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Cyclin and Cyclin-Dependent Kinase Substrate Requirements for Preventing Rereplication Reveal the Need for Concomitant Activation and Inhibition

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ABSTRACT

DNA replication initiation in *S. cerevisiae* is promoted by B-type cyclin-dependent kinase (Cdk) activity. In addition, once-per-cell-cycle replication is enforced by cyclin-Cdk-dependent phosphorylation of the prereplicative complex (pre-RC) components Mcm2-7, Cdc6, and Orc1-6. Several of these controls must be simultaneously blocked by mutation to obtain rereplication. We looked for but did not obtain strong evidence for cyclin specificity in the use of different mechanisms to control rereplication: both the S-phase cyclin Clb5 and the mitotic cyclins Clb1–4 were inferred to be capable of imposing ORC-based and MCM-based controls. We found evidence that the S-phase cyclin Clb6 could promote initiation of replication without blocking reinitiation, and this activity was highly toxic when the ability of other cyclins to block reinitiation was prevented by mutation. The failure of Clb6 to regulate reinitiation was due to rapid Clb6 proteolysis, since this toxic activity of Clb6 was lost when Clb6 was stabilized by mutation. Clb6-dependent toxicity is also relieved when early accumulation of mitotic cyclins is allowed to impose rereplication controls. Cell-cycle timing of rereplication control is crucial: sufficient rereplication block activity must be available as soon as firing begins. DNA rereplication induces DNA damage, and when rereplication controls are compromised, the DNA damage checkpoint factors Mre11 and Rad17 provide additional mechanisms that maintain viability and also prevent further rereplication, and this probably contributes to genome stability.

DNA replication must take place only once per cell cycle in eukaryotes. This mechanism is controlled at the level of prereplicative complex (pre-RC) formation and origin firing. The pre-RC is assembled at replication origins. The origin recognition complex (Orc1-6) is bound to DNA replication origins throughout the cell cycle in *Saccharomyces cerevisiae* (Diffley *et al.* 1994). The Cdc6, Cdt1, and the mini-chromosome maintenance proteins (MCM) Mcm2–7 are sequentially recruited to the origin to form the pre-RC (Cocker *et al.* 1996; Aparicio *et al.* 1997; Devault *et al.* 2002; Tanaka and Diffley 2002; Tanaka *et al.* 1997). The assembled pre-RC further recruits Cdc45, DNA polymerase α, Sld2, and other factors required for initiation and elongation (Bell and Dutta 2002). Activation of two kinases, Dbf4-Cdc7 and cyclin-dependent kinases (CDK), coupled with B-type cyclins are necessary to form replisomes at two nascent replication forks and to initiate DNA replication (Bell and Dutta 2002).

Multiple mechanisms prevent cells from starting a second round of initiation by inhibiting pre-RC formation (Broek *et al.* 1991; Hayles *et al.* 1994; Dahmann *et al.* 1995). In *S. cerevisiae*, phosphorylation of MCMs by Cln-Cdk1 and Clb-Cdk1 kinases causes their exclusion from the nucleus (Labib *et al.* 1999; Nguyen *et al.* 2000; Liku *et al.* 2005). Phosphorylation of Orc2 and Orc6 by Clb-Cdk1 is thought to prevent efficient binding of other pre-RC subunits (Nguyen *et al.* 2001). Binding of Clb5 to Orc6 via its RXL cyclin-binding motif contributes to prevention of rereplication (Wilmes *et al.* 2004). Phosphorylation targets of Clb5-Cdk1 likely include Orc6 itself as well as Orc2 and Orc1 (Nguyen *et al.* 2001; Archambault *et al.* 2004; Wilmes *et al.* 2004). Finally, Cdc6 is inhibited by multiple mechanisms through its N terminus. N-terminal phosphorylation of Cdc6 promotes its degradation by the proteasome (Drury *et al.* 1997; Elsasser *et al.* 1999; Calzada *et al.* 2000; Drury *et al.* 2000), and binding of Cdc6p to mitotic cyclins inactivates Cdc6 for origin loading; Cdc6-cyclin binding is dependent on N-terminal phosphorylation of Cdc6 (Mimura *et al.* 2004). Additionally, Cdc6 has an N-terminal nuclear localization signal, which is critical for its degradation (Luo *et al.* 2003). When multiple disruptions of these mechanisms are combined, cells undergo extensive rereplication (Nguyen *et al.* 2001; Wilmes *et al.* 2004). In other eukaryotes,

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similar but distinct mechanisms combine to control rereplication (Kearsey and Cotterill 2003).

We recently reported that a synthetic genetic array (SGA) analysis identified genes required for viability when rereplication controls were partially compromised by combining the *ORC6-rxl* mutation blocking Clb5 binding to Orc6, and the *CDC6ΔNT* mutation, an N-terminal truncation resulting in abrogation of Cdk control of Cdc6 activity (see above). This background is initially slow growing but viable; we searched the haploid viable deletion set for deletions that would result in tight inviability in an *ORC6-rxl CDC6ΔNT* background. We found two major clusters of genes: one related to DNA damage response and one related to cell-cycle regulation (Archambault *et al.* 2005b). Further experiments suggested that the DNA damage response cluster was detected because cells partially compromised for rereplication control suffered double-strand breaks and required DNA damage surveillance mechanisms for viability (Archambault *et al.* 2005b). Rereplication can induce extensive DNA damage and could lead to genome instability (Archambault *et al.* 2005b; Green and Li 2005).

Here, we investigate the significance of the cell-cycle regulation cluster detected in our SGA analysis.

MATERIALS AND METHODS

Yeast strain construction: Strain list was provided in Table 1. Standard methods were used for mating, tetrad analysis, and transformations. The *ORC6-rxl* allele has mutations at R178A and L180A; these mutations strongly reduce the specific interaction between Clb5 and the ORC complex (Wilmes *et al.* 2004). The *ORC6-ps* allele has phosphorylation site mutations at S106A, S116A, S123A, and T146A (Wilmes *et al.* 2004). *GAL-CDC6ΔNT-HAs* (Δ2-48) (single copy) and *GAL-CDC6ΔNT-HAm* (multiple copy) were constructed by transformation using linearized RS305-based *GAL-CDC6Δ2-48-HA* plasmid (Wilmes *et al.* 2004). These constructs allow strong unregulated expression and accumulation of Cdc6. In all strains with *GAL-CDC6* constructs, the wild-type *CDC6* gene was also present to allow viability on glucose medium where the *GAL-CDC6* constructs were not expressed. The *MCM7-NLS* allele, allowing cell-cycle-constitutive nuclear residence of the Mcm complex, was described in Nguyen *et al.* (2001). *CLB6Δ3P* has three mutations at S6A, T39A, and S147A, stabilizing the protein by blocking Cdk phosphorylation and Skp1/Cull/F-Box protein (SCF)-dependent degradation (Jackson *et al.* 2006).

Serial dilution: Each strain was grown to stationary phase in 3 ml YPD. After the cell concentration was normalized on the basis of OD measurement, 5 μl of 10-fold serial dilutions were spotted onto YEP-D or YEP-G (glucose or galactose) plates. The plates were incubated for 2 days at 30°.

Rereplication assay: For induction of rereplication, cells were grown in liquid YEP-D (glucose) media overnight, washed, and transferred to YEP-R (raffinose) for 8 hr. *GAL-CDC6ΔNT-HAm* was then induced by adding 3% galactose (final concentration) for 4 hr.

Microscopy and DNA flow cytometry analysis: Ddc2-GFP foci were observed under a DeltaVision microscope as described (Archambault *et al.* 2005b). DNA flow cytometry analysis was performed as described (Epstein and Cross 1992).

