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Chromosomal rearrangements and changes in copy number at various genomic loci are hallmarks of cancer cells and may be very early steps in tumorigenesis. The origins of genomic insults are poorly understood and this proposal aims to characterize one potential source of genomic instability, inappropriate DNA re-replication. In a normal eukaryotic cell cycle, the chromosomal DNA of a cell is replicated once, and only once, during S phase to ensure that each daughter cell receives exactly one complement of genomic material. By perturbing the regulation of several proteins involved in replication initiation, our laboratory has been able to conditionally induce varying amounts of re-replication in yeast cells. In this reporting period, we have shown that re-replication induces a rapid and significant decrease in cell viability and a cellular DNA damage response. Strikingly, we have observed DNA damage in the absence of a classical replication stress response. These results indicate that re-replication generates DNA damage, and raise the possibility that this could in turn lead to genomic instability.

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Introduction

Breast cancer is the second most frequently diagnosed cancer in American women and more than a quarter of a million Americans will be diagnosed with this disease in 2003 (American Cancer Society). Clearly, breast cancer is a disease that is in dire need of a cure. Genomic instability is common in breast cancer cells and can lead to loss of heterozygosity (Taback et al., 2003), gene amplifications (Lopez-Guerrero et al., 2003) and other genomic rearrangements. By inducing genomic alterations, genomic instability may promote carcinogenesis or make cancers more resistant to treatment. Currently, the source of genomic instability is unknown, retarding efforts to prevent or cure breast cancer by reducing genomic instability.

Chromosomal rearrangements are hallmarks of cancer cells and may be very early steps in tumorigenesis. The origins of genomic insults are poorly understood and this work aims to characterize one potential source of genomic instability, inappropriate DNA re-replication. In a normal eukaryotic cell cycle, the chromosomal DNA of a cell is replicated once, and only once, during S phase to ensure that each daughter cell receives exactly one complement of genomic material. By perturbing the regulation of several proteins involved in replication initiation, our laboratory has been able to conditionally induce varying amounts of re-replication in yeast cells.

Eukaryotic DNA replication can be divided into three fundamental stages. In the first stage, a pre-replicative complex (pre-RC) is assembled at replication origins through the sequential loading of the initiation proteins ORC, Cdc6, Cdt1, and Mcm2-7. In the second stage, initiation is triggered by the concerted actions of Dbf4-Cdc7 kinase and an S phase cyclin-dependent kinase (CDK). At this stage, additional replication proteins are recruited, some of which are incorporated into a large protein machine that is assembled at newly formed replication forks. In the third stage, replication elongation occurs, during which replication forks progress down chromosomes to duplicate the genome.

In addition to triggering initiation, CDKs play a major role in the block to re-replication by downregulating pre-RC component proteins. In budding yeast, CDKs promote the nuclear exclusion of Mcm2-7, inhibit *CDC6* transcription and promote Cdc6 degradation, and appear to phosphorylate and inactivate ORC. Our lab has been able to abrogate these inhibitory mechanisms by making Mcm2-7 constitutively nuclear, ectopically expressing Cdc6 (under a conditional promoter), and mutating CDK phosphorylation sites on ORC. We have demonstrated that simultaneous disruption of all three mechanisms induces a subset of origins to re-initiate and the DNA surrounding these origins to re-replicate. Effectively, cells enter, but do not complete, a second S phase (Nguyen et al., 2001), because only part of the genome re-replicates.

In this reporting period, we have shown that re-replication induces a rapid and significant decrease in cell viability and a cellular DNA damage response. Strikingly, we have observed DNA damage in the absence of a classical replication stress response. These results indicate that re-replication generates DNA damage, and raise the possibility that this could in turn lead to genomic instability.

Body

During the period from March 24th, 2004 to March 23rd, 2005, significant progress was made on a number of the tasks described in the initial application for this grant. Some of this work was reported in a manuscript, on which I was the first author. This paper was published in January 2005 in the journal Molecular Biology of the Cell (Appendix 1, Green and Li, 2005). Additionally, we have nearly completed a second manuscript, which we expect to submit by May 15th of this year (see figures in Appendix 2). I was also asked to give a talk at the Nucleic Acids Gordon Conference on June 6th, 2004 and present a poster at the Mechanisms of Genomic Integrity Conference on June 22nd, 2004. At these conferences I presented work done with the support of this grant.

The overall purpose of the grant was to study the consequences of re-replication of cellular DNA. During a normal cell cycle, DNA replication is tightly controlled such that the genome is replicated once and only once before each mitosis. Loss of replication control has been proposed to be a source of the genomic instability that is associated with tumorigenesis. Our laboratory, and others, has elucidated many of the mechanisms that prevent re-replication from occurring. In doing so, we have established a yeast system with which we can induce re-replication in a population of cells arrested in metaphase. I have begun to study the consequences of this re-replication.

Task 1 of the initial grant application was to confirm that there was a DNA stress response as a consequence of re-replication. At the time of the initial application, we had preliminary evidence that re-replication caused a cellular stress response. The specific aim of this task was to confirm the presence of this response and characterize the nature of this response. In this project period I have demonstrated and published that re-replication leads to cell death, largely *RAD9* and *RAD53* dependent metaphase arrest, Ddc2-GFP foci formation, *RAD9* dependent Rad53p phosphorylation and DNA double stranded breaks (Green and Li, 2005). We completed this task and have done additional work to study the cellular DNA stress response.

Extra effort was invested in this aim due to the surprising discovery that re-replication leads to a DNA damage response seemingly in the absence of the replication stress response. When DNA damage occurs, a cellular checkpoint response arrests the cell cycle and leads to induction of genes required to repair the damage. This response requires numerous genes, including *RAD9* and *RAD53*. The replication stress response is experimentally triggered by the addition of the drug hydroxyurea which limits the cell for nucleotides. Slowed or stalled forks induce a checkpoint response that is dependent on *MRC1*. I was able to show that although re-replicating cells are capable of signaling through the *MCR1* dependent replication stress pathway, nearly all of the checkpoint response required *RAD9*. This is significant because it suggests DNA damage is induced by re-replication without forks being stalled in a manner recognizable by the normal replication stress checkpoint response pathway.

In the initial grant application I also proposed to study the response to different extents of re-replication. At the time of the initial application, we had preliminary

evidence that we could induce re-replication on a more limited scale by perturbing fewer mechanisms that prevent re-replication. Our previous publications describing re-replication have focused on a strain in which three separate mechanisms to prevent re-replication were disrupted. However, as described in our Molecular Biology of the Cell paper (Green and Li, 2005), extensive re-replication leads to significant cell death. In order to study potential consequences of re-replication, we needed to establish a strain in which the cell death was reduced. We did this by perturbing two, rather than three, mechanisms that block re-replication. We have demonstrated that making these changes does result in reduced re-replication, in fact under some conditions, we are able to observe re-replication primarily from a single origin of replication.

Since my continuing work on the consequences of re-replication will require the use of these limited re-replicating strains, we needed to publish our characterization of their re-replication. Consequently, I delayed work on some of the tasks in my initial proposal in order to prepare this manuscript for publication. This work is nearly done and we intend to submit this manuscript, on which I expect to be a first author, to a journal by May 15th, 2005. I have included the figures and figure legends that we will submit as Appendix 2. I have also shown that limited re-replication leads to a DNA damage response similar to that observed after the more extensive re-replication described in Green and Li, 2005. Limited re-replication leads to cell death, metaphase arrest, Ddc2-GFP foci formation and Rad53p phosphorylation (Figures 1 and 2 in Appendix 3 and data not shown)

In task 2 of the initial application, I intend to determine which stage of re-replication leads to the DNA damage response. Formally, my work published in January 2005 did not demonstrate whether inappropriate pre-replicative complex formation, re-initiation or re-replication lead to a DNA damage response. Clearly distinguishing these possibilities would help to direct further experiments for determining precisely how the DNA damage was generated. In order to address this question, I constructed a strain in which a key protein essential for initiation of DNA replication (Cdc7p) was mutated such that it was temperature sensitive. I was thus able to arrest cells in mitosis and induce re-replication both in the presence and absence of initiation. As can be seen in Figure 1 in Appendix 3, when re-initiation is blocked, there is no cellular DNA damage response. Thus, inappropriate pre-replicative complex formation is not sufficient to cause a checkpoint response, and replication is essential.

My next task (task 3) was to use electron microscopy to determine the nature of the lesions induced by re-replication. The requirement of DNA damage for re-replication initiation described above suggests that electron microscopy will be very useful to visualize the actual DNA lesions induced by re-replication. However, initial attempts to conduct these technically difficult experiments in our laboratory proved to be unfruitful. Consequently, we have established collaboration with Dr. Jose Sogo to help us complete task 3. Dr. Sogo is the world's foremost expert on studying DNA lesions using electron microscopy (Sogo et al., 2002) and has agreed to teach me his electron microscopy technique. Due to his prior collaboration commitments, we have not yet been able to conduct these experiments, but we have tentatively agreed to begin work this summer.

We are confident that I will be able to quickly learn Dr. Sogo's technique in his laboratory and then be able to conduct further experiments in our own laboratory at UCSF.

Since I have not yet been able to generate results for task 3, I began work on several of the other tasks. Specifically, I have generated a strain to use in tasks 5 and 6, which investigate whether re-replication leads to genomic instability. In task 5, I intend to determine if re-replication leads to loss of heterozygosity and in task 6 I intend to determine in re-replication leads to gene amplification. I conducted some initial experiments using a strain deregulated for three mechanisms that prevent re-replication. However, there was such massive cell death that I was unable to detect increased loss of heterozygosity. Re-replication was so extensive that fewer than 1 percent of the cells that re-replicate were able to form colonies. Since colony formation is required to assay for loss of heterozygosity, the vast majority of the cells could not be queried to determine if loss of heterozygosity has occurred.

Clearly, I needed to establish a re-replication system that allowed for a greater fraction of the cells to survive. I decided use a strain in which two, rather than three, inhibitory mechanisms are disrupted. The extent of re-replication in this strain has been characterized in the paper that will be submitted by May 15th, 2005 (see figures in Appendix 2). We have shown that this strain re-replicates predominantly from a single origin of replication. I arrested this strain in metaphase and induced re-replication. I then used the formation of Ddc2-GFP foci as a measure of the minimum percent of cells that re-replicate since, in my system, Ddc2-GFP foci are only appreciably seen when re-replication is induced. Cells were then plated in the absence of re-replication and the ability of cells to form colonies was determined (Figure 2 in Appendix 3). I was able to show that when limited re-replication is induced, most cells suffer DNA damage (Ddc2-GFP foci) but many are able to later form colonies (viability). This strain will thus be ideal for studying loss of heterozygosity and gene amplification, and those experiments are ongoing.

Key Research Accomplishments

I have demonstrated that re-replication leads to DNA damage and specifically, I have shown that:

Extensive re-replication leads to significant cell inviability
Re-replication leads to a *RAD9* and *RAD53* dependent metaphase arrest
Ddc2-GFP foci form in the presence of re-replication
Re-replication leads to Rad53p phosphorylation in a RAD9 dependent manner
Direct evidence of DNA double strand breaks can be observed after re-replication

The DNA damage response due to re-replication requires replication initiation

We have established strains in which re-replication is very limited – largely occurring from a single origin of DNA replication

Limited re-replication from these strains also induces a DNA damage response

Finally, I have demonstrated that cells are capable of surviving limited and transient re-replication, setting the stage for studying genomic instability in these cells

Reportable Outcomes

We have published a manuscript in Molecular Biology of the Cell describing some of the work supported by this grant (Green and Li, 2005, Appendix 1).

We have nearly completed a second manuscript, on which I am also a first author, presenting further work on this project (see figures and figure legends in Appendix 2).

I presented this work in a talk and a poster at the Nucleic Acids Gordon Conference at Salve Regina University on June 6th, 2004.

I also presented this work at a poster presentation at the Mechanisms of Genomic Integrity Conference in Galway, Ireland on June 22nd, 2004.

Conclusions

I have made significant progress in regards to addressing the specific aims proposed in my initial application entitled, "DNA Damage and Genomic Instability Induced by Inappropriate DNA Re-replication." I have published a paper on which I am the first author describing the results supported by this grant. Additionally, we have nearly completed a second paper on this topic. I have also presented this work at two scientific conferences. At one of them, the Nucleic Acids Gordon Conference, I was asked to give a talk describing my work.

To maintain genome stability, the entire genome of a eukaryotic cell must be replicated once and only once per cell cycle. In many organisms, multiple overlapping mechanisms block re-replication, but the consequences of deregulating these mechanisms are poorly understood. I have shown that disrupting these controls in the budding yeast *Saccharomyces cerevisiae* rapidly blocks cell proliferation. Re-replicating cells activate the classical DNA damage-induced checkpoint response, which depends on the BRCT checkpoint protein Rad9p. In contrast, Mrc1p, a checkpoint protein required for recognition of replication stress, does not play a role in the response to re-replication. Strikingly, re-replicating cells accumulate sub-chromosomal DNA breakage products. These rapid and severe consequences suggest that even limited and sporadic re-replication could threaten the genome with significant damage.

We have also shown that limited re-replication can be induced when two mechanisms that block re-replication are deregulated. This has enabled us to establish a system in which the consequences of re-replication on genome stability can be studied. If we are able to demonstrate that re-replication leads to genomic instability, it would be the first proof of this previously underappreciated source of genomic threats. Since most cancers, breast cancer included, show significant genomic instability, it is critical that we understand the source of such changes to the genome. We have made a great deal of progress in this project period and anticipate that this will continue in the next project period.

References

Green BM, Li JJ. (2005) Loss of rereplication control in Saccharomyces cerevisiae results in extensive DNA damage. *Mol Biol Cell.* 16:421-32.

http://www.cancer.org/downloads/STT/CAFF2003PWSecured.pdf, Cancer Facts and Figures 2003. 2003, American Cancer Society.

Lopez-Guerrero, J.A., et al., Histological tumor grade correlates with HER2/c-erB-2 status in invasive breast cancer: a comparative analysis between immunohistochemical (CB11 clone and Herceptest), FISH and differential PCR procedures. *Arkh Patol*, 2003. 65(1): p. 50-5.

Nguyen, V.Q., C. Co, and J.J. Li, Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. *Nature*, 2001. 411(6841): p. 1068-73.

Sogo, J.M., M. Lopes, and M. Foiani, Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. *Science*, 2002. 297(5581): p. 599-602.

Taback, B., et al., Detection of tumor-specific genetic alterations in bone marrow from early-stage breast cancer patients. *Cancer Res*, 2003. 63(8): p. 1884-7.

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Loss of Rereplication Control in *Saccharomyces cerevisiae* Results in Extensive DNA Damage

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To maintain genome stability, the entire genome of a eukaryotic cell must be replicated once and only once per cell cycle. In many organisms, multiple overlapping mechanisms block rereplication, but the consequences of deregulating these mechanisms are poorly understood. Here, we show that disrupting these controls in the budding yeast *Saccharomyces cerevisiae* rapidly blocks cell proliferation. Rereplicating cells activate the classical DNA damage-induced checkpoint response, which depends on the BRCA1 C-terminus checkpoint protein Rad9. In contrast, Mrc1, a checkpoint protein required for recognition of replication stress, does not play a role in the response to rereplication. Strikingly, rereplicating cells accumulate subchromosomal DNA breakage products. These rapid and severe consequences suggest that even limited and sporadic rereplication could threaten the genome with significant damage. Hence, even subtle disruptions in the cell cycle regulation of DNA replication may predispose cells to the genomic instability associated with tumorigenesis.

INTRODUCTION

Eukaryotic DNA replication is tightly controlled such that every segment of the genome is replicated once and only once each cell cycle. This control is primarily exerted at the hundreds to thousands of replication origins where DNA replication initiates. Once an origin initiates in S phase, multiple mechanisms prevent it from reinitiating replication for the remainder of that cell cycle (Gopalakrishnan *et al.*, 2001; Nguyen *et al.*, 2001; Vas *et al.*, 2001; Yanow *et al.*, 2001; Vaziri *et al.*, 2003). Such tight control suggests that even an occasional reinitiation event would be deleterious to cells, and it is readily apparent that, in principle, excessive synthesis of just small segments of the genome could eventually threaten its stable propagation. Nonetheless, a direct analysis of the consequences of rereplication is needed to understand whether and how rereplication contributes to genomic instability. *S. cerevisiae* provides a powerful genetic system for such an analysis, especially as there is considerable understanding of both the mechanisms regulating replication and those protecting genome stability in this organism.

Eukaryotic replication initiation can be divided into two fundamental stages (reviewed in Bell and Dutta, 2002). In the first stage, which occurs in early G1 phase, a prereplicative complex (pre-RC) is assembled at replication origins through the sequential loading of the initiation proteins origin recognition complex (ORC), Cdc6, Cdt1, and Mcm2–7. In the second stage, activation of two kinases, Dbf4-Cdc7 kinase and a cyclin-dependent kinase (CDK), triggers events that culminate in replication initiation and disassembly of the prereplicative complex: additional replication proteins are recruited to the origin, the DNA is unwound, and replisomes are assembled at two nascent replication forks.

In addition to triggering initiation, CDKs also prevent reinitiation of eukaryotic DNA replication (Broek *et al.*, 1991; Dahmann *et al.*, 1995; Sauer *et al.*, 1995; Hua *et al.*, 1997). CDKs do this in part by down-regulating multiple components of the pre-RC, thereby preventing reassembly of these complexes at origins that have initiated. In budding yeast, CDKs promote the nuclear exclusion of Mcm2–7 (Labib *et al.*, 1999; Nguyen *et al.*, 2000), inhibit CDC6 transcription (Moll *et al.*, 1991) and promote its degradation (Drury *et al.*, 1997; Elsasser *et al.*, 1999; Drury *et al.*, 2000), and they seem to inactivate ORC through phosphoryation (Nguyen *et al.*, 2001). Making these three initiation factors refractory to CDK inhibition in metaphase-arrested cells allows a subset of origins to reinitiate and portions of the genome to rereplicate (Nguyen *et al.*, 2001). The limited extent of reinitiation suggests that not all inhibitory mechanisms to block rereplication have been identified. Consistent with this, a recent study indicates that CDK binding to ORC provides an additional mechanism to inhibit pre-RC formation (Wilmes *et al.*, 2004).

