G1-phase and B-type cyclins exclude the DNA-replication factor Mcm4 from the nucleus

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Cyclin-dependent kinases (CDKs) activate the firing of replication origins during the S phase of the cell cycle. They also block re-initiation of DNA replication within a single cell cycle, by preventing the assembly of prereplicative complexes at origins. We show here that, in budding yeast, CDKs exclude the essential prereplicative-complex component Mcm4 from the nucleus. Although origin firing can be triggered by the B-type cyclins only, both G1-phase and B-type cyclins cause exit of Mcm4 from the nucleus. These results suggest that G1 cyclins may diminish the cell's capacity to assemble prereplicative complexes before B-type cyclins trigger origin firing during S phase.

In most eukaryotic cell cycles, the chromosomal DNA is copied just once. Replication begins at multiple sites termed origins, and in the budding yeast *Saccharomyces cerevisiae* the DNA sequences defining origins are well characterized. The origin-recognition complex (ORC) is bound to origins throughout the cell cycle[1–6] and acts as a scaffold around which a larger prereplicative complex (pre-RC) is assembled at the end of mitosis[3]. The assembly of pre-RCs requires the Cdc6 protein[7] which is present in the cell only during early G1 phase[8,9], and involves the loading around origins of a family of six minichromosome-maintenance (Mcm) proteins[5,6] (reviewed in refs 10, 11). Mcm proteins have an important role in origin firing[12], and are displaced from chromatin during S phase as DNA replication proceeds[5,13–16]. This regulated chromatin binding of Mcm proteins ensures that each origin of DNA replication fires just once in each cell cycle. CDKs trigger origin firing[17], at the same time blocking the reformation of new pre-RCs[18,19]. Evidence from both fission and budding yeasts indicates that cyclin-B-associated CDKs perform these roles[18,20–23], as well as triggering mitosis. In human cells, mitotic CDK activity has also been shown to be important for the prevention of rereplication[24].

The substrates of CDK-mediated inhibition of pre-RC formation are poorly characterized, although both Cdc6 and its fission yeast homologue Cdc18 are likely to be major targets[14,25–28]. Immunofluorescence studies of the localization of *S. cerevisiae* Mcm proteins indicated that they are predominantly cytoplasmic after S phase[12,29,30], suggesting an extra mechanism by which CDKs could potentially block the reformation of pre-RCs. Analysis of Mcm localization by subcellular fractionation initially supported this view[12,29,30], but a subsequent study indicated that Mcm proteins may in fact be nuclear throughout the *S. cerevisiae* cell cycle[15] despite the apparent changes seen by immunofluorescence. Moreover, the nuclear localization of Mcm proteins does not show cell-cycle variations in the cells of other eukaryotes (reviewed in ref. 10).

Here we show, using live budding-yeast cells, that Mcm4 is nuclear only before S phase and is excluded from the nucleus later in the cell cycle. Nuclear localization of Mcm4 is regulated by CDK activity in a manner that is independent of Cdc6-dependent chromatin binding, identifying a second mechanism likely to contribute to the cell-cycle control of pre-RC formation. Surprisingly, we find that nuclear exit of Mcm4 is promoted by both G1 cyclins (Clns) and the B-type cyclins (Clbs) that activate CDK activity during S phase and mitosis. This indicates that G1 cyclins may reduce the efficiency of pre-RC formation before B-cyclins trigger origin firing, thereby ensuring that each origin fires just once in each round of the cell cycle.

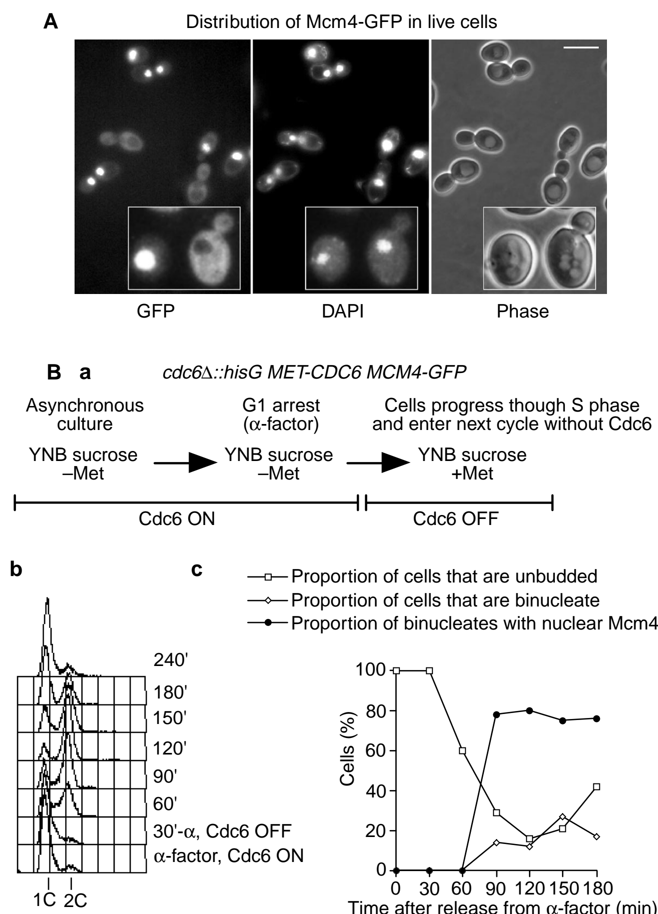

Figure 1 **Mcm4 localization is cell cycle regulated. A**, Live cells of strain YKL142 were treated with the DNA-binding dye DAPI, and the distribution of Mcm4–GFP was determined by fluorescence microscopy. Scale bar represents 10 μm. **B**, Mcm4–GFP accumulates in the nuclei at the end of mitosis, even in the absence of Cdc6. **a**, Experimental procedure; see text for details. **b**, DNA content. **c**, Budding index and the proportion of binucleate cells with nuclear Mcm4–GFP. α, α-factor; Met, methionine; YNB sucrose, YNB medium plus sucrose.

