DNA content, low Cln-cyclin levels and, in yeast, an unbudded morphology (Mendenhall et al., 1987). Indeed, constitutive Cln2 expression prevents entry into G0, suggesting that low cyclin levels are a prerequisite for cell-cycle exit (Hadwiger et al., 1989). Unlike cells in G1, however, it is thought that quiescent cells lack pre-RCs at origins of replication. Studies of the yeast 2μ origin, as well as multiple studies of mammalian cells, suggest that, in G0, ORC remains bound to origins but that the Mcm2-7 complex, while abundant in the cell, is not associated with chromatin (Cocker et al., 1996; Madine et al., 2000; Stoeber et al., 1998; Sun et al., 2000). In mammalian cells, in particular, it has been suggested that the absence of Cdc6 in G0 may explain the lack of assembled pre-RCs and reformation of pre-RCs upon return to growth correlates with accumulation of Cdc6 in these cells (Mailand and Diffley, 2005).

G0 is a reversible arrest-state. In response to improved environmental conditions,
cells re-enter the cell cycle. The transition from G0 to S-phase is similar in many ways to
the passage of cells through G1. As such, cells progressing from G0 to S-phase commit
to a round of division when sufficient G1-cyclins accumulate for cells to pass through
START. However, this transition is very slow, compared to the length of time normally
spent in G1 (Iida and Yahara, 1984) and it remains unclear which processes require such a
long time to complete before cells enter S-phase (Coller, 2007). One possibility is that
one or more of the developmental changes to the cell that occur during entry into G0 take
a long time to reverse as cells return to growth. These physiological changes include
increasing transcription, altering cell-wall composition, and resorting proteins. Another
possibility is that origin licensing is slow when cells exit G0. In cycling cells, pre-RCs
form as cells exit mitosis. Indeed, inactivation of Clb-CDK activity in mitosis is
sufficient to allow pre-RCs to form (Noton and Diffley, 2000). In G0, Clb-CDKs are
inactive, yet pre-RCs form only late into the passage back into the cell-cycle. It is
possible, therefore, that the synthesis of components required for pre-RC formation, or
ever the molecular process itself, are limiting during cell-cycle re-entry.

**Checkpoints regulate cell-cycle progression**

Successful progression through many parts of the cell-cycle is facilitated by the
existence of monitoring mechanisms called checkpoints. A cell-cycle checkpoint delays
the commencement of one cell-cycle event until the completion of a prior event. As
originally defined, checkpoint proteins are only essential under conditions that elicit a
checkpoint response (Hartwell and Weinert, 1989), although more recent work is challenging that view (see below). Checkpoints have been identified that respond to diverse lesions at multiple stages of the cell cycle and can be divided into three categories: factors that sense a checkpoint signal, factors that transduce the signal, and factors, called effectors, that interface with the cell-cycle machinery to arrest cell-cycle progression (Melo and Toczyski, 2002). Like other signal transduction pathways in the cell, transducers of checkpoint signals often amplify the initial signal to allow the cell-cycle to completely arrest even when only one or two events are sensed.

Two major checkpoints monitor DNA during replication: the DNA-damage and intra-S phase checkpoints. The DNA-damage checkpoint senses lesions to DNA and delays the cell-cycle in G1 or in G2, depending on when the damage is sensed. The intra-S phase checkpoint senses blocks to replication fork progression during S-phase and both prevents entry into mitosis pending completion of replication as well as slowing fork progression and stabilizing stalled replication forks. These checkpoints share both a common sensor kinase in Mec1 and a common effector kinase in Rad53, although they utilize different adaptor proteins to transduce the checkpoint signal.

In the case of the DNA-damage checkpoint, the clearest identified checkpoint-eliciting signal is ssDNA coated by a filament of the ssDNA-binding protein, RPA (Branzei and Foiani, 2005). Such DNA can be generated in a number of ways, including
the processing of a double-stranded break or the uncoupling of DNA-unwinding and polymerization during replication. Two protein complexes required for a checkpoint response are independently recruited to the ssDNA region (Kondo et al., 2001): Mec1/Ddc2 (human ATR/ATRIP) and the PCNA-like Rad17/Mec3/Ddc1 (human 9-1-1 complex), loaded by the RFC-like Rad24 (human Rad17)-containing complex. The PCNA-like complex recruits Rad9 to the site of damage (Zou and Elledge, 2003), where it is phosphorylated in a Mec1-dependent manner (Vialard et al., 1998). This phosphorylation induces Rad9 oligomerization. Oligomerized, but not monomeric, Rad9 interacts with and phosphorylates Rad53 at sites of damage (Soulier and Lowndes, 1999; Sun et al., 1998). Subsequent auto-phosphorylation of Rad53 promotes its release from Rad9, thereby providing a mechanism both for signal transduction and amplification (Gilbert et al., 2001).

In the intra-S phase checkpoint, Rad53 is activated primarily by Mrc1, and not by Rad9 (Alcasabas et al., 2001). The intra-S phase checkpoint is engaged when a replication fork encounters an impasse, such as damaged DNA. This block to fork progression can lead to uncoupling of DNA-unwinding and polymerization or of leading- and lagging-strand polymerization to expose ssDNA and elicit a checkpoint response. The Mrc1 adaptor travels with replication forks where it is poised to detect stalled forks (Katou et al., 2003). The intra-S phase checkpoint prevents continued polymerization and the absence of this checkpoint leads to increased fork collapse and recombination (Sogo
et al., 2002).

**Figure 4: Checkpoint pathways monitoring DNA integrity.** Cells arrest in response to DNA damage in either G1 or G2/M depending on when the damage is sensed. Blocks to fork progression during S-phase trigger a checkpoint response that helps ensure the completion of genome duplication prior to mitosis.

Both of these checkpoints act through the Rad53-kinase signal to checkpoint effectors. Although additional targets remain to be identified, elements of the checkpoint response downstream of Rad53 have been enumerated at various points of the cell-cycle (Branzei and Foiani, 2006). Activation of the intra-S phase checkpoint reduces
nucleotide levels through phosphorylation of the ribonucleotide reductase activator Dun1, prevents anaphase through stabilization of the securin, Pds1, and may inhibit initiation from origins of replication through phosphorylation of the Dbf4 subunit of DDK. The only known target of Rad53 during the G1-delay in response to DNA damage is the MBF and SBF subunit, Swi6 (Sidorova and Breeden, 1997). Phosphorylation of Swi6 prevents its chromatin association thereby preventing its activity (Sidorova and Breeden, 2003).

Although originally envisioned as being necessary only when cell-cycle processes are disrupted, a number of checkpoint proteins have proven to play a role in normal cell-cycle progression. In addition to their role in the DNA-damage and intra-S phase checkpoints, Rad53 and Mec1 have an essential function in upregulating nucleotide levels prior to DNA-replication and this function can be bypassed by deletion of the ribonucleotide reductase inhibitor, Sml1 (Zhao et al., 2001). Moreover, the intra-S phase checkpoint is activated to some extent during every cell-cycle, even in the absence of exogenous inhibitors of replication (Shimada et al., 2002). Similarly, in mammalian cells, the spindle checkpoint, which prolongs metaphase in the absence of tension on sister kinetochores, has been shown to influence the timing of an unchallenged anaphase (May and Hardwick, 2006). Thus some cell-cycle checkpoints may play a more general role in coordinating progression through the cell-cycle.

**Checkpoints ensuring replication completeness**
The ability of cells to ensure the completion of DNA replication prior to mitosis remains a poorly understood issue. A role for coordinating the completion of S-Phase with progression through mitosis has been ascribed to three possible checkpoints. First, the intra-S Phase checkpoint inhibits the metaphase to anaphase transition in response to blocked replication forks, as described above. However, because this checkpoint responds to defects encountered during replication, the checkpoint is not triggered in the absence of any DNA-replication (Li and Deshaies, 1993). Therefore, although this checkpoint helps ensure the fidelity of replication is does not monitor the completion of replication in an unperturbed S-phase.

Second, it has been proposed that a separate S/M checkpoint might directly coordinate entry into mitosis with the completion of S-phase (Weinert, 1998). Consistent with this, deletion of both major S-phase cyclins, Clb5 and Clb6, causes a delay in S-phase until Clb3 and Clb4 accumulate. Importantly, this causes both a delay in the onset of DNA-replication as well as a compensatory delay in anaphase (Schwob and Nasmyth, 1993). However, a recent study investigating the consequences of deletion of the cohesin/condensin related SMC5-SMC6 complex, found that the completion of chromosomal duplication was impeded, particularly in ribosomal DNA, but that anaphase proceeded with normal kinetics (Torres-Rosell et al., 2007). Therefore the failure to complete S-phase does not prevent the onset of mitosis in all cases.
Finally, conflicting evidence exists as to whether cells monitor the completion of origin-licensing prior to S-phase (Lau and Jiang, 2006). The *S. pombe* ORC1 homolog, ORP1, was first identified in a genetic screen for temperature-sensitive alleles that arrest with a G1 morphology (Grallert and Nurse, 1996). Reciprocal shift experiments with the isolated *orp1-4* allele demonstrated that Orp1 acts at or before START. Similarly, reduction of ORC2 levels in human cells by RNA interference causes cells to arrest in G1 with low CyclinE-Cdk2 activity and high levels of the CDK-inhibitors, p27 and p21 (Machida et al., 2005). A similar G1-delay was observed in cells containing a hypomorphic ORC2-allele (Teer et al., 2006). In contrast to these results, elimination of other pre-RC subunits argues against the existence of a mechanism that coordinates licensing with S-phase entry. Inactivation of Cdc6 in both *S. pombe* and *S. cerevisiae* allows cells to proceed into mitosis in the absence of any detectable DNA-replication (Kelly et al., 1993; Piatti et al., 1995). Similarly, elimination of Cdt1 in both *S. pombe* and *D. melanogaster* uncouples mitosis from S-phase completion (Hofmann and Beach, 1994; Whittaker et al., 2000).

**Thesis Summary**

In this thesis I examine the coordination between origin licensing and cell cycle progression. I find that in quiescent cells, about half of the origins of replication normally licensed during G1 retain pre-RCs. Pre-RC formation at other origins only occurs after Cdc6, which is absent in G0, accumulates upon return to growth. Moreover,
this additional origin licensing is the rate-limiting step for exit from G0 and re-entry into the cell-cycle and is regulated by the same pre-RC assembly checkpoint present in G1 in cycling cells.

I conduct a screen to identify factors that enhance the replication of a late/inefficient origin. I identify eight candidates and characterize one of these further. I find that the product of the yeast gene \textit{YOR006C} enhanced pre-RC formation at weak origins of replication, causing some changes in origin usage in S-phase. Moreover, I find that cells lacking \textit{YOR006C} are defective in origin licensing upon return to growth from quiescence and defective, in turn, in progression back into the cell cycle. Together these observations contribute to our understanding of the mechanisms that cells have evolved to ensure the efficient and complete duplication of the genome.


