Acknowledgements

We thank D. Bowtell, K. Kaibuchi, J. Miyazaki, N. Minato and N. Nomura for plasmids; H. Mizuno, F. Ohba, N. Otsuka, K. Kimura and K. Okuda for technical assistance; and S. Hattori, M. Dutta and A. Dutta for the critical reading of the manuscript. Supported by grants from the Ministry of Health and Welfare, from the Ministry of Education, Science, Sports and Culture, Mitsui Life Social Welfare Foundation, the Princess Takamatsu Cancer Research Fund and from the Japan Health Science Foundation.

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Cyclin-dependent kinases prevent DNA re-replication through multiple mechanisms

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The stable propagation of genetic information requires that the entire genome of an organism be faithfully replicated once and only once each cell cycle. In eukaryotes, this replication is initiated at hundreds to thousands of replication origins distributed over the genome, each of which must be prohibited from re-initiating DNA replication within every cell cycle. How cells prevent re-initiation has been a long-standing question in cell biology. In several eukaryotes, cyclin-dependent kinases (CDKs) have been implicated in promoting the block to re-initiation[1], but exactly how they perform this function is unclear. Here we show that B-type CDKs in *Saccharomyces cerevisiae* prevent re-initiation through multiple overlapping mechanisms, including phosphorylation of the origin recognition complex (ORC), downregulation of Cdc6 activity, and nuclear exclusion of the Mcm2-7 complex. Only when all three inhibitory pathways are disrupted do origins re-initiate DNA replication in G2/M cells. These studies show that each of these three independent mechanisms of regulation is functionally important.

The mechanism of eukaryotic replication initiation and the role of CDKs in its regulation have been most extensively characterized in the budding yeast *S. cerevisiae* (reviewed in ref. 1). Initiation events at yeast origins can be divided into two fundamental stages: the assembly of pre-replicative complexes (pre-RCs) and the triggering of new DNA synthesis. The assembly of pre-RCs occurs shortly after mitosis and renders origins competent to initiate DNA synthesis. During this assembly ORC, which binds origins throughout the cell cycle, is joined by additional initiator proteins, including Cdc6 and the Mcm2-7 complex. Passage through the G1 commitment point (Start) then activates the kinases Cdc7–Dbf4 and the B-type CDKs Clb–Cdc28, which together trigger origin unwinding, assembly of the replication fork machinery, initiation of daughter strand synthesis, and pre-RC disassembly.

In addition to triggering initiation, Clb–Cdc28 prevents re-initiation, in part by blocking re-assembly of pre-RCs[1]. This block