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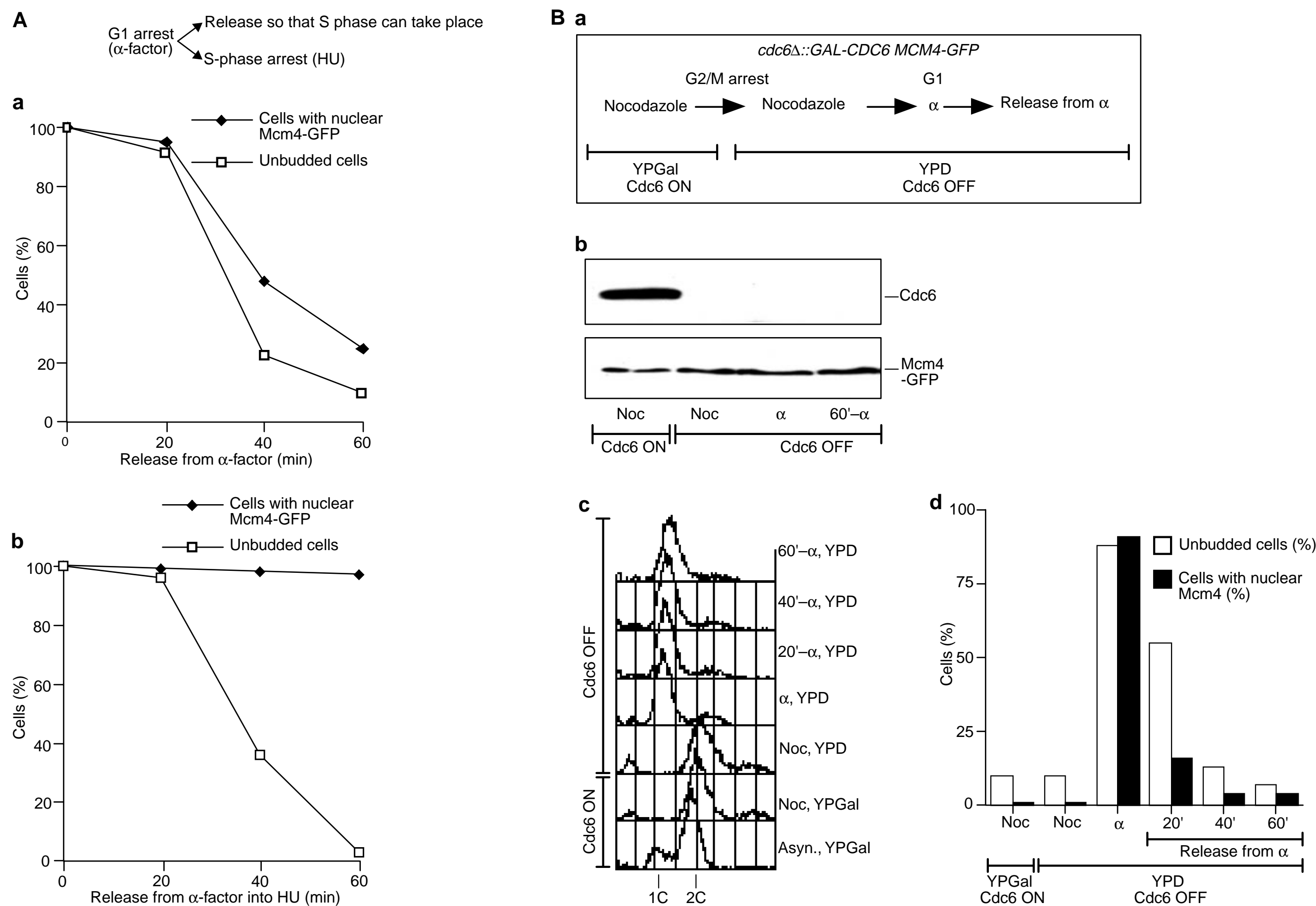

Figure 2 **Exit of chromatin-bound Mcm4 from the nucleus is dependent upon S-phase progression. A**, An asynchronous culture of an *MCM4–GFP* haploid strain was arrested in G1 by treatment with α-factor for 3h at 24 °C, before being released into fresh medium with or without hydroxyurea (HU). Samples were taken every 20 min and either processed for flow cytometry to determine DNA content (see Supplementary Information), or used to measure budding index and the proportion of cells with nuclear Mcm4 by fluorescence microscopy (**a**, **b**). **B**, Nuclear exit of Mcm4 in the absence of Cdc6 function is independent of S-phase progression. **a**, Experimental procedure; see text for details. **b**, Immunoblot analysis of Cdc6 and Mcm4–GFP levels during the course of the experiment. **c**, DNA content. **d**, Budding index and proportion of cells with nuclear Mcm4. Asyn., asynchronous culture; Noc, nocodazole; YPGal, YP medium plus galactose.

Results

Nuclear localization of Mcm4 in live cells. We made haploid and diploid strains in which the *CDC54* locus is altered so that green fluorescent protein (GFP) is expressed as a fusion to the carboxy terminus of Cdc54/Mcm4. The Mcm4–GFP fusion protein is functional and is expressed from the *CDC54/MCM4* promoter as the only copy in the cell. Immunoblot analysis shows that Mcm4–GFP protein levels remain constant at different stages of the cell cycle (see below).

The subcellular localization of Mcm4 in live cells changes dramatically during the cell cycle (Fig. 1A and see Supplementary Information). Mcm4 is almost exclusively nuclear in unbudded G1 cells but cytoplasmic in large-budded cells before anaphase. It appears that Mcm4 is excluded from the nucleus at this stage of the cell cycle (Fig. 1A inset and see Supplementary Information). After nuclear division, Mcm4 returns to the nucleus. Time-lapse analysis of individual live cells confirms that Mcm4 leaves the nucleus around the time of budding and only reaccumulates there at the end of mitosis (see Supplementary Information). It is, therefore, likely that nuclear exclusion of Mcm4 contributes to the inhibition of pre-RC formation from the end of S phase until the end of mitosis.

Mcm4 nuclear accumulation after mitosis does not require Cdc6. Mcm4 accumulates within the nucleus at the end of mitosis (Fig. 1A and see Supplementary Information), around the same time as Mcm proteins become bound to chromatin[13,14,16]. There are two possible explanations for this change in Mcm4 localization. The first is that Mcm proteins can shuttle in and out of the nucleus throughout the cell cycle, so that their nuclear accumulation from late mitosis until S phase results from their Cdc6-dependent binding to chromatin during that period. In this case, changes in Mcm localization would not contribute directly to the regulation of pre-RC assembly. Alternatively, nuclear accumulation of Mcm proteins could be regulated independently of Cdc6-dependent chromatin binding by changes in the rates of import or export (or both). These possibilities can be distinguished by determining whether nuclear accumulation of Mcm4 at the end of mitosis depends upon Cdc6.