Analogous CDK-dependent mechanisms antagonizing Cdc6, ORC, and Cdt1 have been shown to inhibit rereplication in other eukaryotes (Jallepalli *et al.*, 1997; Lopez-Girona *et al.*, 1998; Nishitani *et al.*, 2000; Vas *et al.*, 2001; Wuarin *et al.*, 2002; Zhong *et al.*, 2003). Moreover, a CDK-independent mechanism to prevent pre-RC assembly has been identified in metazoans. Central to this mechanism is the protein Geminin (McGarry and Kirschner, 1998; Tada *et al.*, 2001; Wohlschlegel *et al.*, 2002), which binds to Cdt1 and is thought to sterically inhibit its ability to recruit Mcm proteins to replication origins (Lee *et al.*, 2004; Saxena *et al.*, 2004). Inactivation of geminin can lead to partial rereplication, confirming its role in preventing reinitiation of DNA replication (Quinn *et al.*, 2001; Mihaylov *et al.*, 2002; Melixetian *et al.*, 2004; Zhu *et al.*, 2004).

The partial extent of rereplication that we and others have observed suggests that these rereplicating forks are stalled

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or damaged before they can completely rereplicate the entire genome. Such insults to the rereplicating genome could trigger one or both of the checkpoint pathways that monitor genome integrity (reviewed in Melo and Toczyski, 2002; Nyberg *et al.*, 2002). The replication stress pathway responds to slowed or stalled replication forks, such as those arising from inhibition of nucleotide incorporation. The DNA damage pathway responds to chromosomal insults such as double-stranded breaks generated by ionizing radiation or enzymatic cleavage. These pathways activate proteins that stabilize stalled replication forks and repair DNA damage, respectively. In addition, they provide critical time to complete the replication or repair of DNA by imposing arrests at key cell cycle transitions.

Distinguishing whether the replication stress and/or DNA damage pathway is activated is an important first step in understanding the immediate molecular response to rereplication. This distinction is difficult because many checkpoint proteins and events are shared between the two pathways. For example, in metazoans, both types of genomic insults lead to the induction of p21, p53, and PIG3 protein levels; the phosphorylation of histone H2AX, p53, Cdc2, and the checkpoint kinases Chk1 and Chk2; and the organization of H2AX and Rad51 into subnuclear foci (Haaf *et al.*, 1995; Gottifredi *et al.*, 2001; Saintigny *et al.*, 2001; Ward and Chen, 2001; Brown and Baltimore, 2003). In a few of these responses, the kinetics or degree of change may vary between the two pathways, but overall the events considered to be hallmarks of DNA damage also are observed with replication stress. Complicating the distinction between these two responses is the potential for stalled forks to degenerate into damaged forks, particularly if the stalled forks are not properly stabilized (reviewed in Nyberg *et al.*, 2002).

Two groups have recently reported that the induction of rereplication in human cells induces a checkpoint response. The first group initially reported that rereplication induced by overexpression of Cdc6 and Cdt1 activates a DNA damage response (Vaziri *et al.*, 2003), but they have subsequently observed that overexpression of Cdc6 alone can induce this response in the absence of any detectable rereplication (Zhu *et al.*, 2004). Instead, they now report that rereplication induced by geminin depletion leads to what they suspect is a stalled fork response (Zhu *et al.*, 2004). Thus, they no longer assert that rereplication generates DNA damage. A second group observes similar events during geminin depletion, which they attribute to either a DNA damage or replication stress response (Melixetian *et al.*, 2004). Thus, although a clear assignment of pathways was not possible, the data are consistent with rereplication generating a replication stress-like response.

In *Saccharomyces cerevisiae*, the DNA damage and replication stress responses can be genetically distinguished, because the DNA damage pathway is primarily dependent on the BRCA1 C-terminus checkpoint protein Rad9p (reviewed in Toh and Lowndes, 2003), whereas the replication stress pathway is primarily dependent on Mrc1p (Alcasabas *et al.*, 2001; Osborn and Elledge, 2003). In this work, we take advantage of this genetic distinction to unambiguously determine which response is activated upon rereplication. We present evidence that rereplication leads to significant inviability and the activation of a *RAD53* (budding yeast Chk2)-dependent checkpoint response. The *RAD9* dependence of the signaling pathway suggests that rereplication is triggering a DNA damage response and is not inducing a replication stress pathway. Moreover, we present the first direct evidence for the accumulation of chromosomal damage as a consequence of rereplication. These data indicate that rereplication induces DNA damage and poses an immediate threat to both cell viability and genome integrity.

MATERIALS AND METHODS

Strain Construction

All strains (Table 1) with the exception of YJL310 were derived from YJL1737 (*orc2-cdk6A orc6-cdk4A leu2 ura3–52 trp1–289 ade2 ade3 bar1Δ::LEU2*). The *orc2-cdk6A* and *orc6-cdk4A* alleles encode mutant proteins in which alanine is substituted for the phosphoacceptor serines or threonines in CDK consensus phosphorylation sites (S/T-P-X-K/R). For *orc2-cdk6A*, residues 16, 24, 70, 174, 188, and 206 were mutated and for *orc6-cdk4A*, residues 106, 116, 123, and 146 were mutated. The following plasmids were digested and integrated as follows: pJL806 (*pGAL1, URA3/StuI*; Nguyen *et al.*, 2001), pJL1489 (*pGAL1-Δntcdc6, URA3/StuI*; Nguyen *et al.*, 2001), pRS304-Rad53-HA-HIS (*RAD53-HA-HIS, TRP1/HpaI*; Emili, 1998), YIp22 (*pMET3-HA3-CDC20, TRP1*/MscI; Uhlmann *et al.*, 2000), and pBO1555 (*pMET3-HA3-CDC20, NatMX4*/MscI). pJL1206 (*MCM7–2NLS, URA3/AspI*; Nguyen *et al.*, 2001) was used to replace *MCM7* with *MCM7–2NLS* by two-step gene replacement. The plasmid pBO1555 was generated by subcloning a *BglII* to *SalI pMET3-HA3-CDC20* fragment from YIp22 into pAG25 (Goldstein and McCusker, 1999) cut with *BglII* and *SalI*.

Genomic DNA from yJK7-2 (Melo *et al.*, 2001) was used as a template to generate a *DDC2-GFP, kanMX* PCR fragment by using OJL1404 and OJL1405. Genomic DNA from U973 (*sml1Δ::TRP1 esr1–1*; Rothstein laboratory) was used as a template to generate a *sml1Δ::TRP1* polymerase chain reaction (PCR) fragment by using OJL1110 and OJL1111. Genomic DNA from the yeast haploid deletion collection (ResGen; Invitrogen, Carlsbad, CA) was used as a template to generate a *rad9Δ::kanMX* PCR fragment by using OJL1487 and OJL1488. The entire *RAD53* and *MRC1* open reading frames were deleted using PCR amplification of the kanMX from pAG25 with tagged primers by using the oligonucleotides indicated in Table 2 (Goldstein and McCusker, 1999).

Yeast Media

Cells were grown in YEP, synthetic complete (SC), or synthetic (S broth) medium (Guthrie and Fink, 1990) supplemented with 2% dextrose (wt/vol), 2% galactose (wt/vol), 3% raffinose (wt/vol), or 3% raffinose (wt/vol) + 0.05% dextrose (wt/vol). To obtain reproducible induction of rereplication, cells were inoculated from a culture containing 2% dextrose into a culture containing 3% raffinose + 0.05% dextrose and grown for 12–15 h overnight before the experiment commenced.

Cell Proliferation Assay

Yeast cells were diluted in S broth to OD_{600} measurements of 0.2, and then serially diluted fivefold for six dilutions and spotted onto SDC-Ura or SGalC-Ura plates. For transient pulses of rereplication, cells grown overnight in SRaffC-Ura + 0.05% dextrose were pelleted and resuspended in YEPRaff + 15 μg/ml nocodazole. Once >90% of the cells were arrested as large budded cells, galactose was added to a final concentration of 2%, and samples were removed at various time points, diluted in SD broth, and plated on SDC-Ura plates. Colonies were counted after 72 h at 30°C. All platings were done in triplicate, and two separate experiments were conducted. The mean and SE of the mean are shown. Statistical significance was determined using a Student's *t* test.

Flow Cytometry Analysis

Cells grown overnight in SRaffC-Met,Ura + 0.05% dextrose were pelleted and resuspended in YEPRaff + 2 mM methionine to arrest cells in metaphase by Cdc20p depletion. Once arrested (>90% large budded cells), nocodazole (15 μg/ml) was added for an additional 30 min. Galactose was then added to a final concentration of 2%, and samples were taken every hour. Cells were fixed and stained with 1 μM Sytox Green (Molecular Probes, Eugene, OR) as described previously (Haase and Lew, 1997). Vertical lines indicate median DNA content after gating from 100 to 1000, which captures all whole, unclumped cells.

DDC2-Green Fluorescent Protein (GFP) Foci

Cells grown overnight in YEPRaff + 0.05% dextrose were pelleted and resuspended in YEPRaff + 15 μg/ml nocodazole. Once >90% of the cells were arrested as large budded cells, galactose was added to 2%, and samples were removed at various time points, washed twice in phosphate-buffered saline (PBS), and visualized using an Olympus BX60 microscope. Pictures were recorded using a Hamamatsu Orca-ER camera and OpenLab3.1.7 software. Fluorescent images were taken in three z sections that bracketed the thickness of the cell, and then projected into one image by using ImageJ's maximum pixel intensity function. Between 60 and 120 cells were scored for zero, one, or two or more foci per cell, for each strain for each time point. To obtain hydroxyurea (HU)-treated cells for the experiment in Figure 3A, cells were grown in YEPD. They were then arrested in G1 (>95% unbudded cells) with 50 ng/ml α factor and released into a HU arrest with the addition of pronase to a

Table 1. Strains used in this study

Strain	Source	Genotype
YJL310	Detweiler and Li (1998)	*leu2–3112 ura3–52 trp1–289 bar1Δ::LEU2*
YJL3244	Nguyen *et al.* (2001)	*orc2-cdk6A orc6-cdk4A leu2 ura3–52::{pGAL1, URA3} trp1–289 ade2 ade3 MCM7–2NLS bar1Δ::LEU2 cdc20::{pMET3-HA3-CDC20, TRP1}*
YJL3248	Nguyen *et al.* (2001)	*orc2-cdk6A orc6-cdk4A ura3–52::{pGAL1-Δntcdc6, URA3} trp1–289 leu2 ade2 ade3 MCM7–2NLS bar1Δ::LEU2 cdc20::{pMET3-HA3-CDC20, TRP1}*
YJL3604	This study	*rad53Δ::kanMX6 sml1Δ::TRP1 orc2-cdk6A orc6-cdk4A ura3–52::{pGAL1-Δntcdc6, URA3} trp1–289 leu2 ade2 ade3 MCM7–2NLS bar1Δ::LEU2*
YJL3607	This study	*rad53Δ::kanMX6 sml1Δ::TRP1 orc2-cdk6A orc6-cdk4A leu2 ura3–52::{pGAL1, URA3} trp1–289 ade2 ade3 MCM7–2NLS bar1Δ::LEU2*
YJL5048	This study	*orc2-cdk6A orc6-cdk4A ura3–52::{pGAL1-Δntcdc6, URA3} trp1–289 leu2 ade2 ade3 MCM7–2NLS bar1Δ::LEU2 rad53::{RAD53–2HA6HIS, TRP1}*
YJL5055	This study	*orc2-cdk6A orc6-cdk4A leu2 ura3–52::{pGAL1, URA3} trp1–289 ade2 ade3 MCM7–2NLS bar1Δ::LEU2 rad53::{RAD53–2HA6HIS, TRP1}*
YJL5060	This study	*orc2-cdk6A orc6-cdk4A ura3–52::{pGAL1-Δntcdc6, URA3} trp1–289 leu2 ade2 ade3 MCM7–2NLS bar1Δ::LEU2 rad53::{RAD53–2HA6HIS, TRP1} rad9Δ::kanMX*
YJL5065	This study	*orc2-cdk6A orc6-cdk4A leu2 ura3–52::{pGAL1, URA3} trp1–289 ade2 ade3 MCM7–2NLS bar1Δ::LEU2 rad53::{RAD53–2HA6HIS, TRP1} rad9Δ::kanMX*
YJL5085	This study	*orc2-cdk6A orc6-cdk4A ura3–52::{pGAL1, URA3} trp1–289 leu2 ade2 ade3 MCM7–2NLS bar1Δ::LEU2 rad53::{RAD53–2HA6HIS, TRP1} mrc1Δ::kanMX*
YJL5087	This study	*orc2-cdk6A orc6-cdk4A ura3–52::{pGAL1-Δntcdc6, URA3} trp1–289 leu2 ade2 ade3 MCM7–2NLS bar1Δ::LEU2 rad53::{RAD53–2HA6HIS, TRP1} mrc1Δ::kanMX*
YJL5132	This study	*orc2-cdk6A orc6-cdk4A leu2 ura3–52::{pGAL1, URA3} trp1–289 ade2 ade3 MCM7–2NLS bar1Δ::LEU2 ddc2::{DDC2-GFP, kanMX}*
YJL5135	This study	*orc2-cdk6A orc6-cdk4A leu2 ura3–52::{pGAL1-Δntcdc6, URA3} trp1–289 ade2 ade3 MCM7–2NLS bar1Δ::LEU2 ddc2::{DDC2-GFP, kanMX}*
YJL5408	This study	*rad53Δ::kanMX6 sml1Δ::TRP1 orc2-cdk6A orc6-cdk4A ura3–52::{pGAL1-Δntcdc6, URA3} trp1–289 leu2 ade2 ade3 MCM7–2NLS bar1Δ::LEU2 cdc20::{pMET3-HA3-CDC20, NatMX}*
YJL5411	This study	*orc2-cdk6A orc6-cdk4A ura3–52::{pGAL1-Δntcdc6, URA3} trp1–289 leu2 ade2 ade3 MCM7–2NLS bar1Δ::LEU2 rad53::{RAD53–2HA6HIS, TRP1} rad9Δ::kanMX cdc20::{pMET3-HA3-CDC20, NatMX}*
YJL5441	This study	*orc2-cdk6A orc6-cdk4A ura3–52::{pGAL1-Δntcdc6, URA3} trp1–289 leu2 ade2 ade3 MCM7–2NLS bar1Δ::LEU2 rad53::{RAD53–2HA6HIS, TRP1} mrc1Δ::kanMX cdc20::{pMET3-HA3-CDC20, NatMX}*

final concentration of 100 μg/ml and HU to a final concentration of 0.2 M. Samples were processed for quantification as described above. To obtain phleomycin-treated cells for the experiment in Figure 3A, cells were grown in YEPD, arrested with 15 μg/ml nocodazole (>95% large budded cells), and then treated with phleomycin at a final concentration of 20 μg/ml (Cayla, Toulouse, France). Samples were processed for quantification as described above.

Rad53p Immunoblot

Cells grown overnight in SRaffC-Ura + 0.05% dextrose were pelleted and resuspended in YEPRaff + 15 μg/ml nocodazole. Once >90% of the cells were arrested as large budded cells, galactose was added to a final concentration of 2%, and samples were removed at various time points. Cells (8.5 ml) at OD_{600} 0.5–1.0 were pelleted and lysed by vortex mixing and boiling with 300 μl of 0.5-mm glass beads (Biospec Products, Bartlesville, OK) and 300 μl of SDS-PAGE loading buffer [8% glycerol (vol/vol), 100 mM Tris-HCl, pH 6.8, 1.6% SDS (wt/vol), 1.6 × 10^{-3}% bromphenol blue (wt/vol), 100 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride] with protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml chymostatin, and 1 mM benzamidine) and phosphatase inhibitors (1 mM Na_3VO_4, 50 mM NaF, and 50 mM Na β-glycerophosphate). The soluble protein was quantified using a Bradford assay (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard (Sigma-Aldrich, St. Louis, MO). Forty micrograms of each protein sample was electrophoresed on a 7.5% SDS-PAGE gel and transferred to nitrocellulose (Protran BA85; Applied Scientific, San Francisco, CA). The membrane was probed with anti-HA 16B12 (Covance, Berkeley, CA) at 1:1000, followed by sheep anti-mouse horseradish peroxidase (NA931V; Amersham Biosciences, Piscataway, NJ) at 1:2000. Immunoblots were developed with the SuperSignal system (Pierce Chemical, Rockford, IL).

Assaying Induction of a Metaphase Arrest

Cells grown overnight in SRaffC-Met,Ura + 0.05% dextrose were pelleted and resuspended in YEPRaff + 2 mM methionine to arrest cells in metaphase by Cdc20p depletion. Once arrested (>90% large budded cells), galactose was

Table 2. Oligonucleotides used in this study

Oligo	Purpose	Sequence
OJL1404	*DDC2-GFP*	AAAGGTACGTGGGACAAGAC
OJL1405	*DDC2-GFP*	AGACAGCAACACACATCTAG
OJL1110	*sml1Δ*	ctcgcatcgatAAGGATCACGTTCCTTCTGC
OJL1111	*sml1Δ*	gcgacctcgagGAAGACATTGCGGGTTCAAG
OJL1002	*rad53Δ*	GAGAGAATAGTGAGAAAAGATAGTGTTACACAACATCAACcggatccccgggttaattaa
OJL1003	*rad53Δ*	ctcttaaaaaggggcagcattttctatgggtatttgtcctgaattcgagctcgtttaaac
OJL1487	*rad9Δ*	GCTCCCCATCAAAATAAGGTC
OJL1488	*rad9Δ*	TATGTGTCGTCCCAGTACTC
OJL1497	*mrc1Δ*	AGACAAACAACTAAGGAAGTTCGTTATTCGCTTTTGAACTTATCACCAAATATTTTAGTG-cggatccccgggttaattaa
OJL1498	*mrc1Δ*	CGACTACTTCAAGACAGCTTCTGGAGTTCAATCAACTTCTTCGGAAAAGATAAAAAACCA-catcgatgaattcgagctcg

added to a final concentration of 2% for 2 h, and then the cells were filtered and washed with S broth and resuspended in SGalC-Met,Ura + 50 ng/ml α factor. Samples were fixed in 67% ethanol (vol/vol), washed twice with PBS, and resuspended in 50 ng/ml 4'6-diamidino-2-phenylindole (DAPI). Cells were visualized by fluorescence microscopy on an Olympus BX60 microscope and quantified as pre- or postmetaphase based on nuclear morphology. At least 200 cells were scored for each strain for each time point, and the experiment was executed twice. The mean percentage of postmetaphase cells and the SE of the mean from the two experiments are charted.

