Clb/Cdc28 kinases promote nuclear export of the replication initiator proteins Mcm2–7

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Background: In the budding yeast *Saccharomyces cerevisiae*, the cyclin-dependent kinases of the Clb/Cdc28 family restrict the initiation of DNA replication to once per cell cycle by preventing the re-assembly of pre-replicative complexes (pre-RCs) at replication origins that have already initiated replication. This assembly involves the Cdc6-dependent loading of six minichromosome maintenance (Mcm) proteins, Mcm2–7, onto origins. How Clb/Cdc28 kinases prevent pre-RC assembly is not understood.

Results: In living cells, the Mcm proteins were found to colocalize in a cell-cycle-regulated manner. Mcm2–4, 6 and 7 were concentrated in the nucleus in G1 phase, gradually exported to the cytoplasm during S phase, and excluded from the nucleus by G2 and M phase. Tagging any single Mcm protein with the SV40 nuclear localization signal made all Mcm proteins constitutively nuclear. In the absence of functional Cdc6, Clb/Cdc28 kinases were necessary and sufficient for efficient net nuclear export of a fusion protein between Mcm7 and the green fluorescent protein (Mcm7–GFP), whereas inactivation of these kinases at the end of mitosis coincided with the net nuclear import of Mcm7–GFP. In contrast, in the presence of functional Cdc6, which loads Mcm proteins onto chromatin, S-phase progression as well as Clb/Cdc28 kinases was required for Mcm–GFP export.

Conclusions: We propose that Clb/Cdc28 kinases prevent pre-RC reassembly in part by promoting the net nuclear export of Mcm proteins. We further propose that Mcm proteins become refractory to this regulation when they load onto chromatin and must be dislodged by DNA replication before they can be exported. Such an arrangement could ensure that Mcm proteins complete their replication function before they are removed from the nucleus.

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Received: **27 September 1999**
Revised: **24 December 1999**
Accepted: **24 December 1999**

Published: **8 February 2000**

Current Biology 2000, 10:195–205

0960-9822/00/$ – see front matter
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Background

The faithful transmission of genetic information during cell division requires that the entire genome be replicated once and only once. In eukaryotic cells, DNA replication initiates at multiple replication origins scattered throughout the genome. To ensure that each genomic segment is duplicated exactly once, re-initiation within a cell cycle must be prevented at every origin.

The initiation of eukaryotic DNA replication can be divided into two fundamental stages that are best characterized in the budding yeast *Saccharomyces cerevisiae* [1–4]. In the first stage, which occurs as cells enter G1 phase, pre-replicative complexes (pre-RCs) assemble at origins [2], making them competent to initiate replication [3,4]. A six-protein origin recognition complex (ORC) [5] binds origins throughout the cell cycle [6,7]. During pre-RC assembly, the initiator protein Cdc6 is thought to load a family of six minichromosome maintenance (Mcm) proteins, Mcm2–7, onto the ORC-bound origins [7,8]. The second stage of initiation occurs as cells enter S phase and involves the triggering of initiation by two kinase complexes: the cyclin-dependent kinase (CDK) Cdc28 in association with the B-type cyclins, Clb1–6 [5], and Cdc7 kinase in association with its regulatory subunit Dbf4 [6]. During this stage, origins are unwound and additional proteins required for DNA synthesis are thought to assemble into the elongation machinery at nascent replication forks [1,7]. Importantly, initiation results in disassembly of pre-RCs, leaving only ORC bound to origins for the remainder of the cell cycle [2,7,8]. The Mcm proteins in pre-RCs appear to be incorporated into the elongation machinery and to remain associated with chromatin at moving replication forks until replication terminates [7].

In addition to triggering initiation, Clb/Cdc28 kinases can prevent pre-RC assembly [9,10]. This does not interfere with the initiation of DNA replication in S phase, as these kinases are activated well after pre-RCs assemble in G1 phase. After triggering initiation, however, these kinases are believed to block re-initiation at origins by inhibiting re-assembly of pre-RCs. This block is maintained until the

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end of mitosis, whereupon inactivation of Clb kinases allows pre-RC assembly in the next cell cycle.

Clearly, identifying the relevant inhibitory targets of these kinases is critical for understanding the block to re-initiation. Likely targets include Cdc6 and its *Schizosaccharomyces pombe* homolog Cdc18 [11–13]. Another possible target is the Mcm family of proteins. Mcm2–7 are six sequence-related and evolutionarily conserved proteins, each of which is essential for replication initiation (reviewed in [14]). Indirect immunofluorescence of Mcm2, 3, 5 and 7 indicates that the subcellular distribution of Mcm proteins in *S. cerevisiae* is regulated during the cell cycle. The proteins concentrate in the nucleus in G1 phase, gradually disperse during S phase, and reside predominantly in the cytoplasm during G2 and M phase [15–17]. Given that only a twofold reduction in Mcm2 levels can impair the activity of some origins [18], a more drastic reduction of all Mcm levels in the nucleus during S, G2 and M phase is likely to severely inhibit re-initiation. Hence, regulation of Mcm localization provides a potential mechanism to prevent re-initiation during the cell cycle. Recently, however, this regulation was called into question by a report based on subcellular fractionation that Mcm2 and Mcm3 are constitutively nuclear [19]. To reconfirm this regulation and to examine its mechanism in more detail, we fused Mcm proteins to green fluorescent protein (GFP) and investigated their localization in living cells.

Results

The subcellular distribution of Mcm–GFP fusion proteins is regulated during the cell cycle

Mcm2–4, 6 and 7 were individually fused to GFP, and the fusion constructs under the control of the endogenous *MCM* promoters were substituted for the corresponding wild-type *MCM* genes. The resulting strains all grew at wild-type rates and displayed normal flow cytometry profiles during log-phase growth (data not shown), indicating that these Mcm–GFP fusion proteins could functionally substitute for the wild-type proteins. Appending GFP to either the 5′ or 3′ end of Mcm5 destroyed Mcm function, precluding an examination of Mcm5 localization in living cells.

In asynchronous populations, the Mcm–GFP fusion proteins exhibited a continuous range of subcellular distributions (Figure 1a), roughly correlating with cell-cycle position. GFP fluorescence was primarily nuclear in unbudded cells (100% nuclear, $n > 100$), partially nuclear in small budded cells (35–60% nuclear, $n > 100$) and primarily cytoplasmic in large budded cells (< 15% nuclear, $n > 90$). Similarly, in cells synchronously released from an α-factor arrest in G1 phase (Figure 1b), Mcm7–GFP was nuclear in G1 phase (0 minutes), partly dispersed in S phase (60 minutes), predominantly cytoplasmic in G2/M phase (100 minutes), and nuclear again in the next cell cycle (data not shown). Thus, the Mcm proteins concentrate in the nucleus at the beginning of each cell cycle and gradually disappear from the nucleus during S phase.

To determine more precisely when Mcm proteins change their subcellular distribution, *MCM7–GFP* cells were arrested at various points in the cell cycle (Figure 1c). In cells arrested in early S phase with hydroxyurea (HU), Mcm7–GFP was predominantly nuclear, indicating that progression through S phase and possibly DNA replication is required for the disappearance of Mcm proteins from the nucleus. In cells arrested in G2/M phase with nocodazole (NOC), or in late anaphase at a *dbf2* cell cycle arrest, Mcm7–GFP was primarily cytoplasmic, suggesting that Mcm proteins are fully dispersed after S phase and that reaccumulation in the nucleus requires the completion of mitosis. This reaccumulation was observed within 20 minutes after release from a *dbf2* arrest when nuclei were still at opposite poles of the cell, indicating that Mcm proteins rapidly re-enter the nucleus at telophase or early G1 phase. The localization of Mcm2–GFP, GFP–Mcm3, Mcm4–GFP and Mcm6–GFP exhibited a temporal pattern similar to that of Mcm7–GFP (data not shown). Finally, unlike previous analyses of Mcm localization using immunofluorescence [15–17], we observed nuclear exclusion of Mcm2–GFP, GFP–Mcm3, and Mcm7–GFP in G2/M phase. This exclusion was seen more readily in *MAT***a**/α and *MAT***a**/**a** diploid cells (Figure 1d and data not shown), presumably because of their larger nuclei.