We arrested a strain in which *CDC6* expression is controlled by the methionine-repressible *MET3* promoter in G1 phase by using α-factor (Fig. 1B, a), which prevents activation of the Cln/G1-cyclin-associated forms of the kinase Cdc28. We then washed cells into fresh medium to induce entry into S phase, and included methionine in the medium to repress expression of *CDC6* from this point onwards. New synthesis of Cdc6 is not required for S phase to occur, as the Mcms are already assembled into pre-RCs in α-factor-blocked cells[5,6,13,15]. After passing normally through S phase, mitosis and cytokinesis, absence of *CDC6* expression prevents S phase in the subsequent cell cycle, causing cells to arrest with an unreplicated 1C DNA content (Fig. 1B, b), presumably because of a failure to form pre-RCs. Mcm4 still accumulates within the nuclei of binucleate cells completing mitosis without Cdc6 (Fig. 1B, c), just as it does

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in wild-type binucleate cells expressing CDC6 (Fig. 1A and see Supplementary Information). Nuclear accumulation of Mcm proteins at the end of mitosis is, therefore, independent of Cdc6-mediated chromatin binding. This indicates that regulation of Mcm localization may indeed represent a second mechanism blocking pre-RC assembly late in the cell cycle.

Mcm4 nuclear exit does not require S-phase progression. To determine how the nuclear exit of Mcm4 is regulated as a cell passes from G1 into S phase, we studied a synchronous culture of cells released from G1 arrest (Fig. 2A). Exit of Mcm4 from the nucleus occurred slightly later than bud emergence (Fig. 2A, a) and correlated with progression through S phase (see Supplementary Information). As Mcm proteins become displaced from chromatin only as DNA replication proceeds[5,13–16], nuclear exit of Mcm4 should be dependent upon progression through S phase. Indeed, Mcm4 remains nuclear if the elongation step of DNA replication is blocked by hydroxyurea upon release from G1 arrest (Fig. 2A, b and see Supplementary Information).

It is possible that the signal causing exit of Mcm4 from the nucleus is itself dependent upon progression through S phase. Alternatively, Mcm4 exit could be activated during late G1 phase, independently of DNA replication. To distinguish between these possibilities, we examined cells that had passed through mitosis in the absence of *CDC6* expression before being arrested in the subsequent G1 phase (Fig. 2B, a–c); Mcm4 was nuclear at this stage (Fig. 2B, d). We then released the cells from G1 arrest, to see whether Mcm4 would leave the nucleus. Cells were unable to enter S phase (Fig. 2B, c), showing that Cdc6 had been efficiently inactivated, but nuclear exit of Mcm4 occurred with slightly faster kinetics than those of bud emergence (Fig. 2B, d), even if hydroxyurea were included in the medium (see Supplementary Information). This shows that the signal promoting nuclear exit of Mcm4 is generated independently of S phase, though it does require progression through G1 phase. In a wild-type cell, Cdc6-dependent association of Mcm proteins with chromatin delays their exit from the nucleus until displacement from chromatin occurs during S phase.

B-cyclins prevent nuclear accumulation of Mcm4. In late G1 phase, Cln–Cdc28 kinases cause inactivation of the anaphase-promoting complex (APC)[31] and degradation of the Clb–kinase inhibitor Sic1 (refs 32, 33), thereby allowing activation of Clb–Cdc28 kinases. The latter remain active from S phase until the end of mitosis, and Mcm4 is excluded from the nucleus during the same period of the cell cycle. This indicates that Clb–Cdc28 kinases may prevent nuclear accumulation of Mcm proteins. Overexpression of *SIC1* in G2/M-arrested cells causes inactivation of Clb–Cdc28 kinases and

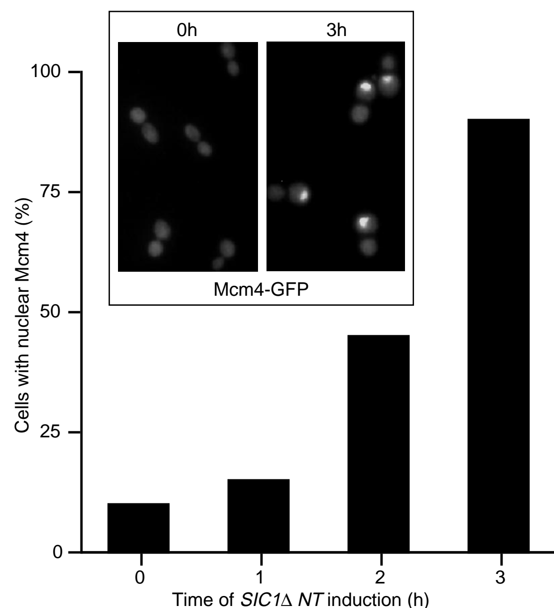

Figure 3 **B-cyclin-associated Cdc28 kinase activity blocks nuclear accumulation of Mcm4 in G2/M-arrested cells.** A *GAL-SIC1ΔNT* strain was blocked in G2/M with nocodazole at 24 °C in YP medium plus raffinose, before addition of galactose to induce expression of Sic1ΔNT, which inhibits the activity of Clb–Cdc28 kinases. The localization of Mcm4–GFP was determined as before.

