An ORC/Cdc6/MCM2-7 complex is formed in a multistep reaction to serve as a platform for MCM double-hexamer assembly

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RUNNING TITLE **Mechanisms in OCM complex assembly and function**

SUMMARY

In *Saccharomyces cerevisiae* and higher eukaryotes the loading of the replicative helicase MCM2-7 onto DNA requires the combined activities of ORC, Cdc6 and Cdt1. These proteins load in an unknown way MCM2-7 into a double-hexamer around DNA. Here we show that MCM2-7 recruitment by ORC/Cdc6 is blocked by an auto-inhibitory domain in the C-terminus of Mcm6. Interestingly, Cdt1 can overcome this inhibitory activity and consequently the Cdt1-MCM2-7 complex activates ORC/Cdc6 ATP-hydrolysis to promote helicase loading. While Cdc6 ATPase activity is known to facilitate Cdt1 release and MCM2-7 loading, we discovered that Orc1 ATP-hydrolysis is equally important in this process. Moreover, we found that Orc1/Cdc6 ATP-hydrolysis promotes the formation of an novel ORC/Cdc6/MCM2-7 (OCM) complex, which functions in MCM2-7 double-hexamer assembly. Importantly, CDK-dependent phosphorylation of ORC inhibits OCM establishment to ensure once per cell cycle replication. In summary, this work reveals multiple critical mechanisms that redefine our understanding of DNA licensing.

HIGHLIGHTS

- **A Cdt1 interaction with MCM2-7 alleviates an auto-inhibitory activity in Mcm6**
- **OCM establishment occurs fast while MCM2-7 double-hexamer formation is slow**
- **Both Orc1 and Cdc6 ATP-hydrolysis promote Cdt1 release and OCM formation**
- **The OCM represents a platform for double-hexamer assembly**

INTRODUCTION

Eukaryotic DNA replication is a faithful and tightly controlled process. It is initiated in G1 phase with the loading of the six-subunit mini-chromosome maintenance (MCM2-7) helicase onto DNA. The DNA-bound helicase complex remains inactive until the cell commits to S phase and the helicase becomes activated in a kinase-dependent process that leads to the formation of replication forks (Masai et al., 2010).

The loading of the MCM2-7 helicase, also known as pre-replicative complex (pre-RC) formation or replication licensing, depends on the combined activities of the six-subunit origin recognition complex (ORC), Cdc6 and Cdt1. This multi-step reaction is initiated in G1 phase, when ORC and Cdc6 form a complex on origin DNA, which then recruits a Cdt1/MCM2-7 complex. Once the initial ORC/Cdc6/Cdt1/MCM2-7 complex is formed (Evrin

et al., 2013; Takara and Bell, 2011), ORC/Cdc6 and Cdt1 cooperate to load the MCM2-7 helicase onto DNA. MCM2-7 loading depends on hydrolysis of ATP by Cdc6 and leads to Cdt1 release from the loading complex (Randell et al., 2006). Interestingly, MCM2-7 is a single-hexamer in solution, but is loaded by ORC, Cdc6 and Cdt1 as a head-to-head double-hexamer around double-stranded DNA (Evrin et al., 2009; Gambus et al., 2011; Remus et al., 2009). During G1-phase the MCM2-7 helicase is inactive and only becomes activated upon binding to Cdc45 and GINS in S-Phase (Masai et al., 2010).

In budding yeast, MCM2-7 and Cdt1 are imported into the nucleus in an interdependent manner during the M-G1 transition (Tanaka and Diffley, 2002). Cdt1 is known to interact with MCM2-7 via a conserved motif in the C-terminus of Mcm6 (Yanagi et al., 2002). Conversely, the C-terminus of Cdt1 contains a binding site for Mcm6 and point mutations in either interaction motif block the nuclear import of the Cdt1/MCM2-7 complex (Wu et al., 2012). Importantly, in the absence of Cdt1, MCM2-7 cannot interact with ORC/Cdc6 (Evrin et al., 2009; Randell et al., 2006). However, an N-terminal Cdt1 deletion mutant (ΔN301) that still contains the MCM2-7 interaction domain, can be recruited to replication origins, but fails to induce MCM2-7 loading (Ferenbach et al., 2005; Takara and Bell, 2011). Cdt1 also interacts with Orc6, and this interaction has been reported to facilitate the initial recruitment of Cdt1/MCM2-7 to ORC/Cdc6 (Chen et al., 2007). On the other hand, CDK dependent phosphorylation of Orc6 inhibits the Cdt1-Orc6 interaction, and as a result both MCM2-7 recruitment and MCM2-7 loading are reduced (Chen and Bell, 2011). How the initial ORC/Cdc6/Cdt1/MCM2-7 complex is transformed into an MCM2-7 double-hexamer is an important unresolved question. Our work reveals that pre-RC formation involves a previously unknown intermediate, the ORC/Cdc6/MCM2-7 (OCM) complex. We show that the OCM is formed upon ATP-hydrolysis dependent Cdt1 release and comprises one ORC, one Cdc6 and one MCM2-7 molecule. Surprisingly, we discovered that not only Cdc6, but also Orc1 promotes ATP-hydrolysis driven Cdt1 release, while an MCM2-7 ATPase mutant did not impact on Cdt1 release or MCM2-7 loading. Importantly, we found that MCM2-7, as part of the OCM, can integrate into the MCM2-7 double-hexamer, showing that the OCM is indeed a pre-RC intermediate. Moreover, we identified an auto-inhibitory domain in the C-terminus of Mcm6, which regulates MCM2-7 recruitment to ORC-Cdc6. In the absence of Cdt1, this domain blocks MCM2-7 binding to ORC/Cdc6, but once it is in complex with Cdt1, the Mcm6 C-terminus not only promotes pre-RC assembly, but also facilitates Orc1/Cdc6 dependent ATP-hydrolysis. On the other hand, we observed that CDK negatively regulates pre-RC assembly by inhibiting OCM establishment. This work uncovers several important mechanisms of pre-RC formation that explain how regulated MCM2-7 assembly on origin DNA occurs.