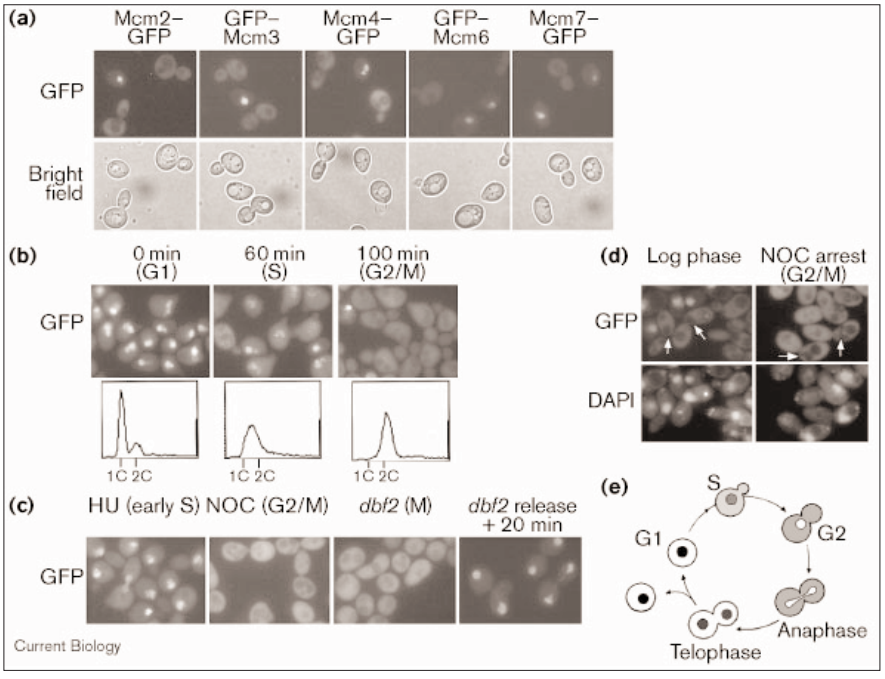
In summary, our analysis of Mcm–GFP localization in living cells corroborates and extends the analysis of Mcm localization determined by indirect immunofluorescence of Mcm2, Mcm3, Mcm5 and Mcm7 in fixed cells. Taken together, these results demonstrate that all six Mcm proteins share the same cell-cycle localization pattern as illustrated schematically in Figure 1e.

The Mcm proteins colocalize as a complex

Complexes containing homologs of all six Mcm proteins have been isolated from *S. pombe* [20], *Xenopus laevis* [21] and humans [22]. Although a comparable complex has yet to be isolated from budding yeast, numerous genetic and biochemical interactions between the budding yeast Mcm proteins [18,23–25] suggest that these proteins also associate with each other *in vivo*. We therefore investigated whether Mcm proteins might colocalize as a complex by determining whether perturbing the localization of one Mcm protein affects the localization of the others. To perturb the localization of an Mcm protein, we fused it to two tandem copies of the SV40 nuclear localization signal ($SVNLS_2$). To monitor the effect of this perturbation on the localization of an Mcm protein, we fused it to GFP.

When GFP and $SVNLS_2$ were attached to the same Mcm protein, the resulting Mcm–GFP–$SVNLS_2$ fusion protein

Figure 1



Mcm–GFP protein localization is regulated across the cell cycle. **(a)** Asynchronous cultures. YJL1969 (*MCM2–GFP*), YJL2160 (*GFP–MCM3*), YJL1973 (*MCM4–GFP*), YJL2163 (*GFP–MCM6*) and YJL1977 (*MCM7–GFP*) cultures were grown exponentially in YEPD containing 2 μg/ml 4,6-diamidino-2-phenylindole (DAPI) for 1 h before being examined by fluorescence and bright-field microscopy. **(b)** Synchronous cultures. YJL1977 (*MAT***a** *MCM7–GFP*) cells growing exponentially in YEPD at 30°C were arrested in α factor for 90 min and synchronously released from the arrest by addition of pronase at 22°C (0 min). Samples were taken at 0, 60 and 100 min and analyzed by fluorescence microscopy and flow cytometry. **(c)** Arrested cultures. YJL1977 cells were released from α arrest as described in (b) and incubated for 90 min at 30°C with HU or NOC. YJL1937 (*dbf2-2 MCM7–GFP*) cells growing exponentially in YEPD at 24°C were arrested in late anaphase by incubation at 37°C for 3 h (*dbf2*), then released from the arrest at 22° for 20 min (*dbf2* release + 20 min). **(d)** Mcm3–GFP is excluded from the nucleus in G2/M phase. YJL2756 (*MAT***a**/*MAT*α *GFP–MCM3/GFP–MCM3*) cells were grown to exponential phase in YEPD at 30°C. DAPI (5 μg/ml) was added to half the culture (log phase), and NOC and DAPI (5 μg/ml) added to the other half (NOC); 90 min later, when the NOC-treated cells had arrested in G2/M phase, cells from both cultures were examined by fluorescence microscopy. Arrows point to examples of nuclei excluding Mcm3–GFP. **(e)** Schematic illustration of Mcm protein localization during the cell cycle. Mcm proteins rapidly accumulate in the nucleus in telophase or early G1 phase, gradually redistribute to the cytoplasm during S phase, and are excluded from the nucleus in G2 and M phase.

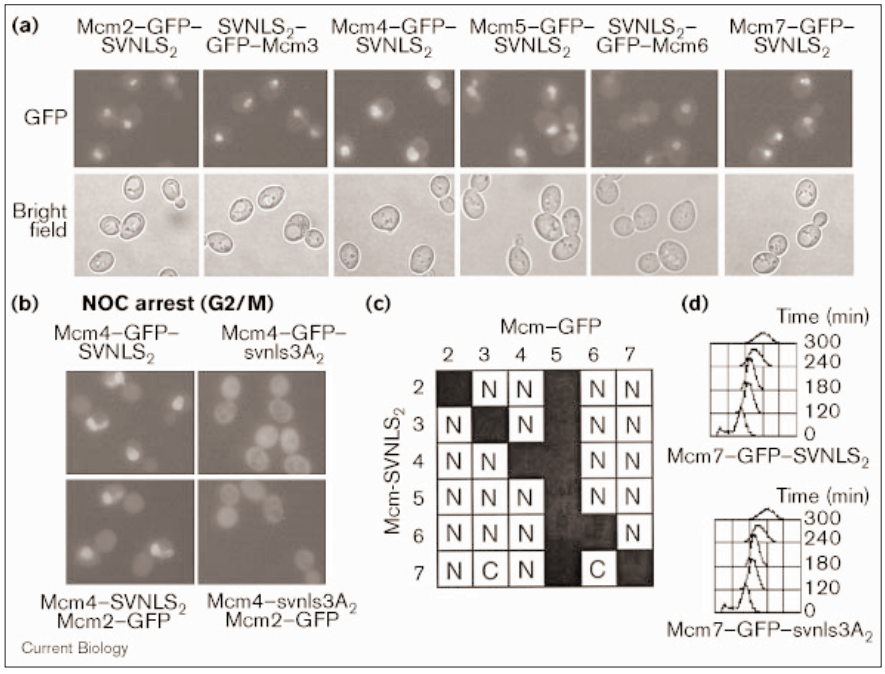
was constitutively nuclear (Figure 2a,b). In contrast, Mcm–GFP–svnls3A2, which contains a mutant NLS, was localized normally (Figure 2b and data not shown). Hence, the $SVNLS_2$ tag was capable of overriding the nuclear export of Mcm proteins observed in S, G2 and M phase. Mcm–GFP fusion proteins were also constitutively nuclear when the $SVNLS_2$ tag was placed on a different Mcm protein.

