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Osaka University

Doctoral Thesis

**Mcm10 plays an essential role in origin DNA unwinding
after assembly of the CMG components**

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Abstract

The CMG complex, composed of Mcm2-7, Cdc45 and GINS, is postulated to be the eukaryotic replicative DNA helicase. Activation of the CMG complex requires sequential recruitment of components onto Mcm2-7. Current models suggest that Mcm10 is involved in assembly of the CMG complex, and in tethering of DNA polymerase α at replication forks. Here I show that Mcm10 is required for origin DNA unwinding after association of the CMG components with replication origins in fission yeast. A combination of promoter shut-off and the auxin-inducible protein degradation (*off-aid*) system efficiently depleted cellular Mcm10 to less than 0.5% of the wild-type level. Depletion of Mcm10 did not affect origin loading of Mcm2-7, Cdc45 or GINS, but impaired recruitment of RPA and DNA polymerase α and δ . Mutations in a conserved zinc finger of Mcm10 abolished RPA loading after recruitment of Mcm10. These results show that Mcm10, together with the CMG components, plays a novel essential role in origin DNA unwinding through its zinc finger function.

General introduction

In this study, I investigated the essential function of Mcm10, which has been screened as an essential replication factor implicated to be involved in the initiation of DNA replication. In Section 1 of the Introduction, I introduce the fundamental scheme of initiation of DNA replication regulated by the cell cycle. In Section 2, I describe the roles of MCM proteins in DNA replication and raise questions about helicase activation and the essential function(s) of Mcm10.

1. Initiation of DNA replication

A. Cell cycle control of initiation of DNA replication

Initiation of DNA replication is a dynamic reaction regulated by progression of the cell cycle in eukaryotic cells. Regarding enzymatic activities, DNA replication requires DNA helicase that unwinds the origin DNA duplex to provide single-stranded DNA (ssDNA) templates for DNA polymerases that synthesize the leading and the lagging strands. In eukaryotes, the heterohexameric mini-chromosome maintenance 2-7 (Mcm2-7) complex comprises the core of replicative DNA helicase (Bell & Dutta, 2002). In G1 phase, when cyclin-dependent kinase (CDK) activity is low, the Mcm2-7 complex is loaded onto replication origins depending on the origin recognition complex (ORC), Cdc6/Cdc18 and Cdt1, forming the pre-replicative complex (pre-RC) (Bell & Dutta, 2002; Diffley et al, 1994). At the onset of S phase, Dbf4-dependent kinase (DDK) phosphorylates the Mcm2-7 and CDK that phosphorylates Sld2 and Sld3 to promote recruitment of several replication factors onto the Mcm2-7 (Sheu & Stillman, 2010; Tanaka et al, 2007; Zegerman & Diffley, 2007).

Thereafter, a single-stranded DNA binding protein complex, RPA, assembles on the unwound DNA by the activated helicase, and then DNA polymerases are loaded onto origin DNA to establish replication forks (Figure 1).

B. Pre-RC formation in G1 phase

Eukaryotic replication origins are recognized by ORC, which is composed of six subunits (Orc1 to 6). In G1 phase, the Mcm2-7 complex is recruited to origins depending on ORC, Cdt1 and Cdc6/Cdc18, forming pre-RC (Figure 1). Although Mcm2-7 comprises the core of replicative helicase, it remains inactive as helicase at this stage. Reconstitution of pre-RC assembly from purified yeast proteins (Evrin et al, 2009; Kawasaki et al, 2006; Remus et al, 2009) has established that ORC, Cdc6 and Cdt1 are necessary and sufficient to load Mcm2-7 onto origin DNA in a reaction requiring ATP hydrolysis. Cdt1 forms a heptameric complex with soluble Mcm2-7, and origin recruitment of the soluble heptamers (Mcm2-7 and Cdt1) leads to ATP hydrolysis by Cdc6, resulting in the loading of Mcm2-7 and disassociation of Cdt1 from origin (Takara & Bell, 2011). Analysis by electron microscopy (EM) has shown that the two Cdt1/Mcm2-7 complexes assemble into an Mcm2-7 double hexamer on double-stranded DNA (dsDNA). The two hexamers are connected via their N-termini with the C-terminal domain facing outward (Evrin et al, 2009; Remus et al, 2009). This symmetry places the two hexamers in the correct orientations to establish bi-directional replication forks (Figure 1).

C. Helicase activation in S phase

During G1 phase, the Mcm2-7 complex is loaded onto replication origins as helicase-inactive

form. It requires assembly of several essential factors onto the Mcm2-7 complex for helicase activation. Current evidence indicates that two additional factors, Cdc45 and the GINS (go-ichi-ni-san) complex, are essential stoichiometric components of replicative DNA helicase (Bochman & Schwacha, 2009; Ilves et al, 2010; Moyer et al, 2006; Pacek et al, 2006; Takahashi et al, 2005). It has been shown that Cdc45, Mcm2-7 and GINS form a stable 'CMG' complex (Cdc45-Mcm2-7-GINS) that translocates on a ssDNA in the 3' to 5' direction (Ilves et al, 2010; Moyer et al, 2006).

At the onset of S phase, when the CDK activity arises, a number of replication factors act on the pre-RCs, converting the helicase-inactive pre-RCs into the CMG helicases. In budding and fission yeast, DDK phosphorylates the Mcm2-7 complex and stimulates loading of Sld3 onto pre-RCs (Heller et al, 2011; Yabuuchi et al, 2006). CDK phosphorylates Sld3 and Sld2/Drc1, promoting formation of a complex of Sld3-Dpb11/Cut5-Sld2 at replication origins (Fukuura et al, 2011; Masumoto et al, 2002; Tanaka et al, 2007; Zegerman & Diffley, 2007). Finally, these factors collectively recruit GINS and Cdc45 onto the Mcm2-7 complex, resulting in full assembly of the CMG helicase (Figure 1) (Araki, 2010; Remus & Diffley, 2009). Requirement of Sld3, Sld2 and Dpb11 for initiation of DNA replication is likely limited at the assembly step of the CMG helicase (Kanemaki & Labib, 2006; Labib, 2010; Taylor et al, 2011). The vertebrate Sld3 orthologue, known as Treslin/ticrr, has been identified (Kumagai et al, 2010; Sanchez-Pulido et al, 2010; Sansam et al, 2010), and it has recently been shown that its phosphorylation by CDK on residues conserved between yeast Sld3 and Treslin/ticrr is required for DNA replication in human cells (Boos et al, 2011; Kumagai et al, 2011). A requirement for phosphorylation of the putative Sld2 orthologue, RecQ4L (Matsuno et al, 2006; Sangrithi et al, 2005), in replication

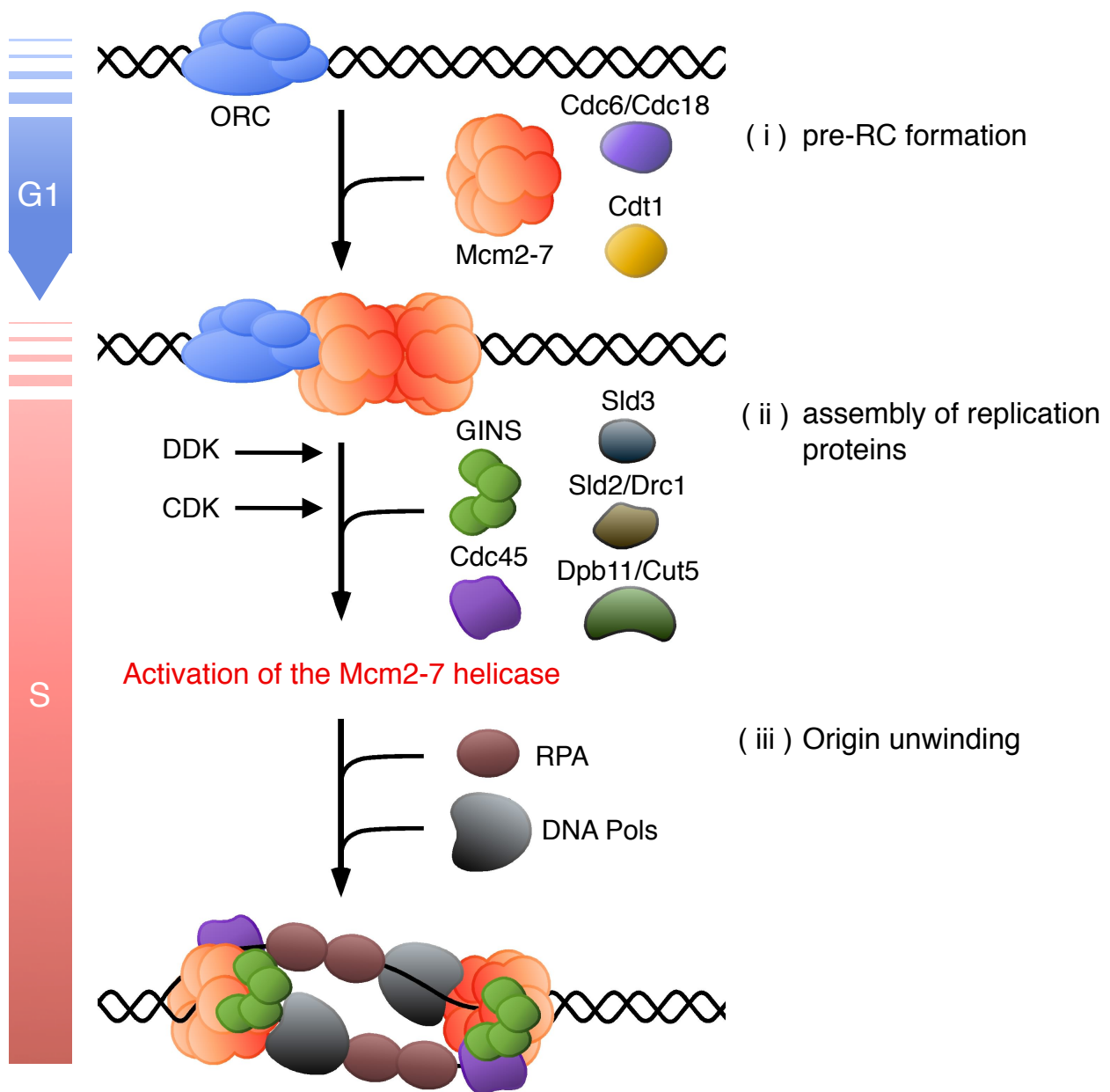


Figure 1. Process of initiation of DNA replication in eukaryotic cells.

(i) In G1 phase, mediated by ORC, Cdt1 and Cdc6/Cdc18, the Mcm2-7 complex that is considered to be a core of replicative helicase is loaded onto replication origin, forming pre-RC. (ii) At the onset of S phase, depending on DDK and CDK activity, several replication factors including GINS and Cdc45 are recruited to pre-RC for activation of the Mcm2-7 helicase. (iii) Then origin DNA is unwound by the activated helicase and RPA, ssDNA binding protein complex, and DNA polymerases are loaded on replication origin to establish replication forks.

has not yet been demonstrated. Origin DNA is then unwound by the activated replicative helicase, and RPA, a eukaryotic ssDNA-binding protein complex, and DNA polymerases are recruited to origins, resulting in assembly of complete replisomes.

2. Roles and functions of MCM proteins

A. The Mcm2-7 complex

The Mcm2-7 complex is a heterohexamer of six essential subunits (numbered 2 through 7), each has a AAA+ ATPase domain at the C-terminus (Forsburg, 2004). Multiple lines of evidence indicate that the Mcm2-7 complex is the engine of the replicative helicase. Mcm2-7 travels with replication forks, as a component of the purified replisome progression complex (RPC) in yeast (Gambus et al, 2006) and vertebrates (Aparicio et al, 2009; Gambus et al, 2011; Im et al, 2009; Pacek et al, 2006). In addition, purified yeast Mcm2-7 complex displays weak helicase activity under appropriate salt conditions (Bochman & Schwacha, 2009). Purification of replisome components from budding yeast cells in S phase has identified a complex containing GINS and Cdc45 as well as Mcm2-7 (Gambus et al, 2006). These factors form a stable complex called ‘CMG’, and the purified CMG complex from *Drosophila* cells has shown to have robust helicase activity (Moyer et al, 2006). *In vitro* experiments have shown that association of GINS and Cdc45 enhances ATP hydrolysis, DNA binding and helicase activity of Mcm2-7 (Ilves et al, 2010).

In vitro experiments have shown that Mcm2-7 is loaded onto dsDNA as helicase-inactive head-to-head double hexamer (Evrin et al, 2009; Remus et al, 2009). In contrast, recent experiments have strongly suggested that the replicative helicase comprises a

single hexamer of Mcm2-7 (with Cdc45 and GINS) bound around ssDNA. The purified *Drosophila* CMG complex, which appears to contain just a single Mcm2-7 hexamer along with Cdc45 and GINS, displaces short oligonucleotide annealed to a single-stranded M13 plasmid in the 3' to 5' direction (Moyer et al, 2006). A 'roadblock' by binding streptavidin to the biotinylated nucleotide in only leading strand roadblocks hampered the advance of the replisome, indicating that Mcm2-7 appears to be a ssDNA translocase, moving 3' to 5' on the leading strand template whilst displacing the lagging strand template (Fu et al, 2011). A structural study using EM has proposed that the central channel of Mcm2-7 in the CMG complex encircles ssDNA and the side channel formed by the GINS-Cdc45 subcomplex, which bridges the Mcm2-5 gap, the weakest point of the Mcm2-7 ring (Davey et al, 2003), likely prevents DNA escape from Mcm2-7 and may help partition the lagging DNA strand from its complement following unwinding (Costa et al, 2011). These findings argue that activation of the replicative DNA helicase involves dynamic structural changes of the Mcm2-7 complex from the dsDNA-bound double hexamer to the ssDNA-bound single hexamer.

B. Mcm10

Mcm10/Cdc23 is an evolutionally conserved protein essential for DNA replication (Aves et al, 1998; Nasmyth & Nurse, 1981). It was identified through genetic screenings for DNA replication defects (Dumas et al, 1982; Solomon et al, 1992) and mini-chromosome maintenance defects (Maine et al, 1984; Merchant et al, 1997). It has been reported that Mcm10 is contained in RPC complex (Gambus et al, 2006), and it localizes at replication fork (Pacek et al, 2006; Ricke & Bielinsky, 2004; Taylor et al, 2011). The central domain of

Mcm10, which contains a zinc finger motif, is required for cell viability in budding yeast (Cook et al, 2003), and is highly conserved among eukaryotes (Homesley et al, 2000; Izumi et al, 2000). *In vitro* studies have shown that purified Mcm10 binds to both ds and ssDNA in human, *Xenopus*, budding yeast, and fission yeast (Eisenberg et al, 2009; Fien et al, 2004; Okorokov et al, 2007; Robertson et al, 2008; Warren et al, 2009; Warren et al, 2008). Although Mcm10 has no sequence homology to Mcm2-7, it physically interacts with the members of the Mcm2-7 complex (Hart et al, 2002; Homesley et al, 2000; Lee et al, 2003; Merchant et al, 1997). Over-expression of Mcm10 partially suppresses the cold sensitivity of the *nda4-108/mcm5* mutant (Hart et al, 2002). Double mutants of *mcm10* with *mcm2-7* mutants exhibit enhanced temperature sensitivity, synthetic lethality or suppression of temperature sensitivity, depending on the mutation alleles (Hart et al, 2002; Homesley et al, 2000; Lee et al, 2010; Liang & Forsburg, 2001). These physical and genetic interactions imply that Mcm10 functions in close coordination with Mcm2-7.

The exact step, at which Mcm10 functions, in the initiation of DNA replication remains enigmatic. It has been reported that Mcm10 is required for recruitment of Cdc45 to activate the Mcm2-7 helicase in budding yeast, fission yeast, and *Xenopus* egg extracts (Gregan et al, 2003; Sawyer et al, 2004; Wohlschlegel et al, 2002). On the other hand, other studies have shown that depletion of Mcm10 does not affect the chromatin association of Cdc45 either *in vivo* or *in vitro* (Heller et al, 2011; Ricke & Bielsky, 2004). Mcm10 has also been shown to be required for tethering DNA polymerase α (Pol α) at replication forks, and for controlling the stability of the catalytic subunit of Pol α in budding yeast and human cells (Chattopadhyay & Bielsky, 2007; Ricke & Bielsky, 2004; Ricke & Bielsky, 2006), whereas siRNA for Mcm10 in human cells does not affect the stability of Pol α (Zhu et al,

2007). Therefore, although these observations consistently suggest that the function of Mcm10 is closely related to the CMG complex and the components of the replisome, the molecular function of Mcm10 is not clearly understood.

Aim of experiments

Activation of the replicative helicase is the most important event to initiate DNA replication. As described above, however, the molecular mechanism how the Mcm2-7 helicase is activated and origin DNA is unwound remains unclear. In this study, I focused on the essential role of Mcm10, which has strong physical and genetic relationship with Mcm2-7. I investigated the essential role of Mcm10 in fission yeast using a recently developed auxin-inducible protein degradation system (*off-aid*) (Kanke et al, 2011; Nishimura et al, 2009). The *off-aid* system is a powerful method for depletion of specific proteins within fission yeast cells and allows me to analyze the null phenotype of the *mcm10*⁺. Based on observations described in this study, I propose a model of helicase activation, in which Mcm10 plays a novel key role in the origin DNA unwinding step for initiation of DNA replication.

Part I: Analysis of roles of Mcm10 in replication initiation

Introduction

DNA helicase activity is essential for initiation and elongation step of DNA replication. In eukaryotes, the Mcm2-7 complex functions as replicative DNA helicase. Mcm2-7 is loaded onto origin DNA in a helicase-inactive form and then activated as a helicase through assembly of other replication factors. Among them, GINS and Cdc45 are the essential co-factors of helicase. They form the CMG complex with Mcm2-7 and travel with replication forks. Interestingly, it has been suggested that a single Mcm2-7 complex (along with GINS and Cdc45) functions as a helicase on ssDNA (Costa et al, 2011; Fu et al, 2011; Moyer et al, 2006), while it is loaded onto dsDNA as a head-to-head double hexamer (Evrin et al, 2009; Remus et al, 2009).