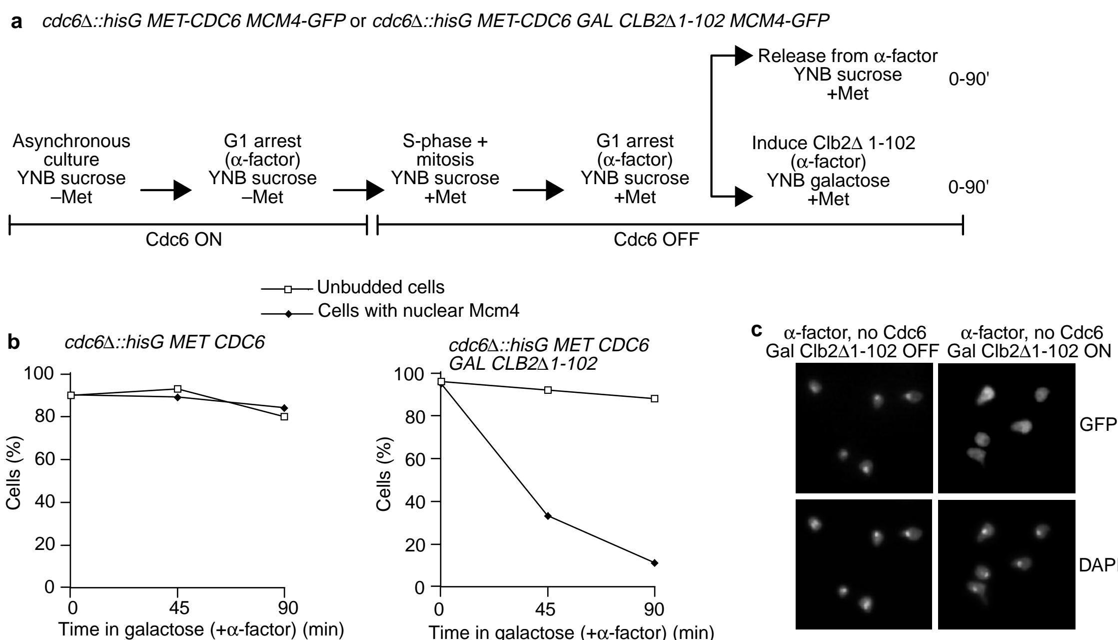

Figure 4 **B-cyclins can promote nuclear exit of Mcm4.** **a**, Experimental protocol; see text for details. **b**, Clb2Δ1-102 promotes exit of Mcm4 from the nucleus without triggering budding. **c**, Micrographs illustrating Mcm4–GFP localization in the presence and absence of *CLB2Δ1-102* expression, in α-factor-blocked cells lacking Cdc6.

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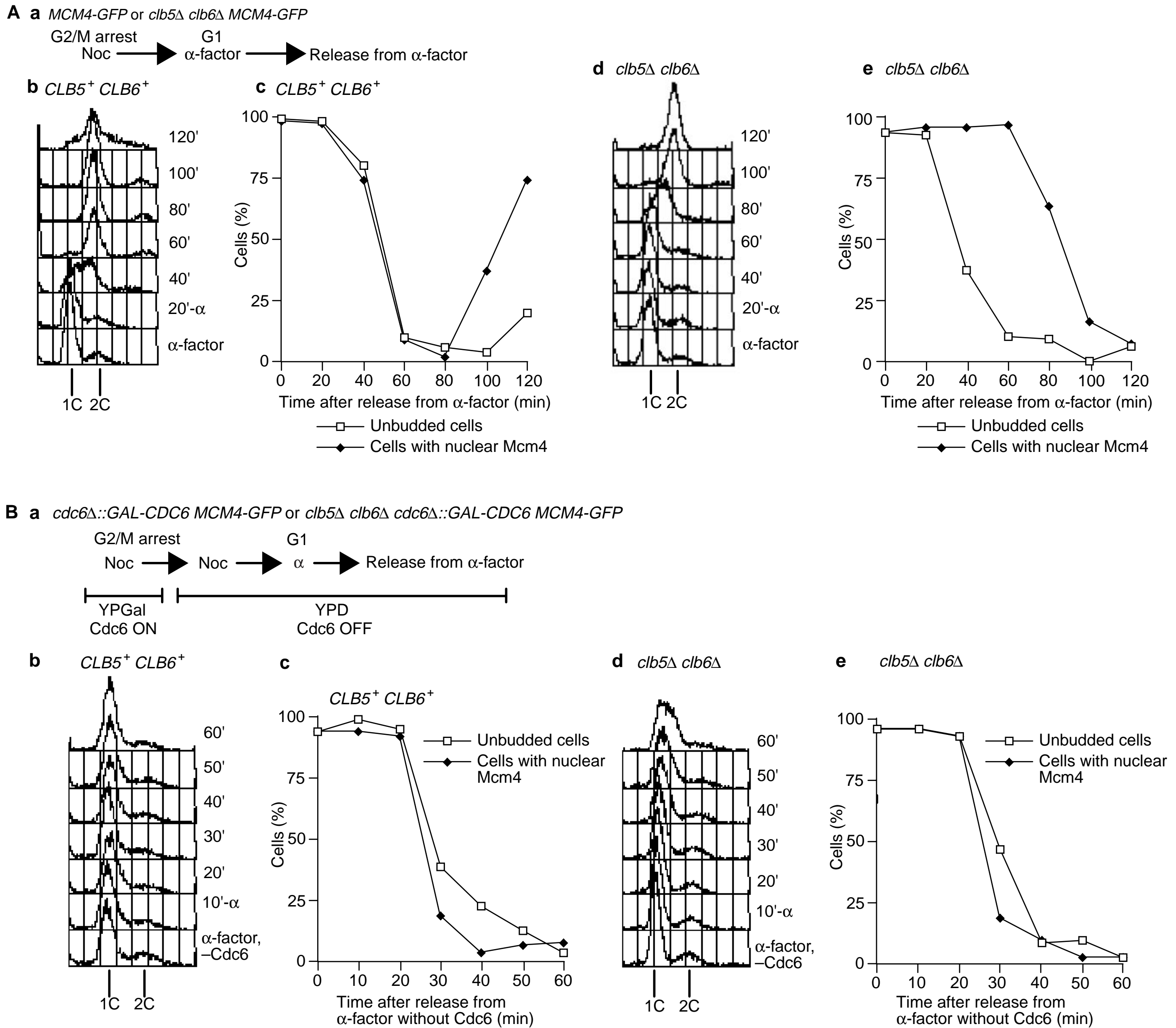

Figure 5 **Nuclear exit of Mcm4 is activated independently of *CLB5,6* expression. A**, In the absence of Clb5 and Clb6, both S phase and nuclear exit of Mcm4 are delayed in cells expressing *CDC6*. **a**, Experimental procedure; see text for details. **b**, **d**, DNA content. **c**, **e**, Budding index and the proportion of cells with nuclear Mcm4–GFP. **B**, Nuclear exit of Mcm4 is not delayed by absence of Clb5 and Clb6 in cells lacking *CDC6*. **a**, Experimental procedure; see text for details. **b**, **d**, DNA content. **c**, **e**, Budding index and the proportion of cells with nuclear Mcm4–GFP.

the reformation of pre-RCs at origins of replication[18]. We have made use of a stabilized version of Sic1 lacking the amino-terminal 50 amino acids[9,34], and find that overexpression of Sic1ΔNT in G2/M-arrested cells is sufficient to cause accumulation of Mcm4 in the nucleus (Fig. 3). This shows that Clb–Cdc28 kinases do indeed inhibit nuclear accumulation of Mcm4 after S phase.