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Chapter II

De Novo Origin Licensing during cell cycle Re-entry
Summary

The efficient duplication of a eukaryotic genome requires the coordinated activation of multiple origins of replication along each chromosome in a cell cycle dependent manner. Central to this coordination is the regulated formation and activation of protein complexes at origins of replication. Upon exit from mitosis, the pre-Replicative Complex (pre-RC) is assembled at origins. Subsequent activation of pre-RCs in S-phase allows the recruitment of additional replication factors at each origin leading to the formation of a pair of bidirectional replication forks. Regulated exit from the cell cycle is thought to be accompanied by a loss of pre-RCs from origins of replication. Here we use genome-wide analysis to show that, in G0, *Saccharomyces cerevisiae* retain pre-RCs at about half of the origins of replication normally licensed during G1 in cycling cells. Pre-RCs form at the remaining unlicensed origins as cells return to the cell cycle and this additional licensing is rate-limiting for cell cycle re-entry. Interestingly, in the absence of the checkpoint protein Rad53, pre-RC formation is no longer rate limiting. Although G0-licensed origins are sufficient to duplicate the genome, complete origin licensing contributes to viability. Together these results extend our understanding of how quiescent cells retain the ability to replicate prior to commitment to the cell cycle re-entry.
Introduction

DNA replication is carefully coordinated with cell division to ensure accurate and efficient genome duplication. Chromatin becomes competent, or “licensed”, to replicate only upon completion of mitosis (Blow and Laskey, 1988). Replication licensing is accomplished through the regulated formation of protein complexes at specific sites, called origins of replication. This process is initiated when the six member Origin Recognition Complex (ORC) binds specifically to origin DNA (Bell and Stillman, 1992). As cells exit mitosis, ORC combines the Cdc6 and Cdt1 to load the Mcm2-7 complex onto origins (Bell and Dutta, 2002). Together these proteins form the pre-Replicative Complex (pre-RC), which marks all potential origins of replication.

Eukaryotic genomes are duplicated using multiple, parallel-acting origins of replication, spaced along each chromosome. These origins are not all activated equivalently. Although the factors governing these differences are poorly understood, it is clear from systematic studies of yeast DNA replication that every origin has a characteristic time in S-phase at which it is replicated and that this property is governed by some combination of the origin's efficiency and the time within S-phase at which it is activated (Friedman et al., 1997; Newlon and Theis, 2002; Yamashita et al., 1997). This temporal pattern of initiation is particularly evident when the ribonucleotide reductase inhibitor, hydroxyurea (HU), is used to engage the intra-S phase checkpoint resulting in the delay of late-origin initiation (Alvino et al., 2007; Santocanale and Diffley, 1998).
Replication is tightly regulated within the cell cycle. Upon entry into S-phase the pre-RC is activated by two kinases, the cyclin-dependent kinase (CDK) and the S-phase specific Dbf4-dependent kinase (DDK or Cdc7/Dbf4). These kinases trigger initiation of replication by stimulating the association of additional replication factors with the pre-RC and these events lead to the assembly of two bidirectional replication forks that carry out DNA synthesis (Takeda and Dutta, 2005). Importantly, high CDK activity also prevents origin licensing through phosphorylation of multiple pre-RC components, ensuring that pre-RCs cannot reform until after chromosome segregation (Arias and Walter, 2007; Nguyen et al., 2001). As a result all origin licensing must occur during G1.

Continued rounds of growth and division depend on the availability of nutrients to the cell. In the absence of sufficient nutrients, cells complete a round of division and exit mitosis, proceeding to a quiescent or G0 state (Gray et al., 2004). Quiescent cells resemble G1 cells in a number of ways. Cells in G0 have low CDK activity, low G1-cyclin levels, unreplicated DNA and, in S. cerevisiae, are unbudded. However, a number of other aspects of G0 make quiescence a unique cell cycle state. Physiologically, yeast cells in G0 have reduced transcription, altered cell wall composition, and increased thermotolerance. Importantly, unlike cycling cells in which low CDK activity is sufficient to allow pre-RCs to form (Noton and Diffley, 2000), cells in G0 appear to lack pre-RCs. A previous study in S. cerevisiae observed the loss of a footprint correlated
with the pre-RC at the 2μ-plasmid origin of replication in quiescent cells (Diffley et al., 1994). Quiescent mammalian cells, despite abundant Mcm2-7 complexes in the cell, lack chromatin-bound Mcm2-7 (Madine et al., 2000; Stoeber et al., 1998; Sun et al., 2000). This lack of pre-RCs is thought to be due to the absence of Cdc6 protein in G0 as the reformation of pre-RCs correlates with the re-accumulation of Cdc6 as cells return to the cell cycle (Mailand and Diffley, 2005).

Quiescent cells remain viable in the absence of growth for extended periods of time. When environmental conditions improve, cells exit G0 and progress through S-phase, back into the cell cycle. Return to the cell cycle involves the reversal of many of the physiological changes that characterize quiescent cells. For example, pre-RCs reform and G1-cyclins accumulate to drive cells back into S-phase. Unlike passage through G1, the G0/S transition is very slow. It is unclear which of the processes involved in cell cycle re-entry cause this transition to proceed so slowly (Coller, 2007).

Here we show that quiescent yeast retain pre-RCs at a subset of origins of replication. Additional origins are licensed in a Cdc6-dependent manner as cells return to growth. Additionally, we find that this additional licensing is the rate-limiting step entry into S-phase and is monitored by a Rad53 dependent cell cycle delay (how confident are you in this data? My recollection is that Andy had a hard time reproducing this aspect of the data.)
Results

A subset of origins retain pre-RCs in G0 cells

To investigate how origin licensing is regulated during exit from the cell cycle in *S. cerevisiae* cells, we first assessed the abundance and distribution of pre-RCs in G0. We transferred asynchronously growing yeast to medium lacking nitrogen for 36 hours to induce quiescence. Quiescent cultures ceased to increase in density, were uniformly unbudded, and contained 1C DNA content as assessed by flow cytometry (data not shown and Fig. 3b, for example). To map sites of pre-RC formation, we performed genome-wide location analysis by combining chromatin immunoprecipitation (ChIP) against the Mcm2-7 complex with hybridization to a high-density tiled microarray. As a control we hybridized Mcm2-7 ChIP samples collected from a cycling cell population arrested in G1 with the mating pheromone α-factor. In G1-arrested cells we detect 359 sites of significant Mcm2-7 enrichment that correspond to origins identified in previous studies (MacAlpine and Bell, 2005; Nieduszynski, et al., 2006). Surprisingly, when we performed the same analysis with cells arrested in G0, we observed significant Mcm2-7 association at 146 out of 359 of these sites (Fig. 1). These G0-retained pre-RCs were distributed throughout the genome and on all chromosomes (Sup. Fig. 1). Consistent with this, we find that the mean distance between G0 pre-RCs is 63.5 kb, about two and half times more distant than that seen in G1 in cycling cells (21.4 kb).
Figure 1: (A) Mcm2-7 association along chromosome X. Mcm2-7 ChIP Signal is plotted versus chromosome position (in kb) for chromosome X. In blue, association of Mcm2-7 in G0 and after 1 and 2 hrs of release back into the cell cycle. In orange, Mcm2-7 association at the G0/S boundary assessed in a \textit{cdc7-1} temperature-sensitive block. In green, a control hybridization of Mcm2-7 from cycling cells arrested in G1 with mating pheromone. (B) As in (A) but for ORC association in G0.

\textbf{Characteristics of G0-licensed origins}

Although we did not find a singular distinguishing characteristic of G0-licensed origins compared to those without pre-RCs, we did identify a number of common characteristics of these origins. Origins can be grouped into two temporal classes based on whether they are able to initiate replication prior to inhibition by the intra-S phase checkpoint in the presence of the replication inhibitor HU (Yabuki et al., 2002). We find that G0-licensed origins are significantly more likely than non-G0-licensed origins to initiate replication early in S-phase in cycling cells (63\% vs. 12\%). When the regulatory mechanisms that prevent pre-RC formation outside of G1 are inhibited, pre-RCs re-form at a subset of origins of replication (Tanny et al., 2006). We find a significant concordance between the subset of re-licensed origins and origins that retain pre-RCs in G0 (Fig. 2a), suggesting the possibility that a shared mechanism governs the selection of these origins.
Figure 2: Replication characteristics of G0-licensed origins. (A) Venn diagram describing the correlation between sites of pre-RC association in G0 and sites observed when control of re-replication is abrogated. (B) Composite profiles of nucleosome occupancy around classes of replication origins. Blue, origins that are licensed in G1 but not in G0. Orange, origins that are licensed in G1 and in G0. (C) DNA enrichment in 200 mM HU in cells entering S-phase from G0 (top) and G1 (bottom). Sites of G0 pre-RCs are indicated with orange dots (D) S-phase profile obtained from synchronized cycling cells released from αF (top) and G0 cells released from a cdc7-1 block (bottom). Colored circles represent pre-RC sites with 7.5 kb of a peak in both profiles and are coded in a yellow-red heat map from earliest to latest replicating on the profile. Black circles represent the position of pre-RCs that are greater than 7.5 kb from any peak in the profile. Triangles represent pre-RCs near a peak in only one profile. Blue line represents relative time of replication in S-phase. Earlier replicating sequences appear higher in the graph.
Figure 2

A. 

\[
\begin{array}{c}
\text{ReReplication} \\
26 & 142 & 29
\end{array}
\]

B. 

\[\text{H3}
\]

C. 

\[
\begin{array}{c}
\text{position (kb)}
\end{array}
\]

D. 

\[
\begin{array}{c}
\text{position (kb)}
\end{array}
\]
Finally, we note that in G0 chromatin is arranged differently around sites that form pre-RCs compared to potential origins that do not. We used genome wide location analysis to measure nucleosome occupancy in both G0 and G1. Consistent with previous observations, we find that histones are depleted near origins in cycling cells. Importantly, this is true in both G1 when pre-RCs are present and in G2/M when they are not. Interestingly, although we observe a similar depletion around sites that form pre-RCs in G0, potential origins that do not assemble pre-RCs fail to demonstrate this depletion. (Fig 2b.)

One reason that a subset of origins might fail to retain licensing in G0 is a loss of ORC binding to origins. To address this possibility, we performed genome-wide location analysis of ORC in G0 cells. Like Mcm2-7, we find that ORC binds only a subset of all potential origins in quiescent cells (Fig. 2c). The majority of sites at which ORC is bound in G0 also show association of Mcm2-7. Therefore, we conclude that origin selection as mediated by the initial binding of ORC, not origin licensing, is the primary limitation in G0 cells.

Additional pre-RCs form as cells return to growth

To better understand how the genome is duplicated when cells return to growth from G0, we measured Mcm2-7 association with origins as cells re-entered the cell cycle. We arrested cells in G0 by nitrogen starvation, as before and then released cells from
One hour after release from G0, Mcm2-7 origin-occupancy was somewhat increased compared to that observed in G0 (Fig. 1). In contrast, two hours after release we detected Mcm2-7 association at many additional origins not licensed at the start of the release. The return of the cells into the cell cycle occurs relatively asynchronously and it is likely that after two hours some cells will have begun S-phase while others are still in G0. To assess the full extent of pre-RC formation prior to the initiation of replication, we blocked cells returning to growth prior to initiation of replication using a cdc7-1 temperature sensitive allele. The distribution of Mcm2-7 binding at the G0/S boundary was very similar to the pattern observed for cycling cells arrested in late G1 with αF (Fig. 1). Therefore, quiescent yeast retain pre-RCs at a subset of origins licensed in cycling-cells and reform pre-RCs at the remaining origins upon return to growth.

We used two approaches to investigate how the pre-RCs formed prior to G0-exit are activated upon cell cycle re-entry. First, we identified early initiating origins by measuring increases in DNA-copy number, genome-wide, in cells arrested early in S-phase with hydroxyurea (HU). In cycling cells, we identify 118 origin-regions that show a significant increase in copy-number in HU (Fig. 2c and Sup. Fig. 2). In contrast, in cells exiting G0, we detect only 55 regions of significant enrichment, 91% of which (50/55) are also sites of enrichment in cycling cells treated with HU. Therefore, despite the fact that an equivalent number of pre-RCs are formed prior to exit from G0 as
compared to in G1, fewer origins escape inhibition by the intra-S-phase checkpoint in these cells.

