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Origin Licensing Requires ATP Binding and Hydrolysis by the MCM Replicative Helicase

Gideon Coster,[1] Jordi Frigola,[1] Fabienne Beuron,[2] Edward P. Morris,[2] and John F.X. Diffley[1,*]
[1]Cancer Research UK London Research Institute, Clare Hall Laboratories, South Mimms, Herts. EN6 3LD, UK
[2]Division of Structural Biology, The Institute of Cancer Research, London SW3 6JB, UK
*Correspondence: John.Diffley@cancer.org.uk
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SUMMARY

Loading of the six related Minichromosome Maintenance (MCM) proteins as head-to-head double hexamers during DNA replication origin licensing is crucial for ensuring once-per-cell-cycle DNA replication in eukaryotic cells. Assembly of these prereplicative complexes (pre-RCs) requires the Origin Recognition Complex (ORC), Cdc6, and Cdt1. ORC, Cdc6, and MCM are members of the AAA+ family of ATPases, and pre-RC assembly requires ATP hydrolysis. Here we show that ORC and Cdc6 mutants defective in ATP hydrolysis are competent for origin licensing. However, ATP hydrolysis by Cdc6 is required to release nonproductive licensing intermediates. We show that ATP binding stabilizes the wild-type MCM hexamer. Moreover, by analyzing MCM containing mutant subunits, we show that ATP binding and hydrolysis by MCM are required for Cdt1 release and double hexamer formation. This work alters our view of how ATP is used by licensing factors to assemble pre-RCs.

INTRODUCTION

DNA replication in eukaryotes initiates from multiple chromosomal locations termed origins, and the stability of the genome is dependent upon each origin firing once and only once per cell cycle. This is achieved by the temporal separation of replication initiation into two distinct steps (reviewed in Costa et al., 2013; Yardimci and Walter, 2014). The first step, origin licensing, involves the loading of the hexameric MCM helicase comprising the six related Mcm2–7 subunits into prereplicative complexes (pre-RCs). This occurs during late mitosis and G1 phase. The second step, origin firing, involves the conversion of the inactive MCM double hexamer into two functional replisomes during S phase.

Origin licensing occurs in an ordered fashion. First, the Origin Recognition Complex (ORC) binds to origin DNA. The budding yeast ORC has inherent DNA sequence specificity, while in metazoans, ORC has little or no specificity and may be recruited to origins by additional factors (Masai et al., 2010; Méchali, 2010; Yekezare et al., 2013). In budding yeast, ATP binding but not hydrolysis by ORC is required for origin binding (Bell and Stillman, 1992; Klemm and Bell, 2001). In metazoans, ATP binding is required for assembly of a stable ORC and can stimulate DNA binding (Chesnokov et al., 2001; Giordano-Coltart et al., 2005; Houchens et al., 2008; Ranjan and Gossen, 2006; Siddiqui and Stillman, 2007; Vashee et al., 2003). Cdc6 is then recruited to form an ORC·Cdc6 complex on origin DNA. The budding yeast MCM forms a complex with the Cdt1 protein (Kawasaki et al., 2006; Tanaka and Diffley, 2002), and this MCM·Cdt1 complex is recruited to ORC·Cdc6 via an essential C-terminal domain in Mcm3 (Frigola et al., 2013). The loading of MCM into salt-resistant double hexamers bound around double-stranded DNA requires ATP and is not supported by the ATP analog ATPγS, indicating a requirement for ATP hydrolysis (Evrin et al., 2009; Randell et al., 2006; Remus et al., 2009). Once MCM is loaded, it no longer requires ORC, Cdc6, or Cdt1 to maintain its origin association.

The six MCM subunits, Cdc6 and five of the six ORC subunits, belong to the large ATPases Associated with various cellular Activities (AAA+) family of ATPases (Erzberger and Berger, 2006). These ATPases typically assemble into oligomers with active sites that are formed at the interface of two adjacent subunits; one subunit contributes Walker A and B motifs, and the adjacent subunit contributes an arginine finger. Specific mutations within these motifs affect most AAA+ ATPases in a predictable manner: mutation of a conserved lysine residue in the Walker A motif prevents binding of ATP; mutation of conserved acidic residues within the Walker B motif can affect ATP binding or hydrolysis, while mutation of the arginine finger hinders ATP hydrolysis.

Mutational analysis has shown that these motifs are important for origin licensing in the budding yeast ORC and Cdc6 (Bowers et al., 2004; Klemm and Bell, 2001; Perkins and Diffley, 1998; Weinreich et al., 1999). The key active site in ORC is formed from the Walker A and B motifs in Orc1 and an arginine finger from Orc4. ORC containing a mutant in the Walker A motif of Orc1 that prevents ATP binding can no longer bind DNA (Klemm et al., 1997), while ORC containing a mutant in the Orc4 arginine finger, which blocks ATP hydrolysis, can bind DNA and load MCM onto DNA but cannot recycle for repeated rounds of MCM loading (Bowers et al., 2004). A Cdc6 mutant in which the conserved Walker A lysine 114 residue was changed to alanine (Cdc6-KA) behaves like a null mutant in vivo (Perkins and Diffley, 1998; Weinreich et al., 1999) and cannot recruit or load MCM in vitro (Evrin et al., 2013). The role of ATP hydrolysis by Cdc6 is less clear. Although the arginine finger required for

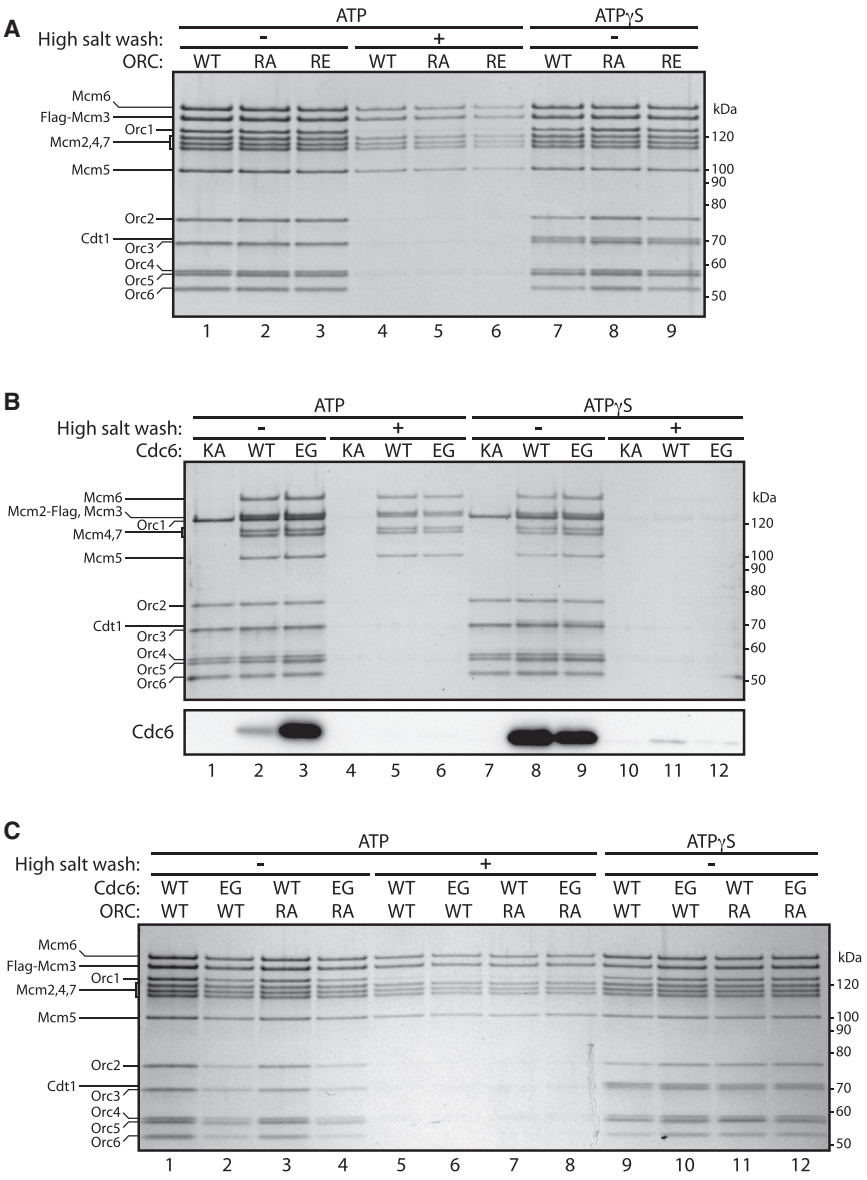

Figure 1. The ATPase Activity of ORC and Cdc6 Is Not Essential for pre-RC Formation In Vitro

(A–C) In vitro loading reactions were carried out using a biotinylated, photocleavable 1 kb double-stranded DNA template coupled to magnetic beads. Purified ORC, Cdc6, and MCM•Cdt1 were mixed with 5 mM of the nonhydrolyzable ATP analog ATPγS or ATP, incubated for 20 min at 30°, and subjected to a mild wash (300 mM KOAc) or a high-salt wash (500 mM NaCl). DNA was released by short exposure to UVA (330 nm), and bound proteins were separated by SDS-PAGE and visualized by silver staining.