RESULTS

Genetic interactions between rereplication controls and the cell-cycle regulators *SIC1* and *CLB5*: The RXL motif of Orc6 interacts with Clb5 to locally prevent origin refiring (Wilmes *et al.* 2004). Combining a mutation disrupting the Orc6-Clb5 interaction (*ORC6-rxl*) with another mutation stabilizing Cdc6 and preventing its sequestration by Clb2-Cdk1 (*CDC6ΔNT*) causes reduced viability and proliferation. Similarly, deletion of *CLB5* in a *CDC6ΔNT* background is lethal in tetrad analysis (Wilmes *et al.* 2004), and *clb5 GAL-CDC6ΔNT* cells were nearly inviable on galactose plates (YEP-G) (Figure 1, A and B). In both experiments, removing Clb5 had a more profound effect than removing the Orc6-RXL (Clb5-binding) in the *CDC6ΔNT* background. Clb5 could engage in residual binding with Orc6-rxl or could have other functions in preventing rereplication that are independent of Orc6 binding (discussed below).

In our previous study, we conducted a genomewide screen using the *ORC6-rxl GAL-CDC6ΔNT-HAs* strains. (*GAL-CDC6ΔNT-HAs* is a single copy of *GAL-CDC6*; in some experiments, we also use a multicopy *GAL-CDC6ΔNT-HAm* allele, as described by Archambault *et al.* 2005b.) We identified a cluster of genes related to DNA damage checkpoints that are strongly required for viability in this background (Archambault *et al.* 2005b). In addition, we detected a cluster of genes that were related to cell-cycle regulation, including *CLB5* and *SIC1* (Figure 1A).

Deletion of *SIC1* in the *ORC6-rxl GAL-CDC6ΔNT-HAs* background markedly decreased cell viability on YEP-G (Figure 1A, top left). The main function of Sic1 is to inhibit Clb5/Clb6-Cdk in late G_1 (Mendenhall 1993; Schwob *et al.* 1994). Since the Clb5-Orc6 interaction is already disabled in the *ORC6-rxl GAL-CDC6ΔNT-HAs* background, lack of Sic1 in this context may be toxic through a mechanism that is independent of this interaction. We speculated that this is due to an elevated or a premature activation of Clb5,6 activity. The *sic1* deletion in *ORC6-ps,rxl GAL-CDC6ΔNT-HAm* [*ORC6-ps,rxl* lacks both the RXL sequence and the Cdk phosphorylation sites described in Nguyen *et al.* (2001) and Wilmes *et al.* (2004)] also reduced viability (Figure 1A, top right). Consistent with the idea that premature Clb5,6 activity was responsible for lethality of *ORC6-ps,rxl GAL-CDC6ΔNT-HAm sic1*, the lethality was rescued by additional deletion of both *CLB5* and *CLB6* (Figure 1A, top right). We speculate that the possible mechanism of premature activation of Clb5,6 activity in the *sic1 ORC6-ps,rxl GAL-CDC6ΔNT-HAm* leads to initiation without blocking origin reloading (due to *ORC6-ps,rxl* mutation), leading to rereplication.

Induction of rereplication requires stabilized Cdc6 (*CDC6ΔNT*) in most genetic combinations tested in this study. This implies that degradation of Cdc6 plays a significant role in the inhibition of DNA rereplication. Interestingly, in tetrad analysis, deletion of *CLB5* in the

TABLE 1

Strains used in this study

Strain	Genotype
RUY121	Wild type; *MAT*α *ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100*
RUY139	*MAT*α *sic1::HIS3*
RUY275	*MAT*α *orc6::HIS3::LEU2::ORC6-ps,rxl URA3::GAL-CDC6ΔNT-HAm*
RUY384	*MAT*? *orc6::HIS3::LEU2::ORC6-ps,rxl URA3::GAL-CDC6ΔNT-HAm sic1::HIS3*
RUY385	*MAT*? *orc6::HIS3::LEU2::ORC6-ps,rxl URA3::GAL-CDC6ΔNT-HAm sic1::HIS3 clb5::URA3 clb6::KanMX*
RUY386	*MAT***a** *orc6::HIS3::LEU2::ORC6-ps,rxl URA3::GAL-CDC6ΔNT-HAm sic1::HIS3 clb6::KanMX*
RUY387	*MAT*α *orc6::HIS3::LEU2::ORC6-ps,rxl URA3::GAL-CDC6ΔNT-HAm clb5::URA3 clb6::KanMX*
RUY140	*MAT*α *cdh1::LEU2*
RUY263	*MAT*α *URA3::GAL-CDC6ΔNT-HAm DDC2-GFP::TRP1*
RUY227	*MAT*α *clb5::URA3 URA3::GAL-CDC6ΔNT-HAm DDC2-GFP::TRP1*
RUY174	*MAT***a** *clb5::URA3 clb6::KanMX URA3::GAL-CDC6ΔNT-HAm DDC2-GFP::TRP1*
RUY228	*MAT***a** *MCM7-NLS URA3::GAL-CDC6ΔNT-HAm DDC2-GFP::TRP1*
RUY179	*MAT***a** *MCM7-NLS clb5::URA3 clb6::KanMX URA3::GAL-CDC6ΔNT-HAm DDC2-GFP::TRP1*
RUY170	*MAT*α *orc6::HIS3::LEU2::ORC6-ps,rxl URA3::GAL-CDC6ΔNT-HAm DDC2-GFP::TRP1*
RUY181	*MAT*α *orc6::HIS3::LEU2::ORC6-ps,rxl clb5::URA3URA3::GAL-CDC6ΔNT-HAm DDC2-GFP::TRP1*
RUY184	*MAT***a** *orc6::HIS3::LEU2::ORC6-ps,rxl clb5::URA3 clb6::KanMX URA3::GAL-CDC6ΔNT-HAm DDC2-GFP::TRP1*
RUY169	*MAT*α *MCM7-NLS orc6::HIS3::LEU2::ORC6-ps,rxl URA3::GAL-CDC6ΔNT-HAm DDC2-GFP::TRP1*
RUY191	*MAT***a** *MCM7-NLS orc6::HIS3::LEU2::ORC6-ps,rxl clb5::URA3 clb6::KanMX URA3::GAL-CDC6ΔNT-HAm DDC2-GFP::TRP1*
RUY325	*MAT*α *MCM7-NLS clb5::URA3 URA3::GAL-CDC6ΔNT-HAm*
RUY326	*MAT*α *clb5::URA3 URA3:: GAL-CDC6ΔNT-HAm MCM7-NLS cdh1::LEU2*
RUY172	*MAT***a** *clb5::URA3 URA3::GAL-CDC6ΔNT-HAm*
RUY329	*MAT***a** *clb5::URA3 URA3::GAL-CDC6ΔNT-HAm cdh1::LEU2*
RUY332	*MAT***a** *clb5::URA3 URA3::GAL-CDC6ΔNT-HAm orc6::HIS3::LEU2::ORC6-ps,rxl*
RUY334	*MAT***a** *clb5::URA3 URA3::GAL-CDC6ΔNT-HAm orc6::HIS3::LEU2::ORC6-ps,rxl cdh1::LEU2*
RUY415	*MAT***a** *MCM7-NLS clb5::URA3 URA3::GAL-CDC6ΔNT-HAm CLB6Δ3p-HA::URA3*
RUY435	*MAT***a** *rad17::KanMX clb5::URA3 URA3::GAL-CDC6ΔNT-HAm*
RUY436	*MAT***a** *rad17::KanMX orc6::HIS3::LEU2::ORC6-ps,rxl URA3::GAL-CDC6ΔNT-HAm*
RUY437	*MAT*α *rad17::KanMX clb5::URA3 orc6::HIS3::LEU2::ORC6-ps,rxl URA3::GAL-CDC6ΔNT-HAm*
RUY438	*MAT*? *mre11::KanMX clb5::URA3 URA3::GAL-CDC6ΔNT-HAm*
RUY439	*MAT*? *mre11::KanMX orc6::HIS3::LEU2::ORC6-ps,rxl URA3::GAL-CDC6ΔNT-HAm*
RUY440	*MAT*? *mre11::KanMX clb5::URA3 orc6::HIS3::LEU2::ORC6-ps,rxl URA3::GAL-CDC6ΔNT-HAm*
RUY299	*MAT*α *orc6::HIS3::LEU2::ORC6-ps,rxl MCM7-NLS URA3::GAL-CDC6ΔNT-HAm rad17::KanMX*
RUY302	*MAT*? *orc6::HIS3::LEU2::ORC6-ps,rxl MCM7-NLS URA3::GAL-CDC6ΔNT-HAm mre11::KanMX*
RUY303	*MAT***a** *orc6::HIS3::LEU2::ORC6-ps,rxl MCM7-NLS URA3::GAL-CDC6ΔNT-HAm mre11::KanMX rad17::KanMX*
RUY280	*MAT***a** *clb1 clb2(ts) clb3::TRP1 clb4::his3::KanMX URA3::GAL-CDC6ΔNT-HAm orc6::HIS3::LEU2::ORC6-ps,rxl*

