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

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The CMG complex composed of Mcm2-7, Cdc45 and GINS is postulated to be the eukaryotic replicative DNA helicase, whose activation requires sequential recruitment of replication proteins 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, we report 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 <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 polymerases. 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.

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Introduction

Initiation of DNA replication requires replicative DNA helicase, which unwinds the origin DNA duplex to provide single-stranded DNA templates for replicative DNA polymerases. In eukaryotes, the heterohexameric mini-chromosome maintenance 2-7 (Mcm2-7) complex comprises the core of replicative DNA helicase (Bell and Dutta, 2002). Current evidence indicates that two additional factors, Cdc45 and the GINS (go–ichi–ni–san) complex, are essential stoichiometric components of replicative DNA helicase (Takahashi *et al*, 2005; Moyer *et al*, 2006; Pacek *et al*, 2006; Bochman and Schwacha, 2009; Ilves *et al*, 2010). It has been shown that Cdc45, Mcm2-7 and GINS form a stable 'CMG' complex (Cdc45–Mcm2-7–GINS) that translocates on a single-stranded DNA in the 3′–5′ direction (Moyer *et al*, 2006; Ilves *et al*, 2010).

Chromatin loading and activation of the replicative DNA helicase are tightly controlled by progression of the cell cycle. In G1-phase, when CDK activity is low, the Mcm2-7 complex is loaded onto replication origins by the origin recognition complex, Cdc6/Cdc18 and Cdt1, forming the pre-replicative complex (pre-RC) (Bell and Dutta, 2002). At this stage, a head-to-head double-hexameric Mcm2-7 encircles the origin DNA duplex as a helicase-inactive form (Evrin *et al*, 2009; Remus *et al*, 2009). 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. The Dbf4-dependent Cdc7 kinase (DDK) phosphorylates the Mcm2-7 complex and stimulates loading of Sld3 onto pre-RCs (Yabuuchi *et al*, 2006; Heller *et al*, 2011). CDK phosphorylates Sld3 and Sld2/Drc1, promoting formation of a complex of Sld3–Dpb11/Cut5–Sld2 at replication origins (Masumoto *et al*, 2002; Tanaka *et al*, 2007; Zegerman and Diffley, 2007; Fukuura *et al*, 2011). Finally, these factors collectively recruit GINS and Cdc45 onto the Mcm2-7 complex, resulting in full assembly of the CMG helicase (Remus and Diffley, 2009; Araki, 2010). Requirement of Sld3, Sld2 and Dpb11 for initiation of DNA replication is likely limited at the assembly step of the CMG helicase (Kanemaki and Labib, 2006; Labib, 2010; Taylor *et al*, 2011). Origin DNA is unwound by the activated replicative helicase, and then RPA, a eukaryotic single-stranded DNA-binding protein complex, and DNA polymerases are recruited to origins, resulting in assembly of complete replisomes. Recent experiments have strongly suggested that a single replisome contains a single CMG complex that translocates on the leading strand template in the 3′–5′ direction (Gambus *et al*, 2006; Moyer *et al*, 2006; Ilves *et al*, 2010; Yardimci *et al*, 2010; Costa *et al*, 2011; Fu *et al*, 2011). These findings argue that activation of the replicative DNA helicase involves dynamic structural changes of the Mcm2-7 complex from the double-stranded DNA (dsDNA)-bound double hexamer to the single-stranded DNA (ssDNA)-bound single hexamer.

Mcm10/Cdc23 is an evolutionally conserved protein essential for DNA replication (Nasmyth and Nurse, 1981; Aves *et al*, 1998). 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). 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 dsDNA and ssDNA in human, *Xenopus*, budding yeast and fission yeast (Fien *et al*, 2004; Okorokov *et al*, 2007; Robertson *et al*, 2008; Warren *et al*, 2008, 2009; Eisenberg *et al*, 2009). Although Mcm10 has no sequence homology to Mcm2-7, it physically interacts with the members of the Mcm2-7 complex (Merchant *et al*, 1997; Homesley

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et al, 2000; Hart *et al*, 2002; Lee *et al*, 2003). 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 (Homesley *et al*, 2000; Liang and Forsburg, 2001; Hart *et al*, 2002; Lee *et al*, 2010). 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 (Wohlschlegel *et al*, 2002; Gregan *et al*, 2003; Sawyer *et al*, 2004). 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* (Ricke and Bielinsky, 2004; Heller *et al*, 2011). 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 (Ricke and Bielinsky, 2004, 2006; Chattopadhyay and Bielinsky, 2007), 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.

To uncover the role of Mcm10 in initiation of DNA replication, we applied a conventional promoter shut-off system combined with a recently developed auxin-inducible protein degradation system (*off-aid*) to Mcm10 (Nishimura *et al*, 2009; Kanke *et al*, 2011). The *off-aid* depletion efficiently removed >99% of Mcm10 from cells, allowing us to determine precisely the step at which Mcm10 executes its essential function. Interestingly, Mcm10 was required for origin DNA unwinding after assembly of the CMG components on replication origins. In addition, initiation-specific factors, such as Sld3, Cut5 and Drc1, were not released from origins in the absence of Mcm10. The zinc finger of Mcm10, whose mutations decreased the self-interaction and ssDNA-binding activity of Mcm10, was essential for origin unwinding but not for association of Mcm10 onto replication origins. Our results demonstrate that Mcm10 plays a novel key role, together with the CMG components, in the origin DNA unwinding step via its zinc-finger motif for initiation of DNA replication.

Results

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. We first examined whether Mcm10 is required for this process. Because the existing mutant allele of Mcm10 was leaky and not suitable for analysis at the molecular level (Liang and Forsburg, 2001), we adopted an approach involving depletion of Mcm10 protein from living cells. For efficient and tight depletion of the protein, we combined an auxin-inducible degron (AID) system (Nishimura *et al*, 2009; Kanke *et al*, 2011) with the thiamine-repressible *nmt81* promoter (P_{nmt81}) (P_{nmt81}-*mcm10-aid*: *mcm10-off-aid*). *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 1 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 (WT) cells, corresponding to <18 molecules per cell at the time of G2/M release (Figure 1A; Supplementary Figure S1). We analysed 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 1B, left). When non-depleted cells were treated with hydroxyurea (HU), which depletes the dNTP pool, DNA replication was blocked at an early stage, generating cells with 1C DNA content (Figure 1B, middle). Mcm10-depleted cells generated a sharp 1C peak similar to HU-arrested cells (Figure 1B, right), indicating arrest of the cell cycle in the early stage of DNA replication.