Figure 2b shows two strains expressing Mcm2–GFP arrested at the G2–M phase boundary. Mcm2–GFP was nuclear when Mcm4 was fused to $SVNLS_2$ but not when Mcm4 was fused to the mutant $svnls3A_2$. Hence, constitutive nuclear localization of Mcm4 resulted in constitutive nuclear localization of Mcm2–GFP. Figure 2c summarizes the G2/M phase localization of Mcm–GFP for all strains constructed with GFP and $SVNLS_2$ attached to different Mcm proteins. (As discussed earlier, strains containing Mcm5–GFP could not be constructed because this fusion is nonfunctional.) In virtually all cases, the Mcm–GFP fusion was nuclear. The only exceptions were two strains, one expressing Mcm7–$SVNLS_2$ and GFP–Mcm3 and the other Mcm7–$SVNLS_2$ and GFP–Mcm6. We suspect that the tags in these strains are positioned in such a way on the Mcm complex that the $SVNLS_2$ tag is masked or inaccessible. Consistent with this idea, Mcm–GFP was constitutively nuclear in the two strains in which the same proteins were tagged but the tags were switched. Finally, in the series of control strains constructed with mutant $svnls3A_2$ tag, Mcm–GFP was always cytoplasmic in G2/M phase (data not shown). Thus, we conclude that the localization of any two Mcm proteins is coupled during S, G2 and M phase in budding yeast, and we propose that all six Mcm proteins colocalize together as a complex.

Nuclear accumulation of Mcm7–GFP in G1 phase is not dependent on Cdc6 or Cdc45 function

The nuclear influx of Mcm–GFP proteins during the M–G1-phase transition could be due to a change in Mcm nucleocytoplasmic transport or to regulated association with a nuclear anchor such as chromatin. To investigate whether Mcm chromatin association might be responsible for this influx, we determined whether the influx was dependent on Cdc6. When Cdc6 was thermally inactivated in *MCM7–GFP cdc6-1^{ts}* cells before the M–G1-phase transition, Mcm7–GFP accumulated in the nucleus to the same extent as the *MCM7–GFP CDC6* control (Figure 3a). If allowed to proceed through G1 phase, these cells failed to replicate their DNA as monitored by flow cytometry (data not shown), confirming that Cdc6 was effectively inactivated. Thus, thermal inactivation of Cdc6 did not prevent Mcm7

Figure 2



Constitutive nuclear localization of one Mcm protein results in constitutive nuclear localization of all Mcm proteins. **(a)** Mcm–GFP proteins fused to $SVNLS_2$ are constitutively nuclear. YJL1987 (*MCM2–GFP–SVNLS$_2$*), YJL2227 (*SVNLS$_2$–GFP–MCM3*), YJL2155 (*MCM4–GFP–SVNLS$_2$*), YJL1991 (*MCM5–GFP–SVNLS$_2$*), YJL2229 (*SVNLS$_2$–GFP–MCM6*) and YJL1981 (*MCM7–GFP–SVNLS$_2$*) cultures were grown to exponential phase in YEPD at 30°C and examined by fluorescence microscopy. **(b)** Mcm4–$SVNLS_2$ directs Mcm2–GFP into the nucleus in G2/M phase. YJL2039 (*MCM4–GFP–SVNLS$_2$*), YJL2221 (*MCM4–GFP–svnls3A$_2$*), YJL2170 (*MCM4–SVNLS$_2$ MCM2–GFP*) and YJL2172 (*MCM4–svnls3A$_2$ MCM2–GFP*) cultures were arrested in G2/M phase with NOC and examined by fluorescence microscopy. **(c)** G2/M-phase localization of Mcm–GFP in NOC-arrested cells expressing a second Mcm fused to $SVNLS_2$. Strains used are listed in the Supplementary material. The numbers 2–7 identify the Mcm proteins fused to either GFP or $SVNLS_2$ in each strain. N, Mcm–GFP was predominantly nuclear; C, Mcm–GFP was predominantly cytoplasmic; shaded box, strain not constructed or not part of this analysis. **(d)** NOC was added (t = 0 min) to exponentially growing cultures of YJL1981 (*MCM7–GFP–SVNLS$_2$*) and YJL1985 (*MCM7–GFP–svnls3A$_2$*), and samples were analyzed by flow cytometry at the indicated times. Cells were > 95% budded by 120 min.

nuclear accumulation in G1 phase. Similar results were obtained when Cdc6 was inactivated by transcriptional repression of the *CDC6* gene (Figure 5a, 60 minutes). Previous experiments have demonstrated that both thermal inactivation and transcriptional depletion of Cdc6 prevent Mcm association with replication origins and chromatin [8,26]. Therefore, the nuclear accumulation of Mcm7 in G1 phase does not require its association with chromatin.

Cdc45 is another potential nuclear anchor for Mcm proteins, as it is constitutively nuclear and has been shown to physically associate with several Mcm proteins [24,25]. We have also shown, however, that Cdc45 is not required for the accumulation of Mcm7 in G1 nuclei (see the Supplementary material). In summary, we have found no evidence to support an anchoring model for the nuclear accumulation of Mcm proteins. It is formally possible that residual Cdc6 or Cdc45 activity facilitated the nuclear accumulation in our experiments or that association with some other nuclear component was responsible for this accumulation. Nonetheless, our data is most consistent with a model in which regulation of Mcm nucleocytoplasmic transport is responsible for the nuclear influx of Mcm proteins at the end of mitosis.

Mcm7–GFP is exported from the nucleus

As first pointed out by Hennessy *et al.* [16], the disappearance of Mcm proteins from the nucleus during S phase could be due either to net export of Mcm proteins from the nucleus or to concomitant nuclear degradation and cytoplasmic synthesis of Mcm proteins (with the newly synthesized Mcms remaining in the cytoplasm). The latter model predicts that Mcm levels should fall during S phase, if protein synthesis is blocked, and that Mcm–GFP protein should still disappear from the nucleus, even if fused to an SV40 NLS.