Reactions were carried out with wild-type ORC and Cdc6 or ORC complexes that harbor a point mutation within the arginine finger motif of the ORC4 subunit (ORC4 R267A or R267E; RA or RE) and/or Cdc6 carrying mutations within the Walker A motif (K114A; KA) or Walker B motif (E224G; EG).

ATP hydrolysis by Cdc6 has not been identified, a double mutant in the Walker B motif of Cdc6 (e.g., $D^{223}E^{224} \rightarrow AA$) is viable, suggesting ATP hydrolysis might not be essential (Weinreich et al., 1999). However, mutation of glutamate 224 of the Walker B motif to glycine (Cdc6-EG) results in a poorly functional protein that is lethal when overexpressed and defective in origin licensing in vivo (Perkins and Diffley, 1998). Randell et al. (2006) showed that combination of Orc4-RA with Cdc6-EG virtually eliminates ATPase activity. Mutation of the Walker A motifs in Mcm6 and Mcm7 did not affect association of MCM with chromatin in *Xenopus* egg extracts but instead blocked subsequent DNA replication, indicating that ATP binding and hydrolysis by MCM are not required for licensing but are required for a downstream step (Ying and Gautier, 2005).

Using purified yeast proteins, we have now systematically analyzed the role of ATP binding and hydrolysis in origin licensing. We found that ORC and Cdc6 mutants defective in ATP hydrolysis are competent for origin licensing, even when combined. This has allowed us to uncover a role for ATP hydrolysis by Cdc6 in release of nonproductive loading intermediates. Surprisingly, we found that ATP binding and hydrolysis by MCM subunits play distinct and essential roles during pre-RC assembly: ATP binding is required for stability of the MCM complex under licensing reaction conditions, and ATP hydrolysis is required for MCM loading and Cdt1 release.

RESULTS

ORC and Cdc6 ATPase Mutants Can Load MCM

To investigate the roles of ATP binding and hydrolysis by ORC and Cdc6 in origin licensing, we generated proteins in which key motifs were mutated. These proteins were tested for their ability to recruit MCM•Cdt1 into a salt-labile complex in ATPγS (recruitment) and for their ability to assemble MCM into salt-resistant pre-RCs in the presence of ATP (loading) using DNA coupled to magnetic beads (Frigola et al., 2013; Remus et al., 2009). Fernández-Cid et al. (2013) previously analyzed the effect of a mutation in the Walker B motif of Orc1 (ORC-d1) on MCM loading; however, because this mutant is defective in both ATP binding and hydrolysis (Klemm and Bell, 2001), we tested mutant ORC complexes in which the arginine finger of Orc4 was mutated to either alanine (Orc4-RA) or glutamic acid (Orc4-RE), which have both been shown to eliminate ORC's ATPase (Bowers et al., 2004). Figure 1A shows that the Orc4-RA and Orc4-RE mutant complexes were functional for both recruitment of MCM in ATPγS (lanes 7–9) and loading of high-salt-wash-resistant MCM complex in ATP (lanes 4–6). Although

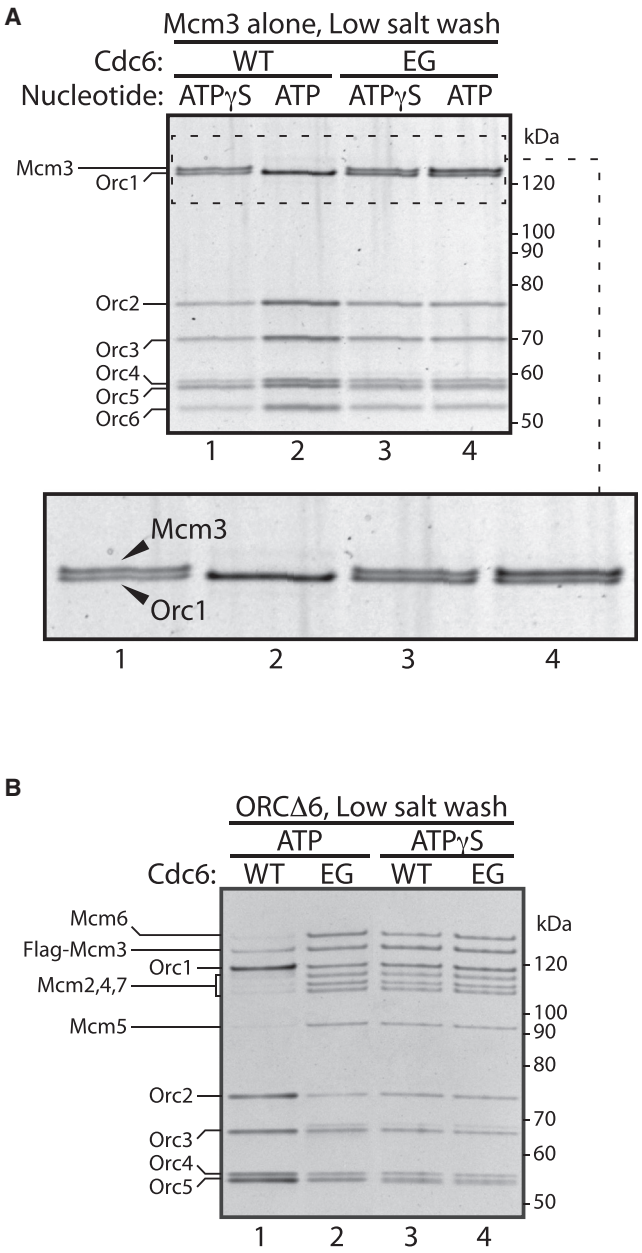

Figure 2. The Cdc6-EG Mutant Is Defective in Release of Nonproductive Loading Intermediates

(A and B) Loading reactions were carried out with either wild-type Cdc6 or Cdc6-EG. In (A), Mcm3 was used instead of the full MCM•Cdt1 complex. In (B), purified ORC lacking the ORC6 subunit (ORCΔ6) was used. The dashed rectangle in (A) highlights the region that is magnified on the bottom.

the Orc4-RE mutant had slightly reduced MCM loading, Orc4-RA, which is unable to hydrolyze ATP (Bowers et al., 2004), loaded MCM as well as the wild-type complex.

The Walker A mutant of Cdc6 (Cdc6-KA) was completely defective in recruiting and loading MCM (Figure 1B, lanes 7 and 4). Cdc6 comigrates in SDS-PAGE with Orc5 and therefore cannot be monitored by silver staining. However, immunoblots show that Cdc6-KA was not recruited to ORC in either ATP or ATPγS. The Walker B E224G mutant (Cdc6-EG) is lethal when overexpressed and shows reduced pre-RC assembly in vivo (Perkins and Diffley, 1998). Moreover, this mutant is defective in ATP hydrolysis when combined with ORC (Randell et al., 2006). Despite these defects, Figure 1B (lanes 6 and 9) shows that this mutant was almost as efficient in both MCM recruitment and loading as the wild-type protein. However, in contrast to the wild-type protein, which was released after loading (compare lanes 2 and 8), Cdc6-EG remained associated with DNA after loading (compare lanes 3 and 9). As shown in Figure 1C, even the combination of the Orc4-RA and Cdc6-EG mutants was still competent to load MCM into salt-resistant complexes almost as well as the wild-type proteins. Therefore, ATP hydrolysis by ORC and Cdc6 does not appear to be required for MCM loading and thus cannot explain why ATPγS does not support MCM loading.