Under Mcm10-depleted conditions, we 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 1C–E, 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 1C–E, 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, we 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. We depleted Psf1 or Cdc45 using the *off-aid* system as described for Mcm10 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 <1% of the original amount, and the cells were arrested with unreplicated DNA (Supplementary Figure S3A–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 2A and C, 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 2B and D, left). In Psf1-depleted or Cdc45-depleted cells, however, Mcm10 was not significantly detected at the origins, whereas Mcm6 accumulated (Figure 2A–D, right). To examine whether Mcm10 localization is dependent on a factor that

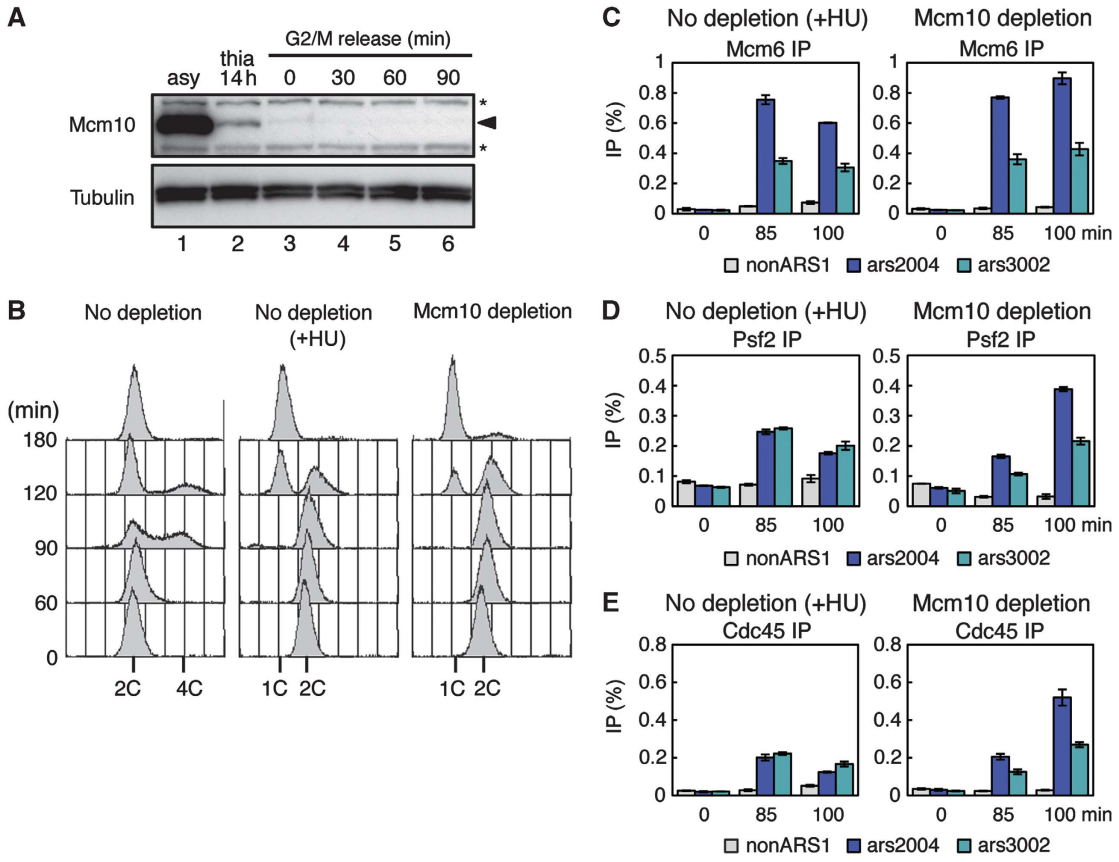
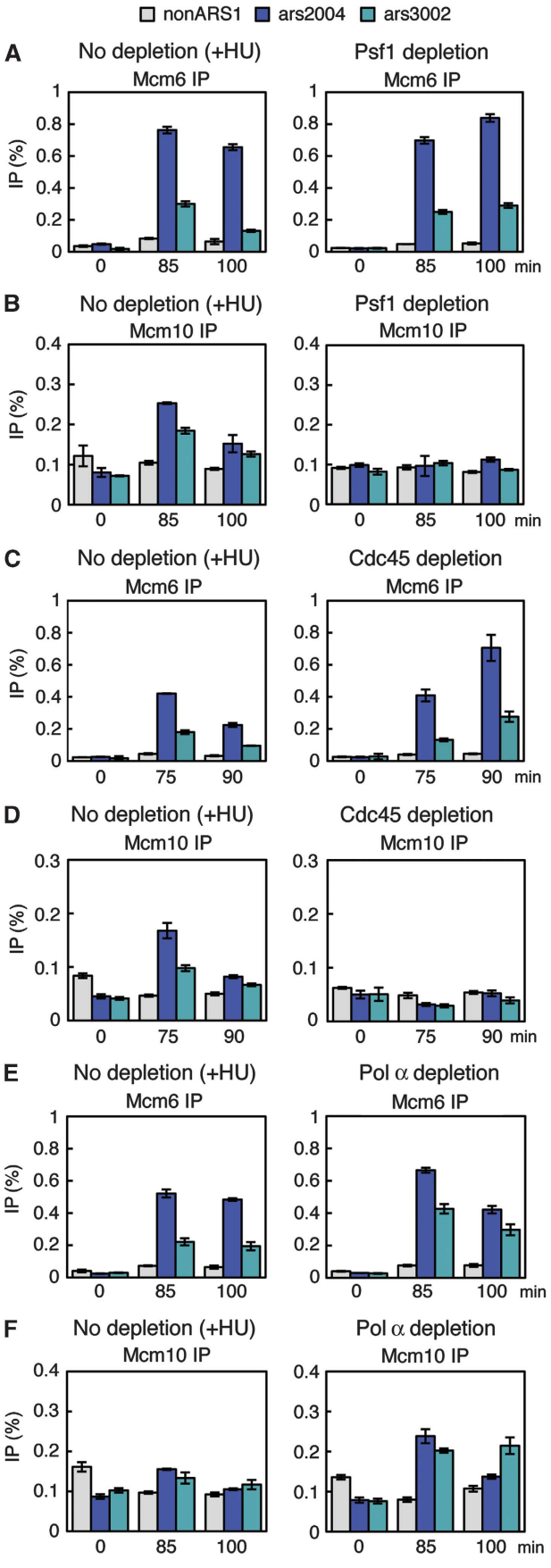