To test these predictions, *MCM7–GFP–SVNLS$_2$* and control *MCM7–GFP–svnls3A2* cells were arrested in early S phase with HU, then released into medium containing cycloheximide to block protein synthesis and NOC to catch cells in G2/M phase (Figure 4). Immunoblot analysis showed that Clb2 failed to accumulate during the release (data not shown), confirming that protein synthesis was blocked. Despite this block, both strains completed S phase and acquired a 2C DNA content within 120 minutes (Figure 4a,b). During this period, *MCM7–GFP7–SVNLS$_2$* cells retained high concentrations of nuclear GFP fluorescence (Figure 4a), and immunoblot analysis showed that they maintained total cellular levels of Mcm7–GFP protein (Figure 4c). Thus, nuclear Mcm proteins are not degraded to any significant extent during S phase. While GFP fluorescence did disappear from the nucleus of *MCM7–GFP–svnls3A2* cells during S phase (Figure 4b), as expected, immunoblot analysis demonstrated that

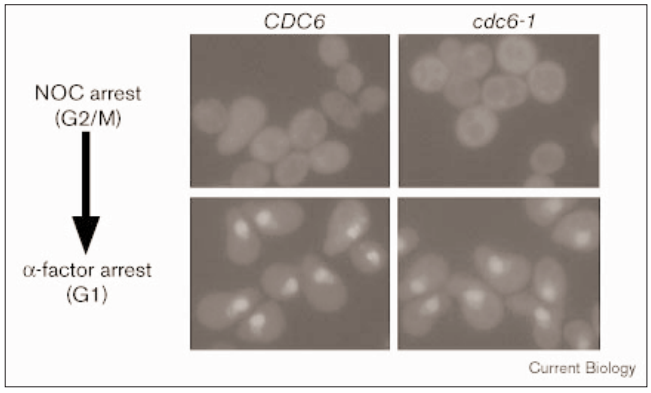
Mcm7–GFP–svnls3A2 was also stably maintained in these cells (Figure 4d). We conclude that the Mcm proteins are stable during S phase and that their disappearance from the nucleus is due to net nuclear export rather than degradation.

Clb/Cdc28 kinases are required for efficient nuclear export of Mcm7–GFP

What cell-cycle signals are responsible for inducing and maintaining the net export of Mcm proteins from the nucleus? Initially, we investigated these signals in the absence of chromatin association by monitoring the localization of Mcm7–GFP in cells depleted for Cdc6 protein (Figure 5a). *MCM7–GFP pMET–CDC6 dbf2-2^{ts}* cells growing in medium containing dextrose and lacking methionine were blocked in late anaphase at a *dbf2* arrest, exposed to methionine for 30 minutes to repress Cdc6 expression, then synchronously released from the arrest at the permissive temperature. Within 60 minutes after the release, cells had entered G1 phase and concentrated Mcm7–GFP in the nucleus. By 100 minutes, however, the cells had passed START (> 80% small budded, $n = 100$) and lost their nuclear concentration of Mcm7–GFP. These cells failed to replicate their DNA (Figure 5b), establishing that Cdc6 had been effectively depleted and that S-phase progression is not intrinsically required for the nuclear exit of Mcm proteins. This exit is dependent on passage through START, however, as cells released from the *dbf2* block into medium containing α factor retained Mcm7–GFP in the nucleus (data not shown).

One possible signal for the net nuclear export of Mcm proteins is the activation of Clb/Cdc28 kinases, which is dependent on and occurs soon after START. These kinases were apparently activated during the course of our experiment, as a congenic *CDC6* strain treated in parallel with the *pMET–CDC6* strain initiated and completed S phase within 100 minutes after release from the *dbf2* block (Figure 5b). To determine whether the nuclear exit of Mcm7–GFP was dependent on Clb/Cdc28 kinases, we examined this exit in the presence of the Clb-kinase-specific inhibitor, Sic1 [5] (Figure 5c). *MCM7–GFP pMET–CDC6 pGAL–sic1-9A dbf2-2^{ts}* and *MCM7–GFP pMET–CDC6 pGAL dbf2-2^{ts}* cells were subjected to the same experimental protocol described above, except cells were grown in raffinose instead of dextrose, and galactose was added 10 minutes after the release from mitotic arrest to induce sic1-9A, a hyperstabilized form of Sic1 [27] (Figure 5c). In the absence of sic1-9A, nuclear fluorescence of Mcm7–GFP began to disappear at 100 minutes and was completely gone by 120 minutes, similar to what was seen in Figure 5a. In the presence of sic1-9A, nuclear fluorescence of Mcm7–GFP began to diminish at 120 minutes, but did not completely disappear and was still faintly detectable in most cells at 160 minutes. Hence, sic1-9A delayed the onset of Mcm7–GFP disappearance from the nucleus and made it less efficient. To confirm that the induction of sic1-9A was effective in inhibiting Clb/Cdc28 kinases, this experiment was repeated with the exception that congenic strains containing wild-type *CDC6* were used instead of *pMET–CDC6* and the cells were analyzed by flow cytometry to see whether they could replicate their DNA. The expression of sic1-9A completely prevented DNA replication (Figure 5d), demonstrating that sic1-9A could effectively inhibit Clb/Cdc28 kinase in our experimental protocol. Thus, Clb/Cdc28 kinases are required for efficient nuclear export of Mcm7–GFP in Cdc6-depleted cells.

Figure 3



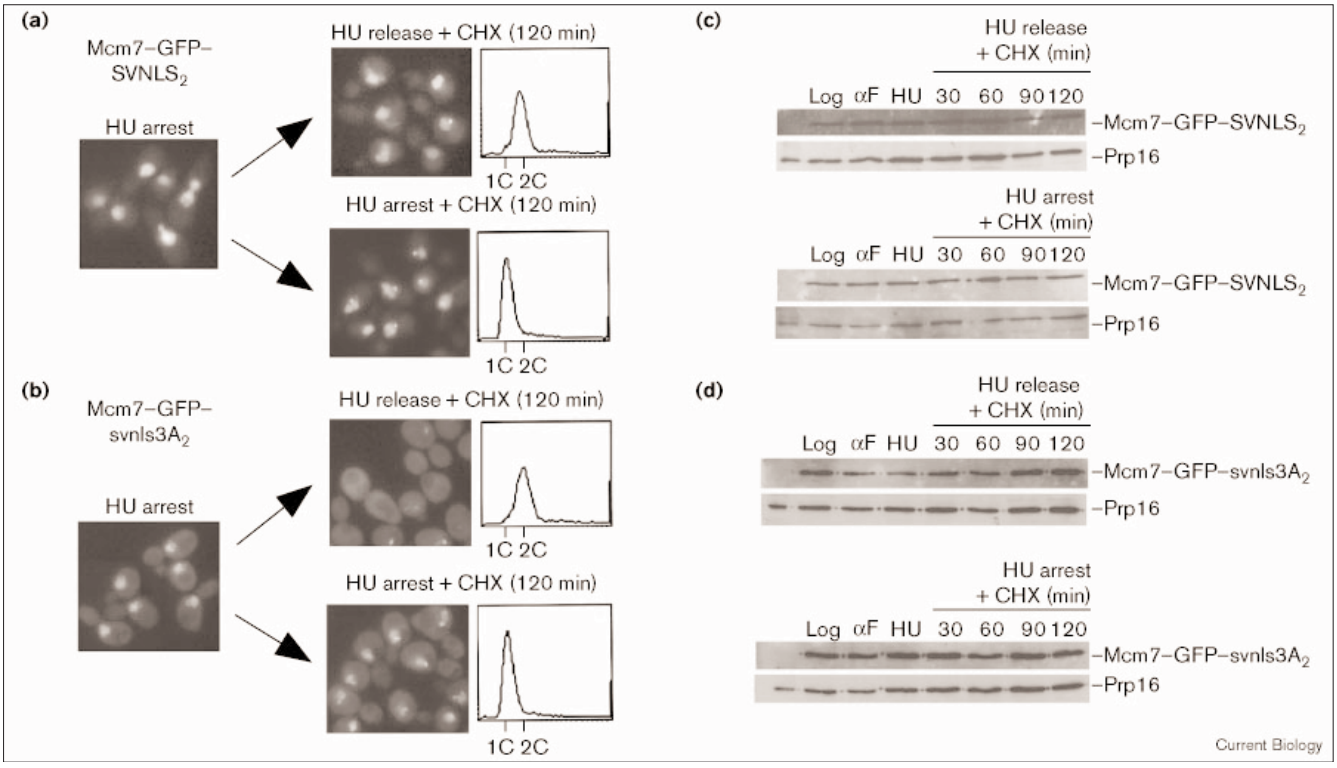
Nuclear accumulation of Mcm7–GFP in G1 phase is independent of Cdc6. *CDC6* (YJL1977, *MAT***a** *CDC6 MCM7–GFP*) and *cdc6-1^{ts}* (YJL1925, *MAT***a** *cdc6-1 MCM7–GFP*) cells growing exponentially in YEPD at 23°C were arrested in G2/M phase by addition of NOC for 3 h, then shifted to 37°C to inactivate *cdc6-1*. After 30 min at 37°C, cells were released from the G2/M phase arrest by filtration and resuspended in prewarmed 37°C YEPD medium containing α factor. Cells were examined by fluorescence microscopy just before release from the G2/M arrest (NOC) and 90 min later when they had fully arrested in G1 phase (α-factor arrest).

