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Research Article

Analysis of Mcm2–7 chromatin binding during anaphase and in the transition to quiescence in fission yeast

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ABSTRACT

Mcm2–7 proteins are generally considered to function as a heterohexameric complex, providing helicase activity for the elongation step of DNA replication. These proteins are loaded onto replication origins in M-G1 phase in a process termed licensing or pre-replicative complex formation. It is likely that Mcm2–7 proteins are loaded onto chromatin simultaneously as a pre-formed hexamer although some studies suggest that subcomplexes are recruited sequentially. To analyze this process in fission yeast, we have compared the levels and chromatin binding of Mcm2–7 proteins during the fission yeast cell cycle. Mcm subunits are present at approximately 1×10^4 molecules/cell and are bound with approximately equal stoichiometry on chromatin in G1/S phase cells. Using a single cell assay, we have correlated the timing of chromatin association of individual Mcm subunits with progression through mitosis. This showed that Mcm2, 4 and 7 associate with chromatin at about the same stage of anaphase, suggesting that licensing involves the simultaneous binding of these subunits. We also examined Mcm2–7 chromatin association when cells enter a G0-like quiescent state. Chromatin binding is lost in this transition in a process that does not require DNA replication or the selective degradation of specific subunits.

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Introduction

Mcm2–7 proteins are likely to function as the replicative helicase in the elongation step of DNA replication (reviewed in [1,2]). These proteins are loaded onto chromatin at replication origins during late mitosis or G1 in a tightly regulated step termed licensing or pre-replicative complex (pre-RC) formation (reviewed in [3,4]). This involves the DNA-associated origin recognition complex (ORC) and two regulatory factors, Cdc18/Cdc6 and Cdt1. Entry into S phase is subsequently triggered by activation of CDK and Hsk1/Cdc7 kinases. During this transition, additional factors bind to origins, such as Cdc45 and GINS, and Mcm2–7 helicase is activated, allowing DNA synthesis by the replicative DNA polymerases. Pre-RCs

are disassembled during S phase and Mcm2–7 proteins are displaced from chromatin, probably when converging replication forks meet. Following onset of S phase, a number of regulatory mechanisms prevent Mcm2–7 proteins from re-associating with origins, thus restricting DNA replication to a single round per cell cycle.

Mcm2–7 proteins themselves are generally thought to function as a complex, most likely as single or double heterohexameric complexes, with each heterohexamer containing one of each subunit (reviewed in [2]). In *S. cerevisiae*, analysis of degron mutants suggests that all Mcm2–7 subunits are required for the elongation step of DNA replication [5], and in *Xenopus* all the Mcm2–7 proteins are needed for licensing [6]. However, Mcm subcomplexes, such as Mcm4, 6 and 7 and

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Mcm3 and 5 can be isolated in fission yeast [7,8] and other organisms [9,10] (reviewed in [2,11]) and only the Mcm(4,6,7)₂ complex has been shown to have helicase activity in vitro [12,13]. In a genome wide study of Mcm3, 6 and 7 localization, all three Mcms were detected at only around 60% of binding sites [14]. It remains an open question whether Mcm2–7 subunits or subcomplexes have specific functions in vivo. Mcm7 may have specific regulatory roles as a recent study showed that this protein has a replication checkpoint signaling function that is not shared with other Mcm subunits [15]. Also, Rb specifically interacts with Mcm7 in mammalian cells, which may provide a mechanism for inhibition of DNA replication [16].

The licensing process, which loads Mcm2–7 proteins onto DNA, requires ATPase activity of ORC and Cdc6 [17,18]. ORC/Cdc6 contains six AAA+ proteins and may function in a way analogous to RF-C [19,20], which loads the ring-shaped PCNA trimer onto DNA. Thus, ORC/Cdc6 may effect topological loading of a pre-formed Mcm2–7 complex onto DNA, perhaps by transient opening of the hexameric ring, which would close around the DNA. A recent study suggests that Mcm2–7 may associate with ORC/Cdc6 via salt-sensitive interactions before being more stably loaded onto DNA by Cdc6-mediated ATP hydrolysis [17]. There is also evidence that Mcm2–7 proteins assemble onto chromatin in a stepwise fashion, which could be a manifestation of sequential association of Mcm subunits with an ORC landing pad (reviewed by [21]). In *Xenopus*, Mcm 2, 4 and 6 appear to bind before Mcm3, 5 and 7, and chromatin association of Mcm4 and 6 is not inhibited by 6-DMAP, unlike the other Mcms [22]. In human cells, Mcm2–7 proteins have been reported to bind to chromatin with different kinetics and deregulation of cyclin E inhibits chromatin association of Mcm subunits differentially, with a dramatic effect on Mcm4 [23,24]. This evidence is controversial, however, as biochemical studies in *Xenopus* indicate that Mcm subcomplexes cannot license DNA when added to chromatin sequentially, and only the complete Mcm2–7 heterohexamer can bind productively to chromatin [6].

In this paper, we have examined the timing of chromatin association of individual Mcm2–7 subunits in the fission yeast cell cycle. Since any attempt to compare the timing of chromatin binding could be affected by large differences in the relative abundance of Mcm2–7 subunits, we first compared total and chromatin bound levels of the proteins. A single cell analysis was used to correlate the timing of chromatin association with progress through mitosis and this showed that Mcm2, 4 and 7 associate with chromatin at a similar time in anaphase B, with no evidence for sequential binding. We also analyzed the chromatin binding of Mcm2–7 proteins during exit from the cell cycle to a quiescent G0-like state and showed that displacement from chromatin occurs in a process that does not require DNA replication.

Materials and methods

Yeast strains

Fission yeast strains used were constructed by standard genetic methods and are shown in Table 1. Strains were

grown in rich medium (YE3S) or minimal medium (EMM) [25].