RESULTS

Cdt1/MCM2-7 associates rapidly with ORC/Cdc6 whereas MCM2-7 double-hexamer loading is a slow process

To understand the temporal order of events during pre-RC formation we have performed time resolved MCM2-7 association and loading experiments on origin DNA (Figure 1 A). It is possible to study complex assembly in the absence of ATP-hydrolysis by employing ATPγS, which can be hydrolyzed only very slowly (Randell et al., 2006). Purified ORC, Cdc6, Cdt1, MCM2-7 and origin DNA were combined in the presence of ATPγS, incubated for 0.5 to 15 minutes and consequently the unbound proteins were removed with a low salt wash. We observed association of Orc3, Cdc6, Cdt1 and Mcm2 with origin DNA (Figure 1A, i), suggesting that an initial complex was formed. Indeed the binding of proteins did not increase significantly after 30 seconds, highlighting rapid complex assembly. When we performed the same experiment in the presence of ATP, we observed DNA binding of Orc3, Cdc6 and Mcm2, but not of Cdt1, as Cdt1 becomes released from DNA in an ATP hydrolysis dependent manner (Randell et al., 2006) (Figure 1A, ii). Interestingly, while the binding of Orc3 and Cdc6 was fast, Mcm2 binding increased over time. Since it has been proposed that Cdc6 ATP hydrolysis promotes both Cdt1 release and the loading of a salt stable MCM2-7 double-hexamer, (Randell et al., 2006; Takara and Bell, 2011) we decided to test this hypothesis by measuring the appearance of salt stable MCM2-7 double-hexamer. We formed a pre-RC complex in the presence of ATP and then removed ORC, Cdc6 and weakly-associated MCM2-7 with a high salt wash, while the salt stable MCM2-7 double-hexamer stays bound to DNA (Evrin et al., 2009; Remus et al., 2009). Interestingly, we observed salt stable MCM2-7 only after 7.5 minutes and the amount increased over time (Figure 1A, iii). In summary, we observed the fast assembly of a salt sensitive pre-RC intermediate using ATP. However, the conversion of this intermediate complex into a salt resistant MCM2-7 double-hexamer is a slow process (>5 minutes). This indicates that ATP-hydrolysis does not immediately produce an MCM2-7 double-hexamer.

An ORC/Cdc6/MCM2-7 (OCM) complex is a novel pre-RC intermediate

To substantiate the hypothesis that ATP-hydrolysis dependent Cdt1 release generates a new pre-RC intermediate and to investigate the composition of such a complex we performed Mcm2 immunoprecipitation experiments (IPs) (Figure 1B-E). We assembled pre-RC complexes in the presence of ATP or ATPγS for 2.5 or 30 minutes, released the protein-complexes from the beads with DNase I and then immunoprecipitated Mcm2 containing complexes. With ATPγS we observed efficient co-precipitation of Orc3, Cdc6 and Cdt1 with Mcm2 at both 2.5 and 30 minutes (Figure 1B and C, lane 4). When we performed the same

reactions with ATP we found efficient co-precipitation of Orc3 and Cdc6 with Mcm2 after 2.5 and 30 minutes of incubation, whilst Cdt1 was undetectable (Figure 1D and E, lane 4). To further characterize the composition of the complex we asked if the OCM contains one or multiple copies of ORC, Cdc6 and MCM2-7. For this analysis we assembled an OCM with mixed populations of MBP-tagged or untagged Mcm5 (Figure 1F), Orc5 (Figure 1G) or Cdc6 (Figure 1H) for 4 minutes and then released the complexes with DNase I and performed an MBP-IP. In the MBP-IP we observed MBP-tagged Mcm5 (Figure 1F, lane 5), MBP-tagged Orc5 (Figure 1G, lane 5) or MBP-tagged Cdc6 (Figure 1H, lane 5), but no co-precipitation of untagged Mcm5, Orc5 or Cdc6, respectively, indicating that the OCM contains only a single copy of MCM2-7, ORC and Cdc6. Importantly, we showed that MBP-Mcm5, MBP-Orc5 and MBP-Cdc6 support salt resistant loading of MCM2-7 (Figure S1), indicating that the proteins are functional. Our analysis shows that in the absence of ATP-hydrolysis an initial ORC/Cdc6/Cdt1/MCM2-7 complex is formed and, upon ATP-hydrolysis, Cdt1 is rapidly released, resulting in the formation of a novel ORC/Cdc6/MCM2-7 (OCM) complex.

Cdt1 is essential for OCM complex formation

We observed that during pre-RC formation an OCM complex is formed. To verify that this complex only forms in the presence of Cdt1 we performed pre-RC assays in the presence and absence of Cdt1 with either ATP or ATPγS (Figure 1I). In the absence of Cdt1, but presence of ATPγS, we observed weak interactions between MCM2-7 and ORC/Cdc6 (Figure 1I, lane 4); with ATP this weak interaction was further decreased (Figure 1I, lane 5) and no high salt resistant MCM2-7 loading occurred (Figure 1I, lane 6). In contrast, in the presence of Cdt1 and ATPγS strong association of Cdt1/MCM2-7 with ORC/Cdc6 occurred (Figure 1I, lane 1). Furthermore, with Cdt1 and ATP we observed very efficient OCM formation and salt resistant MCM2-7 loading (Figure 1I, lane 2 and 3). These results show that MCM2-7 and ORC/Cdc6 interact only very weakly in the absence of Cdt1 and demonstrate that Cdt1 is required for formation of the OCM complex.

The OCM complex is stable

The OCM is formed rapidly during pre-RC formation, whereas MCM2-7 double-hexamer formation is a slow process. These observations suggest that the OCM complex must persist for some time before it is processed into the double-hexamer. To determine the stability of this complex we allowed OCM complexes to form by incubating pre-RC proteins with DNA and ATP for 2.5 minutes, then we washed away the unbound proteins with low salt buffer and incubated the reactions for a further 5, 10, 15 or 20 minutes. Then we analyzed the DNA-bound complexes (bound fraction) and the proteins that were released from DNA into the supernatant during the second incubation (supernatant fraction) (Figure 1J). The DNA-

bound OCM complex did not change significantly over time (Figure 1J, lanes 5-9) and the supernatant fractions did not contain noteworthy amounts of proteins (Figure 1J, lanes 10-14), indicating that the OCM complex is relatively stable. On the other hand, the OCM also needs to be disassembled when it matures into the MCM2-7 double-hexamer and, under these conditions, Cdc6 should be released from the complex. Accordingly, we and others have observed Cdc6 release from DNA in an ATP-hydrolysis dependent manner (Figure 1A, compare I. and II. 15 min Cdc6; Figure 1I, lanes 1 and 2) (Remus et al., 2009).