Alleles used in this study	Description and references
ORC6-rxl	Linealized pRS405 (LEU marked)-based *ORC6-rxl* plasmid was integrated into *orc6::HIS3MX* strain. *ORC6-rxl* contains mutations in RXL motif, which interfere with Clb5 binding (Wilmes *et al.* 2004).
GAL-CDC6ΔNT-HAs	Deletion of N-terminal region of *CDC6* allows its stable expression due to insufficient phosphorylation by CDKs. The plasmid was kindly provided by J. Diffley. The single integration of *GAL-CDC6Δ2-48-HA* was confirmed by Southern blotting. Endogenous copy of *CDC6* exists in addition to *GAL-CDC6Δ2-48* in the strain (Archambault *et al.* 2005a,b).
ORC6-ps,rxl	*ORC6-ps,rxl* contains *ORC6* Cdk phosphorylation site mutations in addition to *ORC6-rxl*. pRS405-based *ORC6-ps,rxl* plasmid was linealized and integrated into the *orc6::HIS3MX* strain (Wilmes *et al.* 2004).
GAL-CDC6ΔNT-HAm	Multiple copy of *GAL-CDC6Δ2-48-HA* was integrated on the basis of Southern blotting. Otherwise the feature of this strain is as the same as that of *GAL-CDC6ΔNT-HAs* (Wilmes *et al.* 2004).
MCM7-NLS	Endogenous copy of Mcm7 was fused at its C terminus to two tandem copies of the SV40 nuclear localization signal allowing constitutive nuclear localization of Mcm7 (Nguyen *et al.* 2001).
CLB6Δ3P	Three serines or threonines of the phosphorylation sites in Clb6 were mutated to alanine, and the resulting *CLB6Δ3P* was expressed from the endogenous *CLB6* promoter (Jackson *et al.* 2006). The *CLB6Δ3P* stabilizes Clb6p and escapes from SCF-dependent degradation.

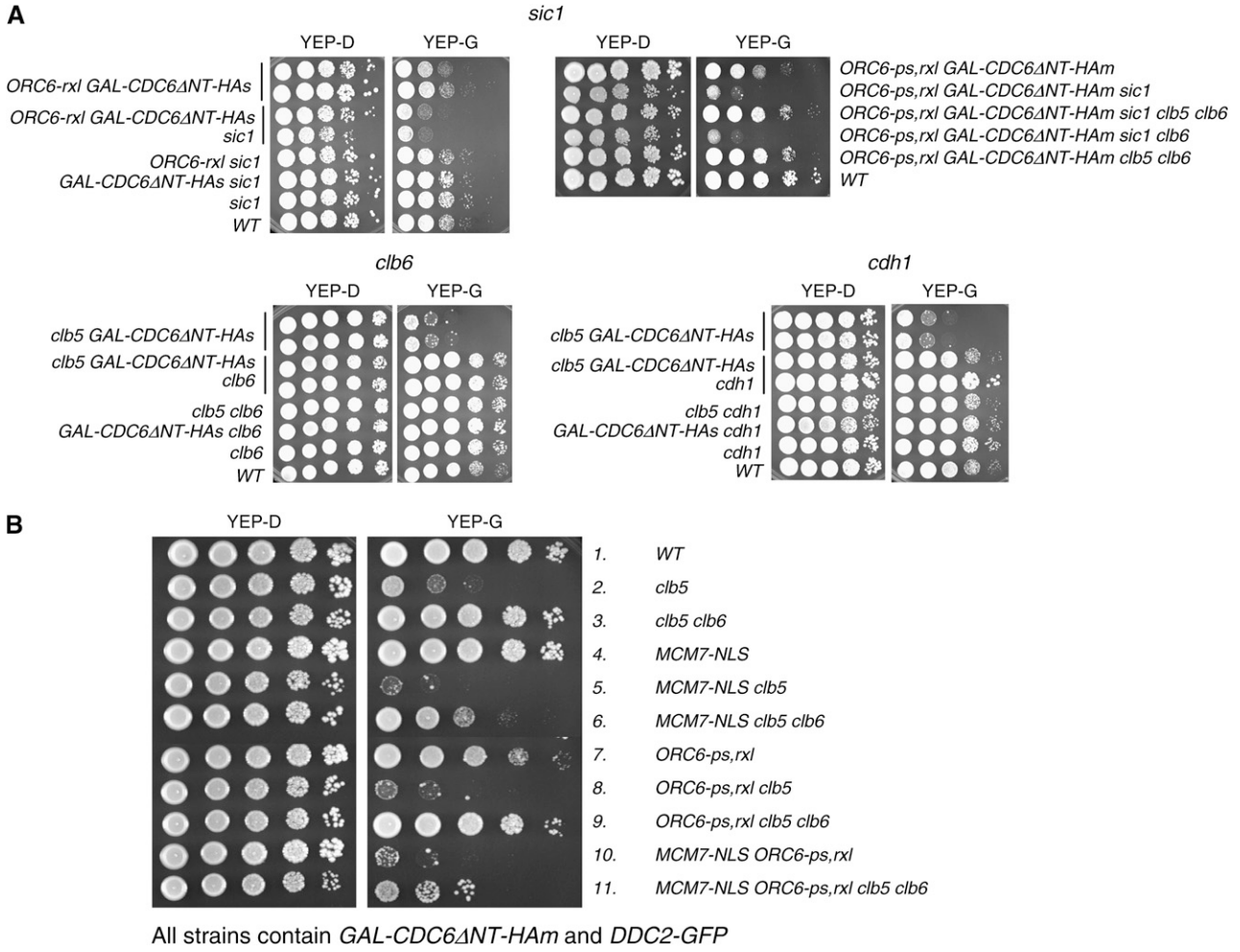


Figure 1.—The survival of cells with disrupted rereplication controls depends on cell-cycle regulators. (A) *ORC6-rxl GAL-CDC6ΔNT-HAs* cells (top left) rely on *SIC1* for their limited survival when *GAL-CDC6ΔNT-HAs* is induced by galactose. Ten-fold serial dilutions on galactose-containing or glucose-containing plates (YEP-G or YEP-D) were performed. Deletion of *SIC1* in *ORC6-ps,rxl GAL-CDC6ΔNT-HAm* caused synthetic lethality. The lethality was rescued by deleting both *CLB5* and *CLB6* (top right). The viability of *clb5 GAL-CDC6ΔNT-HAs* cells (bottom) is rescued by deletion of *CLB6* or *CDH1* on YEP-G. (B) Viability of various strains with disruptions of mechanisms preventing rereplication in the presence or absence of S-phase cyclins. All strains contain *GAL-CDC6ΔNT-HAm* and *DDC2-GFP* (analyzed in Figure 2). Cell viability was tested as in A.

ORC6-ps,rxl sic1 background was lethal (even with wild-type *CDC6* as the sole source of *CDC6*) (data not shown). Clb6p is responsible for this lethality, since additional deletion of *CLB6* in the *ORC6-ps,rxl sic1 clb5* background restored viability (Figure 1A, top right, YEP-D, third row).

Deletion of *CLB6* or *CDH1* rescues defects caused by *clb5 CDC6ΔNT*: The lethality of the *clb5 GAL-CDC6ΔNT-HAs* strain in galactose was efficiently rescued by additional deletion of *CLB6* (Figure 1A, bottom left). The genetic combinations that caused reduced viability involving perturbed rereplication control and *CLB5* deletion (*GAL-CDC6ΔNT clb5 sic1* and *CDC6ΔNT clb5*) were rescued by additional deletion of *CLB6* (data not shown). All of these backgrounds share deregulation of Cdc6 and prevention of Clb5-Orc6 interaction by *clb5* deletion. These results support the idea that in the presence of stabilized Cdc6, Clb6 is toxic when Clb5-based rereplication inhibition mechanisms are abolished. This implies that Clb5 has greater ability to block rereplication than Clb6, despite sequence similarity and the close evolutionary relationship of Clb5 and Clb6 (Schwob and Nasmyth 1993).