Mcm10 is the conserved and essential replication factor. Although Mcm10 has strong genetic and physical interactions with Mcm2-7 (Hart et al, 2002; Homesley et al, 2000; Lee et al, 2010; Lee et al, 2003; Liang & Forsburg, 2001; Merchant et al, 1997), its role(s) for DNA replication event is still enigmatic. To elucidate the essential function(s) of Mcm10 in replication initiation, I employed a recently developed auxin-dependent protein depletion system, AID, in combination with transcriptional repression (the *off-aid* system) (Kanke et al, 2011; Nishimura et al, 2009). The *off-aid* depletion efficiently removed more than 99% of Mcm10 protein from cells, allowing me to determine precisely the step at which Mcm10 executes its essential function. Interestingly, Mcm10 was not required for assembly of the CMG components on replication origins but for unwinding of origin DNA. In addition, initiation-specific factors, such as Sld3, Cut5 and Drc1, were not released from origins in the

absence of Mcm10. Our results demonstrate that Mcm10 plays a novel key role, together with the CMG components, in the origin DNA unwinding step for initiation of DNA replication.

Results

Mcm10 is required in early stage of DNA replication

To analyze an essential role(s) of Mcm10 in DNA replication, I adopted an approach involving depletion of Mcm10 protein from living cells because the existing mutant allele of Mcm10 was leaky and not suitable for analysis at the molecular level (Liang & Forsburg, 2001). For efficient and complete depletion of the protein, I combined an auxin-inducible degron (AID) system (Kanke et al, 2011; Nishimura et al, 2009) with the conventional thiamine-repressible *nmt81* promoter (P_{nmt81}) ($P_{nmt81-mcm10-aid}: mcm10-off-aid$). Addition of an aid-tag at the C-terminus of Mcm10 did not affect the protein expression level whereas replacement of the promoter region by *Pnmt81* causes about 5-time increase in Mcm10 level under the promoter-on conditions in the absence of thiamine (Figure I-1A and B). *mcm10-off-aid* cells were grown in the presence of thiamine for 14 h to repress *mcm10* transcription, and then synchronized using the temperature-sensitive mutation of *cdc25-22*, which causes cell cycle arrest at the G2/M boundary. Auxin was added 1 h prior to G2/M release into 25°C (see Figure legends). Immunoblotting of cell extracts showed that the Mcm10-aid protein was decreased to as little as 0.5% of the amount in wild-type cells (Figure I-1C and D). Because cellular amount of Mcm10 is estimated to be 3,600 molecules/cell (Figure I-1E), Mcm10 at the time of G2/M release corresponds to less than 18 molecules per cell. I analyzed the DNA contents of the cells by flow cytometry to examine the defect in DNA replication. Cells without depletion generated a 4C DNA peak, indicative of DNA replication, because cytokinesis occurs during the period corresponding to S phase in the normal fission yeast cell cycle (Figure I-1F, left). When non-depleted cells were treated with hydroxyurea (HU), which depletes the dNTP pool, DNA replication was blocked at an

Figure I-1

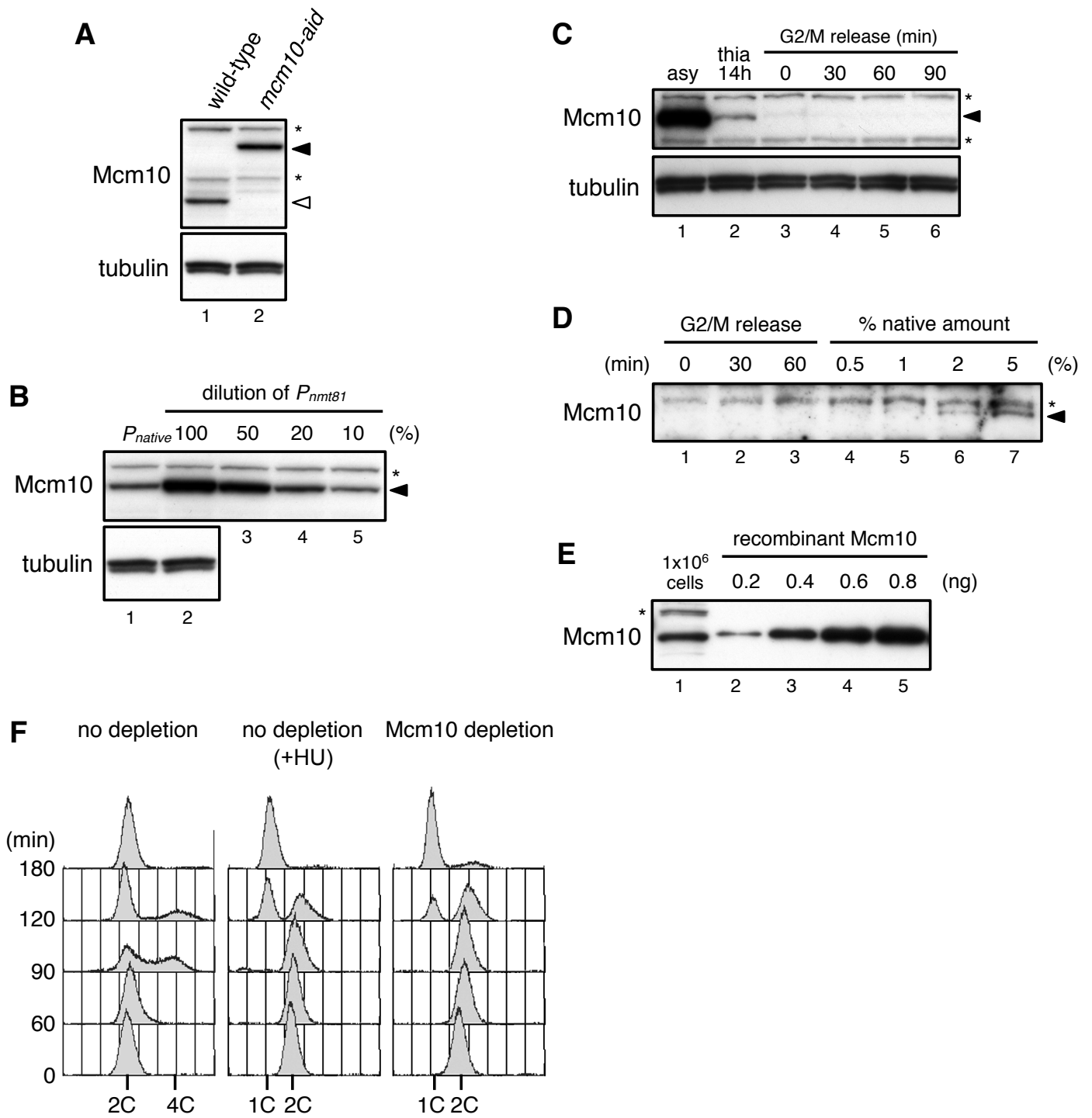


Figure I-1. Depletion of Mcm10 from fission yeast cells.

(A) Proteins in whole-cell extracts from wild-type (lane 1) and *mcm10-aid* cells (lane 2) were analyzed by immunoblotting with anti-Mcm10 and anti- α -tubulin antibodies. Clear and filled arrowheads indicate the positions of Mcm10 and Mcm10-aid, respectively. Non-specific bands are indicated by asterisks. (B) Whole-cell extracts were prepared from the *mcm10-aid* (lane 1) and *mcm10-off-aid* (lane 2) cells and analyzed by immunoblotting as shown in (A). Protein samples from *mcm10-off-aid* cells were diluted by the wild-type (*mcm10*⁺ without an aid-tag) cell extract to the indicated concentrations (lanes 3-5) and compared with the *mcm10-aid* cell extract (lane 1). An arrowhead and an asterisk indicate the positions of Mcm10-aid and a non-specific band, respectively. (C) The *mcm10-off-aid* cells carrying *psf2-flag* and *cdc45-myc* were incubated with thiamine for 14 h and arrested at the G2/M boundary by incubation at 36°C for 3.5 h. Auxin (0.5 mM) was added 1 h before release from G2/M block to 25°C. HU (12 mM) was added to the cells without depletion. The amounts of proteins from asynchronous cells (asy, lane 1), before G2/M arrest (thia 14h, lane 2) and at the indicated time points after release from the G2/M boundary (lanes 3-6) were analyzed by immunoblotting using anti-Mcm10 and anti- α -tubulin antibodies. (D) Immunoblotting analysis with anti-Mcm10 antibody comparing the amount of Mcm10-aid protein after depletion with that of serially diluted Mcm10-aid protein expressed from the native promoter without depletion. The protein sample of *mcm10-aid* was diluted to the indicated concentrations by the wild-type (*mcm10*⁺ without an aid-tag) cell extract. (E) Estimation of the intracellular amount of Mcm10. Extracts from logarithmically growing wild-type cells and purified Mcm10 were analyzed by immunoblotting with anti-Mcm10 antibody. The amount was approximately 0.4 ng per 1×10^6 cells, corresponding to 3,600 molecules per cell. (F) DNA contents of non-depleted cells with (middle) or without (left) HU treatment and Mcm10-depleted cells (right) were analyzed by flow cytometry.

early stage, generating cells with 1C DNA content (Figure I-1F, middle). Mcm10-depleted cells generated a sharp 1C peak similar to HU-arrested cells (Figure I-1F, right), indicating arrest of the cell cycle in the early stage of DNA replication.

Mcm10 is not required for loading of the CMG components onto replication origins

Assembly of the CMG components at replication origins is a key step for initiation of DNA replication. I first examined whether Mcm10 is required for this process. Under Mcm10-depleted conditions, I examined the origin localization of Mcm6, Psf2 (GINS) and Cdc45 by ChIP assay. DNA immunoprecipitated with these factors was quantified by real-time PCR for the *ars2004* and *ars3002* loci, which are efficient replication origins on chromosomes II and III, respectively, and nonARS1, located 30 kb distant from *ars2004*. In HU-arrested cells without depletion, IP recovery of the two origins with Mcm6, Psf2 and Cdc45 was similar to that of nonARS1 at G2/M release (0 min), but was increased at 85 and 100 min (Figure I-2A-C, left), showing that these factors were localized at the origins in S phase. In Mcm10-depleted cells, Psf2 and Cdc45, as well as Mcm6, bound to the origins, as was the case in HU-treated cells (Figure I-2A-C, right). These results show that Mcm10 is not required for origin localization of the CMG components, and suggest that Mcm10 functions downstream of recruitment of the CMG components.

Mcm10 recruitment to replication origins is dependent on the CMG components

Next I examined the dependency of Mcm10 recruitment. If origin loading of Mcm10 was dependent on the CMG components, then depletion of GINS or Cdc45 would impair Mcm10 recruitment. I depleted Psf1 or Cdc45 using the *off-aid* system as described for Mcm10

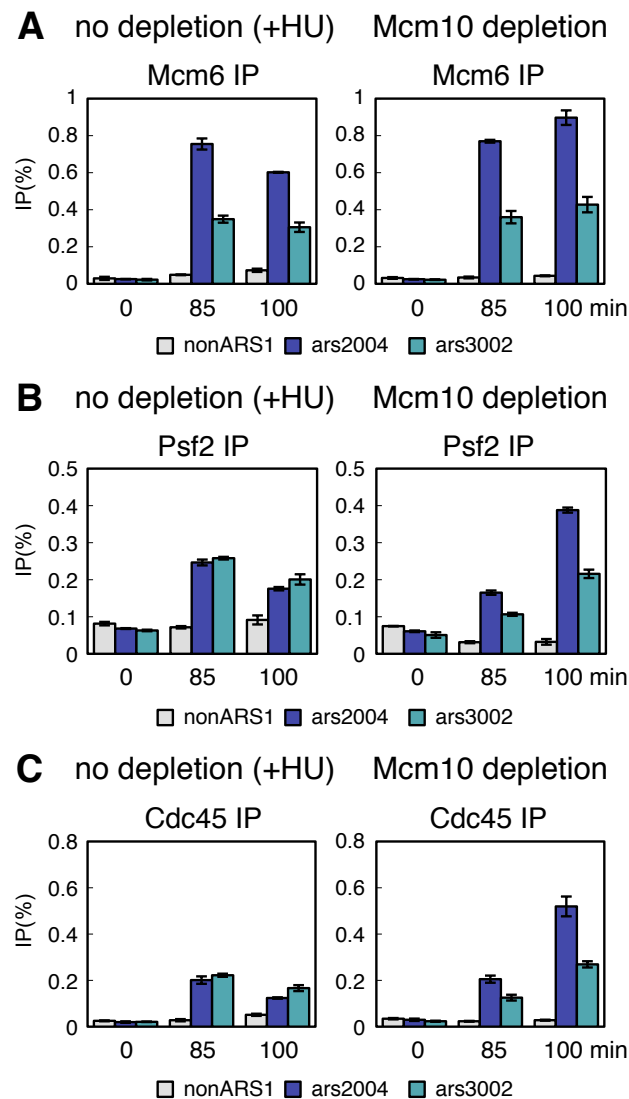


Figure I-2. Localization of the CMG components at replication origins in Mcm10-depleted cells.

(A-C) DNA fragments immunoprecipitated with Mcm6 (A), Psf2-Flag (B) and Cdc45-myc (C) were analyzed by real-time PCR using primer sets for two early origins, *ars2004* (blue) and *ars3002* (cyan), and for the non-origin region, nonARS1 (gray). The columns indicate IP recovery (%) \pm S.D. obtained from triplicate measurements in real-time PCR quantification.

depletion and in the previous study (Kanke et al, 2011). The *psf1-off-aid* or *cdc45-off-aid* cells were incubated with thiamine for transcriptional repression, and auxin was added 1 h prior to G2/M release at 25°C. The amount of Psf1 or Cdc45 after depletion was decreased to less than 1% of the original amount, and the cells were arrested with unreplicated DNA (Figure I-3A-F). Under these conditions, localization of Mcm6 and Mcm10 was examined by ChIP assay. In HU-treated cells without depletion, Mcm6 was preferentially localized at *ars2004* and *ars3002* in S phase (Figure I-3I and K, left). Mcm10, which was not localized at the origins at the G2/M boundary (0 min), was detected at the origins in S phase (Figure 3J and L, left). In Psf1-depleted or Cdc45-depleted cells, however, Mcm10 was not significantly detected at the origins, whereas Mcm6 accumulated (Figure 3I-L, right). To examine whether Mcm10 localization is dependent on a factor that is recruited to origins after assembly of the CMG complex, I depleted Pol α , which binds to chromatin depending on RPA (Walter & Newport, 2000). The catalytic and primase subunits of Pol α (Pol1 and Spp2, respectively) were depleted using the *off-aid* system, resulting in cell cycle arrest at the early stage of DNA replication (Figure 3G and H). The results of ChIP assay showed that Mcm10 bound to the origins in Pol α -depleted cells (Figure 3N, right). These results indicate that GINS and Cdc45, but not Pol α , are required for recruitment of Mcm10 to replication origins, suggesting that Mcm10 is recruited after loading of the CMG components on replication origins.

The CMG components form a complex in the absence of Mcm10

It has been reported that the CMG components form a complex that is stable in the buffer with a high concentration of salt (Gambus et al, 2006). I examined whether the CMG

