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Mcm10 associates with the loaded DNA helicase at replication origins and defines a novel step in its activation

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Mcm10 is essential for chromosome replication in eukaryotic cells and was previously thought to link the Mcm2-7 DNA helicase at replication forks to DNA polymerase alpha. Here, we show that yeast Mcm10 interacts preferentially with the fraction of the Mcm2-7 helicase that is loaded in an inactive form at origins of DNA replication, suggesting a role for Mcm10 during the initiation of chromosome replication, but Mcm10 is not a stable component of the replisome subsequently. Studies with budding yeast and human cells indicated that Mcm10 chaperones the catalytic subunit of polymerase alpha and preserves its stability. We used a novel degron allele to inactivate Mcm10 efficiently and this blocked the initiation of chromosome replication without causing degradation of DNA polymerase alpha. Strikingly, the other essential helicase subunits Cdc45 and GINS were still recruited to Mcm2-7 when cells entered S-phase without Mcm10, but origin unwinding was blocked. These findings indicate that Mcm10 is required for a novel step during activation of the Cdc45–MCM–GINS helicase at DNA replication origins.

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Introduction

The DNA helicase responsible for unwinding the parental DNA duplex during chromosome replication in eukaryotic cells is remarkably complex, and there are many outstanding questions regarding its regulation and mechanism of action (Remus and Diffley, 2009; Araki, 2010). The catalytic core of the helicase comprises a hexameric ring of the six related Mcm2-7 proteins, originally identified in a screen for yeast mutants that had defects in maintaining a minichromosome (Mcm = minichromosome maintenance). Away from DNA, the Mcm2-7 proteins form subcomplexes or a single heterohexamer, but work with budding yeast showed that Mcm2-7 are loaded in an inactive form at origins of DNA replication as a head-to-head double hexamer around double-strand DNA (Evrin *et al*, 2009; Remus *et al*, 2009), and the loaded Mcm2-7 complex in extracts of *Xenopus* eggs also behaves as a double hexamer (Gambus *et al*, 2011). Loading of Mcm2-7 can only occur during the G1-phase of the cell cycle, and the loaded complex is then activated *in situ* during S-phase by recruitment of the Cdc45 protein and the GINS complex to form the active Cdc45–MCM–GINS helicase at nascent DNA replication forks (Moyer *et al*, 2006; Labib, 2010). A variety of regulatory factors assemble around the CMG helicase to produce the 'Replisome Progression Complex' (Gambus *et al*, 2006), which associates with DNA polymerases to form the eukaryotic replisome, the structure of which is still quite poorly defined in comparison with its bacterial counterpart (Yao and O'Donnell, 2010).

The isolated CMG helicase and Replisome Progression Complex contain a single Mcm2-7 ring (Gambus *et al*, 2006; Moyer *et al*, 2006; Costa *et al*, 2011), suggesting that the loaded double hexamer of Mcm2-7 at origins might be broken into two single hexameric rings as part of the initiation process, with each Mcm2-7 ring forming the core of a CMG helicase at the two replication forks established from each origin. This idea is supported by evidence that the two replisomes formed from one origin in a *Xenopus* egg extract can function independently and do not need to be tethered to each other (Yardimci *et al*, 2010). Moreover, very recent work indicates that the replisome can bypass a protein–DNA barrier on the lagging strand DNA template more easily than an identical barrier on the leading strand DNA template (Fu *et al*, 2011), suggesting that the CMG helicase might associate very stably with just one of the two DNA strands at replication forks. The implications of these findings remain to be explored further, but they suggest an attractive model whereby the Mcm2-7 double hexamer around dsDNA is remodelled during initiation, producing two separate Mcm2-7 rings that each encircles just one stand of DNA in an active CMG helicase. The mechanisms and regulation of these presumed transitions are completely unclear at present, and until now the only well-characterised step during the activation of the Mcm2-7 helicase has been the recruitment of Cdc45 and GINS (Remus and Diffley, 2009; Araki, 2010).

The Mcm10 protein was identified in the same screen that yielded the first of the budding yeast Mcm2-7 proteins (Maine *et al*, 1984; Merchant *et al*, 1997), and was also found in a separate screen for temperature-sensitive yeast strains with mutations that caused defects in DNA synthesis (Dumas *et al*, 1982; Solomon *et al*, 1992). Mcm10 is unrelated in primary sequence to the Mcm2-7 proteins and is conserved in most if not all eukaryotes (Liu *et al*, 2009). Orthologues of Mcm10

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have been shown to be essential for DNA synthesis in fission yeast (Nasmyth and Nurse, 1981), *Xenopus* (Wohlschlegel *et al*, 2002) and mouse (Lim *et al*, 2011), although Mcm10 is generally very abundant and so depletion experiments are complicated by the fact that a small fraction of protein can be sufficient to supply function (Christensen and Tye, 2003; Chattopadhyay and Bielinsky, 2007).

Budding yeast Mcm10 was shown to be required for the firing of replication origins (Merchant *et al*, 1997), and work with budding yeast (Ricke and Bielinsky, 2004; Raveendranathan *et al*, 2006), fission yeast (Taylor *et al*, 2011), *Xenopus* (Pacek *et al*, 2006) and human cells (Karnani and Dutta, 2011) then indicated that Mcm10 is also present at DNA replication forks. The molecular role of Mcm10 is still poorly defined, but all Mcm10 orthologues share an 'internal domain' that shows robust binding to ssDNA and can also bind dsDNA under some conditions (Fien *et al*, 2004; Robertson *et al*, 2008; Warren *et al*, 2008, 2009; Eisenberg *et al*, 2009). Budding yeast Mcm10 has the ability to form oligomers (Cook *et al*, 2003), and electron microscopy of human Mcm10 revealed a hexameric ring reminiscent of the Mcm2-7 complex (Okorokov *et al*, 2007), although the significance of this structure remains to be established.

Studies of budding yeast Mcm10 indicated that it can interact with several of the Mcm2-7 proteins (Merchant *et al*, 1997; Gambus *et al*, 2006) and the same was reported for orthologues of Mcm10 in fission yeast (Hart *et al*, 2002; Lee *et al*, 2003), *Drosophila* (Apger *et al*, 2010), *Xenopus* (Zhu *et al*, 2007) and human (Izumi *et al*, 2000; Zhu *et al*, 2007), with an apparent preference for Mcm6 and Mcm2. Subsequently, Mcm10 from yeast (Fien *et al*, 2004; Ricke and Bielinsky, 2004), *Xenopus* (Robertson *et al*, 2008) and human (Chattopadhyay and Bielinsky, 2007; Zhu *et al*, 2007) was also shown to interact with the catalytic subunit of DNA polymerase alpha, as well as the Ctf4/And1 protein that itself is a partner of polymerase alpha (Zhu *et al*, 2007; Wang *et al*, 2010). Moreover, work with budding yeast and human cells indicated that Mcm10 might chaperone polymerase alpha and be required to stabilise the catalytic subunit (Ricke and Bielinsky, 2004, 2006; Chattopadhyay and Bielinsky, 2007).