Figure 1 Localization of the CMG components at replication origins in Mcm10-depleted cells. (**A**) The *mcm10-off-aid* cells carrying *psf2–flag* and *cdc45–myc* were incubated with thiamine for 14 h and arrested at the G2/M boundary by incubation at 36°C for 3.5 h. Auxin (0.5 mM) was added 1 h before 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 14 h, lane 2) and at the indicated time points after release from the G2/M boundary (lanes 3–6) were analysed by immunoblotting using anti-Mcm10 and anti-α-tubulin antibodies. An arrowhead and asterisks (*) indicate the positions of Mcm10-aid and non-specific bands, respectively. (**B**) DNA contents of non-depleted cells with (middle) or without (left) HU treatment and Mcm10-depleted cells (right) were analysed by flow cytometry. (**C**–**E**) DNA fragments immunoprecipitated with Mcm6 (**C**), Psf2–Flag (**D**) and Cdc45–myc (**E**) were analysed by real-time PCR using primer sets for two early origins, *ars2004* (blue) and *ars3002* (cyan), and for the non-origin region, nonARS1 (grey). The columns indicate IP recovery (%) ± s.d. obtained from triplicate measurements in real-time PCR quantification. The results of biologically independent experiments are presented in Supplementary Figure S2.

is recruited to origins after assembly of the CMG complex, we depleted Pol α, which binds to chromatin depending on RPA (Walter and 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 (Supplementary Figure S3G and H). The results of ChIP assay showed that Mcm10 bound to the origins in Pol α-depleted cells (Figure 2F, 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 presence of a high salt concentration (Gambus *et al*, 2006). We examined whether the CMG 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 non-tagged cells (Figure 3A, lanes 3 and 4). Co-precipitation of Mcm6 and Cdc45–myc with Psf2–Flag was specific in S-phase (100 min) but not in G2/M-phase (0 min) cells (Figure 3B, lanes 5 and 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 3B, lanes 7 and 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; Sheu and Stillman, 2006, 2010; Randell *et al*, 2010). We examined whether phosphorylated Mcm4 formed a complex with Psf2 in the absence of Mcm10. Slow-moving forms of Mcm4 preferentially increased in IP fractions at 100 min in both HU-treated and Mcm10-depleted cells (Figure 3B, 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 3B, lanes 6 and 8), probably due to checkpoint-dependent phosphorylations of Mcm4 under HU-arrested conditions (Ishimi *et al*, 2003, 2004; Bailis *et al*, 2008). 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 (Supplementary Figure S5). 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

Because components of the CMG complex, which has been shown to exhibit robust DNA unwinding activity *in vitro* (Moyer *et al*, 2006; Ilves *et al*, 2010), bound to replication origins and formed a complex in the absence of Mcm10, we examined whether origin DNA was unwound. The localization of Rpa2, the second largest subunit of the single-stranded DNA-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 4A, left, 75 and 90 min). In contrast, Rpa2 was hardly detected at the origins in Mcm10-depleted cells (Figure 4A, right). Because HU causes replication fork arrest resulting in accumulation of RPA, we 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 4B–D, left). In Mcm10-depleted cells, origin binding of Rpa2 was greatly decreased, despite accumulation of Mcm6 and Psf2 at the origins (Figure 4B–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. We 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 4E 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 4E, right). No significant localization of Rpa2 was detected at any of the positions examined in the absence of Mcm10 (Figure 4F, right). These results suggest that Mcm10 is required for transition of the assembled CMG components into a translocatable helicase complex.

Figure 2 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 μg/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 μg/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. DNA immunoprecipitated with Mcm6 and Flag–Mcm10 in *psf1-off-aid* cells (**A**, **B**), *cdc45-off-aid* cells (**C**, **D**), or *pol1-off-aid spp2-off-aid* cells (**E**, **F**) at the indicated time points was assayed with a real-time PCR system. Columns indicate IP recovery (%) ± s.d. obtained from triplicate measurements in real-time PCR quantification. The results of biologically independent experiments are presented in Supplementary Figure S4.

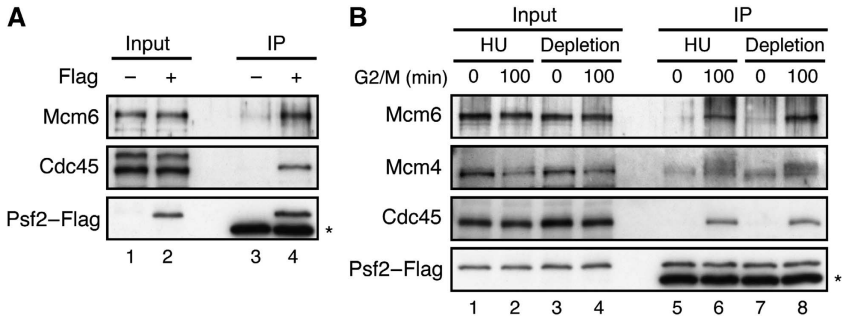


Figure 3 Mcm4, Mcm6 and Cdc45 form a complex with Psf2 (GINS) in the absence of Mcm10. (**A**) Co-precipitation of Mcm6 and Cdc45 with Psf2–Flag. *mcm10-off-aid psf2–flag cdc45–myc* (Flag+) and *mcm10-off-aid cdc45–myc* (Flag–) cells were arrested at the G2/M boundary and released in the presence of 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 and 4) were analysed by immunoblotting using anti-Mcm6, anti-Cdc45 and anti-Flag antibodies. The samples used as input (lanes 1 and 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 analysed 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.