Cdc6-EG Is Defective in Releasing Nonproductive Intermediates

ATP hydrolysis by ORC•Cdc6 is triggered by the binding of a C-terminal domain in the Mcm3 protein. ATP hydrolysis by ORC•Cdc6 can then promote release of MCM from a variety of intermediate complexes that cannot proceed to full double hexamer assembly (Frigola et al., 2013). Because Cdc6-EG was proficient for loading MCM in vitro but was defective in release from the pre-RC (Figure 1B) and for pre-RC assembly in vivo, we considered that it might be defective in releasing nonproductive loading intermediates. Mcm3, in the absence of other MCM subunits, can be specifically recruited to ORC•Cdc6 in ATPγS (Frigola et al., 2013) (Figure 2A, lane 1). In the presence of ATP, Mcm3 is no longer stably bound to wild-type ORC•Cdc6 (Frigola et al., 2013) (Figure 2A, lane 2). The Cdc6-EG mutant recruited Mcm3 as efficiently as wild-type Cdc6 in ATPγS (Figure 2A, lane 3); however, in contrast to the wild-type Cdc6, Mcm3 was retained in the presence of ATP to the same extent it is retained in ATPγS (Figure 2A, lane 4). Figure 2B shows that ORC lacking the Orc6 subunit (ORCΔ6) recruited MCM equally well in ATPγS with wild-type Cdc6 and the Cdc6-EG mutant (Figure 2B, lanes 3 and 4). However, in the presence of ATP (Figure 2B, lanes 1 and 2), MCM was released by wild-type Cdc6 but not Cdc6-EG. We note that ORC appears to be enriched on DNA after ATP-dependent release (e.g., Figure 2A lane 2, Figure 2B lane 1) (Frigola et al., 2013). The reason for this is unclear but may be due to accumulation of ORC on DNA after multiple rounds of Cdc6 and MCM•Cdt1 binding and release, while after normal MCM loading ORC may become destabilized, perhaps due to ATP hydrolysis by ORC (Rowles et al., 1996). We conclude that the Cdc6-EG mutant, while competent for MCM loading, is defective in releasing nonproductive intermediate complexes.

Characterization of MCM Mutants In Vivo

Since neither ORC nor Cdc6 need to hydrolyze ATP in order to load MCM, but ATPγS does not support loading, we turned our attention to ATP binding and hydrolysis by MCM subunits. We generated a full series of mutants in motifs involved in ATP binding and hydrolysis by each of the MCM subunits (Figure 3A). To assess the phenotypes associated with these mutants, we used the conditional protein exchange system outlined in

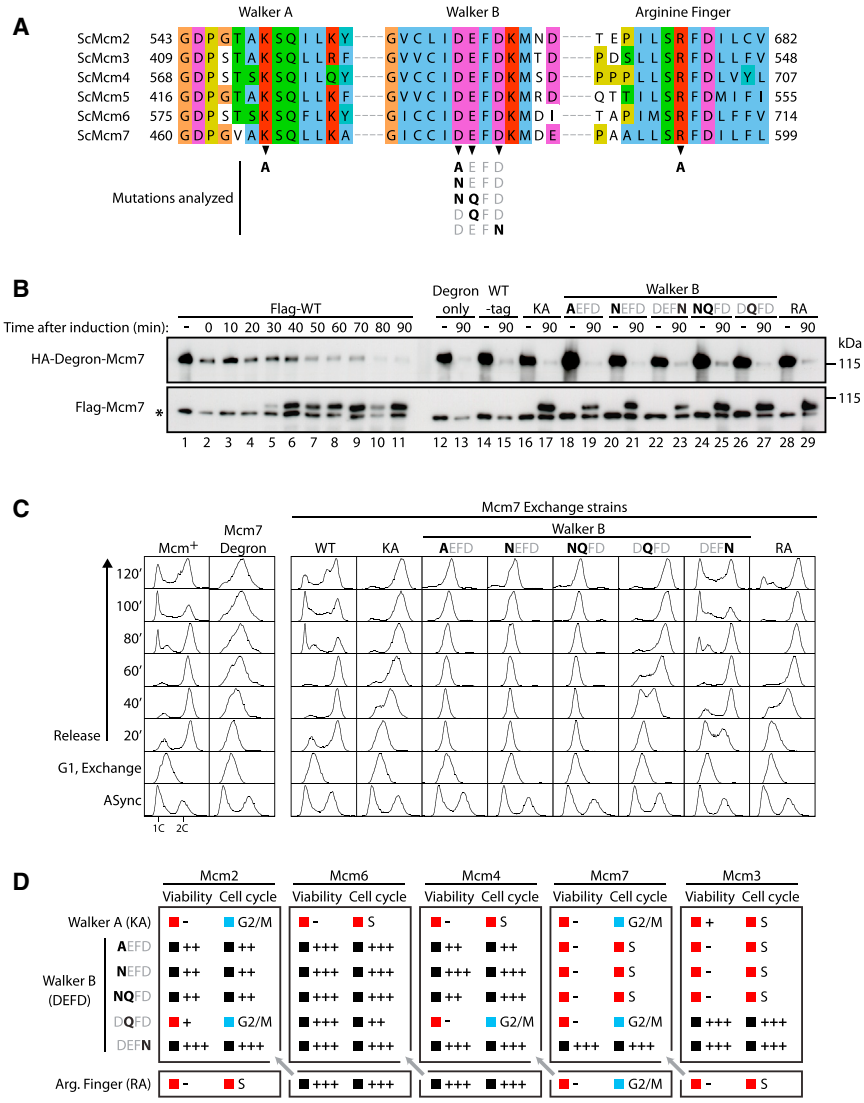

Figure 3. Analysis of MCM ATPase Mutants In Vivo by Conditional Protein Exchange

(A) Sequence alignment of all MCM subunits in *S. cerevisiae*. Mutations explored in this study are depicted below each motif. Alignment was generated and visualized by the JalView software, with standard ClustalW coloring (red = basic residues; magenta = acidic residues). Numbers on both sides signify amino acid positions of the first and last residues shown. The amino acid sequences between these motifs vary in composition and size between subunits and are omitted for simplicity.

(B) Protein exchange is rapid and efficient. Exchange strains were arrested in G1 using the mating pheromone α factor and transferred to restrictive conditions. Samples were collected from the asynchronous culture (–) or at 10 min intervals after induction. Whole-cell extracts were prepared and analyzed by western blotting against the HA or Flag tags, which identify the degron or the second copy, respectively. This panel is representative of the full set of Mcm7 strains. See Figure S1 for all remaining strains. The asterisk denotes a nonspecific signal in the anti-Flag blot, which served as an internal loading control.

(C) MCM ATPase mutants exhibit distinct defects in S phase progression. Time course experiments of wild-type and exchange strains were analyzed by flow cytometry. Asynchronous cultures (ASync) were arrested in G1, and protein exchange was induced for 90 min (G1, Exchange). The resulting G1 exchanged cells were released from α factor under restrictive conditions (Release). Samples were collected every 20 min and fixed immediately, and DNA content was measured by flow cytometry. This panel is representative of the full set of Mcm7 strains. See Figure S3 for all remaining strains.

(D) Table summarizing all of the viability and cell-cycle phenotypes of MCM ATPase mutants. The location of each subunit within the table reflects its relative position within the hexamer. The arginine finger motif of each subunit forms an active site with the Walker A and B motifs of its neighboring subunit, as indicated by arrows. Viability phenotypes: lethal (–), significant growth defect (+), minor growth defect (++), and normal growth (+++). Mutants with a significant growth defect or worse are highlighted in red for simplicity. Cell-cycle phenotypes: S phase arrest (S, red), slow S phase followed by a G2/M block (G2/M, blue), slightly slower cell cycle (++), and normal cell cycle (+++).