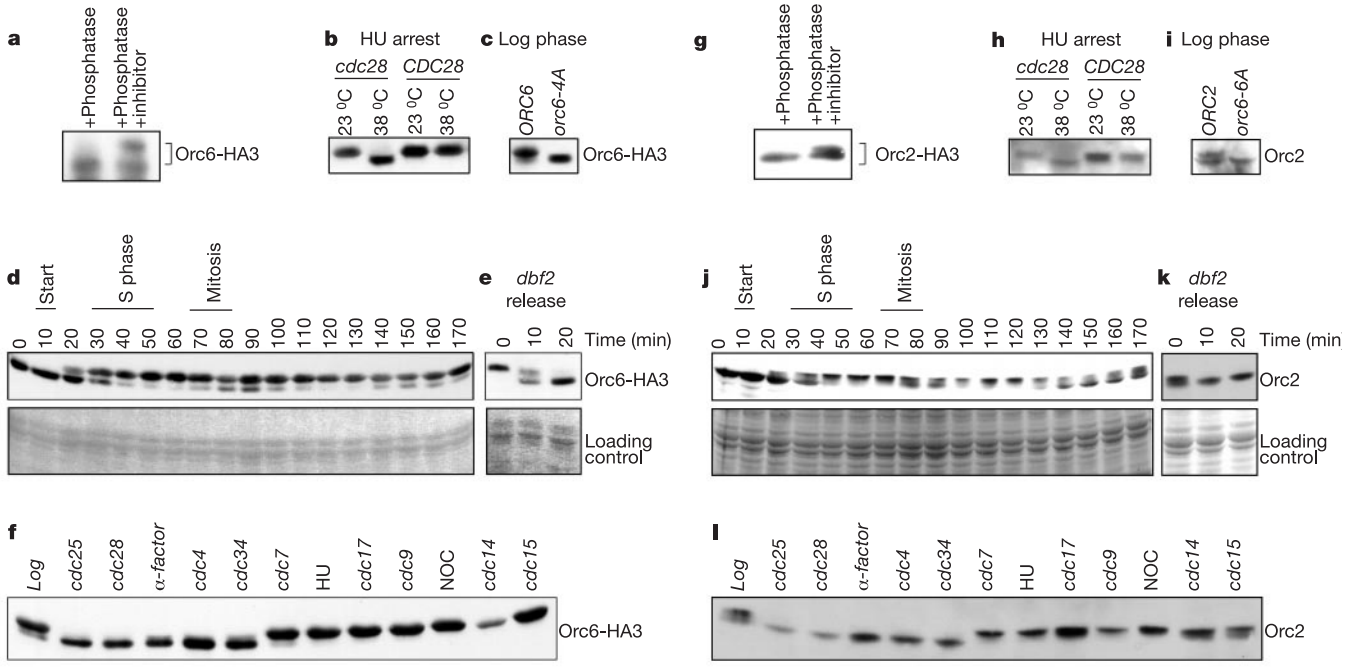

Figure 1 Clb–Cdc28 phosphorylation of Orc6 and Orc2 *in vivo*. **a**–**f**, Immunoblot of Orc6 using anti-haemagglutinin (HA) detection of Orc6-HA3 (**a**–**d**, **f**) or anti-Orc6 (**e**). **g**–**l**, Immunoblot of Orc2 using anti-HA detection of Orc2-HA3 (**g**) or anti-Orc2 (**h**–**l**). Extracts used in **h**, **j**–**l** were identical to those used in **b**, **d**–**f**, respectively. **a**, **g**, Immunoprecipitates from YJL921 (*ORC6-HA3*) (**a**) or YJL963 (*ORC2-HA3*) (**g**) treated with λ-phosphatase with or without phosphatase inhibitors. **b**, **h**, YJL934 (*cdc28-4 ORC6-HA3*) or YJL865 (*CDC28 ORC6-HA3*) grown at 23 °C were arrested in early S phase with hydroxyurea (HU) (after a pre-arrest in G1 with α-factor) then shifted to either 38 °C or kept at 23 °C for a further 3 h. **c**, Log phase YJL865 (*ORC6-HA3*) and YJL1394 (*orc6-4A-HA3*). **i**, Log phase YJL3155 (*ORC2*) and YJL1737 (*orc2-6A*). **d**, **j**, YJL865 (*ORC6-HA3*) cells were released (time 0) from an α-factor arrest in G1 and samples taken every 10 min for analysis by immunoblot, FACS (to determine time of S phase), and budding index with 4,6-diamidino-2-phenylindole (DAPI) staining (to determine time of Start and mitosis). The 80-min time point in **j** is absent. **e**, **k**, YJL1937 (*dbf2-2 ORC6*) cells were grown at 37 °C for 150 min to arrest them in late mitosis, released from the arrest (time 0) by shifting them to 23 °C, and sampled every 10 min for immunoblot analysis. **f**, **l**, immunoblot of *ORC6-HA3 cdc* strains arrested by growth at 37 °C for 2–3 h (until more than 95% have appropriate bud morphology); drug arrests were performed on YJL864 (*ORC6-HA3*) using α-factor, hydroxyurea or nocodazole (NOC).

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is maintained until the kinase is inactivated at the end of mitosis, ensuring that origins initiate only once per cell cycle. CDKs have also been implicated in preventing re-replication in *Schizosaccharomyces pombe*, *Drosophila melanogaster* and *Xenopus laevis*[1]; however, the mechanism by which CDKs prevent pre-RC re-assembly and the identity of their relevant inhibitory targets are poorly understood. Clb–Cdc28 reduces Cdc6 levels through phosphorylation of Cdc6, which promotes its ubiquitin-mediated degradation[2–4], and phosphorylation of the transcriptional activator Swi5, which prevents it from entering the nucleus and inducing Cdc6 expression[5]. Clb–Cdc28 also promotes the net nuclear export of MCM proteins, leading to their exclusion from the nucleus in G2 and M phases[6,7]. However, constitutive expression of stabilized or non-phosphorylatable Cdc6 (ref. 2; and data not shown) or constitutive nuclear localization of Mcm2-7 (ref. 6) do not induce re-replication within a cell cycle. Therefore, it has not been possible to establish the functional importance of these mechanisms in the block to re-replication. Moreover, these results leave open the possibility that Clb–Cdc28 targets additional replication proteins to maintain this block.

Here we have examined the possible regulation of ORC by Clb–Cdc28 and its relevance to the control of replication. Three of the six ORC proteins, Orc1, Orc2 and Orc6, have consensus CDK phosphorylation sites ((S/T)-P-X-(K/R)), indicating that they might be phosphorylated by the kinase *in vivo*. Consistent with this possibility, Orc6 and Orc2 each migrated on SDS–polyacrylamide gel electrophoresis (PAGE) as a doublet, which was converted to the faster-migrating form on phosphatase treatment (Fig. 1a, g). The presence of the slower-migrating hyperphosphorylated form was dependent on both Cdc28 (Fig. 1b, h) and the CDK consensus phosphorylation sites (Fig. 1c, i), and was cell-cycle regulated (Fig. 1d, j). Both Orc6 and Orc2 were hypophosphorylated in G1, became hyperphosphorylated after Start, and remained hyperphosphorylated until the next G1 phase. Incomplete conversion to the hypophosphorylated form in the second and third G1 phases was probably due to loss of cell synchrony, as both proteins showed rapid and complete conversion to the hypophosphorylated form in cells synchronously released into G1 phase from a *dbf2* late mitotic arrest (Fig. 1e, k). These findings indicate that Cdc28 phosphorylates Orc6 and Orc2 on at least some of their CDK consensus sites *in vivo*.