We then asked whether Clb–Cdc28 kinases can trigger nuclear exit of Mcms during G1 phase, by studying the effects of expressing a stable form of Clb2 in α-factor-arrested cells (Fig. 4). Because the APC is still active under such conditions, we used a version of Clb2 (Clb2Δ1–102) that lacks the N-terminal destruction box that normally targets it for APC-mediated degradation[31,35]. Expression of *CLB2Δ1–102* was controlled by the *GAL1-10* promoter, and a strain lacking this construct provided a control. We again used strains with regulatable *CDC6* expression to obtain populations of G1-arrested cells that had passed through mitosis in the absence of Cdc6 (Fig. 4a). Mcm4 was nuclear in G1 (Fig. 4b, 0 min time point), although pre-RC formation and chromatin binding could not occur in the absence of Cdc6. The cultures were then split in two, and one half of each was released from G1 arrest to show that S phase would not take place, confirming that Cdc6 had been inactivated (Fig. 4a and see Supplementary Information). Galactose was added to the other half to induce expression of Clb2Δ1–102 (Fig. 4a and see Supplementary Information). This caused nuclear exit of Mcm4 in the absence of budding and S phase (Fig. 4b, c and see Supplementary Information).

These experiments show that B-cyclins have a crucial role in excluding Mcm4 from the nucleus. Moreover, inhibition of nuclear accumulation of Mcm proteins is likely to be an important reason why pre-RCs cannot form if Cdc6 is expressed after B-cyclin activation in a G1 cell[19].

Mcm4 nuclear exit is activated independently of *CLB5,6* expression. Although Clb–Cdc28 kinases are sufficient to trigger exit of Mcm4 from the nucleus, the experiments described so far do not show that Clbs are necessary for the exit of Mcm4 from the nucleus

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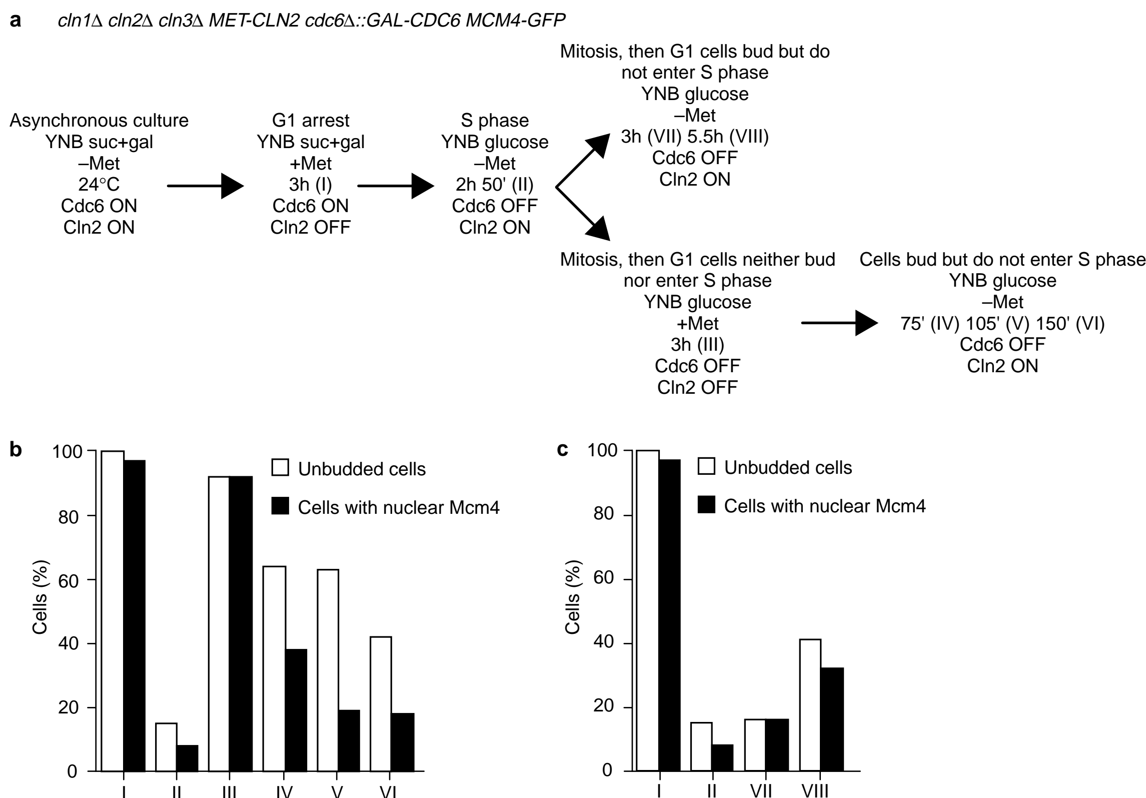

Figure 6 **G1-cyclin-dependent nuclear exit of Mcm4. a**, Experimental protocol. **b**, **c**, Quantification of budding index and the proportion of cells with nuclear Mcm4, at the different stages of the experiment indicated in **a**.

to be activated in late G1 phase. The six *S. cerevisiae* B-type cyclins are expressed in three pairs, firstly Clb5 and Clb6 in late G1, then Clbs 3 and 4 and finally Clb1 and Clb2. If B-cyclin activation is necessary for the nuclear exit of Mcm4, then exit should be delayed in the absence of Clbs 5 and 6, until the later B-cyclins are expressed. As nuclear exit of Mcm4 in a wild-type cell requires its displacement from chromatin, and as this can only occur when B-cyclins trigger origin firing during S phase, both Mcm4 nuclear exit and S phase will be delayed in the absence of Clb5 and Clb6. Thus, to test whether B-cyclins are essential components of the signal that triggers nuclear exit of Mcm4 in late G1, we needed to determine whether exit of Mcm4 from the nucleus is also delayed in a G1 cell that has passed through mitosis in the absence of Cdc6, so that Mcm proteins cannot bind to chromatin.

We first established the duration of time that S phase and the nuclear exit of Mcm4 are delayed by absence of Clbs 5 and 6 in a strain expressing *CDC6*. G1 populations of *CLB5,6*$^+$ and *clb5,6*Δ cells were obtained as described in Fig. 5A, a, and samples were taken every 20 min after release from G1 arrest to analyse DNA content, budding index, and the localization of Mcm4. In the *CLB5,6*$^+$ strain, budding, S phase and nuclear exit of Mcm4 all occurred between 40 and 60 min after release from α-factor (Fig. 5A, b, c). In contrast, although budding was not delayed in *clb5,6*Δ cells, both S phase and exit of Mcm4 from the nucleus took place about 40 min later than in wild-type cells, about 80–100 min after release from G1 arrest (Fig. 5A, d, e). If Clbs are essential to trigger nuclear exit of Mcm proteins, a similar delay should also be observed in the *clb5,6*Δ strain even in the absence of Cdc6.