Figure I-3

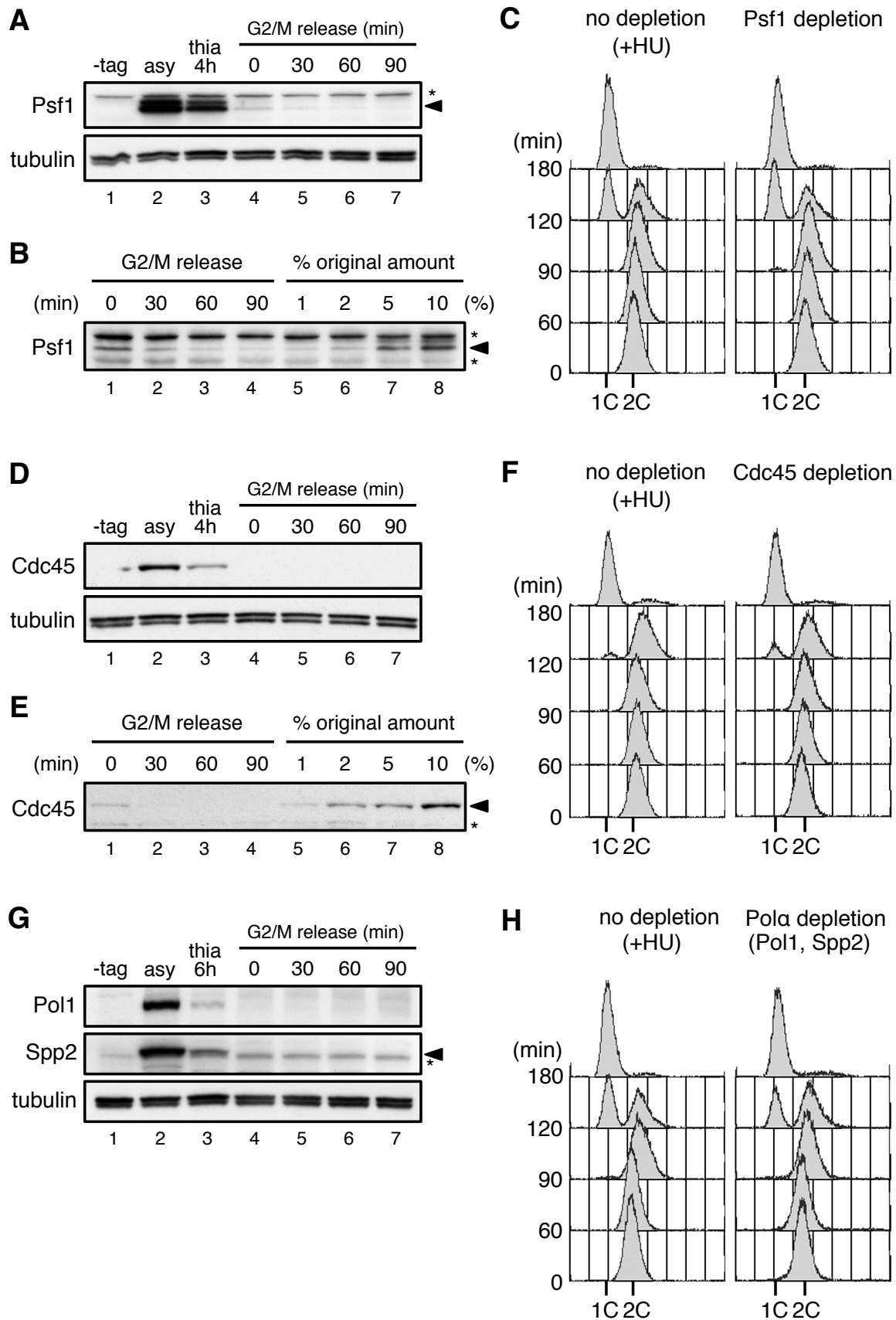


Figure I-3

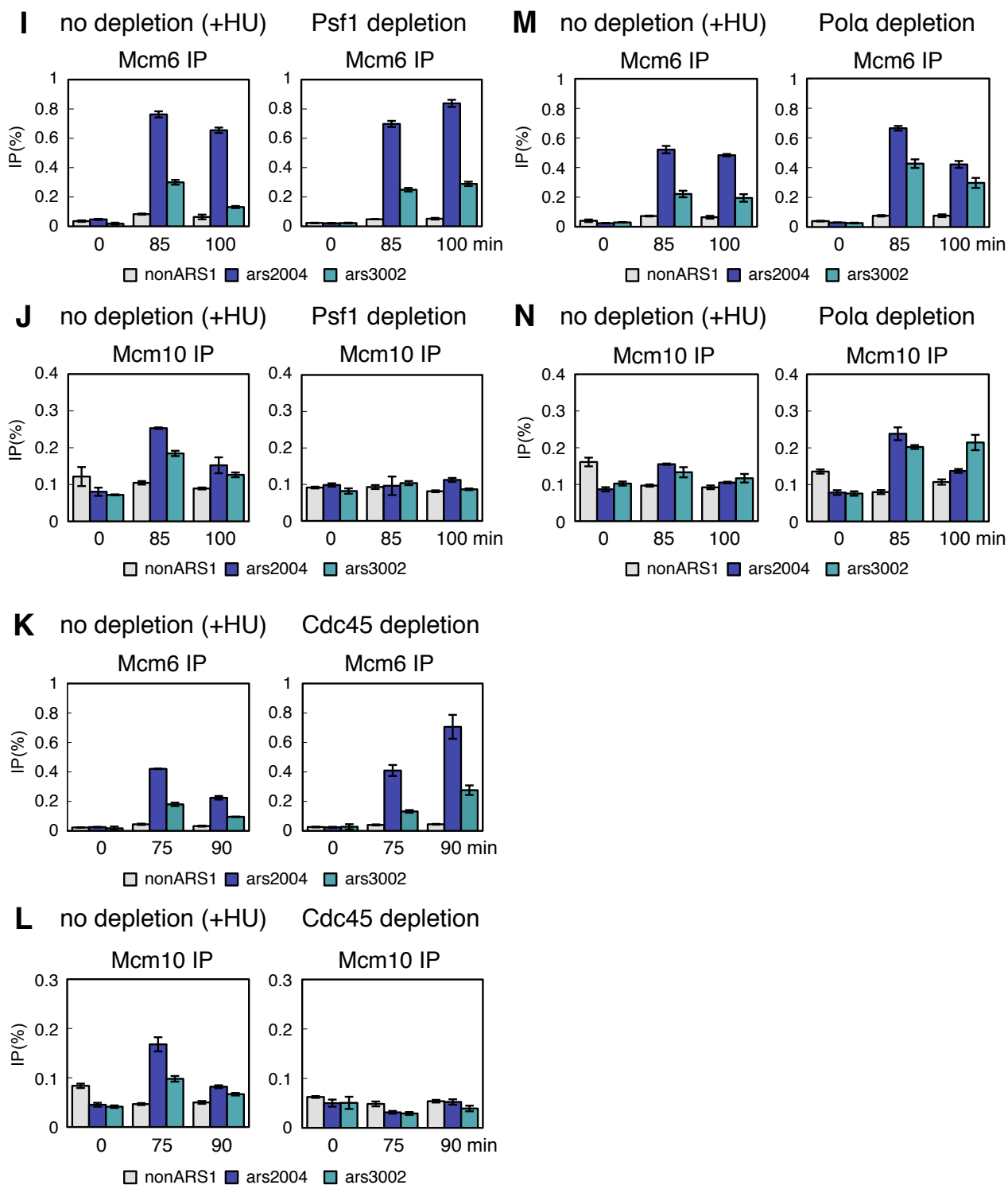


Figure I-3. Mcm10 binds to replication origins depending on GINS and Cdc45, but not on Pol α .

psf1-off-aid cells and *cdc45-off-aid* cells carrying *flag-mcm10* were incubated with thiamine (10 $\mu\text{g/ml}$) for 4 h and then arrested at the G2/M boundary by incubation at 36°C for 3.5 h. *pol1-off-aid spp2-off-aid* cells carrying *flag-mcm10* were incubated with thiamine (10 $\mu\text{g/ml}$) for 6 h and then arrested at the G2/M boundary. Auxin (0.5 mM) was added 1 h before release. The cells were released from G2/M block and HU (12 mM) was added to them without depletion. (A) The amounts of proteins in the cells without an HA-aid-tag on Psf1 (-tag), asynchronous *psf1-off-aid* cells (asy), before G2/M arrest (thia 4h) and at the indicated time points after release from G2/M were analyzed by immunoblotting using anti-HA (Psf1-aid) and anti- α -tubulin antibodies. An arrowhead and an asterisk indicate the positions of Psf1-aid and a non-specific band, respectively. (B) The results of immunoblotting analysis comparing the amount of Psf1-aid protein after depletion with that of serially diluted Psf1-aid protein without depletion are shown. The protein sample from asynchronous cells was diluted to the indicated concentrations by the wild-type (*psf1+* without an aid-tag) cell extract. Psf1-aid protein was decreased to less than 1% of the original amount after G2/M release. (C) The results of flow cytometry analysis of the DNA contents of HU-treated non-depleted cells (left) and Psf1-depleted cells (right) are shown. (D) Proteins in the cells without an aid-tag on Cdc45 (-tag), asynchronous *cdc45-off-aid* cells (asy), before G2/M arrest (thia 4h) and at the indicated time points were analyzed by immunoblotting using anti-Cdc45 and anti- α -tubulin antibodies. (E) Estimation of the amount of Cdc45-aid protein after depletion. The asynchronous protein sample of *cdc45-off-aid* was diluted by the wild-type (*cdc45+* without an aid-tag) cell extract. Cdc45-aid protein was depleted to less than 1% of the original amount after G2/M release. An arrowhead and an asterisk indicate the positions of Cdc45-aid and a non-specific band, respectively. (F) The results of flow cytometry analysis of the DNA contents of HU-treated non-depleted cells (left) and Cdc45-depleted cells (right) are shown. (G) Amounts of protein in the cells without an aid-tag on Pol1 and Spp2 (-tag), asynchronous *pol1-off-aid spp2-off-aid* cells (asy), before G2/M arrest (thia 6h) and at the indicated time points were analyzed by immunoblotting using anti-IAA17 (Pol1-aid and Spp2-aid) and anti- α -tubulin antibodies. An arrowhead and an asterisk indicate the positions of Spp2-aid and a non-specific band, respectively. (H) The results of flow cytometry analysis of the DNA contents of HU-treated non-depleted cells (left) and Pol α -depleted cells (right) are shown. DNA immunoprecipitated with Mcm6 and Flag-Mcm10 in *psf1-off-aid* cells (I and J), *cdc45-off-aid* cells (K and L), or *pol1-off-aid spp2-off-aid* cells (M and N) at the indicated time points was assayed with a real-time PCR system. Columns indicate IP recovery (%) \pm S.D. obtained from triplicate measurements in real-time PCR quantification.

components recruited at the origin in the presence or absence of Mcm10 formed a complex. By immunoprecipitation of Psf2-Flag from HU-treated cell extracts, in which genomic DNA was digested by DNase I, Mcm6 and Cdc45-myc were co-precipitated in the presence of 200 mM NaAc, while these proteins were hardly detected in the precipitates from untagged *psf2*⁺ cells (Figure I-4A, lanes 3-4). Co-precipitation of Mcm6 and Cdc45-myc with Psf2-Flag was specifically observed in S-phase (100 min) but not in G2/M-phase (0 min) cells (Figure I-4B, lanes 5-6). In Mcm10-depleted cells, Mcm6 and Cdc45-myc were co-precipitated with Psf2-Flag, in amounts similar to those in HU-treated cells without depletion (Figure I-4B, lanes 7-8). It has been shown that Mcm4 loaded on chromatin is phosphorylated for initiation of replication at the onset of S phase (Masai et al, 2006; Randell et al, 2010; Sheu & Stillman, 2006; Sheu & Stillman, 2010). I examined whether phosphorylated Mcm4 formed a complex with Psf2 in the absence of Mcm10. Slow-moving forms of Mcm4 were preferentially increased in IP fractions at 100 min in both HU-treated and Mcm10-depleted cells (Figure I-4B, lanes 5-8), suggesting that phosphorylated Mcm4 was enriched in a complex. The mobility of slow-moving Mcm4 differed slightly between HU-treated and Mcm10-depleted cells (Figure I-4B, lanes 6 and 8), probably due to checkpoint-dependent phosphorylations of Mcm4 under HU-arrested conditions (Bailis et al, 2008; Ishimi et al, 2004; Ishimi et al, 2003). The presence of 700 mM NaAc in IP buffer did not cause significant difference in co-IPed Mcm6 or Cdc45 between Mcm10-depleted and HU-treated cells (Figure I-4C). These results suggest that GINS forms a complex with Cdc45 and Mcm2-7 on chromatin in the absence of Mcm10.

Mcm10 is required for origin DNA unwinding

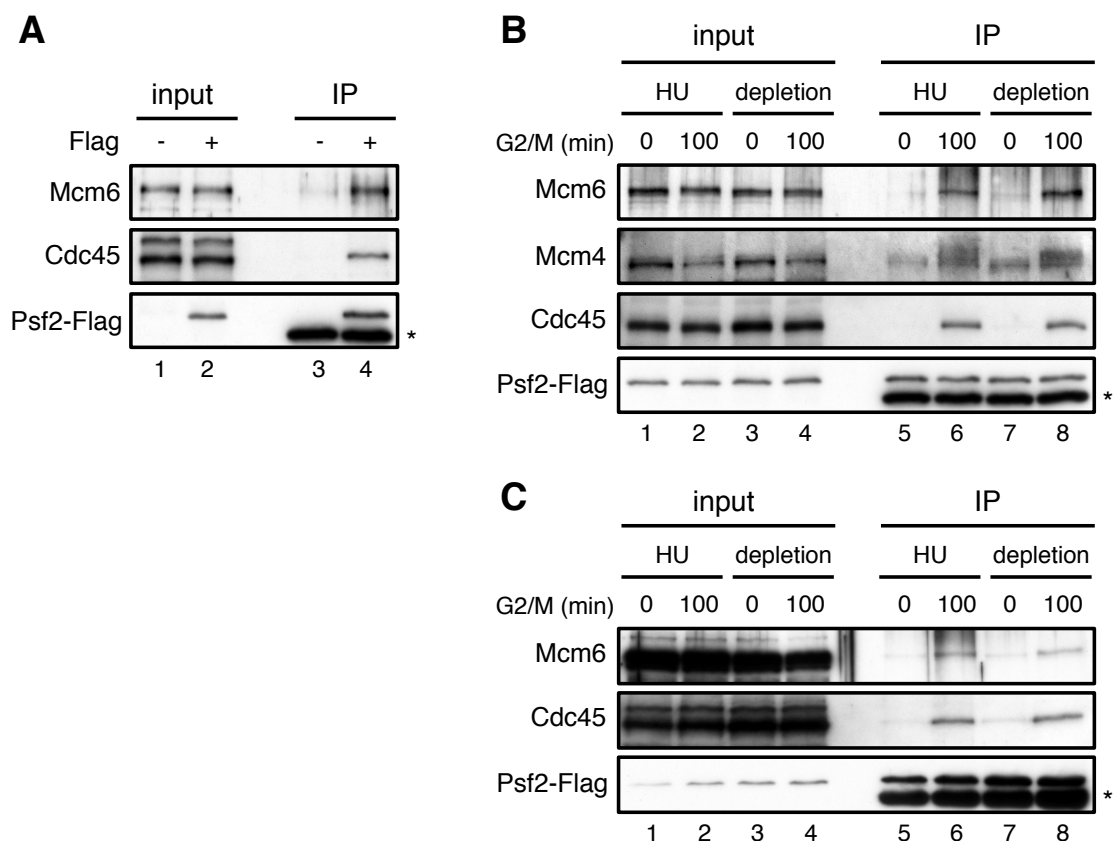


Figure I-4. Mcm4, Mcm6 and Cdc45 form a complex with Psf2 (GINS) in the absence of Mcm10.

(A) Co-precipitation of Mcm6 and Cdc45 with Psf2-Flag. *mcm10-off-aid psf2-flag cdc45-myc* (Flag +) and *mcm10-off-aid cdc45-myc* (Flag -) cells were arrested at the G2/M boundary and released in the presence of HU (12 mM). Cell extracts were prepared at 100 min with HU, and proteins were immunoprecipitated with anti-Flag antibody. Co-immunoprecipitated proteins (lanes 3-4) were analyzed by immunoblotting using anti-Mcm6, anti-Cdc45 and anti-Flag antibodies. The samples used as input (lanes 1-2) corresponded to 0.2%, 1% and 10% of proteins used for immunoprecipitation of Mcm6, Cdc45 and Psf2, respectively. An asterisk indicates the IgG band. (B) *mcm10-off-aid psf2-flag cdc45-myc* cells with or without Mcm10 depletion were synchronously released from G2/M block. HU (12 mM) was added to the cells without depletion. Cell extracts were prepared at G2/M release (0 min, lanes 1, 3, 5 and 7) and at 100 min (lanes 2, 4, 6 and 8), and proteins co-immunoprecipitated with Psf2-Flag (lanes 5-8) were analyzed by immunoblotting with anti-Mcm6, anti-Mcm4, anti-Cdc45 and anti-Flag antibodies. The samples used as input (lanes 1-4) corresponded to 0.2%, 0.1%, 1% and 10% of proteins used for immunoprecipitation of Mcm6, Mcm4, Cdc45 and Psf2, respectively. (C) The same experiments as (B) was performed in the presence of 700 mM NaAc. The samples used for input (lanes 1-4) corresponded to 5% of the proteins used for immunoprecipitation.

Because components of the CMG complex, which has been shown to exhibit robust DNA unwinding activity *in vitro* (Ilves et al, 2010; Moyer et al, 2006), bound to replication origins and formed a complex under depletion of Mcm10, I examined whether origin DNA was unwound. The localization of Rpa2, the second largest subunit of the ssDNA-binding protein complex (RPA), was examined by ChIP assay. In HU-treated cells without depletion, Rpa2 was localized at early origins in S phase (Figure I-5A, left, 75 and 90 min). In contrast, Rpa2 was hardly detected at the origins in Mcm10-depleted cells (Figure I-5A, right). Because HU causes replication fork arrest resulting in accumulation of RPA, I used unperturbed cells to examine the localization of Rpa2, as well as that of Mcm6 and Psf2 (GINS). At 60-70 min after release from G2/M block, Mcm6, Psf2 and Rpa2 were transiently localized to the origin under normal conditions (Figure I-5B-D, left). In Mcm10-depleted cells, origin binding of Rpa2 was greatly decreased, despite accumulation of Mcm6 and Psf2 at the origins (Figure I-5B-D, right). These results suggest that Mcm10 is required for the origin DNA unwinding after loading of the CMG components. Since RPA binding is a proxy for ssDNA formation, it could show, for example, that Mcm10 is required for RPA recruitment itself.

Under conditions where origin unwinding was blocked in the absence of Mcm10, the CMG components would not translocate from replication origins. I examined the distribution of Cdc45 as well as Rpa2 around *ars2004*. In HU-treated cells without Mcm10 depletion, Cdc45 and Rpa2 were widely distributed up to 20 kb, but not at 30 kb (nonARS1) (Figure I-5E and F, left). In sharp contrast, in Mcm10-depleted cells, Cdc45 was localized within a region of 1 kb, but not in distant regions (Figure I-5E, right). No significant localization of Rpa2 was detected at any of the positions examined in the absence of Mcm10

Figure I-5

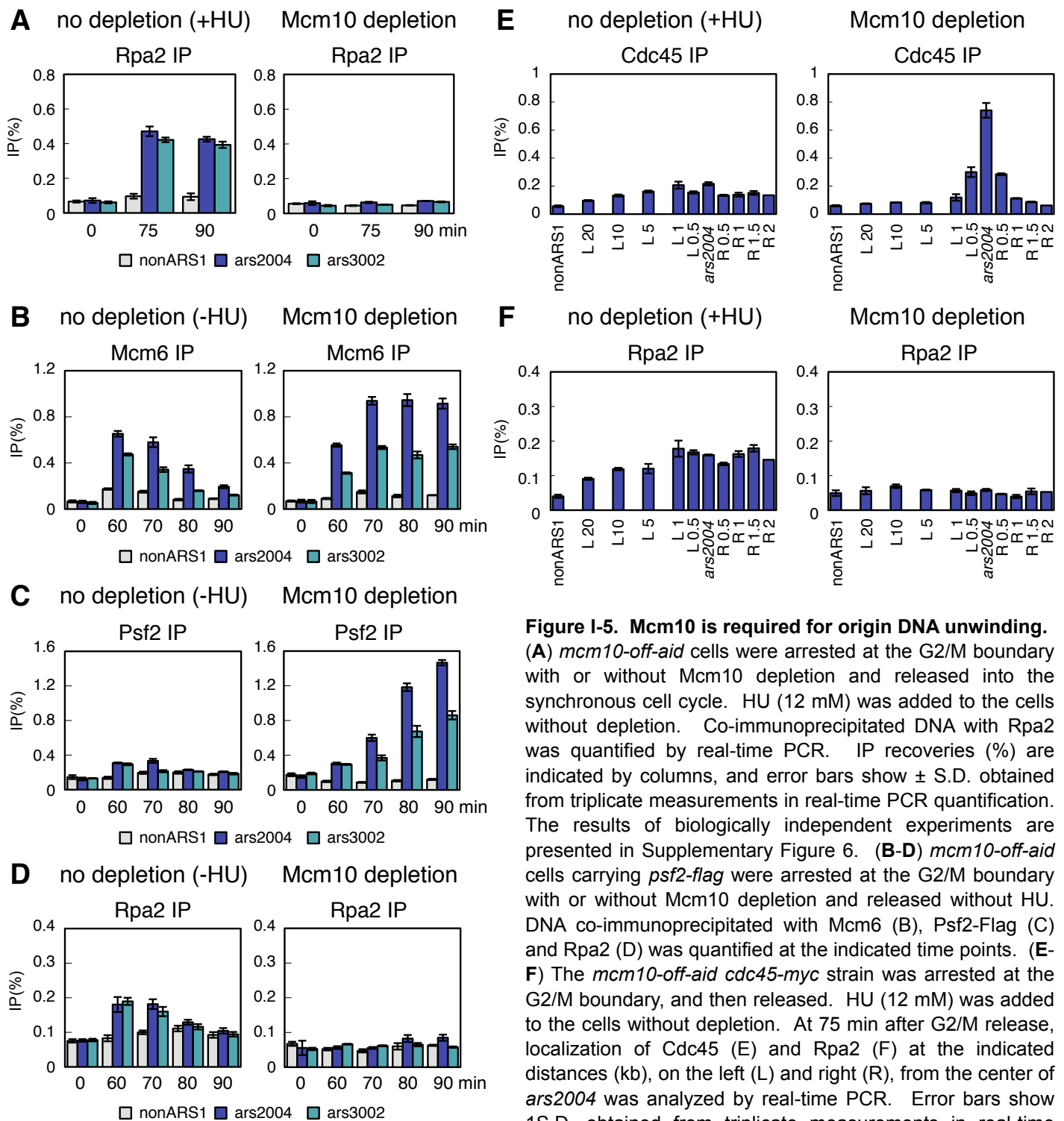


Figure I-5. Mcm10 is required for origin DNA unwinding. (A) *mcm10-off-aid* cells were arrested at the G2/M boundary with or without Mcm10 depletion and released into the synchronous cell cycle. HU (12 mM) was added to the cells without depletion. Co-immunoprecipitated DNA with Rpa2 was quantified by real-time PCR. IP recoveries (%) are indicated by columns, and error bars show \pm S.D. obtained from triplicate measurements in real-time PCR quantification. The results of biologically independent experiments are presented in Supplementary Figure 6. (B-D) *mcm10-off-aid* cells carrying *psf2-flag* were arrested at the G2/M boundary with or without Mcm10 depletion and released without HU. DNA co-immunoprecipitated with Mcm6 (B), Psf2-Flag (C) and Rpa2 (D) was quantified at the indicated time points. (E-F) The *mcm10-off-aid cdc45-myc* strain was arrested at the G2/M boundary, and then released. HU (12 mM) was added to the cells without depletion. At 75 min after G2/M release, localization of Cdc45 (E) and Rpa2 (F) at the indicated distances (kb), on the left (L) and right (R), from the center of *ars2004* was analyzed by real-time PCR. Error bars show 1S.D. obtained from triplicate measurements in real-time PCR quantification.