We determined more precisely the timing of this phosphorylation by examining Orc6 and Orc2 at different cell-cycle arrests (Fig. 1f, l). Both proteins were hypophosphorylated after Start on *cdc4* or *cdc34* arrest, when Cln–Cdc28 is active[8], and only became hyperphosphorylated later in G1 at a *cdc7* arrest, when Clb–Cdc28 is active[8]. Orc2 and Orc6 remained hyperphosphorylated at all arrest points later in the cell cycle, matching the persistence of Clb–Cdc28 kinase activity through late anaphase. These data indicate that Clb–Cdc28 and not Cln–Cdc28 is responsible for the cell-cycle-regulated

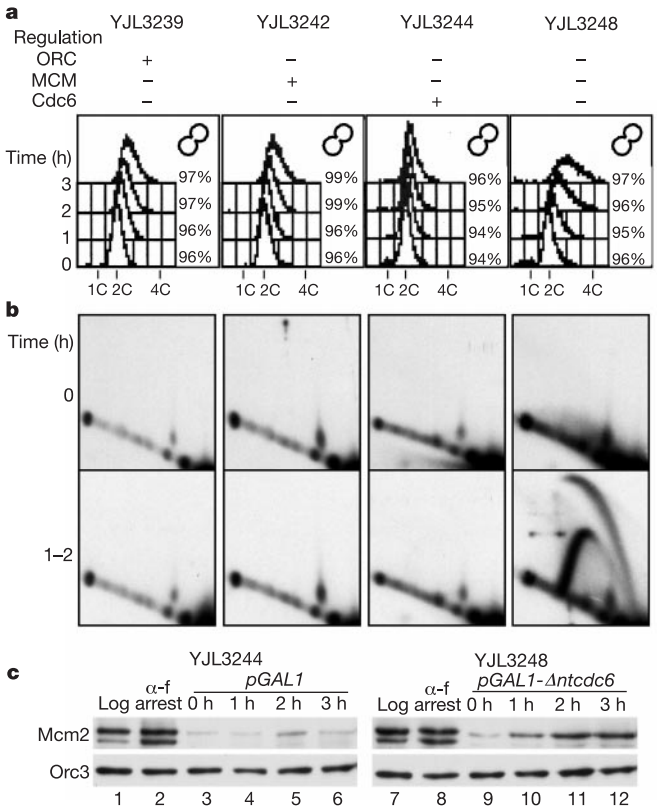

Figure 2 Induction of re-replication and re-initiation in G2/M by deregulation of ORC, Mcm2-7 and Cdc6. ORC phosphorylation, Mcm2-7 localization and Cdc6 expression were deregulated in YJL3239, YJL3242, YJL3244 and YJL3248, as described in the text. Minus, deregulated; plus, regulated. Deregulation of Cdc6 was conditional and dependent on galactose induction of *pGAL1-Δntcdc6*. Cells were initially grown in medium lacking methionine and containing raffinose to prevent expression of ΔntCdc6. They were arrested in G2/M by addition of 2 mM methionine to induce depletion of Cdc20 followed 2.5 h later by 15 μg ml^{-1} nocodazole. After a further 30 min, galactose was added to induce ΔntCdc6 at 0 h. **a**, Budding index and flow cytometry. **b**, Strains were sampled at 0 h or between 1 and 2 h (sampled every 2 min and pooled) for analysis of DNA replication intermediates at *ARS305* by neutral–neutral two-dimensional gel electrophoresis. Similar two-dimensional gel results obtained from cells sampled between 0 and 1 h, 1 and 2 h, and 2 and 3 h were seen with congenic strains that were wild type for *CDC20* and arrested in G2/M solely with nocodazole (data not shown). No replication intermediates were induced in any strain if dextrose instead of galactose was added to the medium to repress the *GAL1* promoter. **c**, Mcm2 reassociates with chromatin during re-replication. Re-replication was induced in YJL3244 and YJL3248 as described above, and chromatin-enriched fractions[20] were analysed at the indicated time points (lanes 3–6, 9–12) by immunoblotting with anti-Mcm2 and anti-Orc3 antibodies. Log phase (lanes 1, 7) and α-factor (α-f; lanes 2, 8) arrested cells were examined together.

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hyperphosphorylation of Orc2 and Orc6. Moreover, the results demonstrate that this phosphorylation is independent of Cdc7–Dbf4.

To determine the function of Orc2 and Orc6 phosphorylation, we constructed a strain in which the phospho-acceptor residues of all CDK consensus sites in these proteins were mutated to alanine. The strain was indistinguishable from its congenic wild-type parent in growth rate, plasmid loss rates (a measure of the efficiency of replication initiation), and flow cytometry profile (data not shown). Similar results were obtained with a strain in which the last remaining CDK consensus site in ORC (on Orc1) was also mutated (data not shown). Thus, phosphorylation of ORC on its CDK consensus sites, like the reduction of Cdc6 levels and the nuclear exclusion of Mcm2-7, is not essential for the block to re-initiation. These experiments also show that phosphorylation of ORC proteins on their CDK consensus sites is not required for the initiation of DNA replication.

To test whether Clb–Cdc28 uses several overlapping mechanisms as a safeguard against re-initiation, we constructed strains that combined various disruptions of ORC, Cdc6 and Mcm2-7 regulation. Phosphorylation of Orc2 and Orc6 on their consensus CDK sites was eliminated by mutating these sites as described above. Nuclear exclusion of Mcm2-7 by Clb–Cdc28 was disrupted by fusing two tandem copies of the SV40 nuclear localization signal (NLS) onto Mcm7 (ref. 6). In both cases, the wild-type genes were precisely replaced by their mutant counterpart. The restriction of Cdc6 expression to G1 phase was overridden by expressing a partially stabilized form of Cdc6, ΔntCdc6, under the control of the galactose-inducible *GAL1* promoter. ΔntCdc6 contains an amino-terminal truncation of amino acids 2–46, which removes sequences that facilitate Cdc6 degradation[2] and are necessary for Clb–Cdc28 association[9]. Despite these mutations, ΔntCdc6 can fully substitute for wild-type Cdc6 (as measured by plasmid loss rates) when expressed from the *CDC6* promoter (data not shown). *pGAL1-Δntcdc6* was introduced in addition to the endogenous *CDC6* gene.