Crucially, specific mutations in budding yeast Mcm2-7 proteins suppressed the lethal defect in chromosome replication at 37°C that is associated with the temperature-sensitive alleles *mcm10-1* and *mcm10-43* (Homesley *et al*, 2000; Liachko and Tye, 2005; Lee *et al*, 2010), indicating that an important role of Mcm10 is intimately related to the function of the Mcm2-7 helicase. The suppressor mutations were dominant, predominantly found in Mcm2, and led to a plasmid-loss phenotype indicating defective Mcm2-7 function (Lee *et al*, 2010). In addition, analogous mutations in an archaeal orthologue of Mcm2-7 reduced the efficiency of helicase action somewhat (Lee *et al*, 2010). Taken together, these various studies led to several related models for Mcm10 function. First, Mcm10 has been proposed to stabilise the catalytic subunit of DNA polymerase alpha at least in budding yeast and humans (Ricke and Bielinsky, 2004; Chattopadhyay and Bielinsky, 2007), though one study attributed this effect to Ctf4 rather than Mcm10 (Zhu *et al*, 2007). Second, Mcm10 was proposed to recruit DNA polymerase alpha to origins during initiation (Zhu *et al*, 2007), and then physically couple Mcm2-7 to polymerase alpha at replication forks together with Ctf4 (Zhu *et al*, 2007; Lee *et al*, 2010). Defective replication associated with the budding yeast alleles *mcm10-1* and *mcm10-43* was attributed in part to degradation of the Pol1 catalytic subunit of polymerase alpha (Ricke and Bielinsky, 2004), and also to a proposed loss of the physical connection between Mcm2-7 and polymerase alpha (Lee *et al*, 2010). According to this view, suppression of *mcm10^{ts}* alleles by the Mcm2 mutations resulted from defects in helicase activity that reduced the rate of unwinding and so reestablished the co-ordination of unwinding with DNA polymerase activity, without restoring physical coupling (Lee *et al*, 2010). It also appears that Mcm10 plays a role at DNA replication forks that is mediated by interaction of diubiquitylated Mcm10 with PCNA (Das-Bradoo *et al*, 2006), and other proposed roles include the maintenance of silent chromatin via interaction with silencing factors (Douglas *et al*, 2005; Liachko and Tye, 2005, 2009), the recruitment of Cdc45 during the initiation of chromosome replication (Wohlschlegel *et al*, 2002), and primase activity (Fien and Hurwitz, 2006).

To explore further how budding yeast Mcm10 functions during chromosome replication, we have examined the preferred interactions of Mcm10 with the DNA replication machinery in yeast cell extracts. Under physiological salt concentrations, we find that Mcm10 interacts preferentially with the loaded Mcm2-7 complex and is not associated stably with the replisome. By combining degron technology with the *mcm10-1* allele, we show that Mcm10 is required for a novel aspect of the initiation reaction, as inactivation of Mcm10 blocks origin unwinding despite the formation of a stable Cdc45–MCM–GINS complex. These findings lead to a revised model for

Figure 1 Mcm10 associates preferentially with the loaded Mcm2-7 complex. (**A**) The unloaded Mcm2-7 complex breaks into subcomplexes in the cell extracts, but can be stabilised by addition of ATP to the extract. The loaded Mcm2-7 complex is stable even without addition of ATP, but must be released from chromatin (by benzonase in our experiments) in order to be isolated. (**B**) *MCM4-9MYC MCM10-5FLAG* (YFJD84) was arrested in G1-phase by addition of mating pheromone and extracts prepared under the indicated conditions, before immunoprecipitation of Mcm4–9MYC. The indicated proteins were monitored by immunoblotting, and the asterisk marks the position of a background band that is recognised in the cell extracts by the anti-FLAG antibody. (**C**) Control (YFJD155) and *cdc28-td* (YFJD154) were grown at 24°C in YPRaff medium and arrested in G2-M-phase with Nocodazole. Cdc28-td was depleted for 1 h at 37°C as described in Materials and methods, maintaining the nocodazole arrest throughout. Extracts of cells at 24 and 37°C were made (–ATP +benzonase) before immunoprecipitation of Mcm4. Stable co-immunoprecipitation of Mcm3/5/7 reflects isolation of the loaded Mcm2-7 complex. (**D**) The same strains as in (**C**) were arrested at 24°C in G1-phase with mating pheromone in YPRaff medium, before inactivation of Cdc28-td at 37°C for 1 h in YPGal medium. Cells were then washed into medium lacking mating pheromone and incubated for the indicated times. DNA content and budding index were monitored throughout the experiment, and the association of Mcm4–9MYC with the indicated proteins was monitored as above, either just before release from G1-arrest at 37°C or 20 min afterwards when control cells were entering S-phase. (**E**) Control (YFJD84) and *cdc7-1* (YFJD133) were grown at 24°C in YPD medium and arrested in G2-M-phase with Nocodazole. Cells were then washed into fresh medium containing alpha factor and incubation continued at 37°C until 90% cells were arrested in G1-phase. Mcm4 was isolated from cell extracts (+benzonase–ATP) by immunoprecipitation. In addition, an aliquot of each culture was released from G1-arrest to confirm by flow cytometry that DNA replication could not occur at 37°C in the *cdc7-1* strain.

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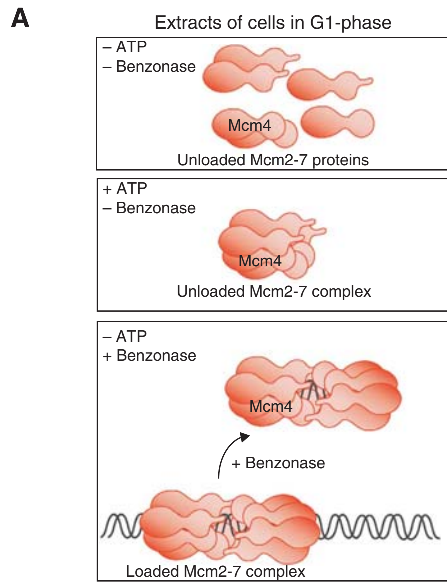
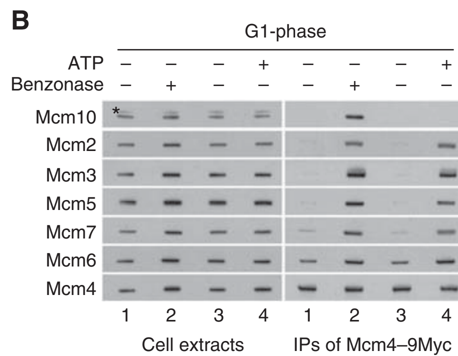
Mcm10 function and point to a previously unanticipated role for Mcm10 in activating the Cdc45–MCM–GINS helicase during the initiation of chromosome replication.

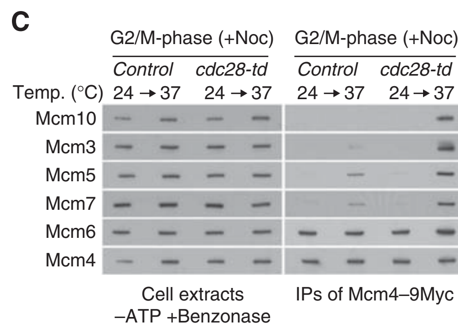
Results

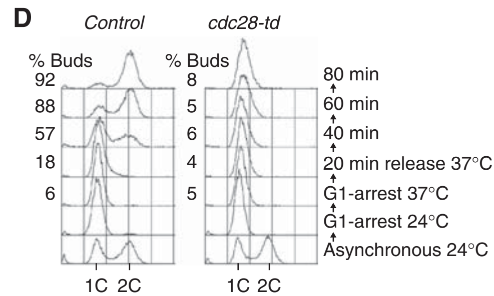
Mcm10 associates preferentially with the loaded form of the Mcm2-7 complex