As a second approach, we compared the temporal pattern of replication across the genome by creating copy-number profiles using DNA collected from a synchronized S-phase. To do this, we synchronized cells with a cdc7-1 temperature-sensitive allele, as before, and collected cells every ten minutes after release from the G0/S block. We compared the profiles obtained from these samples to profiles generated from cycling cells, released from a mating-pheromone arrest in late G1. Although the overall patterns are similar, we notice considerable differences both in the use of particular origins in S-phase and in the relative timing of origin initiation along a chromosome (Fig. 2d and Sup. Fig. 3). Together these results suggest that, although the same origins are licensed upon G0-exit as in G1 in cycling cells, their activation upon S-phase entry differs.

**G0 pre-RCs are sufficient to duplicate the genome**

To determine whether the origins that remained licensed in G0 are sufficient to duplicate the genome we used a methionine-repressible promoter to control Cdc6 expression as quiescent cells return to growth. We grew CDC6 pMet-CDC6 and cdc6Δ pMet-CDC6 cells to log phase in medium lacking methionine to allow for expression from the repressible-Cdc6 allele. We then transferred cells to medium lacking both methionine and nitrogen to induce quiescence. After 36 hours we released cells back into
the cell cycle by transfer to medium containing both nitrogen and methionine. Cells able to express Cdc6 in methionine (CDC6 pMet-CDC6) licensed additional origins upon return to growth, equivalently to wild-type cells. In contrast, cells unable to express Cdc6 (cdc6Δ pMet-CDC6) retained pre-RCs at those origins already licensed in G0 but did not form additional pre-RCs (Fig. 3a). Notably, both strains were able to replicate the bulk of their DNA as measured by flow cytometry, indicating that the origins licensed in G0 are sufficient to replicate the genome (Fig. 3b). In addition, we assessed budding as a replication-independent measure of cell cycle progression. Budding depends on Cln-CDK activity, but not on replication initiation, and occurs as cells exit G1 and enter S-phase. Consistent with pre-RC formation not being required for G0-exit, budding, like DNA replication, occurred fully in cells lacking Cdc6 expression (Fig 3c). We note that in these experiments it appears that both budding and DNA replication in fact occur earlier in cells lacking additional pre-RC formation. Although we cannot say with confidence that this is uniformly the, this finding warrants future investigation.

To confirm that, in the absence of Cdc6 expression and new pre-RC formation, replication occurs from forks established at origins that remain licensed in G0, we used DNA copy-number profiles to measure origin usage in the first S-phase after exit from quiescence. We collected DNA from both Cdc6-expressing and non-Cdc6 expressing cells from a single timepoint during the middle of the first round of DNA replication. Because of the relative asynchrony of cell cycle re-entry upon exit from G0, this
timepoint contains a mix of cells at different points in S-phase, making this sample similar to a pooled S-phase sample conventionally used in experiments done on cycling cells. Cdc6-expressing cells show abundant origin usage that is broadly similar to that seen in S-phase in cycling cells. In contrast, the replication profiles of cells lacking Cdc6 expression used fewer origins to duplicate their genome (Fig. 3d). Although the resolution of this experiment is limited due to the poor synchrony of the cells, the prominent peaks in the profile from non-Cdc6 expressing cells correspond, generally, to sites of G0-licensed origins. Future experiments using better synchronization methods, such as the G0/S block used above will better address the specific origin usage in these cells.

Figure 3: Additional pre-RC formation is not required for genome duplication. (A) Mcm2-7 association in cdc6Δ pMet-CDC6 cells released back into rich medium in the presence of methionine, to repress Cdc6 expression. Samples were taken 0 hr and 2 hr after return to growth. Sites of G0 and G1 pre-RCs are shown in orange and black dots, respectively. (B) Flow cytometry of cells returned to the cell cycle with (blue) or without (orange) Cdc6 expression. (C) Budding index from samples as in B. (D) Copy-number profiles of cells expressing (top) or not expressing (bottom) Cdc6 upon return to growth. Sites of G0 and G1 pre-RCs are shown in orange and black dots, respectively.
Figure 3

A. 

B. position (kb)

C. net-Cdc6 release - 30 degrees

D. position (kb)

with CDC6

without CDC6
**Pre-RC formation is monitored as cells return to growth**

To test whether the delay in G0-exit might be regulated by an active regulatory mechanism, we examined the kinetics of cell cycle entry in cells lacking Rad53, which is required for many cellular checkpoint, including the replication progression checkpoint in S-phase. We found that deletion of the RAD53 gene caused an accelerated S-phase entry as measured both by flow cytometry and budding (Fig. 4a). Rad53 has both checkpoint functions as well as an essential function in regulating nucleotide levels during G1. This essential Rad53 function can be bypassed by deleting the inhibitor of nucleotide biosynthesis, SML1 (Zhao et al., 2001). Importantly, deletion of SML1 on its own did not alter G0-S progression, indicating the effect of eliminating Rad53 was due to its checkpoint function. Additionally, similar results were obtained with a checkpoint-deficient rad53-11 strain (Fig. 4c).

The effect of Rad53 on G0-S progression was not due to it role in other checkpoint pathways. Neither deletion of genes involved in the DNA-damage checkpoint (RAD24 and RAD9, Fig. 4b) nor the intra-S phase checkpoint (MRC1, Fig. 4b) altered the kinetics of cell cycle re-entry. Importantly, deletion of the Mec1 checkpoint-kinase, which acts upstream of Rad53 in both the DNA-damage and intra-S phase checkpoints, also did not alter cell cycle re-entry (MEC1, Fig. 4b). These results indicate that the cell cycle delay that occurs during pre-RC reformation upon G0 exit involves Rad53 but has unique genetic dependencies compared to known checkpoint pathways.
Figure 4: The delay in G0-exit during pre-RC formation has the same genetic dependencies as the G1 pre-RC assembly checkpoint. (A), (B), and (C) Budding indices of the indicated strains upon G0 release.
Additional origin-licensing increases viability

Our data indicate that in the absence of additional pre-RC formation, the origins already licensed in G0 are sufficient to duplicate the bulk of the genome (Fig. 3b). We therefore asked whether replicating the genome from fewer origins was deleterious to cells. To do this, we arrested CDC6 pMet-CDC6 and cdc6Δ pMet-CDC6 in G0 then released these cells back into the cell cycle in medium containing methionine and nocodazole to repress expression from the pMet-promoter and to arrest cells in G2/M after S phase. At one hour intervals, we plated cells back onto plates lacking methionine and lacking nocodazole to assess their ability to continue to grow (Fig. 5). CDC6 pMet-CDC6 cells, that formed pre-RCs at all origins, remained equivalently viable throughout the timecourse. In contrast, cdc6Δ pMet-CDC6 cells remained fully viable if Cdc6 expression was restored during the first two hours of the timecourse, when pre-RC formation could take place when returned to permissive conditions, but remained only about 50% viable if Cdc6-expression was restored after cells had already committed to S-phase. Therefore, entering S-phase with half as many licensed origins as is normally the case allows for duplication of the bulk of the genome, but decreased viability about two-fold.
Figure 5: Additional origin licensing increases viability. (A) Schematic of the experiment. Viability was monitored by plating cells with (blue) or without (orange) Cdc6 expression back to permissive conditions at one hour intervals upon release from G0. (B) Relative viability of cells at indicated times.
Discussion

Here we report the unexpected finding that quiescent yeast retain pre-RCs at about half of the origins of replication normally licensed in G1 in cycling cells. We find that these licensed origins are spaced across the genome. When cells are returned to growth, pre-RCs form at the remaining origins over the course of a number of hours. Even without this additional licensing, however, those pre-RCs that exist in G0 cells are sufficient to duplicate the genome, albeit with reduced success.

**G0-licensed origins are related to re-replicating origins**

Interestingly, those origins that retain pre-RCs in G0 correlate with those sites that re-form pre-RCs in G2/M when the mechanisms that prevent re-replication are abrogated. This finding suggests that these origins may be preferred sites of pre-RC formation or else that some property of those regions that is present both in G2/M in re-replicating cells and in G0 governs origin selection in both cases.

The ability to re-form pre-RCs at an origin was shown to be dependent on chromosomal context (Tanny et al., 2006). That is, an origin capable of re-initiating under conditions that decouple pre-RC formation from cell cycle progression can induce re-replication locally when placed in a region of the genome that otherwise does not re-replicate under these same conditions. It is interesting to note, therefore, that in G0, only those origins that retain pre-RCs are depleted of nucleosomes. Whether these nucleosome-free regions are a determinant of sites of pre-RC formation or a consequence
thereof needs to be addressed in future studies. Because of this observed difference in chromatin structure at these two classes of origins, we additionally examined the levels of histone modifications reported in a genome-wide study (Pokholok et al., 2005) in the vicinity of G0-licensed origins as compared to non-G0-licensed origins. We note that origins that do retain pre-RCs in G0 have higher levels of histone H3 acetylation, particularly on lysine 14. However, because these modification levels were measured in an asynchronous population of cycling cells, this analysis will have to be repeated specifically with G0 chromatin to better understand its relevance to origin selection.

**pre-RC formation is slow during cell cycle re-entry**

As cells exit G0 and re-enter the cell cycle, pre-RCs form at the remaining origins. This additional licensing is dependent on Cdc6 and does not occur until two hours after release of cells back into nutrient-rich medium. This correlates with the time it takes to accumulate Cdc6 as cells exit G0 (data not shown) suggesting the possibility that Cdc6 expression is limiting for pre-RC formation upon re-entry into the cell cycle. Despite the fact that the same origins are licensed upon G0-exit as are licensed in cycling cells in G1, origin usage differs between the subsequent S-phases. Therefore, cell cycle re-entry may serve as a useful model for analyzing sequence-independent differences between origins that contribute to differences in the timing and extent of origin usage.

When Cdc6 expression is inhibited, cells exiting G0 fail to form additional pre-RCs and instead replicate their genomes using only those origins already licensed in G0.
Importantly, cells exit G0 earlier under these conditions as measured both by flow cytometry (Fig. 4a) and by budding (Fig. 5a). The ability of cells to exit G0 without licensing additional origins strongly suggests that all other requirements for cell cycle re-entry have already been met while wild-type cells are forming these additional pre-RCs. Thus, the completion of pre-RC formation appears to be rate-limiting in exit from G0. It remains to be tested if over-production of Cdc6 can reduce the amount of time required to form pre-RCs and whether this would in turn shorten the amount of time cells require to re-enter the cell cycle.

*The rate-limiting step of G0-exit is regulated*

The delay in G0-exit caused by pre-RC formation is dependent on Rad53. Interestingly, this delay is independent of factors involved in other Rad53 functions. Specifically, this cell cycle re-entry delay is not dependent on an essential Rad53 function in regulating nucleotide levels, the G1-DNA damage checkpoint, nor the intra-S phase checkpoint. Further experiments are necessary to determine how and whether Rad53 coordinated cell cycle re-entry with origin licensing.