We constructed three congenic strains (YJL3239, YJL3242 and YJL3244) containing all three possible pairwise combinations of regulatory perturbations described above and examined them for the ability to re-replicate their DNA at a G2/M phase arrest (when replication is complete and Clb–Cdc28 kinase activity is high). To achieve a tight arrest, cells were both depleted of Cdc20, which is required for the metaphase–anaphase transition[10], and exposed to nocodazole, a microtubule-destabilizing agent that disrupts mitotic spindles. Only after cells were arrested was ΔntCdc6 induced by galactose. None of these strains increased their DNA content significantly beyond 2C (as measured by flow cytometry, Fig. 2a) or displayed any actively replicating chromosomes (Supplementary Information Fig. 1), which migrate with retarded mobility during pulsed-field gel electrophoresis (PFGE)[11]. Moreover, *ARS305* (Fig. 2b) and *ARS1* (data not shown), which normally fire in early and early to mid S phase, respectively[12,13], showed no signs of re-initiation or passive re-replication by neutral–neutral two-dimensional gel electrophoresis. We conclude that the block to re-initiation in G2/M phase remains largely intact despite simultaneous disruption of any two of the three regulatory mechanisms described above.

We next tested whether a strain containing disruptions in all three regulatory mechanisms (YJL3248) would undergo re-replication at a G2/M phase arrest. In contrast to the results above, galactose induction of ΔntCdc6 resulted in an increase in DNA content from 2C to ~3C (Fig. 2a) and induction of initiation bubbles at *ARS305*, *ARS121*, *ARS607* (Fig. 2b; see also Supplementary Information Fig. 2b, e, f) and *ARS1* (data not shown). The re-initiation at *ARS305* was dependent on its *ARS* consensus sequence and ORC

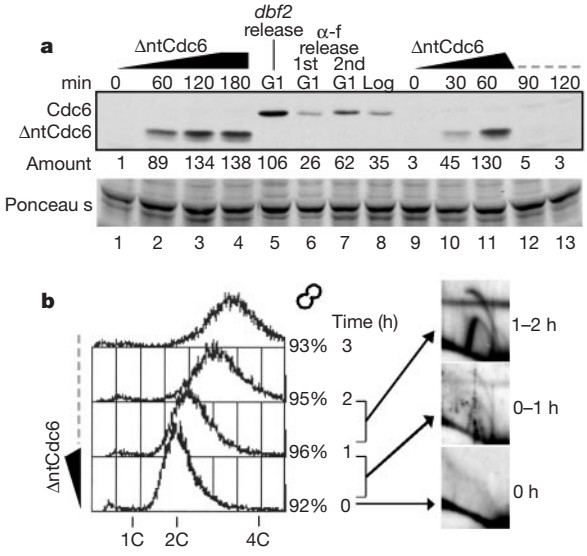

Figure 3 Re-replication and re-initiation are induced with physiological levels of ΔntCdc6. **a**, Immunoblot with anti-Cdc6 antibodies of re-replicating strain YJL3248 (lanes 1–4, 9–13) and *dbf2-2* strain YJL1937 (lanes 5–8). ΔntCdc6 was induced in YJL3248 as described in Fig. 2 (lanes 1–4) or as in Fig. 2 with the addition of dextrose after 60 min to repress further induction (lanes 9–13). Endogenous levels of Cdc6 in YJL1937 were monitored every 10 min after release from a *dbf2-2* late mitotic arrest or every 15 min after release from an α-factor (α-f) arrest. Time points containing the peak levels immediately after *dbf2* release (lane 5, G1) or in the first (lane 6, 1st G1) or second (lane 7, 2nd G1) G1 phases after α-factor release are shown and compared to a log phase population (lane 8). Peak G1 levels in cycling cells seem to be best represented by peak levels after *dbf2* release. Band intensities quantified by densitometry are expressed in arbitrary units (amount). **b**, FACS analysis and budding indices of YJL3248 at indicated times during a transient 1 h induction of ΔntCdc6 as described in **a** (lanes 9–13); two-dimensional gel analysis of *ARS607* taken at 0, 0–1 and 1–2 h (the latter two sampled every 2 min and pooled).