Gene tagging

Mcm3 was tagged with GFP by amplifying a genomic *mcm3* fragment using the oligos 10 (gtaccgggcccttatgcatggtctcagggt-caaagatgcaaaggctgctgg) and 11(cattaagcttcagcaccag-caccggctccggcaccagcaccaccggcaccagatctaccctcagaatacga-taaaccacattatctg), and this product was cloned into pSMUG [26] as an *ApaI*, *HindIII* fragment to generate pSMUG2+Mcm3. The Mcm3 insert in this fragment was subcloned into pSMRG2+ [27] as an *ApaI*, *XhoI* fragment and the resulting plasmid was integrated at the *mcm3*⁺ locus after linearizing with *NheI*.

An Mcm4-CFP expressing strain was constructed by PCR amplification of an *mcm4*-encoding region with the oligos 213 (atagggccatgctacagataggaggtc) and 487 (tttctcgagatcagctgtg-caattgaacgtaca). The product was cloned into pSMUC2+ [28] as an *ApaI*, *XhoI* fragment. The plasmid was integrated at the *mcm4*⁺ locus after linearizing with *EcoNI*.

A strain expressing Mcm7-GFP was constructed by amplifying a genomic *mcm7* fragment with oligos 17 (caagtgggccc-gccgtgcgaacccttata) and 18 (cttaccggggcattctccatgt-taaatccg), and this product was cloned into pSMUG2+ as an *ApaI*, *SmaI* fragment to generate pSMUG2+Mcm7. This was integrated into the *mcm7*⁺ locus after integrating with *MluI*. Mcm7-YFP was constructed by subcloning the *ApaI*, *SmaI* Mcm7 fragment from pSMUC2+Mcm7 [28] into pSMRY2+ [29]. The plasmid was integrated into the *mcm7*⁺ locus after

Table 1 – *S. pombe* strains used

P643	<i>mcm4</i> ⁺ -GFP: :ura4 ⁺ <i>cdc25-22 ura4-D10</i>	This study
P682	<i>mcm4</i> ⁺ -GFP: :ura4 ⁺	[27]
P990	<i>mcm3</i> ⁺ -GFP: :kanMX6 <i>leu1-32 ura4-D18</i>	This study
P992	<i>mcm2</i> ⁺ -GFP: :kanMX6 <i>ade6-M210 leu1-32 ura4-D18</i>	[27]
P1001	<i>mcm6</i> ⁺ -GFP: :kanMX6 <i>ade6-M210 leu1-32 ura4-D18</i>	[27]
P1051	<i>mcm2</i> -CFP: :ura4 ⁺ <i>ade6-M210 leu1-32 ura4-D18 h</i> ⁻	[28]
P1054	<i>mcm7</i> -CFP: :ura4 ⁺ <i>ade6-M210 leu1-32 ura4-D18 h</i> ⁻	[28]
P1057	<i>mcm7</i> ⁺ -GFP: :ura4 ⁺ <i>ade6-M210 leu1-32 ura4-D18</i>	[28]
P1083	<i>sna41(cdc45)</i> -YFP: :ura4 ⁺ <i>ade6-M210 leu1-32 ura4-D18 h</i> ⁻	[28]
P1416	<i>cdc18</i> : :TAP: :kanMX6 <i>pat1-114 h</i> ⁻	D. Hermand
P1424	<i>cdt1-18myc pat1-114 h</i> ⁻	H. Nishitani
P1433	<i>mcm2</i> ⁺ -CFP: :ura4 ⁺ <i>cdc45</i> -YFP: :ura4 ⁺	This study
P1471	<i>mcm7</i> ⁺ -CFP: :ura4 ⁺ <i>ars1(mlu1)</i> : :pREP3X-GFP- <i>atb2</i> : :LEU2 <i>cdc25-22</i>	This study
P1472	<i>mcm2</i> ⁺ -CFP: :ura4 <i>ars1(Mlu1)</i> : :pREP3X-GFP- <i>atb2</i> : :LEU2 <i>cdc25-22</i>	This study
P1513	<i>mcm4</i> ⁺ -CFP: :ura4 ⁺ <i>ars1(mlu1)</i> : :pREP3X-GFP- <i>atb2</i> : :LEU2 <i>cdc25-22</i>	This study
P1525	<i>mcm3</i> ⁺ -GFP: :kanMX6	This study
P1526	<i>mcm6</i> ⁺ -GFP: :kanMX6	This study
P1527	<i>mcm7</i> ⁺ -GFP: :ura4 ⁺	This study
P1545	<i>mcm6</i> ⁺ -GFP: :kanMX6 <i>cdc25-22</i>	This study
P1640	<i>mcm4</i> ⁺ -CFP: :ura4 ⁺ <i>mcm7</i> ⁺ -YFP: :kan ⁺ MX6 <i>ars1(mlu1)</i> : :pREP3X-GFP- <i>atb2</i> : :LEU2 <i>cdc25-22</i>	This study

linearizing with BsaBI. All constructs were verified by sequencing.

Chromatin binding assay

Chromatin binding assay and image analysis were carried out as previously described [26,30]. Filter sets and data collection were as previously described [28,31]. Data shown are the mean of at least two assays.

For quantitation of nuclear fluorescence, cells were fixed by methanol/acetone and mounted on poly-lysine coated slides in PBS-glycerol. Live cells were mounted in agarose. The image field used for data collection was calibrated with InSpeck green (505/515) beads (Molecular Probes) to ensure illumination was even. Images were collected using a $\times 100$ objective and a field size of 768×512 pixels. To minimize photobleaching, cells were only exposed to excitation light during image acquisition. Only one image was collected per field and non-overlapping fields were collected to ensure that cells were only exposed once. NIH Image was used to measure GFP fluorescence. Pixels were averaged over a circular area of $1.5 \mu\text{m}$ diameter centered on each nucleus in binucleate (G1/S) cells and background fluorescence was subtracted. All comparative data were collected in one session.