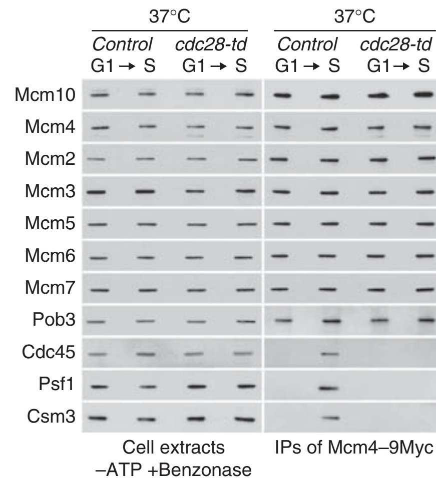
Previously, we isolated the Replisome Progression Complex from DNA replication forks in yeast cell extracts, by digestion of chromosomal DNA followed by consecutive immunoprecipitation of GINS and Mcm2-7, and found that Mcm10 only co-purified at unphysiologically high salt concentrations (Gambus *et al*, 2006, 2009). We also found that Mcm10 co-purified with the Mcm2-7 helicase from extracts of G1-phase cells (Gambus *et al*, 2006), but in this case the association of Mcm10 with Mcm2-7 was salt sensitive and thus appeared to involve a distinct complex (Hiroko Morohashi and Karim Labib, unpublished observations). To study how Mcm10 might interact with Mcm2-7 before loading of the helicase,

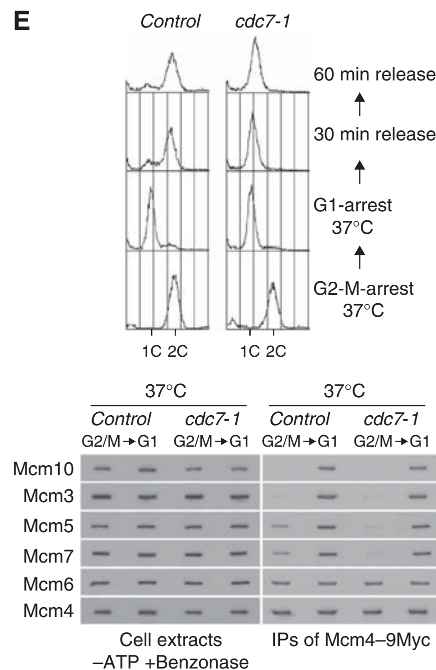












after loading at origins during G1-phase, or following subsequent activation and assembly of the replisome, we developed procedures that allow us to distinguish between these various forms of Mcm2-7 in yeast cell extracts (Figure 1A). All these experiments were performed in the presence of a physiologically plausible concentration of 100 mM salt.

Using our standard extract conditions (see Materials and methods), the loaded Mcm2-7 complex was removed during the centrifugation steps together with almost all of the genomic DNA, whereas the unloaded Mcm2-7 complex was unstable and broke into several subcomplexes, so that isolation of the Mcm4 subunit led to efficient co-immunoprecipitation of Mcm6 but not Mcm2-3-5-7 (Figure 1B, samples 1 and 3). To isolate the unloaded Mcm2-7 complex in a stable form, we made extracts in the presence of ATP (Evrin *et al*, 2009; Remus *et al*, 2009). Under such conditions, Mcm4 associated efficiently with Mcm2-3-5-6-7 (Figure 1B, sample 4). In contrast, we found that the loaded Mcm2-7 complex was equally stable whether or not ATP was added to the extract (FvD and KL, unpublished observations), and we were thus able to isolate specifically the loaded Mcm2-7 complex by prior digestion of chromosomal DNA in extracts lacking ATP (Figure 1B, sample 2). Strikingly, Mcm10 only co-purified with the Mcm2-7 complex that had been released from chromatin (Figure 1B, compare samples 2 and 4), and we confirmed that this was not due to ATP inhibiting the interaction of Mcm10 with the unloaded complex (FvD and KL, unpublished observations). These findings indicated that Mcm10 interacts preferentially with the loaded form of the Mcm2-7 helicase under physiological conditions.

Our data are consistent with previous studies showing that Mcm10 could be detected by chromatin immunoprecipitation at both early and late origins in G1-phase (Ricke and Bielinsky, 2004; Raveendranathan *et al*, 2006), as well as the fact that recruitment of Mcm10 to chromatin in extracts of *Xenopus* eggs requires prior loading of Mcm2-7 (and also the 10-fold concentration of Mcm10 protein that occurs within the nucleus) but is independent of cyclin-dependent kinase (CDK) or Cdc7 kinase (Wohlschlegel *et al*, 2002). To test directly whether the association of Mcm10 with loaded Mcm2-7 in our cell extracts was independent of CDK, which is not active during G1-phase, we used the heat-inducible degron (Dohmen *et al*, 1994; Labib *et al*, 2000) to regulate the level of Cdc28, the budding yeast CDK. We grew control and *cdc28-td* cells at 24°C and then synchronised the cultures in G2-M-phase by addition of nocodazole. Under these conditions, high CDK activity blocks loading of the Mcm2-7 helicase at origins of DNA replication (Dahmann *et al*, 1995; Tanaka *et al*, 1997), so that after digesting chromatin in cell extracts lacking ATP, Mcm4 associated efficiently with Mcm6 but not with Mcm2-3-5-7 or Mcm10 (Figure 1C, IPs of Mcm4 at 24°C). Following inactivation of Cdc28-td at 37°C, however, reloading of Mcm2-7 was reflected by the stable association of Mcm4 with the other Mcm2-7 proteins after chromatin digestion, and Mcm10 co-immunoprecipitated with the loaded Mcm2-7 proteins as predicted (Figure 1C, *cdc28-td* at 37°C). We also confirmed that inactivation of Cdc28-td during G1-phase blocked budding, DNA replication and the stable association of Mcm2-7 with other components of the Replisome Progression Complex such as Cdc45 and GINS, but inactivation of Cdc28-td did not affect the association of Mcm2-7 with Mcm10 in G1-phase or early S-phase (Figure 1D). These findings indicated that Mcm10 could associate with the loaded Mcm2-7 complex in the absence of CDK activity, under these experimental conditions.

To test whether the association of Mcm10 with loaded Mcm2-7 was also independent of Cdc7 kinase, we synchronised control and *cdc7-1* cells in G2-M-phase with nocodazole, before shifting to 37°C. Cells were then washed into fresh medium containing mating pheromone so that they passed through mitosis and arrested in G1-phase of the subsequent cell cycle. Mcm10 still associated with the loaded Mcm2-7 complex in both strains, despite the fact that the subsequent round of replication was blocked in the absence of Cdc7 activity (Figure 1E). Taken together, the above data indicate that budding yeast Mcm10 associates preferentially with the loaded form of the Mcm2-7 complex. These findings are consistent not just with the known regulation of Mcm10 recruitment to chromatin in *Xenopus* egg extracts (Wohlschlegel *et al*, 2002), but also with studies that used chromatin immunoprecipitation to monitor Mcm10 at human replication origins, which showed that Mcm10 is recruited to late origins prior to initiation and thus prior to recruitment of Cdc45, in cells that have low CDK activity due to activation of the S-phase checkpoint response (Karnani and Dutta, 2011).

Mcm10 does not co-purify with DNA polymerase alpha as part of the replisome

We then studied how the association of Mcm10 with Mcm2-7 and other replication proteins might change as cells passed synchronously through one round of the cell cycle (Figure 2A), using cell extracts in which we digested chromosomal DNA to liberate replication proteins from origins and forks. As predicted by the above data, Mcm10 interacted with loaded Mcm2-7 proteins in G1-phase (Figure 2B, 0 min). Upon release from G1-arrest, the complex of Mcm10 with Mcm2-7 diminished by the middle of S-phase and disappeared afterwards, reflecting the displacement of Mcm2-7 from chromatin, before reappearing when cells were blocked in the subsequent G1-phase by readdition of mating pheromone to the cell culture (Figure 2B, 90 min). Despite its robust interaction with loaded Mcm2-7 proteins, Mcm10 did not co-purify from extracts of S-phase cells with replisome components such as Cdc45 and GINS, or with Ctf4 and the Pol1 catalytic subunit of DNA polymerase alpha. We confirmed that polymerase alpha associated with replisome material in yeast cell extracts under identical conditions (Figure 2C). Moreover, Mcm4 associated with Mcm2-7 proteins and Mcm10 during both G1 and S-phase, but with replisome components only during S-phase (Figure 2D). These findings indicate that Mcm10 associates with the loaded Mcm2-7 complex but does not represent an essential link between helicase and DNA polymerase alpha within the replisome.