Cdt1 induces a structural change in MCM2-7 to facilitate direct interactions between ORC/Cdc6 and MCM2-7

Our data indicate that Cdt1 interaction with MCM2-7 is essential to recruit MCM2-7 towards ORC/Cdc6 and to form the OCM. We wanted to map the minimal domain of Cdt1 that allowed OCM formation using the pre-RC assembly assay (Figure 2A). We found that Cdt1 and Cdt1 (306-604) allowed efficient recruitment of MCM2-7 to ORC/Cdc6 in the presence of ATPγS, while Cdt1 (464-604) was less efficient and Cdt1 (1-312) failed to recruit MCM2-7 to ORC/Cdc6. Surprisingly, while Cdt1 and Cdt1 (306-604) formed a stable complex with ORC/Cdc6 and MCM2-7 in ATPγS (Figure 2A, lane 8 and 14), we did not observe Cdt1 (464-604) in complex with ORC/Cdc6 and MCM2-7 (Figure 2A, lane 17). In the presence of ATP, we observed efficient MCM2-7 recruitment and Cdt1 release with Cdt1 and Cdt1 (306-604) (Figure 2A, lane 9 and 15). Although Cdt1 and Cdt1 (306-604) both form an OCM complex in an identical manner, the OCM produced by Cdt1 (306-604) did not promote salt stable MCM2-7 double-hexamer formation (Figure 2A, lane 16). Cdt1 (464-604) recruited MCM2-7 in the presence of ATP (Figure 2A, lane 18) similar as with ATPγS, and did not support loading of salt resistant MCM2-7 (Figure 2A, lane 19). These data indicate that the Cdt1 C-terminus is sufficient for OCM formation. However, the Cdt1 N-terminus is required to form a functional OCM complex that supports MCM2-7 double-hexamer formation. Interestingly, we found that Cdt1 (464-604) could recruit MCM2-7 in the presence of ATPγS, but then the Cdt1 mutant was released in an ATP-hydrolysis independent manner from the complex. This suggests that a transient Cdt1 (464-604) - MCM2-7 interaction is sufficient to promote MCM2-7 recruitment. To test this idea we used two assays to probe the Cdt1 interaction with MCM2-7. The first assay, which employs an MCM2-7 IP (Figure 2B), can detect stable interactions between MCM2-7 and the Cdt1 truncation mutants. The second assay monitors how the MCM2-7 ATPase motor reacts to protein interactions (Figure 2C). It has been shown that AAA+ ATPases, like the Mcm proteins, respond with altered ATPase rate to subtle structural changes, e.g. interaction with a protein factor (Hanson and Whiteheart, 2005). Using the IP assay we observed a very stable interaction between MCM2-7 and Cdt1 (Figure 2B, lane 6). The ATPase assay showed that the binding of Cdt1

to MCM2-7 led to a reduction of 35 % in the ATPase rate (Figure 2C). This change in ATPase activity is very small in comparison to the 300 fold increase of MCM2-7 ATPase activity upon Cdc45 and GINS binding during S-phase (Ilves et al., 2010) and could indicate a slight reorganization of the MCM2-7 complex upon binding to Cdt1. Our data showed that Cdt1 (306-604) was quite efficient in binding to MCM2-7 (Figure 2B, lane 10) and in down regulating the MCM2-7 ATPase activity (about 18 %) (Figure 2C). Meanwhile, Cdt1 (464-604) did not bind MCM2-7 (Figure 2B, lane 12), but reduced the MCM2-7 ATPase activity reproducibly by 10 % (p-value=0.007), suggesting a transient MCM2-7 - Cdt1 (464-604) interaction. Finally, Cdt1 (1-312) did bind weakly to MCM2-7, but failed to alter the MCM2-7 ATPase activity. This analysis indicates that the C-terminal part of Cdt1 contains a domain that can interact with MCM2-7 and promotes OCM formation. However, the Cdt1 N-terminus is essential to produce an OCM complex that is functional for the loading of salt stable MCM2-7 double-hexamers.

The Mcm6 C-terminus contains an inhibitory domain that blocks MCM2-7 association with ORC/Cdc6

Our data indicate that Cdt1 induces a structural change in MCM2-7 which may allow the establishment of direct ORC/Cdc6 interactions with MCM2-7. It has been shown that Cdt1 interacts with MCM2-7 via a C-terminal domain in Mcm6 (Figure 3A) (Zhang et al., 2010). One possibility is that the Mcm6 C-terminal domain inhibits the binding of MCM2-7 to ORC/Cdc6 and that the Cdt1-Mcm6 interaction alleviates this inhibition. To test this idea we created MCM2-7-ΔC6, which is missing the Mcm6 C-terminal extension (amino acids 839-1017), and tested this mutant in the pre-RC assay (Figure 3B). Remarkably, we observed a Cdt1 independent interaction of MCM2-7-ΔC6 with ORC/Cdc6 in the presence of ATPγS (Figure 3B, lane 15). In the presence of ATP we observed the same ORC/Cdc6 interactions with MCM2-7-ΔC6 (Figure 3B, lane 16), but no helicase loading (Figure 3B, lane 17). Importantly, MCM2-7-ΔC6 recruitment was still dependent on ORC and Cdc6 (Figure S2A). This result shows that a deletion of the Mcm6 C-terminus bypasses the Mcm6-Cdt1 interaction requirement during OCM formation. Interestingly, MCM2-7-ΔC6 was still able to interact with Cdt1 (Figure 3C, lane 5) and to form an ORC/Cdc6/Cdt1/MCM2-7-ΔC6 complex in the presence of ATPγS (Figure 3B, lane 12). This suggests that MCM2-7 has more than one Cdt1 binding domain, one in the Mcm6 C-terminus (Liu et al., 2012; Zhang et al., 2010) and at least one more in a different region of MCM2-7. Furthermore, in the presence of ATP and MCM2-7-ΔC6, Cdt1 was not released from the pre-RC complex (Figure 3B, lane 13), indicating that the Mcm6 C-terminus is also important to facilitate Cdt1 release. In addition, MCM2-7-ΔC6 is not functional in salt stable MCM2-7 double-hexamer formation (Figure 3B, lane 14 and 17) and at the same time causes dominant lethality upon overexpression

(Figure 3D). Furthermore, the addition of *wt* MCM2-7 to MCM2-7-ΔC6 did not facilitate MCM2-7-ΔC6 loading (Figure S2B, lane 11), indicating that both MCM2-7 hexamers, which are loaded into a double-hexamer, share the same requirements for a functional Mcm6 C-terminus. In summary, our results indicate that Cdt1 alleviates an auto-inhibitory activity in Mcm6 to facilitate a stable ORC/Cdc6-MCM2-7 interaction and MCM2-7 loading.