The requirement for Clb/Cdc28 kinases to efficiently export Mcm7–GFP from the nucleus does not preclude other cell-cycle signals from sharing a major role in this process. Cdc7/Dbf4 kinase and Cdc45 also act soon after START [1–4] and have been implicated in the regulation of Mcm function. Cdc7/Dbf4 kinase phosphorylates several Mcm proteins *in vitro* [28,29] and interacts genetically with Mcm5 and Mcm2 to trigger initiation [28,30]. Cdc45 interacts genetically and physically with multiple Mcm proteins [23–25,31,32]. Thermal inactivation of Cdc7 or Cdc45, however, did not block the nuclear export of Mcm7–GFP after START in Cdc6-depleted cells (see the Supplementary material). Hence, Cdc7/Dbf4 kinase and Cdc45 do not appear to be required for this export.

Ectopic induction of Clb2 or Clb5 can promote the net nuclear export of Mcm7–GFP

To test whether Clb kinase activity is sufficient to promote the net nuclear export of Mcm proteins, we ectopically expressed Clb2 or Clb5 in G1 phase before START and

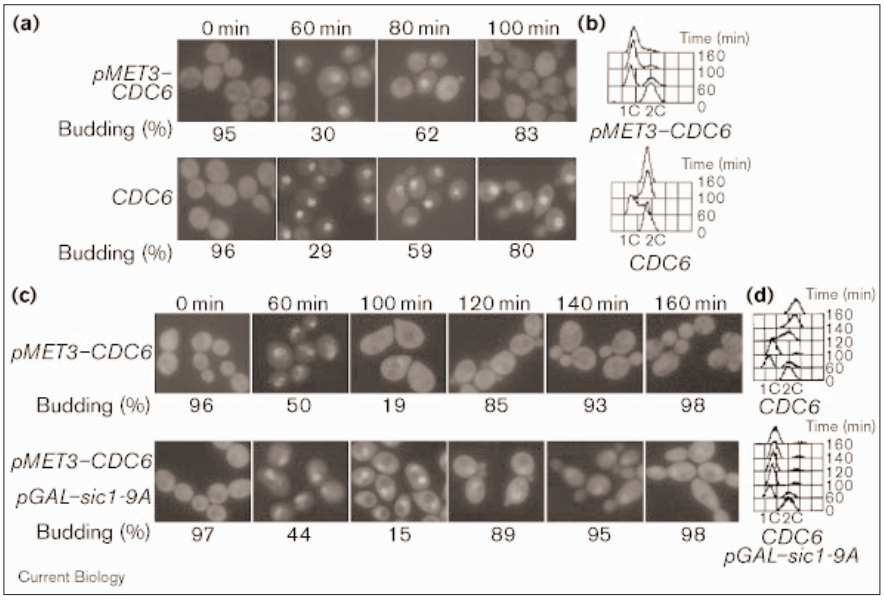
Figure 4



Disappearance of Mcm7–GFP from the nucleus during S phase is not due to Mcm degradation. Cells growing exponentially in YEPD at 30°C were arrested in early S phase by treatment with α factor for 1 h followed by addition of pronase (to inactivate α factor) and HU for 90 min. Half of the cells were released from the early S-phase arrest by filtering the cells and resuspending them in YEPD medium containing cycloheximide to block new protein synthesis. The other half were treated with cycloheximide without releasing them from the arrest. **(a,b)** Samples taken at the early S-phase arrest (HU arrest) and 120 min after treatment with cycloheximide (HU release + CHX, HU arrest + CHX) were analyzed by fluorescence microscopy and flow cytometry. **(c,d)** Samples taken in exponential growth (log), after 1 h α-factor treatment (αF), at the early S-phase arrest, and at 30 min intervals after cycloheximide addition (30–120), were immunoblotted with anti-GFP monoclonal antibodies. Blots were reprobed with anti-Prp16 antibodies as a loading control. Samples from strains containing untagged Mcm7 are shown in the first lane. The yeast strains were YJL1981 (*MAT***a** *MCM7–GFP–SVNLS*$_2$) in (a,c) and YJL1985 (*MAT***a** *MCM7–GFP–svnls3A*$_2$) in (b,d).

determined whether the resulting induction of Clb/Cdc28 kinase activity [33] could cause Mcm7–GFP to disappear from the nucleus (Figure 6a). *MCM7–GFP pMET–CDC6 pGAL–CLB2ΔDB dbf2-2*^{ts} and *MCM7–GFP pMET–CDC6 pGAL–CLB5ΔDB dbf2-2*^{ts} cells growing in medium containing raffinose and lacking methionine were blocked in late anaphase at a *dbf2* arrest and exposed to methionine for 30 minutes to repress Cdc6 expression. Effective depletion of Cdc6 was confirmed by releasing a portion of the cells from the arrest and showing by flow cytometry that the cells fail to replicate their DNA in the next cell cycle (data not shown). The remainder of the cells was released into medium containing α factor, which traps them in the following G1 phase. After 60 minutes, when most of the cells were in G1 phase and had accumulated Mcm7–GFP in their nuclei (Figure 6a, 0 minutes), galactose was added to induce Clb2$^{\Delta db}$ or Cbl5$^{\Delta db}$ (stabilized forms of Clb2 and Clb5 lacking their amino-terminal destruction boxes [33,34]). Loss of GFP nuclear fluorescence could be seen in approximately 20% of cells (n = 100) by 120 minutes and nearly 100% of cells (n = 100) by 180 minutes (Figure 6a), correlating with the increase in Clb levels (Figure 6b). If galactose was not added so that Clb2$^{\Delta db}$ or Clb5$^{\Delta db}$ were not induced, Mcm7–GFP was strongly retained in the nucleus (data not shown). Moreover, control *pMET–CDC6 pGAL MCM7–GFP dbf2-2*^{ts} cells that do not induce any stabilized Clb also retained Mcm7–GFP in the nucleus (Figure 6a). We conclude that ectopic activation of Clb2/Cdc28 or Clb5/Cdc28 kinase in G1 phase can induce the net nuclear export of Mcm proteins. It is likely that the resulting nuclear exclusion of Mcm proteins contributes to the inhibition of pre-RC formation that we previously observed when Clb2$^{\Delta db}$ was ectopically induced before START [9]. In summary, the data from both ectopic and endogenous activation of ClbCdc28 kinases indicate that, in the absence of Cdc6, these kinases are both necessary and sufficient for the efficient export of Mcm proteins from the nucleus.