The essential role of Mcm10 during chromosome replication is independent of the preservation of Pol1 stability

Previous work indicated that budding yeast Mcm10 is important to stabilise the Pol1 catalytic subunit of DNA polymerase alpha, as Pol1 was found to be degraded at 37°C in *mcm10-1* cells and the same was true for *mcm10-td* cells that have the heat-inducible degron fused to Mcm10 (Ricke and Bielinsky, 2004). Unfortunately, we were not able

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to reproduce these findings even with the identical strains (for example, see Supplementary Figure S1). In the original study, Mcm10 was found to interact exclusively with a 140-kDa proteolytic fragment of Pol1 and the authors noted that Pol1 is extremely sensitive to proteolysis *in vitro* (Ricke and Bielinsky, 2004), providing a potential explanation for the discrepancy. To try and inactivate Mcm10 even more efficiently and then examine the consequences for chromosome replication, we added the heat-inducible degron cassette to the *mcm10-1* allele. The resultant *mcm10-1td* strain grew well at 24°C but could not grow at 37°C (Figure 3A), following depletion and inactivation of Mcm10-1td (Figure 3B). Nevertheless, we could not detect any change in the level of Pol1 following inactivation of Mcm10-1td (Figure 3C). These data indicate that Mcm10 has an essential role that is independent of the stabilisation of Pol1.

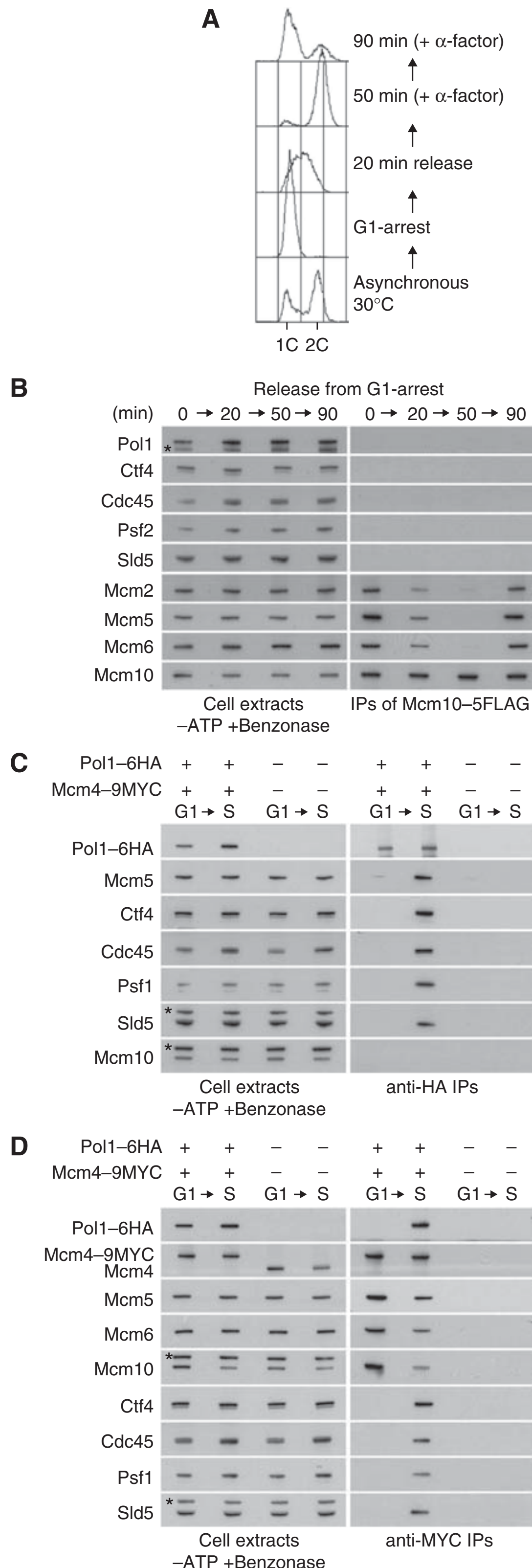

Figure 2 Mcm10 associates stably with the loaded Mcm2-7 complex but not with the replisome. (**A**) Cells of MCM10–5FLAG (CC4972) were arrested in G1-phase at 30°C in YPD medium and then released so that they passed synchronously through one round of the cell cycle. Alpha factor was added again from 50 min onwards so that dividing cells were then blocked in the subsequent G1-phase. DNA content was monitored throughout the experiment by flow cytometry. Note that progression through the cell cycle was faster in this experiment than in Figure 1D, as cells grow more quickly when glucose is used as the carbon source in place of galactose. (**B**) Mcm10–5FLAG was isolated from native cell extracts (+benzonase –ATP) by immunoprecipitation. (**C**, **D**) A similar experiment was performed with *POL1-6HA MCM4-9MYC* (YFJD84) after releasing G1-phase cells for 20 min into S-phase. The asterisks mark the positions of non-specific bands in the cell extracts.

Mcm10 is required for a novel step during the initiation of chromosome replication that is distinct from assembly of the Replisome Progression Complex

If the initiation of chromosome replication is blocked very efficiently, for example by preventing the assembly of the Cdc45–MCM–GINS complex, the absence of DNA replication forks prevents activation of the S-phase checkpoint response that normally protects cells from DNA replication stress (Piatti *et al*, 1996; Tercero *et al*, 2000). In contrast, defects in DNA polymerase alpha lead to very efficient activation of the S-phase checkpoint when budding yeast cells enter S-phase (Marini *et al*, 1997; Pellicioli *et al*, 1999), as the active Cdc45–MCM–GINS helicase unwinds replication origins and generates a defective DNA replication fork that contains the substrates for checkpoint activation such as single-strand DNA.

To compare directly the effects of inactivating Mcm10 or DNA polymerase alpha, we generated a novel strain in which the heat-inducible degron was fused to multiple subunits of DNA polymerase alpha (Pol1, Pol12 and Pri1; see Supplementary Figure S2). Control, *mcm10-1td* and *polα-td* were all synchronised in G1-phase with mating pheromone at 24°C, before depletion of the degron-fusion proteins at 35°C. Whereas control cells replicated rapidly upon transfer to fresh medium lacking pheromone, replication was blocked to a comparable degree in both *mcm10-1td* and *polα-td* (Figure 3D). Strikingly, the Rad53 checkpoint kinase became hyperphosphorylated when *polα-td* entered S-phase and assembled defective DNA replication forks, but a similar indication of checkpoint activation was not detected when *mcm10-1td* entered S-phase. To confirm this result under more stringent conditions, we also released half of each culture in the above experiment from G1-arrest at 35°C into medium containing 0.2 M hydroxyurea, which limits dNTP