Pulsed Field Gel Electrophoresis (PFGE)

YJL3244 and YJL3248 cells grown overnight in SRaffC-Met,Ura + 0.05% dextrose were pelleted and resuspended in YEPRaff + 2 mM methionine to arrest cells in metaphase by Cdc20p depletion. Once arrested (>90% large budded cells), nocodazole was added to a final concentration of 15 μg/ml for 30 min, after which galactose was added to a final concentration of 2% at time 0. To obtain HU-treated cells for the experiment in Figure 5, cells were grown in YEPD. They were then arrested in G1 (>95% unbudded cells) with 50 ng/ml α factor and released into a HU arrest with the addition of pronase to a final concentration of 100 μg/ml and HU to a final concentration of 0.2 M. To obtain phleomycin-treated cells for the experiment in Figure 5, cells were grown in YEPD, arrested with 15 μg/ml nocodazole (>95% large budded cells), and then treated with phleomycin at a final concentration of 20 or 200 μg/ml (Cayla).

To make plugs for PFGE, 6×10^8 cells were washed twice with ice-cold 50 mM EDTA and resuspended to 500 μl with 50°C SCE (1 M sorbitol, 0.1 M Na citrate, and 10 mM EDTA). Lyticase was added to a final concentration of 150 U/ml, and 250 μl of the sample was mixed with 250 μl of molten, 50°C 1% SeaPlaque GTG LMP agarose (FMC Bioproducts, Rockland, ME), and then aliquoted into disposal plug molds (170-3713; Bio-Rad). The plug molds were allowed to solidify at 4°C, and then placed in SCEM + lyticase [1 M sorbitol, 0.1 M Na citrate, 10 mM EDTA, 5% β-mercaptoethanol (vol/vol), and 160 U/ml lyticase] for 24 h at 37°C. Plugs were then washed three times in $T_{10}E_1$ (10 mM Tris, pH 8.0, and 1 mM EDTA) for 15 min each wash and resuspended in proteinase K solution [1% sarcosyl (wt/vol), 0.5 M EDTA, and 2 mg/ml proteinase K] for 48 h at 55°C. Finally, plugs were washed three times in $T_{10}E_1$ for 15 min each wash and left overnight at 37°C in $T_{10}E_1$, which removes background fluorescence during ethidium bromide visualization of the gel.

Plugs were cut in half and loaded on a 1% SeaKem LE agarose (wt/vol) gel in 0.5× TBE (45 mM Tris, 45 mM borate, and 1 mM EDTA). The gel was electrophoresed in 14°C 0.5× TBE on a CHEF DR-III system with initial switch time of 50 s, final switch time of 90 s, run time of 22 h, voltage of 6 V, and angle of 120°. The gel was stained with 0.5 μg/ml ethidium bromide in 0.5× TBE for 1.5 h, destained in deionized water for 2 h, and imaged with an AlphaImager. The DNA was then nicked in 0.5 M HCl for 1 h, denatured in 1.5 M NaCl, 0.5 M NaOH for 40 min, and neutralized in 3M NaCl, 55 mM Tris base, 455 mM Tris-HCl for 40 min. The DNA was then transferred to a GeneScreen Plus nylon membrane and cross-linked with 0.12 J of UV light in a UV Stratalinker 1800 (Stratagene, La Jolla, CA). The membrane was probed with an ARS305 fragment (Nguyen *et al.*, 2001) and imaged and quantified with a Storm 840 (Amersham Biosciences).

RESULTS

Rereplication Rapidly Blocks Cell Proliferation

Previous work in our laboratory established yeast strains in which rereplication can be induced in metaphase-arrested cells (Nguyen *et al.*, 2001). These yeast strains contain genetic alterations that make three replication initiation proteins refractory to the inhibitory effect of the CDK Cdc28p. The CDK phosphorylation of two subunits of the origin recognition complex, Orc2p and Orc6p, was blocked by mutating their CDK consensus phoshorylation sites (*orc2–6A, orc6–4A*). Cdc28p-directed nuclear exclusion of the Mcm2–7p complex (Labib *et al.*, 1999; Nguyen *et al.*, 2000) was prevented by fusing two tandem copies of the simian virus 40 nuclear localization signal to Mcm7p (*MCM7–2NLS*). Finally, CDK regulation of Cdc6p was disrupted by integrating *pGAL1-Δntcdc6*, which expresses an N-terminally truncated and slightly stabilized Cdc6p (Δntcdc6p), under the control of the galactose-inducible *GAL1* promoter (Drury *et al.*, 1997). In this rereplicating strain, rereplication is detectable only after Δntcdc6p is induced by growth in galactose-containing medium. A parallel strain, containing *pGAL1* instead of *pGAL1-Δntcdc6*, does not rereplicate and serves as a negative control strain (Figure 1A).

Further characterization of these strains initially revealed that sustained rereplication leads to a dramatic decrease plating efficiency (Figure 1B). Both the *pGAL1-Δntcdc6* rereplicating strain and *pGAL1* control strain grew with similar efficiency when plated on medium containing dextrose, which represses the *pGAL1* promoter. However, when cells were plated on medium containing galactose, the *pGAL1-Δntcdc6* rereplicating strain showed a decrease in plating efficiency by at least three orders of magnitude. In the absence of perturbations of ORC and MCM, expression of Δntcdc6p had no effect on cell growth as assayed by colony size or plating efficiency on galactose-containing medium (our unpublished data).

Significant inhibition of cell proliferation also could be seen with transient induction of rereplication (Figure 1C). Both the *pGAL1-Δntcdc6* rereplicating strain and *pGAL1* control strain were arrested in metaphase with nocodazole then exposed to galactose to induce rereplication. After varying amounts of time in galactose, cells were plated on dextrose-containing medium to assess the number of cells that could give rise to viable colonies (colony-forming units). Because Δntcdc6p becomes undetectable within 30 min after galactose-induced cells are repressed by the addition of dextrose (Nguyen *et al.*, 2001), we expected reinitiation to end after cell plating. The *pGAL1* control strain showed only a slight decrease in colony-forming units after 3 h in galactose. In contrast, the *pGAL1-Δntcdc6* rereplicating strain showed a fivefold decrease in colony-forming units after only 30 min in galactose and a nearly 50-fold decrease after 3 h, a statistically significant difference ($p < 0.002$).

Rereplication Induces a RAD53-dependent Metaphase Checkpoint Arrest

To determine how rapidly rereplicating cells cease dividing, we examined cells microscopically 2 d after transient exposure to galactose. Most rereplicating cells that did not give rise to colonies also did not rebud (our unpublished data), indicating that the cells could not progress beyond the G1 commitment point of the next cell cycle. To pinpoint where in the cell cycle these cells were blocked, we arrested cells in metaphase by depleting them of Cdc20p, which is required for the metaphase–anaphase transition (Schwab *et al.*, 1997; Visintin *et al.*, 1997), induced rereplication with galactose for 2 h, and then restored Cdc20p expression to remove the original metaphase block. α Factor was added to trap any cells that progressed into G1 phase of the next cell cycle (Figure 2A). Cell and nuclear morphology were used to distinguish between cells that were in metaphase and cells that were postmetaphase (anaphase/telophase or G1 phase). More than 90% of the *pGAL1*-negative control cells proceeded past metaphase and accumulated in G1 phase. In contrast, <20% of the *pGAL1-Δntcdc6*–rereplicating cells had exited metaphase 5 h after Cdc20p expression was restored. Similar results were obtained when these cells were monitored after rereplication was induced for only 1 h instead of 2 h (our unpublished data). Because rereplication was barely detectable by flow cytometry after 1 h of induction (Figure 1A), these data suggest that even limited rereplication induces a metaphase arrest.

In budding yeast, genotoxic stresses such as replication fork stalls or DNA damage induce a metaphase arrest that requires activation of the checkpoint kinase Rad53p (Allen *et al.*, 1994; Weinert *et al.*, 1994; Sanchez *et al.*, 1996; Sun *et al.*, 1996), the homolog of Chk2 in mammalian cells and Cds1 in *Schizosaccharomyces pombe*. To determine whether rereplication might activate these pathways, we induced rereplication in a *rad53Δ* mutant background and monitored the

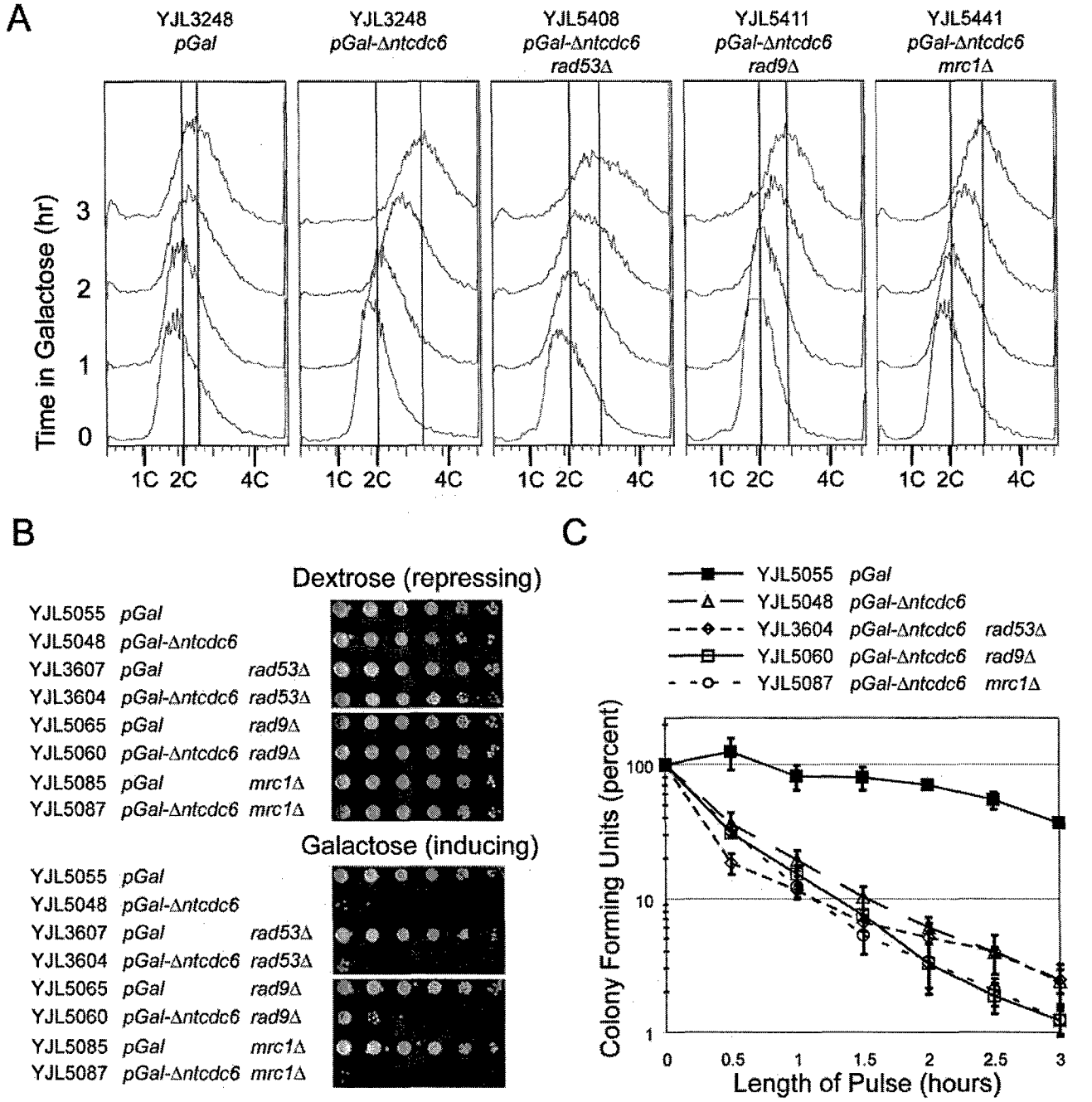

Figure 1. Induction of rereplication rapidly blocks cell proliferation. (A) Checkpoint-deficient strains are capable of rereplicating. Cells with the indicated genotypes plus *pMET3-HA3-CDC20 orc2-cdk6A orc6-cdk4A MCM7–2NLS* were grown in medium containing 3% raffinose + 0.05% dextrose. Metaphase arrest was induced by adding 2 mM methionine, to transcriptionally deplete Cdc20p, and 15 μg/ml nocodazole. Then, 2% galactose was added, and samples were taken hourly for flow cytometry. Vertical lines indicate the median DNA content for the 0- and 3-h time points. (B) Constitutive induction of rereplication prevents cell proliferation. Cells with the indicated genotypes plus *orc2-cdk6A orc6-cdk4A MCM7–2NLS* were grown on plates containing 2% dextrose and serially diluted into S broth with fivefold dilutions. The dilutions were plated on medium containing either 2% dextrose, which represses rereplication, or 2% galactose, which induces rereplication in strains containing *pGAL1-Δntcdc6*. (C) Transient induction of rereplication rapidly inhibits colony forming potential. Cells with the indicated genotypes plus *orc2-cdk6A orc6-cdk4A MCM7–2NLS* were grown in medium containing 3% raffinose plus 0.05% dextrose and arrested in metaphase with addition of 15 μg/ml nocodazole. Galactose (2%) was added for the indicated number of hours to allow for transient induction of rereplication and cells were then plated on medium containing 2% dextrose to score colony-forming units (CFU). For each strain, the CFU is expressed as a percentage of the CFU present at time 0 h. Error bars show SE of the mean from two experiments.

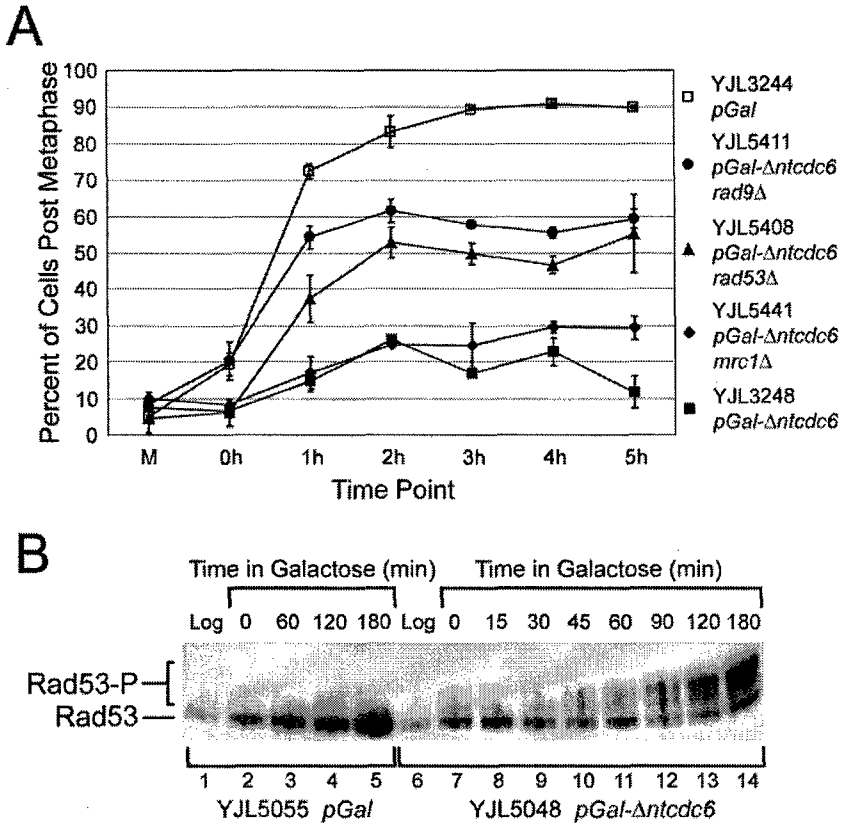

Figure 2. Rereplication induces a *RAD53*-dependent checkpoint response. (A) Rereplication induces a metaphase arrest that is dependent in part on *RAD53* and *RAD9*. Cells with the indicated genotypes plus *pMET3-HA3-CDC20 orc2-cdk6A orc6-cdk4A MCM7–2NLS* were arrested in metaphase by transcriptional depletion of Cdc20p in medium containing 3% raffinose and 2 mM methionine (M). Then, 2% galactose was added for 2 h to allow the induction of rereplication followed, at time 0 h, by release from the Cdc20p depletion arrest by transfer of cells to medium lacking methionine but containing 2% galactose and α factor. At hourly intervals after the release, DAPI-stained cells were scored (n = 300) as pre- or postmetaphase. The percentage of postmetaphase cells is shown for each strain, along with the SE of the mean. (B) Rereplication induces phosphorylation of Rad53p. Cells containing the indicated genotypes plus *RAD53-HA orc2-cdk6A orc6-cdk4A MCM7–2NLS* were grown in medium containing 3% raffinose + 0.05% dextrose and arrested in metaphase with 15 μg/ml nocodazole. Then, 2% galactose was added to allow the induction of rereplication, and at the indicated times samples were harvested for immunoblot analysis of Rad53p-HA. The hypophosphorylated protein is indicated by Rad53 and the hyperphosphorylated protein is indicated by Rad53-P.

ability of these cells to progress past metaphase. Flow cytometry demonstrated that rereplication was still induced in the presence of the *rad53Δ* mutation (Figure 1A), and vital staining with phloxine B showed that most of the cells remained metabolically alive after 3 h of induction (our unpublished data). The percentage of cells that could complete metaphase, however, increased from <20% to nearly 50%. This result suggests that a significant portion of the checkpoint-proficient rereplicating cells were arrested solely in response to a *RAD53*-dependent checkpoint. The remaining 50% of the cells also seemed to activate this checkpoint (see below) but presumably stayed arrested because they were subjected to an additional *RAD53*-independent metaphase block (see *Discussion*).