Cdh1 is known to target residual mitotic B-type cyclins (Clb1-4) for degradation early in the cell cycle (Visintin *et al.* 1997; Schwab *et al.* 2001; Wasch and Cross 2002). Deleting *CDH1* in *clb5 GAL-CDC6ΔNTs* cells completely rescued their proliferation on YEP-G (Figure 1A, bottom right). Deletion of *CDH1* in this background also rescued the rereplication phenotype determined by DNA flow cytometry analysis (see below). Consistently, inviability of *clb5 CDC6ΔNT* (endogenous gene replacement) or slow growth of *ORC6-rxl CDC6ΔNT* segregants in tetrad analysis was efficiently rescued by deletion of *CDH1* (data not shown). In the absence of Cdh1, mitotic

cyclins are present at a nearly constitutive level through the cell cycle, including in early G_1, most likely due in part to inheritance of cyclin from the previous cell cycle; levels later in the cell cycle are quite similar to levels in wild-type cells (Cross *et al.* 2002; Wasch and Cross 2002). Therefore, we attribute the effects of *CDH1* deletion on replication control as being due to early presence of mitotic cyclins. Increasing the level of mitotic cyclins early in the cell cycle may improve the viability of *clb5 CDC6ΔNT* or *ORC6-rxl CDC6ΔNT* cells by eliminating a temporal gap between S-Clb-Cdk and M-Clb-Cdk activation. This could allow a stronger reinitiation block at the time of replication initiation, compensating for the disruption of the Clb5-Orc6 interaction.

The absence of Cdh1 is likely to result in stabilization of several proteins in addition to B-type cyclins. To test whether an enhanced presence of mitotic cyclin in early S-phase could rescue *clb5 CDC6ΔNT* inviability, we placed *CLB2* instead of *CLB5* under the control of the *CLB5* promoter (*CLB5pCLB2*, where *CLB5* is absent) (Cross *et al.* 1999). *CLB5pCLB2 CDC6ΔNT* spores formed small colonies, while *clb5 CDC6ΔNT* spores were completely inviable (supplemental Table S1 at http://www.genetics.org/supplemental/). Thus two independent means of inducing early accumulation of mitotic cyclins relieved inviability of *clb5 CDC6ΔNT*.

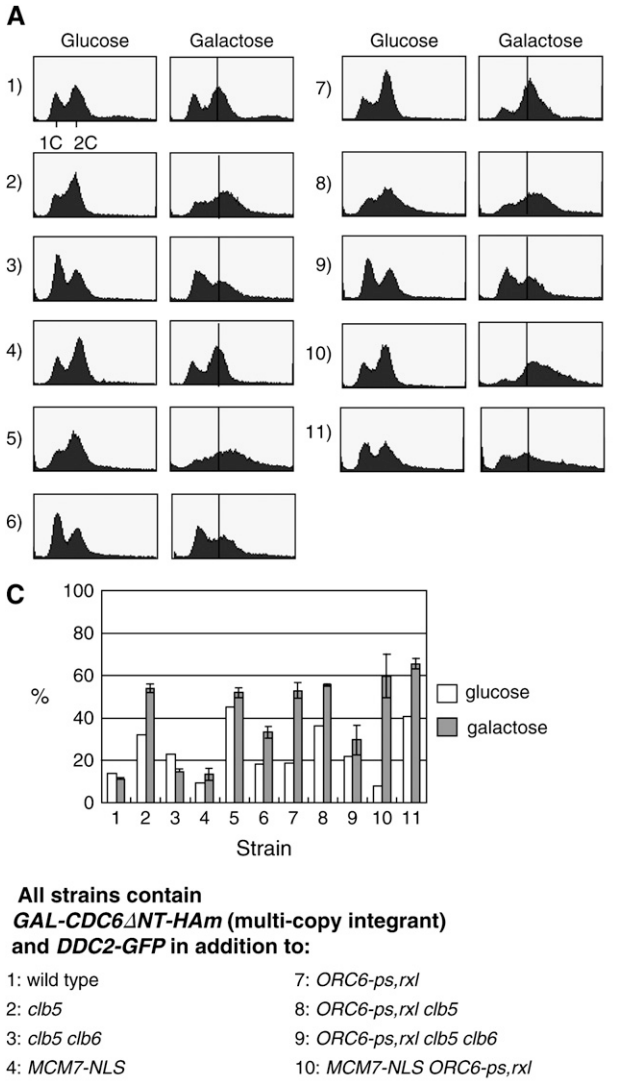
As noted in our previous work (Archambault *et al.* 2005b), a concern with these experiments is that the mutations that we are using to deregulate rereplication control are in proteins (S-phase cyclins, pre-RC components) that also positively control replication, and some of the toxicity that we detect could be due to poor initial origin usage, rather than to rereplication. We tested this by assessing bulk DNA replication in synchronized cells and found at most modest defects in replication initiation (supplemental Figure S1 at http://www.genetics.org/supplemental/). Recently, Green *et al.* (2006) showed little or no replication initiation defects associated with *ORC*, *MCM*, and *CDC6* mutations deregulating replication control by a microarray-based assay. Thus, overall we consider it unlikely that replication initiation defects contribute significantly to the results presented here.

Rereplication and accumulation of Ddc2-GFP DNA damage foci: The previous results suggest that the relative timing of onset of S-phase cyclin and mitotic cyclin activities is crucial for viability in strains in which specific mechanisms preventing rereplication have been disrupted. To confirm this idea, we examined DNA rereplication directly by DNA flow cytometry and indirectly by accumulation of Ddc2-GFP (DNA damage foci) in the presence or absence of S-phase cyclin, accompanied by inactivation of various rereplication controls. We and others showed previously that Ddc2-GFP foci accumulate under rereplication conditions, even in the absence of overt rereplication detectable by DNA flow cytometry (Archambault *et al.* 2005b; Green and Li 2005). In the following sections, we describe how we used these assays to examine rereplication and accumulation of DNA damage in various backgrounds.

Clb5 blocks Clb6-dependent rereplication in cells expressing stable Cdc6: DNA flow cytometry analysis showed an accumulation of cells with a signal increased to slightly more than 2C DNA content in *clb5 GAL-CDC6ΔNT-HAm* when cells were incubated in galactose (Figure 2A, 2), indicating moderate rereplication. We were concerned that this apparent rereplication signal could be spurious and due to drifts in the DNA flow cytometry signal in cells that enlarged due to cell-cycle arrest, even without actual rereplication. We observed, however, that Ddc2-GFP foci also accumulated in these cells (Figure 2B, 2, and 2C, 2), consistent with activation of rereplication (Archambault *et al.* 2005b; Green and Li 2005). Also, no apparent rereplication signal was detected using these methods with the *clb1,3,4-del clb2-ts* block, previously characterized as a G_2 block without rereplication (Amon *et al.* 1993) (supplemental Figure S2 at http://www.genetics.org/supplemental/). For these reasons, we consider that this DNA flow cytometry signal likely represents authentic rereplication, but we recognize that in borderline cases it is hard to be sure of this. Recently, Green *et al.* (2006), using a microarray method, showed that rereplication at some specific loci could be detected even in cells without DNA flow-cytometry-detectable rereplication, and such an assay may ultimately be necessary to definitively prove rereplication in cases that are borderline by DNA flow cytometry.

These interpretations led to the idea that Clb5 blocks rereplication in cells with stabilized Cdc6. The DNA flow cytometry phenotype, as well as the Ddc2-GFP accumulation, was rescued by additional deletion of *CLB6*, implying that, in *clb5 GAL-CDC6ΔNT-HAm* cells, Clb6 induces inviability, rereplication, and DNA damage (Figure 1B; compare Figure 2A, 2 with 3; 2B, 2 with 3; and 2C, 2 with 3). This result implies that Clb6 can induce replication without blocking rereplication.