For analysis of the timing of Mcm2–7 chromatin binding during anaphase, *cdc25ts* strains expressing CFP and YFP-tagged Mcms and GFP-tagged α -tubulin were grown in YES at 26°C for 24 h, transferred to EMM for 20 h and arrested at 35.5°C for 255 min. Cells were released from the block by shifting down to 26°C for 20–40 min. Cells were processed for the chromatin binding assay and imaged to show Mcm protein, GFP- α -tubulin and nuclei (DAPI). Nuclear separation distance was used to determine progress through anaphase and 150 cells were measured for each analysis. Only cells showing mitotic spindles were analyzed (cells only expressing α -GFP-tubulin do not show nuclear fluorescence after detergent extraction at any stage of the cell cycle). The *cdc25* block and release procedure was used simply to enrich for anaphase cells and the time after the release of the block was not used to determine the timing of Mcm chromatin binding. The strains used in these experiments (Mcm2, 4, 7-CFP and Mcm4-CFP, Mcm7-YFP) showed no growth defects. Unlike the GFP-tagged versions, cells expressing Mcm3-CFP and Mcm6-CFP were elongated compared to wild-type cells and were therefore not used in the timing analysis.

Flow cytometry

Flow cytometry was carried out using sytox green ($1 \mu\text{m}$) as previously described [28].

Western blotting

Protein extracts for Western blotting were made by trichloroacetic acid extraction as previously described [32]. Cell breakage was $>95\%$. Purified EGFP (BD Biosciences) was diluted in BSA carrier before gel analysis. For quantitative measurements of Mcm2–7 levels, we checked that proteins had been transferred efficiently by staining the gels after transfer. For Western blot analysis, antibodies against GFP (3E1 monoclo-

nal) and α -tubulin (Sigma T5168) were used. Detection was performed using the chemiluminescence procedure and images were collected with a CCD camera.

Results

Comparative levels of Mcm2–7 proteins in fission yeast

We constructed strains where the endogenous Mcm2–7 genes, expressed from the native promoter, were modified at the C-terminus with GFP. Mcm2, Mcm3, Mcm4, Mcm6 and Mcm7-GFP strains grew at the same rate as wild type at temperatures in the normal range (25 – 36°C) and showed normal flow cytometry profiles and a wild-type cell size distribution (Supplementary Fig. 1). These GFP-tagged proteins show the expected S phase-dependent chromatin association ([26–28] and data not shown). We also compared levels of GFP-tagged Mcm4, Mcm6 and Mcm7 with the untagged proteins, using Mcm-specific antibodies, and this showed that the GFP tag does not affect the protein level (Supplementary Fig. 2). Mcm5-GFP was not fully functional after C-terminal tagging, so this protein was excluded from further analysis.

We compared the levels of Mcm2–7 subunits by Western blotting of total cell extracts with loadings of purified GFP (Fig. 1A). Three independent analyses indicated that Mcm subunits are present at comparable levels, at around 10^4 molecules/cell, although the most abundant, Mcm4, is approximately fourfold more abundant than the least abundant, Mcm6. One possible explanation for this disparity could be cell cycle changes in Mcm6 levels, given that these have been reported to fluctuate during the mammalian cell cycle, in contrast to other Mcm subunits [33]. However, fission yeast Mcm4 and Mcm6 levels were constant following *cdc25* block and release analysis (Fig. 1B), which is consistent with constant transcript levels [34]. We also compared the levels of chromatin-associated Mcm4 and Mcm6, since it is possible that these proteins bind to DNA with an equivalent stoichiometry even if there are differences in total protein levels. To carry out this analysis, we used fluorescence microscopy with a single cell chromatin-binding assay to quantitate Mcm4 and Mcm6-GFP. This method uses detergent extraction to remove non-chromatin-associated Mcm-GFP proteins and while, for instance, Mcm4 is constitutively nuclear during the fission yeast cell cycle, only binucleate (late M/G1/S) phase cells show Mcm4 after extraction, which is dependent on ORC and Cdc18 [26]. We found that chromatin-bound Mcm4 and Mcm6 were present at approximately equal levels (1.5:1, Figs. 1C, D), indicating that the ratio of levels of total protein is more extreme than for the chromatin-associated forms.

Relative timing of Mcm2–7 chromatin association during licensing

Given earlier reports suggesting that Mcm2–7 proteins bind to chromatin asynchronously [22–24], we analyzed the situation in fission yeast. Earlier, we showed that Mcm4 and Mcm7 bind to chromatin during anaphase [26,28], but we have not carefully compared the timing of binding. We therefore used