*Complete pre-RC formation and origin function are important for viability*

Although cells are able to complete the bulk of DNA-replication with only the origins licensed in G0, fewer of these cells are viable than in a population of cells that replicate using a full complement of origins. Previous studies have suggested that significantly more origins are licensed than are required during S-phase (Dershowitz et
al., 2007; Okuno et al., 2001; Walter and Newport, 1997). It is reasonable to suggest, as well, that there is a lower limit to that origin redundancy, below which replication with fewer origins can have deleterious consequences to the cell. Some previous studies have used mutations that reduce pre-RC formation to study the effect of the number of activated replication forks on genomic instability (Ekholm-Reed et al., 2004; Lengronne and Schwob, 2002; Pflumm and Botchan, 2001; Tanaka and Diffley, 2002). It is difficult, however, to distinguish in these cases between the direct effect of reducing the number of origins and the possible effects of formation of improper replication complex formation or deregulation of cell cycle progression. Release of cells from G0 in the absence of Cdc6 provides a distinct situation in which to analyze the consequences of a fifty percent reduction in the number of replication forks. It will be interesting to exploit this system to study the intrinsic processivity of replication forks as well as the cellular mechanisms that safeguard fork progression.
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Materials and Methods

Cell growth

To obtain quiescent cells, yeast were grown in YPD to an OD600 of 0.2, spun down, washed twice with water, and resuspended in G0 medium. G0 medium was prepared as described (Diffley et al., 1994). Cells were released by washing once with water and then resuspending in rich medium.

Genome-wide location analysis and copy-number profiles

Location analysis was performed as previously described (Tanny, et al., 2006). Labeled samples were co-hybridized to custom microarrays from Agilent Technologies containing 44,290 probes spanning the yeast genome and analyzed as described.

For copy-number profiles in figure 2, DNA was collected every 5 minutes in a span encompassing S-phase. For profiles in figure 3, a single timepoint in mid-S-phase was used.

Data analysis was done partly using the R statistical environment (http://www.r-project.org) and partly using custom scripts written in Perl. Scripts and detailed statistical methods available upon request.

Flow cytometry

Fixed, RNased cells stained with Sytox Green (Invitrogen). Data were plotted using FlowJo™ software.
Supplementary Material

Supplemental Figure 1 – Data from all chromosomes as in Figure 1.

Supplemental Figure 2 – Data from all chromosomes as in Figure 2b.

Supplemental Figure 3 – Data from all chromosomes as in Figure 2c.

Supplemental Figure 4 – Analysis of chromatin modifications surrounding origins that retain pre-RCs in G0 (orange) and those that do not (blue). ACS positions are taken from (Nieduszynski et al., 2006). Origins are aligned so that the ACS is at position 0 and extends in the positive direction. The average density of histone modifications is calculated using data from (Pokholok et al., 2005).
Supplemental Figure 1
Supplemental Figure 2

chromosome 1

chromosome 2

chromosome 3

chromosome 4
Supplemental Figure 4
Chapter III

A screen for enhanced replication of a late-initiating origin
Summary

To better understand the coordination of replication initiation from diverse classes of origins in a eukaryotic S-phase, we conducted a screen for factors that enhance the replication of a plasmid-based, late-initiating origin. This screen yielded eight candidate genes, one of which was characterized further. *YOR006C* is a non-essential gene of unknown function. Deletion of this gene increases retention of a late-replicating plasmid under mildly-inhibitory conditions. Both genome-wide and global measurements of replication timing do not reveal gross changes in origin initiation in these cells. Instead, measurements of origin efficiency, global measurements of replication factor binding, and directed genetic assessments suggest that the absence of *YOR006C* stimulated initiation of weak origins, at least in part by increasing the efficiency of pre-Replicative Complex formation.
Introduction

The efficient duplication of a eukaryotic genome requires the coordinated activation of origins of replication spaced along each dividing chromosome. Potential origins of replication are marked throughout the cell-cycle by their association with the the six member Origin Recognition Complex (ORC) (Bell, 2002). ORC binding in the yeast *Saccharomyces cerevisiae*, requires a match to the 11-bp ARS consensus sequence (ACS). ORC binding in other organisms shows sequence preference but less sequence specificity (Gilbert, 2004). However, even in yeast, the sequence determinants of ORC binding are not sufficient to explain the distribution of ORC binding across the genome. Instead, additional poorly understood determinants of ORC binding – which may include chromatin structure and additional specificity factors – appear to play an important role. These non-sequence determinants of origin selection are also likely to be important in altering origin usage in response to environmental or developmental cues (Gilbert, 2005).

Origin selection by ORC binding to a sequence of DNA is not sufficient to ensure that replication initiate locally from that origin during S-phase. For a potential origins to initiate in S-phase it must also be rendered competent. This origin licensing is accomplished by the regulated loading of the Mcm2-7 helicase complex onto ORC-bound origin DNA (Diffley et al., 1994). This loading reaction requires the combined activity of ORC, the Mcm2-7 helicase and two helicase loading factors – Cdc6 and Cdt1.

Licensed origins are activated in S-phase by the combined activity of two kinases – the cyclin dependent kinase CDK and Dbf4-dependent kinase DDK. Interestingly, initiation events at different
origins occur at different times throughout S-phase (Brewer et al., 1993). Although origin usage differs between individual cells in a population, both local and global measurements of the time at which different regions of the genome are duplicated demonstrate a reproducible pattern of overall replication kinetics (Newlon and Theis, 2002). This replication profile is determined by the interplay of two factors. First, origins can differ in the efficiency with which they are utilized. If an origin fails to initiate in a given cell cycle, then that sequence will be replicated by a replication fork emanating from a nearby active origin. Regions of the genome containing consistently less efficient origins will therefore be duplicated later in S-phase, on average, than regions containing more active origins. Second, different active origins may differ in the time within S-phase at which they initiate replication (Brewer et al., 1993). Studies of replication intermediates in yeast have revealed origins that initiate in a majority of cell cycles, but whose initiation events tend to occur late in S-phase, leading to the suggestion that origins may have an intrinsic time within S-phase at which they can initiate. When initiation occurs at a given origin appears to be dependent on chromosomal context more than on the sequence of the origin itself (Ferguson and Fangman, 1992), although the relevant aspects of chromatin structure that may influence replication timing have not yet been identified.

The temporal distribution of initiation events in S-phase is particularly evident when the S-phase checkpoint is engaged. When replication forks are stalled or encounter lesions during replication, a number of mechanisms that prevent inappropriate S-phase progression are triggered. Among these mechanisms is the inhibition of replication initiation from origins that have not yet initiated. Treatment with a high dose of a genotoxic agent, such as Hydroxyurea (HU), effectively divides origins into two classes – those that initiate early enough to escape checkpoint control and those that are inhibited from
More recent studies suggest that this repression of replication initiation can be better understood as an overall retardation of initiation events rather than an absolute division between two classes of origins (Alvino et al., 2007). Nonetheless, the behavior of origins under genotoxic conditions provides a useful differentiation scheme between active/early origins and less active/later ones.

Although individual origins vary in when and if they initiate replication in a given cell cycle, origin timing and efficiency combine to produce a reproducible pattern of replication in a population. This profile, however, is dependent on developmental and environmental conditions. For instance, early in Xenopus development, initiation occurs randomly at sites spaced evenly across the genome, becoming restricted to specific sites as development progresses (Gilbert, 2001). The time of replication of an origin often correlates with the transcriptional state of the region. However, little is known about the determinants of origin usage in different cellular states (MacAlpine and Bell, 2005).

Here we describe a screen for factors that enhance the replication of a late/inefficient origin. We identify eight candidate genes that allow a late replicating plasmid to be duplicated despite inhibition by the S-phase checkpoint. We focussed our characterization on one candidate, the previously unstudied yeast ORF \textit{YOR006C}. We find that deletion of this gene does not generally alter replication timing, but instead influences replication efficiency. Deletion of \textit{YOR006C} enhances origin licensing at sites that normally for pre-RCs weakly. Finally, we show that \textit{YOR006C} is important for genome replication in cells returning to the cell cycle from quiescence.
Results

Screen rationale

To identify factors involved in pre-RC activation, we designed a screen for factors that, when deleted, enhance replication of a late/inefficient origin. The observation that some origins initiate early and efficiently and others do not can be explained with two models. Either some positive factor required to stimulate initiation preferentially acts on early/efficient origins or a negative active factor selectively inhibits initiation from later/inefficient origins. We designed a screen based on the latter possibility.

To conduct this screen we made use of an origin of replication, ARS301, that initiates late in S-phase both in its endogenous chromosomal context and when the minimal origin is on a plasmid, pARS301 (Bousset and Diffley, 1998; Santocanale et al., 1999; Santocanale and Diffley, 1996). We reasoned that if the ARS301 origin were regulated similarly to other late initiated chromosomal origins when it is on a plasmid, then activation of the S-phase checkpoint would inhibit replication of the plasmid. To test this hypothesis, we measured the rate of loss of the pARS301 plasmid with and without the addition of a sublethal dose of HU (50mM). Consistent with decreased replication of pARS301 when the S-phase checkpoint is engaged, the measured plasmid loss rate is significantly higher in 50 mM HU than in 0 mM HU (22% vs 7%, p<ZZ, figure 1a). In contrast, a plasmid harboring the early replicating origin ARS1 showed no change in plasmid stability when cells were grown in HU (Fig. 1A).
a.

![Graph](image1)

- % plasmid loss/generation
- HU (mM)
- pARS1 (early origin)
- pARS301 (late origin)

b.

- Growth
- Transformed
- HU1
- HU2
- HU3

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![Plate](image2)
Because ARS301 is located at the edge of the HML silent mating locus, we sought to determine if the inefficient replication of pARS301 in HU was dependent on the specialized chromatin that forms at the chromosomal HML locus. To this end, we repeated the plasmid loss measurements described above in cells lacking the silent mating chromatin component, Sir3. Although all plasmid were slightly more stable in sir3Δ cells, pARS301 was still lost at a higher rate in cells grown in 50 mM HU, suggesting that this property is independent of the silent mating locus chromatin (data not shown).

Replication enhancement screen

To identify factors that negatively regulate pARS301 duplication, we screened an ordered library containing all 3,995 non-essential yeast deletion strains. Factors of interest were defined as those that when deleted allowed pARS301 to be retained in 50 mM HU better than in wild-type cells. To conduct this screen we grew the deletion strains in 96-well plates to saturation. We then transformed pARS301 into each strain. Successful URA-marked transformants were selected by two rounds of growth in selective medium. Aliquots of transformed cells were then transferred into new plates containing non-selective medium with or without 50 mM HU. After two days of growth, cells from each plate were spotted onto both selective and non-selective petri dishes, and allowed to grow for an additional 36
hours (Fig. 1b). We monitored cell growth at each step by optically measuring the size of colonies formed under each condition. 95.6% of the strains that we grew were successfully transformed with pARS301. Of these 97.8% were able to grow both in 0 mM and 50 mM HU. A previous systematic test of this library identified 103 strains that are sensitive to the genotoxic agent MMS (Chang et al., 2002). Of those, 91 were found to be sensitive to HU and 12 were not. We also observe HU sensitivity for 51/62 of those 91 strains and for none of the 12 resistant strains. In addition we found 31 strains, not tested in the previous study that are sensitive to HU.

We found eight strains that maintained pARS301 even when grown in HU (table 1). We termed this phenotype “Replication of Late-origin Enhanced” (RLE). To confirm the RLE phenotype, we deleted the eight candidate genes in a clean genetic background and measured the plasmid loss directly with and without HU (Fig 1c). All eight strains showed a similar suppression of the high plasmid loss in HU. Two of the candidate genes, AHC1 and SET6, have described or implied roles in chromatin structure. Two of the candidate genes, SIM1 and XBP1, have been implicated in regulating cyclin levels under certain conditions. Three of the eight RLE genes are uncharacterized ORFs. We decided to focus our studies on one RLE gene in particular – YOR006C (RLE2). RLE2 encodes for a protein of unknown function with a predicted molecular mass of 36 kDa. Rle2 has one predicted protein-protein interaction with Cdc7 as determined by yeast two-hybrid.