(Figure I-5F, right). These results suggest that Mcm10 is required for transition of the assembled CMG components into a translocatable helicase complex.

Mcm10-dependent origin unwinding is prerequisite for replisome assembly

Absence of origin DNA unwinding would impair assembly of the replisome complex. I examined the localization of the catalytic subunits of Pol α (Pol1), Pol δ (Cdc6) and Pol ϵ (Cdc20), respectively, in the absence of Mcm10. In HU-treated cells without depletion, *ars2004* and *ars3002*, but not nonARS1, were enriched by Pol1-, Cdc6- or Cdc20-IP at 75-90 min, indicating that these factors bound to replication origins (Figure I-6A-C, left). In contrast, neither Pol1 nor Cdc6 was localized at the origins under conditions of Mcm10 depletion (Figure I-6A and B, right). These results suggest that Mcm10-dependent origin unwinding is prerequisite for replisome assembly. Although these results are consistent with previous reports indicating that Mcm10 is required for chromatin binding of Pol α (Ricke & Bielinsky, 2004; Zhu et al, 2007), I did not observe any decrease in the cellular amount of the catalytic subunit in the absence of Mcm10 (Figure I-6D), unlike the reported requirement of Mcm10 for stabilization of Pol α in budding yeast and human cells (Chattopadhyay & Bielinsky, 2007; Ricke & Bielinsky, 2004; Ricke & Bielinsky, 2006). In contrast to Pol1 and Cdc6, Cdc20 accumulated at origins under Mcm10-depleted conditions (Figure I-6C, right). Pol ϵ and GINS are mutually required for assembly onto replication origins before initiation of DNA replication in budding yeast (Muramatsu et al, 2010) and fission yeast (Handa et al, 2012). The results that Mcm10 is not required for recruitment of Pol ϵ is consistent with the observation that Mcm10 is not required for loading of GINS. Therefore, Mcm10 is not required for origin recruitment of Pol ϵ but prerequisite for recruitment of Pol α

Figure I-6

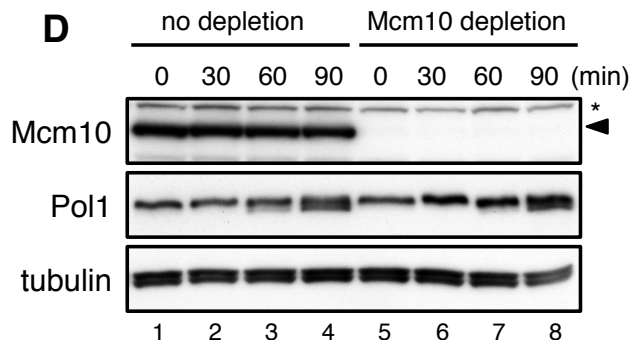
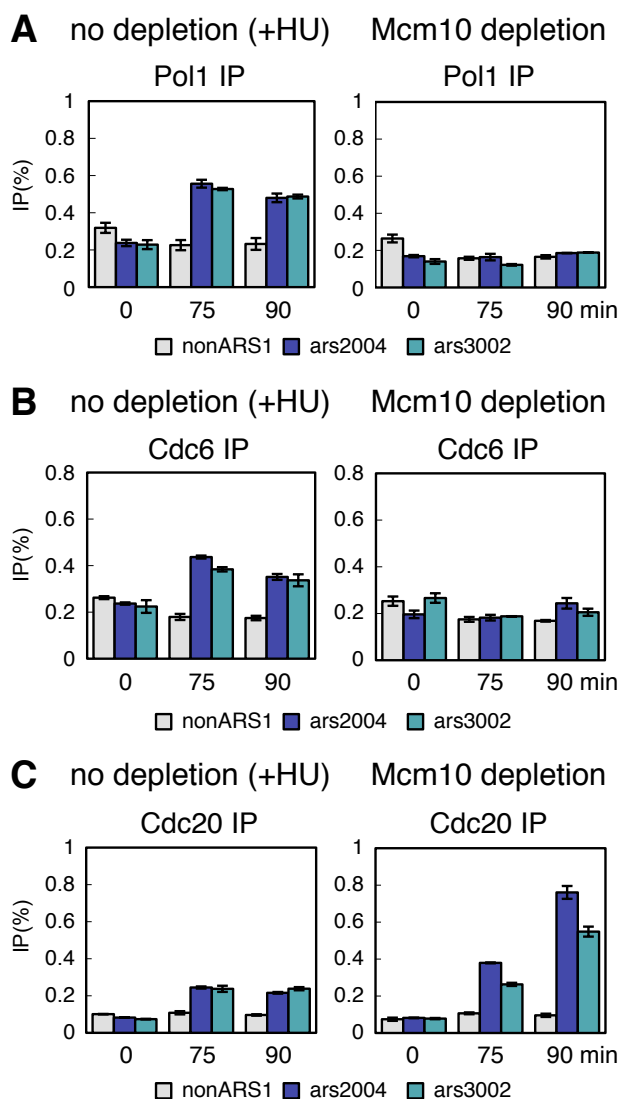


Figure I-6. Replisome assembly is dependent on Mcm10.

(A-C) *mcm10-off-aid* cells carrying *pol1-flag*, *cdc6-flag* or *cdc20-flag* were arrested at the G2/M boundary with or without Mcm10 depletion and released into the synchronous cell cycle. HU (12 mM) was added to the cells without depletion. DNA co-immunoprecipitated with Pol1-Flag (Pol α) (A), Cdc6-Flag (Pol δ) (B) and Cdc20-Flag (Pol ϵ) (C) was measured by real-time PCR. IP recoveries (%) are indicated by columns, and error bars show S.D. obtained from triplicate measurements in real-time PCR quantification. (D) The amounts of Mcm10-aid, Pol1 and α -tubulin in the HU-treated cells without depletion (lanes 1-4) and in the Mcm10-depleted cells (lanes 5-8) used in Figure 5A were analyzed by immunoblotting with anti-Mcm10, anti-Flag (Pol1) and anti- α -tubulin antibodies. An arrowhead and an asterisk indicate the position of Mcm10-aid and a non-specific band, respectively.

and Pol δ , which occurs after origin unwinding.

Mcm10 seems to be essential specifically in initiation of DNA replication

The results presented above suggest that Mcm10 plays an essential role in initiation of DNA replication, which is prerequisite for origin assembly of RPA and DNA polymerases. It is possible that Mcm10 plays a role, in addition to that in origin unwinding, in the association of Pol α with the replisome, because Mcm10 physically interacts with the catalytic subunit of Pol α (Chattopadhyay & Bielsky, 2007; Fien et al, 2004; Ricke & Bielsky, 2004; Robertson et al, 2008; Warren et al, 2009). Since Mcm10 is localized at replication forks in budding yeast, fission yeast and *Xenopus* egg extracts (Gambus et al, 2006; Pacek et al, 2006; Ricke & Bielsky, 2004; Taylor et al, 2011), Mcm10 may also play some essential roles at replication forks. To test this possibility, I depleted Mcm10 from HU-arrested cells and examined whether DNA replication resumed after HU removal. As shown in Figure I-7A, *mcm10-off-aid* cells were arrested at G2/M boundary and transcription of Mcm10 was repressed by addition of thiamine. Then, the cells were released in the presence of HU and auxin was added to deplete the protein. The results of immunoblotting show that, while a substantial level of Mcm10-aid protein remained in HU-arrested cells with thiamine repression (Figure I-7B, lane 3), it was decreased by auxin-induced degradation to an invisible level at the time of HU release (Figure I-7B, lane 4). The amount of Mcm10-aid after depletion was as little as 0.5% of the amount in the wild-type, corresponding to 18 molecules per cell (Figure I-7C). The number is much smaller than expected number of active replication forks (about 200 forks per cell), which was estimated by the assumption that bidirectional replication initiated from 307 early origins at the average firing efficiency of

Figure I-7

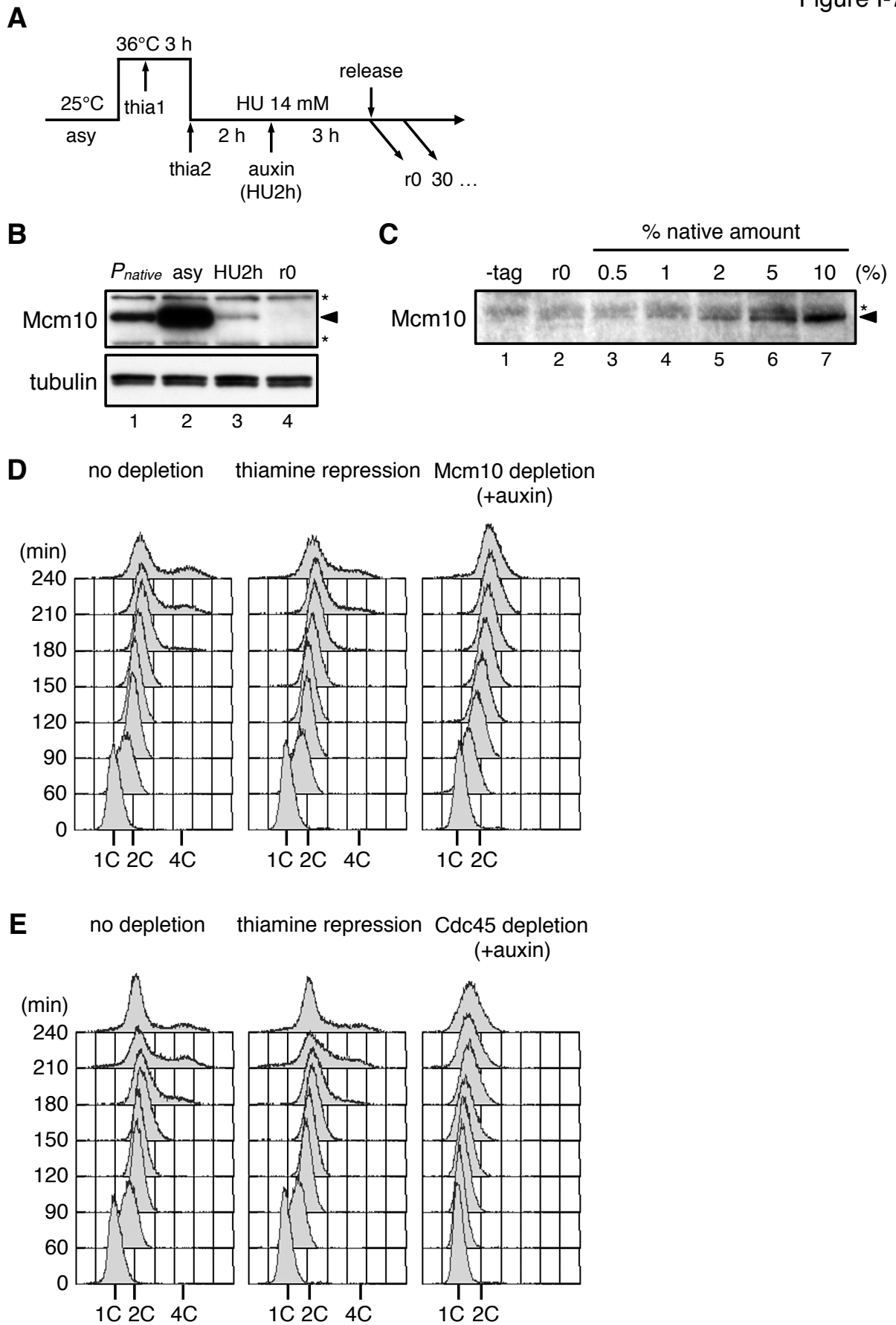


Figure I-7. Mcm10 is not essential after initiation of DNA replication.

(A) Experimental scheme is shown. *mcm10-off-aid* or *cdc45-off-aid* cells were arrested at G2/M boundary by incubation at 36°C for 3 h. To repress transcription of *mcm10-off-aid* or *cdc45-off-aid*, thiamine (10 µg/ml) was added 2 h before (thia1) or just at (thia2) G2/M release, respectively. The cells released from G2/M block were cultured for 2 h with HU (14 mM) to allow initiation from early origins but limit progression of replication. Auxin (0.5 mM) was added to the culture (HU2h) and cells were incubated for 3 h to degrade Mcm10 or Cdc45, and then released into fresh media without HU (r0) and collected at indicated time points for flow cytometry analysis. (B) Whole-cell extracts were prepared from *mcm10-aid* cells (P_{native} , lane 1), the asynchronous *mcm10-off-aid* cells before depletion in (A) (asy, lane 2), after 2 h incubation with HU (HU2h, lane 3) and after 3 h with auxin (r0, lane 4), and analyzed by immunoblotting with anti-Mcm10 and anti- α -tubulin antibodies. An arrowhead and an asterisk indicate the positions of Mcm10-aid and non-specific bands, respectively. (C) The amount of Mcm10-aid after depletion was estimated. Protein samples from *mcm10-aid* cells (lane 1 in (B)) were diluted by the wild-type (*mcm10*⁺ without an aid-tag) cell extracts to the indicated concentrations (lanes 3-7) and compared with the extract prepared at the time of HU release (r0, lane 2). The wild-type extract was loaded in lane 1 (-tag). An arrowhead and an asterisk indicate the positions of Mcm10-aid and a non-specific band, respectively. (D) The results of flow cytometry analysis of *mcm10-off-aid* cells after HU release without depletion (left), with thiamine repression (middle) and with Mcm10 depletion (right) are shown. (E) The results of flow cytometry analysis of *cdc45-off-aid* cells after HU release without depletion (left), with thiamine repression (middle) and with Cdc45 depletion (right) are shown.

30% (Hayashi et al, 2007). Upon HU release, the DNA contents of Mcm10-depleted cells increased to 2C DNA as non-depleted and thiamine-repressed cells (Figure I-7D, left and middle), although Mcm10-depleted cells did not enter the next cell cycle (Figure I-7D, right). The lack of origin firing after HU removal in Mcm10-depleted cells probably resulted in a slight delay in increase of DNA contents and failure in entry into the next cell cycle. On the other hand, DNA contents did not significantly increase from 1C DNA when Cdc45, the essential co-factor of the replicative helicase, was depleted from the cells (Figure I-7E), indicating failure in elongation of replication, while non-depleted and thiamine-treated cells resumed DNA replication. These results suggest that Mcm10 is not essential after initiation of DNA replication.

Initiation-specific factors accumulate at replication origins in the absence of Mcm10

Sld3, Dpb11/Cut5 and Sld2/Drc1 are localized at replication origins and required for loading of GINS and Cdc45 to replication origins (Fukuura et al, 2011; Masumoto et al, 2002; Tanaka et al, 2007; Yabuuchi et al, 2006; Zegerman & Diffley, 2007). Because these factors do not translocate with the CMG in the replisome progression complex (Kanemaki & Labib, 2006; Labib, 2010; Taylor et al, 2011), they appear to be released from the CMG at a specific step during or after initiation. To examine whether these factors are released by recruitment of GINS and Cdc45 in the absence of Mcm10, I examined the localization of Sld3, Cut5 and Drc1 by ChIP assay. As a control condition for Cut5 localization, cells were not treated with HU to avoid loading of Cut5 onto the stalled replication forks (Taylor et al, 2011). Sld3 and Cut5 were localized only transiently at origins in cells without HU (Figure I-8A and B, left). Drc1 was transiently detected in HU-treated cells without Mcm10 depletion (Figure I-8C,

left). In contrast, all of these factors were highly accumulated at the replication origins in Mcm10-depleted cells (Figure I-8A-C, right). These observations suggest that loading of GINS and Cdc45 does not cause release of Sld3, Cut5 or Drc1, and that they dissociate from origins upon or after recruitment of Mcm10.