Mcm10-dependent origin unwinding is prerequisite for replisome assembly

Absence of origin DNA unwinding would impair assembly of the replisome complex. We 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 5A–C, left). In contrast, neither Pol1 nor Cdc6 was localized at the origins under conditions of Mcm10 depletion (Figure 5A and B, right). These results show that Mcm10 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 and Bielinsky, 2004; Zhu *et al*, 2007), we did not observe any decrease in the cellular amount of the catalytic subunit in the absence of Mcm10 (Supplementary Figure S8), unlike the reported requirement of Mcm10 for stabilization of Pol α in budding yeast and human cells (Ricke and Bielinsky, 2004, 2006; Chattopadhyay and Bielinsky, 2007). In contrast to Pol1 and Cdc6, Cdc20 accumulated at origins under Mcm10-depleted conditions (Figure 5C, right). Pol ε is essential for assembly of GINS to replication origins before initiation of DNA replication in budding yeast (Muramatsu *et al*, 2010) and fission yeast (T Handa and H Masukata, unpublished observations). These results suggest that Mcm10 is not required for origin recruitment of Pol ε but prerequisite for recruitment of Pol α and Pol δ, which occurs after origin unwinding.

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 (Masumoto *et al*, 2002; Yabuuchi *et al*, 2006; Tanaka *et al*, 2007; Zegerman and Diffley, 2007; Fukuura *et al*, 2011). Because these factors do not translocate with the CMG in the replisome progression complex (Kanemaki and 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, we 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 6A and B, left). Drc1 was transiently detected in HU-treated cells without Mcm10 depletion (Figure 6C, left). In contrast, all of these factors were highly accumulated at the replication origins in Mcm10-depleted cells (Figure 6A–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.

To confirm that the above observations are not a side effect of depletion of other protein with Mcm10, we isolated a novel temperature-sensitive mutant, *mcm10-5* (Supplementary Figure S10). Using this mutant, we found that Mcm6, Sld3, Cdc45 and Dpb2, the second largest subunit of Pol ε, but not Rpa2, were assembled at replication origins (Supplementary Figure S11), thus supporting the contention that Mcm10 is required for origin unwinding after recruitment of the CMG components.

A conserved zinc-finger motif of Mcm10 is essential for origin DNA unwinding

To understand the molecular mechanism of origin unwinding that requires Mcm10, we focused on the role of the central domain of Mcm10 that is highly conserved among eukaryotes and contains a zinc-finger motif (Figure 7A) (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 been shown to reduce 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, we constructed a mutant Mcm10 protein bearing three amino-acid substitutions at the conserved residues (Mcm10^{ZA}; Figure 7A). We examined the effects of the mutations on ssDNA-binding activity using recombinant proteins expressed by an *in vitro* transcription-coupled translation (IVT) system. When the WT or mutant protein was incubated with ssDNA-coated beads, the amount of beads-bound Mcm10^{ZA} was reduced to about half that of Mcm10^{WT} (Supplementary Figure S12). These results suggest that the zinc-finger motif is required for ssDNA binding, although the possibility remains that the ssDNA binding of Mcm10 with a high isoelectric point (pI = 10.1) was affected by the mutation. We next examined the effects of zinc-finger mutations on the interactions of Mcm10 with Mcm10 as well as other replication factors using the yeast two-hybrid assay (Figure 7B). WT Mcm10 interacted with Mcm2, Mcm4 and Mcm6, as described in previous studies (Merchant *et al*, 1997; Hart *et al*, 2002). In addition, we observed interactions of Mcm10 with all of the four subunits of GINS, Cut5, Drc1, Dpb2 (Pol ε), Cdc20 (Pol ε), and Mcm10 (Figure 7B; Supplementary Table S1). Interestingly, the interaction of Mcm10^{ZA} with Mcm10 was specifically decreased, while Mcm10^{ZA} interacted with the other factors as the WT protein (Figure 7B). These results suggest that the zinc finger of Mcm10 is involved in self-interaction and ssDNA binding.

To elucidate the role of the zinc-finger motif in the functions of Mcm10 in fission yeast cells, we 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 plates containing thiamine and auxin, the Mcm10^{ZA}-expressing strain did not grow (Figure 7C), suggesting that the mutations impair the essential function of Mcm10. To examine the defects in DNA replication, the DNA contents of *mcm10-off-aid*, Mcm10^{WT} and Mcm10^{ZA} cells were analysed by flow cytometry under conditions of Mcm10 depletion (see Figure 7 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