Clb5 blocks Clb6-dependent rereplication in cells containing a constitutively nuclear Mcm complex: We tested the cyclin requirements for rereplication control when Mcm7 is constitutively localized in the nucleus. The *MCM7-NLS* mutation prevents cell-cycle-dependent Clb-Cdk regulation of Mcm complex localization. Mcm localization contributes to control of rereplication in parallel to Cdc6- and ORC-based mechanisms (Labib *et al.* 1999; Nguyen *et al.* 2000, 2001; Wilmes *et al.* 2004). *GAL-CDC6ΔNT-HAm MCM7-NLS* cells were viable on YEP-G, and these cells did not exhibit DNA rereplication or Ddc2-GFP foci (Figure 1B and Figure 2A, 4, 2B, 4, and 2C, 4), consistent with previous results (Nguyen *et al.* 2001). Deleting *CLB5* in this background caused rereplication, DNA damage, and inviability (Figure 1B and Figure 2A, 5, 2B, 5, and 2C, 5). Consistent with the results above, additional deletion of *CLB6* partially rescued inviability, largely prevented DNA rereplication,



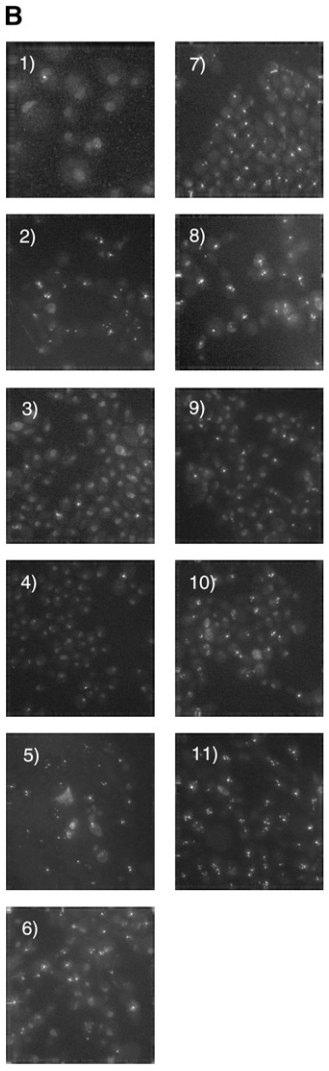


FIGURE 2.—Induction of DNA rereplication and recruitment of Ddc2-GFP foci. (A) Cells were grown in YEP-D and transferred to YEP-R for 8 hr. Galactose at the final concentration of 3% was added for 4 hr. Cells were fixed and DNA content was measured by DNA flow cytometry analysis. The approximate position of 2C DNA content was determined on the basis of the major 2C peak in the control (1; *GAL-CDC6ΔNT-HAm* strain). This position is indicated by a bar. All samples were processed in parallel, and data were collected using the same set up (voltage 780 and total cell number 20,000 cells) and gating. As a rough empirical guide, we consider the DNA flow cytometry profile to represent over-replication if the main peak is shifted rightward compared to the bar. Each histogram is numbered on the basis of the strain numbering in Figure 1B. (B) The same strains, numbered as in A, were induced with galactose for 4 hr, and cells were observed under a DeltaVision microscope. Image stacks were deconvolved and processed at maximum projection. (C) Percentages of the cells showing DDC2-GFP foci in the presence of glucose or galactose. At least 100 cells were counted for each experiment, and three experiments were repeated. Average number and standard deviation were plotted. Open bars show percentages of DDC2-GFP foci when incubated in glucose, and shaded bars show the same when incubated in galactose.

and reduced accumulation of Ddc2-GFP foci (Figure 1B, 2A, 6, 2B, 6, and 2C, 6). Inducing early accumulation of the mitotic cyclins Clb1–4 by deletion of *CDH1* partially rescued the lethality in *clb5 GAL-CDC6ΔNT-HAm MCM7-NLS* and largely rescued the rereplication profile (Figure 3, A and B, top).

Clb5 blocks Clb6-dependent rereplication in cells with unphosphorylatable Orc6 lacking a cyclin-binding motif: We next examined the consequences of deleting S-phase cyclins in the presence of the *ORC6-ps,rxl* mutation, which eliminates cyclin binding and phosphorylation of Orc6 (WILMES *et al.* 2004). Introducing *ORC6-ps,rxl* in *GAL-CDC6ΔNT-HAm* cells caused an accumulation of 2C DNA cells (Figure 2A, 7) and moderate slow growth (Figure 1B). Additionally deleting *CLB5* in this background induced complete inviability (Figure 1B) and overt rereplication (Figure 2A, 8), indicating that Clb5 has Orc6-independent targets preventing rereplication. Potential additional targets for Clb5 include Orc2 phosphorylation and Mcm complex phosphorylation leading to Mcm nuclear export (see above). Lethality, rereplication, and DNA damage in this background were dependent on Clb6 (Figure 1B; compare Figure 2A, 8 with 9, and 2C, 8 with 9).

Cyclin requirements for rereplication in cells lacking both Mcm control and Orc control: *MCM7-NLS* in a *clb5 ORC6-ps,rxl* background led to lethality in tetrad analysis even without overexpression of stabilized Cdc6 (data not shown); thus these strains could not be assayed directly for the rereplication phenotype, since in all other experiments we used conditional expression of stabilized Cdc6 to trigger rereplication and lethality. As in other contexts, however, additional deletion of *CLB6* rescued the inviability of this background, in the absence

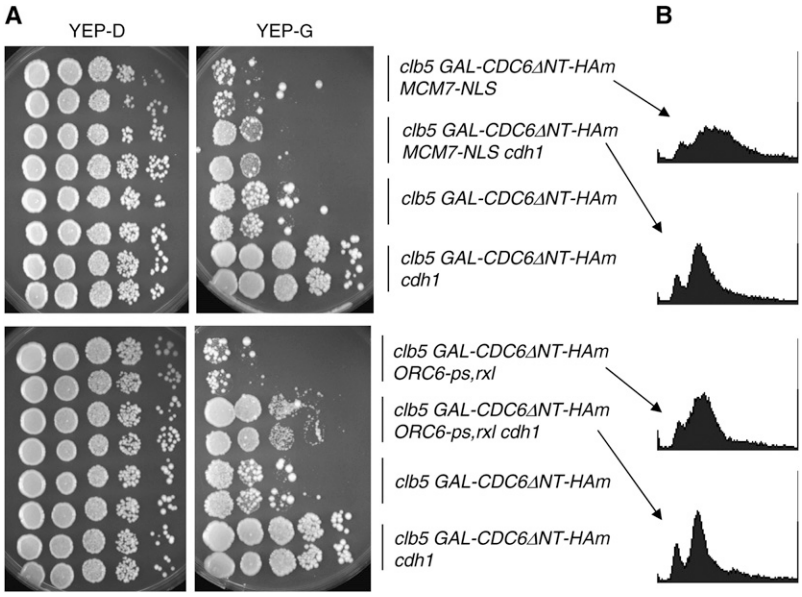


FIGURE 3.—Cdh1 can promote inviability and rereplication in the absence of specific replication control mechanisms. (A) Serial dilutions as in Figure 1 for *clb5 GAL-CDC6ΔNT-HAm ORC6-ps,rxl* and *clb5 GAL-CDC6ΔNT-HAm MCM7-NLS*, with and without *CDH1*. (B) DNA flow cytometry analysis performed for selected strains after *GAL-CDC6ΔNT-HAm* induction, as in Figure 2.

but not in the presence of *GAL-CDC6ΔNT* expression (Figure 1B). These results broaden our findings to a context not requiring Cdc6 stabilization.

GAL-CDC6ΔNT-HAm MCM7-NLS ORC6-ps,rxl induced lethality, extensive rereplication, and multiple Ddc2-GFP foci per cell (Figure 1B; Figure 2A, 10, and 2B, 10), as reported previously (NGUYEN *et al.* 2001; WILMES *et al.* 2004; ARCHAMBAULT *et al.* 2005b; GREEN and LI 2005). Additional deletions of both *CLB5* and *CLB6* in this background did not rescue these phenotypes efficiently (Figure1B; Figure 2A, 11, 2B, 11, and 2C, 11), indicating that rescue by *CLB5* and *CLB6* deletions in other backgrounds was largely dependent on regulation of Cdc6, MCM, or ORC.