We considered three ways in which deletion of RLE2 could affect the plasmid loss rate of pARS301. First, the absence of Rle2 could eliminate the S-phase checkpoint response to HU, thereby bypassing the normal inhibition of initiation of origins under these conditions. Second, deletion of RLE2 could advance late origin timing, thereby exempting the normally late replicating pARS301
plasmid from S-phase checkpoint control. Third, deleting \textit{RLE2} could increase the efficiency of the \textit{ARS301}, thereby lowering its loss rate. We tested each of these possibilities.

\textit{S-phase checkpoint response}

To test whether cells lacking Rle2 are defective in engaging the S-phase checkpoint, we measured the sensitivity of cells to genotoxic agents. The design of the screen ensured that any candidate we isolated must be able to grow in 50 mM HU. We took three additional approaches to test the ability of \textit{rle2} cells to respond to S-phase perturbations. First we measured the growth rate of cells grown in 50 mM HU. Second, we measured the ability of cells to return to growth after exposure to a high dose of HU (200 mM). Third, we tested the ability of cells to recover after exposure to uv-irradiation. We found no differences between wild-type and \textit{rle2} cells in any of these assays. Therefore we considered it unlikely that pARS301 is retained in \textit{rle2} cells due to a failure to activate an otherwise inhibitory checkpoint.

\textit{Replication Timing in rle2}\Delta

To measure replication timing in cells lacking \textit{RLE2}, we performed both local and global timing assays. First we measured the time of replication of specific early and late origins of replication using Heavy-Light density transfer assays. Briefly, cells are grown in medium containing heavy isotopes of Carbon and Nitrogen. These isotopes are incorporated into both strand of DNA over time. Cells are then allowed to pass synchronously through S-phase in medium lacking heavy isotopes. Newly synthesized DNA contains one heavy strand and one light strand. This heavy-light DNA can be
separated from the unreplicated heavy-heavy DNA by ultracentrifugation. Measurement of the ratio of heavy-heavy to heavy-light DNA at intervals reveals the time at which a particular fragment of the genome was replicated. Using wild-type and \textit{rle2Δ} cells, we performed density-transfer assays and examined the replication of two representative early- and late-initiating origins of replication (Fig. 2a). We failed to detect a significant difference in the time of replication of these origins between the two strains.

To comprehensively assess the affect of Rle2 on replication timing, we took an additional lower-resolution, genomic approach to measuring replication timing. Here DNA samples are collected at intervals in S-phase and pooled. Early replicating sequences are present in two copies in more of these samples than later replicating sequences, causing them to be over-represented in the pool as a whole. Relative abundance of each fragment of the genome is assessed by hybridizing the pooled DNA sample onto a genomic microarray, relative to an unreplicated standard sample. Plotting relative abundance against chromosome position gives a complete replication profile of the genome. Although there were some local differences in replication timing in \textit{rle2Δ} cells as compared to wild-type cells (see below), we did not observe any systematic differences between the time of replication of early or later origins between the two strains (Fig. 2b). Specifically, peaks in the profile represents sites of local replication initiation and hence origins of replication. The height of a peak corresponds to its relative time of replication in S-phase. Times of replication of origins in wild-type cells distribute bi-modally. The distribution of origin replication times in \textit{rle2Δ} cells does not significantly differ from this (Fig. 2c), arguing that the division of replication origins into those that are replicated early in S-phase and those replicated later is preserved.
Figure 2

ARS305 - normalized

wild-type

rle2Δ

ARS609 - normalized

wild-type

rle2Δ

b. frequency

chromosomal position

0 200 400 600

2 1 0 -1

c. frequency

wild-type

rle2Δ

relative time
Figure 2: (A) Density Transfer timing measurements of the early replicating ARS305 origin and late replicating ARS609. For each timepoint the abundance of signal is given in fractions ranging from unreplicated (HH) on the left to replicated (HL) on the right. Timepoints are minutes after αF release at 25°C. (B) Replication profile of chromosome X. Wild-type profile in blue is overlayed with rle2Δ profile in orange. (C) Distribution of times of replication of origins (profile peaks) for each strain.

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**Origin efficiency**

We took three complementary approaches to address whether Rle2 influences the efficiency of origin usage. First, we asked if deleting *RLE2* affects pre-RC formation. To do this we assessed Mcm2-7 association with origin DNA using genome wide location analysis (Fig. 3a). Wild-type cells show robust association with Mcm2-7 at previously identified origins of replication. Mcm2-7 associates with the majority of these same sites in *rle2Δ* cells. Interestingly, however, we noticed increased Mcm2-7 association in *rle2Δ* cells at sites where we detect only weak MCM association in wild-type cells. This difference is particularly evident when we plot ratio of Mcm2-7 signal between *rle2Δ* and wild-type cells for each origin as a function of the strength of Mcm2-7 signal in wild-type cells (Fig. 3b). From this analysis it is evident that the relative signal in *rle2Δ* cells (y-axis) is much higher for those origins that have weak Mcm2-7 signal in wild-type cells (x-axis, left side).

Figure 3: (A) Binding of Mcm2-7 across chromosome X in αF. Blue, wild-type and Orange, rle2Δ cells. (B) Comparison of the relative binding in rle2Δ cells compared to wild-type cells as a function of signal in wild-type cells.
Mcm2-7 signal in wild-type
ratio of signal – rle2Δ/wild-type

Figure 3

a. Chromosome X

b.
As a second approach to examining the effect of Rle2 on origin efficiency, we made use of previously characterized mutants of the well-studied ARS1 origin (Marahrens and Stillman, 1992; Wilmes and Bell, 2002). We measured the plasmid loss rate of plasmids containing ARS1-derived, mutant origins in wild-type and rle2Δ cells. The rate of plasmid loss was similar in both strains when a wild-type origin was used (Fig. 4a). The rate of loss is greatly increased when an ARS1-B1 mutant is used. This increased loss is partially suppressed, however, in cells lacking Rle2. To a lesser extent, deletion of RLE2 also decreased the plasmid loss in B2 mutant origins. We found no difference in the rate of plasmid loss in ARS1-B3 mutant origins.

Finally, we asked whether RLE2 displayed genetic interactions with several factors involved in pre-RC formation or activation. We combined a deletion of RLE2 with temperature sensitive mutations in ORC2, ORC5, CDC6, and CDC7. Cells containing an orc2-1 mutation failed to grow at any temperature above 24°C, with or without a deletion of RLE2. Deletion of RLE2 had little effect on the temperature sensitivity of cdc6-1 and appears to somewhat exacerbate the temperature sensitivity of cdc7-4 cells. In contrast, deletion of RLE2 enhanced the ability of orc5-1 cells to grow at both 30°C and 34°C. Thus rle2Δ was able to partially rescue the poor growth of an orc5-mutant, whose growth was previously shown to be rescued by over-expression of Cdc6 (Liang et al., 1995).
Figure 4: (A) Plasmid loss measurements for the indicated plasmids in the indicated strains. (B) 5-fold serial dilutions of the indicated strains grown at different temperatures.
Phenotypes associated with RLE2 deletion

To better understand the role of Rle2 in replication, we looked for phenotypes associated with deletion of RLE2. First we measured S-phase progression by flow cytometry. We observed only slight differences in S-phase progression between wild-type and rle2Δ cells. Furthermore, cells rle2Δ double at the same rate as wild-type cells, and do not demonstrate impaired growth at either high or low temperature.

We recently characterized origin licensing in quiescent yeast cells. Contrary to previous suggestions, we find that pre-RCs are maintained in G0 at about half of the origins that are licensed in cycling cells in G1. When cells return to growth from G0, pre-RCs form at the remaining, un-licensed origins. Unlike in cycling cells where pre-RC formation occurs within minutes of exit from mitosis, pre-RC formation transpires over the course of a few hours as cells re-enter the cell cycle. Therefore we were interested in whether RLE2 influences the retention of pre-RCs in G0 or the reformation of pre-RCs in the more limiting conditions that exist as cells exit G0. We induced quiescence in a logarithmically growing culture of wild-type or rle2Δ population of cells by depletion of nitrogen from the medium. Both populations entered G0 as evidenced by a 1C DNA content and uniformly unbudded cells.

We performed genome-wide location analysis to test the distribution of pre-RCs in G0. We observe robust binding of Mcm2-7 in rle2Δ cells as in wild-type cells (Fig 5a). We then examined the re-formation of pre-RCs upon G0-exit by repeating the location analysis two hours after cells were
returned to rich medium. Consistent with our previous observations, we find Mcm2-7 assembled on origins across the genome at this two hour timepoint (Fig. 5b). Pre-RC re-assembly also occurs in \( rle2\Delta \) cells, by two hours, but to a noticeably lesser extent. This is particularly evident when we overlay the Mcm2-7 signal in these two strains (Fig. 5b). In addition, we assessed the kinetics of cell cycle re-entry in wild-type and \( rle2\Delta \) cells. We find that both DNA replication, as measured by flow cytometry, and cell cycle progression, as assessed by budding, are delayed in \( rle2\Delta \) cells compared to wild-type cells by about thirty minutes to sixty minutes.

Figure 5: (A) Budding upon return to growth from quiescence. (B) Mcm2-7 association with chromosome X in G0 (top) and two hours after release from G0 (bottom). Overlay of wild-type (blue) and \( rle2\Delta \) cells (orange).
Figure 5

a. % budded

b. Mcm2-7 binding

G0

G0 + 2 hrs
Discussion

Here we describe a screen for factors that whose deletion enhanced the replication of a late-origin containing plasmid. We further investigated one candidate from this screen, the previously uncharacterized ORC \textit{YOR006C}, which we termed \textit{RLE2}. We found that while a deletion of \textit{RLE2} did enhance the maintenance of pARS301 in 50 mM HU, cells lacking Rle2 had no gross differences in replication timing nor in the ability to engage an S-phase checkpoint signal. Instead we found that in the absence of Rle2, pre-RC formation was increased at origins of replication where origin licensing is normally weak. Finally we find that disruption of Rle2 impairs the ability of cells to recover from cell cycle exit.

\textit{Rle2 is not involved in initiation}

Our initial hypothesis was that Rle2 might be a factor that prevented late origins from initiating early in S-phase. We reasoned that, if this is the case, then deletion of Rle2 would increase the stability of a late origin containing plasmid when grown in sub-lethal levels of Hydroxyurea by allowing the plasmid to replicate prior to inhibition by the S-phase checkpoint. However, we do not find gross differences in replication timing between \textit{rle2}\textDelta and wild-type cells. Our initial interest in Rle2 was also based on a reported yeast two-hybrid interaction with the initiation kinase Cdc7 (Uetz et al., 2000). However, we detect only a faint interaction with Cdc7 by co-Immunoprecipitation (data not shown). Instead, our data are consistent with a model in which the primary influence of Rle2 on DNA replication is by altering origin efficiency.
Deletion of RLE2 improves origin function of pARS301. A seminal screen for factors required for plasmid maintenance - the Mini-Chromosome Maintenance (MCM) screen - identified important components of the essential replication machinery (Maine et al., 1984). The screen carried out here is opposite in design to the MCM screen in that it sought to isolate factors in whose absence replication is enhanced. Thus the normal function of RLE genes appears to be to inhibit or restrain DNA replication. Indeed, we find that in the absence of Rle2, pre-RC formation is enhanced at sites that normally support only weak MCM loading. Additionally, we detect increased initiation from some these enhanced origins. This enhanced initiation from weak origins may underlie the ability of rle2Δ to partially rescue the temperature-sensitivity of an orc5-1 mutation.

Interestingly, we also observe decreased pre-RC formation at many origins at which pre-RC formation is robust in wild-type cells. This suggests the possibility that ORC binding in rle2Δ cells is redistributed from stronger origins to weaker origins. We therefore propose a model in which Rle2 acts as a specificity factor to preferentially direct pre-RC formation to a subset of efficient origins.