Figure S1A (available online). Briefly, a second copy of each MCM subunit gene, either wild-type or containing the mutations shown in Figure 3A, was cloned and expressed from a galactose-inducible promoter in strains harboring the cognate MCM subunit fused to a degron cassette (*mcm-td*) (Labib et al., 2000). Under permissive conditions (glucose, 24°C), the degron-fused wild-type protein is expressed, and the second copy is repressed. After shift to restrictive conditions for the degron alleles, the degron-fused protein is degraded while the second copy is expressed. Figures 3B and S1B show that each of the degron-fused proteins was largely degraded by 90 min after shifting to restrictive conditions. Similarly, by 90 min each of the second copy proteins, including the wild-type control and all of the ATPase mutants, were expressed. Figure S2 shows that all of the degron fusions on their own were viable under permissive conditions and inviable under restrictive conditions (compare rows 1 and 2 in Figures S2A and S2B). Expression of the wild-type protein suppressed inviability for each of the degron strains, and the presence of a FLAG tag did not affect this suppression (rows 3 and 4, Figure S2B). Figure S2B rows 5–11 shows the ability of various mutants to support viability under restrictive conditions. Because the Mcm5 degron fusion is inviable at all temperatures (Labib et al., 2000), we were unable to analyze *mcm5* ATP binding and hydrolysis mutants by this method. Most of the MCM subunit mutants had previously been analyzed for their ability to complement null mutants in budding and fission yeast (Bochman et al., 2008; Gómez et al., 2002; Schwacha and Bell, 2001), and our viability results are broadly consistent with this previous analysis, with the exception of the *mcm6-RA* mutant. Bochman and colleagues found this mutant could not complement a null mutant, while we

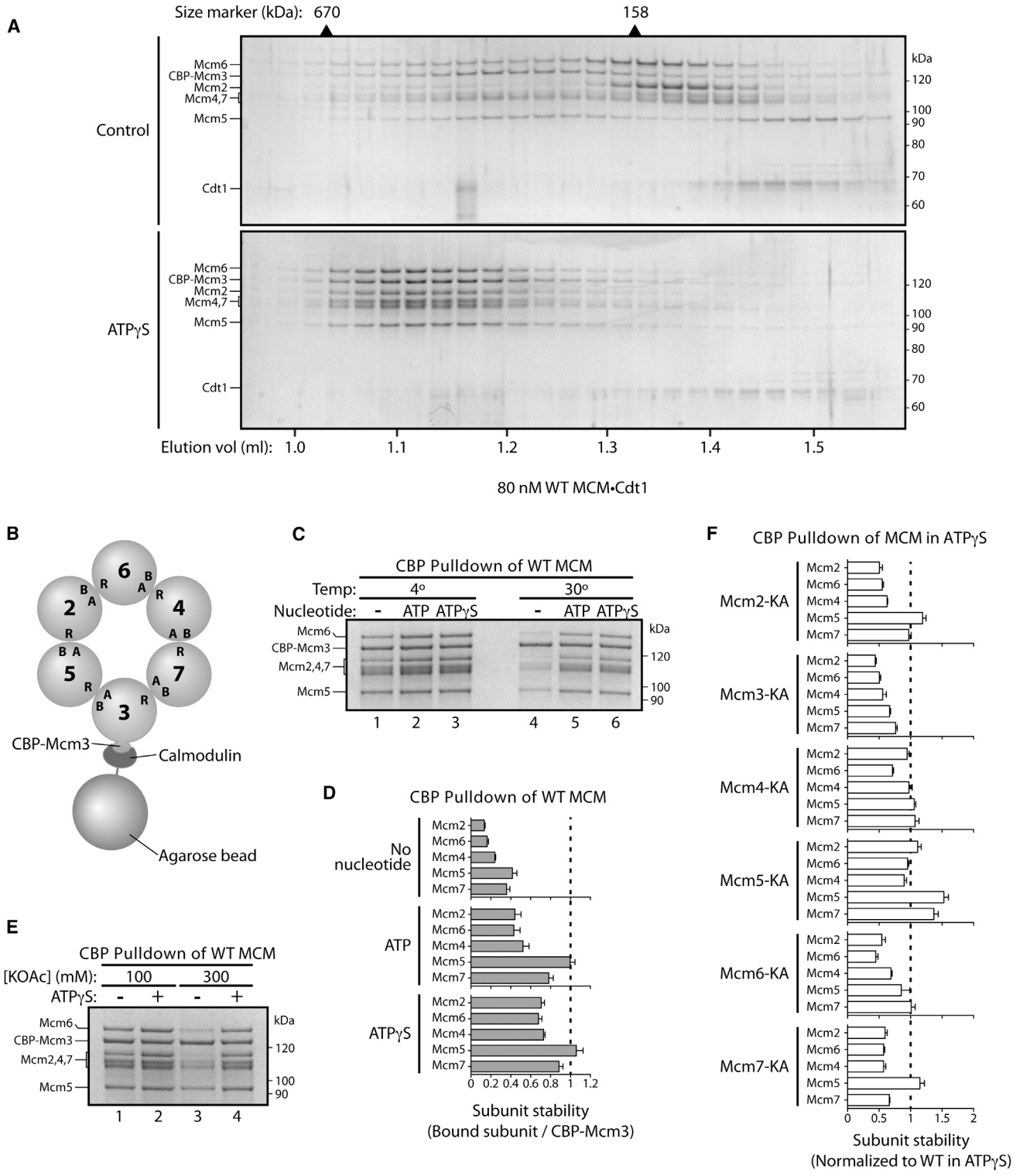

Figure 4. MCM Oligomerization Requires ATP Binding

(A) The stability of MCM•Cdt1 was analyzed by analytical gel filtration as a function of nucleotide. Purified wild-type MCM•Cdt1 was diluted at 80 nM in a low-salt wash buffer (300 mM KOAc) in the absence (top panel) or presence (bottom) of 5 mM ATPγS. Samples were incubated without mixing at 30° for 20 min and then immediately loaded onto a 2.4 ml Superdex 200 column. Fractions were separated by SDS-PAGE and visualized by silver staining. The elution volume of gel filtration standards—Thyroglobulin (670 kDa) and γ-globulin (158 kDa)—is denoted above the corresponding fractions, and the elution volume is denoted below. Note that in both cases the gel filtration buffer did not contain any nucleotide. See also Figure S4.

(legend continued on next page)

found it supported growth under restrictive conditions (Figure S2B, row 11). To investigate this further, we examined the growth of this strain in galactose-containing medium at different temperatures. Surprisingly, we found that this mutant could support growth at 37°C but exhibited a severe growth defect when expressed at 24°C (Figure S2C). Therefore, the *mcm6-RA* mutant is likely to be nonfunctional at this lower temperature. This temperature effect likely explains the discrepancy between our results and those of Bochman et al. (2008).

With the conditional protein exchange strategy, we can examine the effects of lethal mutations within a single cell cycle. To do this, cells were first arrested with α factor mating pheromone under permissive conditions, shifted to degron-restrictive conditions for 90 min while expressing the second copy genes, and then released from the α factor block under restrictive conditions. Figure 3C shows flow cytometry for such an experiment using the *mcm7-td* strain and various Mcm7 second copy constructs. Analysis of the other MCM subunits is shown in Figure S3. Figure 3C shows that the *mcm7-td* strain enters but does not complete S phase even 2 hr after release. Expression of the wild-type Mcm7 protein suppresses this defect, and cells complete S phase around 60 min after release, similar to the strain lacking the degron fusion. Some mutants, for example, the *mcm7-DEFN* mutant, progressed through S phase and into the next G1 phase, consistent with the fact that this mutant supported viability under restrictive conditions (Figure S2B, row 8). Other Walker B mutants, like the *mcm7-AEFD*, *mcm7-NEFD*, and *mcm7-NQFD* mutants, caused an early S phase arrest, even earlier than the degron by itself. Finally, some mutants, like the Walker A mutant *mcm7-KA* and the Walker B *mcm7-DQFD* mutant, progressed slowly through S phase but arrested in G2/M. Figure 3D summarizes this analysis for all *mcm* mutants tested. The strong correlation between cell-cycle phenotypes and viability suggested that most if not all of the observed growth defects were due to defects in DNA replication.