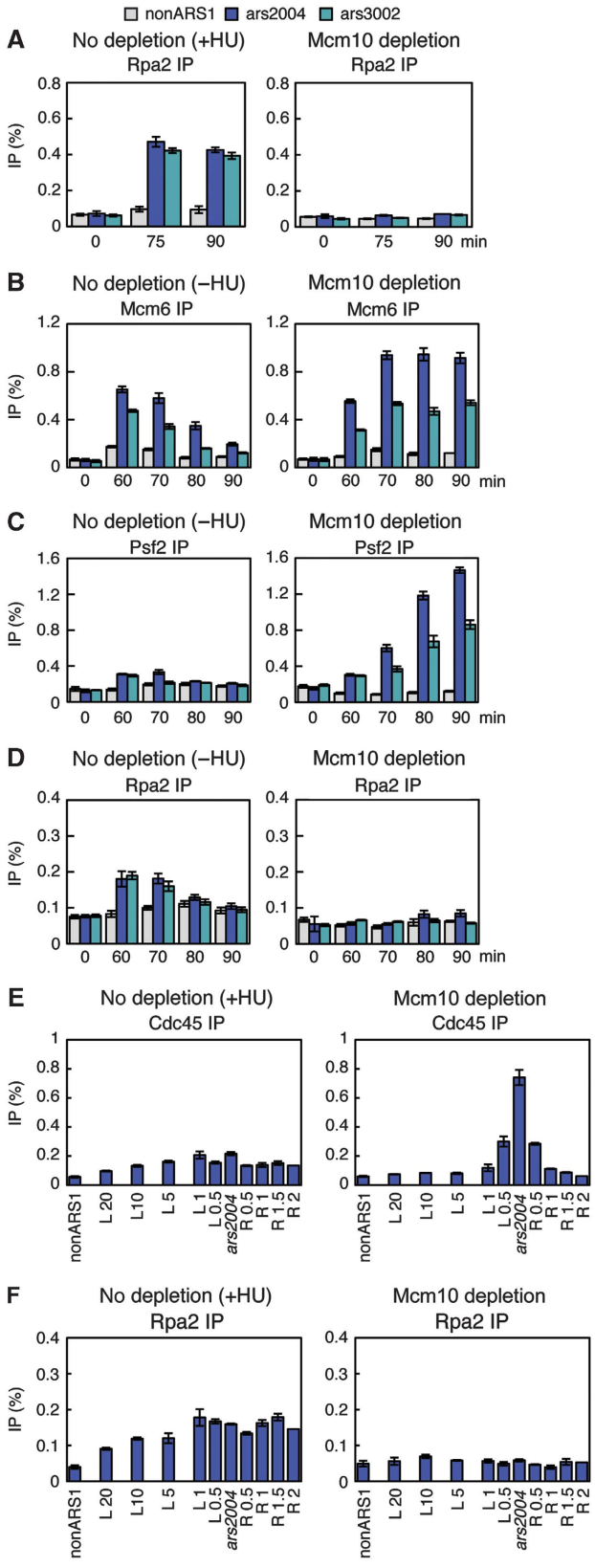


Figure 4 Mcm10 is required for origin DNA unwinding. (**A**) *mcm10-off-aid* cells were arrested at the G2/M boundary with or without Mcm10 depletion and released into the synchronous cell cycle. HU (12 mM) was added to the cells without depletion. Co-immunoprecipitated DNA with Rpa2 was quantified by real-time PCR. IP recoveries (%) are indicated by columns, and error bars show ± s.d. obtained from triplicate measurements in real-time PCR quantification. The results of biologically independent experiments are presented in Supplementary Figure S6. (**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 centre of *ars2004* was analysed by real-time PCR. Error bars show s.d. obtained from triplicate measurements in real-time PCR quantification. The results of biologically independent experiments are presented in Supplementary Figure S6.

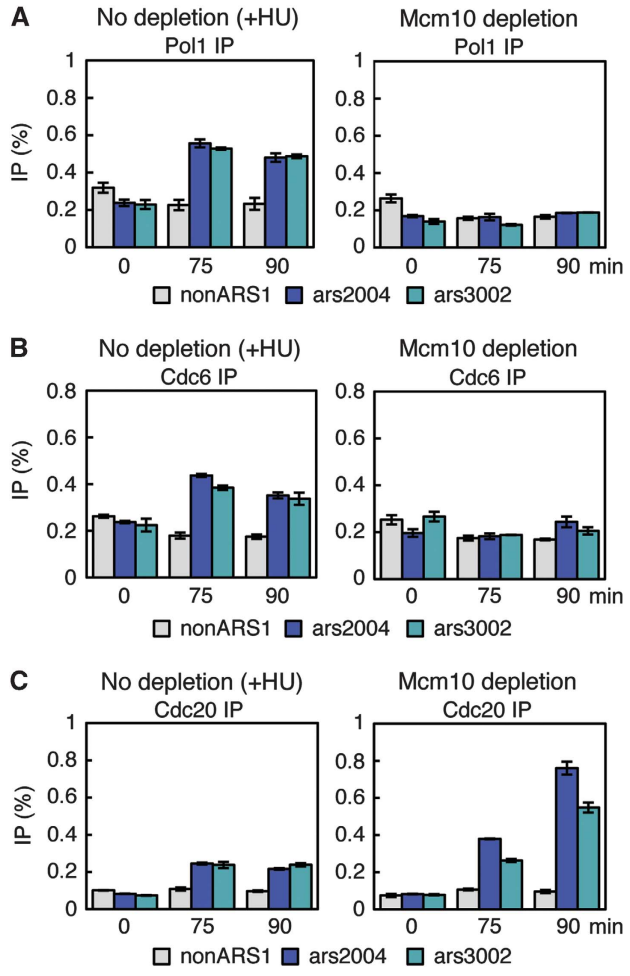


Figure 5 Replisome assembly is dependent on Mcm10. (**A**–**C**) *mcm10-off-aid* cells carrying *pol1–flag*, *cdc6–flag* or *cdc20–flag* were arrested at the G2/M boundary with or without Mcm10 depletion and released into the synchronous cell cycle. HU (12 mM) was added to the cells without depletion. 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. The results of biologically independent experiments are presented in Supplementary Figure S7.