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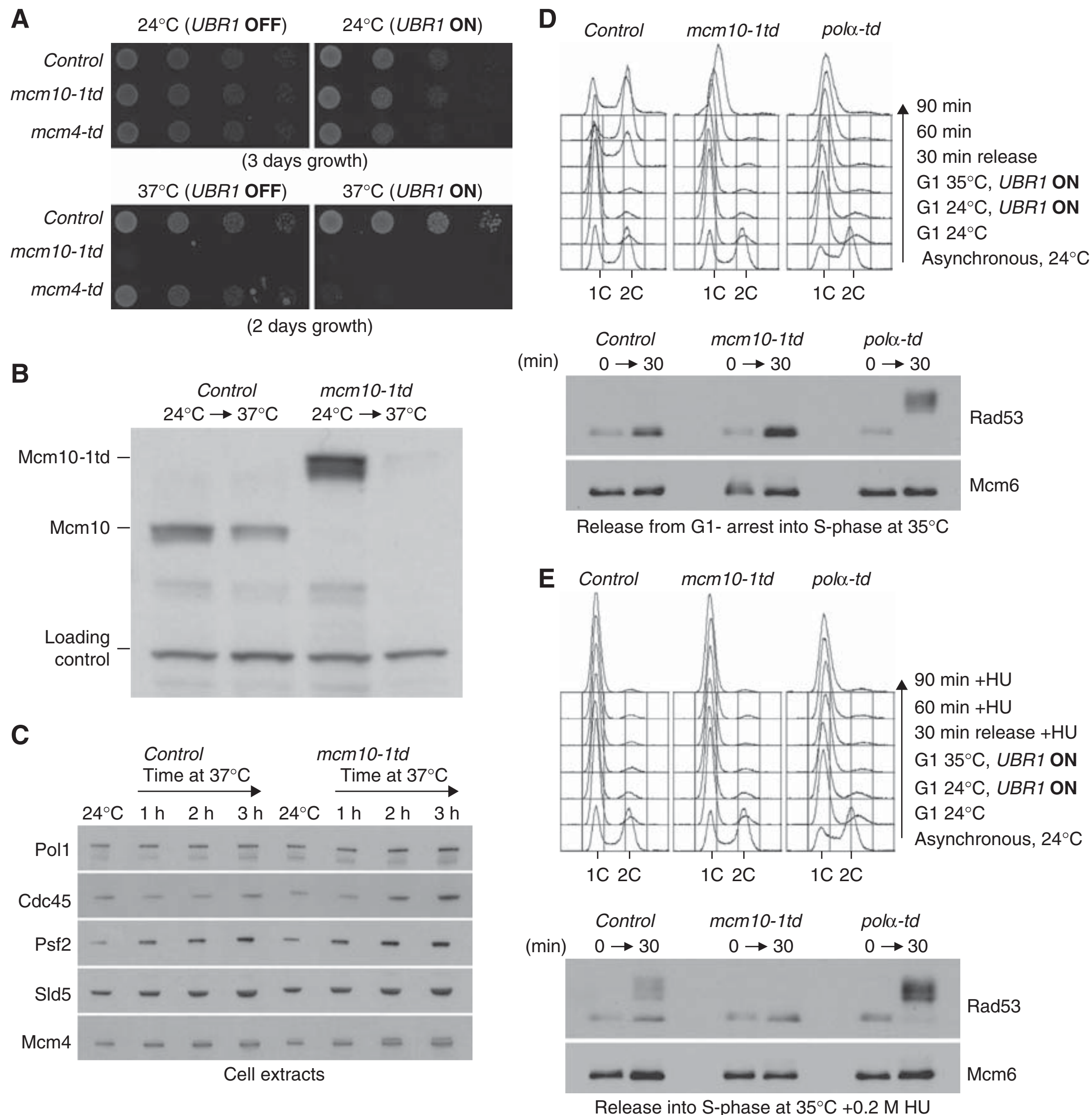

Figure 3 Inactivation of Mcm10-1td blocks the initiation of chromosome replication without causing degradation of Pol1. (**A**) Serial dilutions of the indicated strains were grown on YPDCu (*UBR1* **OFF**) or YPGal (*UBR1* **ON**) as shown. (**B**) The level of Mcm10 or Mcm10-1td was monitored by immunoblotting at 24°C, or following induction of *GAL-UBR1* and incubation at 37°C for 60 min. (**C**) Asynchronous cultures of control (YFJD74) and *mcm10-1td* (YFJD77) were grown in YPRaffCu medium at 24°C before shifting to YPGal medium at 24°C for 1 h and then incubating at 37°C for the indicated times. Samples were used to prepare extracts in the presence of Tricholoracetic acid, before immunoblotting to monitor the level of the indicated proteins. (**D**, **E**) Control (YMK302), *mcm10-1td* (YAG458-1) and *polα-td* (CC1235) were synchronised in G1-phase at 24°C before inactivation of Mcm10-1td and Polα-td at 35°C for 1 h. Cells were then washed into fresh medium without (**D**) or with (**E**) 0.2 M Hydroxyurea and incubated for the indicated times. DNA content was monitored by flow cytometry and hyperphosphorylation of the Rad53 checkpoint kinase was assayed by immunoblotting.

levels by inhibition of ribonucleotide reductase and thus provides a powerful signal for checkpoint activation at defective replication forks (we used 35°C as budding is inhibited at higher temperatures in the presence of hydroxyurea). As shown in Figure 3E, hyperphosphorylation of Rad53 was induced rapidly in control cells and *polα-td* under such conditions, but was defective in *mcm10-1td*. These data demonstrated that inactivation of Mcm10 or DNA polymerase alpha caused distinct phenotypes, and suggested that the initiation of chromosome replication might be defective in *mcm10-1td*.

Previous studies indicated that the major regulated step in the initiation of chromosome replication is the assembly of the Cdc45–MCM–GINS helicase (Labib, 2010), which forms the core of the Replisome Progression Complex. We therefore repeated the above experiment and monitored assembly of Cdc45–MCM–GINS by immunoprecipitation of Mcm4 from yeast cell extracts after digestion of chromosomal DNA. As before, hyperphosphorylation of Rad53 was greatly defective when cells entered S-phase in the presence of hydroxyurea following inactivation of Mcm10-1td, in comparison with control cells (Figure 4A). Strikingly, however, Mcm4 associated not just with the other Mcm2-7 proteins but also with Cdc45, GINS, Ctf4 and Csm3 (Figure 4B), indicating that assembly of the Replisome Progression Complex could still occur in the absence of Mcm10.

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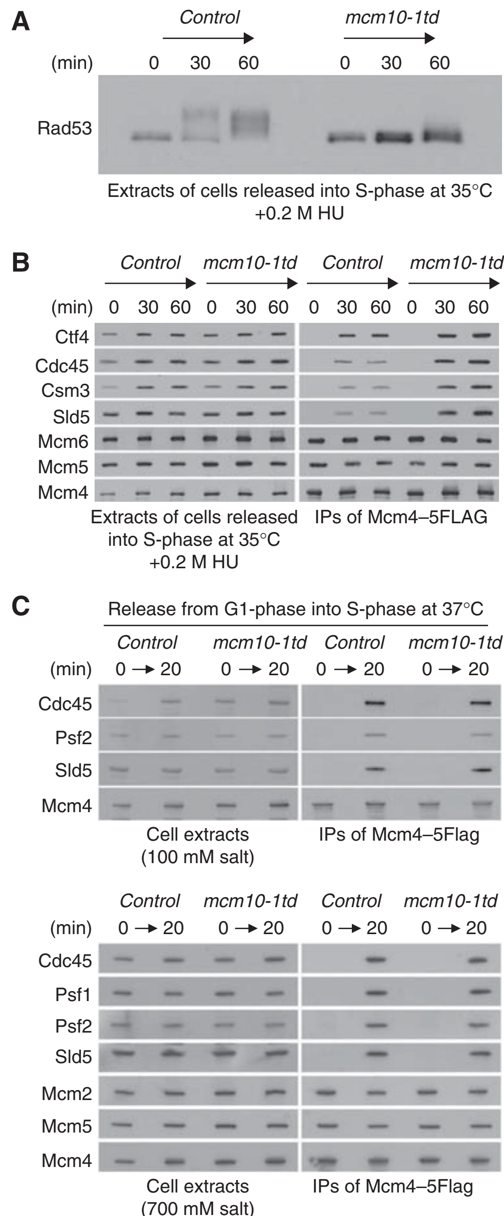

Figure 4 Mcm10 is required for the initiation of chromosome replication independently of assembling the Cdc45–MCM–GINS helicase complex. (**A**, **B**) Cells were grown as in Figure 3E and used to analyse the phosphorylation status of Rad53 (**A**) or the co-immunoprecipitation of the indicated proteins with Mcm4 (**B**). (**C**) Control (YAG458-1) and *mcm10-1td* (YMP174-1) were released from G1-arrest for 20 min at 37°C and native cell extracts (+benzonase –ATP) were prepared in the presence of 100 or 700 mM potassium acetate, before isolation of Mcm4–5FLAG.