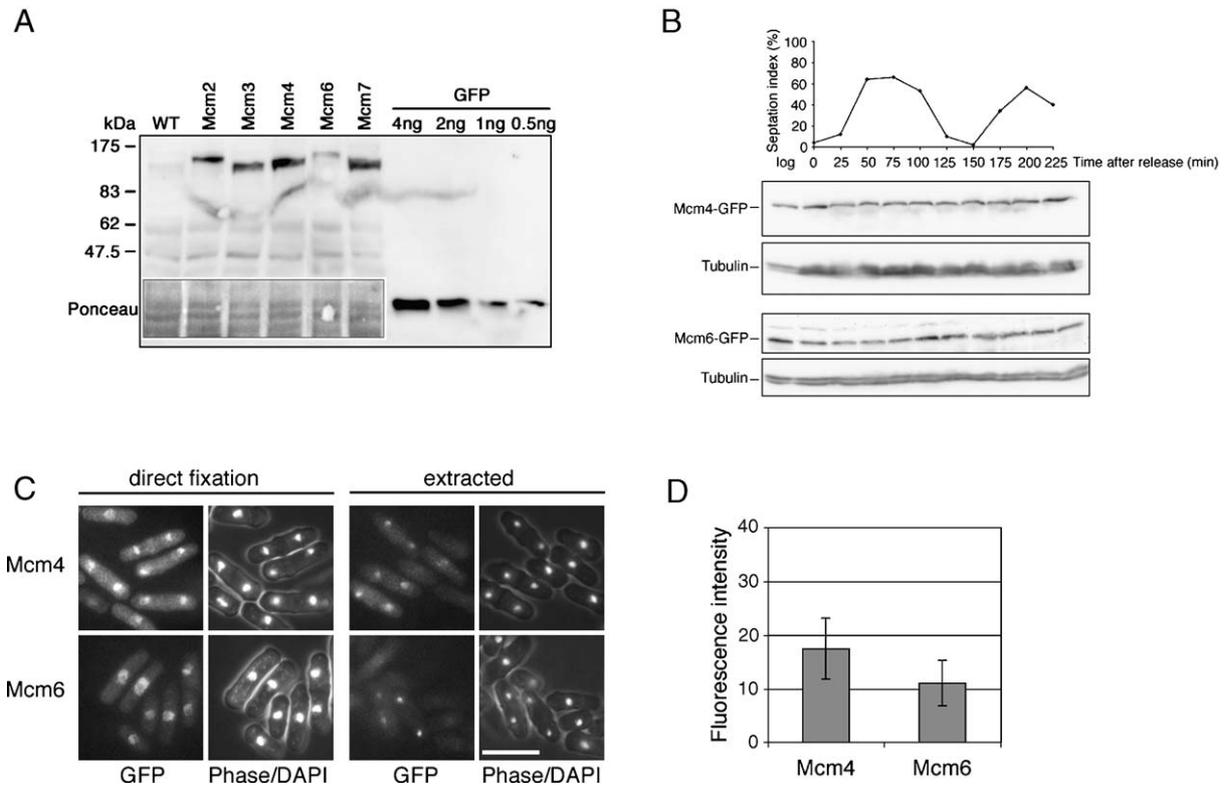


Fig. 1 – Comparison of Mcm2–7 levels in fission yeast. (A) Protein extracts from strains expressing GFP-tagged Mcm2–7 protein (10^7 cells per lane) were analyzed by Western blotting, and compared with known amounts of purified GFP. The blot was probed with an anti-GFP monoclonal antibody and the images were collected with a CCD camera. Three independent analyses gave the following average estimates of the number of Mcm2–7 proteins per cell: Mcm2, 8×10^3 ; Mcm3: 9×10^3 ; Mcm4, 1.5×10^4 ; Mcm6: 4×10^3 ; Mcm7: 8×10^3 . Strains used were P138, P992, P990, P682, P1001 and P1057. **(B)** Septation index and Western analysis of Mcm4-GFP (P643) and Mcm6-GFP (P1545) levels in cells synchronized by *cdc25* block and release. α -tubulin is shown as a loading control. **(C)** Comparison of Mcm4-GFP (P682) and Mcm6-GFP (P1001) fluorescence in directly fixed cells, and cells processed for the chromatin binding assay. Mcm4-GFP cells show higher cytoplasmic fluorescence (37 ± 10) compared to Mcm6-GFP (10 ± 6) after direct fixation (also with live cells, data not shown). Scale bar, $10 \mu\text{m}$. **(D)** Analysis of fluorescence intensity of nuclear Mcm4 and Mcm6-GFP after detergent extraction. Cells expressing Mcm4-GFP (P682) or Mcm6-GFP (P1001) were grown to log phase and processed for the chromatin binding assay. Following imaging, the fluorescence intensity (arbitrary units) of 100 nuclei in binucleate cells was quantitated for each strain (error bars show the SD). The Mcm4:Mcm6 ratios were 1.5:1 and 1.6:1 in two independent experiments.

the single cell assay to correlate the chromatin binding of GFP-tagged Mcm2, Mcm4 (both binding early to chromatin in *Xenopus*) and Mcm7 (late in *Xenopus*) with the separation distance between the dividing nuclei in anaphase. This allows us to use progress through anaphase as an internal clock (nuclear separation occurs at about $1.6 \mu\text{m}/\text{min}$) and the experiment is not dependent on the degree of cell synchronization that can be achieved. As in previous studies of the timing of Mcm2–7 chromatin association, this approach follows licensing at all origins, but this event appears to be synchronous [35].

Analysis of anaphase cells for each strain (Figs. 2A, B, Supplementary Fig. 3) showed that the chromatin association of the three proteins occurred at an overlapping range of nuclear separations ($6\text{--}9 \mu\text{m}$ for Mcm4, $6\text{--}12 \mu\text{m}$ for Mcm7 and $7\text{--}12 \mu\text{m}$ for Mcm2). With cells released from a *cdc25* block, the anaphase spindle remains intact until the nuclear separation distance is $20\text{--}25 \mu\text{m}$ and thus Mcm2–7 chromatin association is occurring at an early phase of anaphase B. Since Mcm

subunits are binding over a range of nuclear separations it is difficult to say whether the small temporal differences in the data shown in Fig. 2 are significant. We therefore carried out a similar analysis using cells expressing both Mcm4-CFP (early in *Xenopus*) and Mcm7-YFP (late in *Xenopus*). In this analysis, cells showed chromatin association of both proteins occurring when nuclear separation was in the range of $4\text{--}9 \mu\text{m}$ (Fig. 3). Thus, Mcm2–7 chromatin binding is not precisely correlated with the nuclear separation distance in anaphase B, which may indicate some cell-to-cell variation as to when licensing occurs. Out of approximately 50 cells with a nuclear separation of $4\text{--}9 \mu\text{m}$, none showed binding of one tagged Mcm subunit, but not the other, which is consistent with simultaneous chromatin binding of Mcm4 and 7. Thus, although a subset of Mcm proteins has been analyzed, these data provide no evidence for an intermediate stage in licensing where only a specific Mcm subunit or subcomplex has bound to chromatin.