MCM Requires ATP for Stability under Loading Reaction Conditions

None of the Walker A mutants supported viability, while two of the arginine finger mutants (Mcm4 and 6) were viable under restrictive conditions, suggesting that ATP binding and hydrolysis might play distinct roles in MCM function. Based on previous work with the SV40 large T antigen (TAg) (Dean et al., 1992), we considered that ATP binding might contribute to the stability of the MCM hexamer. When purified in the absence of added nucleotide under conditions of low temperature (4°C) and high protein concentration, MCM•Cdt1 elutes from preparative gel filtration columns close to the 670 kDa marker, suggesting it is an intact heptamer (Figure S4A). However, the stability of MCM has not been tested under the conditions used to assemble pre-RCs. To examine this, we preincubated the MCM•Cdt1 complex at a concentration of 80 nM and a temperature of 30°C either with or without ATPγS for 20 min prior to fractionating the complex by gel filtration. Figure 4A shows that, after preincubation in the absence of nucleotide, the MCM subunits were primarily found in low-molecular-weight fractions with very little present in high-molecular-weight fractions. Nearly all Mcm2 and Mcm6 were present in fractions smaller than 158 kDa; some Mcm5 was present in low-molecular-weight fractions, and some cofractionated with Mcm3 and Mcm7 between the 158 and 670 kDa markers. Therefore, preincubation under conditions similar to those used in pre-RC assembly is sufficient to cause disassembly of the MCM complex in the absence of nucleotide. The bottom panel of Figure 4A shows that the presence of ATPγS during preincubation greatly reduced complex disassembly. Under these conditions, all of the MCM subunits cofractionated at a higher molecular weight. This peak was still somewhat smaller than 670 kDa, suggesting that even in the presence of nucleotide during preincubation, the hexamer isn't completely stable. Because the gel filtration was performed without nucleotide in the buffer, it is possible that some disassembly of the complex occurred during fractionation. Note that Cdt1 dissociates from the complex even in the presence of ATPγS, indicating that its association with the complex is not affected by nucleotide but is sensitive to dilution. MCM•Cdt1 was still unstable without added nucleotide at the higher concentration of 320 nM (Figure S4B); however, when MCM•Cdt1 was preincubated at a much higher concentration (1.9 μM), the complex was stable in the presence or absence of ATPγS. In addition, Cdt1 remained associated with the complex under these conditions (Figure S4C).

Gel filtration is useful to see the extent of complex disassembly. However, because the fractionation takes considerable time, involves further dilution of the complex during fractionation, and cannot be performed under many different conditions, we developed a simple pull-down assay to test stability of the

(B–D) The stability of the MCM complex is affected by nucleotide and temperature. A schematic of a pull-down-based stability assay of MCM is shown in (B), and a representative result is shown in (C). In this schematic, the position of the Walker A, Walker B, and arginine finger motifs within each subunit are indicated by "A," "B," and "R," respectively. Purified MCM was diluted to 100 nM in a low-salt wash buffer (300 mM KOAc) and incubated with calmodulin beads in the presence or absence of 5 mM ATP or ATPγS at the indicated temperatures for 20 min. Beads were washed, and bound proteins were separated by SDS-PAGE and visualized by Coomassie blue staining. Quantification of the stability assay shown in (C) at 30° (lanes 4–6) is shown in (D). Multiple independent experiments were carried out and quantified as follows: the staining intensity for each of the six MCM subunits was measured. We defined "Subunit stability" as the ratio between each bound subunit relative to bound CBP-Mcm3 within the same lane. This ratio represents the relative stability of each subunit while accounting for potential differences in pull-down efficiency. The data of six independent experiments is shown as mean ±SEM.

(E) The stability of MCM is also affected by ionic strength. A pull-down-based stability assay was carried out as in (C) at 30° with a buffer containing the indicated salt concentration in the absence or presence of 5 mM ATPγS.

(F) Walker A mutants exhibit structural defects even in the presence of a nucleotide. Stability assay of wild-type or mutant MCM complexes was carried out with ATPγS, 300 mM KOAc and at 30°. The "Subunit stability" value was calculated as in (D) and normalized to the wild-type value within the same experiment. Therefore, a ratio of one means that a mutant exhibits the same stability (or instability) as wild-type, and values that are lower or higher than one signify a complex that is less or more stable than wild-type, respectively. The data of three independent experiments is shown as mean ±SEM. Figure S5 shows the complete set of experiments with or without a nucleotide, as well as experiments with all arginine finger mutants.

complex. MCM bound to calmodulin beads via the CBP tag on the N terminus of Mcm3 is incubated under different conditions, beads are isolated, and bound proteins are examined (Figure 4B). Figure 4C shows that, after incubation at 30°C in the absence of nucleotide, CBP-Mcm3 was retrieved, but the other MCM subunits were present at substoichiometric levels (lane 4). The presence of either ATP (lane 5) or ATPγS (lane 6) during the incubation greatly suppressed complex disassembly as seen by the elevated levels of the other five MCM subunits. These effects were quantified for each MCM subunit and shown in Figure 4D. Under these same conditions, ORC was completely stable with or without nucleotide (Figure S4D). We consistently see slightly more Mcm5 and Mcm7 associated with Mcm3 than Mcm2, Mcm4, or Mcm6, even in the presence of nucleotide, consistent with the fact that Mcm5 and Mcm7 are the immediate neighbors of Mcm3 in the MCM ring. In addition, the complex was slightly more stable in ATPγS than in ATP, suggesting that ATP hydrolysis may have a destabilizing effect (compare the middle and bottom panels of Figure 4D). The complex was substantially more stable when the incubation was performed at 4°C (Figure 4C, lanes 1–3) in the absence of the added nucleotide. The complex was also more stable at 0.1 M potassium acetate than 0.3 M, in the absence of nucleotide (Figure 4E). Although pre-RC assembly is usually performed in 0.1 M potassium acetate, it works equally well in 0.3 M potassium acetate (Figure S4E). In addition, our standard low-salt wash buffer contains 0.3 M potassium acetate, indicating that nucleotide-dependent stabilization is likely to be important under standard conditions. Figures S4F and S4G show that both complex disassembly in the absence of nucleotide and reassembly after adding nucleotide happen within a few minutes, suggesting that it is a dynamic process. Taken together, these results indicate that stability of the MCM complex is affected by protein concentration, temperature, and ionic strength. Most significantly, the presence of nucleotide stabilizes the complex under a variety of conditions compatible with pre-RC assembly.

We examined the stability of MCM complexes containing mutations in the Walker A motif of individual MCM subunits in the presence of ATPγS. Figure 4F shows that most of the mutant complexes showed significant instability compared to the wild-type complex. Mcm3-KA and Mcm7-KA mutants had the most severe defects. Mcm2-KA and Mcm6-KA mutants did not reduce the levels of Mcm5 or Mcm7 pulled down but did result in reduced levels of Mcm2, Mcm4 and Mcm6, consistent with their positions in the MCM ring. The Mcm5-KA mutant was the only Walker A mutant that did not show detectable MCM instability and, in fact, reproducibly showed enhanced stability, suggesting that ATP bound at the Mcm2–5 interface may be destabilizing. The complete set of stabilities in ATP, ATPγS, and without added nucleotide are presented in Figure S5. These experiments show that nucleotide binding by the MCM subunits plays a role in hexamer stability.

MCM ATP Binding and Hydrolysis Mutants Are Defective in MCM Loading

We tested the ability of MCM complexes containing mutant subunits that showed early S phase arrest (Figure 3D) to recruit and load MCM. Figure S6A shows that these mutant complexes were purified with subunit stoichiometry indistinguishable from the wild-type complex. Figure 5A shows that these mutants were able to recruit MCM to ORC•Cdc6 in ATPγS to at least some extent; however, Figure 5B shows that all of these mutants were defective to varying degrees in MCM loading. There are Walker A, Walker B, and arginine finger mutants among this group. Because mutations in the Walker B motif can affect ATP binding and/or ATP hydrolysis and because the effects of these mutants on viability was complex and different for different MCM subunits, we analyzed the Walker A and arginine finger mutants in detail.