The molecular mechanism by which Rle2 affects replication remains unclear. Chromatin spin-down assays demonstrate chromatin association of a sizable fraction of the cellular Rle2 (data not shown), suggesting the possibility that Rle2 affects pre-RC formation directly at origins. We do not detect an interaction with ORC by co-Immunoprecipitation (data not shown) although other interactions between Rle2 and pre-RC components remain possible. It is also possible that Rle2 affects pre-RC
formation indirectly by influencing chromatin structure near origins.

**Function of Rle2 in replication progression**

Although Rle2 is dispensable for growth in cycling cells, we note that *rle2Δ* cells are markedly delayed in returning to growth from quiescence. Although we find that *rle2Δ* cells retain a subset of pre-RCs in G0 similarly to wild-type cells, they are impaired for re-formation of pre-RCs at the remaining origins upon return to growth. Origin licensing upon G0-exit takes long compared to the very rapid pre-RC formation upon exit from mitosis in cycling cells. In particular, it is presumed that Cdc6 is more limiting for pre-RC formation upon cell cycle re-entry than it is in cycling cells. It is an interesting possibility that under these more limiting conditions, Rle2 might play an important role in directing pre-RC formation to more robust origins. If this is the case, then the delay in pre-RC re-formation in *rle2Δ* cells could be due to the dispersal of Mcm2-7 loading to what are normally less efficient origins.

**Other RLE screen candidates**

We have focussed our attention on characterizing the role in replication of one particular candidate from the screen. It will be interesting to see whether other candidates have similar influences on pre-RC formation and replication progression. In particular, we note that two of the screen candidates, the histone acetyl-transferase component, Ahc1, and the histone methyl-transferase, Set1, have the potential to influence chromatin structure around origins of replication (Eberharter et al., 1999; Santos-Rosa et al., 2002), although both remain poorly characterized. Origin timing and efficiency are,
in most cases, heavily influenced by chromosomal context, and it is an interesting possibility that these chromatin factors might influence origin behavior locally.
Materials and Methods

Screen

A tiled array encompassing all non-essential yeast deletions (Winzeler et al., 1999) was grown in 96-well plates in 1 mL YPD without agitation for 2 days at 30°C, until large plaques formed. Media was removed by inversion of the plate and blotting. Cells were resuspended in 150 µL transformation mix (0.8 mL 50% PEG, 0.1 mL 1M LiAc, 0.01 mL 1M Tris pH 7.6, 0.002 mL 0.5M EDTA pH 8.0, water to 1 mL), 10 µL ssssDNA, and 1 µL plasmid (100ng/µL). Cells were then incubated at 30°C for 5 hrs. 17 µL DMSO were added to each well. Cells were then transferred to 42°C for 40-60 minutes. 900 µL of selective medium was added to each well. 25 µL of resuspended cells were then diluted into an additional 900 mL selective medium in a fresh plate and grown for 2 days at 30°C. After sufficient growth cells were frogged onto selective plates for subsequent screening.

To screen cells were replica plated three times onto both selective and non-selective plates containing 50 mM HU. Plates were then grown for 36-48 hours at 30°C. At each step the plates were recorded digitally and cell growth assessed by densitometry. After three rounds of growth, candidate genes were selected that retained significant growth on selective HU plates.

Strain construction and candidate verification

Candidate gene deletions were transferred into a W303 background by amplification of the longtine deletion module with primers 500 bp upstream and downstream of the candidate gene and confirmed by PCR. Plasmid loss was performed as previously described (Wilmes and Bell, 2002).
Cell growth

To obtain quiescent cells, yeast were grown in YPD to an OD600 of 0.2, spun down, washed twice with water, and resuspended in G0 medium. G0 medium was prepared as described (Diffley et al., 1994). Cells were released by washing once with water and resuspending in rich medium.

Genome-wide location analysis and copy-number profiles

Location analysis was performed as previously described (Tanny et al., 2006). Labeled samples were co-hybridized to custom microarrays from Agilent Technologies containing 44,290 probes spanning the yeast genome and analyzed as described.

For copy-number profiles in figure 2, DNA was collected every 5 minutes in a span encompassing S-phase. For profiles in figure 3, a single timepoint in mid-S-phase was used.

Data analysis was done partly using the R statistical environment (http://www.r-project.org) and partly using custom scripts written in Perl. Scripts and detailed statistical methods available upon request.

Density Transfer

Density transfer timing assays were performed as previously described (Raghuraman et al., 2001).
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Chapter IV

Discussion and Future Directions
**Key conclusions**

In this thesis I address the question of how cells ensure that the capacity to replicate has been established prior to S-phase in two systems. In cycling cells, I show that a novel yeast gene, *YOR006C*, alters origin licensing throughout the genome by enhancing pre-RC formation at weak origins. This redistribution leads to changes in S-phase, which are particularly evident as cells re-enter the cell cycle from quiescence. Yeast cells that have entered quiescence ensure the ability to replicate upon return to the cell-cycle in two ways. First, G0 cells retain pre-RCs at about half of the origins used in cycling cells. Secondly, upon G0-exit additional origins are licensed and this licensing is monitored by the checkpoint factor Rad53. Interestingly, the half of the origins already licensed are sufficient to duplicate the genome and the additional pre-RC formation that occurs as cells return to growth is rate-limiting for cell-cycle re-entry. Together these results provide new insights into how DNA-replication is coordinated with cell-cycle progression.

**Three-step model of origin usage**

A major focus in the field of DNA replication for many years has been understanding how sites of replication initiation are determined across the genome under a given condition. Much of this work has been focussed on elucidating both the sequence-dependent and sequence-independent determinants of ORC binding (Gilbert, 2004). Although ORC binding is a prerequisite for the establishment of an origin locally, a more complete view of origin determination must take into account three steps. First, origins must be selected by ORC binding. Second, a subset of these sites are then licensed to replicate by the loading of Mcm2-7 helicase complex. Third, a subset of licensed origins are then activated – to varying degrees – in S-phase.
In this thesis, I identify a factor, \textit{YOR006C (RLE2)}, that affects origin usage by altering origin licensing throughout the genome. Of particular note is that deletion of \textit{RLE2} does not uniformly decrease pre-RC formation, like mutation of an origin licensing factor does. Instead, pre-RC formation is increased at origins that normally load Mcm2-7 only weakly and decreased at sites where loading is normally robust. We do not see significant differences in ORC binding across the genome. Thus, we envision that Rle2 determines origin usage by directing Mcm2-7 loading to certain origins at the expense of others.

It remains to be investigated how, mechanistically, Rle2 alters origin licensing. Chromatin spin-down assays show Rle2 to be chromatin associated, although this association is not ORC-dependent. Thus it is possible that Rle2 acts, locally, at efficient origins to recruit pre-RC components. It is also possible that Rle2 acts at inefficient origins to inhibit pre-RC formation. It is interesting to remark that two of the other candidate genes isolated in the same screen are thought to affect chromatin structure (\textit{AHC1} and \textit{SET6}). Thus it is a possibility that local chromatin sets a threshold for pre-RC formation at different ORC sites.

**Correlation between origin licensing and initiation**

The altered distribution of Mcm2-7 in \textit{rle2Δ} cells does not result in systematically a altered replication profile in cycling cells. The most significant change that we observe is increased initiation from weak origins that have increased pre-RC formation in \textit{rle2Δ} cells. There has been recent speculation as to whether origins of replication actually have an intrinsic time on initiation in S-phase or whether differences in the time of replication of different origins can be explained by differences in origin efficiency alone (Rhind, 2006). While our data do not address this question directly, the earlier
time of replication of origins with increased pre-RC formation does suggest that changes in the replication profile of a genome can, under some circumstances, be driven largely by changes in origin efficiency.

We do not observe differences in the replication profile of \textit{rle2}\textgreek{A} cells at many origins despite changes in Mcm2-7 loading at those sites. It has been appreciated for some time that the level of Mcm2-7 detected at origins in G1 does not correlate with the time or efficiency of origin activation in S-phase (Newlon and Theis, 2002). Our data suggest, additionally, that reducing Mcm2-7 loading at specific origins does not reduce their ability to efficiently initiate. In a number of systems investigated, Mcm2-7 complexes have been found to assemble in a stoichiometric excess to the number of ORC molecules bound (Edwards et al., 2002; Lei et al., 1996). It is unclear how many of the loaded Mcm2-7s are functional and whether this apparent excess of assembled molecules is required for efficient replication. Our data suggest that, at specific origins, reduction of Mcm2-7 loading by as much as 2-fold has no apparent consequence to origin usage, within the range of the assays we used.

\textbf{Yeast retain a subset of pre-RCs in G0}

It is a surprising finding that quiescent yeast retain a subset of licensed origins. Although we find some differences between those origins with pre-RCs and those without, it is unclear what the fundamental difference between these origins is. We note a concordance between these origins and origins which reform pre-RCs under re-replicating conditions. The susceptibility to pre-RC reformation is a property that is retained when an origin is moved to an ectopic location. It will be interesting to see whether the presence of a pre-RC in G0 is similarly context-independent.
In cycling cells, pre-RCs form as cells exit mitosis. Cells enter quiescence from mitosis. The presence of pre-RCs at a subset of origins in G0 implies either that when cells commit to cell-cycle exit pre-RCs only form at some origins or else that pre-RCs form at all origins but are only retained at some. During *Drosophila* embryogenesis, cells switch at the 16th division from a cycle lacking G1 to one in which G1 follows mitosis. During this transitionary division, pre-RCs do not form at the exit from mitosis, suggesting that this temporal coupling is not obligatory (Su and O'Farrell, 1997). In contrast, examination of a fluorescently-labeled MCM subunit in *S. pombe* starved of nitrogen suggests that pre-RCs are first formed coming from a final mitosis and then lost as cells enter G0 (Namdar and Kearsey, 2006). Furthermore, nuclei from human Swiss 3T3 cells loose the ability to replicate in a *Xenopus* extract slowly, over the course of many days (Sun et al., 2000). This residual replicative capacity is independent of exogenous MCMs, implying a gradual loss of MCMs over time. However in both fission yeast and in human cells pre-RCs are absent from all origins in G0 and it is unclear whether budding yeast regulate pre-RC loss similarly.

It is interesting to note that cells arrested in late-G1 with the mating pheromone αF require continued Cdc6 function to maintain Mcm2-7 association with origins (Aparicio et al., 1997; Donovan et al., 1997). Preliminary evidence suggests that inactivation of Cdc6 does not cause a equivalent loss of MCMs at all origins, suggesting that some pre-RCs may be more stable in the absence of Cdc6 than other (data not shown). It would be interesting to test whether ectopic expression of Cdc6 in G0 is sufficient to induce formation of pre-RCs at those origins not normally licensed in G0.

The presence of pre-RCs in G0 in yeast differs from the previously observed absence of pre-RCs
in quiescent mammalian cells. Many differentiated cells lose the capacity to divide over time. This capacity has been shown to correlate with a reduced amount of pre-RC components in cells. In contrast to yeast, differentiated mammalian cells that lack mitogenic signal are less likely to return to nutrient rich growth conditions. One exception in multi-cellular organisms is adult stem cells, which retain the ability to both self-duplicate and generate differentiated progeny through the organism's life. While in some tissues, such as the skin, stem cells are continuously dividing, in other tissues, such as the liver and blood, the stem cells are kept in a quiescent state until stimulated to divide. It is an interesting proposition that these quiescent stem cells may be regulated differently than differentiated cells induced into G0 in culture with respect to DNA-replication.