To exclude the possibility that Cdc45 and GINS only formed a very unstable complex with the loaded Mcm2-7 helicase in the absence of Mcm10, we repeated the above experiment but released cells into S-phase at 37°C without hydroxyurea, and then made cell extracts containing either 100 or 700 mM potassium acetate. In both cases, the interaction of GINS and Cdc45 with Mcm2-7 was comparable in extracts of control or *mcm10-1td* cells (Figure 4C), reflecting the inherent stability of the Cdc45–MCM–GINS helicase assembly within the Replisome Progression Complex (Gambus *et al*, 2006). Considered together with the above

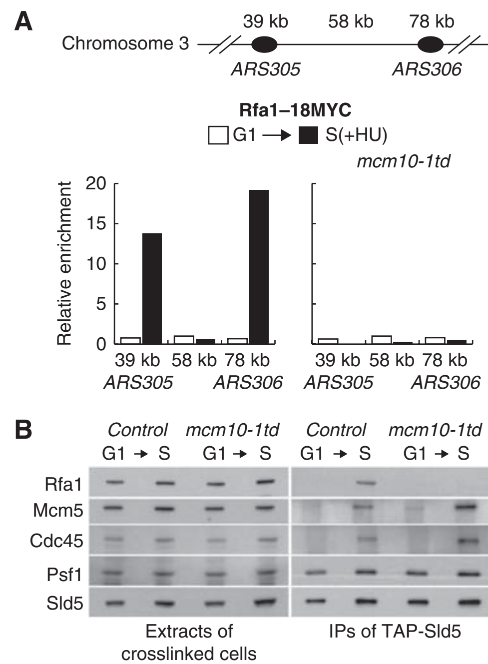

Figure 5 DNA unwinding is defective when cells enter S-phase after inactivation of Mcm10. (**A**) Cells were grown as in Figure 3E and the association of Rfa1–18MYC with the indicated DNA sequences was monitored by chromatin immunoprecipitation after formaldehyde crosslinking, followed by real-time PCR analysis. (**B**) Control (YFJD168) and *mcm10-1td* (YFJD151) were treated as in (**A**), but after immunoprecipitation of TAP-Sld5 and reversal of crosslinking, the association of the indicated proteins with GINS was monitored by immunoblotting.

data, these findings indicated that Mcm10 is required for a novel step during the initiation of chromosome replication that is independent of the recruitment of Cdc45 and GINS to the loaded Mcm2-7 complex at replication origins.

Mcm10 is required for unwinding of DNA replication origins during the initiation of chromosome replication

In previous studies, very severe defects in the loading or activation of the Mcm2-7 helicase in budding yeast were found to impede checkpoint activation (Piatti *et al*, 1996; Tercero *et al*, 2000), as they produced a very tight block to the unwinding of replication origins. Our finding that checkpoint activation was defective following the depletion of Mcm10, despite efficient assembly of the Cdc45–MCM–GINS helicase complex, suggested that Mcm10 might be needed for a previously unidentified step in origin unwinding. To test this directly, we released control or *mcm10-1td* cells into S-phase at 35°C in the presence of 0.2 M hydroxyurea, and used chromatin immunoprecipitation after formaldehyde crosslinking to monitor the association with origin DNA of the Rfa1 subunit of Replication Protein A, which binds single-strand DNA at eukaryotic replication forks. When control cells entered S-phase in the presence of hydroxyurea, Rfa1 associated with the replication origins *ARS305* and *ARS306* but not with a distal sequence located midway between the two origins (Figure 5A). In contrast, the enrichment of Rfa1 at replication origins was not detected when the *mcm10-1td* strain entered S-phase (Figure 5A), indicating that origin unwinding was defective in the absence of Mcm10.

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We also performed an analogous chromatin immunoprecipitation experiment in which we released cells into S-phase at 37°C in the absence of hydroxyurea, and then monitored the association of the Sld5 subunit of GINS with replisome proteins and associated factors in extracts of crosslinked cells (Kanemaki and Labib, 2006; Muramatsu *et al*, 2010). Whereas Rfa1 co-immunoprecipitated with GINS from extracts of crosslinked control cells, reflecting the assembly of DNA replication forks containing ssDNA, Rfa1 was not detected in the analogous material isolated from *mcm10-1td*, despite the association of GINS with Cdc45 and Mcm2-7 (Figure 5B). Taken together, these data indicate that Mcm10 is required for a novel step in the unwinding of replication origins during the initiation of chromosome replication in budding yeast, distinct from the recruitment of GINS and Cdc45 to the loaded and inactive Mcm2-7 complex.

Discussion

Our data support a revised model for the essential role of Mcm10 during chromosome replication in budding yeast (Figure 6). Mcm10 is recruited to the loaded Mcm2-7 complex at origins of DNA replication and is then essential for its activation, independently of the known activation step involving the recruitment of GINS and Cdc45 to Mcm2-7. We have found that Mcm10 does not associate stably with the Cdc45–MCM–GINS helicase in isolated replisome material under physiological conditions, consistent with a previous study indicating that Mcm10 is not stably associated with the active Cdc45–MCM–GINS helicase in *Xenopus* (Pacek *et al*, 2006). Moreover, studies of the Cdc45–MCM–GINS helicase from *Drosophila* show that it can function *in vitro* without any apparent requirement for Mcm10 (Ilves *et al*, 2010). It thus seems unlikely that Mcm10 is an essential subunit of the activated Cdc45–MCM–GINS helicase.