We next focused on the full set of Walker A mutants. Figure 5C shows that at least some MCM subunits were recruited in the presence of ATPγS by all the Walker A mutant complexes after low-salt wash (0.3 M potassium acetate); although, as shown in Figure 4F, most of these mutants show some instability in ATPγS (see also Figure S6B). Recruitment levels of individual MCM subunits in each of the Walker A mutants were quantified (Figure S6C). Some mutants, like Mcm5-KA, recruited all MCM subunits with wild-type stoichiometry, but to somewhat reduced levels. Other mutants showed clear defects in recruitment of intact MCM. For example, Mcm2-KA recruited Mcm3, Mcm5, and Mcm7 to near-wild-type levels but had reduced levels of Mcm2, Mcm4, and Mcm6 (lane 2, Figure 5C). This is very similar to the stability of the complex seen in Figure 4F, where tagged Mcm3 is recruited to beads directly rather than by its interaction with ORC•Cdc6. On the other hand, Mcm6-KA showed similar defects to Mcm2-KA in Figure 4F but much milder defects in the ATPγS complex (lane 6, Figure 5C). It may be that additional contacts between ORC•Cdc6 and MCM (Sun et al., 2013) can stabilize some complexes even when they have subunits that cannot bind ATP. Figure 5D shows that none of the Walker A mutants was able to load the MCM complex efficiently, including mutants that recruited MCM in ATPγS relatively well, suggesting that ATP binding plays an essential role in licensing independent of hexamer stabilization.

We next tested arginine finger mutants. As shown in Figure S5B, all of the arginine finger mutants except Mcm5 and to a lesser extent Mcm4 showed wild-type hexamer stability. Moreover, most of the arginine finger mutants recruited MCM to wild-type levels in ATPγS (Figure 6A). However, as shown in Figure 6B, all of these mutants except Mcm4 showed very substantial defects in MCM loading in ATP. The *mcm4-RA* mutant was one of two arginine finger mutants that could support viability at 37°C (Figure 3D). As described above, the *mcm6-RA* mutant was also viable at 37°C, but expression of this mutant inhibited growth at lower temperatures. In contrast to Mcm4-RA, however, Mcm6-RA did not load MCM in Figure 6B. We tested whether Mcm6-RA mutant complex could be loaded at 37°C; however, we found that even the wild-type MCM complex was very poorly loaded at this temperature. We have not pursued this further. Fernández-Cid et al. (2013) showed that Mcm3-RA could load in their system. In our hands, this mutant shows a defect in loading, though it is not as defective as other mutants like Mcm2-RA and Mcm5-RA.

Figures 6C and 6D show a time course analysis for the MCM complex containing the *mcm2-RA* mutant. The loading of wild-type MCM was complete by 5–15 min, but MCM loading by

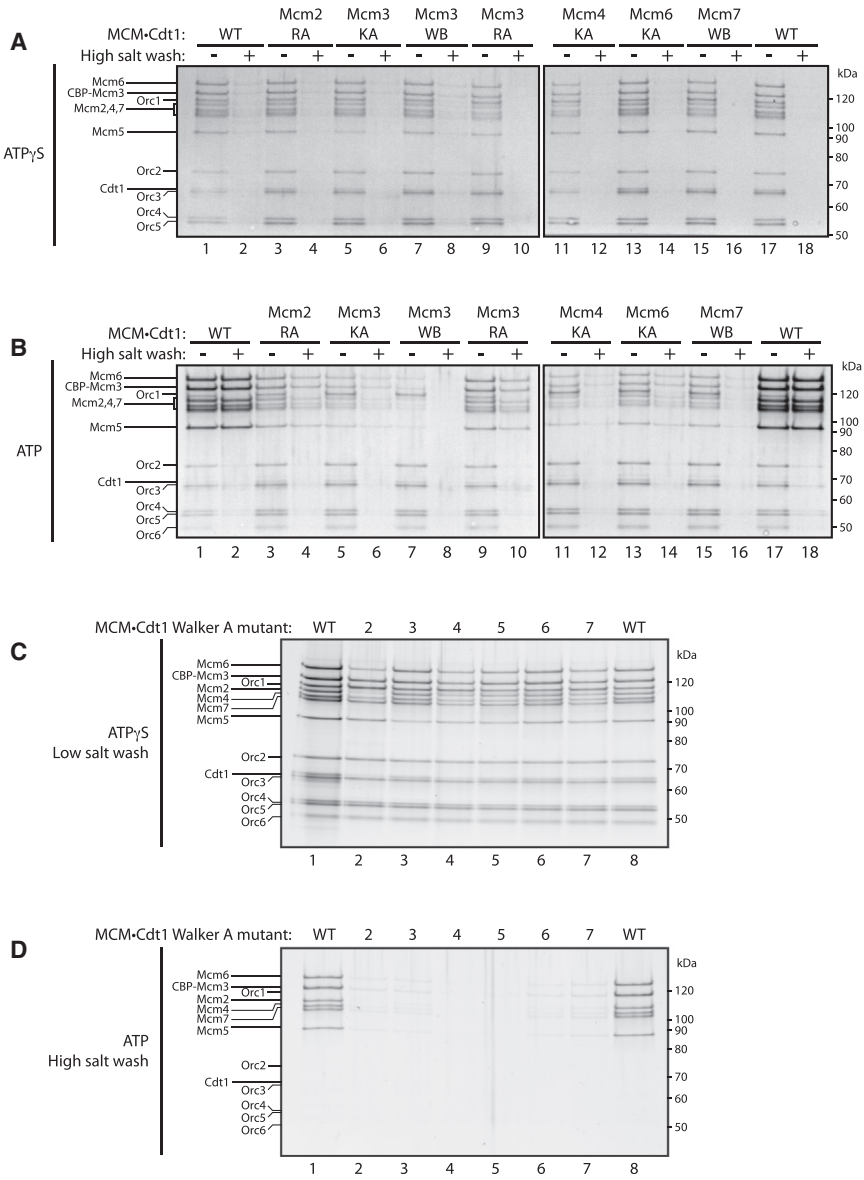

Figure 5. Lethal MCM ATPase Mutants In Vivo Are Defective in Licensing In Vitro

(A–D) Loading reactions were carried out with wild-type or mutant MCM•Cdt1 complexes in ATPγS or ATP and a low- or high-salt wash, as indicated. See also Figures S6A and S6B. The Walker B mutants of Mcm3 and Mcm7 are *NEFD* mutants.

the Mcm2-RA complex showed less than 20% of the loading of the wild-type protein, even after 4 hr. This experiment also shows that we see no detectable lag before wild-type MCM loading, in contrast to the 10–15 min lag seen by Fernández-Cid et al. (2013).

Our assay for MCM loading involves a high-salt wash. Thus, it was possible that mutants could assemble double hexamers but that these double hexamers might be unstable—and consequently removed by the high-salt treatment. We used electron microscopy to examine and quantify double hexamer formation in the presence of ATP with wild-type ORC and Cdc6 after low-salt wash. Figure 6E shows that, in the midst of other smaller complexes, MCM double hexamers could be readily identified with wild-type MCM under these conditions. Although all mutants tested were bound to ORC•Cdc6 similar to wild-type (Figure S6D), double hexamers were rarely seen with the Mcm2-RA and Mcm3-RA mutants and were not seen at all with the other mutants. To quantify pre-RC assembly, double hexamers were counted on EM grids containing products from the loading reactions using a single-blind protocol (see Experimental Procedures). The densities shown below each of the representative images indicate that the Mcm2 and Mcm3 arginine finger mutants generated 20–30 times fewer double hexamers than the wild-type protein after 20 min, consistent with results from the high-salt washed samples (Figures 6C and 6D). Therefore, the arginine finger mutants show significant defects in pre-RC assembly, even in low-salt buffer.

If only one of the two MCM hexamers in the double hexamer needs to bind and hydrolyze ATP, one might predict that the presence of wild-type MCM could promote the loading of mutant MCM into hybrid complexes. Wild-type MCM complex containing Flag-tagged Mcm3 was mixed in different ratios with a complex containing the NEFD Walker B mutant Mcm3, which was CBP tagged. The *mcm3-NEFD* mutant exhibited a tight S phase arrest (Figure S3) and was recruited as well as wild-type Mcm3 complex in ATPγS (Figure S6E; compare lanes 1 and 7). However, the mutant Mcm3 was not loaded into a salt-resistant complex in ATP at any ratio of complexes (Figure S6E, lanes 9–14). Indeed, the presence of the mutant MCM complex appeared to inhibit the loading of the wild-type complex.