Figure I-8

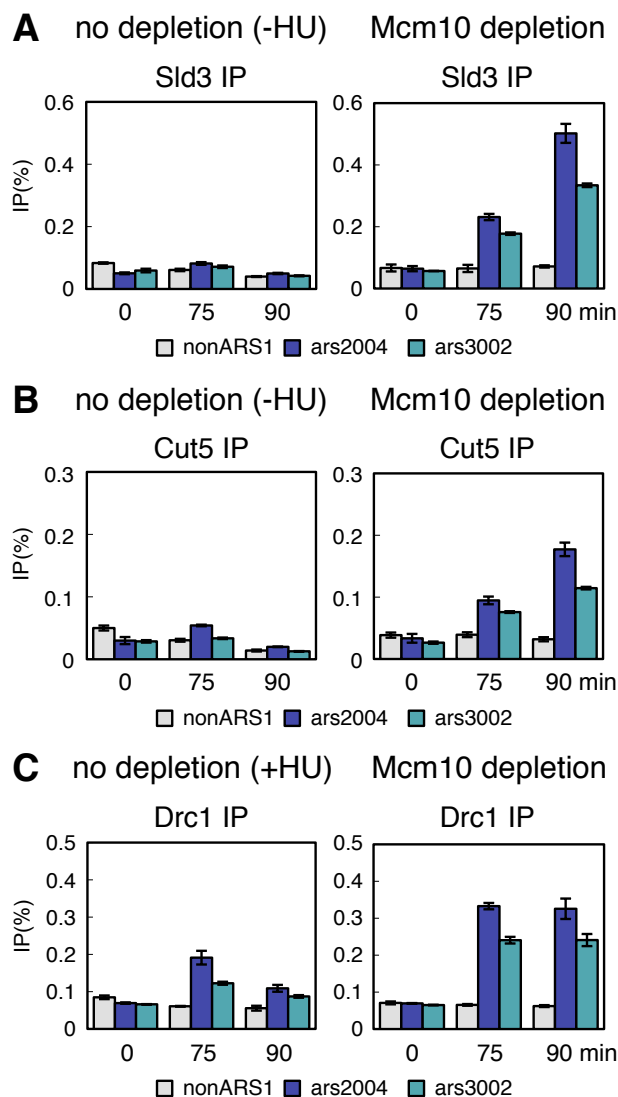


Figure I-8. Sld3, Cut5 and Drc1 accumulate at replication origins in the absence of Mcm10.

(A-B) *mcm10-off-aid* cells carrying *sld3-flag* and *cut5-myc* were arrested at the G2/M boundary with or without Mcm10 depletion and released into the synchronous cell cycle. DNA co-immunoprecipitated with Sld3-Flag (A) and Cut5-myc (B) was measured by real-time PCR. IP recoveries (%) are indicated by columns, and error bars show S.D. obtained from triplicate measurements in real-time PCR quantification. (C) *mcm10-off-aid* cells carrying *drc1-flag* were arrested at the G2/M boundary with or without Mcm10 depletion and released into the synchronous cell cycle. HU (12 mM) was added to the cells without depletion. DNA co-immunoprecipitated with Drc1-Flag was quantified by real-time PCR.

Discussion

Mcm10 is not required for assembly of the CMG components

At the onset of S phase, replication factors assemble to replication origins through an ordered process under regulation by CDK and DDK. First, DDK promotes loading of Sld3 to the origins, and this step is prerequisite for loading of other replication factors (Heller et al, 2011; Yabuuchi et al, 2006). CDK is required for origin loading of GINS, Pol ϵ , Cut5 and Drc1, which are dependent on each other (Fukuura et al, 2011; Handa et al, 2012; Muramatsu et al, 2010; Yabuuchi et al, 2006)). Stable binding of Cdc45 depends on CDK and the other replication factors, including Sld3, Cut5, Drc1 and GINS (Kamimura et al, 2001; Kanemaki & Labib, 2006; Yabuuchi et al, 2006; Yamada et al, 2004). Using the *off-aid* system that decreases Mcm10 to less than 0.5% of the wild-type amount (Figure I-1), I demonstrated that Mcm10 is not required for recruitment of GINS, Cdc45 or the other factors described above (Figures I-2 and 8). In addition, Mcm6, phosphorylated Mcm4 and Cdc45 were co-immunoprecipitated with Psf2 from Mcm10-depleted cells at efficiencies similar to those from HU-treated early S-phase cells (Figure I-4), suggesting that they form a complex in the absence of Mcm10. However, the complex might differ from the helicase-active CMG complex, because the initiation-specific factors, such as Sld3, Cut5 and Drc1, associated with the origins under the conditions employed (Figure I-8). Mcm10 function may be required for conversion of the origin-assembled complex into the active replicative helicase (see below). It is unlikely that residual Mcm10 molecules support the assembly of the CMG and the other factors, because Mcm10 was depleted to less than 18 molecules per cell by the *off-aid* system (Figure I-1), which is far below the number of active replication origins in fission yeast (Feng et al, 2006; Hayashi et al, 2007; Segurado et al, 2003). My results are

consistent with an *in vitro* study demonstrating that Mcm10 is not required for assembly of Dpb11, GINS and Cdc45 at origins in budding yeast cell extracts (Heller et al, 2011). However, the possibility that Mcm10 may affect the conformation and/or stability of the CMG complex after initiation event remains, because previous studies have shown that Mcm10 promotes stable chromatin binding of Cdc45 in *Xenopus* egg extracts, budding yeast and fission yeast (Gregan et al, 2003; Sawyer et al, 2004; Wohlschlegel et al, 2002).

Origin binding of Mcm10 is dependent on GINS and Cdc45 (Figure I-3), which is consistent with a budding yeast *in vitro* study (Heller et al, 2011) and with a recent *in vivo* study (Watase et al, 2012). However, work from another group has shown that Mcm10 interacts with chromatin-loaded Mcm2-7 even in G1 (van Deursen et al, 2012). Because purified Mcm10 interacts with subunits of Mcm2-7 *in vitro* (Lee et al, 2003), Mcm10 may transiently associate with Mcm2-7 in G1, which would not be detected by ChIP assay.

Mcm10 is required for unwinding of origin DNA

Under Mcm10 depletion, RPA was not localized at replication origins despite accumulation of the CMG components (Figures I-2 and 5). This suggests that origin DNA was not significantly unwound, although I cannot exclude the possibility that Mcm10 is required for recruitment of RPA to unwind DNA. I propose that Mcm10 plays an essential role in origin DNA unwinding that is catalyzed by the CMG complex. Similar results indicating that Mcm10 is required for origin DNA unwinding, but not for recruitment of GINS or Cdc45, have been obtained in two independent *in vivo* studies in budding yeast (van Deursen et al, 2012; Watase et al, 2012), suggesting that the essential function of Mcm10 required for origin DNA unwinding is conserved.

My results showed that origin localization of Pol α and Pol δ was also impaired in the absence of Mcm10. These observations are consistent with previous *in vivo* and *in vitro* studies (Heller et al, 2011; Ricke & Bielsky, 2004; Zhu et al, 2007), although I did not observe any destabilization of the catalytic subunit of Pol α (Figure I-6D). Pol α might fail to bind to the origins due to reduction of RPA binding, because depletion of RPA reduces chromatin binding of Pol α in *Xenopus* egg extracts (Walter & Newport, 2000). However, it is possible that Mcm10 tethers Pol α to replication forks after initiation of DNA replication because Mcm10 physically interacts with the catalytic subunit and localizes at replication forks (Chattopadhyay & Bielsky, 2007; Fien et al, 2004; Gambus et al, 2006; Pacek et al, 2006; Ricke & Bielsky, 2004; Robertson et al, 2008; Taylor et al, 2011; Warren et al, 2009). Even in such case, the roles of Mcm10 at replication forks do not seem to be essential for elongation of DNA replication, because depletion of Mcm10 from HU-arrested fission yeast cells did not cause severe inhibition of DNA replication after HU removal (Figure I-7). These observations suggest that Mcm10 plays the essential role specifically in the initiation of DNA replication.

Mcm10 function is required for dissociation of initiation-specific factors from origins

I observed that initiation-specific factors such as Sld3, Cut5 and Drc1, which do not travel with replication forks (Kanemaki & Labib, 2006; Labib, 2010; Taylor et al, 2011), accumulated at replication origins in the absence of Mcm10 (Figure I-8). These results suggest that Mcm10 is required for a step at which initiation-specific factors are released from origins. Release of Sld3, Cut5 and Drc1 from origins requires the essential function of Mcm10. Mcm10 may enforce conformational changes of the Mcm2-7 complex allowing

origin DNA unwinding, which indirectly causes release of initiation-specific factors from origins, although it remains possible that dissociation of these factors is needed for the change in the helicase.

Part II: Roles of the zinc finger of Mcm10 in initiation of DNA replication

Introduction

Mcm10 is an evolutionally conserved protein that is essential for cell viability. Several studies have reported that Mcm10 physically interacts with Mcm2-7 as well as other replication factors (Apger et al, 2010; Christensen & Tye, 2003; Gouge & Christensen, 2010; Hart et al, 2002; Homesley et al, 2000; Izumi et al, 2000; Lee et al, 2003; Merchant et al, 1997; Sawyer et al, 2004; Zhu et al, 2007). Substantial sequence similarities among Mcm10 homologs are restricted to a conserved internal domain of about 210 amino acid residues (Homesley et al, 2000; Izumi et al, 2000), suggesting a critical role for this domain in Mcm10 function. Indeed, several mutations that induce cell lethality or temperature sensitivity have been identified within this domain (Cook et al, 2003; Grallert & Nurse, 1997; Izumi et al, 2000; Liang & Forsburg, 2001; Maine et al, 1984; Nasmyth & Nurse, 1981). In fission yeast, the internal 318 amino acid region supports cell growth of *mcm10 ts* mutant (Lee et al, 2003). Because it has been shown that fission yeast or *Drosophila* Mcm10 support growth of budding yeast *mcm10* mutant (Aves et al, 1998; Christensen & Tye, 2003), the essential role of Mcm10, specifically of the conserved internal domain, seems to be conserved among eukaryotes. This domain contains an oligonucleotide/oligosaccharide (OB)-fold and a conserved zinc finger motif, both of which are the classic DNA binding motif (Warren et al, 2008), and Mcm10 has been shown to bind both dsDNA and ssDNA (Eisenberg et al, 2009; Fien et al, 2004; Okorokov et al, 2007; Robertson et al, 2008). Study in budding yeast has shown that mutations in the zinc finger motif impair self-interaction of Mcm10 and lead cell

lethality or temperature sensitivity (Cook et al, 2003), indicating crucial function(s) of the motif.

In Part I, I showed that Mcm10 is required for origin DNA unwinding after assembly of the CMG components. In this part, I focus on the conserved zinc finger motif of Mcm10 and examined the effects of mutations on protein-protein interaction and ssDNA binding activity of Mcm10. The results show that the mutations in the conserved zinc finger reduced ssDNA binding activity and self-interaction of Mcm10. The mutant protein failed to support initiation of DNA replication. Interestingly, the mutant Mcm10 protein did not promote origin DNA unwinding, although it bound to replication origin. These results demonstrate the novel role of conserved zinc finger motif of Mcm10 that functions for origin DNA unwinding but not for origin binding of Mcm10.

Results

A conserved zinc finger motif is involved in self-interaction and single-stranded DNA binding activity of Mcm10

To understand the molecular mechanism of origin unwinding that requires Mcm10, I focused on the role of the central domain of Mcm10 that is highly conserved among eukaryotes and contains a zinc finger motif (Figure II-1A) (Homesley et al, 2000; Izumi et al, 2000). In budding yeast, mutations in the zinc finger motif abolish homo-oligomerization of Mcm10 and result in cell lethality (Cook et al, 2003). On the other hand, studies on the crystal structure of *Xenopus* Mcm10 have suggested that the zinc finger domain functions in ssDNA binding (Warren et al, 2008), and a mutation in the zinc finger of human Mcm10 has reduced the ssDNA binding activity (Okorokov et al, 2007). To elucidate the role of the zinc finger motif in the essential function of Mcm10 in DNA replication, I constructed a mutant Mcm10 protein that has three amino-acid substitutions at the conserved residues (Mcm10^{ZA}; Figure II-1A). I examined the effects of mutations on interactions of Mcm10 with Mcm10 as well as other replication factors using the yeast two-hybrid assay (Figure II-1B). Wild-type Mcm10 interacted with Mcm2, Mcm4 and Mcm6, as described in previous studies (Hart et al, 2002; Merchant et al, 1997). In addition, I observed interactions of Mcm10 with all of the four subunits of GINS, Cut5, Drc1, Dpb2 (Pol ε), Cdc20 (Pol ε), and Mcm10 (Figure II-1B and Table 1). Interestingly, the interaction of Mcm10^{ZA} with Mcm10 was specifically decreased, while Mcm10^{ZA} interacted with the other factors as the wild-type protein (Figure II-1B). These results suggest that the zinc finger of Mcm10 is specifically required for self-interaction of Mcm10.

I next examined the effects of zinc finger mutations on ssDNA binding activity. I

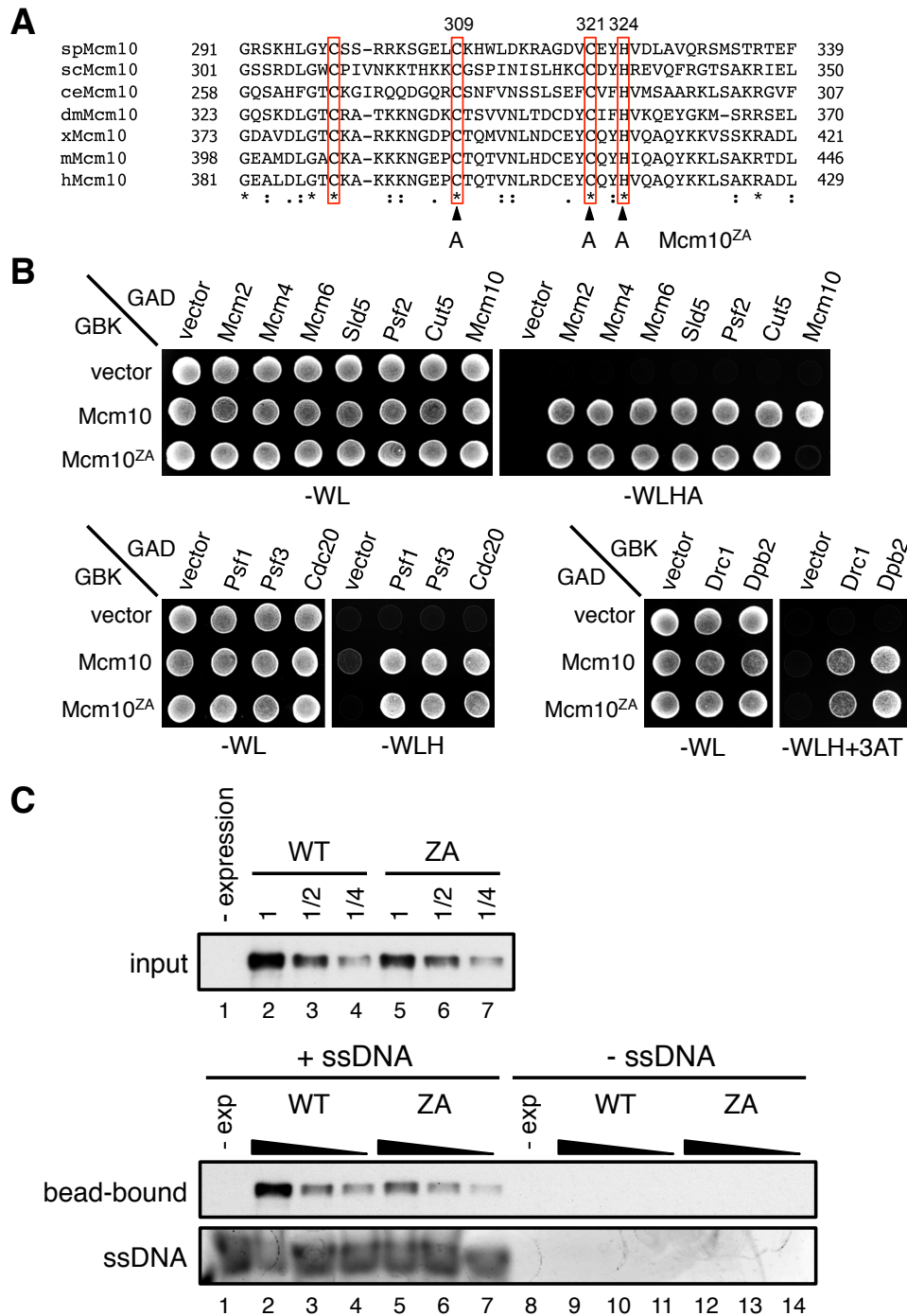


Figure II-1. Mutations in a conserved zinc finger motif of Mcm10 decrease self-interaction and ssDNA-binding.

(A) The amino acid sequence around the conserved CCCH zinc-finger motif (red boxes) of Mcm10 of *Shizosaccharomyces pombe* (sp), *Saccharomyces cerevisiae* (sc), *Caenorhabditis elegans* (ce), *Drosophila melanogaster* (dm), *Xenopus laevis* (x), *Mus musculus* (m) and *Homo sapiens* (h). Two conserved Cys residues and a His residue that were substituted by alanines are indicated with arrowheads. Two conserved Cys residues and a His residue that were substituted by alanines are indicated by arrows. (B) The results of the yeast two-hybrid assays of interactions of Mcm10^{WT} or Mcm10^{ZA} with other replication factors are presented. (C) N-terminally 5S-tagged Mcm10^{WT} and Mcm10^{ZA} proteins were expressed using the IVT system and diluted to 1, 1/2, or 1/4 concentrations (upper panel). Diluted lysates were incubated with streptavidin-beads coated with (lanes 1-7) or without (lanes 8-14) 28-nt ssDNA. Bead-bound proteins and ssDNA were analyzed by immunoblotting using anti-S antibody and TBE-PAGE, respectively (lower panels).

expressed the wild-type (Mcm10^{WT}) and Mcm10^{ZA} proteins by an *in vitro* transcription-coupled translation (IVT) system (Figure II-1C, upper panel), and the IVT-lysate was incubated with ssDNA-coated beads. The amount of beads-bound Mcm10^{ZA} was found to be about a half of Mcm10^{WT}, suggesting that the zinc finger motif contributes to ssDNA binding (Figure II-1C, lower panels, compare lanes 2-4 and 5-7). It might be possible that the apparent decrease in ssDNA binding activity of Mcm10^{ZA} is due to the defect in oligomerization. It has been reported that Mcm10 binds to as short as ~12 nt long ssDNA (Eisenberg et al, 2009). Because I used 28-mer ssDNA in the binding assay, to which less than three Mcm10 can bind, the requirement of oligomerization might be marginal.

To examine the property of Mcm10 precisely, I tried to purify recombinant Mcm10 protein from *E. coli*. I designed N-terminally Flag-tagged and C-terminally 6His-tagged Mcm10 and introduced the expression plasmid into *E. coli*. However, purification was unsuccessful because expression level of the protein was low and, in addition, only small amount of the protein bound to Flag-agarose beads (data not shown).