Clb6-dependent rereplication can be rescued by early accumulation of mitotic cyclins: Above, we described three backgrounds with impaired control of rereplication in which Clb6 was deduced to cause rereplication, DNA damage foci, and loss of viability: *clb5 GAL-CDC6ΔNT-HAm*, *clb5 GAL-CDC6ΔNT-HAm MCM7-NLS*, and *clb5 ORC6-ps,rxl GAL-CDC6ΔNT-HAm*. In all of these backgrounds, additional deletion of *CDH1* significantly rescued the phenotypes (Figures 1 and 3). Thus, we infer that the damaging rereplication induced by Clb6 in these backgrounds is suppressed by the simultaneous presence of mitotic cyclins along with Clb6, and we infer that the mitotic cyclins must therefore be able to effectively impose rereplication control by either the ORC-based or the Mcm-based mechanisms.

Stabilized Clb6 substitutes efficiently for Clb5 in regulating replication: Clb5 and Clb6 degradation are regulated by distinct mechanisms (JACKSON *et al.* 2006). Clb6p is mostly degraded at the G_1/S transition through the SCF^{Cdc4} ubiquitin ligase complex, while Clb5 is stable until mitosis and is degraded by APC^{Cdc20}. Mutation of three phosphorylation sites in Clb6 (*CLB6Δ3P*) leads to Clb6 stabilization by preventing SCF^{Cdc4} binding (JACKSON *et al.* 2006). As shown above, inviability and rereplication in the *MCM7-NLS clb5 GAL-CDC6ΔNT-HAm* background are strongly dependent on the absence of *clb5* (Figures 1 and 2). We found that substitution of *CLB6Δ3P* for *CLB6* in the *MCM7-NLS clb5 GAL-CDC6 ΔNT-HAm* background completely rescued its strong lethality and also rescued its apparent rereplication defect as judged by DNA flow cytometry (Figure 4). Thus, Clb6 and Clb5 probably have similar intrinsic abilities to regulate rereplication, but Clb6 is normally unable to carry out this role due to its rapid degradation early in the cell cycle.

A DNA damage response including Mre11 and Rad17 functions to limit overt rereplication when cyclin-dependent mechanisms fail: We previously showed that viability of *ORC6-rxl GAL-CDC6ΔNTs* cells depends on genes required for DNA damage response (ARCHAMBAULT *et al.* 2005b). Ddc2-GFP foci occurred in *clb5 GAL-CDC6ΔNT-HAm* and *ORC6-ps,rxl GAL-CDC6ΔNT-HAm* strains, suggesting that the DNA damage checkpoint might be activated (Figure 2B, 2 and 7, and 2C, 2 and 7). To test if the viability of *clb5 GAL-CDC6ΔNT-HAm* and *ORC6-ps,rxl GAL-CDC6ΔNT-HAm* depends on DNA damage signaling through the MRX complex or the 9-1-1 complex (LOWNDES and MURGUIA 2000), cell viability was examined with or without *MRE11* (MRX complex) or *RAD17* (9-1-1 complex). The *ORC6-ps,rxl GAL-CDC6ΔNT-HAm* strain showed reduced viability in the absence of *MRE11* or *RAD17* (Figure 5A), suggesting that DNA damage occurs and is signaled or repaired by MRX and 9-1-1 complexes in those strains. Deleting *MRE11* or *RAD17* in the *clb5 GAL-CDC6ΔNT-HAm* had little effect, perhaps because the viability of *clb5 GAL-CDC6ΔNT-HAm* is already quite low. Deleting *MRE11* or *RAD17* had little effect on replication profiles in either background

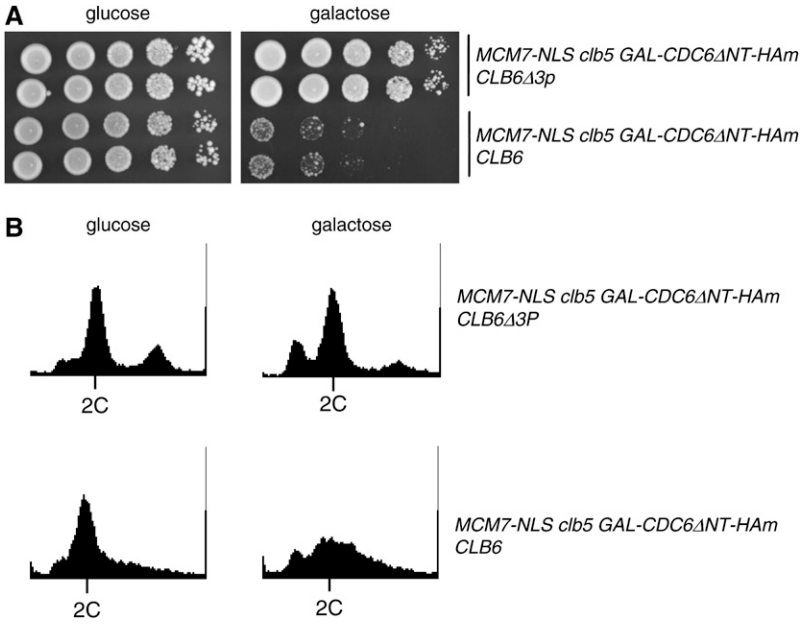


FIGURE 4.—Stabilized Clb6 reverses loss of viability and replication control due to absence of Clb5. (A) Serial dilutions for *MCM7-NLS clb5 GAL-CDC6ΔNT-HAm* with *CLB6Δ3P* [gene replacement, encoding Clb6 stabilized due to mutation of phosphorylation sites (JACKSON *et al.* 2006)] or *CLB6-wt*. (B) DNA content was measured by DNA flow cytometry analysis, as in Figure 2A. There is a minor peak at 4C DNA content in the *CLB6Δ3P MCM7-NLS clb5 GAL-CDC6ΔNT-HAm* strain. Microscopically, we did not detect significant clumping or division problems in this strain (data not shown). At present we do not know the explanation of this peak.

(Figure 5, B and C), suggesting that the increased inviability in the *ORC6-ps,rxl GAL-CDC6ΔNT-HAm* background caused by the *MRE11* or *RAD17* background is not caused by further deregulation of rereplication, but probably rather by response to DNA breaks generated by S-phase-cyclin-dependent rereplication.

In contrast, in the *ORC6-ps,rxl MCM7-NLS GAL-CDC6ΔNT-HAm* background (shown above to induce rereplication largely independent of S-phase cyclins), deleting *MRE11* or *RAD17* caused increased rereplication (Figure 5D), as reported previously (ARCHAMBAULT *et al.* 2005b).

The double deletion of *MRE11* and *RAD17* did not significantly enhance the rereplication seen in single deletion of either *MRE11* or *RAD17* (Figure 5D). We conclude that *MRE11* and *RAD17* are in largely overlapping pathways that can contribute to limit the extent of DNA rereplication when cyclin-Cdk-dependent rereplication blocks at the pre-RC level fail.

We assessed the ability of the MRX or 9-1-1 complexes to protect against the deleterious effects of loss of replication control by pulsing *ORC6-ps,rxl MCM7-NLS GAL-CDC6ΔNT-HAm* in galactose for short periods, followed by plating on glucose medium to assess viability. The *MRE11 RAD17* controls showed little lethality after 4 hr in galactose medium, despite some amount of detectable overreplication (Figure 5E). Deletion of *MRE11* in this background resulted in a 100-fold loss of viability upon short galactose pulses, while deletion of *RAD17* resulted in at most a 10-fold viability loss. *rad17 mre11* double mutants were not more sensitive than *mre11* single mutants.

DISCUSSION

Cell-cycle regulators determine the viability of cells with compromised rereplication controls: All of the mutant data presented in this article are summarized in Table 2, which emphasizes deductions that can be made from single additional deletions even in complex genetic backgrounds.