Finally, we asked whether mutant MCM complexes were able to release Cdt1 and form an OCM complex, which normally happens very quickly after ATP binding (Fernández-Cid et al., 2013). Although Cdt1 is released efficiently in the presence of ATP by the wild-type MCM complex (Figure 6F, lane 4), mutant MCM complexes containing either the Mcm2, Mcm3, or Mcm5 arginine finger mutant were defective in release of Cdt1 (Figures 6F and S6F) even after 20 min. Therefore, ATP hydrolysis by MCM appears to be required for formation of the OCM complex.

DISCUSSION

Based on previous work and the work presented here, the model in Figure 6G summarizes our current view of how ATP binding

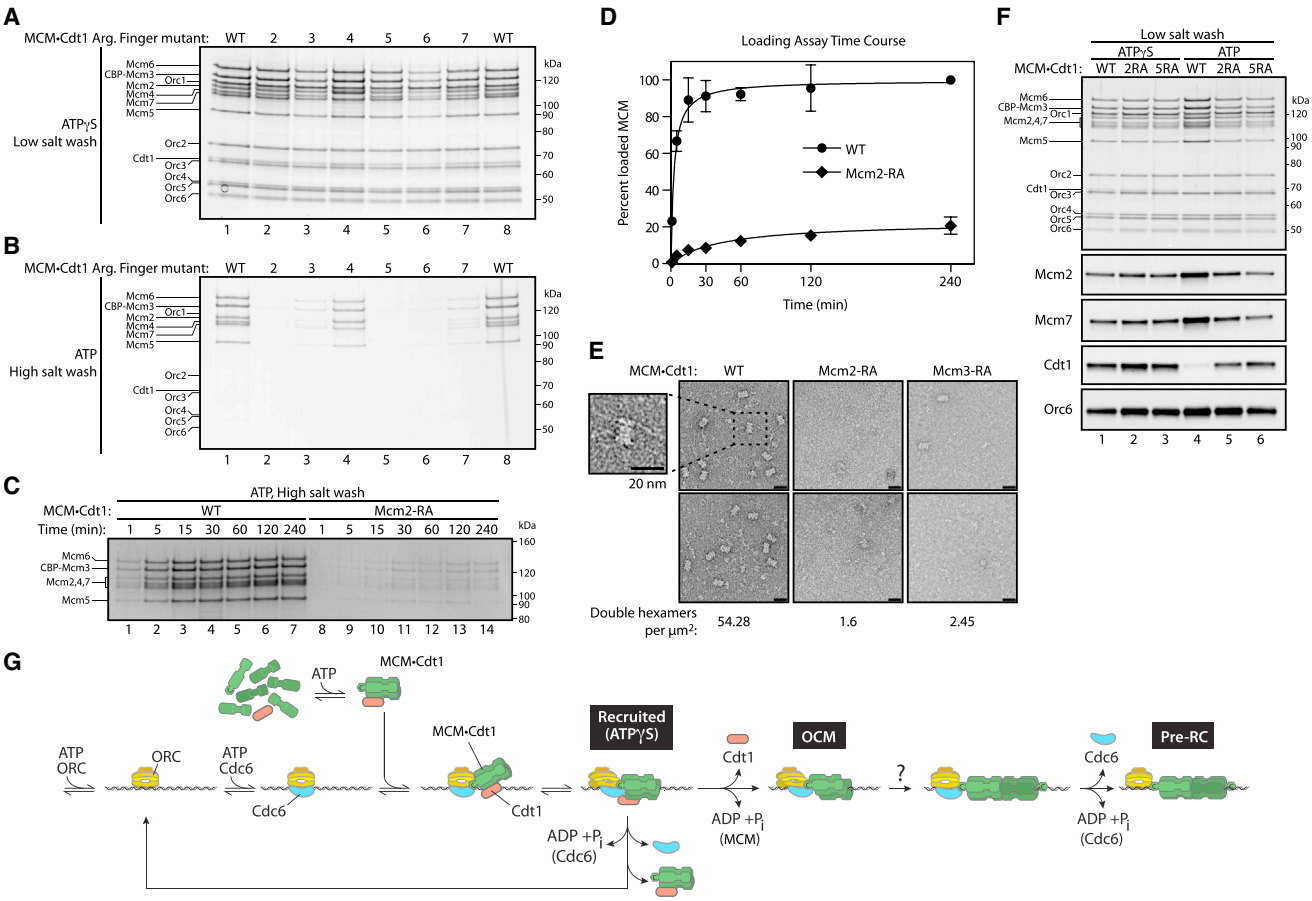

Figure 6. MCM ATP Hydrolysis Mutants Are Defective in Formation of MCM Double Hexamers and Cdt1 Release

(A and B) MCM arginine finger mutants are defective in licensing in vitro. Loading reactions were carried out with wild-type or mutant MCM•Cdt1 complexes in ATPγS or ATP and a low or high-salt wash as indicated.

(C and D) Time course loading assay with wild-type or *mcm2-RA* MCM•Cdt1 complexes. Loading reactions were carried out in ATP and a high-salt wash for the indicated times. Shown are a representative experiment (C) and quantification of three independent experiments (D). Data is represented as mean ±SEM, fitted with nonlinear Michaelis-Menten curves.

(E) Even under very mild conditions, MCM ATPase mutants fail to form double hexamers. Wild-type or mutant MCM•Cdt1 complexes were used in loading reactions with ATP but were only washed with a mild wash buffer (100 mM KOAc). Each reaction was split in two, with one half analyzed by SDS-PAGE and silver staining (Figure S6D) and the other half analyzed by electron microscopy. Images of representative fields are shown for wild-type MCM•Cdt1 and the Mcm2 and 3 arginine finger mutants. In our imaging conditions, wild-type MCM double hexamers are almost exclusively side views of overall dimensions ~13 × 20 nm. Only particles fitting this description were counted as double hexamers. The average density per surface area of particles with dimensions and morphology compatible with a double hexamer was calculated and is noted below each set. All other samples apart from the ones shown did not have particles that had double hexamer dimensions. Scale bar, 20 nm.

(F) MCM mutants are defective in ATP-hydrolysis-dependent release of Cdt1. Loading reactions were carried out with wild-type or arginine finger mutants of Mcm2 or Mcm5. Samples were split in two and analyzed by silver staining and western blotting. See also Figure S6F.

(G) An updated model of origin licensing. The first two steps are the ATP-dependent sequential binding of ORC and Cdc6 to DNA. MCM•Cdt1 assembles in an ATP-dependent manner and is then recruited to ORC•Cdc6 via the C terminus of Mcm3. ATP hydrolysis by ORC, Cdc6, and MCM leads to the formation of double hexamers and release of Cdc6 and Cdt1. The order of hydrolysis by ORC, Cdc6, and MCM is unknown and denoted by a question mark. See Discussion for details.

and hydrolysis contribute to origin licensing. ATP binding plays multiple roles early in the licensing reaction. ATP binding by the Orc1 subunit of ORC is required for it to bind origin DNA (Bell and Stillman, 1992; Klemm and Bell, 2001). Though ATP does not contribute appreciably to the stability of the yeast ORC (Figure S4D), it is required to stabilize human ORC (Ranjan and Gossen, 2006; Siddiqui and Stillman, 2007). Cdc6 must also bind ATP to interact stably with origin-bound ORC, as demonstrated by the fact that the Cdc6-KA Walker A mutant is not recruited in either ATP or ATPγS (Figure 1B) (Evrin et al., 2013; Frolova et al., 2002).

We have found that ATP binding also plays a role in stabilizing the MCM complex. Although MCM is stable at high protein concentration and low temperature (4°C), it is unstable in the absence of nucleotide at the lower protein concentration and elevated temperature (30°C) used in pre-RC assembly reactions.

The oligomeric state of the SV40 large TAg initiator/helicase is also regulated by ATP: TAg is monomeric, but preincubation with ATP at 37°C induces assembly into a hexamer (Dean et al., 1992). Because MCM•Cdt1 is initially recruited to ORC•Cdc6 by a single MCM subunit (Mcm3), we propose that maintaining the hexameric MCM complex is important during the early steps in pre-RC assembly.