Figure 5



Clb/Cdc28 kinases are necessary for efficient net nuclear export of Mcm7–GFP in Cdc6-depleted cells. **(a,b)** The congenic strains YJL1945 (*MAT***a** *dbf2-2 pMET–CDC6 MCM7–GFP*) and YJL1937 (*MAT***a** *dbf2-2 CDC6 MCM7–GFP*) were grown to exponential phase in SDC–Met (see Materials and methods) at 23°C and arrested in late anaphase by incubation at 37°C for 2 h. The arrested cells were then filtered and resuspended in prewarmed (37°C) YEPD medium containing methionine to repress *pMET–CDC6* transcription. After 30 min, the cells were released into the next cell cycle at 23°C and split into two cultures (t = 0 min). (a) HU was added to one culture to prevent DNA replication and live cells were examined by fluorescence microscopy at the indicated times. NOC was also added to prevent any inappropriate mitosis in the next cell cycle arising from Cdc6 depletion [39]. (b) NOC was added to the second culture and samples were analyzed at the indicated times by flow cytometry. **(c,d)** Cells were arrested in late anaphase, treated with methionine to repress *pMET–CDC6*, and released into the next cycle (t = 0 min) as described above except the medium contained raffinose instead of dextrose. At 0 min, α factor was added to transiently block cells before START. After 10 min, galactose was added to induce sic1-9A. After 60 min, pronase was added to degrade the α factor, allowing cells to proceed through START, and NOC was added to block any inappropriate mitosis [39]. Samples were analyzed at the indicated times by fluorescence microscopy or flow cytometry. (c) Fluorescence micrographs and the percentage of budding of the congenic *pMET–CDC6* strains YJL2962 (*MAT***a** *dbf2-2 pMET–CDC6 MCM7–GFP pGAL*) and YJL2971 (*MAT***a** *dbf2-2 pMET–CDC6 MCM7–GFP pGAL–sic1-9A*). (d) Flow cytometry of the congenic *CDC6* strains YJL2951 (*MAT***a** *dbf2-2 CDC6 MCM7–GFP pGAL*) and YJL2959 (*MAT***a** *dbf2-2 CDC6 MCM7–GFP pGAL–sic1-9A*).

Clb/Cdc28 kinase activity is not sufficient to induce Mcm disappearance from the nucleus

The data implicating Clb/Cdc28 kinases in the net nuclear export of Mcm7–GFP were obtained in the absence of Cdc6 to remove any potential influence of chromatin association on this export. When these same studies were repeated in the presence of Cdc6 by using congenic *CDC6* strains, no nuclear export of Mcm7–GFP was observed (Figures 5a and 6c). Hence, when Cdc6 functions normally, activation of Clb/Cdc28 kinases is not sufficient to induce net nuclear export of Mcm7–GFP; passage through S phase is now also required (Figure 1c). The simplest interpretation of these data is that chromatin association promoted by Cdc6 makes Mcm proteins refractory to the nuclear export induced by Clb/Cdc28 kinases, and S-phase progression (and possibly DNA replication) is required to dissociate Mcm proteins from chromatin before they can be exported.

Regulation of Mcm localization is not essential for the prevention of re-initiation

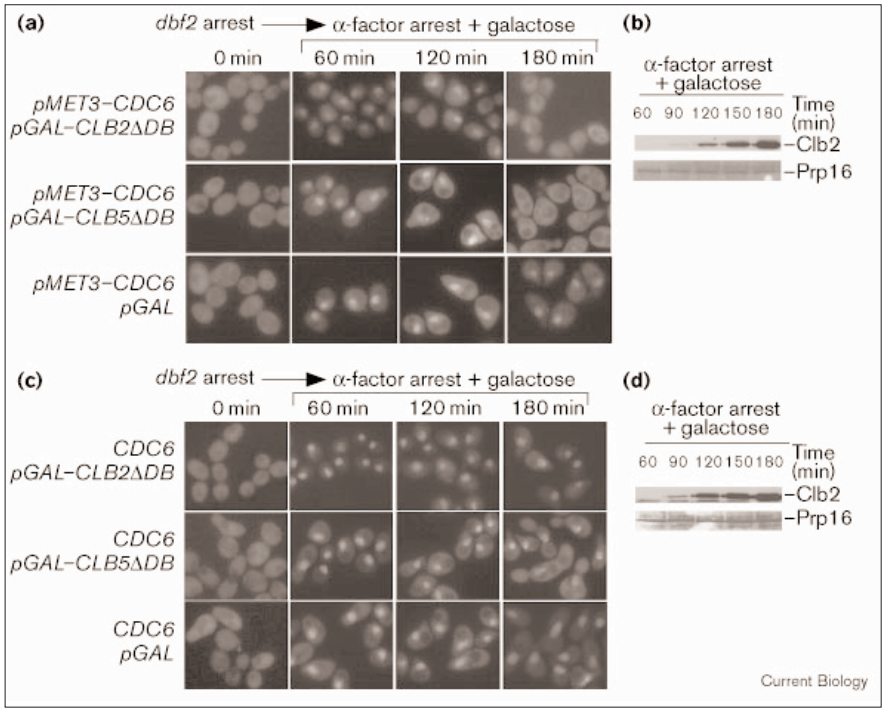
Exclusion of Mcm proteins from the nucleus during S, G2 and M phase may be sufficient to inhibit re-initiation of DNA replication during the cell cycle. It is unlikely, however, to be the sole mechanism for preventing re-initiation, given the need to strictly enforce this block at hundreds of origins in the budding yeast genome. Consistent with this notion, *MCM7–GPF–SVNLS*$_2$ cells, in which all six Mcm proteins are constitutively nuclear (Figure 2c), showed no signs of re-replicating. The cells displayed a normal distribution of 1C and 2C DNA content during exponential growth and maintained a stable 2C DNA content (matching the DNA content of *MCM7–GFP* control cells) when arrested at the G2–M boundary with NOC (Figure 2d). Moreover, these cells divided and maintained plasmids at wild-type rates (data not shown), indicating that the initiation of DNA replication was normal in these cells. We propose that the exclusion of Mcm proteins from the nucleus is only one of several overlapping mechanisms preventing re-initiation within a cell cycle.

Discussion

Cell-cycle regulation of Mcm localization in budding yeast

We have shown here that the subcellular distribution of budding yeast Mcm2, Mcm3, Mcm4, Mcm6 and Mcm7 is regulated during the cell cycle as illustrated in Figure 1e. Our data confirm and extend previous immunofluorescence analyses of Mcm2, Mcm3, Mcm5 and Mcm7 localization in fixed cells [15–17] and finally demonstrate that the localization of all six Mcm proteins shares the same