A specific Mcm6-Cdt1 interaction is required to allow recruitment of MCM2-7 to ORC/Cdc6

Our data show that a structural change in the Mcm6 C-terminus is important to establish direct ORC/Cdc6-MCM2-7 interactions. Structural work has established 5 specific amino acids in Mcm6 (E945, D947, L951, E953 and Y954) and 3 amino acids in Cdt1 (R486, L487 and R490) to be important for functional Mcm6-Cdt1 interaction (Liu et al., 2012). *In vivo* analysis of an Mcm6-5A mutant (E945A, D947A, L951A, E953A and Y954A) and Cdt1-5A mutant (R486A, R488A, R490A, R501A and K512A) established that the Mcm6-Cdt1 interaction is essential for the nuclear import of Cdt1/MCM2-7 (Wu et al., 2012) and in consequence Cdt1-5A and Cdt1-3A (R486A, L487A and R490A) both block chromatin binding of MCM2-7 (Liu et al., 2012). However, it is not known if these mutants can function in pre-RC assembly. In order to clarify this we analyzed the ability of the MCM2-7-(6-5A) and Cdt1-3A mutants to form MCM2-7-Cdt1 complexes and to participate in pre-RC formation (Figure 4). MCM2-7-(6-5A) was able to interact with Cdt1 (Figure 4A, lane 6), which is consistent with the finding that MCM2-7 has more than one Cdt1 binding site. As observed with MCM2-7, MCM2-7-(6-5A) interacted weakly with Cdt1 (1-312) (Figure 4A, lane 8). Like MCM2-7, we found that MCM2-7-(6-5A) failed to interact with Cdt1 (464-604) (Figure 4A, lane 12). While MCM2-7 interacted with Cdt1 (306-604) efficiently (Figure 2B, lane 10), we observed a slightly reduced interaction with MCM2-7-(6-5A) (Figure 4A, lane 10 and Figure S3A), indicating that the mutation in Mcm6 has only a small influence on the interaction. We obtained similar results with MCM2-7-Δ6, which is also missing the Mcm6 binding site for Cdt1 (Figure S3B). Next we analyzed the functionality of MCM2-7-(6-5A) in the presence and absence of Cdt1 in the pre-RC assay. We observed that MCM2-7-(6-5A) failed to be recruited to ORC/Cdc6 in the presence of Cdt1 (Figure 4B, lanes 12-14) or absence of Cdt1 (Figure 4B, lanes 15-17). We conclude that MCM2-7-(6-5A) can interact with Cdt1, but that this interaction is not productive and in consequence Cdt1/MCM2-7-(6-5A) cannot be recruited to ORC/Cdc6. In contrast to MCM2-7-(6-5A), MCM2-7-ΔC6 is missing the inhibitory Mcm6 C-terminal domain completely, and therefore can bind directly to ORC/Cdc6.

In addition we were interested in understanding if Cdt1-3A is able to interact with MCM2-7, MCM2-7-(6-5A) or MCM2-7-ΔC6 and if Cdt1-3A is able to form a pre-RC complex.

We found that Cdt1-3A and Cdt1 interacted in a very similar manner with MCM2-7, MCM2-7-(6-5A) and MCM2-7-ΔC6 (Figure 4C and D). However, in the pre-RC assay, Cdt1-3A failed to promote MCM2-7 recruitment to ORC/Cdc6 and consequently did not support the loading of a salt stable MCM2-7 complex onto DNA (Figure 4E, lanes 13-15). In contrast, Cdt1-3A formed a complex with MCM2-7-ΔC6 and ORC/Cdc6 in ATPγS (Figure 4E, lane 16), but Cdt1-3A was not released from DNA in the presence of ATP nor was MCM2-7-ΔC6 loaded as a salt stable complex on DNA (Figure 4E, lane 17-18). We conclude that a specific Cdt1-Mcm6 interaction, involving 5 amino acids of Mcm6 (E945, D947, L951, E953 and Y954) and three amino acids of Cdt1 (R486, L487 and R490), is required to alleviate the inhibitory effect of the Mcm6 C-terminus.

Cdc6 and Orc1 produce pre-RC induced ATP-hydrolysis

We showed that ORC/Cdc6 recruits a Cdt1/MCM2-7 complex to origin DNA and once the initial ORC/Cdc6-Cdt1/MCM2-7 complex is formed, Cdt1 is released from DNA in an ATP-hydrolysis dependent manner. To address the role of ATP-hydrolysis during OCM formation we established a pre-RC ATP-hydrolysis assay (Figure 5A) employing an origin DNA fragment of 150 base pairs, which contains all essential motifs for MCM2-7 loading *in vivo* (Marahrens and Stillman, 1992). We found that the rate of ATP-hydrolysis by ORC/Cdc6 is very low (Randell et al., 2006; Speck and Stillman, 2007), while MCM2-7 had a significantly higher ATPase activity (Schwacha and Bell, 2001), and the MCM2-7 ATPase-activity was reduced upon Cdt1 binding (Figure 5A, compare lane 5 and 6). Importantly, we saw that ORC/Cdc6 addition to Cdt1/MCM2-7 increased the ATP hydrolysis rate significantly (Figure 5A, lane 7), which is consistent with induction of ATP-hydrolysis during pre-RC formation. We define the 'pre-RC induced ATPase activity' as the ATPase activity of the full pre-RC reaction with the ATPase activity of the individual components (ORC/Cdc6 + Cdt1/MCM2-7) subtracted. The individual ATPase activities of ORC/Cdc6 and Cdt1/MCM2-7 are superimposed on the graph in Figure 5A (lane 8) whereas the pre-RC induced ATPase activity is shown in red (Figure 5A, lane 9). On the other hand, ORC/Cdc6 and MCM2-7, in the absence of Cdt1, promoted only weak induction of ATP-hydrolysis (Figure 5B, lane 5) (ORC/Cdc6 MCM2-7 induced ATPase activity = ORC/Cdc6 MCM2-7 ATPase activity – (ORC/Cdc6 + MCM2-7 ATPase activity)). Among the pre-RC proteins Cdc6, Orc1 and MCM2-7 are the active ATPases (Masai et al., 2010). However, only Cdc6 mediated ATP-hydrolysis was shown to be important for Cdt1 release and MCM2-7 loading (Evrin et al., 2013; Perkins and Diffley, 1998; Randell et al., 2006). An Orc1 ATPase mutant with a mutation in an Orc4 arginine finger (ORC-4R) supports a single round of MCM2-7 loading (Bowers et al., 2004; Evrin et al., 2013). Another Orc1 ATPase mutant with a mutation in the Walker B ATPase motif of Orc1 (ORC-d1 – Orc1 D569Y) has not yet been analyzed for