Additional evidence that rereplication activates a *RAD53*-dependent checkpoint response was obtained by examining Rad53p directly. Activation of Rad53p protein kinase is tightly correlated with its hyperphosphorylation (Allen *et al.*, 1994; Weinert *et al.*, 1994; Sanchez *et al.*, 1996; Sun *et al.*, 1996), a modification that retards Rad53p mobility during gel electrophoresis. After inducing rereplication with galactose in metaphase-arrested cells, we monitored the phosphorylation state of Rad53p by immunoblotting total cell lysates (Figure 2B). In the *pGAL1* control strain, Rad53p remained hypophosphorylated for the duration of the galactose induction, consistent with the absence of any checkpoint arrest of the cell cycle. In the *pGAL1-Δntcdc6*–rereplicating strain, however, Rad53p hyperphosphorylation was detected within 45 min of induction, and the majority of the protein became hyperphosphorylated by 120 min. Together, the metaphase arrest and Rad53p hyperphosphorylation indicate that Rad53p is activated as part of a checkpoint response triggered by rereplication. The nearly complete conversion of Rad53p to the hyperphosphorylated form (Figures 2B and 4A) further suggests that this response was activated in almost all rereplicating cells.

Rereplication Induces Formation of Ddc2-GFP Foci

Because the genome is only partial rereplicated in our strains, many rereplication forks cannot be properly terminating with a converging fork from the adjacent replicon. This suggests that many of the rereplication forks must be stalled or disrupted, potentially signaling replication stress, DNA damage, or both. Analysis of the Ddc2p response to rereplication provided an initial hint that rereplication elicits a checkpoint response to DNA damage. Like Rad53p, Ddc2p is required for the response to both DNA damage and replication stress. Ddc2p in complex with Mec1p is recruited to both sites of double-strand breaks (Kondo *et al.*, 2001; Melo *et al.*, 2001) and stalled replication forks (Katou *et al.*, 2003; Osborn and Elledge, 2003) as part of the sensing of these lesions by the checkpoint pathways. Previous studies established that Ddc2p relocalizes from a diffuse nuclear distribution to punctate subnuclear foci in response to DNA damage (Melo *et al.*, 2001). We observed that similar foci are not generated in response to HU in our strains, thereby providing a possible way to distinguish between the two responses (Figure 3A).

This distinction was demonstrated in a *pGAL1-Δntcdc6* rereplicating strain where *DDC2* was replaced by *DDC2-GFP*. Initial experiments were performed in dextrose-containing medium to ensure tight repression of *pGAL1-Δntcdc6*. The rereplicating strain was arrested in metaphase with nocodazole, exposed to 20 μg/ml of the DNA damaging agent phleomycin, and examined by fluorescence mi-

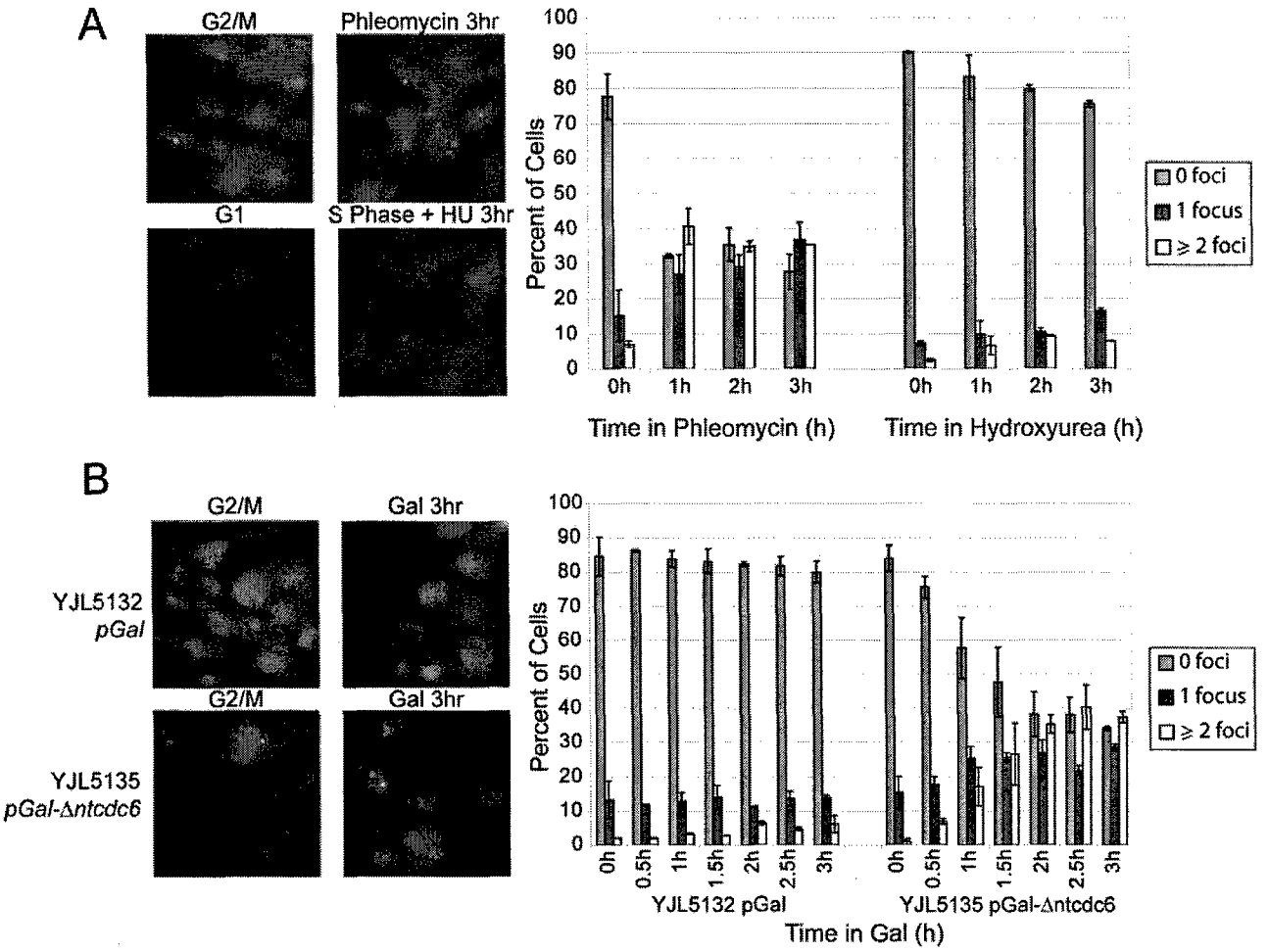

Figure 3. Subnuclear Ddc2p foci consistent with DNA damage are formed when rereplication is induced. (A) HU-induced replication stress does not induce subnuclear Ddc2p foci to the same extent as DNA damage. YJL5135 (*ddc2:DDC2-GFP pGAL1-Δntcdc6 orc2-cdk6A orc6-cdk4A MCM7–2NLS*) growing in medium containing 2% dextrose was arrested in metaphase with 15 μg/ml nocodazole followed by treatment with 20 μg/ml phleomycin to induce DNA damage. A parallel culture was arrested in G1 phase with α factor and released from the arrest into 0.2 M HU to induce replication stress. At hourly intervals after either phleomycin addition or release into HU, cells were scored for subnuclear GFP foci, and the number of cells with zero foci, one focus, or two or more foci was quantified. Representative images at 0 and 3 h are shown. Error bars show SE of the mean from two experiments (n = 60–120 per experiment). (B) Rereplication induces Ddc2p foci. YJL5135 and YJL5132 (*ddc2:DDC2-GFP pGAL1 orc2-cdk6A orc6-cdk4A MCM7–2NLS*) growing in medium containing 3% raffinose + 0.05% dextrose were arrested in metaphase by the addition of 15 μg/ml nocodazole. Then, 2% galactose was added to induce rereplication in YJL5135 and at 30-min intervals the number of foci per cell was quantified (n = 60–120 per experiment). Representative images and quantification are shown as in A.

croscopy. Within 1 h, one or more subnuclear foci of Ddc2p-GFP were observed in most cells (Figure 3A), consistent with previously published observations. In contrast, when these cells were released from a G1 arrest into S phase in the presence of 0.2 M HU, there was little induction of Ddc2p-GFP subnuclear foci even 3 h after imposition of the replication block (Figure 3A). If phleomycin is added to these cells, subnuclear Ddc2p-GFP foci occur within an hour, indicating that damage-induced foci are observable in HU-arrested cells (our unpublished data). Similar results were observed in wild-type cells not containing any perturbations of ORC, Mcm2–7, or Cdc6.

To examine the localization of Ddc2p after rereplication, the *pGAL1-Δntcdc6* rereplicating and *pGAL1* control strains containing *DDC2-GFP* were arrested in metaphase, induced with galactose, and examined at 30-min intervals by fluorescence microscopy. In the *pGAL1-Δntcdc6* strain, within 1 h of induction of rereplication, there was a significant increase in Ddc2p-GFP subnuclear foci (Figure 3B). Within 2 h, the number of cells with foci and the number of foci per cell were quantitatively similar to the response observed with the addition of the DNA damaging agent phleomycin. Little increase in Ddc2p-GFP foci was observed in the *pGAL1* control strain. Thus, these findings suggest that rereplication induces a DNA damage checkpoint.

Rereplication Induces a DNA Damage Response

For a more definitive examination of whether rereplication was triggering a DNA damage response, a replication stress response, or both, we took advantage of the genetic distinction between these two checkpoint pathways in budding yeast. Both pathways converge on *RAD53* and induce a metaphase arrest. However, upstream of *RAD53*, the DNA

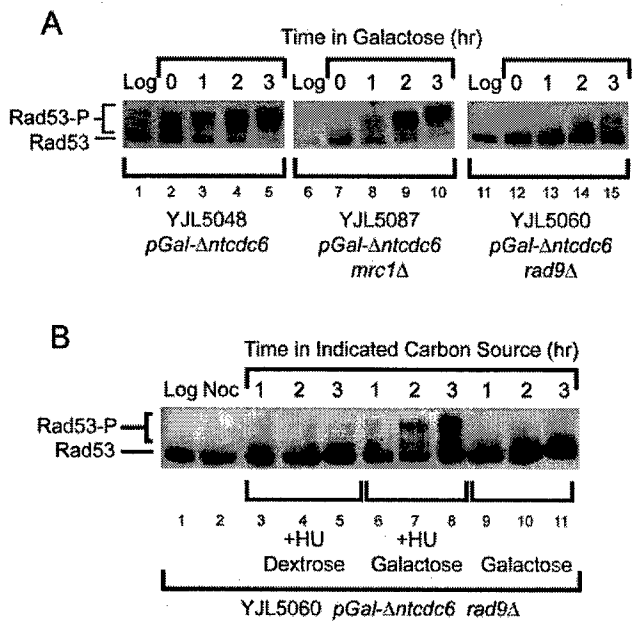

Figure 4. The checkpoint response induced by rereplication is dependent on Rad9p and not Mrc1p. (A) Cells with the indicated genotypes plus *orc2-cdk6A orc6-cdk4A MCM7–2NLS* were grown in 3% raffinose + 0.05% dextrose and arrested in metaphase by the addition of 15 μg/ml nocodazole. Then, 2% galactose was added, and at the indicated times samples were harvested for immunoblot analysis of Rad53p-HA. The hypophosphorylated protein is indicated by Rad53 and the hyperphosphorylated protein is indicated by Rad53-P. (B) The *rad9Δ* cells are capable of responding to stalled rereplication forks. YJL5060 (*rad9Δ pGAL1-Δntcdc6 orc2-cdk6A orc6-cdk4A MCM7–2NLS*) grown in medium containing 3% raffinose + 0.05% dextrose was arrested at metaphase with 15 μg/ml nocodazole and split into three cultures: 0.2 M HU and 2% dextrose were added to the first culture; 0.2 M HU and 2% galactose were added to the second; and 2% galactose was added to the third. Immunoblot analysis was performed as described in A.

damage response is predominantly dependent on *RAD9*, whereas the replication stress response is predominantly dependent on *MRC1*. We individually deleted each gene in the *pGAL1-Δntcdc6* rereplicating strain and the *pGAL1* control strain and investigated whether the metaphase arrest and Rad53p hyperphosphorylation induced by rereplication was dependent on either gene. Initial experiments established that rereplication was still induced on all chromosomes in the *mrc1Δ* and *rad9Δ* mutants (Figure 1A; our unpublished data).

As described above, the proportion of cells arrested in metaphase due to rereplication was approximately halved when *RAD53* was deleted. A slightly higher reduction was observed when *RAD9* was deleted, whereas a much smaller reduction was observed upon deletion of *MRC1* (Figure 2A). Thus, nearly half of the rereplicating cells that are arrested in metaphase are solely held at that arrest by a *RAD9*-dependent pathway. The remainder, as discussed previously, seem to be subjected to an additional metaphase block. The hyperphosphorylation of Rad53p induced during rereplication (Figure 4A, lanes 1–5) also was dramatically reduced in a *rad9Δ* mutant background (Figure 4A, lanes 11–15). The simplest interpretation of these results is that the Rad53p phosphorylation and *RAD53*-dependent metaphase arrest induced by rereplication is primarily triggered through the *RAD9*-dependent DNA damage response pathway.

The virtually complete dependence of Rad53p hyperphosphorylation on *RAD9* suggests that rereplication generates little or no *MRC1*-dependent signaling of replication stress. Alternatively, one could hypothesize that the *rad9Δ* mutation, the metaphase state of the cell, or an insufficient number of rereplicating forks, somehow prevents the detection of replication stress in our rereplicating cells. For example, if Mrc1p did not properly assemble onto rereplication forks during reinitiation as it normally does at replication forks during normal initiation, the *rad9Δ* cells would be unable to signal the presence of stalled forks.

To demonstrate that we can indeed detect replication stress during rereplication in a *rad9Δ* mutant, the mutant strain was arrested in metaphase, split into three separate culture conditions, and each harvested for immunoblot analysis of Rad53p. Galactose was added to one culture to induce rereplication. As described above, there was little Rad53p hyperphosphorylation because of the *rad9Δ* mutation (Figure 4B, lanes 9–11). Galactose and HU were added to a second culture to induce replication stress during rereplication. In these cells, robust Rad53p hyperphosphorylation could now be observed (Figure 4B, lanes 6–8), presumably through activation of the *MRC1*-dependent replication stress response pathway. Finally, dextrose and HU were added to the third culture. Dextrose represses the *pGAL1* promoter and stifles any induction of rereplication. No Rad53p hyperphosphorylation was observed in this culture (Figure 4B, lanes 3–5), confirming that rereplication forks were generating the HU-induced replication stress response observed in the second culture. Thus, the *MRC1*-dependent replication stress response pathway is capable of sensing stalled rereplication forks during a metaphase arrest in a *rad9Δ* background. The lack of any significant activation of this pathway in the absence of HU suggests that stalled rereplication forks are not triggering the checkpoint response observed in rereplicating cells. Consistent with this conclusion is the observation that the extent and kinetics of Rad53p hyperphosphorylation induced by rereplication are unchanged by deletion of *MRC1* (Figure 4A, lanes 6–10). Together, our data suggest that DNA damage, and not replication stress, is the predominant genotoxic insult accumulating as a consequence of rereplication.

Rereplication Induces Double-stranded Breaks

Given the induction of a DNA damage response, we looked for direct evidence of DNA damage induced by rereplication. We assayed whether rereplication results in double-stranded breaks by monitoring the appearance of subchromosomal fragments by PFGE. To verify that PFGE can detect chromosome fragmentation, we examined yeast chromosomes from metaphase-arrested cells treated with phleomycin, which generates double-stranded breaks. At high doses of phleomycin, all chromosomes were converted to a heterogeneous pool of subchromosomal fragments (Figure 5A, lanes 4–6). These results were confirmed by Southern blot analysis of these gels, by using *ARS305* to probe for chromosome III (Figure 5A, lanes 15–17).

Similar chromosome fragmentation was not observed in cells arrested in S phase with HU (Figure 5A, lanes 1–3 and 12–14). Replicating structures, such as replication bubbles and forks, are thought to significantly retard DNA mobility during PFGE, and whole chromosomes with many replicating structures are retained in gel loading wells (Hennessy *et al.*, 1990). Nonetheless, the absence of any significant subchromosomal fragments even after prolonged HU arrest suggests that there is no rapid or widespread degeneration of stressed replication forks to double-stranded breaks.