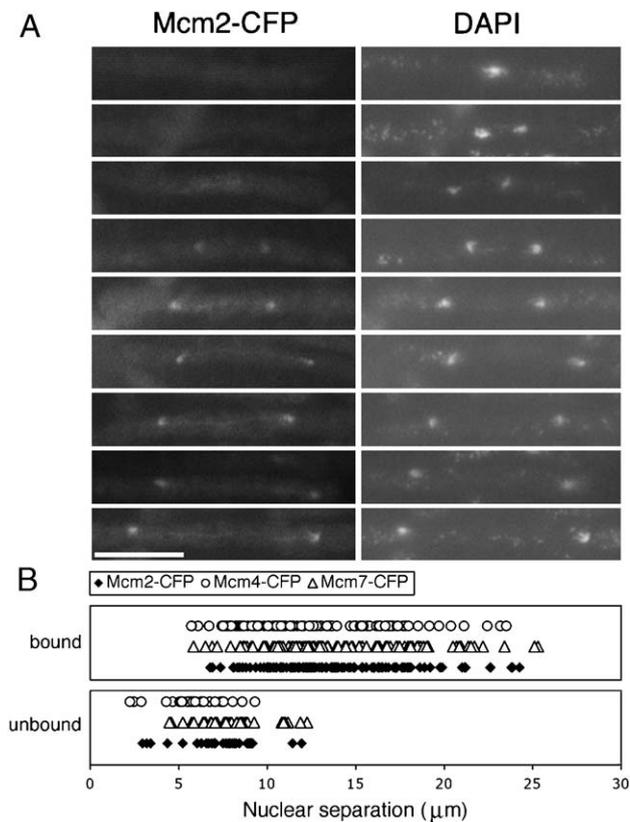


Fig. 2 – Analysis of timing of Mcm2 chromatin binding in anaphase. Cells expressing Mcm2-CFP (P1472) were enriched in anaphase and processed for the chromatin binding assay as described in the Materials and methods. Cells were imaged for chromatin-associated Mcm2-CFP and progress through anaphase was determined by measuring the nuclear separation distance. Similar data were obtained for Mcm4 and Mcm7 (see Supplementary Fig. 3). Scale bar, 10 μ m. (B) Timing of Mcm2, Mcm4 and Mcm7 chromatin binding during anaphase. Each symbol shows whether chromatin binding of Mcm2, 4 or 7 was detected in an anaphase cell, plotted against the nuclear separation distance.

Regulation of Mcm2–7 chromatin association during G0 arrest

In proliferating mammalian cells, association of Mcm2–7 with chromatin occurs during late mitosis [23] and displacement occurs during S phase. On entry into the quiescent G0 state, Mcm2–7 are displaced from chromatin [36–38] and on prolonged arrest, levels decline in most but not all tissues ([39], reviewed by [40]). Since Mcm2–7 chromatin binding normally occurs before the restriction point in proliferating cells, this suggests that chromatin disassociation can occur in G1 in the absence of DNA replication. However, studies of *Drosophila* embryos have shown that cells committed to exit from the cell cycle show a block to Mcm2–7 chromatin association in late mitosis [41], in other words exit from the cell cycle is not accompanied by an abortive round of licensing that is reversed in G1 phase. In fission yeast, we have previously shown that Mcm2, 4 and 6 are not chromatin-associated after nitrogen starvation [27] but the kinetics of

displacement have not been addressed. We therefore analyzed whether Mcm2–7 displacement from chromatin requires S phase followed by a block to chromatin binding in mitosis, or chromatin binding in mitosis followed by displacement in G1 without DNA replication (Fig. 4A).

We first checked whether levels of Mcm subunits are dramatically affected by nitrogen starvation, since complete loss of one Mcm protein could account for chromatin displacement [42]. Although GFP-tagged Mcm2–4, 6 and 7 proteins become somewhat less abundant during nitrogen starvation (approximately 50% of log phase level at 16 h) they are clearly all present even after 32 h (Figs. 4B–D). We also analyzed untagged proteins using Mcm-specific antibodies and this showed a change in Mcm4 levels similar to that observed with the GFP-tagged strain at 16 h, but Mcm6 and 7 were little changed (Supplementary Fig. 2). Thus, although this analysis has not included Mcm5, complete selective degradation of an Mcm subunit does not appear to occur during G1 arrest over the time scale analyzed.

We then investigated the kinetics of Mcm2–7 chromatin association during the transition to quiescence. We found that after 7 h of nitrogen starvation, there is an increase in the percentage of uninucleate cells with chromatin-associated Mcm4, which correlates with an increase in the proportion of

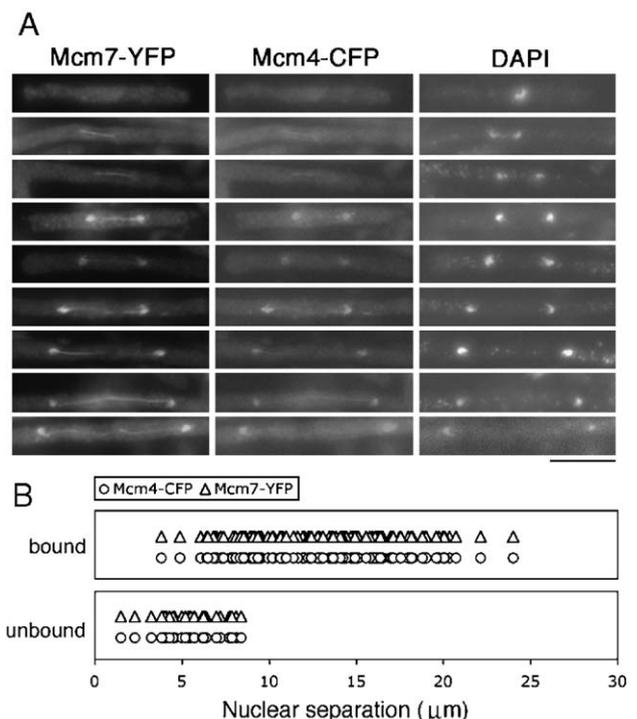


Fig. 3 – Simultaneous analysis of Mcm4-CFP and Mcm7-YFP chromatin binding during anaphase. Strain P1640 was processed as described in Fig. 2 and imaged to determine if chromatin-associated Mcm4 or Mcm7 could be detected. Scale bar, 10 μ m. (B) Timing of Mcm4 and Mcm7 chromatin binding during anaphase. Each symbol shows whether chromatin binding of Mcm4 or 7 was detected in an anaphase cell, plotted against the nuclear separation distance (data for Mcm4-CFP and Mcm7-YFP for the same cell are vertically aligned in each box).