**Pre-RC formation is monitored as cells exit G0**

It has been an open question for some twenty years, why quiescent cells, stimulated to divide, spend so much long in a G1-like state than cycling cells. Here I showed that in the absence of Cdc6-expression, cells exiting G0 replicate with half as many licensed origins of replication. Interestingly, these cells exit G0, as assessed both by budding and by flow cytometry, more rapidly than wild-type cells. The ability of these cells to enter the cell-cycle and replicate their DNA implies that all other aspects of preparing a cell for growth have been completed and that the slowest process involved in cell-cycle re-entry is additional origin licensing. It is likely that other aspects of the G0/S transition are still slow, compared to G1 cells, as even in the absence of Cdc6-expression cells still take a number of hours to enter the cell-cycle. However, it will be interesting to test whether induction of Cdc6-expression early in G0 can promote additional pre-RC formation and whether this would, in turn, reduce the time it takes for cells to re-commence the division cycle.
Regulation of cell cycle re-entry

The additional pre-RC formation that occurs late in G0 is not only rate-limiting for cell-cycle re-entry, but it is also monitored by a cell-cycle checkpoint. The delay in G0-exit induced by origin licensing is bypassed by inactivation of the checkpoint kinase Rad53. However, factors that act with Rad53 in other cell-cycle checkpoints are not. It is likely, therefore, that as yet unidentified factors mediate checkpoint signaling in this case. Specifically, the DNA-Damage and Intra-S Phase checkpoints each have unique adaptor proteins that activate Rad53, and such a factor is likely to exit here as well. It may be possible to identify such a factor by testing the involvement of factors that interact genetically or biochemically with Rad53 but are uncharacterized in this checkpoint response.

Although Rad53 may interact with unique partners in regulating G0 exit, it is likely to affect a S-phase re-entry delay in a manner similar to the Rad53-dependent G1-delay induced by DNA-damage. The only identified target of Rad53 during G1-damage is the transcription factor subunit Swi6. Phosphorylation of Swi6 leads to loss of chromatin-binding and therefore down-regulation of G1/S transcripts. It will be interesting to test whether Swi6 phosphorylation is involved in this G0-exit regulation as well. A subset of Rad53-dependent phosphorylation sites on Swi6 have been identified, and mutation of these sites leads to partial loss of a G1-damage response. Although the phosphorylation of Swi6 has only been detected by 2-D gel, it would be very feasible to examine the chromatin binding of Swi6, and in fact its promoter association, during checkpoint engagement. In addition, inhibition of other elements of the G0/S transition machinery may be involved in this arrest.
Although complete duplication of the genome is possible from half as many origins, I observed that these cells are less viable than cells that replicate using all of their origins. Thus although quiescent yeast retain the ability to replicate without the need for additional licensing, they also ensure efficient duplication by monitoring additional pre-RC formation upon return to nutrient-rich conditions.

Finally, it is interesting to note that, in G0, although cell cycle re-entry is delayed in rle2Δ cells, where pre-RC formation is delayed, the complete absence of pre-RC formation does not cause a re-entry delay. Similarly, the inability of cells to form any pre-RCs in G1, such as when cells lack Cdc6 activity, does not prevent cell cycle progression. This failure to engage a checkpoint leads cells to commit to a fatal cell-cycle during which the lack of replication is followed by an aberrant mitosis and rapid cell death. While the inability of a checkpoint to prevent this catastrophe may seem to significantly reduce the value of such a monitoring mechanism to preserving genomic integrity, it might be explained by noting that evolution does not act on boundary cases. If a cell cannot form any pre-RCs, then a delay in G1 is not likely to prevent subsequent cell death. In contrast, if a cell has formed some pre-RCs but is delayed in the completion of this process, then selective pressure might favor the evolution of such a control mechanism.
Bibliography


Appendix 1

A pre-RC Assembly Checkpoint Coordinates Origin Licensing with Cell-Cycle Progression
Note:

The experiments described in this appendix were not sufficiently reproducible at the time of publication of this thesis to warrant inclusion as a chapter. Nevertheless, some of the observations may prove of use in future studies of the fundamental question addressed here: How cells coordinate cell cycle entry with origin licensing. Two conditions of these experiments require further investigation, in particular. First, many of these experiments use a temperature sensitive allele, *orc1-161*, of the gene encoding the largest ORC subunit. This allele contains a number of mutations and the ability to create strains that uniformly bear the same genetic alterations has been, at times, challenging. Secondly, the experiments described were performed under very particular growth conditions. For G2/M release experiments cells were grown to exponential phase in glucose, shifted to the non-permissive temperature for one hour, and released at the non-permissive temperature in raffinose. Release into a better metabolized sugar such as glucose or perturbation of the growth conditions altered the results obtained. Given the sensitivity of the G1 phase of the cell cycle to growth and metabolism, more should be done to understand the influence of environmental conditions on these experiments.
Summary

Eukaryotic DNA replication must be closely coordinated with cell cycle progression to ensure genome stability. This coordination requires pre-replicative complex (pre-RC) formation at origins of replication in G1 phase followed by the activation of these licensed origins in S phase. Here we describe experiments in *S. cerevisiae* cells consistent with a checkpoint that delays the G1/S transition in response to ongoing pre-RC formation. Elimination of pre-RC formation expedites G1 exit whereas incomplete pre-RC formation causes a G1 delay. This delay depends on the Rad53 but not the Mec1 checkpoint kinase and is distinct from known DNA-damage checkpoints. The resulting G1-delay is mediated by down regulation of G1 cyclins. In the absence of the checkpoint, defective pre-RC formation leads to cell death. These observations reveal a regulatory mechanism that contributes to genome stability by increasing the fidelity of DNA replication events prior to commitment to S-phase.
Accurate and complete duplication of eukaryotic genomes requires the precisely controlled assembly of replication forks at numerous origins of replication. Origins are initially identified and bound by the six-member origin recognition complex (ORC). Upon exit from mitosis, Cdc6 and Cdt1 combine with ORC to license origins by loading the Mcm2-7 replicative helicase onto origins to form pre-replicative complexes (1). As cells enter S-phase, two kinases, cyclin-dependent kinase (CDK) and Dbf4-dependent kinase combine to activate pre-RCs and, through the recruitment of many additional proteins, to establish bidirectional replication forks at origins (2).

The separation of origin licensing and origin activation is central to the coordination of DNA replication with the cell cycle. The separation of these two events is primarily controlled at the level of pre-RC formation, which is tightly restricted to the G1 phase of the cell cycle. Exit from G1 is marked by an increase in CDK activity that triggers initiation from licensed origins. Importantly, the same CDK activity, together with other mechanisms, inhibits new pre-RC formation during the S, G2, and M phases of the cell cycle (3). This control ensures that origins cannot be re-licensed or re-initiate within the same cell cycle, an event that would result in DNA damage and aneuploidy (4).

Proliferating eukaryotic cells must balance strict control over re-initiation with the need to license sufficient origins. Entering S-phase with an insufficient number of licensed origins causes increased mutation, genomic instability and lethality (5, 6). Previous studies have left unclear whether cells monitor the completion of pre-RC formation before CDK activity increases at the end of G1 (7). Studies of S. pombe and human cells suggest that such a mechanism might exist, as partial inactivation of ORC in these systems delays exit from G1 (8-10). In contrast, other mutants that inactivate pre-RC
components cause cells to progress through mitosis without replicating their DNA, suggesting that the absence of licensed origins fails to restrain cell-cycle progression (11-14). Here, we present data to suggest that ongoing pre-RC formation, but not the absence of pre-RCs, generates a signal that delays G1-exit.

To understand how origin licensing affects cell-cycle progression we monitored G1-exit in synchronized cells after inactivation of pre-RC components. We arrested wild-type cells and cells containing a temperature-sensitive mutation in the largest ORC subunit, orc1-161, at G2/M by the addition of nocodazole. We then released cells from the G2/M block at the non-permissive temperature in raffinose-containing medium to slow cell-cycle transit. Upon release, we monitored the M-G1 transition (by the disappearance of large-budded cells and the appearance of unbudded cells with G1 DNA content) and G1 exit (by the appearance of small budded cells, Fig. 1a).

Inactivation of Orc1 caused cells to delay in G1, consistent with results in S. pombe and human cells (8-10). When released from nocodazole at the non-permissive temperature, orc1-161 cells failed to form small buds and instead accumulated as unbudded cells with unreplicated DNA (Fig. 1b and Supp. Fig. 1). This delay is not specific to the Orc1 allele as temperature-sensitive orc2-1 cells also show a G1 delay, although of shorter duration (Fig. 1c). The G1-delay exhibited by orc1-161 cells can be bypassed by elimination of the checkpoint kinase Rad53 (Fig 1d.). Checkpoint deficient orc1-161 rad53-11 cells do not accumulate as unbudded cells and instead exit G1. Consistent with the bypass of a G1 arrest, flow cytometry of orc1-161 rad53-11 cells reveals a pattern of DNA content (including a subset of cells with sub-G1 DNA content) consistent with passage through S-phase in the absence of
DNA replication, followed by a reductional anaphase (13) (Supp. Fig. 2).

Rad53 functions to arrest the cell cycle at multiple points in response to DNA damage or replication stress (15). We asked if other proteins involved in the Rad53-dependent response to these lesions eliminated the orcl-161-dependent G1 delay. Interestingly, unlike the Rad53 mutation, inactivation of Rad24, Rad9, Mrc1, and Mec1 did not bypass the arrest (Fig. 1d and Supp. Fig. 2), indicating that this control is distinct from the established DNA-damage and S-phase checkpoint pathways.

**Figure 1**: Mutation of ORC subunits causes a Rad53-dependent G1-delay. (A) Schematic of the experiment. Cells arrested in G2/M by the addition of nocodazole are large budded (plotted in blue). As cells are released from nocodazole, they transition into G1 and appear unbudded (plotted in green). Exit from G1 is marked by the appearance of small buds (plotted in orange). (B) Budding indices are plotted for wild type and orcl-161 cells released from nocodazole at 37°C in raffinose. (C) As in (B) with wild type and orc2-1 cells. (D) Budding indices of double mutants of orcl-161 with known checkpoint factors released from nocodazole as in (B).
In contrast to mutation of ORC, elimination of another pre-RC component, Cdc6, did not result in G1-arrest. Rather, when *cdc6-1* cells were released from a G2/M block at the non-permissive temperature they showed a more rapid and synchronous G1 exit than wild-type cells (Fig. 2a), raising the possibility that pre-RC formation is rate-limiting in wild-type cells. As has been described previously, flow cytometry of the *cdc6-1* cells is consistent with nuclear division in the absence of DNA replication (Supp. Fig. 3) and is similar to *orc1-161 rad53-11* cells.

The discrepancy between the *orc1-161* and *cdc6-1* arrests is reminiscent of the distinct cell cycle arrest points previously observed when different pre-RC mutants were analyzed. Prior characterization suggested that, although the Cdc6 allele used here prevents all replication (13, 16), whereas many Orc-alleles, including those used in this study, are incompletely penetrant (17, 18). In addition, we noted that the *cdc6-1* cells lacking pre-RCs showed similar properties to *orc1-161 rad53-11* cells that lacked the G1-delay. Therefore, we considered the hypothesis that incomplete pre-RC formation in the ORC-mutant cells generates a Rad53-dependent signal that delays G1-exit whereas the complete absence of pre-RC formation in the Cdc6 mutant cells fails to generate such a signal.