Figure 6



Ectopic Clb2$^{\Delta db}$ and Clb5$^{\Delta db}$ induction before START can induce net nuclear export of Mcm7–GFP in Cdc6-depleted cells. **(a,b)** The congenic strains YJL1939 (*MAT***a** *dbf2-2 pMET–CDC6 MCM7–GFP pGAL–CLB2ΔDB*), YJL2964 (*MAT***a** *dbf2-2 pMET–CDC6 MCM7–GFP pGAL–CLB5ΔDB*) and YJL2962 (*MAT***a** *dbf2-2 pMET–CDC6 MCM7–GFP pGAL*) were grown to exponential phase in SRC–Met (see Materials and methods) at 23°C and arrested in late anaphase by incubation at 37°C for 2 h. The arrested cells were then filtered and resuspended in prewarmed (37°C) YEPR medium containing additional methionine to repress *pMET–CDC6* transcription. After 30 min (t = 0 min), the cells were released into the next cell cycle at 23°C and α factor was added to re-arrest them before START of the next cell cycle. HU was also added to mirror the treatment in **(c,d)**, where it was added to prevent S-phase progression. After 60 min, the cells had progressed into G1 phase, and galactose was added to induce Clb2$^{\Delta db}$ or Clb5$^{\Delta db}$. Samples were taken at the indicated times for (a) fluorescence microscopy and (b) in the case of YJL1939, for immunoblot analysis of total Clb2 levels (with Prp16 serving as a loading control). (c,d) The congenic strains YJL1935 (*MAT***a** *dbf2-2 CDC6 MCM7–GFP pGAL–CLB2ΔDB*), YJL2954 (*MAT***a** *dbf2-2 CDC6 MCM7–GFP pGAL–CLB5ΔDB*) and YJL2951 (*MAT***a** *dbf2-2 CDC6 MCM7–GFP pGAL*) were treated as described in (a,b). HU was added to prevent cells that were inducing Clb2$^{\Delta db}$ and Clb5$^{\Delta db}$ from replicating their DNA [33]. Samples were taken at the indicated times for (c) fluorescence microscopy and (d) in the case of YJL1935, for immunoblot analysis of total Clb2 levels.

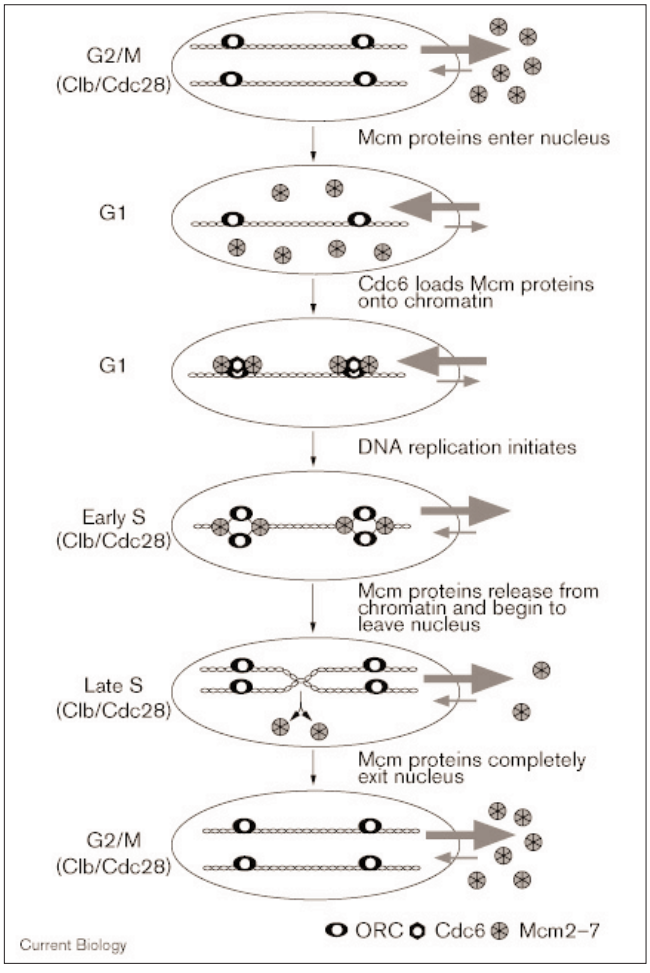
cell-cycle regulation. In addition, we have shown that these proteins are actually exported from the nucleus and that this export results in nuclear exclusion of Mcm proteins in G2 and M phase. This exclusion makes the control of Mcm localization particularly compelling as a means of regulating initiation (see below). Our data also suggest that the six Mcm proteins colocalize as a complex. They not only share identical localization during the cell cycle but are also dependent on each other for this localization. The simplest interpretation of these findings is that the Mcm proteins are transported through the nuclear pore as a complex comprising all six Mcm proteins. Our results, however, do not rule out the possibility that the Mcm proteins are transported across the nuclear envelope as individual proteins or subcomplexes and then assembled into a full complex on either side. Nonetheless, because Mcm proteins colocalize, we believe that our more detailed analysis of Mcm7 localization can be extended to the other Mcm proteins.

Regulation of Mcm localization by Clb/Cdc28 kinases and chromatin association

Our data suggest that cell-cycle regulation of Mcm localization is driven by Clb kinase modulation of Mcm nucleocytoplasmic transport. In the absence of Cdc6 function, induction of these kinases is both necessary and sufficient to induce efficient net export of Mcm proteins from the nucleus. Moreover, a drop in Clb kinase activity at the end of mitosis (after the *dbf2* arrest point) appears to be necessary for the rapid entry of Mcm proteins into G1 nuclei.

While our manuscript was undergoing review, it was reported that Cln kinases promote the nuclear exit of Mcm4, based on the observation that Clb5 and Clb6 are not essential for Mcm4 nuclear exit during the passage through START [35]. We also observed some Clb-kinase-independent nuclear export of Mcm proteins during the passage through START (Figure 5c), but this export was both slow and incomplete, as evidenced by the persistence of Mcm protein in the nucleus (which we could see in live but not fixed cells). Although it is tempting to speculate that Cln/Cdc28 kinases induce this export because of a weak overlap in substrate specificity, a role for Cdc7/Dbf4 or Cdc45 cannot be ruled out as these components have not been inactivated in the absence of Clb kinase activity. More importantly, the inefficiency of this Clb-kinase-independent export suggests that it may not be potent enough to exclude Mcm proteins from the nucleus and may not be a major determinant in the block to re-initiation. This view is consistent with

Figure 7



Model for regulation of Mcm localization during the cell cycle. See text for detailed explanation. In accordance with the work of Aparicio *et al.* [7], we have depicted Mcm proteins as loading onto origins before initiation, shifting their association to replication forks after initiation, and dissociating from chromatin when forks collide and disassemble. Although the model shows both import (left-pointing arrows) and export (right-pointing arrows) rates being regulated by Clb/Cdc28 kinases, a shift in net transport direction is possible with changes in only one of these rates.

the previously published observation that Clb kinase activity is required for the block to pre-RC assembly induced after START [8,10]. It is also consistent with the observation that the Clb-kinase-independent (but not the Clb-kinase-dependent) export of Mcm4 can be overridden by expression of Cdc6 [35], which presumably counteracts the export by loading Mcm4 in the nucleus onto chromatin. Hence, we believe that Clb kinases are required for the efficient and effective exclusion of Mcm proteins from the nucleus.

Our experiments do not address the mechanism by which Clb kinases promote Mcm nuclear exit. We do not know whether the kinases phosphorylate the Mcm proteins and/or other proteins that mediate Mcm transport, nor do we know whether Clb kinases inhibit Mcm import rates, stimulate Mcm export rates, or both. Identification of the transport signals and transport receptors for the Mcm complex will help address these questions in the future.

Although Clb/Cdc28 kinases can promote net nuclear export of Mcm proteins, once Cdc6 loads Mcm proteins onto chromatin, activation of these kinases is not sufficient for this export to occur; progression through S phase is also required. Previous work has shown that the association of Mcm proteins with chromatin is regulated during the cell cycle in budding yeast [7,8,26] and other eukaryotes (reviewed in [14]). Mcm proteins are tightly associated with chromatin in G1 phase, gradually dissociate during S phase, and remain unassociated in G2 and M phase. These observations raise the possibility that chromatin association prevents Mcm nuclear export and that Mcm dissociation from chromatin during S phase is required before Clb/Cdc28 kinases can expel Mcm proteins from the nucleus.