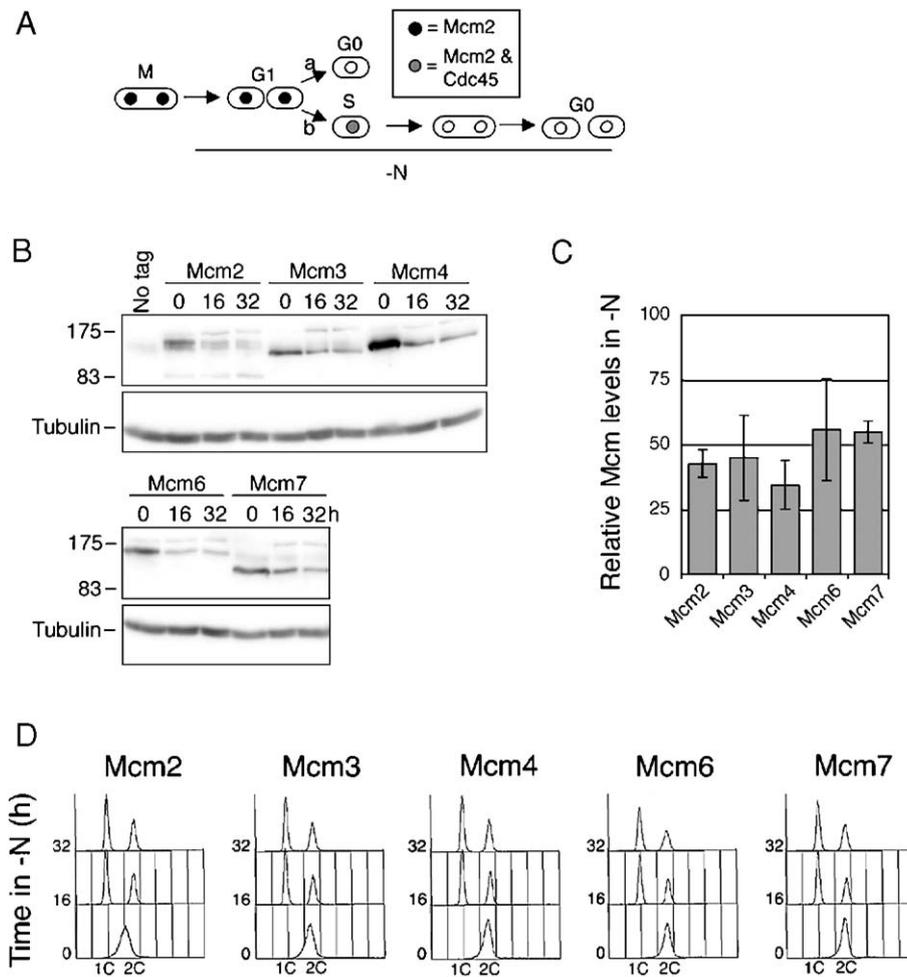


Fig. 4 – Analysis of Mcm2–7 during nitrogen starvation. (A) Possible mechanisms for loss of chromatin-associated Mcm2–7 during nitrogen starvation: (a) dissociation from chromatin in the absence of DNA replication; (b) loss via DNA replication followed by inhibition of chromatin association in mitosis. (B) Western analysis of Mcm-GFP levels during nitrogen starvation. Prototrophic strains P1433 (Mcm2-GFP), P1525 (Mcm3-GFP), P682 (Mcm4-GFP), P1526 (Mcm6-GFP) and P1527 (Mcm7-GFP) were grown to log phase in EMM medium then transferred to EMM lacking nitrogen for 16–32 h and analyzed by Western blotting. (C) Mcm levels after 16 h of nitrogen starvation (100=level of Mcm protein during log phase); error bars show statistical range. (D) Flow cytometry profile of cells used for (B).

1C cells (Figs. 5A, B). This reflects G1-arrested cells which have carried out pre-RC formation but which have not proceeded to DNA replication, due to CDK inhibition by Rum1 stabilization [43]. During 7–16 h, there is a decrease in the percentage of cells with chromatin-associated Mcm4, but during this interval the septation index is very low, while the 1C/2C cell distribution and cell number hardly changes (Figs. 5C, D) indicating that cells are not carrying out S phase followed by mitosis and cytokinesis during this interval. Mcm4 remains nuclear in cells that have not been detergent extracted (Fig. 5B, -triton). Overall, this suggests that cells lose chromatin-associated Mcm4 (or it becomes less tightly bound) in the absence of DNA replication during nitrogen starvation.

To confirm that Mcm2–7 is being displaced from chromatin in the absence of DNA replication, we carried out a similar experiment using a strain expressing both Cdc45-YFP and Mcm2-CFP, so that chromatin association of both proteins could be simultaneously monitored. Cdc45 binds to chromatin

at around initiation of DNA replication and remains bound until termination [44]. It thus serves as an S phase marker in detergent-extracted cells [28]. Using this strain, we saw an increase in the percentage of uninucleate cells with chromatin-associated Mcm2 after around 7 h of nitrogen starvation, then a decrease over 7–16 h, as with Mcm4 (Figs. 6A, B). Chromatin-associated Cdc45 is only detectable up to 4 h (Figs. 6A, B, D), indicating that DNA replication is complete by 7 h of nitrogen starvation. Although chromatin-associated Cdc45 is lost at an early stage of nitrogen starvation, the total level remains constant and it remains in the nucleus. Taken together, these results indicate that during nitrogen starvation, cells generated by the last mitosis initially have chromatin-associated Mcm2–7 protein, but this is then lost in a process that does not require DNA replication. We also examined levels of Cdt1 and Cdc18 after nitrogen starvation. While Cdt1 is present after 16 h of nitrogen starvation, Cdc18 is not detectable (Fig. 6D) and this could be relevant to loss of chromatin-associated Mcm2–7.