To test this prediction, we made use of *CLB5,6*$^+$ and *clb5,6*Δ strains expressing *CDC6* under the control of the *GAL1-10* promoter (Fig. 5B, a), and performed a similiar experiment to that shown in Fig. 5A. Cells were grown in galactose-containing medium and blocked in G2/M; *CDC6* expression was then repressed and cells were allowed to pass through mitosis and were arrested in G1 with α-factor. Upon release from α-factor, both strains budded between 20 and 40 min later (Fig. 5B, c, e), but in the absence of Cdc6 neither strain entered S phase (Fig. 5B, b, d). Strikingly, nuclear exit of Mcm4 occurred slightly earlier than budding, regardless of the presence or absence of Clbs 5 and 6 (Fig. 5B, c, e). Thus, although Clb–Cdc28 kinases are capable of promoting nuclear exit of Mcm4 (Fig. 4) and are important in blocking nuclear accumulation of Mcm4 after S phase (Fig. 3), nuclear exit is activated independently of *CLB5,6* expression. In the absence of Clbs 5 and 6, there appears to be a delay in the activation of any of the later Clbs that can block pre-RC assembly[36]. This suggests, therefore, that there is another activity that is dependent on passage through 'Start' which is capable of triggering Mcm4 nuclear exit in late G1 phase.

G1-cyclin-dependent exit of Mcm4 from the nucleus. As a cell completes mitosis, Mcm4 accumulates in the nucleus, even in the absence of Cdc6 (Fig. 1B). Under such conditions, Mcm4 remains nuclear if α-factor is added to prevent progression through G1 (Fig. 2B). This suggests that nuclear exit of Mcm4 is dependent upon the activation of Cln–Cdc28 (G1) kinases. It is, therefore, possible that exit of Mcm4 from the nucleus can be caused by either Cln- or Clb-associated forms of Cdc28, so that Cln–Cdc28 kinases represent the activity that is essential for Mcm4 nuclear exit in the absence of pre-RC formation. To test this possibility, we used a strain in which the only copy of *CLN2* is expressed from the *MET3* promoter, the *CLN1* and *CLN3* genes have been deleted, and *CDC6* is expressed from the *GAL1-10* promoter. This allows us to determine whether G1-cyclin expression is essential for Mcm4 nuclear exit in late G1, after a cell has passed through mitosis in the absence of Cdc6.

We synchronized cells in G1, with nuclear Mcm4 assembled into pre-RCs, by repressing expression of the G1 cyclin *CLN2* (Fig. 6a, b, stage I). G1-cyclin expression was subsequently induced to allow cells to enter S phase (Fig. 6a, b, stage II), causing Mcm4 to become cyto-

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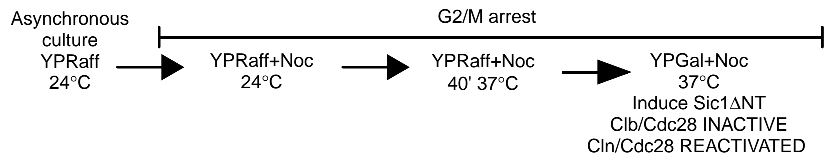

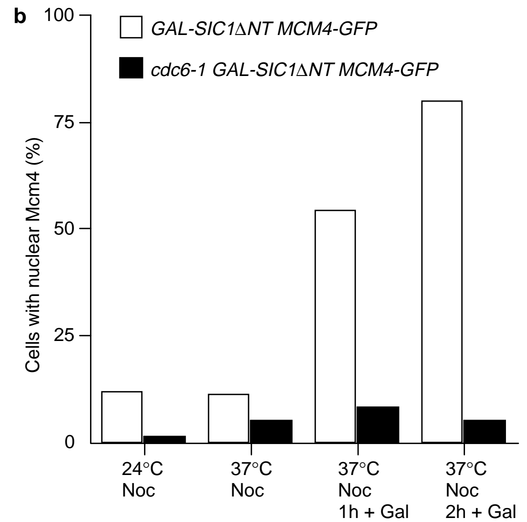

Figure 7 **Cdc6 is required for nuclear accumulation of Mcm4 when Clb–Cdc28 kinases are inactivated in G2/M-arrested cells. a**, Experimental procedure; see text for details. **b**, Proportion of cells with nuclear Mcm4–GFP. YPRaff, YP medium plus raffinose.

plasmic (Fig. 6b, stage II), and expression of *CDC6* was repressed. The culture was then split in two, and one half served as a control to show that, in the absence of *CDC6* expression, cells did not enter S phase in the next cell cycle (data not shown), nor did they retain nuclear Mcm4 (Fig. 6a, c, stage VII). The expression of *CLN2* was repressed for a second time in the other half of the culture, so that, after completing mitosis in the absence of Cdc6 and Cln2, cells became arrested in the subsequent G1 phase without forming a bud (Fig. 6a, b, stage III). Mcm4 accumulated in the nuclei of these G1-arrested cells (Fig. 6b, stage III). Thus the Clns are indeed necessary for nuclear exit of Mcm4 in the absence of Cdc6, in contrast to Clbs 5 and 6 (Fig. 5B). To confirm that G1 cyclins can promote exit of Mcm4 from the nucleus, we induced expression of *CLN2* once again, causing Mcm4 nuclear exit with slightly faster kinetics than those of bud formation (Fig. 6a, b, stages IV–VI).

Exit of Mcm4 from the nucleus in late G1 is therefore either directly triggered by Cln–Cdc28 kinases, or rapidly promoted by an activity downstream of Cln–Cdc28 kinases that is not dependent upon Clbs 5 and 6. One such downstream activity is the Cdc7–Dbf4 kinase, but we found that nuclear exit of Mcm4 still occurs in the absence of Cdc7 function when cells pass through mitosis without Cdc6 (see Supplementary Information). This indicates that Cln–Cdc28 kinases activate nuclear exit of Mcm4 by an independent mechanism, possibly by directly phosphorylating Mcm proteins or factors mediating their nuclear transport.

Dual regulation of Mcm4 nuclear localization. Our data indicate that Clns promote the nuclear exclusion of Mcm4 independently of Clbs 5 and 6, either by activating nuclear export of Mcm4 or by inhibiting its nuclear import (or by both mechanisms). At first sight such dual regulation of Mcm localization would appear to contradict the fact that inactivation of Clb–Cdc28 kinases after S phase is sufficient to cause nuclear accumulation of Mcm4 (Fig. 3). Inactivation of Clb–Cdc28 kinases in G2/M-arrested cells causes expression of *CLN1* and CLN2 (refs 18, 37), so why does this not block the nuclear accumulation of Mcm proteins? In addition, B-cyclin expression marks a 'point of no return' in late G1, after which ectopic expression of *CDC6* is unable to promote S phase[36]. This implies that *CDC6* expression is still able to promote binding of Mcm to chromatin and pre-RC formation after Cln–Cdc28 kinase activation but before B-cyclin expression. In contrast, our data show that Mcm proteins are largely cytoplasmic after Cln activation if cells have passed from mitosis to G1 in the absence of Cdc6.