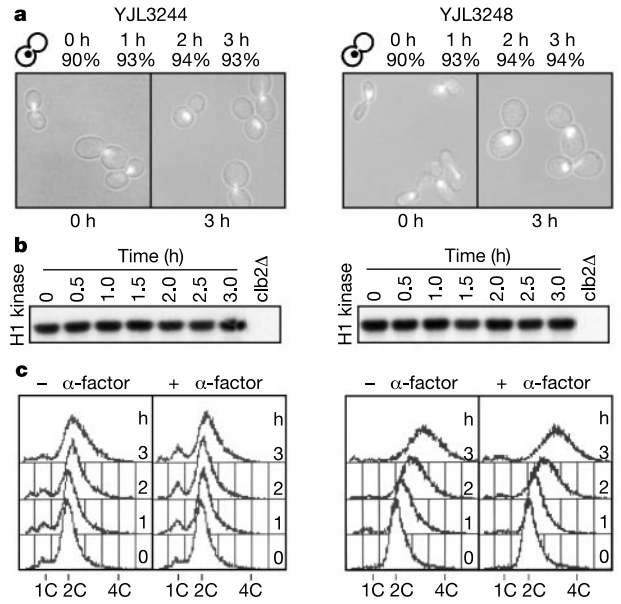

Figure 4 Re-replicating cells remain in G2/M phase. YJL3244 and YJL3248 are a control and re-replicating strain, respectively, induced to re-replicate as described in Fig. 2. **a**, Percentage of cells that are large-budded with a single nucleus. Pictures show DAPI fluorescence overlaying bright field microscopy of cells at 0 and 3-h time points. **b**, H1 kinase assays performed on anti-Clb2 immunoprecipitates taken every 0.5 h after galactose induction of re-replication. Measurements were performed in the linear range of the assay. **c**, Galactose induction of re-replication was performed in the presence or absence of α-factor (to arrest any cells entering G1 phase) and samples were taken every hour for flow cytometry.

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binding site[14] (Supplementary Information Fig. 2a), suggesting that re-initiation occurred through the same ORC-dependent mechanism as S-phase initiation. Re-replication was also accompanied by re-association of Mcm2 with chromatin (Fig. 2c), suggesting that MCM complexes reloaded onto origins to re-initiate replication. Furthermore, the mobility of all chromosomes was retarded during PFGE (Supplementary Information Fig. 1a), with Southern analysis confirming that both large (chromosomes 4 and 7, Supplementary Information Fig. 1b) and small (chromosome 3; data not shown) chromosomes experienced difficulty entering the gel. Hence, all chromosomes seemed to participate in the re-replication. Together our results indicate that ORC, Cdc6 and Mcm2-7 must be simultaneously deregulated to re-initiate DNA replication.

Given that re-initiation arose after induction of a partially stabilized form of Cdc6 from the strong *GAL1* promoter, we wished to confirm that the re-initiation was due to ectopic expression and not massive overexpression of ΔntCdc6 (which might cause additional, unknown perturbations). Western analysis indicated that the level of ΔntCdc6 induced in our re-replicating strain after 2 h in galactose was less than twofold higher than peak levels of endogenous Cdc6 expressed in early G1 phase (Fig. 3a). We also induced ΔntCdc6 synthesis for only 1 h to mimic the normally transient G1 expression of Cdc6. Considerable re-replication and re-initiation was still observed after this transient induction, and some re-initiation was detected during the induction before ΔntCdc6 had fully accumulated (Fig. 3b). Induction of the fully unstable wild-type Cdc6 in G2/M also triggered re-replication (Supplementary Information Fig. 3). Thus, ectopic expression of ΔntCdc6 without any significant overexpression was sufficient to induce re-replication in the triply deregulated strain.

Despite re-initiating DNA replication, the triply deregulated strain did not completely duplicate its DNA, suggesting that we had not removed all restraints on re-replication. Although several origins re-initiated efficiently, some origins did not, including two early (*ARS306* and *ARS307*; Supplementary Information Fig. 2c, d) and two late (*ARS501* and *ARS1413*; Supplementary Information Fig. 2h, i) origins. Two-dimensional gel analysis of *ARS305*, *ARS306* and *ARS307* demonstrated that they all initiated efficiently during the S phase preceding the induced re-replication (data not shown). These observations suggest that additional mechanisms prevent re-initiation of some origins and raise the question of what distinguishes these origins from those that do re-initiate. Y arcs were clearly induced at origins that failed to re-initiate, indicating that these origins were still passively re-replicated, presumably by replication forks that had re-initiated from neighbouring origins. The weaker intensity of these arcs, however, suggests that re-elongation may also have been partially inhibited. Forks originating from *ARS305* and *ARS607* seemed to have some difficulty re-replicating fragments only 30–35 kilobases (kb) away (see Supplementary Information Fig. 2c, g), even though replication forks can travel at least 100–200 kb in S phase[12]. A more quantitative genome-wide analysis of re-replication will be needed to confirm and fully characterize any remaining inhibition of re-initation and/or re-elongation in our triply deregulated strain. Nonetheless, additional mechanisms besides those we specifically deregulated are likely to restrict re-replication within a single cell cycle. Some of these mechanisms may provide further means of inhibiting ORC, Cdc6 or Mcm2-7 (just as Cdc18 in *S. pombe* is independently restrained by both decreased expression and phosphorylation[15]), whereas others may target additional replication proteins.

Unscheduled DNA replication has also been reported to arise from transient inactivation of CDK activity in G2 or G2/M-arrested cells[16–19]. This CDK inactivation, however, resets the cell cycle to G1 phase in the absence of mitosis, effectively inducing S phase in a new cell cycle and not re-initiation within G2 or G2/M phase of the original cell cycle. In *S. cerevisiae*, for example, transient inactivation of Clb–Cdc28 in G2/M-arrested cells, by overexpression of the CDK inhibitor Sic1, induces transcription of G1-specific genes and triggers the G1-specific event of budding[17]. Consistent with the cell cycle being reset to G1 phase, the new round of replication that ensues on release of Clb–Cdc28 inactivation can be blocked by the mating pheromone α-factor which arrests cells in G1 (J. Diffley,