MCM2-7 loading defects *in vitro*, but blocks MCM2-7 loading upon overexpression (Klemm and Bell, 2001). Finally, Xenopus MCM2-7 ATPase mutants are competent for MCM2-7 loading, suggesting that MCM2-7 ATPase is dispensable for pre-RC formation (Ying and Gautier, 2005). In budding yeast the role of MCM2-7 ATPase activity during pre-RC formation has not been analyzed, but several MCM2-7 ATP-hydrolysis mutants have been identified (Bochman et al., 2008; Schwacha and Bell, 2001). One of these mutants carrying a mutation in an arginine finger (R542A) of Mcm3 (3RA) reduces the ATPase activity of the MCM2-7 complex considerably (Bochman et al., 2008). To identify which ATPase is responsible for OCM formation, we investigated the influence of Cdc6 N263A (sensor-1) (Evrin et al., 2013; Schepers and Diffley, 2001; Takahashi et al., 2002), Cdc6 E224G (Walker B) (Perkins and Diffley, 1998; Randell et al., 2006), ORC-d1 (Klemm and Bell, 2001), ORC-4R (Bowers et al., 2004) and MCM2-7-3RA (Bochman et al., 2008) ATPase mutants on pre-RC induced ATPase activity. Initially we verified the ATPase defects of these proteins (Figure S4). Then we analyzed the mutants in the pre-RC induced ATPase assay. We found that Cdc6 N263A led to strong reduction of pre-RC induced ATPase activity (Figure 5C), but this reduction was less pronounced with Cdc6 E224G (Figure S5A). Interestingly, ORC-d1 also led to a reduction of pre-RC induced ATPase activity (Figure 5C). On the other hand, ORC-4R had no influence on pre-RC induced ATPase activity (Figure S5B), suggesting that the arginine finger of Orc4 is not required to promote ATP hydrolysis during OCM formation. Consistent with previous observations in Xenopus (Ying and Gautier, 2005), an MCM2-7 ATPase mutant had no influence on pre-RC induced ATPase activity (Figure 5D). Finally, Cdc6 N263A and ORC-d1 in combination suppressed pre-RC induced ATPase activity almost completely (Figure 5C).

To examine if ORC-d1 and Cdc6 N263A also have an influence on Cdt1 release, we analyzed these mutants in the pre-RC assay. We found that the mutants individually reduced Cdt1 release and MCM2-7 loading (Figure 5E and S5C), and the mutant combination blocked Cdt1 release and MCM2-7 loading completely (Figure 5E, lanes 16-18). On the other hand, ORC-4R (Figure S5D) and MCM2-7-3RA (Figure S5E) were found to be competent for Cdt1 release and MCM2-7 loading. In summary, these results highlight that both Cdc6 and Orc1 contribute to pre-RC induced ATPase activity and promote Cdt1 release.

The Cdt1/MCM2-7 complex is required to induce ATP-hydrolysis during pre-RC formation

Next we were interested in finding factors that are required to activate ORC/Cdc6 dependent ATP hydrolysis during OCM formation. As expected, Cdt1-3A or MCM2-7-(6-5A), which do

not support pre-RC formation (Figure 4), led to significantly reduced pre-RC induced ATPase activity (Figure 5F). However with MCM2-7-ΔC6 or MCM2-7-ΔC6/Cdt1, which support complex formation, we did not observed any pre-RC induced ATPase activity either (Figure 5G), indicating that a functional Mcm6-Cdt1 interaction is crucial to induce ATP-hydrolysis. Consistent with this, Cdt1 addition to ORC/Cdc6 was not able to induce ATP hydrolysis (Figure 5H). To better understand how Cdt1 is promoting ATP hydrolysis during pre-RC formation we tested several Cdt1 truncation mutants in the ATPase assay (Figure 5I). Cdt1 (1-312) failed to induce pre-RC ATP-hydrolysis, which was expected since Cdt1 (1-312) did not support OCM formation. Furthermore, we observed robust ATPase activity with Cdt1 (306-604), consistent with the ATP-hydrolysis dependent Cdt1 release we observed earlier (Figure 2A, lane 14 and 15). Still, Cdt1 (464-604), which allows MCM2-7 recruitment to ORC/Cdc6, but is released from DNA in an ATP-hydrolysis independent way (Figure 2A) did not promote ATPase induction. We conclude that a Cdt1-MCM2-7 complex in which Mcm6 interacts with the Cdt1 C-terminus is required to induce ATP-hydrolysis during OCM formation.

Orc6 is essential for OCM establishment

It was found that Orc6 interacts with Cdt1 and that an Orc1-5 complex does not support MCM2-7 loading (Chen et al., 2007), suggesting that Orc6 could be involved in OCM formation. To test this idea we analyzed an Orc1-5 complex in the context of the pre-RC assay (Figure 5J) and the ATPase assay (Figure 5K). Orc1-5 is known to bind DNA and to interact with Cdc6 (Chen et al., 2007; Chen et al., 2008). Consistent with that we observed an Orc1-5-Cdc6 interaction on DNA. This complex was able to recruit Cdt1/MCM2-7 in the presence of ATPγS (Figure 5J, lane 4) and this recruitment was Cdc6 dependent (Figure 5J, lane 7). However, in the presence of ATP we did not observe MCM2-7 binding (Figure 5J, lane 5), suggesting that ATP hydrolysis during Orc1-5 induced OCM formation leads to simultaneous Cdt1 and MCM2-7 release. To test this hypothesis we measured the pre-RC induced ATP-hydrolysis with Orc1-5 and ORC (Figure 5K). Interestingly, Orc1-5 led to very strong ATP-hydrolysis. This suggests that fast and repetitive assembly and ATPase dependent disassembly of the complex leads to the high ATP-hydrolysis rate. These results show that Orc6 is not necessary for the initial recruitment of Cdt1/MCM2-7 by ORC/Cdc6, but for successful OCM establishment.