The zinc finger motif of Mcm10 is essential for origin DNA unwinding

To elucidate the role of zinc finger motif in Mcm10 functions in fission yeast cells, I first examined whether the Mcm10^{ZA} mutant could complement the depletion of Mcm10. The N-terminally Flag-tagged Mcm10^{WT} or Mcm10^{ZA} was ectopically expressed at the *ura4*⁺ locus under control of the constitutive *P_{adh81}* promoter in the *mcm10-off-aid* strain. While the *mcm10-off-aid* cells expressing Mcm10^{WT} grew normally, like the cells without the aid-tag (*mcm10*⁺), on the plate containing thiamine and auxin, the Mcm10^{ZA}-expressing strain did not grow (Figure II-2A), suggesting that the mutations impair the essential function of

Mcm10. To examine the defects in DNA replication, DNA contents of *mcm10-off-aid*, Mcm10^{WT} and Mcm10^{ZA} cells were analyzed by flow cytometry under conditions of Mcm10 depletion (see Figure legends). The cells expressing Mcm10^{WT} appeared to undergo DNA replication, as judged by an increase in their DNA content at 90 min after release from G2/M block. In contrast, *mcm10-off-aid* alone and Mcm10^{ZA}-expressing cells were arrested with a 1C DNA peak, showing that Mcm10^{ZA} protein did not support DNA replication (Figure II-2B). These results suggest that the zinc finger plays an essential role in DNA replication.

Next I examined whether the zinc finger mutant protein bound to replication origin and supported origin unwinding. Immunoblotting of Flag-Mcm10^{WT/ZA} using anti-Flag antibodies showed that Mcm10^{ZA} protein was decreased to about a half after depletion of Mcm10 (Figure II-2C), suggesting that Mcm10^{ZA} protein is slightly unstable in the absence of the functional Mcm10. Under these conditions, the origin localizations of Mcm6, Rpa2 and Flag-Mcm10^{WT/ZA} were examined by ChIP assay at 75 min after G2/M release. Mcm6-IP preferentially recovered the *ars2004* fragment in the all three strains (Figure II-2D). Rpa2 was localized at the origin in Mcm10^{WT} cells but not in Mcm10^{ZA} cells, similar to the situation in the *mcm10-off-aid* cells (Figure II-2F). Interestingly, Mcm10^{ZA} protein was localized at the origin more efficiently than Mcm10^{WT} protein (Figure II-2G). These results show that the zinc finger motif is not required for recruitment of Mcm10 to the origin, but is essential for the function of Mcm10 in origin unwinding. When I examined whether recruitment of Mcm10 to the origin caused release of Sld3, Sld3 accumulated at the origin in Mcm10^{ZA} cells, as was the case in *mcm10-off-aid* cells (Figure II-2E). These results suggest that Sld3 is released during or after Mcm10-dependent origin unwinding.

Figure II-2

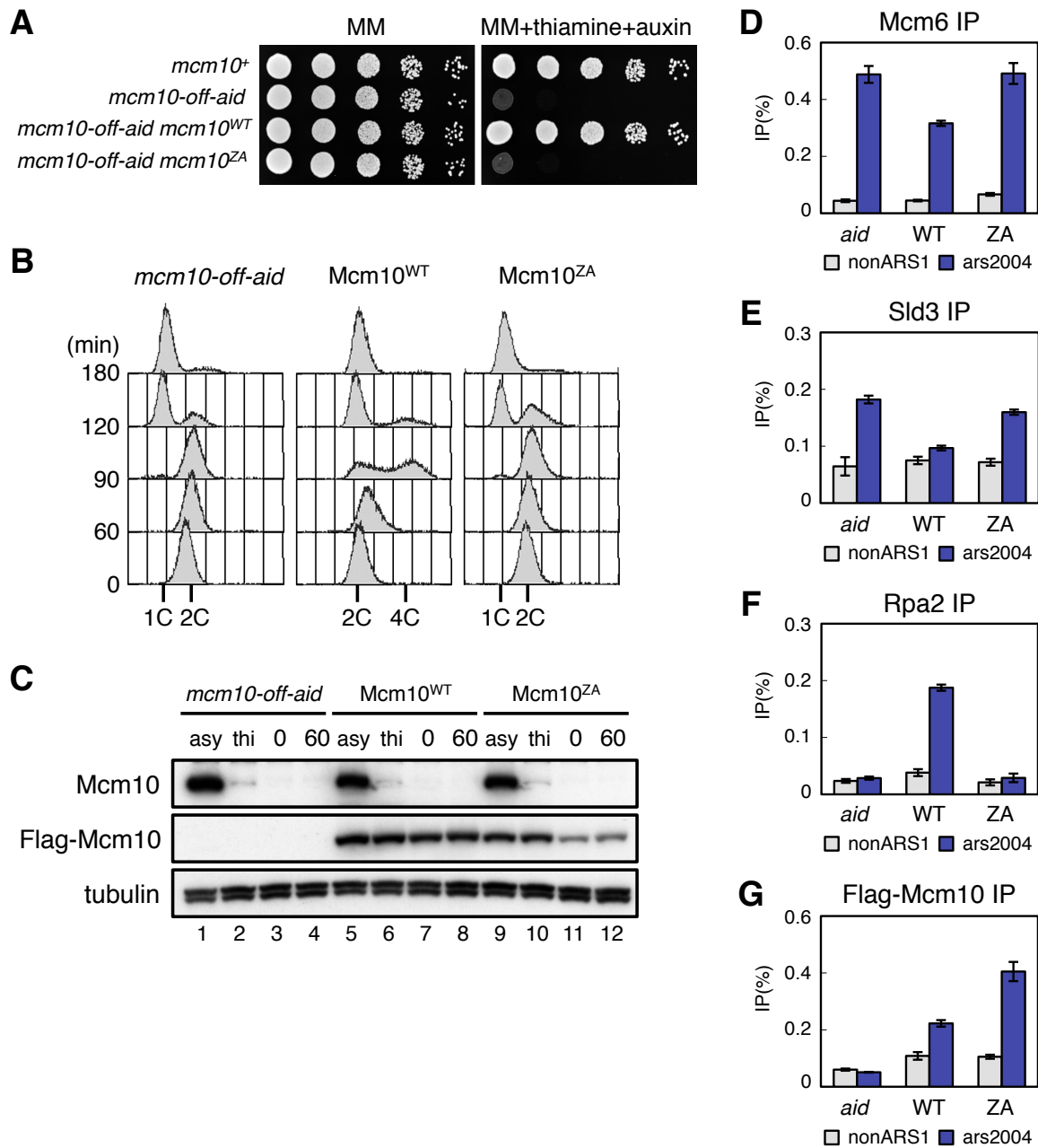


Figure II-2. The zinc finger of Mcm10 is essential for origin DNA unwinding.

(A) Growth of the Mcm10^{ZA} strain. Cells with the indicated genotypes in addition to *cdc25-22 TIR1* were spotted onto plate media containing thiamine (10 µg/ml) and auxin (0.5 mM) and incubated at 25°C. (B) Cells carrying *mcm10-off-aid* and those with ectopically expressed Mcm10^{WT} and Mcm10^{ZA} were arrested at the G2/M boundary under Mcm10-depletion and then released. The results of flow cytometry analysis are shown. (C) The amounts of proteins from asynchronous cells (asy), cells incubated with thiamine for 14 h before G2/M arrest (thia), G2/M release after depletion (0) and at 60 min after release (60) in experiment (B) were analyzed by immunoblotting using anti-Mcm10, anti-Flag and anti-α-tubulin antibodies. (D-G) Co-immunoprecipitated DNA with Mcm6 (D), Sld3 (E), Rpa2 (F) and Flag-Mcm10 (G) from cells under Mcm10 depletion was quantified at 75 min after G2/M release. HU (12 mM) was added to the Mcm10^{WT} strain. IP recoveries (%) are indicated by columns, and error bars were obtained by real-time PCR quantification. The results were reproduced in two independent experiments.

The mutation in the zinc finger could not be bypassed by fusion of Mcm10^{Z^A}

Because Mcm10^{Z^A} reduced self-interaction with Mcm10, it was possible that fusion protein of Mcm10^{Z^A} bypassed the defects of the mutation. To examine this possibility, I constructed plasmids that express Mcm10^{Z^A}-Mcm10^{Z^A} fusion protein (28-aa linker GYQASGGRPA GGSQIYPFLYKVVISH was placed between Mcm10s), Mcm10^{Z^A}x3, Mcm10^{Z^A}x4, and Mcm10^{Z^A}x6 and examined whether the fusion rescues defective growth of *mcm10-off-aid* strain. Expression of these fusion proteins, except for Mcm10^{Z^A}x4, was confirmed by immunoblotting with anti-Mcm10 antibody (Figure II-3B), where the intensity of the band increased by the number of fused Mcm10. The *mcm10-off-aid* strain harboring each plasmid was spotted on media containing auxin and thiamine (Figure II-3A). The *mcm10-off-aid* cells expressing Mcm10^{WT}-Mcm10^{WT} fusion grew normally (Figure II-3A), suggesting that the fusion does not inhibit Mcm10 functions. However, none of Mcm10^{Z^A} fusions supported the growth better than the vector (Figure II-3A). Because multimerization of Mcm10^{Z^A} did not bypass the essential function of the zinc finger motifs, it is difficult to distinguish possibilities such as the self-interaction of Mcm10 via the zinc finger motifs is important or that the zinc finger has some other functions.

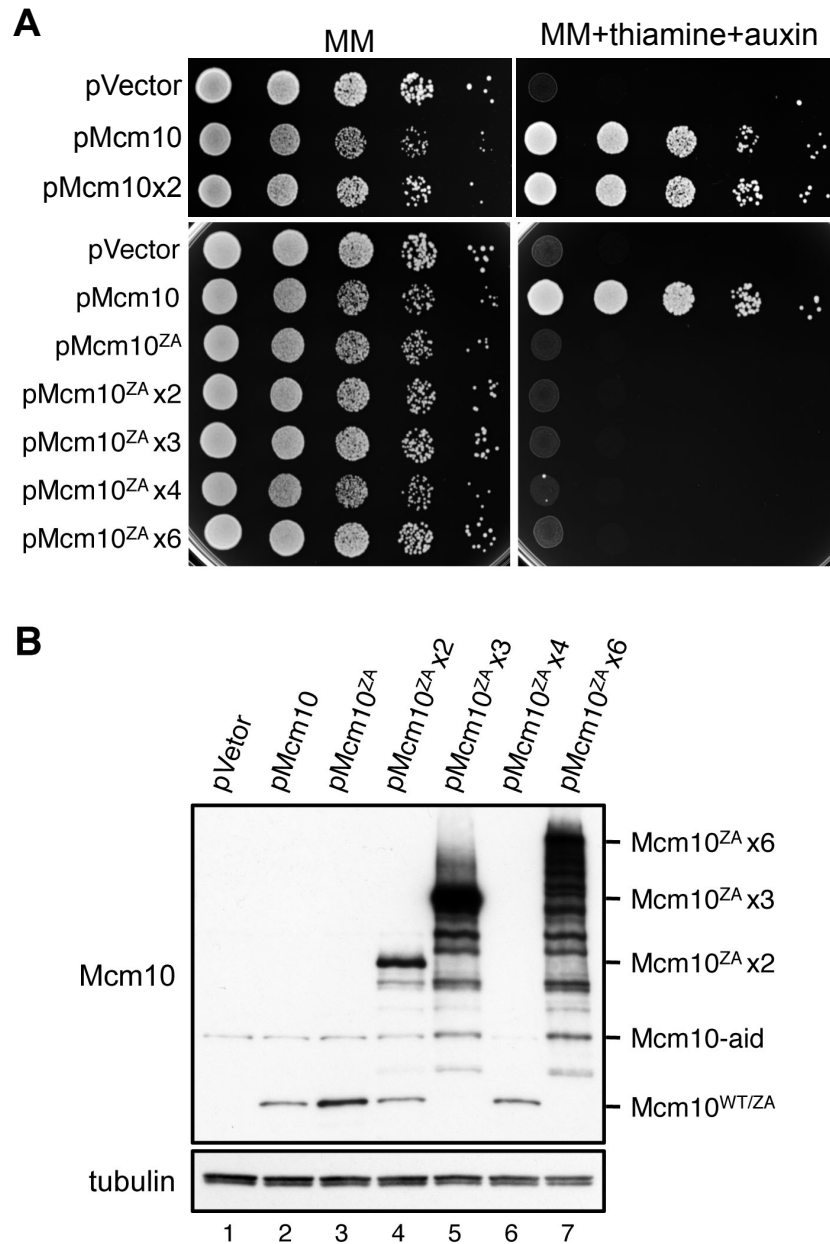


Figure II-3. Fusion of Mcm10ZA did not complement depletion of Mcm10.

(A) *mcm10-off-aid leu1-32* strain carrying pVector, pMcm10, pMcm10x2, pMcm10^{ZA}, pMcm10^{ZA}x2, pMcm10^{ZA}x3, pMcm10^{ZA}x4 and pMcm10^{ZA}x6 were spotted on media containing thiamine (10 µg/ml) and auxin (0.5 mM) and incubated at 25 °C. Mcm10^{ZA}-fusion proteins did not support cell growth of *mcm10-off-aid leu1-32* strain. (B) Whole-cell extracts were prepared from the cells carrying pVector, pMcm10, pMcm10^{ZA}, pMcm10^{ZA}x2, pMcm10^{ZA}x3, pMcm10^{ZA}x4 and pMcm10^{ZA}x6 and analyzed by immunoblotting using anti-Mcm10 and anti- α -tubulin antibodies. All of the proteins except for Mcm10^{ZA}x4 were expressed in the cells. Positions of each protein are indicated on the left of the panel.

Discussion

An essential role of Mcm10 in origin DNA unwinding

During the process of initiation of DNA replication, Mcm2-7 changes its structure dynamically. The Mcm2-7 is loaded onto origins as a head-to-head double hexamer that encircles dsDNA in G1 phase (Evrin et al, 2009; Remus et al, 2009). On the other hand, a single hexamer of Mcm2-7 encircles ssDNA in the active replicative helicase (Fu et al, 2011; Ilves et al, 2010; Moyer et al, 2006; Yardimci et al, 2010). Therefore, conversion of pre-RC into the active helicase requires dynamic structural changes such as separation of a double hexamer into two single hexamers and extrusion of ssDNA through the gate between Mcm2 and Mcm5 (Costa et al, 2011).

In Part I, I demonstrated that Mcm10 has an essential and initiation specific role in origin DNA unwinding after the CMG components as well as initiation specific factors assembled onto replication origin. Here, I showed that essential function of Mcm10 in DNA replication requires its zinc finger motif (Figure II-2). It is likely that the zinc finger motif is required for origin unwinding process after origin binding of Mcm10, because the Mcm10^{ZA} mutant protein was detected at origin but RPA was not (Figure II-2F and G). The yeast two-hybrid assay showed that the zinc finger mutation specifically impaired the interaction of Mcm10 with Mcm10 itself (Figure II-1B), consistent with results indicating that the zinc finger motif of budding yeast Mcm10 is required for formation of a homo-multimeric complex (Cook et al, 2003). Together, multimeric Mcm10 complex associated with the CMG at replication origin may promote origin DNA unwinding.

How is the mechanism of origin DNA unwinding by Mcm10? There are following four possibilities. First, Mcm10 is required for separation of Mcm2-7 double

hexamer into two single hexamers. Second, it functions for ssDNA extrusion. Third, it is involved both in separation of hexamers and in ssDNA extrusion. Fourth, it is required for extensive origin DNA unwinding after separation of hexamers and extrusion of ssDNA. Among these, I propose the first possibility for the role of Mcm10. Because the EM observation has been shown that the structure of Mcm2-7 dramatically changes by binding of GINS and Cdc45 (Costa et al, 2011), it is possible that the origin bound Mcm2-7 is converted into ssDNA-bound form upon assembly of GINS and Cdc45 in the absence of Mcm10. Initiation specific factors, which are accumulated at replication origin in the absence of Mcm10, may help Mcm2-7 to open the gate and exclude ssDNA because Sld3 and Sld2 have been reported to bind ssDNA *in vitro* (Bruck & Kaplan, 2011; Kanter & Kaplan, 2011). It has been shown that Mcm10 interacts with the N-terminal half of Mcm4 (Hart et al, 2002), and my yeast two-hybrid results also show that it interacts with N-terminal regions of Mcm2, 4 and 6 (data not shown). Mcm10 in oligomeric forms, which is recruited to origin depending on interactions with several factors, as detected in yeast two-hybrid assay (Figure II-1B), interacts with multiple subunits of Mcm2-7 at their N-terminus, may cause decrease in head-to-head interaction of the two hexamers. This process may also release the initiation specific factors, such as Sld3, Sld2/Drc1 and Dpb11/Cut5 from Mcm2-7. Alternatively, these factors may have inhibitory activity on Mcm2-7 and Mcm10 may be required for release of them from Mcm2-7. However, overproduction of Sld3, Sld2 and Dpb11 promote, rather than inhibit, origin firing (Mantiero et al, 2011), suggesting that these factors have acceleratory effects on Mcm2-7 activation. The strong genetic and physical interactions of Mcm10 between MCM subunits (Hart et al, 2002; Homesley et al, 2000; Lee et al, 2010; Lee

et al, 2003; Liang & Forsburg, 2001; Merchant et al, 1997) also support the model that Mcm10 directly works on the Mcm2-7 complex and promote initiation of DNA replication.

Although Mcm10 is found in RPC complex and localizes at replication fork (Gambus et al, 2006; Pacek et al, 2006; Ricke & Bielinsky, 2004; Taylor et al, 2011), it seems not to be an integral component of replicative helicase, because depletion of Mcm10 after replication initiation did not cause arrest of replication (Figure I-7). Although it is likely that the fork associated Mcm10 facilitate association of DNA Pol α with replication forks (Lee et al, 2010; Ricke & Bielinsky, 2004), the function might be redundant with other factors such as Ctf4/Mcl1 (Tanaka et al, 2009; Zhu et al, 2007).

To understand Mcm10 function further, *in vitro* reconstitution experiments will be needed for understand the molecular mechanism of origin DNA unwinding by Mcm10. EM observation or single molecule measurement approach will be effective to reveal how the Mcm10 molecules interact with the subunits of Mcm2-7. These experiments will clarify the mechanism of origin DNA unwinding promoted by Mcm10.