Several controls are known to function in preventing rereplication in *S. cerevisiae*. Cdk-dependent phosphorylation of Cdc6 on its N terminus promotes ubiquitination and degradation (DRURY *et al.* 1997, 2000; NGUYEN *et al.* 2001) and Cdc6 sequestration by Clb2-Cdk1 (MIMURA *et al.* 2004); Cdk-dependent phosphorylation of Orc6 and Orc2 is thought to hinder pre-RC formation (NGUYEN *et al.* 2001); and Cdk-dependent phosphorylation of MCM proteins causes cytoplasmic retention of the complex (LABIB *et al.* 1999; NGUYEN *et al.* 2000, 2001; LIKU *et al.* 2005). In addition, binding of Clb5 to Orc6 via a defined RXL motif contributes to preventing rereplication (WILMES *et al.* 2004).

Our results support the idea that the timing of Clb5 and Clb6 activity relative to other cyclins is crucial in determining the viability of rereplication-sensitized strains, in which Cdc6 is stabilized (*CDC6ΔNT*) and Clb5-dependent replication control mechanisms are disrupted (*ORC6-rxl* or *clb5*). In such strains, Clb6 may activate an additional round of replication from some reloaded origins, since Clb1-4, which can efficiently limit rereplication even in these partially compromised strains, only accumulate later [their accumulation may be additionally delayed in *clb5* mutants due to prolonged Cdh1 activity (YEONG *et al.* 2001)]. In this interpretation, the earlier that Clb5,6 activity appears relative to mitotic cyclins in cells lacking Clb5-dependent mechanisms of control, the more damage is likely to be done. This could account for the enhanced lethality caused by deletions of *SIC1*. Inversely, the less time there is between Clb5,6 activity onset and mitotic cyclin accumulation, the less damage is done. This could account for the

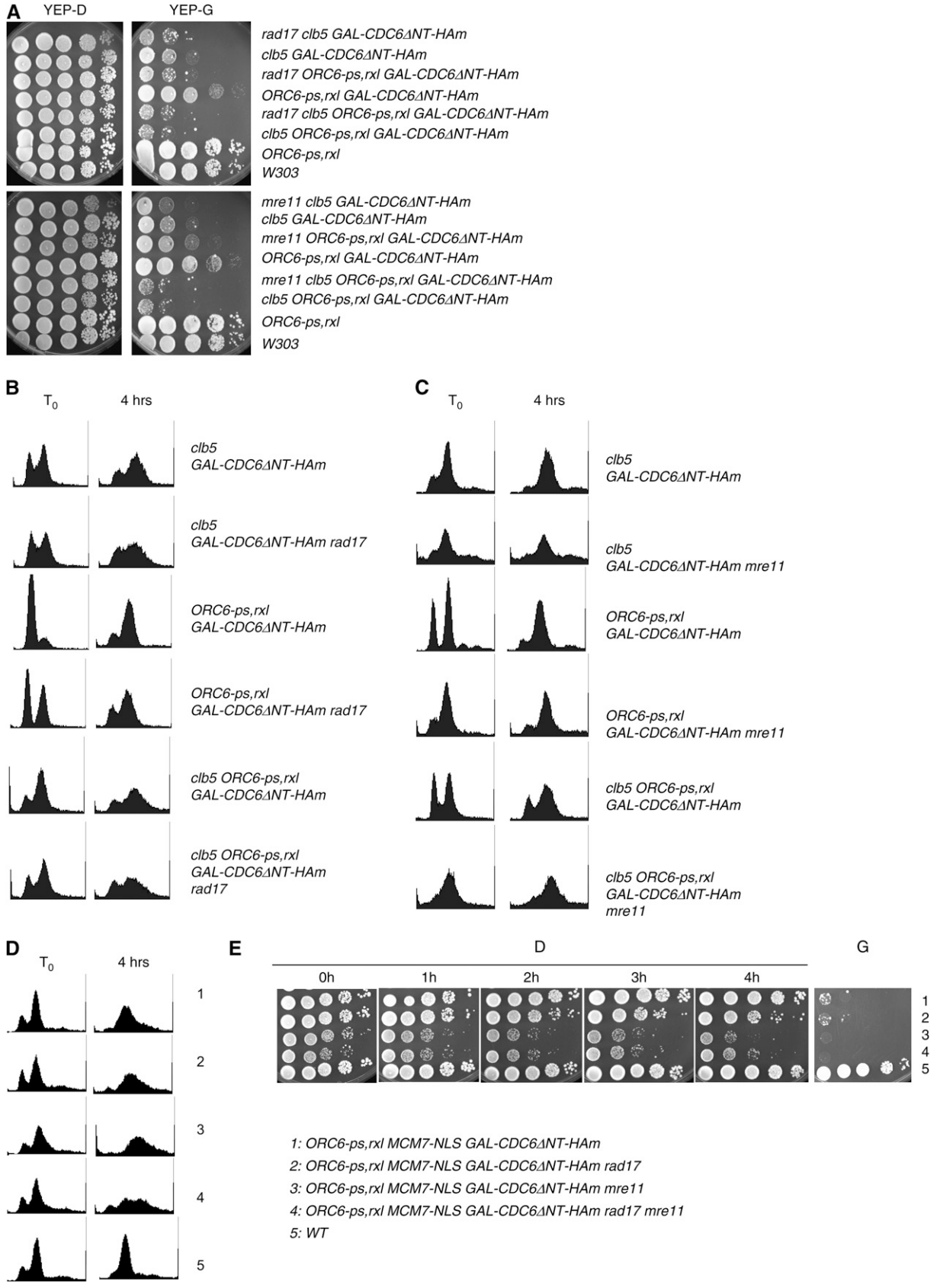


Figure 5.—Requirement for DNA damage response genes *MRE11* and *RAD17* in rereplicating cells. (A) Cell viability was tested in the presence or absence of *MRE11* or *RAD17* in cells of the indicated genotypes, as in Figure 1. (B–D) DNA content was analyzed by DNA flow cytometry after galactose induction as in Figure 2. (E) Cells were grown in YEP-D and transferred to YEP-R for 8 hr.

Continued

TABLE 2

Summary of conclusions derived from genetic interactions and comparisons

Strains and additional mutations (underlined)	Viability (serial dilution)	Rereplication (DNA flow cytometry analysis)
In *CDC6ΔNT* (no. 1)	Viable	No rereplication
clb5 from no. 2	Clb5 stays alive	Clb5 blocks rereplication
MCM7-NLS from no. 4	Mcm regulation has no effect	Mcm regulation has no effect
ORC6-ps,rxl from no. 7	Orc regulation helps to stay alive	Orc regulation has no effect
In *CDC6ΔNT clb5* (no. 2)	Reduced viability	Moderate rereplication
clb6 from no. 3	Clb6 kills	Clb6 may drive rereplication
MCM7-NLS from no. 5	Mcm regulation helps viability	Mcm regulation blocks rereplication
ORC6-ps,rxl from no. 8	Orc regulation helps viability	Orc regulation may block rereplication
cdh1 from Figure 1	Cdh1 kills	No data
In *ORC6-ps,rxl CDC6ΔNT* (no. 7)	Moderate reduced viability	No rereplication (2C DNA accumulation)
clb5 from no. 8	Clb5 stays alive	Clb5 blocks rereplication
MCM7-NLS from no. 10	Mcm regulation stays alive	Mcm regulation blocks rereplication
sic1 from Figure 1	Sic1 regulation stays alive	No data
In *ORC6-ps,rxl clb5 CDC6ΔNT* (no. 8)	Lethal	Rereplication
clb6 from no. 9	Clb6 kills	Clb6 drives rereplication
MCM7-NLS from tetrad	Mcm regulation stays alive	Mcm cannot test
cdh1 from Figure 3	Cdh1 may kill	Cdh1 drives rereplication
In *MCM7-NLS CDC6ΔNT* (no. 4)	Viable	No rereplication
clb5 from no. 5	Clb5 stays alive	Clb5 blocks rereplication
ORC6-ps,rxl from no. 10	Orc regulation stays alive	Orc regulation blocks rereplication
In *MCM7-NLS clb5 CDC6ΔNT* (no. 5)	Lethal	Rereplication
clb6 From #6	Clb6 helps killing	Clb6 drives rereplication
CLB6Δ3p from Figure 4	Clb6 kills because of early proteolysis	Clb6 cannot block rereplication because of early proteolysis
cdh1 from Figure 3	Cdh1 helps to kill	Cdh1 drives rereplication
In *ORC6-ps,rxl MCM7-NLS CDC6ΔNT* (no. 10)	Lethal	Rereplication
clb5 clb6 from no. 11	Clb5,6 have little effect	Clb5,6 have little effect
In *ORC6-ps,rxl MCM7-NLS* (no. 10 on YEP-D)	Viable	No rereplication
clb5 from tetrad	Clb5 stays alive	Clb5 cannot be tested
In *ORC6-ps,rxl MCM7-NLS clb5*	Lethal	Cannot test
clb6 from no. 11 on YEP-D	Clb6 kills	Clb6 cannot be tested
In *clb5 clb6 GAL-CDC6ΔNT* (no. 3)	Viable	No rereplication
MCM7-NLS from no. 6	Mcm regulation may stay alive	Mcm regulation has no effect
ORC6-ps,rxl from no. 9	Orc regulation has no effect	Orc regulation has no effect