We propose the following model for the regulation of Mcm localization during the cell cycle (Figure 7). In G2 and M phases, Clb/Cdc28 kinases maintain Mcm proteins in the cytoplasm by promoting their net nuclear export. At the end of mitosis, the precipitous drop in Clb/Cdc28 kinase activity allows the Mcm proteins to revert to a default state of net nuclear import. Once the Mcm proteins enter the nucleus, Cdc6 loads them onto chromatin in preparation for replication initiation. Although this chromatin association is not necessary for the accumulation of Mcm proteins in the nucleus, it does affect their subsequent relocalization to the cytoplasm by making them refractory to nuclear export. Consequently, Mcm proteins are not exported as soon as Clb/Cdc28 kinases are activated in late G1 phase but instead remain on chromatin, poised to initiate DNA replication. After initiation, Mcm proteins are still tightly associated with chromatin (possibly at replication forks [7]) and are retained in the nucleus. Only during the course of S phase do Mcm proteins gradually dissociate from chromatin and become susceptible to nuclear export. Hence, both passage through S phase and Clb/Cdc28 kinase activation are normally required for nuclear exclusion of Mcm proteins.

Role of regulating Mcm localization in the block to re-initiation

Clb/Cdc28 kinases play a pivotal role in the block to re-initiation. We and others have shown that these kinases can block re-initiation by preventing the re-assembly of pre-RCs at origins that have already fired [8–10]. Exactly how these kinases prevent pre-RC assembly is not understood. In this report we present one likely mechanism — the export and exclusion of Mcm proteins

from G2 and M phase nuclei. Net nuclear export of Mcm proteins could also discourage re-initiation during S phase, despite the persistence of Mcm proteins in S-phase nuclei. Presumably this persistent population is tightly associated with chromatin, while the free nucleoplasmic pool of Mcm proteins (the pool likely involved in pre-RC assembly) is significantly reduced by Clb kinase-induced export.

Multiple overlapping mechanisms are used to ensure reliable, complete and decisive execution of many key cell-cycle events. For example, both Clb destruction and Sic1-mediated kinase inhibition ensure the exit from mitosis in budding yeast [36,37]. Although neither mechanism is essential, as either can drive mitotic exit in the absence of the other, each is considered a crucial component of this pathway. It also makes sense for Clb/Cdc28 kinases to use multiple overlapping mechanisms to guarantee the block to re-initiation, as this block must be strictly enforced at hundreds of origins scattered throughout the budding yeast genome. In support of this idea, we have shown that constitutive nuclear localization of all six Mcm proteins does not result in any detectable re-replication, indicating that the regulation of Mcm localization is not the sole mechanism for preventing re-initiation.

Another likely mechanism is the regulation of Cdc6 levels. Cdc6 is an unstable protein whose levels peak during G1 phase when pre-RC assembly occurs, and plummet in S, G2 and M phase when pre-RC reassembly is prevented [38–40]. Clb/Cdc28 kinases maintain these low levels in part by negatively regulating *CDC6* transcription [39]. The potential importance of this regulation for the block to re-initiation is highlighted by the observation that constitutive overexpression of the Cdc6 homolog Cdc18 results in re-replication in *S. pombe* [11,12]. In *S. cerevisiae*, however, constitutive overexpression of a stabilized Cdc6 protein does not lead to re-replication (C.S. Detweiler and J.J.L., unpublished data; [8,38]). We suspect that the regulation of Mcm localization helps to prevent re-initiation in these cells. Interestingly, we do not observe any re-replication when both Mcm localization and Cdc6 expression are deregulated (V.Q.N. and J.J.L., unpublished data). This suggests that additional mechanisms, possibly acting on other initiator proteins like ORC, block re-initiation in budding yeast.

Similar overlapping regulatory mechanisms probably block re-initiation in other eukaryotes, although the way in which these mechanisms are implemented may differ. For example, although Mcm proteins are constitutively nuclear in most eukaryotes (reviewed in [14]), Cdc6 is constitutively expressed in human cells and resembles budding yeast Mcm proteins in its cell cycle localization [41–44]. Hence, the general strategy of excluding initiator proteins from the nucleus to prevent re-initiation may be conserved among eukaryotes.

Conclusions

We investigated one mechanism by which CDKs can inhibit re-initiation of DNA replication within a cell cycle. Our results suggest that all six members of the Mcm2–7 family of initiator proteins in budding yeast accumulate in the nucleus in G1 phase, are gradually exported from the nucleus in S phase, and are finally excluded from the nucleus in G2/M phase (Figure 1e). Mcm proteins are dependent on each other for this regulated localization, consistent with the proteins colocalizing as a complex. Clb/Cdc28 kinases play a major role in this regulation by promoting the efficient nuclear export of Mcm2–7. Although this nuclear exclusion of Mcm proteins may be sufficient to prevent re-initiation, it is not necessary, suggesting that Clb/Cdc28 kinases have additional overlapping mechanisms to ensure that origins fire at most once per cell cycle.

Materials and methods

Yeast media, growth and arrest

YEP medium and synthetic complete medium lacking methionine [3] were supplemented with 2% dextrose (YEPD; SDC–Met), or 2% raffinose (YEPR; SRC–Met). The *GAL1* promoter (*pGAL*) was induced by addition of 2% galactose and the *MET* promoter (*pMET*) was repressed by the addition of 2 mM methionine.

To arrest cells, α factor was added to 50 ng/ml (all strains were *bar1*), hydroxyurea was added to 0.2 M, and nocodazole was added to 10 μg/ml. These cell-cycle blocks were released by filtering the cells, washing them three times with an equal volume of resuspension medium pre-warmed to the appropriate temperature, then resuspending them in the appropriate medium. In some cases, α-factor-arrested cells were released by the addition of pronase to 100 μg/ml to degrade the α factor. To inhibit protein synthesis during S phase, cycloheximide was added to 100 μg/ml. This inhibition blocks entry into S phase and M phase but not progression through S phase.

Flow cytometry, immunoblot analysis and fluorescence microscopy

Flow cytometry was performed as described in [39]. Immunoblot analysis was performed as described in [3]. Blots were probed with B34 monoclonal anti-GFP antibodies at a 1:40 dilution (a gift of E. O'Shea, University of California, San Francisco), anti-Prp16 antibodies at a 1:5000 dilution (a gift of C. Guthrie, University of California, San Francisco), or anti-Clb2 antibodies at a 1:1200 dilution (a gift of D. Morgan, University of California, San Francisco). For fluorescence microscopy of live cells, cells were rapidly washed with PBS and photographed within 3 min of sampling using a Leica DMLB fluorescence microscope with a 100 × PL Fluotar oil immersion objective. Images were acquired with an Optronics DEI-750 CCD camera using the Scion Image Software program. Panels were assembled with Adobe Photoshop software.

Supplementary material

Supplementary material including additional methodological detail and two figures showing that nuclear accumulation of Mcm7–GFP is independent of Cdc45, and that Cdc7 and Cdc45 are not necessary for Mcm7–GFP export in Cdc6-depleted cells is available at http://current-biology.com/supmat/supmatin.htm.

Acknowledgements

We thank Erin O'Shea, Carol Gross, Elizabeth Blackburn, Catherine Takizawa, Douglas Crawford, Alexander Johnson and Anita Sil for their comments on the manuscript. This work was supported by the Searle Scholars Program, the Rita Allen Foundation, the Markey Charitable Trust Fund and the American Cancer Society (RPG-99-169-01-CCG).

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