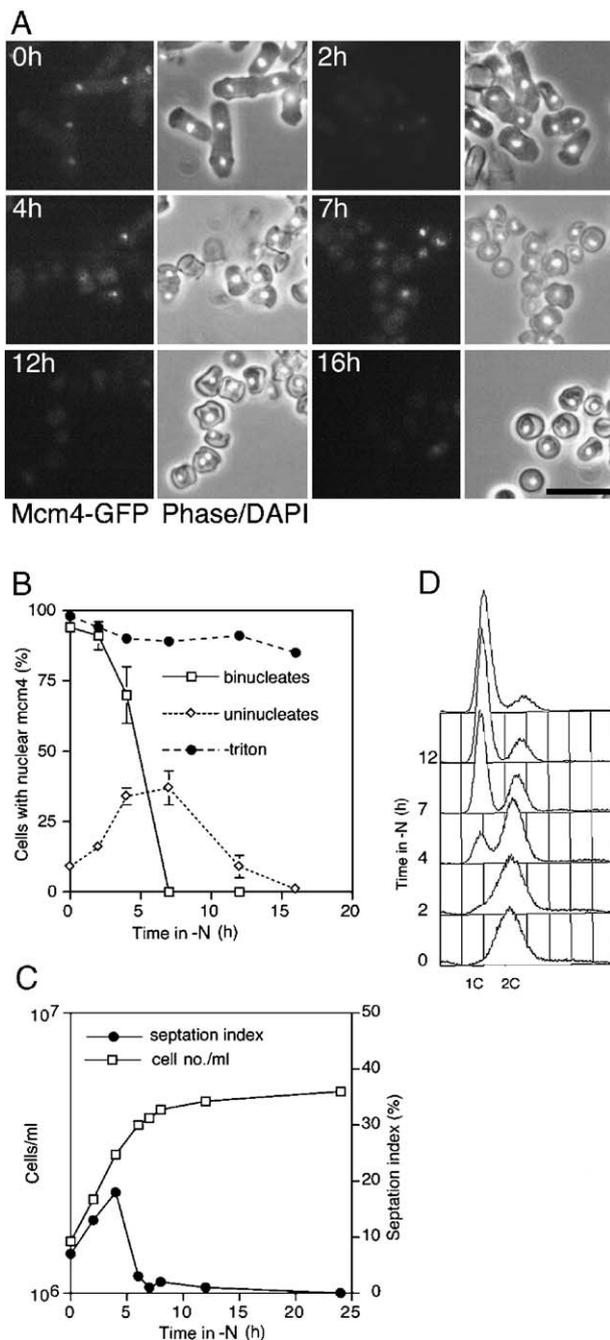


Fig. 5 – Mcm4 chromatin binding during a G1-phase arrest induced by nitrogen starvation. Mcm4-GFP strain (P682) was grown into exponential phase in EMM and transferred to EMM medium lacking nitrogen at 25°C. (A) Chromatin binding assays showing Mcm4-GFP chromatin association (left-hand panels) and phase/DAPI (right-hand panels) during nitrogen starvation. (B) Quantitation of chromatin binding assay, showing percentage of uninucleate and binucleate cells positive for Mcm4-chromatin binding. “-triton” shows percentage of cells with nuclear localization of Mcm4 when triton-extraction step is omitted. Error bars show statistical range. Scale bar, 10 μ m. (C) Cell concentration and septation index after transfer to nitrogen-deficient medium. (D) Flow cytometric analysis of DNA content of cells shown in panel A.

Discussion

Mcm2–7 proteins are generally believed to function as a helicase in the elongation step of DNA replication, and most studies suggest that the active form of the complex is a heterohexamer containing one of each Mcm2–7 subunit. In this study, Mcm2–7 proteins are found to be present at around 10^4 molecules/cell in fission yeast, which is comparable to an estimate of Mcm2–7 abundance in *S. cerevisiae* ($1\text{--}3 \times 10^4$ molecules/cell; [45]) and approximately tenfold more abundant than fission yeast Orc6 [28]. An earlier report indicated that there are 3000 Mcm2–7 complexes per cell in fission yeast, but this was estimated to be lower than the total amount of Mcm protein [46]. The number of potential replication origins in fission yeast has been estimated at about 385 [47], although only around a third of these are active in a single S phase [48], thus there is approximately 100:1 excess of Mcm2–7 proteins per active origin.

Comparing individual subunits, total Mcm4 is more abundant than Mcm6, with Mcm2, 3 and 7 present at similar intermediate levels, but a comparison of Mcm4 and Mcm6 on chromatin indicated that they are bound in approximately equal (1.5:1) stoichiometry. These results suggest that there may be a pool of free Mcm4 present in the cell and we note that non-extracted Mcm4-GFP cells show higher cytoplasmic fluorescence compared to Mcm6-GFP cells (Fig. 1C). Intact Mcm2–7 complexes are required for retention in the nucleus and this may be a mechanism to ensure the correct stoichiometry of the subunits, even if the expression levels of the individual Mcm2–7 proteins differ [42,49,50]. The significance of differences in total levels of Mcm subunits is unclear, but this could be relevant to involvement of these proteins in processes other than DNA replication, such as transcription [2,51,52].

Two possible models for the chromatin loading of the Mcm2–7 complex at ORC are sequential association of Mcm subunits or subcomplexes, or the simultaneous chromatin binding of a pre-formed Mcm2–7 complex analogous to the RF-C loading of PCNA. Analysis using a single cell method showed no clear differences in the timing of chromatin binding of Mcm2, 4 and 7, which occurs during early anaphase B. Although we are unable to rule out small temporal differences, the simplest interpretation of our data is that licensing does not involve an intermediate stage where only a specific subset of Mcm proteins is chromatin-associated. This finding is consistent with biochemical studies of licensing in *Xenopus* [6] and the finding that Mcm2–7 complex must be intact for maintenance in the nucleus in fission yeast [49].