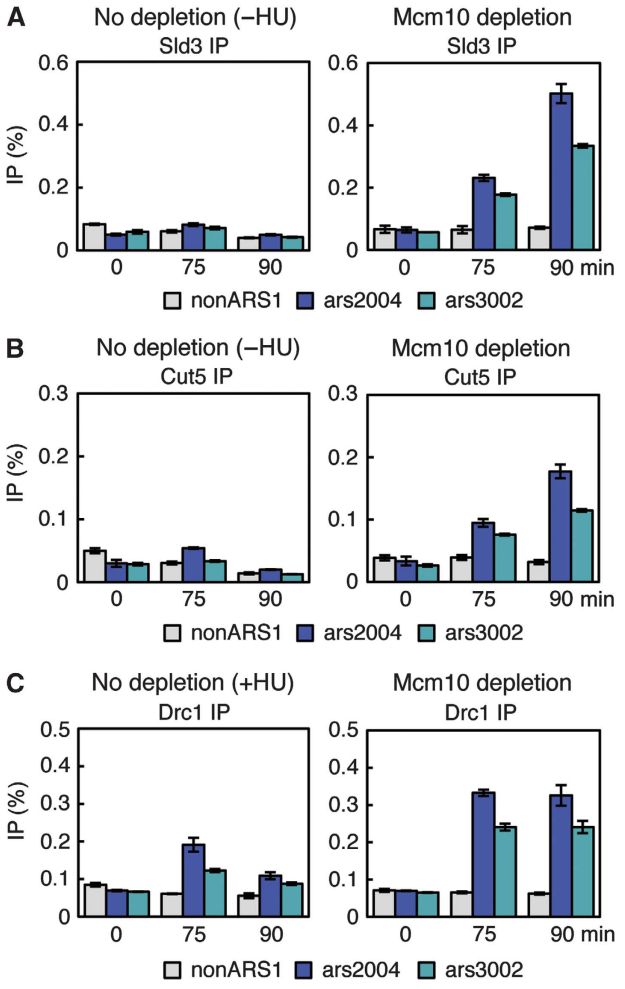


Figure 6 Sld3, Cut5 and Drc1 accumulate at replication origins in the absence of Mcm10. (**A**, **B**) *mcm10-off-aid* cells carrying *sld3–flag* and *cut5–myc* were arrested at the G2/M boundary with or without Mcm10 depletion and released into the synchronous cell cycle. DNA co-immunoprecipitated with 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. The results of biologically independent experiments are presented in Supplementary Figure S9. (**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.

(Figure 7D). These results suggest that the zinc finger plays an essential role in DNA replication.

Next, we examined whether the zinc-finger mutant protein bound to replication origins and supported origin unwinding. Immunoblotting of Flag–Mcm10$^{WT/ZA}$ using anti-Flag antibodies showed that Mcm10^{ZA} protein was decreased to about half after depletion of Mcm10 (Figure 7E), 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 all three strains (Figure 7F). 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 7H). Interestingly, Mcm10^{ZA} protein was localized at the origin more efficiently than Mcm10^{WT} protein (Figure 7I). 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 we examined whether recruitment of Mcm10 to the origin caused release of Sld3, Sld3 was found to accumulate at the origin in Mcm10^{ZA} cells, as was the case in *mcm10-off-aid* cells (Figure 7G). These results suggest that Sld3 is released during or after Mcm10-dependent origin unwinding.

Discussion

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

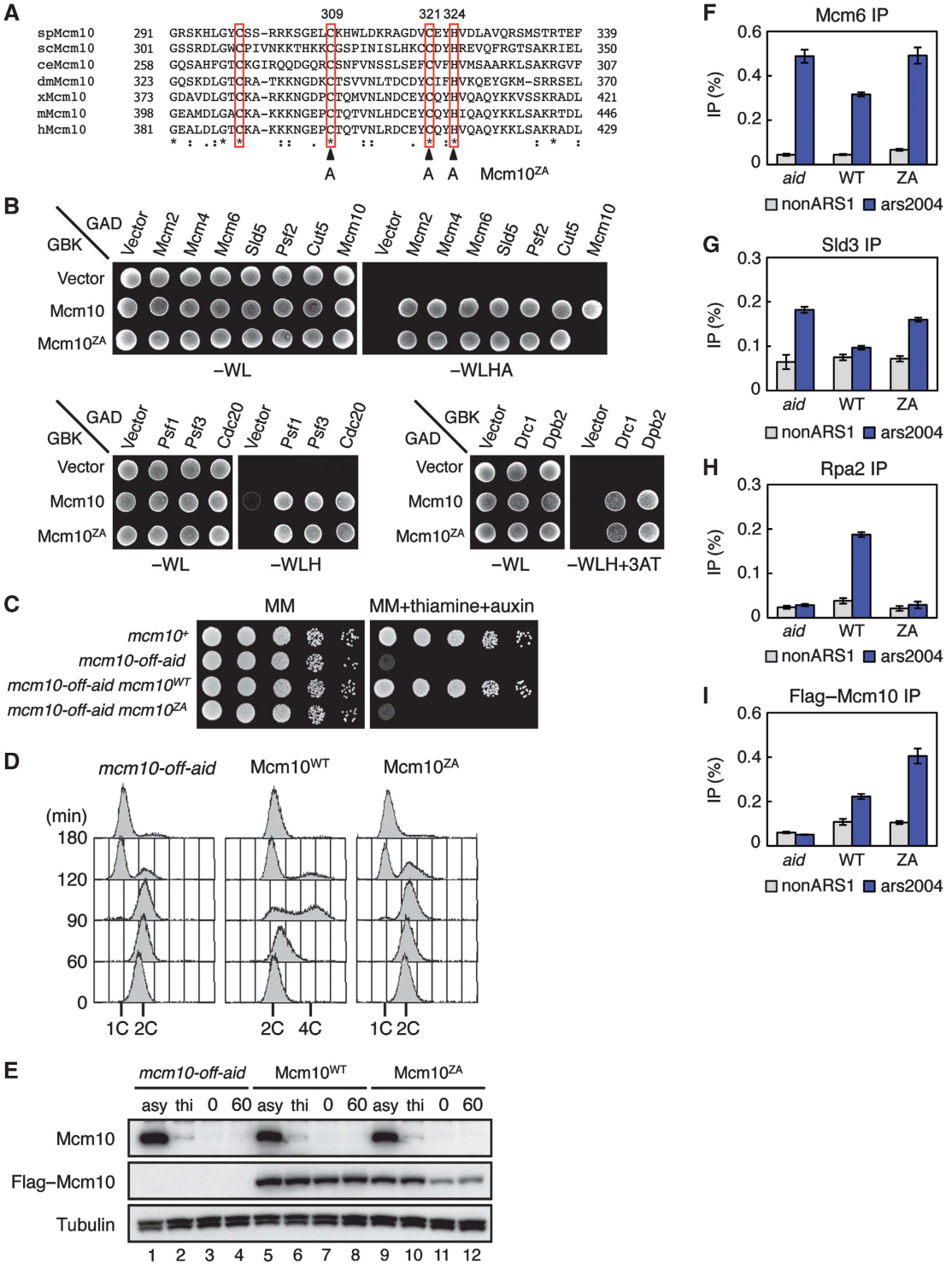


Figure 7 A conserved zinc-finger motif of Mcm10, which functions in self-interaction, is essential for origin DNA unwinding. (**A**) The amino-acid sequence around the conserved CCCH zinc-finger motif (red boxes) of Mcm10 of *Shizosaccharomyces pombe* (sp), *Saccharomyces cerevisiae* (sc), *Caenorhabditis 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. (**B**) The results of the yeast two-hybrid assays of interactions of $Mcm10^{WT}$ or $Mcm10^{ZA}$ with other replication factors are presented (see Materials and methods). (**C**) 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. (**D**) 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. (**E**) 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 (**D**) were analysed by immunoblotting using anti-Mcm10, anti-Flag and anti-α-tubulin antibodies. (**F–I**) DNA co-immunoprecipitated with Mcm6 (**F**), Sld3 (**G**), Rpa2 (**H**) and Flag–Mcm10 (**I**) 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 show s.d. obtained from triplicate measurements in real-time PCR quantification. The results of biologically independent experiments are presented in Supplementary Figure S13.