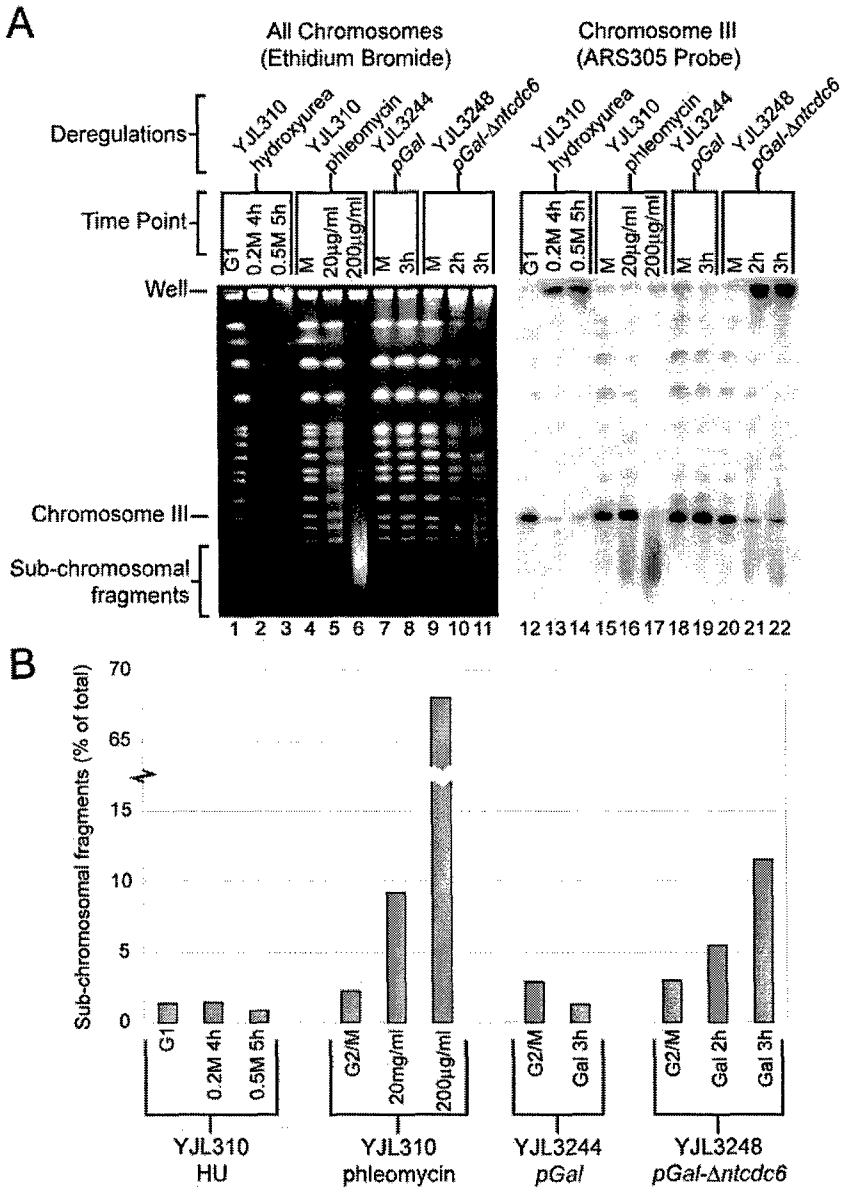

Figure 5. Rereplication induces double-stranded breaks. (A) Rereplication generates subchromosomal fragments. (Lanes 1–11) PFGE stained with ethidium bromide. (Lanes 12–22) Southern blot of PFGE probed with *ARS305* fragment to detect chromosome III. (Lanes 1–3, 12–14) YJL310 (*CDC6 ORC2 ORC6 MCM7*) was arrested in G1 phase with α factor and then released from the arrest into the indicated amounts of HU for the indicated times. (Lanes 4–6, 15–17) YJL310 was arrested in metaphase with 15 μg/ml nocodazole and then treated for 2 h with the indicated amount of phleomycin. (Lanes 7–8, 18–19) YJL3244 (*pGAL1 orc2-cdk6A orc6-cdk4A MCM7–2NLS pMET3-HA3-CDC20*) was arrested in metaphase in medium containing 3% raffinose and 2 mM methionine. Once arrested, galactose was added to 2%. (Lanes 9–11, 20–22) YJL3248 (*pGAL1-Δntcdc6 orc2-cdk6A orc6-cdk4A MCM7–2NLS pMET3-HA3-CDC20*) was treated as for YJL3244. DNA from equivalent numbers of cells were loaded in each lane, except twice as many cells were loaded in lanes 1–3 and 12–14 to compensate for their G1 or nearly G1 DNA content. (B) Quantification of subchromosomal fragment from Southern blot in A. The intensity of subchromosomal signal is shown as a percentage of the total signal for each lane.

Like HU treatment, rereplication caused the majority of each chromosome to be retained in the wells. However, rereplication also generated subchromosomal fragments, which looked like a smear of DNA migrating from below the smallest chromosome up toward the well (Figure 5A, lanes 9–11). This could be seen more clearly by Southern blot analysis, which showed an accumulation of chromosome III fragments migrating faster than the smallest full-length chromosome (Figure 5A, lanes 20–22) in amounts comparable with those generated by 20 μg/ml phleomycin (Figure 5B). This induction of subchromosomal fragments was specific to rereplicating cells, because no such induction was seen in the control strain (Figure 5A, lanes 18–19). Similar subchromosomal fragments were observed when the Southern blots were probed for chromosome 4 and 7 (our unpublished data). Thus, rereplication, but not replication stressed by HU, generates double-stranded DNA breaks.

Checkpoint Responses Do Not Reduce the Lethality Induced by Rereplication

By mobilizing a corrective response and delaying the cell cycle, checkpoint pathways help to protect cells from insults that would disrupt the proper transmission of genetic information. In some cases, however, recovery from the insult may not be possible despite the activation of a checkpoint. For example, degradation of Mcm proteins in the middle of S phase disrupts active replication forks and seems to activate the replication stress response: Rad53p is hyperphosphorylated and cells experience a *RAD9*-independent metaphase arrest (Labib *et al.*, 2001). Despite the activation of this checkpoint, cells are unable to recover their ability to replicate after Mcm proteins are restored (Labib *et al.*, 2001), presumably because Mcm proteins cannot be reloaded onto the disrupted replication forks. To determine whether the DNA damage response is able to protect cells from the amount and type of DNA lesions generated by rereplication,

we examined the viability of rereplicating cells that harbor deletions in *RAD53*, *RAD9*, or *MRC1*. Strains deleted for any of these genes showed similar decreases in viability as checkpoint-proficient strains when subjected to constitutive or transient ($p > 0.35$ at 3 h) rereplication (Figure 1, B and C). This suggests that the extent of rereplication in these cells generates an amount or type of lethal genotoxic stress that is irreparable.

DISCUSSION

Eukaryotic cells use multiple overlapping mechanisms to prohibit reinitiation of DNA replication within a single cell cycle. An obvious reason why cells might impose such extensive and layered safeguards is that even a low frequency and amount of extra DNA synthesis could eventually alter genome content. We report here that rereplication can induce an immediate and severe threat to the cell. Rereplicating cells rapidly and permanently cease cell division. They phosphorylate Rad53p in a *RAD9*-dependent manner and arrest in metaphase. This checkpoint response is unlikely to be a novel "rereplication checkpoint." Rather, we infer from the stereotypical DNA damage response that rereplication rapidly generates DNA lesions that are recognized by the cell as DNA damage. Thus, the use of multiple mechanisms to prevent rereplication not only preserves genome content in the long-term but also protects cells from lethal genomic insults in the short-term.

Surprisingly, we have been able to demonstrate that rereplication triggers little or no replication stress response, even though rereplication forks fail to complete a full round of replication. The Rad53p phosphorylation observed during rereplication was almost exclusively dependent on *RAD9*, which signals DNA damage, and was independent of *MRC1*, which signals replication stress. Similarly, the metaphase arrest induced by rereplication was more dependent on *RAD9* than on *MRC1*. Importantly, the absence of a replication stress response was not due to an inability to respond to replication stress. In a *rad9Δ* mutant background, where rereplication by itself failed to induce Rad53p phosphorylation, the addition of HU to stress the rereplicating forks leads to robust and persistent Rad53p phosphorylation. The simplest interpretation of these data is that rereplicating forks fail to complete a full round of replication, not because they eventually stall, but because they somehow degenerate into DNA lesions that are recognized as DNA damage. These results contrast with those obtained in human cells depleted of geminin, where the resulting rereplication can be associated with the replication stress response (Melixetian *et al.*, 2004; Zhu *et al.*, 2004). Whether these contrasting results reflect differences in species or protocol for inducing rereplication remains to be addressed in the future.

A key question raised by these findings is how rereplication generates DNA lesions without inducing a stalled fork response. Because a prompt DNA damage response is observed in almost all cells in the presence of the microtubule depolymerizing agent nocodazole, the lesions are unlikely to be a consequence of spindle tension on partially replicated chromosomes. Consistent with this, we can induce rereplication and observe the attendant DNA damage response during S phase (our unpublished data), suggesting that a mitotic state is not required to generate the lesion. Moreover, preliminary evidence suggests that elongation is restrained during rereplication (our unpublished data), raising the possibility that rereplicating replisomes encounter problems that could lead to DNA lesions. We therefore suspect that the lesions are generated by the act of rereplication itself.

Any molecular model for how these lesions are generated must explain why they are generated during rereplication and not during normal replication. One possible explanation is that the first round of replication structurally alters chromosomes in a manner that interferes with their rereplication within the same cell cycle; sister chromatid cohesion, which is established during DNA replication, provides precedence for such a replication-coupled change in chromosome state (reviewed in Nasmyth, 2001). Other possible explanations include hypothetical problems specific to rereplication such as poor coordination of histone synthesis and/or nucleosome assembly with rereplication (Verreault, 2003), rereplicating forks from later rounds of rereplication overtaking rereplicating forks from earlier rounds, or defective assembly of replisomes during reinitiation.

An important approach to understanding how rereplication generates DNA damage is to characterize the molecular structure of the primary lesions that are induced. Importantly, these primary lesions may not be the chromosomal breaks that we observed by PFGE. Other abnormal DNA structures that could trigger the DNA damage response might be generated earlier before degenerating into chromosomal breaks. Fork collapse, for example, can generate "chicken feet" structures (Sogo *et al.*, 2002), which expose free double-stranded DNA ends without cleaving the chromosome. Further analysis of rereplicating DNA will hopefully yield more insight into the structure of these primary lesions and the molecular mechanisms by which they are generated.

Although rereplication induces a *RAD9*-dependent checkpoint response, this response offers little protection against the lethal consequences of rereplication (Figure 1B). This lack of protection is reminiscent of the futile induction of a *RAD9*-independent checkpoint response after complete Mcm degradation in S phase (Labib *et al.*, 2001). Loss of Mcm proteins from replication forks is apparently irreparable even after resynthesis of the proteins, because there is no efficient mechanism to reload Mcm proteins at forks. Similarly, in our rereplicating cells the damage induced by rereplication may be irreparable and overwhelm any possible protective effect of the DNA damage response. Additionally, other lethal problems may arise from rereplication that are not dependent on DNA damage and cannot be corrected by the DNA damage response. Such additional problems might account for the partial persistence of metaphase-arrested cells when rereplication is induced in the absence of *RAD53* or *RAD9* (Figure 2A). Fully understanding the lethal consequences of rereplication will require further molecular characterization of the terminal phenotype of rereplicating cells.

The extra copies of genes that are generated by rereplication have long been considered a possible source of genomic instability. Our observation that DNA damage is generated during rereplication suggests an additional way by which rereplication might generate genomic changes. Interestingly, in mammalian cells, overexpression of a single replication initiation protein Cdt1 can induce subtle rereplication (Vaziri *et al.*, 2003) and has been implicated in tumorigenesis (Arentson *et al.*, 2002). Thus, rereplication may be another potential source for the genomic instability associated with tumorigenesis.

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REFERENCES

Alcasabas, A. A., Osborn, A. J., Bachant, J., Hu, F., Werler, P. J., Bousset, K., Furuya, K., Diffley, J. F., Carr, A. M., and Elledge, S. J. (2001). Mrc1 transduces signals of DNA replication stress to activate Rad53. Nat. Cell Biol. *3*, 958–965.

Allen, J. B., Zhou, Z., Siede, W., Friedberg, E. C., and Elledge, S. J. (1994). The SAD1/RAD53 protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. Genes Dev. *8*, 2401–2415.

Arentson, E., Faloon, P., Seo, J., Moon, E., Studts, J. M., Fremont, D. H., and Choi, K. (2002). Oncogenic potential of the DNA replication licensing protein CDT1. Oncogene *21*, 1150–1158.

Bell, S. P., and Dutta, A. (2002). DNA replication in eukaryotic cells. Annu. Rev. Biochem. *71*, 333–374.

Broek, D., Bartlett, R., Crawford, K., and Nurse, P. (1991). Involvement of p34cdc2 in establishing the dependency of S phase on mitosis [see comments]. Nature *349*, 388–393.

Brown, E. J., and Baltimore, D. (2003). Essential and dispensable roles of ATR in cell cycle arrest and genome maintenance. Genes Dev. *17*, 615–628.

Dahmann, C., Diffley, J. F., and Nasmyth, K. A. (1995). S-phase-promoting cyclin-dependent kinases prevent re-replication by inhibiting the transition of replication origins to a pre-replicative state. Curr. Biol. *5*, 1257–1269.

Detweiler, C. S., and Li, J. J. (1998). Ectopic induction of Clb2 in early G1 phase is sufficient to block prereplicative complex formation in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA *95*, 2384–2389.

Drury, L. S., Perkins, G., and Diffley, J. F. (1997). The Cdc4/34/53 pathway targets Cdc6p for proteolysis in budding yeast. EMBO J. *16*, 5966–5976.

Drury, L. S., Perkins, G., and Diffley, J. F. (2000). The cyclin-dependent kinase Cdc28p regulates distinct modes of Cdc6p proteolysis during the budding yeast cell cycle. Curr. Biol. *10*, 231–240.

Elsasser, S., Chi, Y., Yang, P., and Campbell, J. L. (1999). Phosphorylation controls timing of Cdc6p destruction: a biochemical analysis. Mol. Biol. Cell *10*, 3263–3277.

Emili, A. (1998). MEC1-dependent phosphorylation of Rad9p in response to DNA damage. Mol. Cell *2*, 183–189.

Goldstein, A. L., and McCusker, J. H. (1999). Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. Yeast *15*, 1541–1553.

Gopalakrishnan, V., Simancek, P., Houchens, C., Snaith, H. A., Frattini, M. G., Sazer, S., and Kelly, T. J. (2001). Redundant control of rereplication in fission yeast. Proc. Natl. Acad. Sci. USA *98*, 13114–13119.

Gottifredi, V., Shieh, S., Taya, Y., and Prives, C. (2001). p53 accumulates but is functionally impaired when DNA synthesis is blocked. Proc. Natl. Acad. Sci. USA *98*, 1036–1041.

Guthrie, C., and Fink, G. (eds.) (1990). Guide to Yeast Genetics and Molecular Biology, New York: Academic Press.

Haaf, T., Golub, E. I., Reddy, G., Radding, C. M., and Ward, D. C. (1995). Nuclear foci of mammalian Rad51 recombination protein in somatic cells after DNA damage and its localization in synaptonemal complexes. Proc. Natl. Acad. Sci. USA *92*, 2298–2302.

Haase, S. B., and Lew, D. J. (1997). Flow cytometric analysis of DNA content in budding yeast. Methods Enzymol. *283*, 322–332.

Hennessy, K. M., Clark, C. D., and Botstein, D. (1990). Subcellular localization of yeast *CDC46* varies with the cell cycle. Genes Dev. *4*, 2252–2263.

Hua, X. H., Yan, H., and Newport, J. (1997). A role for Cdk2 kinase in negatively regulating DNA replication during S phase of the cell cycle. J. Cell Biol. *137*, 183–192.

Jallepalli, P. V., Brown, G. W., Muzi-Falconi, M., Tien, D., and Kelly, T. J. (1997). Regulation of the replication initiator protein p65cdc18 by CDK phosphorylation. Genes Dev. *11*, 2767–2779.

Katou, Y., Kanoh, Y., Bando, M., Noguchi, H., Tanaka, H., Ashikari, T., Sugimoto, K., and Shirahige, K. (2003). S-phase checkpoint proteins Tof1 and Mrc1 form a stable replication-pausing complex. Nature *424*, 1078–1083.

Kondo, T., Wakayama, T., Naiki, T., Matsumoto, K., and Sugimoto, K. (2001). Recruitment of Mec1 and Ddc1 checkpoint proteins to double-strand breaks through distinct mechanisms. Science *294*, 867–870.

Labib, K., Diffley, J. F., and Kearsey, S. E. (1999). G1-phase and B-type cyclins exclude the DNA-replication factor Mcm4 from the nucleus. Nat. Cell Biol. *1*, 415–422.

Labib, K., Kearsey, S. E., and Diffley, J. F. (2001). MCM2–7 proteins are essential components of prereplicative complexes that accumulate cooperatively in the nucleus during G1-phase and are required to establish, but not maintain, the S-phase checkpoint. Mol. Biol. Cell *12*, 3658–3667.

Lee, C., Hong, B., Choi, J. M., Kim, Y., Watanabe, S., Ishimi, Y., Enomoto, T., Tada, S., and Cho, Y. (2004). Structural basis for inhibition of the replication licensing factor Cdt1 by Geminin. Nature *430*, 913–917.

Lopez-Girona, A., Mondesert, O., Leatherwood, J., and Russell, P. (1998). Negative regulation of Cdc18 DNA replication protein by Cdc2. Mol. Biol. Cell *9*, 63–73.

McGarry, T. J., and Kirschner, M. W. (1998). Geminin, an inhibitor of DNA replication, is degraded during mitosis. Cell *93*, 1043–1053.

Melixetian, M., Ballabeni, A., Masiero, L., Gasparini, P., Zamponi, R., Bartek, J., Lukas, J., and Helin, K. (2004). Loss of Geminin induces rereplication in the presence of functional p53. J. Cell Biol. *165*, 473–482.

Melo, J., and Toczyski, D. (2002). A unified view of the DNA-damage checkpoint. Curr. Opin. Cell Biol. *14*, 237–245.

Melo, J. A., Cohen, J., and Toczyski, D. P. (2001). Two checkpoint complexes are independently recruited to sites of DNA damage in vivo. Genes Dev. *15*, 2809–2821.

Mihaylov, I. S., Kondo, T., Jones, L., Ryzhikov, S., Tanaka, J., Zheng, J., Higa, L. A., Minamino, N., Cooley, L., and Zhang, H. (2002). Control of DNA replication and chromosome ploidy by Geminin and Cyclin A. Mol. Cell. Biol. *22*, 1868–1880.

Moll, T., Tebb, G., Surana, U., Robitsch, H., and Nasmyth, K. (1991). The role of phosphorylation and the CDC28 protein kinase in cell cycle-regulated nuclear import of the *S. cerevisiae* transcription factor SWI5. Cell *66*, 743–758.

Nasmyth, K. (2001). Disseminating the genome: joining, resolving, and separating sister chromatids during mitosis and meiosis. Annu. Rev. Genet. *35*, 673–745.

Nguyen, V. Q., Co, C., Irie, K., and Li, J. J. (2000). Clb/Cdc28 kinases promote nuclear export of the replication initiator proteins Mcm2–7. Curr. Biol. *10*, 195–205.

Nguyen, V. Q., Co, C., and Li, J. J. (2001). Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms. Nature *411*, 1068–1073.

Nishitani, H., Lygerou, Z., Nishimoto, T., and Nurse, P. (2000). The Cdt1 protein is required to license DNA for replication in fission yeast. Nature *404*, 625–628.

Nyberg, K. A., Michelson, R. J., Putnam, C. W., and Weinert, T. A. (2002). Toward maintaining the genome: DNA damage and replication checkpoints. Annu. Rev. Genet. *36*, 617–656.

Osborn, A. J., and Elledge, S. J. (2003). Mrc1 is a replication fork component whose phosphorylation in response to DNA replication stress activates Rad53. Genes Dev. *17*, 1755–1767.

Quinn, L. M., Herr, A., McGarry, T. J., and Richardson, H. (2001). The *Drosophila* Geminin homolog: roles for Geminin in limiting DNA replication, in anaphase and in neurogenesis. Genes Dev. *15*, 2741–2754.