Instead, our data point to a previously unappreciated role for Mcm10 in activating the Mcm2-7 helicase in the context of replication origins *in vivo*. A very attractive possibility for future investigation would be that Mcm10 is required for one or more of the transformations that are thought to convert the inactive double hexamer of Mcm2-7 around dsDNA at origins into two CMG complexes at replication forks, each with an Mcm2-7 ring around ssDNA. The step mediated by Mcm10 is not essential for recruitment of Cdc45 and GINS in budding yeast, and might instead involve direct remodelling of the Mcm2-7 complex to facilitate correct interaction of the latter with the ssDNA template at the nascent replication fork. Further investigation of this process will be dependent upon an unambiguous determination of how the Cdc45–MCM–GINS helicase interacts with the two parental DNA strands at replication forks, as well as a more detailed analysis of how Mcm10 interacts with the inactive Mcm2-7 double hexamer. In a variety of species, Mcm10 has been found to interact with several of the Mcm2-7 proteins (Merchant *et al*, 1997; Izumi *et al*, 2000; Hart *et al*, 2002; Lee *et al*, 2003; Gambus *et al*, 2006; Zhu *et al*, 2007; Apger *et al*, 2010), with most evidence for Mcm6 and Mcm2. We have found in a yeast two-hybrid screen that Mcm10 interacts with the winged-helix domain at the carboxyl terminus of Mcm6 (Supplementary Figure S3), and it thus shares at least one interaction site on Mcm2-7 with the Cdt1 protein that

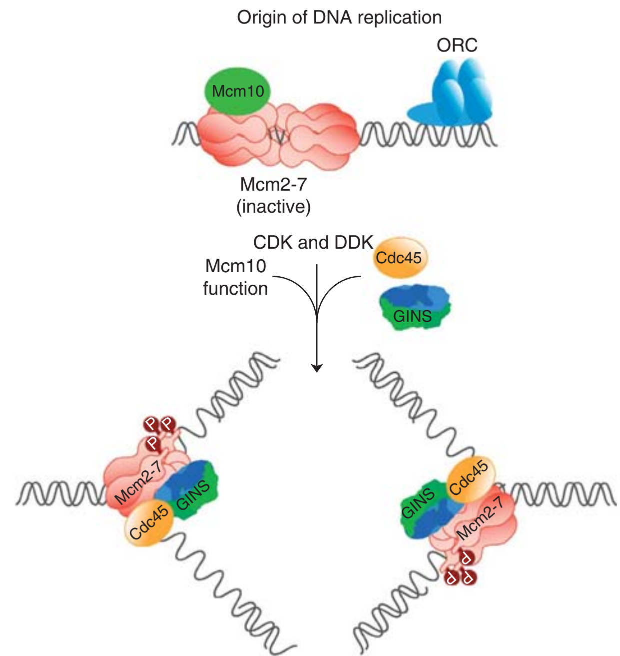

Figure 6 Model for the activation of Cdc45–MCM–GINS helicase during the initiation of chromosome replication. Mcm10 associates with the loaded Mcm2-7 complex at origins of DNA replication. As cells enter S-phase, activation of cyclin-dependent kinase (CDK) and Cdc7 kinase mediates the recruitment of GINS and Cdc45, respectively (Heller *et al*, 2011). Mcm10 is dispensable for the recruitment of GINS and Cdc45 to the loaded Mcm2-7 complex in budding yeast, and does not appear to be a stable subunit of the active Cdc45–MCM–GINS helicase at replication forks. Instead, we propose that Mcm10 is required for a novel step of the initiation reaction that is required for activation of the loaded Mcm2-7 complex at replication origins *in vivo*—see text for further details. For the sake of simplicity, other replisome components have been omitted, and the presumed association of Mcm10 with replication forks is not shown.

plays a central role in loading of the Mcm2-7 rings at origins of replication.

Though any model must of necessity remain speculative at present, it is possible that Mcm10 might be required to stabilise an open form of the Mcm2-7 complex during initiation to allow remodelling of the Mcm2-7 rings from dsDNA to ssDNA. This idea could provide a neat explanation for the very interesting observation that defects in budding yeast Mcm10 are suppressed by particular mutations in Mcm2-7 proteins (Homesley *et al*, 2000; Liachko and Tye, 2005; Lee *et al*, 2010). A screen for spontaneous suppressors of *mcm10-1* identified mutations in Mcm2, and further such mutations were found by random mutagenesis of the *MCM2* gene (Lee *et al*, 2010). Nine out of ten were clustered at two sites in Mcm2, between amino acids 399–401 and 617–619. Intriguingly, the two equivalent sites in the single Mcm protein of the archaeon *Sulfolobus solfataricus* were mapped onto a three-dimensional model of the homo-hexameric complex, revealing that the first site in one subunit was immediately adjacent to the second site in an adjacent subunit, across the intersubunit interface (Lee *et al*, 2010). The mutations in Mcm2 seem likely to weaken or otherwise alter the interface of Mcm2 with adjacent subunits of Mcm2-7, and it was proposed that this might compromise helicase activity and thus restore the coupling between DNA

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unwinding and DNA synthesis that was thought to be disrupted in *mcm10-1* (Lee *et al*, 2010).

Our data suggest an alternative explanation of the same data, by which the mutations in Mcm2 alter the interface with other Mcm2-7 proteins and thereby mimic the action of Mcm10 during initiation. This would explain why all of the *mcm2* mutations are dominant suppressors of defects in Mcm10 and why they are able to suppress both *mcm10-1* and *mcm10-43*, despite these two alleles affecting different parts of Mcm10. Suppression of *mcm10* mutations probably occurs at a price, however, as the analogous mutations that alter the intersubunit interface of *Sulfolobus* MCM correlate with reduced helicase activity (though the comparison is unfair as the effect will be amplified in the homo-hexamer, since all subunits are mutated and not just one), and the yeast *mcm2* mutations have a plasmid-loss phenotype (Lee *et al*, 2010).

Our finding that budding yeast Mcm10 is required for replication independently of the recruitment of Cdc45 and GINS to the loaded Mcm2-7 complex, agrees with very recent observations of plasmid replication in an *in-vitro* system based on extracts of budding yeast cells (Heller *et al*, 2011), although a potential role for Mcm10 in DNA unwinding was not reported. Moreover, a previous study found that Cdc45 is still recruited to chromatin in budding yeast cells lacking Mcm10 activity (Ricke and Bielinsky, 2004), although the replication defect of *mcm10* mutants was attributed in that study to degradation of Pol1. Furthermore, two other groups have recently found that Mcm10 is required for origin unwinding independently of the assembly of Cdc45–MCM–GINS, both in studies of budding yeast (Masato Kanemaki, personal communication) and fission yeast (Hisao Masukata, personal communication). We note that Mcm10 was previously found to be required for stable association of Cdc45 with chromatin during the initiation of replication in extracts of *Xenopus* eggs (Wohlschlegel *et al*, 2002; Zhu *et al*, 2007), and so perhaps the intermediates of the initiation reaction are less stable in the absence of Mcm10 in frog than in budding or fission yeasts, making it harder to distinguish a role for Mcm10 that is independent of recruiting Cdc45 and GINS. It remains to be determined whether the action of Mcm10 at replication origins is also dependent upon the presence of other initiation factors.

Our data indicate that Mcm10 is not required to couple Mcm2-7 to polymerase alpha within the replisome and indeed Mcm10 is not a stable component of isolated replisome material unlike polymerase alpha (Gambus *et al*, 2009). We cannot exclude, however, that Mcm10 makes a redundant contribution to the coupling of helicase and polymerase *in vivo*, in parallel with the Ctf4/And1 protein. Work with various species indicates that Mcm10 is present at or near replication forks after initiation (Ricke and Bielinsky, 2004; Pacek *et al*, 2006; Karnani and Dutta, 2011; Taylor *et al*, 2011), although our analysis of the *mcm10-1td* allele did not identify an essential role for Mcm10 at DNA replication forks (Supplementary Figure S4).