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.

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 (Yabuuchi *et al*, 2006; Heller *et al*, 2011). CDK is required for origin loading of GINS, Pol ε, Cut5 and Drc1, which are dependent on each other (Yabuuchi *et al*, 2006; Muramatsu *et al*, 2010; Fukuura *et al*, 2011; T Handa and H Masukata, unpublished observations). Stable binding of Cdc45 depends on CDK and the other replication factors, including Sld3, Cut5, Drc1 and GINS (Kamimura *et al*, 2001; Kanemaki and Labib, 2006; Yabuuchi *et al*, 2006; Yamada *et al*, 2004). Using the *off-aid* system that decreases Mcm10 to $<0.5\%$ of the WT amount (Supplementary Figure S1), we demonstrated that Mcm10 is not required for recruitment of GINS, Cdc45 or the other factors described above (Figures 1 and 6). 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 3), 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 6). 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 <18 molecules per cell by the *off-aid* system (Figure 1A; Supplementary Figure S1), which is far below the number of active replication origins in fission yeast (Segurado *et al*, 2003; Feng *et al*, 2006; Hayashi *et al*, 2007). Our 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 remains, because previous studies have shown that Mcm10 promotes stable chromatin binding of Cdc45 in *Xenopus* egg extracts, budding yeast and fission yeast (Wohlschlegel *et al*, 2002; Gregan *et al*, 2003; Sawyer *et al*, 2004).

Origin binding of Mcm10 is dependent on GINS and Cdc45 (Figure 2), which is consistent with a budding yeast *in vitro* study (Heller *et al*, 2011) and with a recent *in vivo* study (Masato Kanemaki, personal communication). However, work from another group has shown that Mcm10 interacts with chromatin-loaded Mcm2-7 even in G1 (Karim Labib, personal communication). 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. At the onset of S-phase, interactions of Mcm10 with GINS, Cut5, Drc1 and Pol ε, as detected using the yeast two-hybrid assay (Figure 7B), may enhance the binding of Mcm10 to Mcm2-7.

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 1 and 4). This suggests that origin DNA was not significantly unwound, although we cannot exclude the possibility that Mcm10 is required for recruitment of RPA to unwound DNA. Thus, we propose that Mcm10 plays an essential role in origin DNA unwinding that is catalysed 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 (Karim Labib and Masato Kanemaki, personal communications), suggesting that the essential function of Mcm10 required for origin DNA unwinding is conserved.

Our 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 (Ricke and Bielinsky, 2004; Zhu *et al*, 2007; Heller *et al*, 2011), although we did not observe any destabilization of the catalytic subunit of Pol α (Supplementary Figure S8). Pol α might fail to bind to the origins due to a reduction of RPA binding, because depletion of RPA reduces chromatin binding of Pol α in *Xenopus* egg extracts (Walter and Newport, 2000). On the other hand, we could not exclude the possibility 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 (Fien *et al*, 2004; Ricke and Bielinsky, 2004; Chattopadhyay and Bielinsky, 2007; Robertson *et al*, 2008; Warren *et al*, 2009). Since Mcm10 is localized at replication forks in budding yeast, fission yeast and *Xenopus* egg extracts (Ricke and Bielinsky, 2004; Gambus *et al*, 2006; Pacek *et al*, 2006; Taylor *et al*, 2011), Mcm10 may play some roles at replication forks. However, the roles of Mcm10 at replication forks do not seem to be essential for elongation of DNA replication, because depletion of Mcm10 to 0.5% of the WT level from HU-arrested fission yeast cells did not cause severe inhibition of DNA replication after HU removal (Supplementary Figure S14). 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

We observed that initiation-specific factors such as Sld3, Cut5 and Drc1, which do not travel with replication forks (Kanemaki and Labib, 2006; Labib, 2010; Taylor *et al*, 2011), accumulated at replication origins in the absence of Mcm10 (Figure 6). These results suggest that Mcm10 is required for a step at which initiation-specific factors are released from origins. It is unlikely, however, that origin binding of Mcm10 is directly responsible for their removal, because Sld3 accumulated at the origin together with $Mcm10^{ZA}$ (Figure 7G and I). 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