Saintigny, Y., Delacote, F., Vares, G., Petitot, F., Lambert, S., Averbeck, D., and Lopez, B. S. (2001). Characterization of homologous recombination induced by replication inhibition in mammalian cells. EMBO J. *20*, 3861–3870.

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

Sauer, K., Knoblich, J. A., Richardson, H., and Lehner, C. F. (1995). Distinct modes of cyclin E/cdc2c kinase regulation and S-phase control in mitotic and endoreduplication cycles of *Drosophila* embryogenesis. Genes Dev. *9*, 1327–1339.

Saxena, S., Yuan, P., Dhar, S. K., Senga, T., Takeda, D., Robinson, H., Kornbluth, S., Swaminathan, K., and Dutta, A. (2004). A dimerized coiled-coil domain and an adjoining part of geminin interact with two sites on Cdt1 for replication inhibition. Mol. Cell *15*, 245–258.

Schwab, M., Lutum, A. S., and Seufert, W. (1997). Yeast Hct1 is a regulator of Clb2 cyclin proteolysis. Cell *90*, 683–693.

Sogo, J. M., Lopes, M., and Foiani, M. (2002). Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. Science *297*, 599–602.

Sun, Z., Fay, D. S., Marini, F., Foiani, M., and Stern, D. F. (1996). Spk1/Rad53 is regulated by Mec1-dependent protein phosphorylation in DNA replication and damage checkpoint pathways. Genes Dev. *10*, 395–406.

Tada, S., Li, A., Maiorano, D., Mechali, M., and Blow, J. J. (2001). Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin. Nat. Cell Biol. *3*, 107–113.

Toh, G. W., and Lowndes, N. F. (2003). Role of the *Saccharomyces cerevisiae* Rad9 protein in sensing and responding to DNA damage. Biochem. Soc. Trans. *31*, 242–246.

Uhlmann, F., Wernic, D., Poupart, M. A., Koonin, E. V., and Nasmyth, K. (2000). Cleavage of cohesin by the CD clan protease separin triggers anaphase in yeast. Cell *103*, 375–386.

Vas, A., Mok, W., and Leatherwood, J. (2001). Control of DNA rereplication via Cdc2 phosphorylation sites in the origin recognition complex. Mol. Cell. Biol. *21*, 5767–5777.

Vaziri, C., Saxena, S., Jeon, Y., Lee, C., Murata, K., Machida, Y., Wagle, N., Hwang, D. S., and Dutta, A. (2003). A p53-dependent checkpoint pathway prevents rereplication. Mol. Cell *11*, 997–1008.

Verreault, A. (2003). Histone deposition at the replication fork: a matter of urgency. Mol. Cell *11*, 283–284.

Visintin, R., Prinz, S., and Amon, A. (1997). CDC20 and CDH *1*, a family of substrate-specific activators of APC-dependent proteolysis. Science *278*, 460–463.

Ward, I. M., and Chen, J. (2001). Histone H2AX is phosphorylated in an ATR-dependent manner in response to replicational stress. J. Biol. Chem. *276*, 47759–47762.

Weinert, T. A., Kiser, G. L., and Hartwell, L. H. (1994). Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. Genes Dev. *8*, 652–665.

Wilmes, G. M., Archambault, V., Austin, R. J., Jacobson, M. D., Bell, S. P., and Cross, F. R. (2004). Interaction of the S-phase cyclin Clb5 with an "RXL" docking sequence in the initiator protein Orc6 provides an origin-localized replication control switch. Genes Dev. *18*, 981–991.

Wohlschlegel, J. A., Kutok, J. L., Weng, A. P., and Dutta, A. (2002). Expression of geminin as a marker of cell proliferation in normal tissues and malignancies. Am. J. Pathol. *161*, 267–273.

Wuarin, J., Buck, V., Nurse, P., and Millar, J. B. (2002). Stable association of mitotic cyclin B/Cdc2 to replication origins prevents endoreduplication. Cell *111*, 419–431.

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

Zhong, W., Feng, H., Santiago, F. E., and Kipreos, E. T. (2003). CUL-4 ubiquitin ligase maintains genome stability by restraining DNA-replication licensing. Nature *423*, 885–889.

Zhu, W., Chen, Y., and Dutta, A. (2004). Rereplication by depletion of Geminin is seen regardless of p53 status and activates a G2/M checkpoint. Mol. Cell. Biol. *24*, 7140–7150.

Whole genome analysis of DNA replication by microarray competitive genomic hybridization permits detection of rereplication from a single origin of replication.

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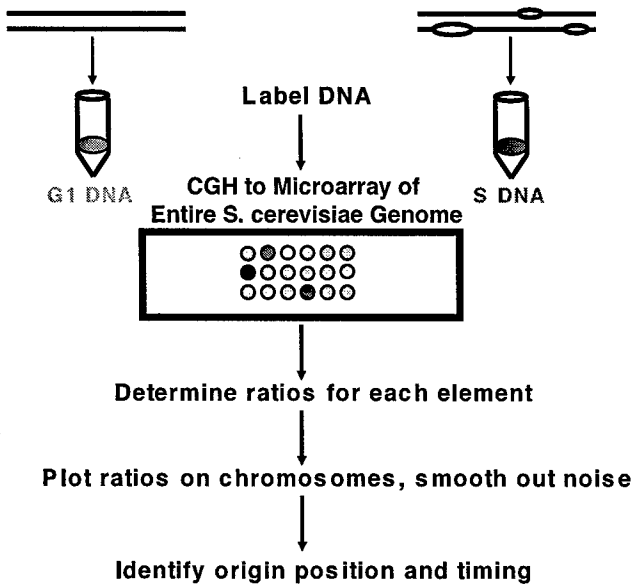
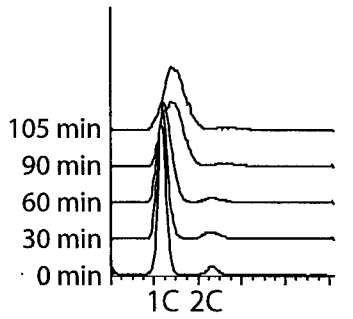
Figure 1

A



C



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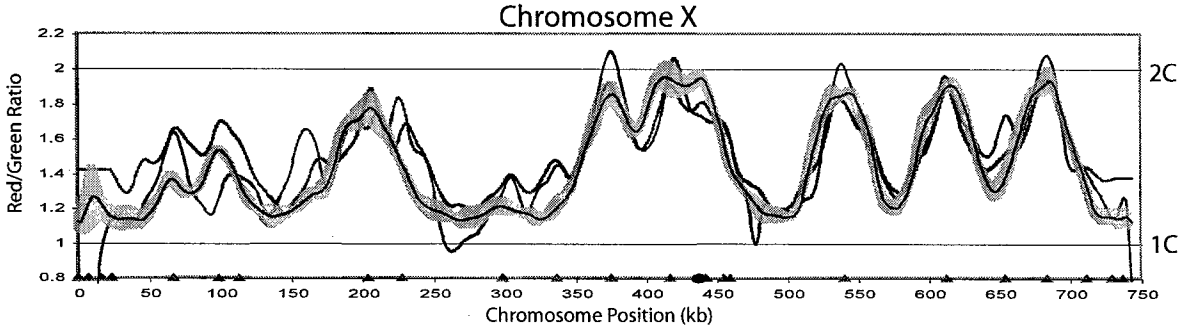
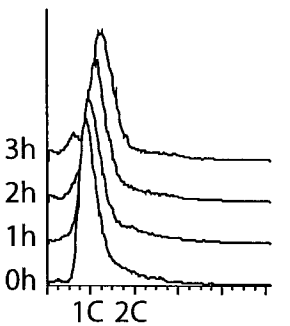


D

	Mean distance
Raghuraman et al. (2001)	6.5 kb
Wyrick et al. (2001)	3.9 kb
Yabuki et al. (2002)	3.5 kb
Our Data	3.1 kb
Our Data, One Hyb	4.2 kb

F



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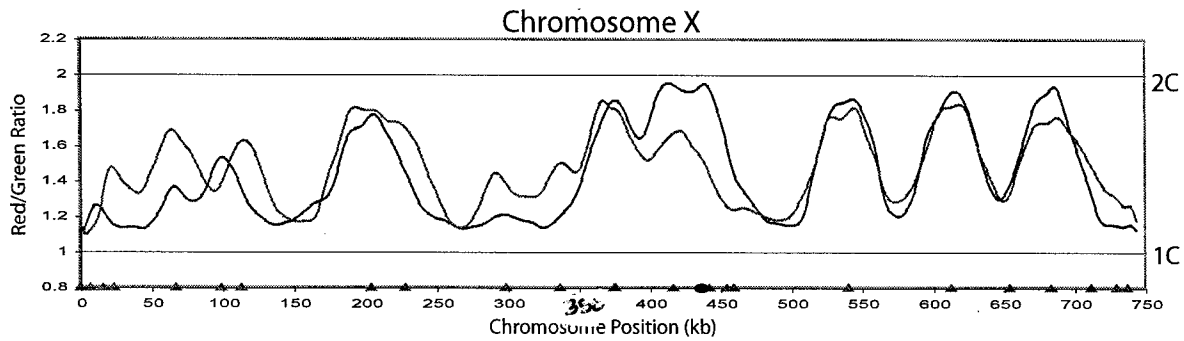

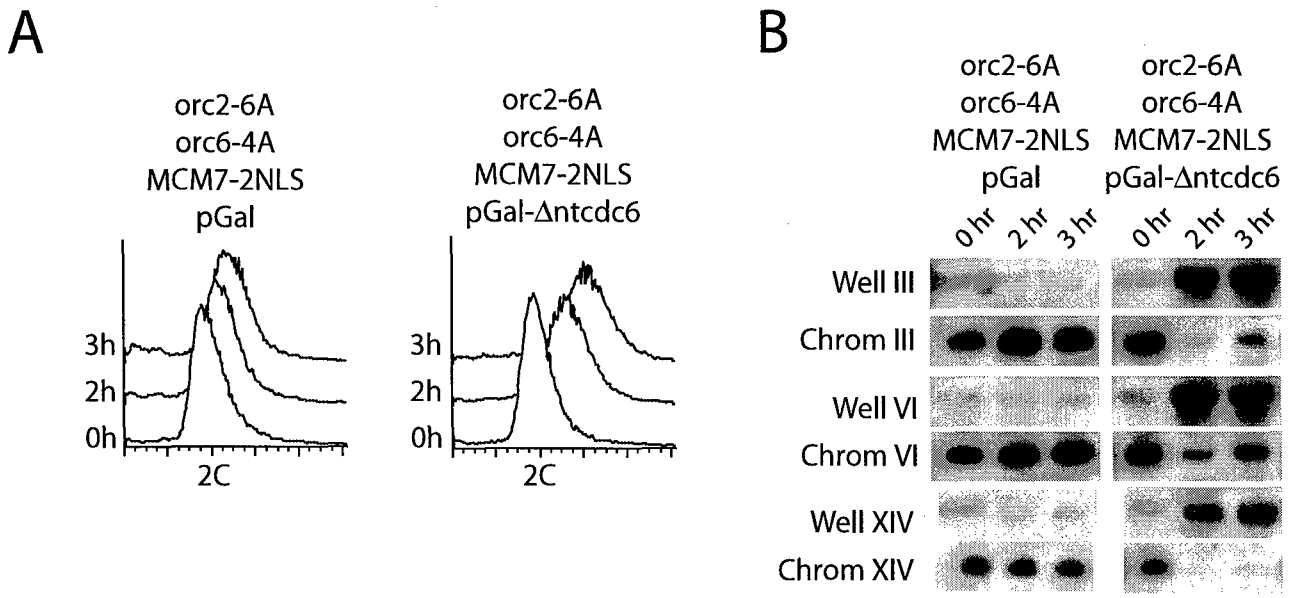
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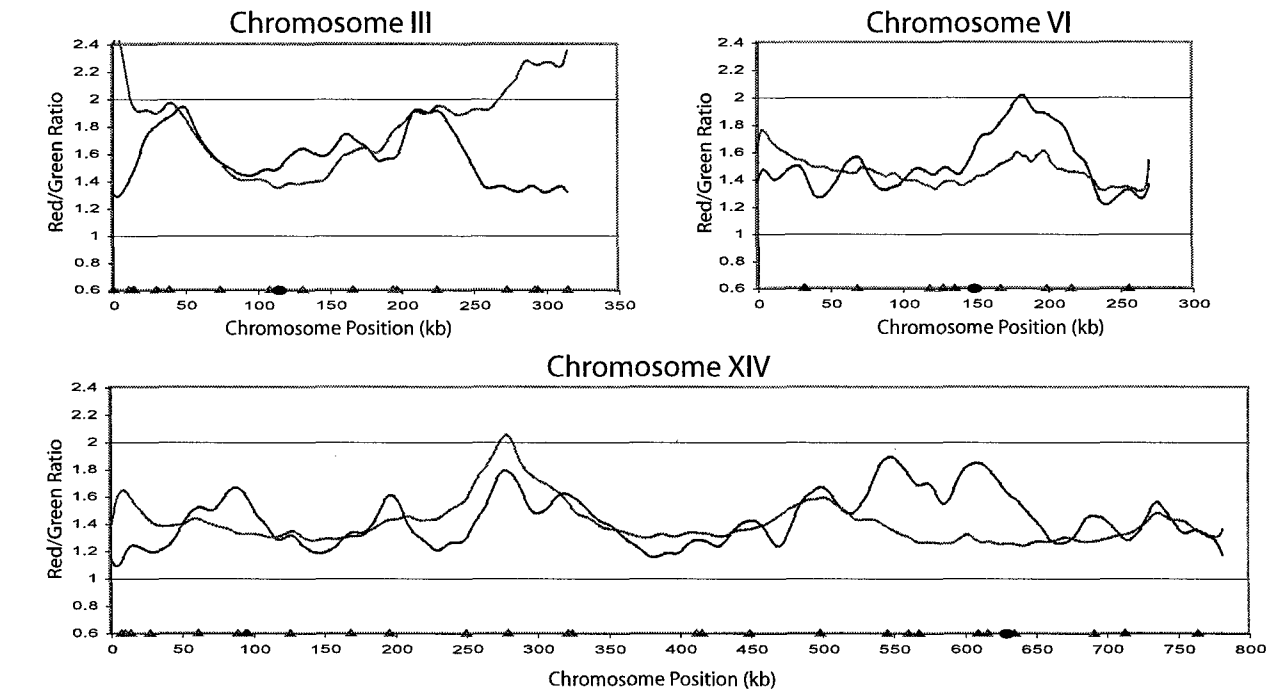
orc6-4A
MCM7-2NLS
pGal
orc2-6A
orc6-4A
MCM7-2NLS
pGal-Δntcdc6
3h
2h
0h
2C
B
orc2-6A
orc6-4A
MCM7-2NLS
pGal
orc2-6A
orc6-4A
MCM7-2NLS
pGal-Δntcdc6
0 hr
2 hr
3 hr
Well III
Chrom III
Well VI
Chrom VI
Well XIV
Chrom XIV


C
Chromosome III
Chromosome VI
Chromosome XIV
Red/Green Ratio
Chromosome Position (kb)

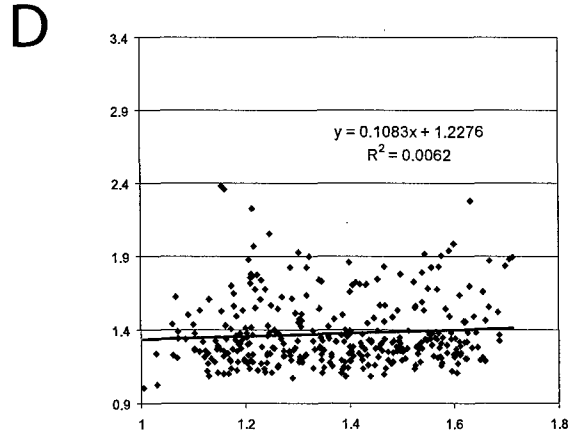
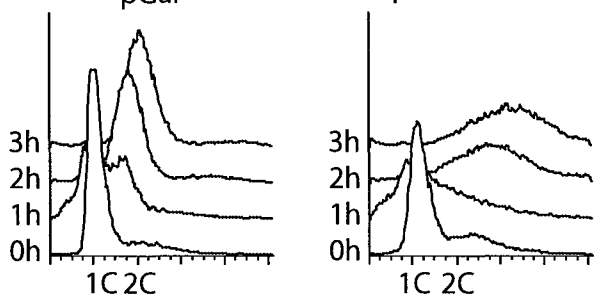

D
y = 0.1083x + 1.2276
R2 = 0.0062

Figure 3

A



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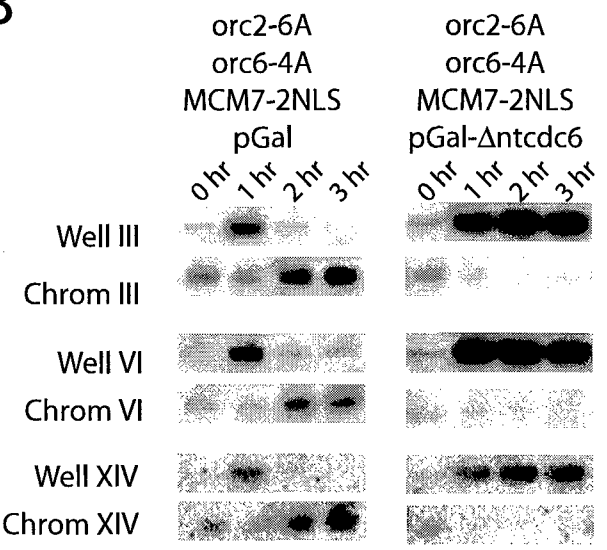