The OCM is a true intermediate of the pre-RC assembly process

Our work established that an OCM complex is formed in an ATP-hydrolysis dependent manner, but it is not yet clear if MCM2-7, as part of the OCM, can also integrate into the MCM2-7 double-hexamer. To test this idea, we used a two-step pre-RC assay with untagged

and MBP-tagged MCM2-7 (Figure 6A). Untagged MCM2-7 was used during the initial OCM formation, which lasted for 2.5 minutes. Then, unbound proteins were washed away and replaced with buffer, Cdt1, MBP-tagged MCM2-7, Cdt1/MBP-tagged MCM2-7 (Figure 6B) or Cdt1 (306-604)/MBP-MCM2-7 (Figure S6). After a second incubation of 15 minutes, the reactions were washed with low-salt or high-salt buffer and DNA bound proteins were analyzed. Consistent with previous results we found that the OCM complex formed during the first incubation was not high-salt stable (Figure 6B, lane 1 and 2). The addition of buffer, Cdt1, MBP-MCM2-7 (Figure 6B) or Cdt1 (306-604)/MBP-MCM2-7 (Figure S6) in the second incubation did not promote loading of a salt stable MCM2-7 double-hexamer. However, the addition of Cdt1/MBP-tagged MCM2-7 promoted the loading of a salt stable MCM2-7 double-hexamer (Figure 6B, lane 10). Subsequently, we repeated the experiment to identify if untagged MCM2-7 did integrate with tagged MCM2-7 (MBP-Mcm3) into the double-hexamer (Figure 6C). In brief, the OCM was formed, and then either tagged or untagged MCM2-7 was added, followed by a high-salt wash. The resulting complex was released from DNA with DNase I, immunoprecipitated with an anti-MBP antibody and probed for the presence of tagged and untagged Mcm3 by western blot (Figure 6D). Consistent with the OCM representing a platform for double-hexamer formation we observed that untagged Mcm3 co-precipitated with tagged MCM2-7 (Figure 6D, lane 8). Importantly, we did not observe untagged MCM2-7 in the MBP-IP missing MBP-tagged MCM2-7 (Figure 6D, lane 7). We conclude that the OCM complex is a true intermediate of the pre-RC formation reaction and it represents a platform for MCM2-7 double-hexamer formation.

CDK regulates OCM establishment

MCM2-7 loading onto chromatin is tightly regulated by CDK (Chen and Bell, 2011; Nguyen et al., 2001). Using an extract based system it was shown that phosphorylation of ORC reduces the association of Cdt1/MCM2-7 with ORC/Cdc6 and also hinders MCM2-7 loading (Chen and Bell, 2011). We wondered if OCM formation is regulated in a CDK-dependent manner. To address this question we bound ORC to DNA and then phosphorylated ORC with a range of kinase concentrations. Afterwards CDK was washed away and Cdc6, Cdt1 and MCM2-7 were added in the presence of ATP or ATPγS (Figure 7A). With ATPγS we observed efficient Cdt1/MCM2-7 association with ORC/Cdc6 both in the presence and absence of CDK (Figure 7, lanes 6, 9, 12 and 15), indicating that initial complex formation is not impaired by CDK. However, OCM formation was severely reduced in the presence of CDK phosphorylated ORC (Figure 7, lanes 10, 13 and 16), showing that the establishment of an OCM is blocked by this kinase. Furthermore, loading of salt stable MCM2-7 was completely blocked in the presence of CDK phosphorylated ORC (Figure 7, lanes 11, 14 and 17). CDK inhibition of OCM formation was observed over a large range of CDK

concentrations. This work establishes that OCM establishment is a target for negative regulation, suggesting that OCM formation is a critical step in MCM2-7 loading.

DISCUSSION

In all eukaryotes Cdt1 is essential for the binding of MCM2-7 to ORC/Cdc6 at replication origins. However, after initial complex formation, Cdt1 is rapidly released from DNA in an ATP-hydrolysis dependent manner (Randell et al., 2006) (Figure 1). Our data demonstrate now that ATP-hydrolysis dependent Cdt1 release generates the OCM complex. Since ORC/Cdc6 and MCM2-7 do not form a complex on DNA, MCM2-7 must undergo a structural change to be stably integrated in the OCM complex. Here, we provide two lines of evidence for such a structural change: 1.) MCM2-7 does not bind to ORC/Cdc6, but MCM2-7-ΔC6, missing the Cdt1 binding domain of Mcm6, binds to ORC/Cdc6. This indicates that a structural change in Mcm6 is sufficient to allow direct interactions between ORC/Cdc6 and MCM2-7. 2.) MCM2-7-(6-5A), which has 5 mutations in its Cdt1 binding motif in Mcm6 (Liu et al., 2012), is unable to promote helicase recruitment to ORC/Cdc6, showing that a specific Cdt1-Mcm6 interaction is required to induce a structural change in MCM2-7. Based on these observations, we conclude that Cdt1 provokes a reorganization of the Mcm6 C-terminus during pre-RC formation, which in turn alleviates an auto-inhibitory activity of this domain and allows the binding of MCM2-7 to ORC/Cdc6. In the absence of Cdt1 the inhibitory function of Mcm6 prevents the formation of an ORC/Cdc6/MCM2-7 complex. Indeed, this seems important, as MCM2-7-ΔC6, which is missing the inhibitory domain, can block pre-RC formation even in the presence of MCM2-7.

Here we found that the addition of Cdt1/MCM2-7 to ORC/Cdc6 leads to a strong induction of ATP hydrolysis. In contrast, the addition of MCM2-7 towards ORC/Cdc6 only promotes weak ATP-hydrolysis. Furthermore, Cdt1 and Mcm6 mutants that affect the Cdt1-Mcm6 interaction and interfere with pre-RC formation also significantly weaken ATP-hydrolysis. This shows that pre-RC assembly and ATP-hydrolysis are closely linked to each other. Still, the addition of MCM2-7-ΔC6 to ORC/Cdc6 allows Cdt1-independent complex formation, but does not induce ATPase activity. Furthermore, Cdt1 (464-604) promotes MCM2-7 recruitment to ORC/Cdc6, but is unable to sustain the interaction with ORC/Cdc6/MCM2-7 and does not promote pre-RC induced ATP-hydrolysis. This shows that MCM2-7-ΔC6 or MCM2-7 interaction with ORC/Cdc6 is not sufficient to induce ATP hydrolysis. Moreover, Cdt1 addition to ORC/Cdc6 does not activate ATPase activity either. Even an ORC/Cdc6/Cdt1/MCM2-7-ΔC6 complex fails to induce ATP hydrolysis, indicating

that a functional Cdt1-Mcm6 interaction is required to promote ATP-hydrolysis during pre-RC formation.