We used several approaches to test this hypothesis. First, we asked whether the precocious G1-exit observed for *cdc6-1* cells requires complete inactivation of Cdc6. To accomplish this, we arrested wild-type and *cdc6-1* cells at G2/M and then released them at a semi-permissive (30°C) temperature. Consistent with this being a semi-permissive temperature, we observed intermediate levels of MCM origin association at this temperature compared to no association at 37°C (Fig. 2c). Unlike cells released at 37°C, *cdc6-1* cells released at 30°C did not show a precocious G1-exit. Instead, these cells
consistently show a modest delay in G1-exit compared to wild-type cells as evidenced by the delay in the peak appearance of small budded cells (Fig. 2d, maximum small budded cells at 2.5 hrs. for cdc6-1 vs. 2 hrs. for WT).

If the hypothesis is correct, we would expect eliminating the ability to detect ongoing pre-RC formation would mimic elimination of pre-RCs. To test this possibility, we assessed progression through G1 for cells containing the rad53-11 allele that bypass the orc1-161 G1-delay. Indeed, when tested in the same protocol, rad53-11 cells exhibited more rapid and synchronous passage through G1 compared to wild type cells (Fig. 2b and Supp Fig. 4).

Fig. 2: Mutation of Cdc6 does not cause a G1-delay. (A) Nocodazole release of wild-type and cdc6-1 cells at 37°C in raffinose. (B) Association of Mcm2-7 with the ARS305 origin (top band) and the non-origin URA3 locus (bottom band) in wild-type and cdc6-1 cells at the indicated temperatures. (C) Nocodazole release of wild-type and cdc6-1 cells at 30°C in raffinose. (D) Nocodazole release of wild-type and rad53-11 cells at 30°C in galactose. (This leads to a slower G1 in wild-type cells than in raffinose.)
If incomplete pre-RC formation is the signal for G1 delay, then the effect of a complete inhibition of pre-RC formation should be dominant to the effect of a partially penetrant mutation. To test this, we investigated the G1-arrest phenotype in \textit{orc1-161 cdc6-1} double mutant cells. When released from nocodazole \textit{orc1-161 cdc6-1} double mutants readily pass through G1 (Fig. 3a) and undergo nuclear division in the absence of DNA replication (Supp. Fig. 3). This result indicates that Cdc6 function is required for the G1 arrest and strongly suggests that the G1 delay observed in \textit{orc1-161} cells depends on ongoing pre-RC formation and not on a pre-RC independent function of ORC in G1-exit.

If partial penetrance of the \textit{orc1-161} allele leads to incomplete pre-RC formation, then we would expect these cells to retain some level of ORC association. To test this, we arrested WT and \textit{orc1-161} cells at the non-permissive temperature in G1 and used genome-wide location analysis to measure ORC association with origins. Wild-type cells showed robust association of ORC with origins of replication whereas \textit{orc1-161} cells showed reduced, but significant, ORC association at a subset of origins (Fig. 3b). Interestingly, \textit{orc1-161} cells do not show significant MCM association in G1 (Supp. Fig. 5), suggesting that the residual chromatin-associated ORC in these cells cannot support complete pre-RC formation. Together, these experiments are consistent with incomplete pre-RC formation, but not a complete absence of pre-RC formation, inducing a Rad53-dependent G1-arrest.
Figure 3: G1-delay of orc1-161 cells depends on ongoing pre-RC forming activity. (A) orc1-161 and orc1-161 cdc6-1 cells released from nocodazole at 37° C in raffinose. The orc1-161 control is from the same experiment as in Figure 1b. (B) Genome-wide location analysis of ORC in wild-type and orc1-161 cells. ORC ChIP-signal versus chromosome position (in kb) is plotted for chromosome X.
We next examined the mechanism of G1 arrest in response to incomplete origin licensing. We first examined expression of a yeast G1-cyclin, Cln2. G1 cyclins are necessary for cells to exit G1 and wild-type cells released from nocodazole express Cln2 as they pass through late G1 (Fig. 4a). In contrast, orc1-161 cells released at the non-permissive temperature showed dramatically reduced Cln2 expression. To test whether reduced levels of G1 cyclins are responsible for the G1 arrest in orc1-161 cells, we expressed Cln2 under a galactose-inducible promoter after wild-type or orc1-161 cells were released from nocodazole arrest at the non-permissive temperature. Consistent with reduced G1-cyclin expression being responsible for the G1 arrest, orc1-161 cells released with induced Cln2 expression (in galactose) showed no delay in G1-exit (Fig. 4b). We observed no change in the orc1-161 G1-arrest when Cln2 expression was not induced (in glucose). Additionally, orc1-161 cells undergo nuclear division in the absence of replication only when Cln2 is induced (Supp. Fig. 7). Therefore, orc1-161 mutants released into G1 fail to complete pre-RC formation, accumulate as unbudded cells and fail to express Cln2.

To test the biological significance of a prolonged G1 in response to delayed pre-RC formation, we asked if bypassing the G1 delay in orc1-161 cells decreased cell viability. We released wild-type and orc1-161 cells at 37° either with or without Cln2 induction from a G2/M block as above. At intervals, we plated serial dilutions of cells back to the permissive temperature without induced Cln2 expression to assess viability. Orc1-161 released with Cln2 induction (galactose) rapidly lost viability compared to cells released without Cln2 induction (glucose) (Fig. 4c). Wild-type cells remained viable under both conditions. This finding suggests that prolonging G1 in response to incomplete pre-RC formation prevents deleterious cell-cycle progression in the absence of sufficient pre-RC formation.
Fig. 4: Incomplete origin licensing inhibits cyclin accumulation at G1-exit. (A) CLN2-HA and orc1-161 CLN2-HA cells released from nocodazole at 37°C in glucose. Cells were assessed for budding and for the accumulation of Cln2-HA by immunoblotting of cell extracts. Pgk1 was used as a loading control. (B) pGAL1-CLN2 and orc1-161 pGAL1-CLN2 cells released from nocodazole at 37°C in either glucose (left) or galactose (right) to induce Cln2 expression. (C) Five-fold dilutions of cells in (B) were spotted at indicated times onto YPD plates and colonies allowed to form at 25°C.
Together our data support the existence of a checkpoint that delays the G1/S transition in response to incomplete pre-RC formation, thereby coupling origin licensing with cell cycle progression. The signal activating the checkpoint depends on Cdc6 activity, but cannot detect the complete absence of pre-RCs. This is analogous to the intra-S phase checkpoint that readily detects defects in ongoing replication but does not slow the cell cycle when replication is completely inhibited (19). Based on studies of the G1-DNA damage checkpoint, we suspect that Rad53 mediates this G1 arrest by inhibiting transcription of G1-specific target genes including Cln-type cyclins (20). CDK activity inhibits pre-RC formation to restrict origin licensing to a window between mitotic exit and the end of G1-phase. Thus, preventing Cln-CDK expression in response to incomplete pre-RC formation would provide additional time for origin licensing prior to the irreversible commitment to DNA replication.

We find that elimination of Cdc6 advances G1-exit relative to wild-type cells, suggesting that origin licensing is a rate-limiting event for cells passing through G1. Intriguingly, eliminating Rad53, and presumably the ability to detect incomplete pre-RC formation, also causes a similar advance in G1-exit. This raises the possibility that the checkpoint we have identified influences the timing of an unperturbed G1 similar to the role of the spindle checkpoint during an unchallenged anaphase in mammalian cells (21).

A similar checkpoint is likely to regulate cell cycle progression in other organisms. A reduction of Orc2 levels in human cells reduces pre-RC formation and results in both slower G1/S progression and decreased cyclin E levels (10). Similar to the effect of Cln2 induction (Fig. 4b), abrogation of this G1-delay by depletion of CDK inhibitors causes increased apoptosis, suggesting that a cell-cycle delay
caused by reduced origin licensing contributes to cell-viability (9). Finally, over-expression of cyclin E in human cells results in reduced association of pre-RC components with chromatin and accelerated entry into S phase (22). The effect of cyclin E over-expression on pre-RC formation has been proposed to be due to direct inhibition of pre-RC formation by cyclin E-Cdk2. Our data suggest that increased cyclin E may also bypass a pre-RC checkpoint allowing cells to enter S phase prior to complete pre-RC formation. Cyclin E over-expression is observed in many cancer cells and is associated with chromosome instability (23). It is an intriguing possibility that bypassing a pre-RC assembly checkpoint cells would allow cells to enter S phase with insufficient licensed origins and that this could contribute to genomic instability in cancer cells.
References and Notes

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Randell for critical reading of the manuscript. S.P.B. is an investigator of the Howard Hughes Medical Institute.
Materials and Methods

**cell growth and synchronization**

For arrest and release experiments cells were grown at 25° C in YPD to an OD\textsubscript{600} of 0.3 and arrested in G2/M by the addition of nocodazole (10 µg/ml). After 3.5 hours cells were shifted to 37° C (or 30° C in figure 2 as indicated) for 1 hour while maintaining the G2/M arrest. Next, cells were washed twice with water and resuspended in fresh medium containing either raffinose, glucose, or galactose as indicated at 37° C. Budding samples were sonicated briefly and at least 200 cells were counted per sample. For MCM and ORC chromatin immunoprecipitation, cells were arrested as above and released into medium containing α-factor (10 ng/mL). Probes as in Aparicio, et al., 1997.

For Cln2 over-expression experiments, \textit{pGAL1-CLN2} and \textit{orc1-161 pGAL-CLN2} strains were grown at 25° C in YP+raffinose and arrested by the addition of nocodazole. Cells were shifted to 37° C for one hour. Half of the culture was released from nocodazole into YP+glucose and the other half released into YP+galactose, both at 37° C. To assess subsequent cell viability, five-fold serial dilutions were spotted onto YPD plates at 25° C at the indicated intervals. Colonies were allowed to grow for two days.

**genome-wide location analysis**

Location analysis was performed as previously described (Tanny, et al., 2006). Labeled samples were co-hybridized to custom microarrays from Agilent Technologies containing 44,290 probes spanning the
yeast genome and analyzed as described. Complete data sets are available upon request.

**immunoblotting**

Total cellular protein was collected by standard trichloroacetic acid (TCA) precipitation. Proteins were separated by electrophoresis on 10% polyacrylamide gels. Cln2-HA was detected using 12CA5, α-HA antibody. A monoclonal antibody (α-Pgk1 A 6457, Molecular Probes) was used to detect 3-phosphoglycerate kinase as a loading control.

**flow cytometry**

Fixed, RNased cells stained with Sytox Green (Invitrogen). Data were plotted using FlowJo™ software.


Supplementary Material

Supp. Fig. 1: Flow cytometry of wild-type and orc1-161 cells corresponding to timecourses shown in Fig. 1a.
Supp. Fig. 2: Flow cytometry of *orc1-161*, *orc1-161 rad9Δ*, *orc1-161 mec1Δ sml1Δ*, *orc1-161 rad53-11*, *orc1-161 mrc1Δ*, and *orc1-161 rad24Δ* cells corresponding to timecourse shown in Fig. 1b.
**Supp. Fig. 3:** Flow cytometry of *cdc6-1* and *orc1-161 cdc6-1* cells corresponding to timecourses shown in Fig. 2a and Fig. 3a.

**Supp. Fig. 4:** Flow cytometry of wild-type and *rad53-11* cells corresponding to timecourses shown in Fig. 2b.
Supp. Fig. 5: Genome-wide location analysis of Mcm2-7 in wild-type and orcl-161 cells. Mcm2-7 ChIP-signal versus chromosome position (in kb) is plotted for chromosome X.
Supp. Fig. 6: Flow cytometry of $cln2$-HA and $orc1$-$161$ $cln2$-HA cells corresponding to timecourses shown in Fig. 4a.
Supp. Fig. 7: Flow cytometry of pGAL-CLN2 and orc1-161 pGAL-CLN2 cells released in glucose (left) and galactose (right) corresponding to timecourse shown in Fig. 4b.