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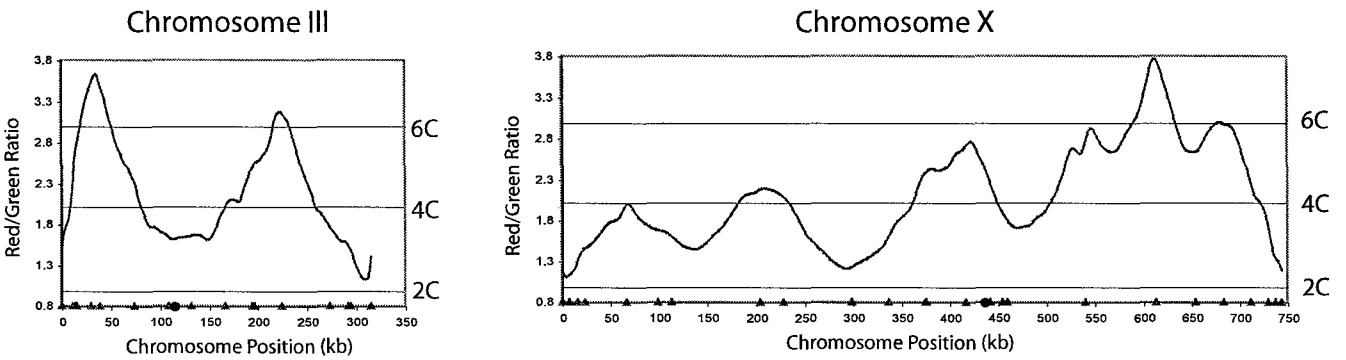
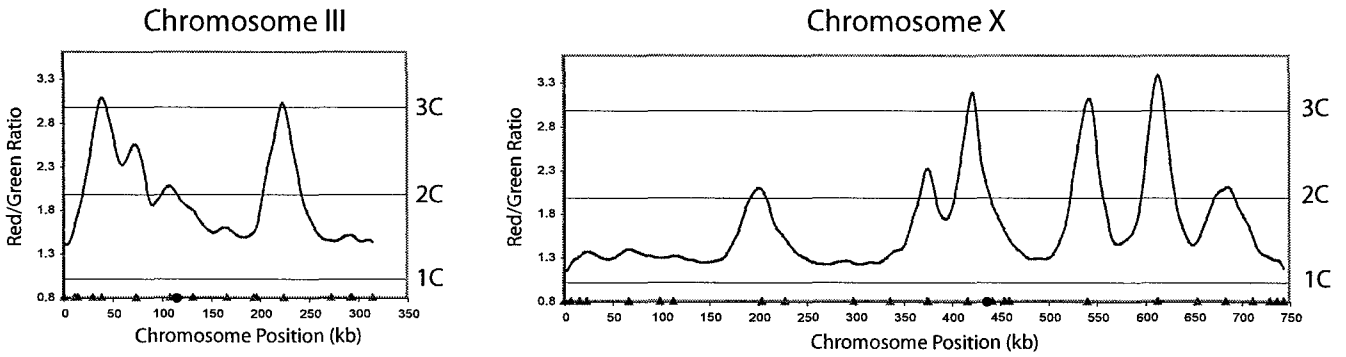


D



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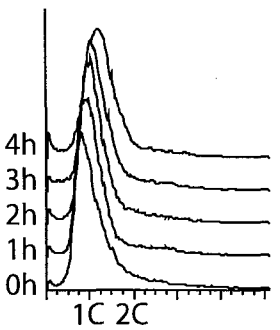

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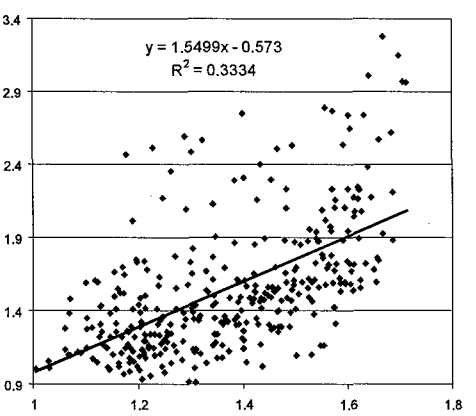
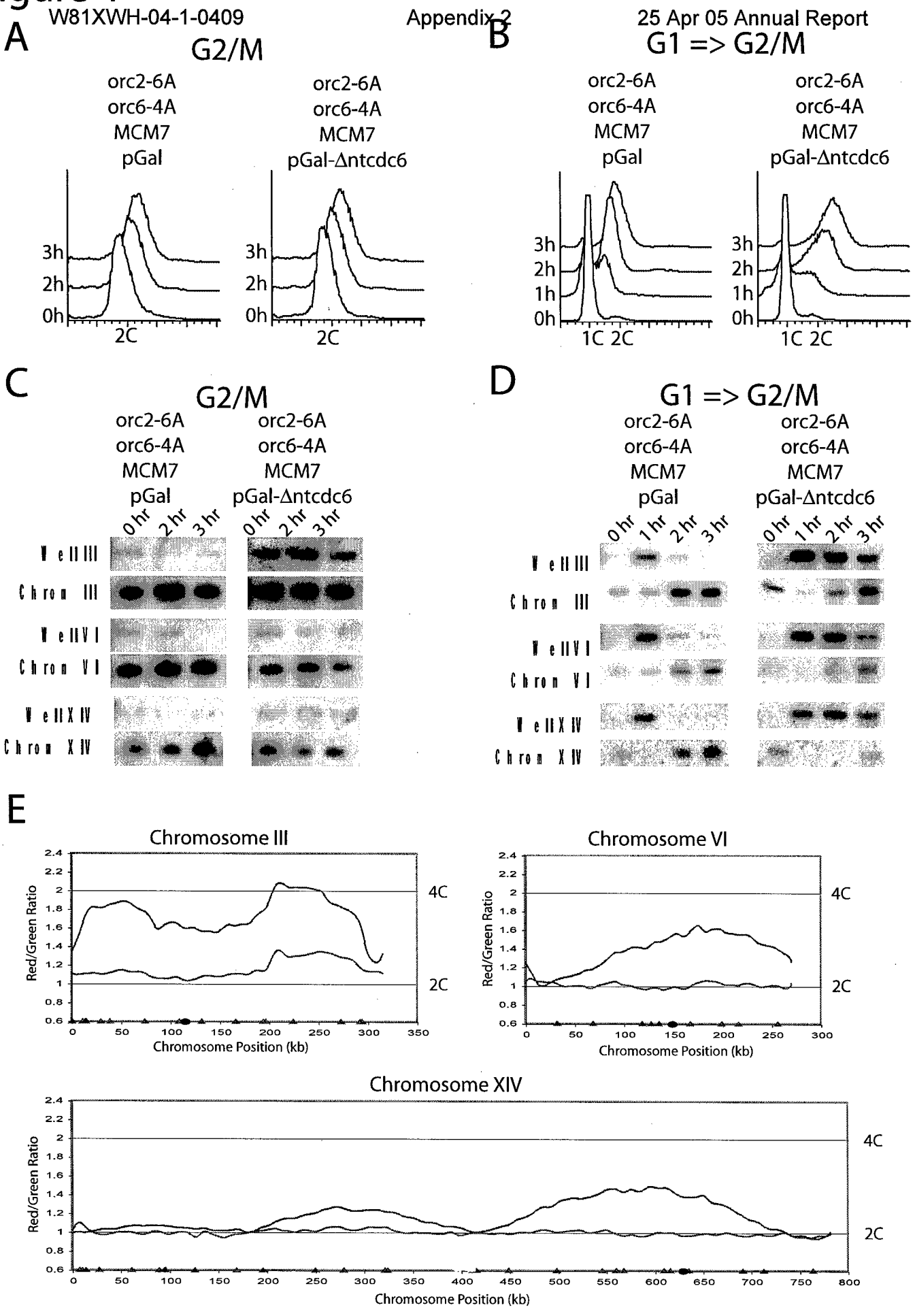


Figure 4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G2/M
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orc6-4A
MCM7
pGal
orc2-6A
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MCM7
pGal-Δntcdc6
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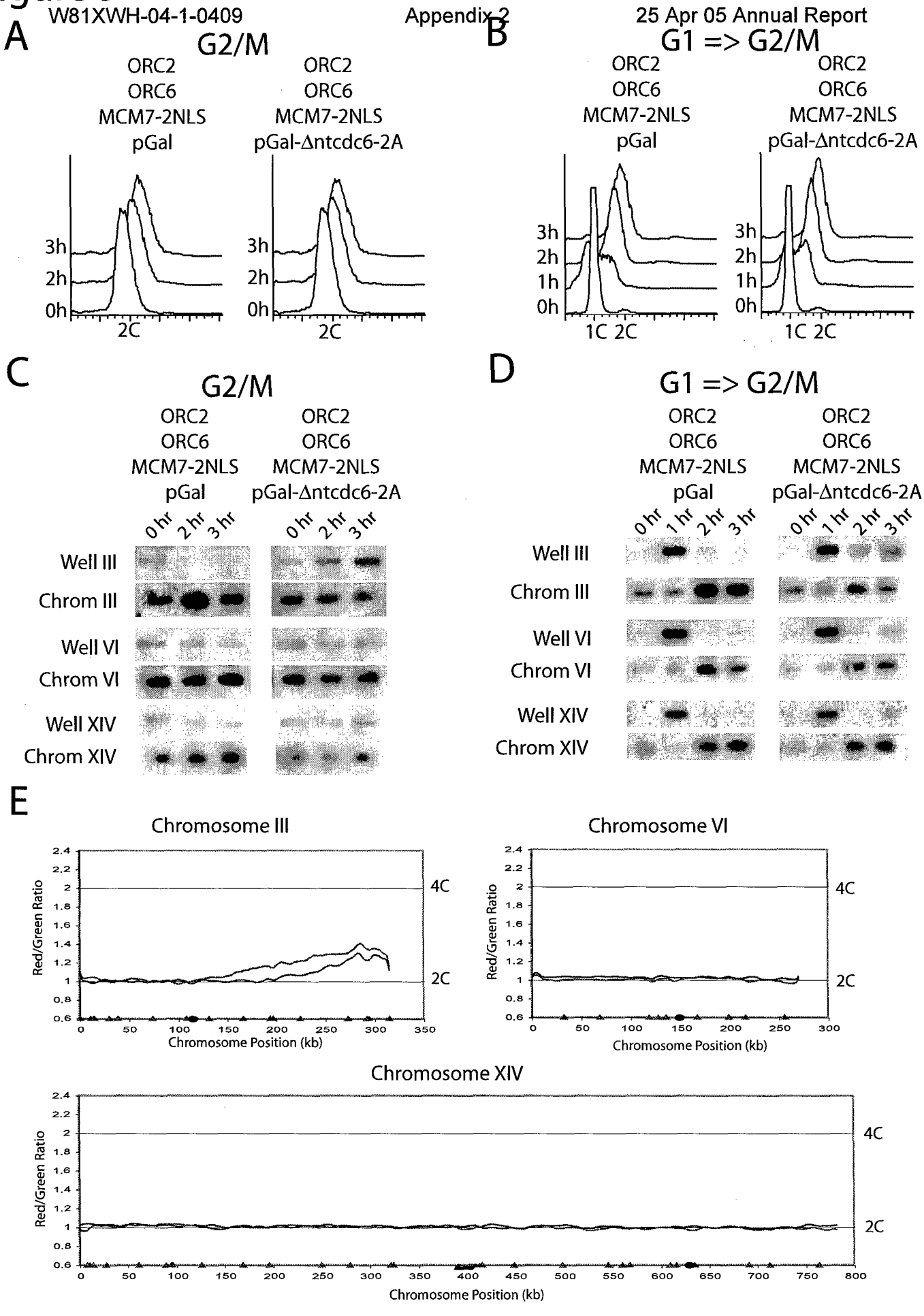
2h
0h
2C
B
G1 => G2/M
orc2-6A
orc6-4A
MCM7
pGal
orc2-6A
orc6-4A
MCM7
pGal-Δntcdc6
3h
2h
1h
0h
1C 2C
C
G2/M
orc2-6A
orc6-4A
MCM7
pGal
orc2-6A
orc6-4A
MCM7
pGal-Δntcdc6
0 hr
2 hr
3 hr
Chrom III
Chrom VI
Chrom XIV
D
G1 => G2/M
orc2-6A
orc6-4A
MCM7
pGal
orc2-6A
orc6-4A
MCM7
pGal-Δntcdc6
0 hr
1 hr
2 hr
3 hr
Chrom III
Chrom VI
Chrom XIV
E
Chromosome III
Red/Green Ratio
4C
2C
Chromosome Position (kb)
Chromosome VI
Red/Green Ratio
4C
2C
Chromosome Position (kb)
Chromosome XIV
Red/Green Ratio
4C
2C
Chromosome Position (kb)

Figure 5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G2/M
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ORC6
MCM7-2NLS
pGal
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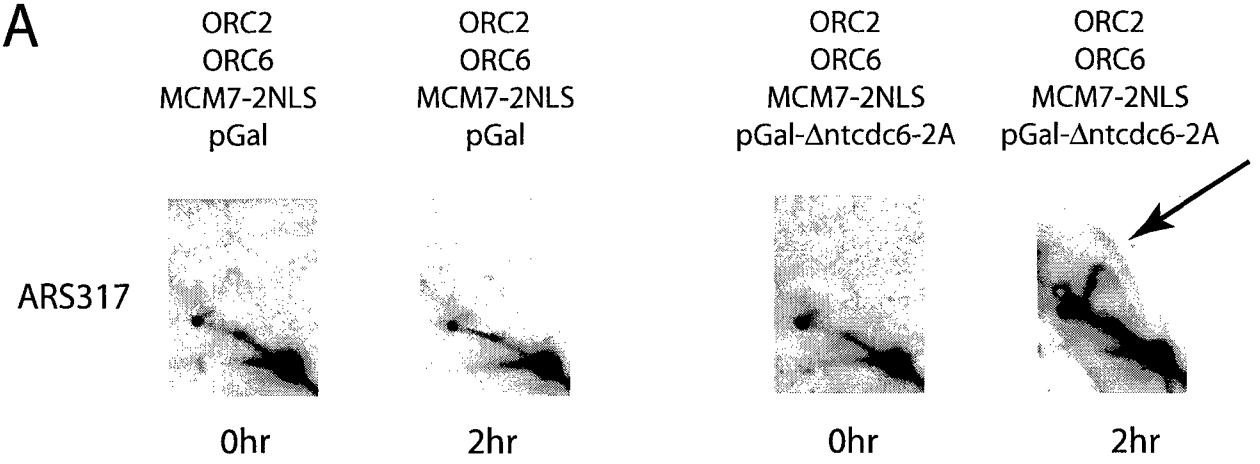
ORC6
MCM7-2NLS
pGal-Δntcdc6-2A
3h
2h
0h
2C
B
G1 => G2/M
ORC2
ORC6
MCM7-2NLS
pGal
ORC2
ORC6
MCM7-2NLS
pGal-Δntcdc6-2A
3h
2h
1h
0h
1C 2C
C
G2/M
ORC2
ORC6
MCM7-2NLS
pGal
ORC2
ORC6
MCM7-2NLS
pGal-Δntcdc6-2A
0 hr
2 hr
3 hr
Well III
Chrom III
Well VI
Chrom VI
Well XIV
Chrom XIV
D
G1 => G2/M
ORC2
ORC6
MCM7-2NLS
pGal
ORC2
ORC6
MCM7-2NLS
pGal-Δntcdc6-2A
0 hr
1 hr
2 hr
3 hr
Well III
Chrom III
Well VI
Chrom VI
Well XIV
Chrom XIV
E
Chromosome III
Red/Green Ratio
4C
2C
Chromosome Position (kb)
Chromosome VI
Red/Green Ratio
4C
2C
Chromosome Position (kb)
Chromosome XIV
Red/Green Ratio
4C
2C
Chromosome Position (kb)

A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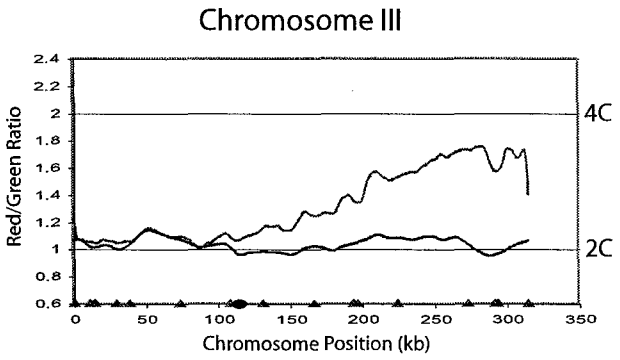
ORC6
MCM7-2NLS
pGal
ORC2
ORC6
MCM7-2NLS
pGal
ORC2
ORC6
MCM7-2NLS
pGal-Δntcdc6-2A
ORC2
ORC6
MCM7-2NLS
pGal-Δntcdc6-2A
ARS317
0hr
2hr
0hr
2hr

B


Chromosome III
Red/Green Ratio
Chromosome Position (kb)
4C
2C

C

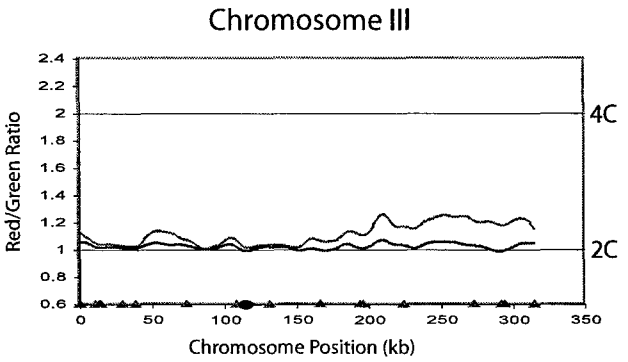

Chromosome III
Red/Green Ratio
Chromosome Position (kb)
4C
2C

Figure 1 Replication profiles generated by competitive genomic hybridization.

A Schematic representation of our modified competitive genomic hybridization protocol. G1 arrested and replicating cells are harvested and genomic DNA is prepared. The genomic DNA is differentially labeled with Cy3 (G1, "green") and Cy5 (S phase, "red") and hybridized to a microarray containing 13,165 ORF and intergenic PCR products. Flow cytometry is used to determine a red to green ratio normalization factor that reflects the extent of replication, then normalized ratios are plotted and mathematically smoothed. Peaks on the replication profile are identified as origins.

B Our modified competitive genomic hybridization protocol accurately assays replication timing. Wild type S288c yeast were synchronized in G1 by the addition of alpha factor. Cells were then either maintained in the alpha factor arrest or released into S phase in the presence of 100 mM hydroxyurea (HU) for 110 minutes. Cells were then harvested and their genomic DNA purified. Genomic DNA from G1 arrested and S phase cells were differentially labeled with Cy3 and Cy5 and competitively hybridized. The red to green normalization factor for this experiment was 1.3. Shown is chromosome 10 (blue line) average values from four independent experiments. Each data point is shown plus and minus the standard deviation (light blue band) among the four experiments. For comparison, previously published data from Raghuraman et al. (violet line) and Yabuki et al. (brown line) are shown, scaled to appear within the range of the graphs. Previously identified origins cataloged in SGD (red triangles) and the centromere (black circle) are plotted along the X-axis.