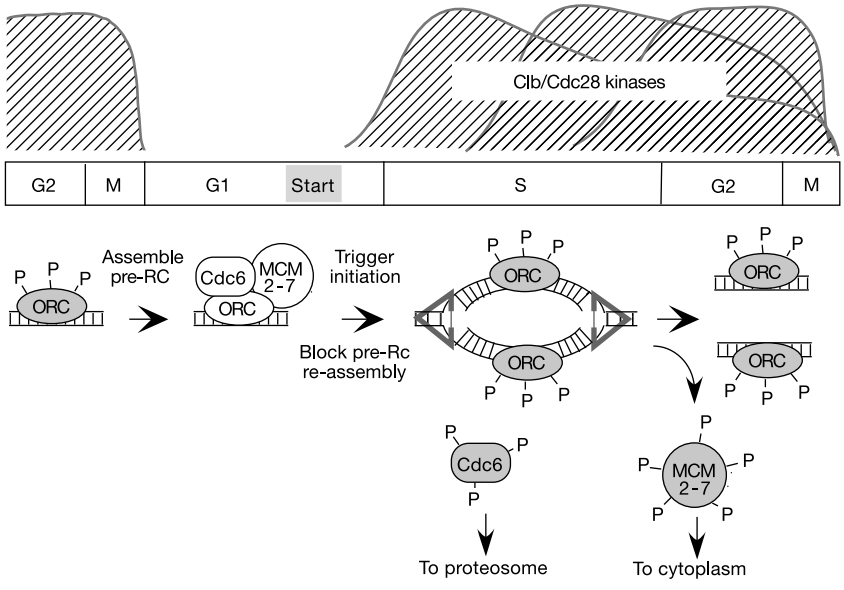

Figure 5 Model for Clb–Cdc28 inhibition of re-replication through several overlapping mechanisms. Mcm2-7 and Cdc6 join ORC at the origin to form the pre-replicative complex (pre-RC) in G1 phase when Cdc28 kinase activity is low. Induction of Clb–Cdc28 after Start helps to trigger initiation, resulting in assembly of the replication fork machinery (triangles) and disassembly of the pre-RC. The kinase simultaneously prevents re-initiation by at least three overlapping mechanisms: (1) phosphorylating Cdc6 and facilitating its polyubiquitination and degradation[3,9]; (2) promoting nuclear exclusion of MCM proteins[6,7], most likely by phosphorylating the MCM proteins (A. Rosales and J.J.L., unpublished data); and (3) inhibiting ORC function through phosphorylation. No single mechanism is individually essential to prevent re-replication in G2/M cells, as each is sufficient to maintain this block. Additional replication proteins involved in pre-RC assembly or triggering (not shown) may also be inhibited by Clb–Cdc28.

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personal communication). Thus, although this unscheduled DNA replication confirms that cell-cycle position is determined by CDK activity[18], it does not address how this CDK activity prevents re-replication within a cell cycle.

Our triply deregulated strain did not bypass mitosis and enter a G1-like state before they re-replicated. The cells did not rebud (Figs 2a and 4a), maintained high mitotic levels of Clb2–Cdc28 kinase activity (Fig. 4b), and were able to re-replicate in the presence of α-factor (Fig. 4c). They also did not break through the mitotic arrest and enter S phase of the next cell cycle, as cells maintained a single, undivided nucleus while they re-replicated (Fig. 4a). Furthermore, by inducing ΔntCdc6 rather than the full-length protein, we avoided Cdc6 association with, and possible inhibition of, Clb–Cdc28 (ref. 9). We conclude that the block to re-initiation was removed in the triply deregulated strain because we had simultaneously rendered ORC, Cdc6 and Mcm2-7 refractory to the inhibitory action of Clb–Cdc28, and not because we had inadvertently inactivated Clb–Cdc28.

These findings indicate that Clb–Cdc28 uses at least three overlapping inhibitory pathways involving phosphorylation of ORC, decreased expression of Cdc6, and nuclear exclusion of Mcm2-7 to prohibit re-initiation of DNA replication in budding yeast (Fig. 5). Because any one of these mechanisms is sufficient to block re-initiation in the absence of the others (Figs 2 and 3, strains YJL3239, YJL3242 and YJL3244) no single mechanism is individually essential for this block. We propose that budding yeast uses a combination of overlapping mechanisms targeting distinct initiation proteins to ensure that none of its hundreds of replication origins re-initiate within a cell cycle. Although no overt re-initiation was seen when any single mechanism was disrupted, each mechanism may be important for preserving long-term genome stability by keeping the frequency of re-initiation events extremely low over the course of many cell divisions. Hence these overlapping mechanisms should be considered mutually reinforcing and not necessarily redundant.

Although multiple mechanisms targeting distinct proteins must be disrupted to induce re-initiation, given that these proteins work together in a complex, it is conceivable that mutation or perturbation of one of these proteins could override enough mechanisms to trigger re-initiation. This may account for reports of re-replication arising from perturbation of just *CDC6* or its *S. pombe* orthologue *CDC18*. In one report, *cdc6-3* (a mutant severely defective for initiation) accumulated a 2.5–3C DNA content in G2/M phase and exhibited both persistent initiation intermediates and persistent association of MCM proteins with chromatin[20]. Although there was no direct demonstration that these persistent signs of replication arose from re-initiation, it is possible that the *cdc6-3* mutation induced re-initiation by counteracting mechanisms inhibiting ORC and MCM proteins as well as those inhibiting Cdc6. Similarly, whereas massive overexpression of Cdc18 is sufficient to induce repeated rounds of re-replication in *S. pombe*[21,22], more modest overexpression does not do so unless a second initiation protein, Cdt1, is simultaneously overexpressed[23] or CDK phosphorylation of Orc2 is prevented[24]. These results suggest that, in addition to overriding the G1-specific expression of Cdc18, massive overexpression of Cdc18 may overwhelm other mechanisms that prevent re-replication in *S. pombe*.