A Cdc6 sensor-1 ATPase mutant (Cdc6 N263A) and the Walker B ATPase mutant (Cdc6 E224G) cause mild growth defects *in vivo* (Perkins and Diffley, 1998; Schepers and Diffley, 2001; Takahashi et al., 2002), display a weak ATPase activity in complex with ORC and origin DNA (Randell et al., 2006; Speck and Stillman, 2007), and result in reduced MCM2-7 loading *in vitro* (Randell et al., 2006). On the other hand, an arginine finger mutant in Orc4 (ORC-4R) has been show to block Orc1 ATPase activity (Bowers et al., 2004). Yet, this mutant allowed a single round of MCM2-7 loading and only blocks repetitive MCM2-7 loading. Based on this work it was suggested that Cdc6 is the main ATPase involved in pre-RC assembly, while Orc1 facilitates repetitive MCM2-7 loading. Here, we systematically analyzed the influence of Cdc6, Orc1 and MCM2-7 proteins on pre-RC induced ATPase activity (Figure 5 and S5). Our analysis revealed that Cdc6 ATPase and surprisingly also Orc1 ATPase make up the pre-RC induced ATPase activity. On the other hand, an ORC-4R ATPase mutant or a Mcm3 ATPase mutant (MCM2-7-3RA) had no influence on pre-RC induced ATP-hydrolysis, Cdt1 release or MCM2-7 loading. Interestingly, ORC-d1 or Cdc6 N263A on their own only reduced pre-RC induced ATP-hydrolysis and MCM2-7 loading, while the combination of the two blocked Cdt1 release and MCM2-7 loading completely. Potentially both ATPases generate a similar structural change during OCM formation and in consequence both Orc1 and Cdc6 can facilitate Cdt1 release and weak MCM2-7 loading. However, if only one ATPase is active, the reaction becomes less efficient – like an engine firing only on two out of four cylinders. This arrangement could be considered a redundant system, which facilities MCM2-7 loading even if one ATPase should fail.

We found that Orc1-5 can recruit Cdc6 and Cdt1/MCM2-7 to DNA in the presence of ATPγS, however, the complex fell apart upon ATP-hydrolysis, indicating that OCM establishment is not possible in the absence of Orc6. Likewise, we found that ORC, when it is phosphorylated by CDK, can recruit Cdc6 and Cdt1/MCM2-7 efficiently in the presence of ATPγS, but fails to establish an OCM complex with ATP. Interestingly, it is known that CDK phosphorylates Orc6 and that this phosphorylation blocks an interaction with Cdt1 (Chen and Bell, 2011). Therefore, the experiments using Orc1-5 or phosphorylated ORC identify an important role for Orc6 in OCM establishment. Similar results were reported very recently by another group (Frigola et al., 2013), further confirming that Cdt1-MCM2-7 recruitment by ORC/Cdc6 does not depend on Orc6. However, our work showed that Cdt1 is essential for MCM2-7 recruitment and Frigola et al reported that a point mutation in the Mcm3 C-terminus blocks Cdt1-MCM2-7 binding to ORC/Cdc6 (Frigola et al., 2013). In the same study Mcm3 was shown to facilitate ORC/Cdc6 ATP-hydrolysis, which promotes MCM2-7 release in the

absence of Cdt1. This result fits with our observation that MCM2-7 addition to ORC/Cdc6 promotes weak ATP-hydrolysis (Figure 5B), and MCM2-7 release (Figure 1I, lane 5). In summary, Mcm3 and the Mcm6/Cdt1 interaction enable Cdt1/MCM2-7 recruitment towards ORC/Cdc6.

A model describing pre-RC formation in the context of the OCM complex

Based on the presented data, summarized in part in Figure 7B, we propose a model describing the pre-RC formation process (Figure 7C). ORC is chromatin bound throughout the cell cycle (1) (Weinreich et al., 1999). In late M phase Cdc6 binds ORC to form an ORC/Cdc6 complex on origin DNA (2) (Cocker et al., 1996; Weinreich et al., 1999). Meanwhile, Cdt1 and MCM2-7 form a complex in the cytoplasm, which is required for their nuclear import (Tanaka and Diffley, 2002). Our work has revealed that Cdt1 has at least two MCM2-7 interaction sites, one in the N-terminus and one in the C-terminus. The C-terminal MCM2-7 binding site changes the structure of MCM2-7, alleviating an inhibitory function in the Mcm6 C-terminus, which in turn allows the recruitment of the first Cdt1/MCM2-7 heptamer by ORC/Cdc6 to origin DNA (3). Once the ORC/Cdc6/Cdt1/MCM2-7 complex is formed, Orc1 and Cdc6 ATP-hydrolysis lead to rapid Cdt1 release and establishment of an OCM complex (4). Interestingly, we found that ATP-hydrolysis is only triggered by an intact Cdt1/MCM2-7 complex, but not by MCM2-7 or Cdt1 individually. Furthermore, successful OCM formation is inhibited by CDK phosphorylation of ORC. Importantly, we established that an OCM complex is the platform for MCM2-7 double-hexamer formation (5). While the OCM is formed within seconds, MCM2-7 double-hexamer formation takes several minutes. The mechanism of the OCM to double-hexamer transition is still unknown, but we found that Cdt1 is required for the recruitment of the first and second MCM2-7 hexamer. MCM2-7 double-hexamer formation could either involve one OCM, which then could recruit directly a second MCM2-7 hexamer. Alternatively, one OCM could recruit a second OCM and consequently both complexes could fuse to form an MCM2-7 double-hexamer. It will be exciting to discover the mechanism involved in the OCM - double-hexamer transition.

EXPERIMENTAL PROCEDURES

Pre-RC assay

The pre-RC assay was performed as described with minor modifications (Evrin et al., 2009). Here a one-step reaction was used. 40 nM ORC, 80 nM Cdc6, 40 nM Cdt1, 40 nM MCM2-7 (*wt* or mutant) in buffer A [50 mM Hepes-KOH pH 7.5, 100 mM KGlu, 10 mM MgAc, 50 µM ZnAc, 3 mM ATP or ATPγS, 5 mM DTT, 0.1% Triton, and 5% Glycerol] were added to 6 nM linear pUC19-ARS1 DNA coupled to magnetic beads for 15 min at 24 °C. In the case of MCM2-7-3RA 300 mM KGlu was used during the loading assay to stabilize the complex. Beads were washed 3 times (time course experiment 2 washes) with buffer A plus 1 mM EDTA or buffer B [50 mM Hepes-KOH pH 7.5, 1 mM EDTA, 500 mM NaCl, 10% Glycerol, 0.1% Triton X-100, and 5 mM DTT] before digestion with 1 U of DNase I in buffer A plus 5 mM $CaCl_2$ for 2 min (time course experiment 1 minute) at 24 °C. The samples were separated by SDS-PAGE and analyzed by silver staining or by western blot.