The answer to this conundrum is suggested by the following observation. Although inactivation of Clb–Cdc28 kinase in G2/M-arrested cells does indeed cause nuclear accumulation of Mcm4, this is entirely dependent upon the function of Cdc6, which accumulates under such conditions (L. Drury and J.F.X.D., unpublished data). This contrasts with the nuclear accumulation of Mcm4 at the end of mitosis, which is independent of Cdc6 (Fig. 1B). We arrested *CDC6*^{+} *GAL-SIC1ΔNT* and *cdc6-1 GAL-SIC1ΔNT* strains in G2/M with nocodazole at 24 °C, before shifting them to 37 °C for 40 min to inactivate the temperature-sensitive Cdc6-1 protein (Fig. 7a). We then added galactose to induce expression of Sic1ΔNT. Although Clb–Cdc28 inactivation causes nuclear accumulation of Mcm4 in the strain with wild-type *CDC6*, this does not occur in the absence of Cdc6 function (Fig. 7b). Thus Cdc6 has an essential role in Mcm4 nuclear accumulation when B-cyclin kinases are inhibited by Sic1. As Cdc6 is not required for Mcms to enter the nucleus at the end of mitosis (Fig. 2), it is possible that Mcm4 shuttles between the nucleus and the cytoplasm when Clb–Cdc28 kinases are inactive and Cln–Cdc28 kinases become reactivated. Nuclear accumulation of Mcm4 under such conditions might, therefore, be a consequence of Cdc6-dependent pre-RC assembly, causing retention of Mcm proteins on chromatin.

Once Clb–Cdc28 kinases are active, constitutive expression of *CDC6* from the *GAL1-10* promoter does not prevent nuclear exclusion of Mcm4 (see Supplementary Information). This is likely to be an important reason why ectopic expression of *CDC6* does not promote pre-RC reformation or the loading of Mcm proteins around origins after S phase[6,9,19]. However, Cdc6 does not accumulate to high levels within the nucleus under such conditions[36], probably because of Cdc4-dependent proteolysis. Expression of *CDC6* from the *GAL1-10* promoter does produce high levels of nuclear Cdc6 after S phase in a *cdc4* temperature-sensitive mutant[36]. Even so, this still does not lead to nuclear accumulation of Mcm4 in G2/M-arrested cells (see Supplementary Information). Cdc6 cannot, therefore, promote nuclear accumulation of Mcm4 once Clb–Cdc28 kinases are active. This further highlights the importance of regulated Mcm localization for the cell-cycle control of DNA replication in budding yeast.

Nuclear accumulation of Mcm4 at the end of mitosis is independent of Cdc6 function, and our data predict that this independence results from the inactivity of both Clb- and Cln-associated forms of Cdc28. In this case, inactivation of all forms of Cdc28 should cause Cdc6-independent nuclear accumulation of Mcm4, even in G2/M-arrested cells. To test this idea, we used a strain that

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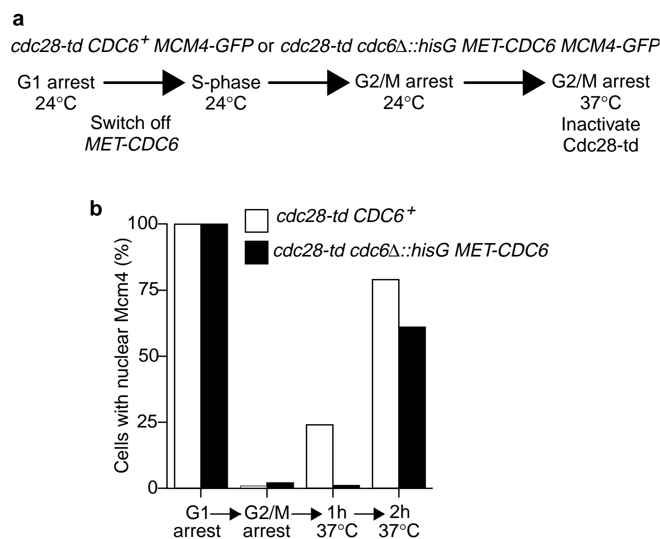

Figure 8 **Inactivation of Cdc28 in G2/M-arrested cells is sufficient to cause Cdc6-independent accumulation of Mcm4 in the nucleus. a**, Experimental procedure. **b**, Proportion of cells with nuclear Mcm4.

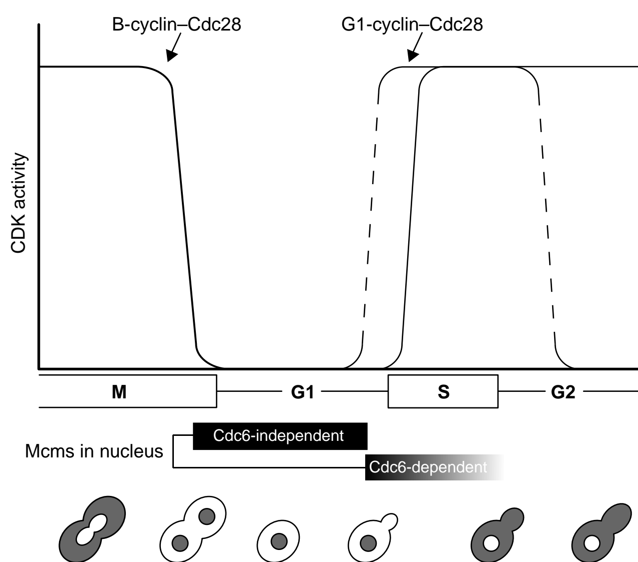

Figure 9 **Regulation of the nuclear localization of Mcm proteins during the budding-yeast cell cycle.** Dark shading shows Mcm localization. See text for details.

has a temperature-sensitive version of Cdc28 that is specifically degraded at high temperatures[38] (see Methods) and in which *CDC6* is expressed from the *MET3* promoter. This allowed us to repress expression of Cdc6 after synchronizing the cells in G1, then to arrest cells in G2/M and subsequently inactivate all forms of Cdc28. As a control we used a strain that also had the *cdc28-td* allele but in which *CDC6* was expressed from its own promoter. Asynchronous cultures of the *cdc28-td CDC6*+ and *cdc28-td MET-CDC6* strains were synchronized in G1 and then allowed to enter S phase (Fig. 8a). Expression of *MET-CDC6* was repressed from this point onwards and nocodazole was included in the medium so that, after passing through S phase, cells arrested in G2/M with cytoplasmic Mcm4 (Fig. 8b). The cultures were then shifted to 37 °C to inactivate Cdc28, as indicated by dephosphorylation of the B subunit of DNA polymerase-α (see Supplementary Information), a previously characterized substrate of Clb–Cdc28 kinases[34]. Although inactivation of Cdc28 caused the reappearance of Cdc6 protein in the *CDC6*+ strain only (see Supplementary Information), nuclear accumulation of Mcm4 occurred in both strains, regardless of the presence or absence of Cdc6 (Fig. 8b). Cdc28 inactivation is, therefore, sufficient to explain Cdc6-independent nuclear accumulation of Mcm4 as a cell completes mitosis.