C Samples for flow cytometry were taken at the times indicated during the experiment described in Figure 1B.

D Origins can be accurately identified from peaks in the replication profile. Origins on chromosomes 3, 5, 6 and 10 have been comprehensively mapped and cataloged in SGD. The mean distance from each origin identified in our data set, as well as several other published sets, to an origin cataloged in SGD is listed in the table. Our data, one hyb indicates the results obtained from a single microarray hybridization from a single experiment.

E The normal S phase replication timing profile of the rereplication competent mutant is similar to wild type. Cells containing *MCM7-2NLS orc2-cdk6A orc6-cdk4A pMET3-HA3-CDC20 pGal-Δntcdc6*, but not rereplicating, were synchronized in G1 by the addition of alpha factor. Cells were then either maintained in the alpha factor arrest or released into 100 mM hydroxyurea (HU) for 180 minutes. Genomic DNA purification and hybridization was carried out as described in Figure 1A. The red to green normalization factor for this experiment was 1.45. The replication profile of *orc2-cdk6A orc6-cdk4A MCM7-2NLS pMET3-HA3-CDC20* (red line) and wild type replication profile from Figure 1B (blue line) are shown. Previously identified origins cataloged in SGD (red triangles) and the centromere (black circle) are plotted along the X-axis.

F Samples for flow cytometry were taken at the times indicated during the experiment described in Figure 1E.

Figure 2 Complete analysis of rereplication in metaphase arrested *orc2-cdk6A orc6-cdk4A MCM7-2NLS pGal-Δntcdc6* cells.

A Significant rereplication can be induced in the metaphase arrested *orc2-cdk6A orc6-cdk4A MCM7-2NLS pGal-Δntcdc6* mutant. Cells with the indicated genotypes plus *pMET3-HA3-CDC20* were grown in medium containing 3% raffinose + 0.05% dextrose. Cells were arrested in metaphase by adding 2mM methionine, to transcriptionally deplete Cdc20p, and 15μg/ml nocodazole. Then, 2% galactose was added to induce Δntcdc6p in the strain containing *pGal-Δntcdc6*, and samples were taken for flow cytometry at 0, 2 and 3 hours.

B Pulsed field gel electrophoresis shows all chromosomes rereplicate. Cells were induced to rereplicate as in Figure 2A. Southern blots of a pulsed field gels were probed for an ARS305 fragment to detect chromosome III, ARS607 to detect chromosome 6 and ARS1413 to detect chromosome 14. For each blot the well and the indicated chromosome are shown.

C Rereplication occurs on all chromosomes, but different origins initiate in rereplication than replication. Cells were induced with galactose as in Figure 2A. At 3 hours after galactose addition, cells were harvested and their genomic DNA was purified. Genomic DNA from *orc2-cdk6A orc6-cdk4A MCM7-2NLS pGal* and *orc2-cdk6A orc6-cdk4A MCM7-2NLS pGal-Δntcdc6* strains were differentially labeled with Cy3 and Cy5 and competitively hybridized to a microarray as in Figure 1A. The red to green normalization factor for this experiment was 1.35. The mitotic rereplication profile (red line) is plotted along with a normal S phase replication profile from Figure 1D (blue line) for chromosomes 3, 6 and 14. Previously identified origins (red triangles) either cataloged in SGD (chromosomes 3 and 6) or from Wyrick et al. (chromosome 14) and the centromere (black circle) are plotted along the X-axis.

D Replication timing does not correlate with efficiency of mitotic rereplication. For 351 proARSes defined by Wyrick et al., the red to green ratio of the rereplication profile from Figure 2C is plotted versus the red to green ratio of the replication profile from Figure 1D.

Figure 3 Rereplication induced as cells pass through S phase is more extensive than rereplication induced in metaphase arrested cells.

A Increased rereplication can be observed by flow cytometry. Cells with the indicated genotypes plus *pMET3-HA3-CDC20* were grown in medium containing 3% raffinose + 0.05% dextrose then were arrested in G1 by the additional of alpha factor. Galactose was added to 2% for 30 minutes to induce Δntcdc6p in the strain containing *pGal-Δntcdc6*. Cells were then released from the G1 arrest and rearrested in metaphase with the addition of 2mM methionine, to transcriptionally deplete Cdc20p, and 15μg/ml nocodazole. Samples were taken for flow cytometry at 0, 1, 2 and 3 hours.

B PFGE shows that all chromosomes rereplicate. Cells were induced as in Figure 3A and samples for PFGE were taken every hour. Southern blots of PFGE gels were probed as in Figure 2B.

C In some regions of the genome, more than one round of rereplication can be induced. Cells were induced to rereplicate as in Figure 3A. Cells were harvested at 3 hours. Genomic DNA from metaphase arrested *orc2-cdk6A orc6-cdk4A MCM7-2NLS pGal* and *orc2-cdk6A orc6-cdk4A MCM7-2NLS pGal-Δntcdc6* strains was purified, differentially labeled with Cy3 and Cy5, and competitively hybridized to a microarray. The red to green normalization factor was 1.6. The profile of rereplication (green line) is plotted for chromosomes 3 and 10. Previously identified origins cataloged in SGD (red triangles) and the centromere (black circle) are plotted along the X-axis. Red to green ratios indicative of different genome copy numbers (C) are shown to the right of each graph.

D Rereplication can occur while cells are still within S phase. Cells containing *orc2-cdk6A orc6-cdk4A MCM7-2NLS pGal-Δntcdc6 pMET3-HA3-CDC20* grown in medium containing 3% raffinose + 0.05% dextrose were arrested in G1 by the additional of alpha factor. To induce Δntcdc6p, 2% galactose was added to 30 minutes. Cells were then either maintained in the alpha factor arrest or released to rereplicate during S phase in the presence of 100 mM hydroxyurea (HU) for 240 minutes. Genomic DNA from G1 arrested and S phase cells was purified, differentially labeled with Cy3 and Cy5, and competitively hybridized to a microarray. The red to green ratio normalization factor was 1.4. The profile of rereplication during S phase (blue line) is shown for chromosomes 3 and10. Red triangles, black circles and genomic copy number (C) are indicated as in Figure 3C.

E Flow cytometry on samples taken every 60 minutes during the experiment described in Figure 3D indicates that cells are still within S phase.

F Rereplication induced as cells transit through S phase shows a preference for early origins. For 351 proARSes defined by Wyrick et al., the red to green ratio of the rereplication profile from Figure 3C is plotted versus the red to green ratio of the replication profile from Figure 1D

Figure 4 Under certain conditions, rereplication can be induced when only ORC and Cdc6 are deregulated.

A Rereplication is undetectable by flow cytometry when Δntcdc6p is induced in metaphase arrested cells containing *orc2-cdk6A orc6-cdk4A*. Cells with the indicated genotypes plus *pMET3-HA3-CDC20* were arrested in metaphase and induced with galactose as described in Figure 2A. Samples were taken for flow cytometry at 0, 2 and 3 hours.

B Significant rereplication can be induced in *orc2-cdk6A orc6-cdk4 pGal-Δntcdc6* cells while they are progressing through S phase. Cells with the indicated genotypes plus *pMET3-HA3-CDC20* were arrested in G1 and induced with galactose as described in Figure 3A. Samples were taken for flow cytometry at 0, 1, 2 and 3 hours.

C Limited rereplication, primarily on chromosome III, is detectable using PFGE when *orc2-cdk6A orc6-cdk4A pGal-Δntcdc6* cells are induced from a metaphase arrest. Cells were

induced with galactose in metaphase as in Figure 4A. Samples for PFGE were taken at 0, 2 and 3 hours. Southern blots of PFGE gels were probed as described in Figure 2B.

D PFGE shows rereplication on most chromosomes as cell pass from G1 into metaphase. Cells were induced as in Figure 4B and samples for PFGE were taken every hour. Southern blots of PFGE gels were probed as described in Figure 2B.

E Rereplication induced during progression through S phase occurs at many chromosomal locations and limited rereplication can be detected on chromosome III in metaphase. Rereplication profile of *orc2-cdk6A orc6-cdk4 pGal-Δntcdc6* cells induced to rereplicate in metaphase (red line) and from G1 into metaphase (blue line) are shown for chromosomes 3, 6 and 14. The red to green ratio normalization factor for the metaphase induction was 1.0 and for the G1 to metaphase induction was 1.3. Red triangles, black circles and genomic copy number (C) are indicated as in Figure 3C.

Figure 5 When Mcm2-7 are constitutively nuclear and Cdc6 is significantly overexpressed, limited rereplication can be induced.

A Rereplication is undetectable by flow cytometry when Δntcdc6-2Ap is induced in metaphase arrested cells containing *MCM7-2NLS*. Cells with the indicated genotypes plus *pMET3-HA3-CDC20* were arrested in metaphase and induced with galactose as described in Figure 2A. Samples were taken for flow cytometry at 0, 2 and 3 hours.

B Rereplication is also undetectable by flow cytometry when Δntcdc6-2Ap is induced as cells containing *MCM7-2NLS* transit through S phase. Cells with the indicated genotypes plus *pMET3-HA3-CDC20* were arrested in G1 and induced with galactose as described in Figure 3A. Samples were taken for flow cytometry at 0, 1, 2 and 3 hours.

C PFGE indicates that chromosome III rereplicates in metaphase arrested *MCM7-2NLS pGal-Δntcdc6-2A* cells. Cells were induced with galactose as in Figure 5A. Samples for PFGE were taken at 0, 2 and 3 hours. Southern blots of PFGE gels were probed as described in Figure 2B

D PFGE indicates that chromosome III also rereplicates in *MCM7-2NLS pGal-Δntcdc6-2A* cells transiting through S phase when Δntcdc6-2Ap is induced. Cells were induced with galactose as in Figure 5B. Samples for PFGE were taken every hour. Southern blots of PFGE gels were probed as described in Figure 2B

E Rereplication is limited to chromosome III when *MCM7-2NLS pGal-Δntcdc6-2A* cells rereplicate. Rereplication profiles of cells induced to rereplicate in metaphase (red line) and from G1 into metaphase (blue line) are shown for chromosomes 3, 6 and 14. The red to green ratio normalization factor for both experiments was 1.0. Red triangles, black circles and genomic copy number (C) are indicated as in Figure 3C.

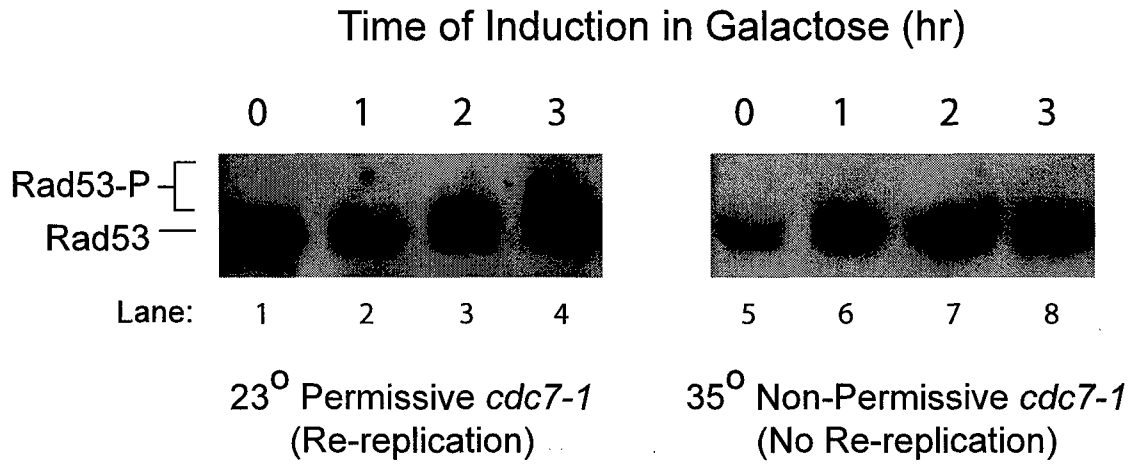
Figure 6 ARS317 initiates when cells containing *MCM7-2NLS pGal-Δntcdc6-2A* rereplicate.

A 2D gel electrophoresis indicates that ARS317 initiates when rereplication is induced in metaphase. Cells containing the indicated genotypes and *pMET3-HA3-CDC20* were arrested in metaphase and induced with galactose as described in Figure 2A. Samples were taken for 2D gel at 0 and 2 hours. Southern blots of 2D gels were probed with an ARS317 fragment.

B Microarray analysis confirms that the ARS317 sequence is required for rereplication when Mcm2-7p and Cdc6p is deregulated in metaphase. Cells containing *MCM7-2NLS pMET3-HA3-CDC20 pGal-Δntcdc6-2A, MCM7-2NLS pMET3-HA3-CDC20 pGal-Δntcdc6-2A ΔARS317,* and their respective *pGal* control strains were arrested in metaphase and induced with galactose for 3 hours as in Figure 2A. Rereplication profile of *MCM7-2NLS pGal-Δntcdc6-2A* (red line) and *MCM7-2NLS pGal-Δntcdc6-2A ΔARS317* (blue line) are shown for chromosome 3. The red to green ratio normalization factor for both experiments is 1.0. Red triangles, black circles and genomic copy number (C) are indicated as in Figure 3C.

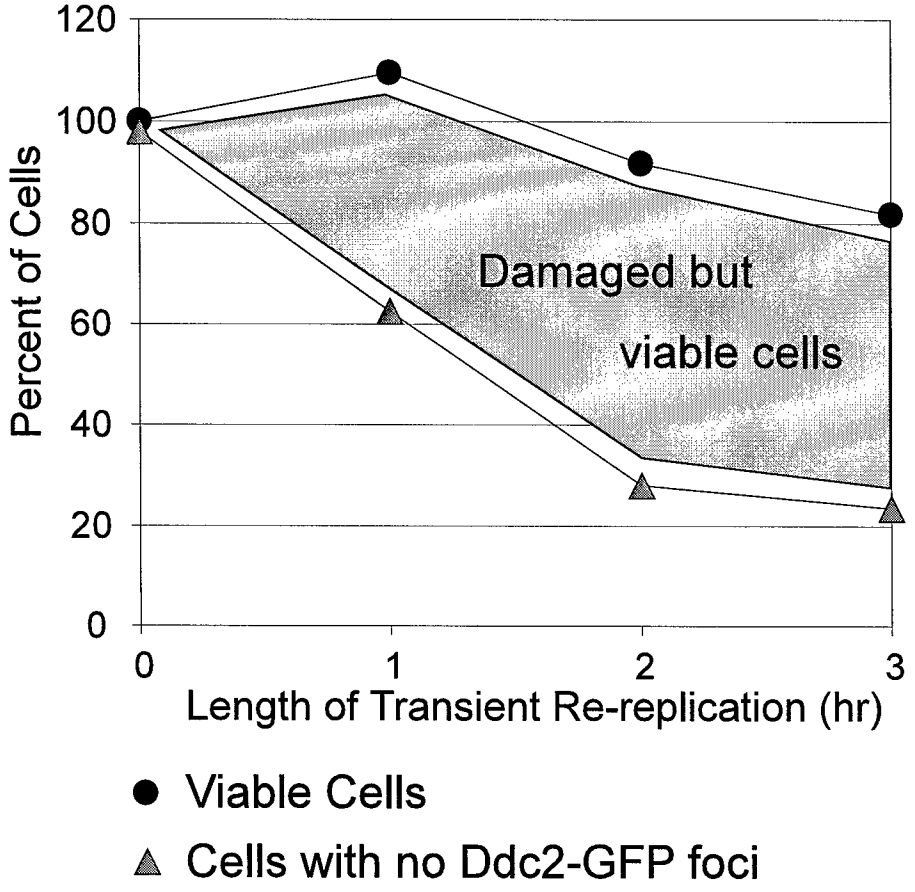
C Rereplication requires Cdc7p, a kinase required for S phase initiation. Cells containing *MCM7-2NLS pMET3-HA3-CDC20 pGal-Δntcdc6-2A CDC7, MCM7-2NLS pMET3-HA3-CDC20 pGal-Δntcdc6-2A cdc7-1* and their respective *pGal* control strains were grown in medium containing 3% raffinose and 0.05% dextrose. Cells were then arrested in metaphase at 23°C with the addition of 2mM methionine, to transcriptionally deplete Cdc20p, and 15μg/ml nocodazole. The temperature of the cultures was then changed to 35°C for 1 hour. Then, galactose was added to induce Δntcdc6-2Ap in the *pGal-Δntcdc6-2A* strains and cells were harvested 4 hours after galactose addition. Rereplication profile of *MCM7-2NLS pGal-Δntcdc6-2A CDC7* (red line) and *MCM7-2NLS pGal-Δntcdc6-2A cdc7-1* (blue line) are shown for chromosome 3. The red to green ratio normalization factor for both experiments is 1.0. Red triangles, black circles and genomic copy number (C) are indicated as in Figure 3C.

Figure 1



Deregulation of two mechanisms that act to block re-replication leads to a DNA damage response. This response requires re-replication and not simply inappropriate pre-replicative complex formation. Cells deregulated for Mcm2-7p inhibition (MCM7-2NLS) and Cdc6p downregulation (*pGal-Δntcdc6-2A*) were arrested in metaphase. These cells also contain *cdc7-1*, which produces a temperature sensitive version of a protein required for DNA replication. Cells kept at a permissive temperature for *cdc7-1* re-replicated primarily from a single orign and this lead to Rad53p phosphorylation (lanes 1-4). However, cells shifted to a non-permissive temperature for *cdc7-1* were unable to re-replicate and no DNA damage response was observed (lanes 5-8).

Figure 2



Transient induction of re-replication in a strain that primarily re-replicates from a single origin generates widespread DNA damage but limited cell death. Cells expressing Ddc2-GFP were induced to re-replicate at time 0. At the indicated times, cells were plated to quantify colony forming units and examined by fluorescence microscopy to determine the fraction of cells with Dd2-GFP foci formation, a marker of the DNA damage induced by re-replication.