It seems likely that the recruitment of Mcm10 to origins of DNA replication is driven by at least two key factors: first, the preferential association of Mcm10 with the loaded form of the Mcm2-7 complex; and second, the high concentration of Mcm10 within the nucleus. The same applies to the recruitment of Mcm10 to chromatin in extracts of *Xenopus* eggs, which is independent of CDK or Cdc7 kinases but requires the prior loading of Mcm2-7 and also the concentration of Mcm10 within the nucleus (Wohlschlegel *et al*, 2002). This latter feature might help to explain why *in-vitro* recruitment of Mcm10 to plasmid DNA occurred at a later stage in extracts of budding yeast than would have been predicted by our experiments, and was found to require the activation of CDK and Cdc7 (Heller *et al*, 2011). There is evidence that Mcm10 interacts with other factors at forks including Cdc45 (Sawyer *et al*, 2004), and these interactions might contribute to recruitment of Mcm10 when the concentration of the latter is too low to mediate its efficient association with the loaded Mcm2-7 complex. We note that two previous studies used chromatin immunoprecipitation to show that budding yeast Mcm10 could be observed at replication origins before CDK activation (Ricke and Bielinsky, 2004; Raveendranathan *et al*, 2006), and these findings agree with an analogous study of human Mcm10 (Karnani and Dutta, 2011). However, others have observed CDK-dependent recruitment of Mcm10 to replication origins in budding or fission yeasts by chromatin immunoprecipitation (Masato Kanemaki and Hisao Masukata, personal communication). Whereas Mcm2-7 can only function if loaded before CDK activation, we have found that Mcm10 can function even if its recruitment is delayed until after CDK activation (Supplementary Figure S5). It thus seems that the timing of recruitment of Mcm10 is not a critical determinant of function. Whatever the true timing of recruitment at replication origins, our data indicate that the loaded Mcm2-7 complex is likely to be the preferred substrate for Mcm10 during the initiation of chromosome replication.

The conserved interaction of Mcm10 with Mcm2-7 proteins in various eukaryotic species indicates that the underlying biology of Mcm10 function will be fundamentally similar. Recent progress in studying the earliest steps of chromosome replication has started to provide new tools that should facilitate a more detailed analysis in the coming years of how Mcm10 helps activate the replicative DNA helicase at eukaryotic replication origins.

Materials and methods

Yeast strains and growth

The strains used in this study are listed in Supplementary Table SI. Yeast cultures were grown in rich medium (1% Yeast Extract, 2% peptone, 0.1 mg/ml adenine) supplemented with 2% glucose (YPD), 2% Raffinose (YPRaff) or 2% Galactose (YPGal) as the carbon source. For experiments involving degron strains, exponential cultures were grown at 24°C in YPRaff medium containing 0.1 mM $CuSO_4$ (YPRaffCu). As necessary, cells were synchronised in G1-phase by addition of 7.5 μg/ml alpha factor mating pheromone, in S-phase by addition of 0.2 M Hydroxyurea, or in G2-M-phase by addition of 5 μg/ml Nocodazole. Cells were released from arrest by washing twice in fresh medium. To degrade proteins fused to the heat-inducible degron (Sanchez-Diaz *et al*, 2004), cells were transferred to YPGal medium at 24°C for 35 min to induce expression of *GAL-UBR1*, and then transferred to 37°C for 1 h (or longer as indicated in the figures).

Preparation of native cell extracts and isolation of Mcm2-7 complexes

Native cell extracts were made from 250 ml mid-log culture as described previously (Gambus *et al*, 2009; Morohashi *et al*, 2009), in the presence of 100 mM potassium acetate unless otherwise specified. To stabilise the unloaded form of the Mcm2-7 complex in the extracts we included 3 mM ATP in all the buffers and used 100 mM potassium glutamate instead of potassium acetate (as we

found that stability of the unloaded Mcm2-7 complex was slightly more robust in the presence of potassium glutamate compared with potassium acetate). To liberate the loaded Mcm2-7 complex into the extract and prevent it being lost during the centrifugation steps, chromosomal DNA was digested for 30 min at 4°C with 800 units of Benzonase (71206-3; Merck Biosciences). After centrifugation at 25 000 *g* for 30 min and then 100 000 *g* for 60 min, immunoprecipitations were performed with magnetic beads coupled to M2 anti-FLAG monoclonal antibody (F3165; Sigma), 9E10 anti-MYC antibody (CRUK), 12CA5 anti-HA antibody (CRUK), or rabbit IgG (S1265; Sigma). Replisome proteins were detected by immunoblotting with previously described antibodies (Gambus *et al*, 2006). For the experiment in the lower panels of Figure 4C, we used 700 mM potassium acetate in all buffers apart from one final wash in the presence of 100 mM potassium acetate after immunoprecipitation of Mcm4.

Chromatin immunoprecipitation

For the experiments in Figure 5, formaldehyde was added to cell cultures to a final concentration of 1%. The samples were incubated for 25 min at 24°C and the crosslinking reaction was then terminated with 120 mM glycine for 5 min, before washing cells once with 20 mM Hepes-KOH pH 7.9 and once with lysis buffer (50 mM Hepes-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA). Cell pellets were then resuspended in three volumes of lysis buffer supplemented with Complete Protease Inhibitor (Roche), Protease inhibitor cocktail (Sigma), 2 mM sodium β-glycerophosphate and 2 mM sodium fluoride, before freezing droplets in liquid nitrogen. Frozen cells were ground in the presence of liquid nitrogen using a SPEX SamplePrep LLC 6850 freezer/mill. After grinding, we added 0.25 volumes of extraction buffer (50 mM Hepes-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 5% Triton X-100, 0.5% sodium deoxycholate, plus protease inhibitors as above), which we showed in analogous experiments was sufficient to disrupt replication complexes if the cells had not been treated with a crosslinking agent (Giacomo de Piccoli and KL, unpublished data). Samples were then sonicated and insoluble material removed by centrifugation at 16 000 *g* for 15 min. We then incubated the cell extracts with antibody-coated magnetic beads for 2 h, before washing twice each with buffer 1 (50 mM Hepes-KOH pH 7.5, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM EDTA, plus protease inhibitors), buffer 2 (50 mM Hepes-KOH pH 7.5, 500 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM EDTA, plus protease inhibitors) and buffer 3 (10 mM Tris–HCl pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA). After a final wash with TE pH 8.0, we incubated the immunoprecipitated protein samples at 65°C for 10 min with elution buffer (50 mM Tris–HCl pH 8.0, 10 mM EDTA, 1% SDS). For experiment in Figure 5A, the samples were then treated overnight with 100 μg/ml RNase A in elution buffer, before extraction of DNA and analysis by real-time PCR as described previously (Calzada *et al*, 2005). The specific enrichment of Rfa1 at each tested sequence was calculated according to the formula $2^{(Ct\ 12CA5 - Ct\ 9E10)}$, where 'Ct 12CA5' is the threshold cycle number (Ct) for real-time PCR analysis of DNA purified from a control immunoprecipitate with 12CA5 monoclonal antibody, and 'Ct 9E11' is the corresponding Ct value for the immunoprecipitate of Rfa1–18MYC with 9E10 monoclonal antibody (Calzada *et al*, 2005). The specific enrichments for each time point were then normalised relative to the background value observed in G1-arrested cells for the non-origin sequence. For the experiment in Figure 5B, Laemmli buffer was added to the eluted sample, before incubation for a further 30 min at 95°C to reverse the crosslinks (Kanemaki and Labib, 2006). The associated proteins were then analysed by SDS–PAGE.

Flow cytometry

Samples were fixed with 70% ethanol and then processed for flow cytometry as previously described (Kanemaki *et al*, 2003), before analysis with a Becton Dickinson FACScan machine and CellQuest software.

Two-hybrid screen for partners of Mcm10

A two-hybrid screen with full-length Mcm10 as bait was performed by the company Hybrigenics, using a library of random fragments of yeast genomic DNA.

Supplementary data

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

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Author contributions: FvD performed all the experiments except for the following: SS developed the assays shown in Figure 1 and performed the experiment in Figure 1D; GDP performed the experiments in Figure 3D–E, Figure 4A, B and Supplementary Figure S2; ASD made the *polα-td* strain used in Figure 3D–E. FvD and KL designed the experiments, and KL wrote the manuscript together with FvD, SS and GDP.

Conflict of interest

The authors declare that they have no conflict of interest.

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