Conclusions

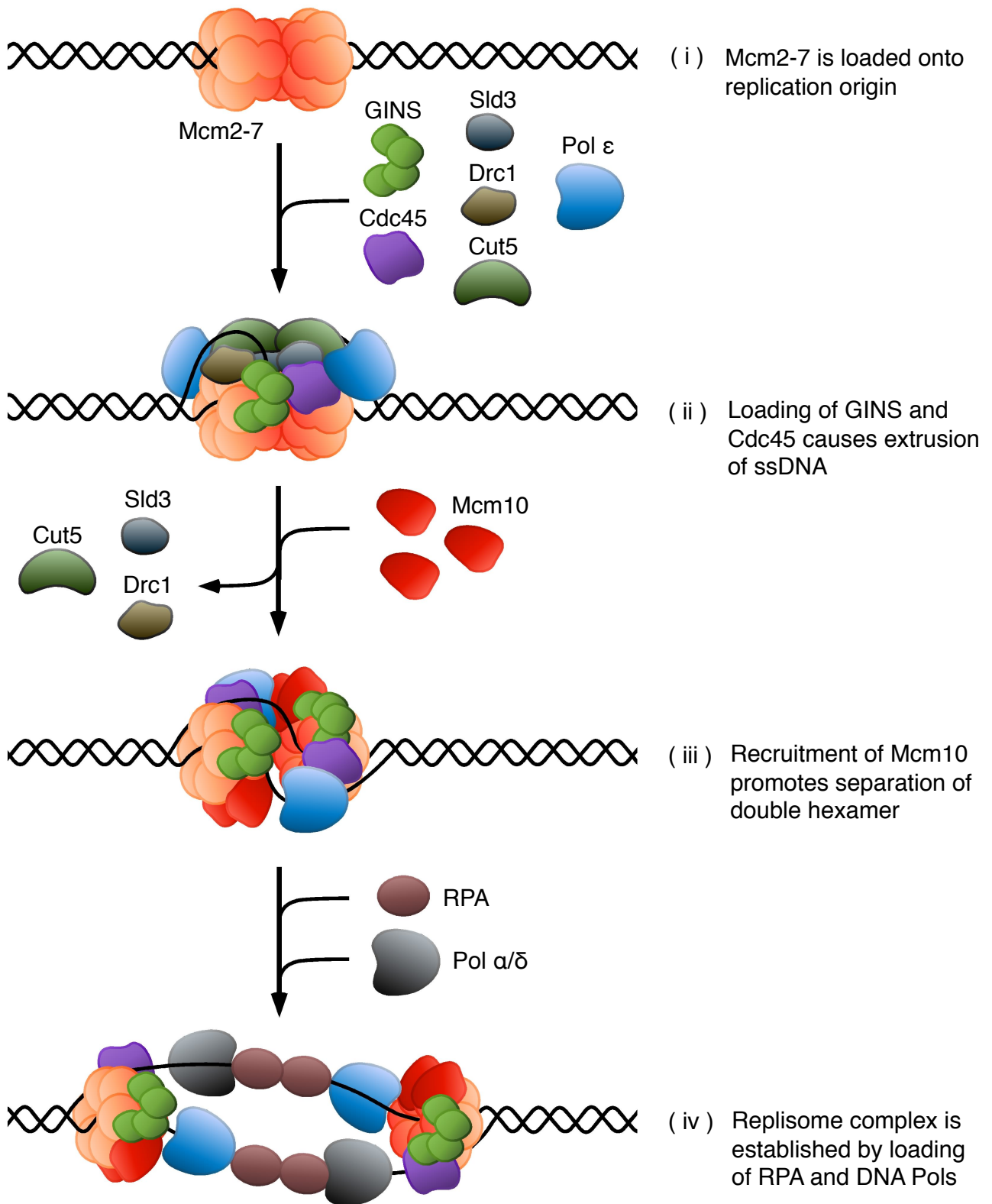
Activation of the replicative helicase is a key reaction for initiation of DNA replication at the onset of S phase. Although the CMG complex composed of Mcm2-7, GINS and Cdc45 exhibits robust helicase activity *in vitro*, it is not known whether loading of GINS and Cdc45 to Mcm2-7, dependently on Sld3, Dpb11/Cut5 and Sld2/Drc1, is sufficient for unwinding of origin DNA, and for subsequent recruitment of DNA polymerases that initiate DNA synthesis. In this study, using efficient depletion of replication factors in a transcription-coupled auxin-inducible protein degradation (*off-aid*) system, we demonstrated that Mcm10, which is loaded onto replication origins dependently on the CMG components, plays an essential role in a novel step that is required for origin DNA unwinding.

In Part I, I examined precisely the initiation step in which Mcm10 functions. The results showed that, interestingly, origin DNA unwinding was inhibited while the co-factors for the replicative helicase, GINS and Cdc45, assembled at replication origin in the absence of Mcm10. Depletion of Mcm10 greatly reduced association of the single strand binding protein complex RPA to replication origin, suggesting that Mcm10 plays an essential role in origin DNA unwinding after assembly of the CMG complex. As a consequence, recruitment of the components of replisome, Pol α and Pol δ (but not Pol ϵ) was impaired, and the CMG complex did not dislocate from replication origin. In addition, initiation specific Sld3, Cut5 and Drc1 accumulated at replication origin.

In Part II, I focused on the conserved zinc finger motif of Mcm10. The mutations in the zinc finger abolished Mcm10 function in initiation of DNA replication. I found that, interestingly, the mutant Mcm10 protein bound to replication origin, suggesting that the

mutation specifically impaired its function for origin DNA unwinding. The mutation was found to reduce self-interaction of Mcm10.

Base on observations described in this study, I propose a model of the role of Mcm10 in origin DNA unwinding. (i) In G1 phase, the double-hexameric Mcm2-7 is loaded onto origin dsDNA to form pre-RC. (ii) At the onset of S phase, Sld3, Cut5, Drc1, GINS, Pol ϵ and Cdc45 assemble onto the Mcm2-7. Binding of GINS and Cdc45 causes conversion of the structure of Mcm2-7 from dsDNA-bound form to ssDNA-bound form. At this stage, DNA replication is not initiated because two Mcm2-7 molecules connected via its N-terminus. (iii) Depending on assembly of the CMG components, Mcm10 binds to the complex as homo-multimers. Mcm10 interacts with multiple subunits of Mcm2-7 at their N-terminal regions and promotes separation of the two Mcm2-7 molecules, generating the two CMG complexes. These processes may cause release of Sld3, Cut5 and Drc1 from origins. (iv) RPA is recruited to unwound origin DNA, and then DNA Pol α and Pol δ are loaded to establish the replisome complex. After initiation, Mcm10 translocates with the replisome complex and may stimulate helicase activity and/or facilitate association of DNA Pol α with replication forks.



Materials and methods

Strains

Fission yeast strains used in this study are listed in Table 2. Details of strain construction are described below. Strains were cultured in complete YE medium and minimal EMM medium (Moreno et al, 1991). All solid media contained 2.0% agar. Transcription from the *nmt* promoter was induced in the absence of thiamine. Transformation of *S. pombe* was performed by the lithium acetate method (Forsburg, 2003) and hygromycin B (Wako) and G418 (Nacalai Tesque) were used for selection of transformants at final concentrations of 150 µg/ml. PMG medium was used for selection because of higher sensitivity to the antibiotics on PMG plates than EMM plates. A synthetic auxin, NAA (1-naphthaleneacetic acid), (Nacalai Tesque) was added at a concentration of 0.5 mM to EMM medium.

Construction of off-aid strains

The *mcm10-off-aid* ($P_{nmt81-mcm10-IAA17}$) strain was constructed as follows. A 0.15-kb fragment containing the upstream non-coding region of *mcm10*⁺ together with the *hphMX6* marker gene was cloned into the *EcoRI-ClaI* sites of pBluescript II SK+ to create pBS-mcm10up-hphMX6. A 1.9-kb *SpeI-SacI* fragment from pBS-mcm10up-hphMX6, a 1.2-kb *SacI-NdeI* fragment containing the *nmt81* promoter (P_{nmt81}), and a 0.44-kb *NdeI-BamHI* fragment of the N-terminal region of *mcm10*⁺ were introduced into the *SpeI-BamHI* sites of pBluescript II SK+ to create pBS-hphMX6-Pnmt81-mcm10N. Then pBS-hphMX6-Pnmt81-mcm10N was *EcoRV*-digested and used for transformation of the *mcm10-aid* strain, HM2550 (Kanke et al, 2011). Among hygromycin B-resistant transformants, integration of $P_{nmt81-mcm10}$ at the *mcm10*⁺ locus was confirmed by PCR.

The *psf1-aid* strain was constructed as described below. A 0.28-kb fragment containing the 3'-UTR of *psf1*⁺ was cloned into the *Bam*HI-*Eco*RI sites of pKM40 (Kanke et al, 2011). Then a *Spe*I-*Nsi*I fragment containing the *psf1*⁺ ORF was introduced into the *Spe*I-*Pst*I sites, resulting in pBS-*psf1*-2HA-IAA17-*ura4-psf1dw*. pBS-*psf1*-2HA-IAA17-*ura4-psf1dw* was digested with *Bam*HI and used for transformation. Among *Ura*⁺ transformants, insertion of the 2HA-*aid*-tag at the *psf1*⁺ locus was confirmed by PCR.

To obtain the *psf1-off-aid* strain, a 0.65-kb fragment containing the *psf1*⁺ ORF together with *P_{nmt81}* were cloned into the *Pst*I-*Kpn*I sites of pBluescript II SK+ to generate pBS-Pnm81-*psf1*. A 0.27-kb fragment containing the upstream non-coding region of *psf1*⁺ was cloned into the *Bam*HI-*Pst*I sites of pBluescript II SK+ followed by insertion of a 1.7-kb *Sma*I-*Eco*RV fragment containing the *hphMX6* gene into the *Eco*RV site, resulting in pBS-*psf1up-hphMX6*. Then a 1.8-kb *Sma*I-*Kpn*I fragment containing *P_{nmt81-psf1}* was inserted into the *Eco*RV-*Kpn*I sites of pBS-*psf1up-hphMX6* to create pBS-*hphMX6-Pnmt81-psf1*. Then pBS-*hphMX6-Pnmt81-psf1* was *Nsi*I-digested and used for transformation of the *psf1-aid* strain.

The *aid*-tag was added at the C-terminus of *spp2*⁺ by a two-step PCR method, as described by Kanke et al (Kanke et al, 2011) using the primers *spp2*-F, *spp2*+*ad*-R, *spp2*+*ad*-F and *spp2*-R (Table 3). The *spp2-off-aid* strain was obtained as follows. A 0.33-kb fragment containing the N-terminal region of *spp2*⁺ together with *P_{nmt81}* was cloned into the *Pst*I-*Xho*I sites of pBluescript II SK+ to create pBS-Pnm81-*spp2N*. A 0.28-kb fragment containing the upstream non-coding region of *spp2*⁺ was cloned into the *Sac*I-*Spe*I sites of pBluescript II SK+, resulting in pBS-*spp2up*. Then a 1.5-kb *Bgl*III-*Spe*I fragment containing the *kanMX6* marker gene and a 1.6-kb *Spe*I-*Kpn*I fragment from pBS-Pnm81-*spp2N* were

introduced into the *Bam*HI-*Kpn*I sites of pBS-spp2up to obtain pBS-kanMX6-Pnmt81-spp2. pBS-kanMX6-Pnmt81-spp2 was *Xho*I-digested and used for transformation of the *spp2-aid* strain. Among G418-resistant transformants, integration of *P_{nmt81}-spp2* at the *spp2*⁺ locus was confirmed by PCR.

Construction of Mcm10^{WT/ZA} strains

To insert the *mcm10^{WT}* or *mcm10^{ZA}* genes at the *ura4*⁺ locus, the *kanMX6* marker gene was introduced into the *Cla*I-*Hind*III sites of pMH4 (Hayashi et al, 2007), resulting in pBS-ura4updw-kanMX6. An *Nde*I-*Bam*HI fragment containing the N-terminally 6Flag-tagged *mcm10^{WT}* or *mcm10^{ZA}* gene was inserted at the *Nde*I-*Bam*HI sites between the *P_{adh81}* promoter and *nmt* terminator on pBS-Padh81, resulting in pBS-Padh81-6FLmcm10 or pBS-Padh81-6FLmcm10ZA, respectively. A *Not*I fragment from pBS-Padh81-6FLmcm10 or pBS-Padh81-6FLmcm10ZA containing the *P_{adh81}*, *mcm10^{WT}* or *mcm10^{ZA}* and *nmt* terminator was introduced into the *Not*I site of pBS-ura4updw-kanMX6 to obtain pBS-ura4::kan-Padh81-6FLmcm10 or pBS-ura4::kan-Padh81-6FLmcm10ZA, respectively. These plasmids were digested with *Sse*8387I and used for transformation. Among the G418-resistant transformants, integration of *P_{adh81}-6flag-mcm10* or *P_{adh81}-6flag-mcm10^{ZA}* at the *ura4*⁺ locus was confirmed by PCR.

Construction of fusion proteins of Mcm10^{ZA}

To construct fusion proteins of *Mcm10^{ZA}*, the plasmid containing *mcm10^{ZA}* gene with 28-aa linker at the C-terminus (GYQASGGRPAGGSQIYPAFLYKVVSSH) between *Nhe*I-*Xba*I sites was constructed (pBS-mcm10^{ZA}-NheXba). A *Nhe*I-*Xba*I fragment from

pBS-mcm10^{Z^A}-NheXba was introduced into *NheI* site of pBS-mcm10^{Z^A}-NheXba to construct pBS-mcm10^{Z^A}_{x2}-NheXba and pBS-mcm10^{Z^A}_{x3}-NheXba. A *NheI-SmaI* fragment containing *mcm10^{Z^A}* gene was introduced into *NheI-SmaI* site between *adh81* promoter and *nmt* terminator sequence on pRAD81-Nhe to generate pRAD81-mcm10^{Z^A}. Then *NheI-XbaI* fragment from pBS-mcm10^{Z^A}-NheXba, pBS-mcm10^{Z^A}_{x2}-NheXba and pBS-mcm10^{Z^A}_{x3}-NheXba were introduced into *NheI* site of pRAD81-mcm10^{Z^A} to create pRAD81-mcm10^{Z^A}_{x2}, pRAD81-mcm10^{Z^A}_{x3} and pRAD81-mcm10^{Z^A}_{x4}, respectively. pRAD81-mcm10^{Z^A}_{x6} was obtained by insertion of *NheI-XbaI* fragment from pBS-mcm10^{Z^A}_{x2}-NheXba fragment into *NheI* site of pRAD81-mcm10^{Z^A}_{x4}. The plasmid pRAD81-mcm10 and pRAD81-mcm10_{x2} were constructed in the same way. Constructed plasmids were used for transformation.

Polyclonal antibody against Mcm10

To express the N-terminally 6His-tagged Mcm10 fragment (1-146 a.a) in *Escherichia coli*, a 0.46-kb DNA encoding the fragment was PCR-amplified using the primers Cdc23N-f and Cdc23N-r (Table 3). The PCR product digested with *BamHI* was cloned into pUC119 and the sequence was confirmed. The *NdeI-BamHI* fragment encoding His6-Mcm10N was cloned into pET21a to create pET21-His6-Mcm10N. His6-tagged Mcm10N polypeptide was expressed in *E. coli* BL21 (DE3), purified as recommended by the manufacturer (Qiagen), and used to immunize rabbits (Hokudo Inc.). Anti-Mcm10 polyclonal antibodies were affinity-purified as recommended by the manufacturer using a Hi-Trap NHS-activated column (GE Healthcare).

Purification of recombinant Mcm10

To express the N-terminally His6-tagged full-length Mcm10 in *Escherichia coli*, a 2.0-kb fragment encoding *mcm10*⁺ was introduced into pDONR201 (Life Technologies), resulting in pDONR-Mcm10. Then the *mcm10*⁺ fragment of pDONR-Mcm10 was transferred into the pETDuet-1 vector (Merck) containing a Gateway vector conversion cassette using the Gateway system (Life Technologies) to obtain pETD-Mcm10. Protein expression was induced in the *E. coli* BL21 (DE3) strain carrying pETD-Mcm10. Cells were then harvested and suspended in buffer A (10 mM Tris-HCl [pH 8.0], 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM benzamidine). After addition of lysozyme at a final concentration 2.5 mg/ml, sonication buffer (100 mM NaPi [pH 8.0], 1 M NaCl, 2% Triton X-100, 10 mM β -mercaptoethanol, 2 mM PMSF, 4 mM benzamidine) was added and the cells were lysed by sonication. The lysate was centrifuged and the pellet was washed twice with wash buffer A (1 M NaCl, 50 mM NaPi [pH 8.0], 1% Triton X-100, 1 mM EDTA, 1 mM PMSF, 2 mM benzamidine), twice with wash buffer B (1 M urea, 50 mM NaPi [pH 8.0], 1% Triton X-100), and finally suspended in urea buffer (8 M urea, 50 mM NaPi [pH 8.0]). Mcm10 protein was identified by SDS-PAGE and visualized by staining with Coomassie brilliant blue. The concentration of recombinant Mcm10 protein was estimated by comparison with BSA.

Cell cycle synchronization and flow cytometry

To synchronize the cell cycle, derivatives carrying *cdc25-22* were incubated at 36°C for 3.5 h for arrest at the G2/M boundary, and then released at 25°C. To repress the *nmt* promoter, thiamine was added at a final concentration of 10 μ g/ml at the indicated time points before G2/M arrest (see Figure legends). Synthetic auxin, NAA (1-naphthaleneacetic acid, Nacalai Tesque), was added 1 h before the release from G2/M to induce protein degradation. Cells

were fixed with 70% ethanol and incubated with 0.5 µg/ml PI and 50 µg/ml RNase A in 50 mM sodium citrate for 1 h at 37°C. Samples were then measured using a FACScan (Becton, Dickinson and Company).

Preparation of cell extracts and immunoblotting

Fission yeast cell extracts were prepared as described previously (Kanke et al, 2011). Proteins in the extracts were separated by SDS-PAGE and transferred to PVDF membranes (Immobilon, Millipore Corp). The membranes were incubated for 1 h at room temperature in PBST containing 5% skim milk and reacted in PBST containing 0.5% skim milk overnight at 4°C with rabbit anti-Mcm6 (1:3,000) (Ogawa et al, 1999), rabbit anti-Mcm4 (1:2,000) (Sherman et al, 1998), rabbit anti-Mcm10 (1:2,000), rabbit anti-Cdc45 (1:1,000) (Nakajima & Masukata, 2002), rabbit anti-IAA17 (1:2,000) (Nishimura et al, 2009), mouse anti-FLAG M2 (1:3,000; Sigma-Aldrich), mouse anti-HA 16B12 (1:1,000; Covance), or mouse anti-TAT1 (1:2,000) (Woods et al, 1989) antibodies. HRP-conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody (1:10,000; Jackson ImmunoResearch). Binding was visualized with West Pico and Femto Chemiluminescent Substrate (Thermo Scientific).