CDKs in other eukaryotes may also depend on several downstream inhibitory targets to prevent re-replication within a single cell cycle. Such a model could help explain why disruption of Cdc6 regulation in humans[25] and *Xenopus*[26] is not sufficient to induce re-replication. Basic mechanistic strategies used by *S. cerevisiae* to prevent re-replication, such as destruction, re-localization, or modification of replication proteins, are likely to be conserved. The precise implementation of those strategies, however, may vary. For example, although in most eukaryotes MCM proteins are not excluded from the nucleus in S and G2 phase[1], in mammalian cells Cdc6 is excluded after G1 phase[27–30]. The relative importance of various strategies may also differ in different organisms and at different times in the cell cycle. Nonetheless, by using multiple inhibitory mechanisms to target more than one replication protein, eukaryotic cells can maintain a tight block to re-replication at the hundreds to thousands of replication origins in their genomes. □

Methods

Details of plasmid/strain construction and experimental assays can be found in Supplementary Information. Yeast growth, galactose induction, methionine repression and cell cycle arrest/release were performed as described[6].

The strains with deregulated ORC phosphorylation, Mcm2-7 localization and Cdc6 expression (Fig. 2) were: YJL3239 (*ORC2 ORC6 MCM7-2NLS CDC6 pGAL1-Δntcdc6 pMET3-CDC20*); YJL3242 (*orc2-6A orc6-4A MCM7-2nls3A CDC6 pGAL1-Δntcdc6 pMET3-CDC20*); YJL3244 (*orc2-6A orc6-4A MCM7-2NLS CDC6 pGAL1 pMET3-CDC20*); and YJL3248 (*orc2-6A orc6-4A MCM7-2NLS CDC6 pGAL1-Δntcdc6 pMET3-CDC20*).

Received 9 April; accepted 31 May 2001.

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Acknowledgements

We thank T. Wang, S. Chu and J. Whangbo for early observations about Orc2 and Orc6 phosphorylation; U. Tran for analysis of ΔntCdc6 plasmid loss rates; L. Huang and J. Gitschier for aid with the PFGE analysis; B. Stillman, C. Liang, S. Bell, J. Ubersax, A. Rudner, M. K. Raghuraman, F. Uhlman, D. Morgan and D. Toczyski for advice and reagents; and A. Sil, I. Herskowitz, A. Johnson, D. Morgan, P. O'Farrell, E. Blackburn, D. Toczyski, B. Thorner, J. Ubersax and C. Takizawa for critical reading of the manuscript. This work was supported by ACS and NIH (J.J.L.) and an NIH training grant (V.Q.N.). Part of this work was performed when J.J.L. was a Markey, Searle and Rita Allen Foundation Scholar.

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Multiple pathways cooperate in the suppression of genome instability in *Saccharomyces cerevisiae*

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Gross chromosome rearrangements (GCRs), such as translocations, deletion of a chromosome arm, interstitial deletions and inversions, are often observed in cancer cells[1–3]. Spontaneous GCRs are rare in *Saccharomyces cerevisiae*; however, the existence of mutator mutants with increased genome instability suggests that GCRs are actively suppressed[4,5]. Here we show by genetic analysis that these genome rearrangements probably result from DNA replication errors and are suppressed by at least three interacting pathways or groups of proteins: S-phase checkpoint functions[5], recombination proteins[4] and proteins that prevent *de novo* addition of telomeres at double-strand breaks (DSBs). Mutations that inactivate these pathways cause high rates of GCRs and show synergistic interactions, indicating that the pathways that suppress GCRs all compete for the same DNA substrates.

The pathways that suppress GCRs have not been well characterized. Using a mutator assay that detects GCRs in *S. cerevisiae*, we demonstrated that mutations in some DNA repair genes and genes encoding S-phase checkpoint functions cause increased rates of accumulating GCRs[4,5]. Three types of GCRs were seen including translocations, deletions and terminal deletion of the ends of chromosomes with addition of a new telomere (telomere additions). All of the mutator mutants had increased rates of telomere additions, whereas only a subset had increased rates of translocations, suggesting that telomerase activity has a role in the formation of GCRs. Thus, mutations in genes that affect telomerase activity were tested for their effect on GCR rates (Table 1). Mutations that inactivated the catalytic activity of telomerase (*est1, est2, est3, cdc13-2, tlc1*) or inactivated proteins that affect telomeres (*tel1, sir1-4, yku70, yku80, rif1, rif2*)[6,7] had no significant effect on the GCR rate. The *stn1-13* mutation, which causes longer telomeres[8], resulted in a small increase in the GCR rate. A *pif1Δ* deletion mutation caused an approximately 1,000-fold increase in the GCR rate. PIF1 is a DNA helicase that functions in both mitochondria and the nucleus, and *pif1* mutations result in both longer telomeres and loss of mitochondrial function[9–11]. The *pif1-m2* allele, which only inactivates the nuclear function of PIF1, increased the GCR rate, whereas the *pif1-m1* allele, which only inactivates the PIF1 mitochondrial function, had no effect. This indicates that the nuclear PIF1 has a principal function in suppression of GCRs.