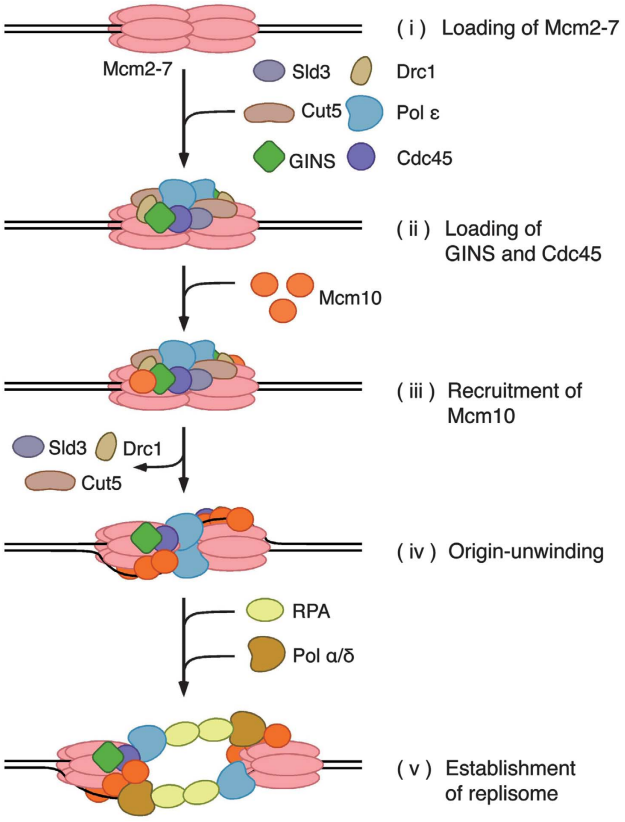


Figure 8 A model for the essential role of Mcm10 in origin DNA unwinding. The process of initiation of replication where Mcm10 plays an essential role in origin DNA unwinding is presented. (i) In G1-phase, the double-hexameric Mcm2-7 is loaded onto origin dsDNA to form pre-RC. (ii) At the onset of S-phase, Sld3, Cut5, Drc1, GINS, Pol ε and Cdc45 assemble onto the Mcm2-7. (iii) Mcm10 binds to the complex depending on assembly of the CMG components. Loading of Mcm10 does not cause release of Sld3, Cut5 or Drc1. (iv) Mcm10 forms homo-multimers and promotes conversion of the Mcm2-7 complex from the dsDNA-bound double hexamer into two ssDNA-bound single hexamers through its interactions with multiple subunits of Mcm2-7. These structural changes may cause release of Sld3, Cut5 and Drc1 from origins. Alternatively, Mcm10 captures ssDNA extruded from the gate between Mcm2 and Mcm5, to stabilize the ssDNA-bound form of Mcm2-7. Sld3, Cut5 and Drc1 are released from Mcm2-7 during the conversion. (v) 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.

release of initiation-specific factors from origins (Figure 8 and see below), although it remains possible that dissociation of these factors is needed for the change in the helicase.

Essential roles of the zinc-finger motif of Mcm10 in origin DNA unwinding

We showed that the zinc-finger motif of Mcm10 is essential for cell viability and DNA replication (Figure 7). It is likely that the mutations in the zinc finger specifically impaired the function of Mcm10 in origin DNA unwinding, because the Mcm10^{ZA} mutant protein bound to replication origins but did not promote binding of RPA (Figure 7H and I). What is the function of the zinc finger of Mcm10 in origin DNA unwinding for initiation of DNA replication? The zinc finger of Mcm10 may play a role in a step required for the conversion of Mcm2-7 (Figure 8). 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 (Moyer *et al*, 2006; Ilves *et al*, 2010; Yardimci *et al*, 2010; Fu *et al*, 2011). 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). The yeast two-hybrid assay showed that the zinc-finger mutation specifically impaired the interaction of Mcm10 with Mcm10 itself (Figure 7B). This is consistent with results that the zinc-finger motif of budding yeast Mcm10 is required for formation of a homo-multimeric complex (Cook *et al*, 2003). Mcm10 in oligomeric forms may interact with multiple subunits of Mcm2-7 and enforce structural changes in Mcm2-7, such as 'gate opening' between Mcm2 and Mcm5, for possible extrusion of the ssDNA (Costa *et al*, 2011) (Figure 8). Alternatively, the zinc finger of Mcm10 may participate in capturing the ssDNA that is extruded through the gate, and stimulating conversion of dsDNA-bound Mcm2-7 into the ssDNA-encircling complex (Figure 8). Further analysis will be needed to clarify the mechanism of origin DNA unwinding promoted by Mcm10.

Materials and methods

Strains

Fission yeast strains used in this study are listed in Supplementary Table S2. Details of strain construction are provided in the Supplementary data.

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 (Supplementary Table S3). The PCR product digested with *Bam*HI was cloned into pUC119 and the sequence was confirmed. The *Nde*I–*Bam*HI 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).

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 μg/ml at the indicated time points before G2/M arrest (see Figure 1, 2 and 7 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 (1:3000) (Ogawa *et al*, 1999), rabbit anti-Mcm4 (1:2000) (Sherman *et al*,

1998), rabbit anti-Mcm10 (1:2000), rabbit anti-Cdc45 (1:1000) (Nakajima and Masukata, 2002), rabbit anti-IAA17 (1:2000) (Nishimura *et al*, 2009), mouse anti-FLAG M2 (1:3000; Sigma-Aldrich), mouse anti-HA 16B12 (1:1000; Covance) or mouse anti-TAT1 (1:2000) (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*-α-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 μl of 10% Triton X-100 and 54 μl 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 r.p.m. for 10 min was used for immunoprecipitation with 10 μl 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 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 PMSF and proteinase inhibitor cocktail (Sigma-Aldrich)) and disrupted with glass beads using a Micro Smash (TOMY SEIKO). After addition of 50 μl 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 r.p.m. 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 analysed 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 nonARS-F and nonARS-R for the non-origin region. The primer sets used for real-time PCR are listed in Supplementary Table S3.

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, an activation-domain (AD) vector, and pGBKT7, a Gal4-DNA-binding domain (BD) vector, were constructed as follows. The *Nde*I–*Bam*HI fragment containing the full-length *mcm10*$^+$, *mcm10*^{2A}, *drc1*$^+$ or *dpb2*$^+$ was introduced into pGBKT7. Similarly, the *Nde*I–*Bam*HI 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 *Saccharomyces cerevisiae* AH109 (*MATa*, *trp1-901*, *leu2-3*, *112*, *ura3-52*, *his3-200*, *gal4*Δ, *gal80*Δ, *LYS2::GAL1*$_{UAS}$*-GAL1*$_{TATA}$*-HIS3*, *GAL2*$_{UAS}$*-GAL2*$_{TATA}$*-ADE2*, *URA3::MEL1*$_{UAS}$*-MEL1*$_{TATA}$*-lacZ*, *MEL1*) cells. Trp$^+$ Leu$^+$ transformants harbouring both plasmids were selected on synthetic growth medium (SD) lacking tryptophan and leucine (–WL) and the interaction was analysed 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).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: MK carried out the molecular genetic studies, participated in the design of the study and drafted the manuscript. YK participated in the molecular genetic studies. TST and TN participated in the design of the study and contributed to analysis and interpretation of the data. HM conceived the study and participated in its design and coordination. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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