Discussion

We have shown using live cells that Mcm4 accumulates in the nucleus during late mitosis and G1 phase, the period during which pre-RC assembly occurs at origins of DNA replication, but is then excluded from the nucleus after S phase. Our data support the following model for the regulation of the nuclear localization of Mcms by Cdc28 during the *S. cerevisiae* cell cycle (Fig. 9). At the end of mitosis, Cdc28's kinase activity falls to very low levels, and this causes Mcm proteins to accumulate in the nucleus, while expression of *CDC6* leads to Mcms becoming bound to chromatin around origins of DNA replication. As a G1 cell passes Start, any Mcm proteins that are not bound to chromatin are excluded from the nucleus. This is dependent upon Cln activation but is independent of Clbs 5 and 6. Chromatin-bound Mcms remain in the nucleus until they are displaced from chromatin during S phase, after which they are excluded from the nucleus by the Clbs until the end of the subsequent mitosis.

Several lines of evidence indicate that the Clns promote the nuclear exclusion of Mcm4 independently of Clb function. Mcm4 nuclear exclusion is Cln dependent in G1 cells lacking pre-RCs (Fig. 6), and occurs slightly more quickly than budding (Fig. 2). This is true even in the absence of Clbs 5 and 6 (Fig. 5), whereas the activation of any of the later Clbs that can block pre-RC assembly appears to be significantly delayed under such conditions[36]. Furthermore, Clb–Cdc28 kinase inactivation by Sic1 does not cause nuclear accumulation of Mcm4 in G2/M-arrested cells lacking Cdc6 activity (Fig. 7). These results indicate that Clns promote nuclear exclusion of Mcm4 independently of B-cyclins. Clns also appear to promote Cdc6 degradation during late G1 phase (L. Drury and J.F.X.D., unpublished observations). This indicates that pre-RC assembly is blocked by the Clns from this point onwards, before the Clbs trigger origin firing during S phase. By inhibiting pre-RC formation before origin firing occurs, the cell has evolved an efficient mechanism to ensure that each origin of replication fires just once in each round of the cell cycle. The Clbs subsequently maintain the block to pre-RC assembly until the end of the cell cycle, by exclusion of Mcms from the nucleus and by prevention of Cdc6 accumulation.

Expression of *CDC6* messenger RNA normally occurs at the end of mitosis, but a second burst occurs upon release from G1 arrest[8]. This will be of particular importance to cells returning to the G1 period of the cell cycle from stationary phase, as these cells lack pre-RCs[3]. The second burst of *CDC6* expression occurs before *CLN1* expression[8,39,40] and the involvement of Cdc28 in nuclear exit of Mcm4 and proteolysis of Cdc6 may help to explain why this is so.

Increasing amounts of evidence indicate that the cell-cycle control of DNA replication in other eukaryotes also involves the inhibition of pre-RC formation from the time of S phase until the end of mitosis. A role for regulated nuclear localization of replication proteins in this process was first suggested by Blow and Laskey[41]. These authors proposed that replication competence in *Xenopus* is established by an essential 'licensing factor' that is inactivated during S phase and can only reaccumulate in the nucleus at the end of mitosis. It has since been shown that Mcm proteins are essential for the licensing reaction[42], although it is possible that changes in the nuclear localization of CDKs, rather than of Mcm proteins themselves, are responsible for the regulation of licensing-factor activity[43]. In human cells, Cdc6 is excluded from the nucleus after S phase in what appears to be a CDK-dependent fashion[44–47]. Together with our data regarding budding-yeast Mcm proteins, this result

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shows that CDK-dependent nuclear exclusion of pre-RC components may be a conserved mechanism by which eukaryotic cells limit chromosome replication to a single round in each cell cycle. □

Methods

Strains, media, microscopic analysis and flow cytometry.

For details of the strains and media used in this study, together with procedures for microscopic analysis and flow cytometry, see Supplementary Information.

Tagging of Mcm4 with GFP.

The F64L S65T variant of GFP (Clontech) was expressed as a fusion to the C terminus of Mcm4, using a linker comprising five consecutive Gly–Ala repeats. See also ref. 48.

Other plasmid and strain constructions.

The strain YKL145 contains a construct expressing *GAL–CLB2Δ1-102*, with a copy of the c-Myc epitope at the C terminus.

Strains in which expression of the *CDC6* gene was under the control of the *GAL1-10* promoter were made by integrating pGP15 at the *CDC6* locus. The construction of pGP15 will be described elsewhere (G. Perkins *et al.*, personal communication). The construction of a strain in which the only copy of *CDC6* is under the control of the MET3 promoter has already been described[7].

To make the *cdc28* degron mutant described in Fig. 6, we integrated pPW66R[38] at the *CDC28* locus. As will be described elsewhere (K.L. and J.F.X.D., unpublished observations), we made use of *GAL*-controlled expression of the *UBR1* gene to enhance inactivation of the temperature-sensitive degron fusion.

Immunoblotting.

Proteins were transferred to Hybond ECL membrane which was then blocked with 5% dried milk in Tris-buffered saline containing 0.1% Tween-20. Detection of Cdc6 was performed using monoclonal antibody 9H8/5 (5μg ml^{-1}); Mcm4–GFP was detected using monoclonal antibody 3E1 (5μgml^{-1}; provided by S. Geley and J. Gannon); Myc-tagged Clb2Δ1-102 was detected using the monoclonal antibody 9E10 (5μgml^{-1}). Horseradish-peroxidase-coupled anti-mouse secondary antibodies were then used, before visualization of immunoreactive bands by enhanced chemiluminescence (Amersham).

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