PIF1 is an inhibitor of telomerase that suppresses telomere additions at HO-induced DSBs by about 14-fold[10,11]. The GCRs observed in the *pif1-m2* mutant were telomere additions (Table 2b). Mutations that inactivated the catalytic activity of telomerase (*est1, est2, est3, cdc13-2, tlc1*) reduced the GCR rate caused by the *pif1-m2* mutation to wild-type levels (Table 1), indicating that telomere additions in *pif1* mutants require telomerase. A *tel1* mutation had no effect on the GCR rate caused by the *pif1-m2* mutation, and 80% of the GCRs seen in the double mutant were telomere additions (Table 2b). This indicates that there is sufficient telomerase activity in a *tel1 pif1* double mutant for *de novo* telomere addition to occur. Mutations in *RIF1* or *RIF2*, which encode proteins that interact with RAP1 (refs 6 and 7), had no effect on the GCR rate caused by the *pif1-m2* mutation. In contrast, a *rif2* mutation increased the length of telomeres added at an HO break-site adjacent to a 81-base-pair (bp) TG repeat[12], a difference that may reflect the fact that the telomere additions seen here do not occur at long TG repeats[4,5,13]. Mutations in *yKU70* or *yKU80*, which encode the Ku70/80 heterodimer that binds to DSBs[6], reduced the GCR rate caused by the *pif1-m2* mutation to almost wild-type levels. Mutations in *SIR2*, *SIR3* or *SIR4*, but not *SIR1*, reduced the GCR rate caused by the *pif1-m2* mutation. The effect of *sir* is probably indirect, as a mutation in *HMLα* significantly reduced the defect caused by *sir2* (ref. 14). The *stn1-13* mutation reduced the GCR rate caused by the *pif1-m2* mutation, a result that may reflect the reduced end protection that occurs in both *stn1* and *cdc13* mutants[15]. These results indicate that the GCRs that occur in the *pif1-m2* mutant require telomerase and other proteins (Ku70/80, CDC13) that interact with DSBs and effect telomeres.

Mutations that inactivate S-phase checkpoint functions cause an increase in the rate of telomere-addition GCRs[5]. This has been suggested to occur because of errors that normally take place during DNA replication, and S-phase checkpoint defects either cause reduced repair or allow repair to occur in a phase of the cell cycle where telomere addition is the principal outcome[5,12]. To investigate the role of telomerase in the GCRs resulting from S-phase checkpoint defects, interactions between selected mutations that affect

Table 1 Mutations that alter telomerase activity effect the GCR rate

	PIF1		*pif1-m2*	
Relevant genotypes	Strain number	Mutation rate (Can^r 5-FOA^r)	Strain number	Mutation rate (Can^r 5-FOA^r)
Wild type	3615	3.5×10^{-10} (1)	4343	8.3×10^{-6} (237)
est1Δ	4345	1.5×10^{-10} (0.4)	4365	2.3×10^{-10} (0.7)
est2Δ	4347	1.2×19^{-10} (0.3)	4367	5.8×10^{-10} (1.7)
est3Δ	4349	1.5×10^{-10} (0.4)	4369	2.3×10^{-10} (0.7)
cdc13-2	4351	4.6×10^{-10} (1.3)	4371	4.4×10^{-10} (1.3)
tlc1Δ	4224	3.1×10^{-10} (0.9)	4373	3.3×10^{-10} (0.9)
tel1Δ	3731	2.0×10^{-10} (0.6)	4375	8.2×10^{-8} (234)
sir1Δ	4353	8.9×10^{-10} (2.5)	4377	8.9×10^{-8} (254)
sir2Δ	4355	2.5×10^{-10} (0.7)	4379	6.0×10^{-9} (17)*
sir3Δ	4357	5.0×10^{-10} (1.4)	4381	9.0×10^{-9} (26)
sir4Δ	4359	8.4×10^{-10} (2.4)	4383	3.8×10^{-8} (109)
yku70Δ	3639	4.1×10^{-10} (1)	4385	9.04×10^{-10} (3)
yku80Δ	3640	7.8×10^{-10} (2)	4387	4.0×10^{-9} (11)
rif1Δ	4361	9.9×10^{-10} (3)	4389	7.05×10^{-8} (201)
rif2Δ	4363	5.0×10^{-9} (14)	4391	8.3×10^{-8} (237)
stn1-13	4554	5.3×10^{-9} (15)	4552	2.3×10^{-8} (66)

The GCR rate of a *pif1* deletion strain (RDKY4399) is a 3.53×10^{-7}, which is about four times higher than the GCR rate of the *pif1-m2* strain. Deletion of *PIF1* resulted in a petite phenotype owing to the loss of both the nuclear and the mitochondrial function of PIF1. Loss of mitochondrial function of PIF1 did not cause genome instability; a spontaneously arising petite but otherwise wild-type strain (RDKY4397) did not have an increased GCR rate, and a *pif1-m1* strain (RDKY4393) that only lost the mitochondrial function of PIF1 did not have an increased GCR rate. Mutation of *RRM3* (RDKY4395), which encodes another PIF1-like helicase in *S. cerevisiae*, did not affect the GCR rate. Numbers in parentheses indicate the GCR rate relative ot the wild-type GCR rate.
* The GCR rate of the *hmlα::Leu2 sir2Δpif1-m2* strain (RDKY4547) was 4.3×10^{-8} (121).
Can^r 5-FOA^r is resistant to both canavanine and 5-fluoroorotic acid.

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