Consistent with this, Walker A mutants in several MCM subunits show defects in recruitment in ATPγS under conditions in which nucleotide binding is required for complex stability (Figure 4F). Hexamerisation of SV40 Tag by ATP prevents association with origin DNA, perhaps because DNA cannot gain access to the central channel of the helicase when it is present as a topologically closed ring. In contrast to TAg, the inherent instability of the Mcm2-5 "gate" may allow access of DNA into the central channel of MCM even in the presence of ATPγS (Figure 4F) (Bochman and Schwacha, 2008; Costa et al., 2011; Sun et al., 2013).

ATP binding by ORC, Cdc6, and MCM are all required to generate the "recruited" complex, seen in ATPγS. ATP hydrolysis by these proteins then plays separate downstream roles. Previous analysis of the Orc4-RA mutant in extracts showed that it was capable of a single round of MCM loading but was defective in iterative MCM loading (Bowers et al., 2004). The amount of MCM that is loaded with purified proteins doesn't greatly exceed the amount of ORC bound, and EM analysis under conditions of DNA excess indicated that most DNA molecules had no more than one double hexamer (Remus et al., 2009), suggesting that ORC may not load MCM repeatedly in our system. Perhaps, analogous to *E. coli* dnaA (Skarstad and Katayama, 2013), additional factors may be required for ORC to exchange ADP and ATP.

Cdc6-EG can also load MCM, even together with Orc4-RA. However, our experiments show it cannot release nonproductive intermediates. We propose that one of the key roles of ATP hydrolysis by Cdc6 during licensing is to prevent ORC•Cdc6 from being trapped in dead-end complexes, which may explain why the *cdc6-EG* mutant is lethal in vivo when overexpressed. Cdc6-EG is also defective in MCM loading in extracts (Randell et al., 2006). We suggest this may indicate a greater requirement for this proofreading function of Cdc6 in extracts than we see with purified proteins. Nonproductive complexes may form more readily in extracts, perhaps because of competing nonspecific DNA binding proteins that block the completion of the loading reaction. Fernández-Cid et al. (2013) saw reduced loading with Cdc6-EG using purified proteins. We suggest some difference between our reaction conditions makes their system more dependent on efficient release of nonproductive intermediates. Our results show that the ATPases of Cdc6 and MCM subunits play somewhat opposing roles, and it will be interesting to see how they are regulated during pre-RC assembly. The ATPase of Cdc6 may be actively suppressed during assembly of productive MCM•Cdt1 complexes or may be relatively slow, acting as a timer that promotes release of MCM after some suitable period has elapsed. Further work is also required to determine whether the defect in releasing nonproductive intermediates by Cdc6-EG is solely due to defects in ATPase activity or whether this mutant has a more complex defect, perhaps mimicking or stabilizing some intermediate in ATP hydrolysis.

Our results show that ATP hydrolysis by MCM plays an essential role in assembling the MCM double hexamer on DNA. Previous work showed that Walker A mutants in Mcm6 and Mcm7 bound to chromatin but did not support replication in *Xenopus* egg extracts. In our system, these mutants are recruited in ATPγS but are not loaded into double hexamers. It is possible that the wash conditions used in the *Xenopus* experiments are not stringent enough to distinguish recruitment of a single hexamer from topological loading of the double hexamer. Alternatively, it is possible that the *Xenopus* proteins behave differently. Nonetheless, our analysis of the yeast Mcm-RA mutants has indicated that ATP hydrolysis is required for release of Cdt1 and assembly of the double hexamer. We note that, by contrast, SV40 TAg can assemble double hexamers in the presence of nonhydrolyzable ATP analogs (Mastrangelo et al., 1989). Aside from the Mcm4-RA mutant, which is viable and can load MCM, the other Mcm-RA mutants behaved similarly; they showed severe defects in MCM loading and Cdt1 release. Thus, we cannot at present ascribe different roles for the different MCM active sites during licensing. The defect in Cdt1 release suggests that ATP hydrolysis occurs before recruitment of the second hexamer, but further work is required to firmly establish this point.

ATP hydrolysis by MCM is also essential for its DNA helicase activity (Bochman and Schwacha, 2008; Ilves et al., 2010). It is interesting that there is a strong correlation between the severity of phenotypes in vivo and the severity of defects in MCM loading for the *mcm-RA* mutants. This may indicate that ATP hydrolysis plays similar roles in origin licensing and helicase activity. In this regard, an Mcm7-RA mutant in the *Drosophila* CMG complex reduced helicase activity by only approximately 50% (Ilves et al., 2010), but an Mcm2-RA mutant in the yeast MCM complex eliminated helicase activity (Bochman and Schwacha, 2008). Similarly, the Mcm7-RA mutant was less defective in licensing than the Mcm2-RA mutant (Figure 6B). Alternatively, it has been shown that the archaeal MCM helicase can tolerate the presence of catalytically inactive subunits (Moreau et al., 2007), suggesting ATP hydrolysis by the helicase is not strictly sequential. Thus, it is possible that the eukaryotic MCM helicase can also tolerate inactive subunits, but the licensing reaction cannot. A more comprehensive analysis of helicase and licensing activities using the Mcm-RA mutants may help resolve this issue.

EXPERIMENTAL PROCEDURES

Plasmids and Yeast Strains

Table S1 contains a full list that details the cloning vector, insert source, and restriction sites used for plasmid construction. For a full list of yeast strains, see Table S2.

Protein Expression and Purification

Expression and purification of ORC, Cdc6, Mcm3, and Flag-tagged MCM•Cdt1 was carried out as previously described (Frigola et al., 2013). For ORC4 mutants, endogenous ORC4 was Flag-tagged and depleted during purification. For details on purification of CBP-tagged MCM•Cdt1 complexes, see Supplemental Information.

Loading Assay

Loading assays were performed as described previously (Frigola et al., 2013). For details, see Supplementary Information.

Analysis of Viability and Cell-Cycle Phenotypes

Viability of yeast strains was analyzed by plating each strain in a series of 10-fold dilutions under permissive (YP + 2% glucose, 24°) or restrictive (YP + 2% galactose, 37°) conditions and scoring for growth after 4 days. For full details on time course experiments, see Supplemental Information.

Analysis of Mcm2–7 Stability by Analytical Gel Filtration

Purified MCM•Cdt1 was diluted to 80 nM in a final volume of 30 μl of (45 mM HEPES-KOH [pH 7.6], 0.3 M KOAc, 5 mM MgOAc, 10% Glycerol, and 0.02% NP-40) in the absence or presence of 5 mM ATPγS (Roche) on ice. Samples were then incubated without mixing at 30° for 20 min and immediately loaded onto a 2.4 ml Superdex 200 column (GE Healthcare) using an Ettan FPLC instrument (GE Healthcare) at 4°. Columns were equilibrated with the same buffer used to dilute the MCM•Cdt1 complex. In all cases, the column did not contain any added nucleotide.

Stability Assay

Purified MCM•Cdt1 was diluted to 100 nM in 50 μl of (45 mM HEPES-KOH [pH 7.6], 0.3 M KOAc, 5 mM MgOAc, 2 mM $CaCl_2$, 10% Glycerol, and 0.02% NP-40) in the absence or presence of 5 mM ATP (GE Healthcare) or ATPγS (Roche) on ice. Samples were then applied to 15 μl Calmodulin affinity resin (Stratagene) and incubated 20′ with mixing at 1,250 RPM at 30°. Beads were then collected, washed once, resuspended with sample buffer, boiled, and analyzed by SDS-PAGE. Gels were stained with InstantBlue (Expedeon), scanned, and quantified using ImageJ.

Electron Microscopy

Samples were applied directly onto glow-discharged carbon-coated copper grids (Quantifoil, GmbH) and stained with 2% uranyl acetate. Images were collected with a Tecnai TF20 electron microscope (FEI Company) operating at 200 kV on a 4k × 4k CCD detector (TVIPS, GmbH) at a nominal magnification of 50,000× and an under focus level of ~900 nm. Images were collected at random in areas of suitable stain thickness. Particles selection was carried out using Boxer, part of EMAN (Tang et al., 2007).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2014.06.034.

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