All of the mutant strains presented in this study are summarized. Strains that are underlined were compared to those with a single additional mutation. Results from tetrad analysis, serial dilution (Figures 1B, 3, and 4), and rereplication analysis (Figure 2A) are considered. "no." refers to the strain from Figure 1B and Figure 2. "Orc regulation" refers to the ability to bind to and/or phosphorylate Orc6 and is based on the effects of the *ORC6-ps,rxl* mutation. "Mcm regulation" refers to the ability to regulate nuclear localization of the Mcm complex and is based on the effects of the *MCM7-NLS* mutation. See text.

rescue caused by simultaneous deletion of *CLB5* and *CLB6* (in which case both initiation and regulation of rereplication can be carried out by Clb1-4) and by deletion of *CDH1* or by replacement of *clb5* with *CLB5pCLB2* (in these cases, the putative unregulated replication initiation driven by Clb6 is temporally accompanied by accumulation of some of Clb1-4, which can block reinitiation). This interpretation also implies that origin reloading must be blocked throughout S-phase, not merely in the extended G_2 postreplicative period; this was the biological rationale that we proposed previously for efficient Clb5 binding locally to fired origins in mid-S-phase, even while other neighboring origins remained unfired (Wilmes *et al.* 2004). Recent results with a very different approach, genome-wide mapping of DNA synthesis (Green *et al.* 2006), independently support the conclusion that control of origin reloading must function throughout S-phase.

Our results suggest that while Clb6 is active in promoting replication initiation, at least at some origins

Galactose at the final concentration of 3% was added. The samples were collected every hour after the galactose induction and were subjected to serial dilution experiments. The serial dilution was performed on YEP-D plates after the incubation in YEP-G media. As a control, cells were plated on YEP-G after the incubation in YEP-D media. Genotypes are as indicated.

(Schwob and Nasmyth 1993; Donaldson *et al.* 1998), it is apparently much less able to block rereplication, due to its early, rapid proteolysis by SCF^{Cdc4} ubiquitin ligase (Jackson *et al.* 2006) (Figure 4).

Previously, we concluded that the mechanism by which Clb5 blocks rereplication requires binding specifically to the regulated origin (Wilmes *et al.* 2004). There is not a large excess of Clb5 over the probable number of replication origins, and Clb6 is present at only ~10% the level of Clb5 (Cross *et al.* 2002), presumably due to its early proteolysis (Jackson *et al.* 2006); therefore, Clb6 may simply not be present at a high-enough level to stoichiometrically inhibit origin reloading. The mechanism of promotion of origin firing, in contrast, probably does not involve stoichiometric Clb5,6 origin binding (Wilmes *et al.* 2004) and may instead rely on catalytic phosphorylation of *trans*-acting replication factors such as Sld2 (Masumoto *et al.* 2002), allowing even low levels of Clb6, which escape SCF^{Cdc4}-dependent proteolysis later in S-phase, to positively promote rereplication from reloaded origins.

Multiple levels of control of replication reinitiation: In early S-phase, Clb5 and Clb6 initiate replication, and Clb5 simultaneously inhibits rereplication, probably acting through phosphorylation of Mcm and the Orc complex, since both are likely to be favored phosphorylation targets of Clb5 (Wilmes *et al.* 2004; Archambault *et al.* 2005a; Loog and Morgan 2005).

A *clb1 clb2(ts) clb3 clb4* strain (Amon *et al.* 1993) additionally containing *ORC6-ps,rxl GAL-CDC6ΔNT(m)* showed G_2 accumulation and no rereplication at a nonpermissive temperature in galactose (supplemental Figure S2 at http://www.genetics.org/supplemental/). In this strain, replication is driven exclusively by Clb5,6 (Schwob *et al.* 1994). This supports the hypothesis that S-phase cyclins, in addition to targeting Orc6, can regulate other rereplication-limiting substrates independently of Orc6 binding or Orc6 phosphorylation, such as the Mcm complex (Loog and Morgan 2005). In the converse situation, when initiation is driven in the absence of *CLB5,6* and all replication is under control of Clb1-4 (Schwob *et al.* 1994), mitotic cyclins inhibit rereplication, presumably also through largely redundant regulation of Mcm and Orc (Figure 2; compare strains 3, 6, 9, and 11). Thus, there is redundancy among the B-type cyclins, and additionally among the multiple phosphorylation targets Cdc6, Orc2, Orc6, and the Mcm complex, and many of these cyclins and/or controls can be eliminated without strong induction of rereplication.

While our results clearly imply that different B-type cyclins are substantially redundant for rereplication control by multiple mechanisms, we were interested in the possibility of some degree of specificity. One result suggestive of specificity was the finding that *MCM7-NLS GAL-CDC6ΔNT* cells exhibited normal growth rates, but deletion of *CLB5,6* in this background caused reduced viability (compare strains 4 and 6 in Figure 1B); in sharp contrast, *ORC6-ps,rxl GAL-CDC6ΔNT* cells exhibit slow growth, and deletion of *CLB5,6* resulted in complete rescue to normal growth rates (compare strains 7 and 9 in Figure 1B). The opposite effects of *CLB5,6* deletion in these backgrounds could be explained if replication under control of Clb5,6 specifically involved rereplication control via ORC, while replication under control of Clb1-4 specifically involved rereplication control via MCM. However, the fact that these viability phenomena were not reflected in DNA flow-cytometry-detectable rereplication (Figure 2A) reduces our ability to simply interpret these results. As discussed above, the results with DNA flow-cytometry-detectable rereplication are most consistent with all B-type cyclins (except Clb6, due to its rapid proteolysis) having the ability to regulate rereplication via either ORC or MCM.

Finally, we showed that viability of *ORC6-ps,rxl CDC6ΔNT-HAm* relies on the DNA damage checkpoint genes *RAD17* and *MRE11*. However, the rereplication profile by DNA flow cytometry analysis in this background did not change when *RAD17* or *MRE11* was removed (Figure 5, B and C). This suggests that inhibition of rereplication by mitotic Clb1-4 kinases, and not the DNA damage response, is the primary mechanism preventing rereplication when Clb5-dependent mechanisms are disrupted. On the other hand, removing DNA damage signaling in *MCM7-NLS ORC6-ps,rxl CDC6ΔNT-HAm* enhanced rereplication (Figure 5D). Therefore, it appears that restraint of rereplication by a DNA-damage response pathway acts as a "last resort" mechanism when cyclin-CDK-dependent mechanisms fail.

Sensitive assays of the extent of rereplication of specific genomic regions under conditions of complete or partial deregulation of replication control have indicated that additional levels of control exist. Not all origins are equally used for rereplication, and some are used multiple times (Green *et al.* 2006; Tanny *et al.* 2006); even with only a few replication controls removed, where no detectable rereplication exists by DNA flow cytometry, rereplication of some regions can still be detected (Green *et al.* 2006). The latter result emphasizes the likely evolutionary reason for the apparent multiplicity of controls; it is unknown if different regions differ in their cyclin requirements for regulation of rereplication. Many origins are not used detectably for rereplication; additionally, it was shown that origin reloading with the Mcm complex may be necessary but not sufficient for overt rereplication (Tanny *et al.* 2006). Thus, there are probably additional controls blocking rereplication that remain to be identified.

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