We analyzed chromatin association of Mcm2–7 proteins as cells exit the vegetative cycle upon nitrogen starvation and arrest in a quiescent G0-like state. In fission yeast, Mcm2–7 proteins are not chromatin-associated in quiescent cells but remain in the nucleus [27]. Mcm2–7 proteins normally disassociate from chromatin during S phase, but we found that during exit from the cell cycle into quiescence, binding of Mcm2–7 to chromatin occurs during the last mitosis followed by displacement in G1 in the absence of DNA replication (Figs. 5 and 6). This situation is similar to that in *S. cerevisiae*, where the pre-RC footprint at origins reverts to the post-RC state in

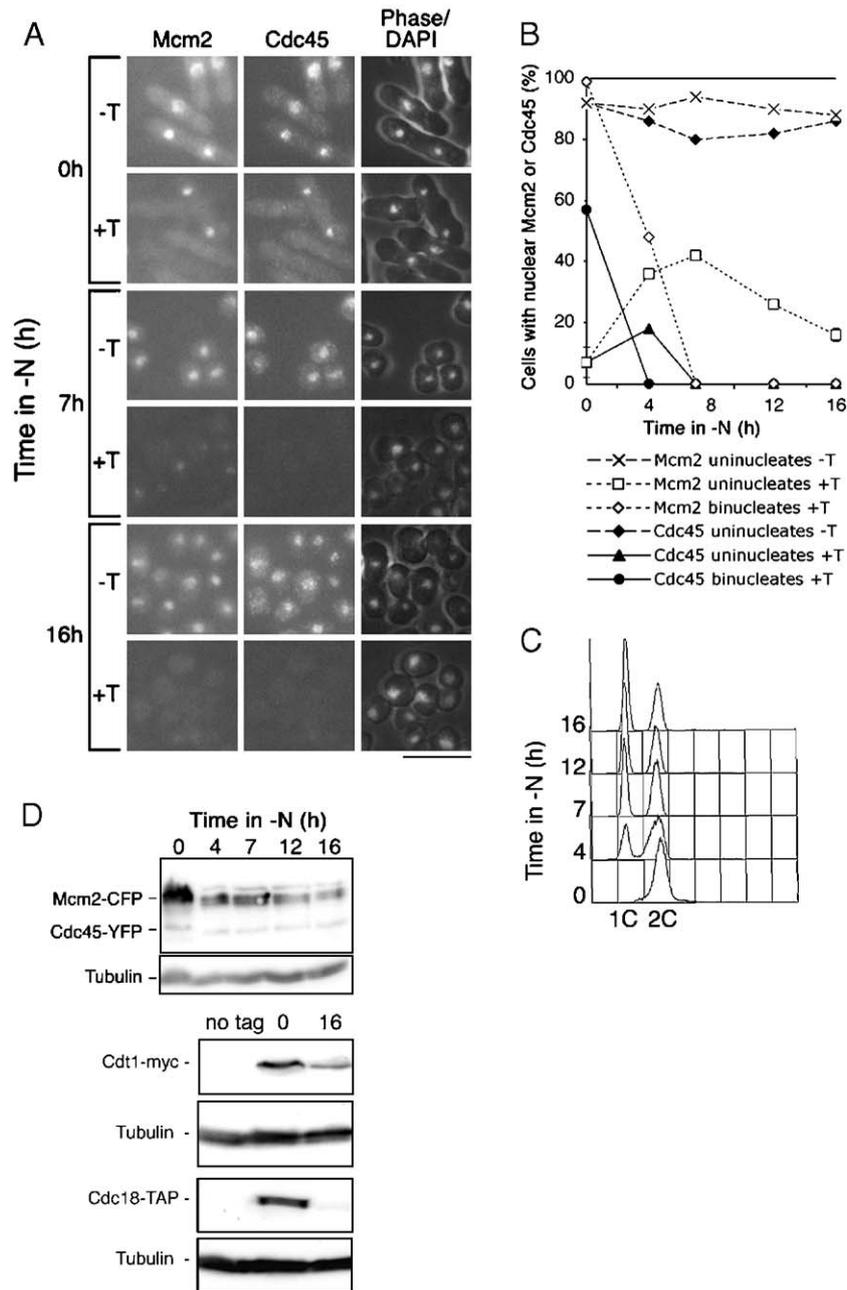


Fig. 6 – Analysis of Mcm2 and Cdc45 chromatin binding during nitrogen starvation. Strain P1433 was grown to log phase then transferred to EMM medium lacking nitrogen for 16 h at 25°C. (A) Analysis of Mcm2-CFP and Cdc45-YFP with (+T) and without (-T) detergent extraction during nitrogen starvation. Scale bar, 10 μ m. (B) Quantitative analysis of cells showing nuclear Mcm2 and/or Cdc45 during nitrogen starvation with (+T) and without (-T) detergent extraction. Values are the mean of two experiments. (C) Flow cytometry analysis of cells shown in panels A and B. (D) Western analysis of Mcm2, Cdc45, Cdt1 and Cdc18 levels during 0–16 h nitrogen starvation at 25°C. α -tubulin is shown as a loading control.

the absence of DNA replication [53]. However, the mitotic block to Mcm2-7 chromatin binding seen in *Drosophila* when cells exit the cell cycle [41] suggests that in contrast to our findings there can be developmental situations where there is a programmed block to licensing in mitosis, in preparation for the cell cycle arrest. Conceivably the process we have described in fission yeast may reflect the fact that Mcm2-7 chromatin binding occurs earlier than the point in the cell cycle at which nutritional cues lead to a G1 arrest.

The mechanism causing displacement of Mcm2-7 from chromatin in G1 is likely to differ from the process in S phase. In contrast to Cdt1, Cdc18 is not detectable in nitrogen-starved cells and loss of this factor would prevent further chromatin association of Mcm2-7 proteins. Consistent with this, in *S. cerevisiae* inactivation of Cdc6 in G1-arrested cells leads to loss of the pre-RC footprint [54]. However, the absence of this factor alone may not be sufficient to explain how loss of chromatin-associated Mcm2-7 proteins occurs during quiescence, since

DNA binding of Mcm2–7 in vitro is stable after removal of Cdc6 [45,55–57]. Conceivably, disassembly of Mcm2–7 from chromatin during quiescence may be an active process that does not simply reflect instability of pre-RCs during cell cycle arrest. A further question is why Mcm2–7 proteins do not remain associated with chromatin, given that licensing must be re-established when cells re-enter the cell cycle? It is possible that restoration of chromatin to an unlicensed state is important for maintaining genome stability, by, for instance, eliminating the possibility for inappropriate activation of replication origins in quiescent cells. In quiescent cells, reversion of origins to an unlicensed state, while maintaining unbound nucleoplasmic Mcm2–7, may represent a compromise between ensuring genome stability and facilitating rapid re-establishment of licensed origins on re-entry to the cell cycle.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2006.07.001.

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