Co-immunoprecipitation of the CMG components

Fission yeast cells (3×10^8) were suspended in 720 µl of breaking buffer (50 mM Hepes-KOH [pH 7.4], 1 mM EDTA, 140 mM NaCl, 0.1% sodium deoxycholate, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride [PMSF], 4.5 µg/ml N-alpha-Tosyl-L-lysine chloromethyl ketone [TLCK], 5 µg/ml aprotinin, 5 µg/ml leupeptin and proteinase inhibitor cocktail [Sigma-Aldrich]) and disrupted with glass beads using a Micro Smash (TOMY

SEIKO). After addition of 80 μ l of 10% Triton X-100 and 54 μ l of 3 M NaAc (final concentration 200 mM), genomic DNA was digested with DNase I (TaKaRa) by incubation at 4°C for 45 min with gentle shaking. The supernatant obtained by centrifugation at 15,000 rpm for 10 min was used for immunoprecipitation with 10 μ l of mouse anti-FLAG M2 antibody-conjugated agarose beads (Sigma-Aldrich).

Chromatin immunoprecipitation assay

ChIP assays were performed as described previously (Kanke et al, 2011). Cells (1.5×10^8) were fixed in 1% formaldehyde for 15 min and then in 125 mM glycine for 5 min. After being washed once with cold water, the cells were suspended in 450 μ l of breaking buffer (50 mM HEPES-KOH [pH 7.4], 1 mM EDTA, 140 mM NaCl, 0.1% sodium deoxycholate, 0.1% Triton X-100, 1 mM PMSF, and proteinase inhibitor cocktail [Sigma-Aldrich]) and disrupted with glass beads using a Micro Smash (TOMY SEIKO). After addition of 50 μ l of 10% Triton X-100, the samples were sonicated four times for 10 s each time (Sonifier, Branson). The supernatant obtained by centrifugation at 15,000 rpm for 10 min was used for immunoprecipitation with magnetic beads (Life Technologies) conjugated with rabbit anti-Mcm6 (1:400) (Ogawa et al, 1999), rabbit anti-Rpa2 (1:400) (Yabuuchi et al, 2006), mouse anti-FLAG M2 (1:333; Sigma-Aldrich), or mouse anti-Myc 9E11 (1:267; Lab Vision) antibodies. DNA prepared from whole-cell extracts or immunoprecipitated fractions was analyzed by real-time PCR using SYBR green I in a 7300 real-time PCR System (Applied Biosystems) or by agarose gel electrophoresis of the PCR products of the *ars2004* and non-origin fragments using the primers *ars2004F* and *ars2004R* for *ars2004* and non-ARS-F

and non-ARS-R for the non-origin region. The primer sets used for real-time PCR are listed in Table 3.

Yeast two-hybrid assay

Yeast two-hybrid assays were performed as described previously (Fukuura et al, 2011). The BD MATCHMAKER GAL4 2-Hybrid System 3 (BD Biosciences) was used for yeast two-hybrid analysis. Derivatives of pGADT7, a Gal4-DNA-binding-domain (BD) vector, and pGBKT7, an activation-domain (AD) vector, were constructed as follows. The *NdeI-BamHI* fragment containing the full-length *mcm10*⁺, *mcm10*^{Z^A}, *drc1*⁺ or *dpb2*⁺ was introduced into pGBKT7. Similarly, the *NdeI-BamHI* fragment containing *cdc20*⁺ was introduced into pGADT7. The pGADT7 derivatives carrying *mcm2*⁺, *mcm3*⁺, *mcm4*⁺, *mcm6*⁺, *mcm7*⁺, *sld5*⁺, *psf1*⁺, *psf2*⁺, *psf3*⁺, *sld3*⁺, *cut5*⁺, *cdc45*⁺, *mcm10*⁺, and pGBK-Mcm5 were constructed as described previously (Fukuura et al, 2011). A pair of pGBKT7 and pGADT7 derivatives was introduced into *S. cerevisiae* AH109 (*MATa*, *trp1-901*, *leu2-3, 112*, *ura3-52*, *his3-200*, *gal4Δ*, *gal80Δ*, *LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3*, *GAL2_{UAS}-GAL2_{TATA}-ADE2*, *URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ*, *MEL1*) cells. Trp⁺ Leu⁺ transformants harboring both plasmids were selected on synthetic growth medium (SD) lacking tryptophan and leucine (-WL) and the interaction was analyzed using growth media lacking histidine (-WLH) or histidine and adenine (-WLHA) at 30°C for 2-3 days. When indicated, the -WLH medium was supplemented with 2 mM 3-aminotriazole (-WLH+3AT).

In vitro DNA binding assay

To express N-terminally 5S-tagged Mcm10 or Mcm10^{ZA} in an *in vitro* transcription-coupled translation (IVT) system from rabbit reticulocyte lysates, a fragment containing *mcm10*⁺ or *mcm10*^{ZA} was cloned into the pDONR201 vector (Life Technologies) to obtain pDONR-mcm10 or pDONR-mcm10^{ZA}. The *mcm10*⁺ or *mcm10*^{ZA} gene was then transferred into the pCITE4a vector (S-tag) (Merck) by the Gateway LR reaction (Life Technologies). Proteins were expressed using the TNT T7 Coupled Reticulocyte Lysate System (Promega) in accordance with the manufacturer's protocol. To prepare ssDNA-coated streptavidin beads, 5'-biotin-conjugated 28-mer oligoDNA (M13-biotin; listed in Table 3) was incubated with My oneTM streptavidin T1 beads (Life Technologies) in binding buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 2 M NaCl, 0.1% Triton X-100) at 4°C over-night. For an *in vitro* DNA-binding assay, 5 µl of IVT-lysate was incubated with ssDNA-beads at 30 °C for 30 min in zinc binding buffer A (ZBA) (10 mM Tris-HCl [pH 7.4], 90 mM KCl, 1 mM MgCl₂, 90 µM ZnCl₂, 5 mM DTT, 0.05% NP-40, 1% BSA). After incubation, the beads were washed three times with ZBA and bead-bound proteins were analyzed by immunoblotting using mouse anti-S antibody (1:5,000; Bethyl Laboratories).

Table 1. Summary of the yeast two-hybrid assay

activation domain		DNA binding domain		
		vector	Mcm10	Mcm10 ^{ZA}
vector		-	-	-
Mcm2-7	Mcm2	-	+++	+++
	Mcm3	-	-	NT
	Mcm4	-	+++	+++
	Mcm5*	-	-	NT
	Mcm6	+	+++	+++
	Mcm7	-	-	NT
GINS	Sld5	+	+++	+++
	Psf1	-	+	+
	Psf2	-	+++	+++
	Psf3	-	+	+
Pol ϵ	Cdc20	-	+	+
	Dpb2*	+	++	++
others	Sld3	-	-	NT
	Cut5	-	+++	+++
	Drc1*	+	++	++
	Cdc45	-	-	NT
	Mcm10	-	+++	+

+: growth on SD-WLH; ++: on SD-WLH+3AT; +++: on SD-WLHA; NT: not tested.

* Combination of a pGBK-derivative with pGAD-Mcm10 or Mcm10^{ZA} was tested.

Table 2. Fission yeast strains used in this study

Strain	Genotype	Source
972	<i>h⁻</i>	Lab stock
HM83	<i>h⁻ ura4-D18</i>	Lab stock
HM1527	<i>h⁻ mcm10-5-ura4⁺</i>	This study
HM1529	<i>h⁻ nda3-KM311 mcm10-5-ura4⁺</i>	This study
HM1544	<i>h⁺ mcm10::ura4⁺-6flag-mcm10</i>	This study
HM1554	<i>h⁻ nda3-KM311 mcm10-5-ura4⁺ sld3::sld3-5flag-ura4⁺</i>	This study
HM1559	<i>h⁻ nda3-KM311 mcm10-5-ura4⁺ cdc45::flag-cdc45</i>	This study
HM1562	<i>h⁻ nda3-KM311 mcm10-5-ura4⁺ dpb2::4flag-dpb2-sup3-5</i>	This study
HM2550	<i>h⁻ mcm10::mcm10-IAA17-ura4⁺</i>	Kanke et al, 2011
	<i>ade6::ade6⁺-P_{adh15}-skp1-AtTIR1-2NLS-9myc</i>	
HM3021	<i>h⁻ cdc25-22</i>	This study
	<i>ade6::ade6⁺-P_{adh15}-skp1-OsTIR1-natMX6-P_{adh15}-skp1-AtTIR1-2NLS</i>	
HM3056	<i>h⁻ cdc25-22 mcm10::hphMX6-P_{nmt81}-mcm10-IAA17-ura4⁺</i>	This study
	<i>ade6::ade6⁺-P_{adh15}-skp1-OsTIR1-natMX6-P_{adh15}-skp1-AtTIR1-2NLS</i>	
HM3095	<i>h⁻ cdc25-22 mcm10::hphMX6-P_{nmt81}-mcm10-IAA17-ura4⁺</i>	This study
	<i>ade6::ade6⁺-P_{adh15}-skp1-OsTIR1-natMX6-P_{adh15}-skp1-AtTIR1-2NLS</i>	
	<i>psf2::psf2-3flag-kanMX6</i>	
HM3110	<i>h⁻ cdc25-22 mcm10::hphMX6-P_{nmt81}-mcm10-IAA17-ura4⁺</i>	This study
	<i>ade6::ade6⁺-P_{adh15}-skp1-OsTIR1-natMX6-P_{adh15}-skp1-AtTIR1-2NLS</i>	
	<i>cdc45::cdc45-9myc-ura4⁺</i>	
HM3238	<i>h⁻ cdc25-22 mcm10::hphMX6-P_{nmt81}-mcm10-IAA17-ura4⁺</i>	This study
	<i>ade6::ade6⁺-P_{adh15}-skp1-OsTIR1-natMX6-P_{adh15}-skp1-AtTIR1-2NLS</i>	
	<i>sld3::sld3-5flag-ura4⁺ cut5::cut5-13myc-kanMX6</i>	
HM3276	<i>h⁻ cdc25-22 mcm10::hphMX6-P_{nmt81}-mcm10-IAA17-ura4⁺</i>	This study
	<i>ade6::ade6⁺-P_{adh15}-skp1-OsTIR1-natMX6-P_{adh15}-skp1-AtTIR1-2NLS</i>	
	<i>drc1::drc1-3flag-kanMX6</i>	
HM3288	<i>h⁻ cdc25-22 mcm10::hphMX6-P_{nmt81}-mcm10-IAA17-ura4⁺</i>	This study
	<i>ade6::ade6⁺-P_{adh15}-skp1-OsTIR1-natMX6-P_{adh15}-skp1-AtTIR1-2NLS</i>	
	<i>cdc20::cdc20-3flag-kanMX6</i>	
HM3338	<i>h⁻ cdc25-22 mcm10::hphMX6-P_{nmt81}-mcm10-IAA17-ura4⁺</i>	This study
	<i>ade6::ade6⁺-P_{adh15}-skp1-OsTIR1-natMX6-P_{adh15}-skp1-AtTIR1-2NLS</i>	
	<i>psf2::psf2-3flag-kanMX6 cdc45::cdc45-9myc-ura4⁺</i>	
HM3347	<i>h⁻ cdc25-22 cdc45::hphMX6-P_{nmt81}-cdc45-IAA17-ura4⁺</i>	This study
	<i>ade6::ade6⁺-P_{adh15}-skp1-OsTIR1-natMX6-P_{adh15}-skp1-AtTIR1-2NLS</i>	
HM3390	<i>h⁻ cdc25-22 cdc45::hphMX6-P_{nmt81}-cdc45-IAA17-ura4⁺</i>	Kanke et al, 2011
	<i>ade6::ade6⁺-P_{adh15}-skp1-OsTIR1-natMX6-P_{adh15}-skp1-AtTIR1-2NLS</i>	
	<i>mcm10::ura4⁺-6flag-mcm10</i>	
HM3747	<i>h⁻ cdc25-22 mcm10::mcm10-IAA17-ura4⁺</i>	This study
	<i>ade6::ade6⁺-P_{adh15}-skp1-OsTIR1-natMX6-P_{adh15}-skp1-AtTIR1-2NLS</i>	
HM3817	<i>h⁻ cdc25-22 mcm10::hphMX6-P_{nmt81}-mcm10-IAA17-ura4⁺</i>	This study
	<i>ade6::ade6⁺-P_{adh15}-skp1-OsTIR1-natMX6-P_{adh15}-skp1-AtTIR1-2NLS</i>	
	<i>pol1::pol1-3flag-kanMX6</i>	
HM3818	<i>h⁻ cdc25-22 mcm10::hphMX6-P_{nmt81}-mcm10-IAA17-ura4⁺</i>	This study
	<i>ade6::ade6⁺-P_{adh15}-skp1-OsTIR1-natMX6-P_{adh15}-skp1-AtTIR1-2NLS</i>	
	<i>cdc6::cdc6-3flag-kanMX6</i>	

HM3940	<i>h⁻ cdc25-22 psf1::hphMX6-P_{nmt81}-psf1-2HA-IAA17-ura4⁺ ade6::ade6⁺-P_{adh15}-skp1-OsTIR1-natMX6-P_{adh15}-skp1-AtTIR1-2NLS mcm10::ura4⁺-6flag-mcm10</i>	This study
HM3966	<i>h⁻ cdc25-22 pol1::hphMX6-P_{nmt81}-pol1-IAA17-ura4⁺ spp2::kanMX6-P_{nmt81}-spp2-IAA17-ura4⁺ ade6::ade6⁺-P_{adh15}-skp1-OsTIR1-natMX6-P_{adh15}-skp1-AtTIR1-2NLS mcm10::ura4⁺-6flag-mcm10 cdc45::cdc45-9myc-ura4⁺</i>	This study
HM4230	<i>h⁻ cdc25-22 mcm10::hphMX6-P_{nmt81}-mcm10-IAA17-ura4⁺ ade6::ade6⁺-P_{adh15}-skp1-OsTIR1-natMX6-P_{adh15}-skp1-AtTIR1-2NLS ura4::kanMX6-P_{adh81}-6flag-mcm10</i>	This study
HM4233	<i>h⁻ cdc25-22 mcm10::hphMX6-P_{nmt81}-mcm10-IAA17-ura4⁺ ade6::ade6⁺-P_{adh15}-skp1-OsTIR1-natMX6-P_{adh15}-skp1-AtTIR1-2NLS ura4::kanMX6-P_{adh81}-6flag-mcm10^{ZA}</i>	This study
HM4364	<i>h⁻ cdc25-22 mcm10::hphMX6-P_{nmt81}-mcm10-IAA17-ura4⁺ ade6::ade6⁺-P_{adh15}-skp1-OsTIR1-natMX6-P_{adh15}-skp1-AtTIR1-2NLS ura4::kanMX6-P_{adh81}-mcm10 sld3::sld3-5flag-ura4⁺</i>	This study
HM4365	<i>h⁻ cdc25-22 mcm10::hphMX6-P_{nmt81}-mcm10-IAA17-ura4⁺ ade6::ade6⁺-P_{adh15}-skp1-OsTIR1-natMX6-P_{adh15}-skp1-AtTIR1-2NLS ura4::kanMX6-P_{adh81}-mcm10^{ZA} sld3::sld3-5flag-ura4⁺</i>	This study

Table 3. Primers used in this study

Name	Sequence
Cdc23N-f	5'-CGGGATCCATATGCACCATCACCATCACCATGATCCCTTCAT-3'
Cdc23N-r	5'-CGGGATCCTATTTCTCCTCTTCACTTTTTTCC-3'
mcm10UPF1	5'-CTGTAGACACATCTTTTATG-3'
mcm10UPHdR1	5'-AAAAAGCTTAATGTAGTGCATAAACAAGC-3'
mcm10UPHdF2	5'-AAAAAGCTTCCTTAAACACATGTCCTTTAG-3'
mcm10NR2	5'-GCAAGTCGTTTCGTGAAATGG-3'
mcm10muF2	5'-GTGATATCGAACGTGT-3'
mcm10muR2	5'-GCAAAGTGTGTCGCATG-3'
spp2-F	5'-GGTCCTGGAGATGCCACGGTT-3'
spp2+ad-R	5'-TCGACACTGCCCATCATTGATTCTAAACTAAGTTG-3'
spp2+ad-F	5'-GTTTCGTCAATATCACAAGCGCTAAGATACTTTTAGTTC-3'
spp2-R	5'-GGAAGTGTGTTTCGGAAGATTGC-3'
M13-biotin	5'-biotin-CAGGAAACAGCTATGACCATGATTACGC-3'
ars2004F	5'-CTTTTGGGTAGTTTTTCGGATCC-3'
ars2004R	5'-ATGAGTACTTGTCACGAATTC-3'
non-ARS-F	5'-TCGAAGATCCTACCGCTTTC-3'
non-ARS-R	5'-CTTGCGCTGAAGCTTTAGTAAAAG-3'
ars2004 region-273F	5'-CGGATCCGTAATCCCACAAA-3'
ars2004 region-338R	5'-TTTGCTTACATTTTCGGGAACCTA-3'
nonARS1 region-514F	5'-TACGCGACGAACCTTGCATAT-3'
nonARS1 region-583R	5'-TTATCAGACCATGGAGCCCAT-3'
ars3002-F	5'-TCATTAGCAAACAAAAGCAATTGAG-3'
ars3002-R	5'-AATTTCCGGGCATTA AAAACG-3'
ars2004+L20kb-F	5'-CCTTTGCATCCGCCTCCTA-3'
ars2004+L20kb-R	5'-AATCCTCCCCAAAAACAATTCTC-3'
ars2004+L10kb-F	5'-GCTGGTGTCTTAGTTGTTGGA-3'
ars2004+L10kb-R	5'-CCCTTGGTCAAACCTTGCATCT-3'
ars2004+L5kb-F	5'-GCAACAACATTAAACCAATTGAAACT-3'
ars2004+L5kb-R	5'-AAAGACGATGCTTGGAAAAACAA-3'
ars2004+L1kb-F	5'-GAAGAAGCACGGTCAAAGTAT-3'
ars2004+L1kb-R	5'-CCAATTTGCTGGCGAGCTA-3'
ars2004+L0.5kb-F	5'-GCTTTCCATATTCGGACAACCA-3'
ars2004+L0.5kb-R	5'-ATAACCCGTTTCTCCATCTACCAT-3'
ars2004+R0.5kb-F	5'-GGCACTATACACAAACTCACCATTTTAA-3'
ars2004+R0.5kb-R	5'-TCATGGAGTGCGTGCTTTTTTA-3'
ars2004+R1kb-F	5'-GAACGATTGAATTGACGAAAACC-3'
ars2004+R1kb-R	5'-ATGATGAAAAGACTCGGTCAACTG-3'
ars2004+R1.5kb-F	5'-TTTCTCGGAAGTGAATAAACGCAC-3'
ars2004+R1.5kb-R	5'-CCAAAAACGAGACAAAACGGGTT-3'
ars2004+R2kb-F	5'-CTCCTTAATCTCCTCCTTTTT-3'
ars2004+R2kb-R	5'-GTAATAACGGCTGATAAAGCAGAAA-3'

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