ACKNOWLEDGMENTS

We would like to thank Bruce Stillman for Mcm2, Mcm3, Cdt1 (CS1411/CS1415) and Orc3 (SB3) antibodies, Julian Blow, Aloys Schepers and Luis Aragon for comments on the manuscript and MRC for funding.

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FIGURE LEGENDS

Figure 1. ATPase dependent formation of a stable OCM complex

(**A**) Pre-RC complexes were formed for 0.5 to 15 minutes, washed with low salt buffer (i and ii) or high salt buffer (iii), released from DNA with DNase I and then analyzed by western blot. The asterisk indicates the area of the Cdt1 signal. (**B**-**E**) Complexes were formed using ATPγS (B and C) or ATP (D and E), incubated for the indicated time points, washed, released from DNA with DNase I and then immunoprecipitated with an anti-Mcm2 antibody or with plain protein G beads (mock). The dilution series represents X % of the proteins added into the pre-RC assay. (**F**) Only one MCM2-7 molecule is present in the OCM. MBP-tagged MCM2-7, MCM2-7 or an equimolar mixture of both were used in pre-RC assays. Reactions were assembled on DNA for four minutes, washed, eluted with DNase I and then immunoprecipitated with an anti-MBP antibody. The hash symbol marks a nonspecific band originating from the anti-MBP antibody. (**G**) Only one ORC molecule is present in the OCM. These reactions were prepared as described in (F), using MBP-tagged ORC. (**H**) Only one Cdc6 molecule is present in the OCM. These reactions were prepared as described in (F), using MBP-tagged Cdc6. (**I**) Pre-RC reactions were assembled in the presence of ATPγS or ATP, washed with low [L] or high salt [H] and analyzed by silver staining. The smallest subunit of the Orc1-6 complex stains only weakly during silver staining. (**J**) Pre-RC reactions were assembled for 2.5 minutes, washed and incubated further for 0 up to 20 minutes. Subsequently, the DNA bound proteins (bound fraction) and the proteins dissociated from DNA into the supernatant (supernatant fraction) were analyzed by silver staining. See also Figure S1.

Figure 2. Identification of Cdt1 domains involved in OCM establishment

(**A**) Analysis of pre-RC assembly in the presence of Cdt1 or Cdt1 mutants. (**B**) MCM2-7-Cdt1 interaction analysis employed IgG control beads or MCM2-7 (HA-Mcm3) coupled to anti-HA beads. A 14% input is shown along with 100% of the IP. (**C**) Analysis of Cdt1 and MCM2-7 ATPase activities in the absence of DNA. Data are represented as mean +/- SD.

Figure 3. Cdt1 alleviates an inhibitory function in the Mcm6 C-terminus to promote pre-RC formation.

(**A**) The organization of *Saccharomyces cerevisiae* Mcm6 and *Methanothermobacter thermautotrophicus* Mcm is shown. The Mcm proteins contain an N-terminal domain, an ATP binding AAA+ domain and a C-terminal extension. The relative position of the conserved

motifs including Zinc-finger, N-C linker, Walker A [WA], Walker B [WB], arginine finger [RF] and C-terminal extension [c-t] is indicated. The asterisk marks the position of the Cdt1 binding motif in Mcm6. (**B**) Analysis of pre-RC assembly in the presence and absence of Cdt1 using MCM2-7 or MCM2-7-ΔC6. (**C**) Cdt1/MCM2-7 interaction analysis using MBP and MBP-Cdt1 coupled to anti-MBP beads. A 2% input is shown along with 100% of the IP. (**D**) An MCM2-7-ΔC6 mutant was overexpressed (galactose) from 2-micron plasmids to test for a dominant lethal effect. In plates containing glucose the expression is repressed. Serial dilutions of cells ranging from $2x10^6$ to $3.2x10^3$ were spotted on selective plates. See also Figure S2.

Figure 4. A specific Cdt1-Mcm6 interaction is required to alleviate an inhibitory function in Mcm6

(**A**) IgG control beads or anti-HA beads coupled with MCM2-7-(6-5A) were incubated with purified Cdt1 or Cdt1 mutants, washed and analyzed by western blotting. A 14% input is shown along with 100% of the IP. (**B**) Analysis of pre-RC assembly in the presence or absence of Cdt1 using MCM2-7 or MCM2-7-(6-5A). Interaction analysis of MCM2-7, MCM2-7-(6-5A) (**C**) and ΔC6-MCM2-7 (**D**) with Cdt1 and Cdt1-3A. Anti-IgG control beads or anti-HA beads coupled with MCM2-7 (*wt* or mutants) were incubated with purified Cdt1 or Cdt1-3A, washed and analyzed by western blot. (**E**) Analysis of pre-RC assembly in the presence of MCM2-7 or MCM2-7-ΔC6 using Cdt1 or Cdt1-3A. See also Figure S3.

Figure 5. Cdt1 and MCM2-7, are both required to induce ATP-hydrolysis during pre-RC formation

The ATP hydrolysis rates were determined for the indicated proteins in the presence of ARS1 origin DNA. (**A**) Analysis of ATPase activities during pre-RC formation. In the presence of ORC, Cdc6, Cdt1 and MCM2-7 a strong ATPase activity is induced. Lane 8 shows the ORC/Cdc6 ATPase activity in dark grey, the Cdt1/MCM2-7 ATPase activity in light grey and the pre-RC induced ATPase activity in red. Lane 9 shows only the pre-RC induced ATPase activity. (**B**) Analysis of the ATPase activity with ORC/Cdc6 and MCM2-7. Lane 4 shows the ORC/Cdc6 ATPase activity in dark grey, the MCM2-7 ATPase in light grey and the ORC/Cdc6 MCM2-7 induced ATPase activity in red. Lane 5 shows only the ORC/Cdc6 and MCM2-7 induced ATPase activity. (**C**) Analysis of the pre-RC induced ATPase activity with Cdc6 N263A and ORC-d1. (**D**) Analysis of the pre-RC induced ATPase activity with MCM2-7-3RA. (**E**) Analysis of pre-RC assembly using ORC-d1 and Cdc6 N263A ATPase mutants. The reactions were assembled, washed with low salt [L] or high salt [H] and analyzed by silver staining. Only the low salt reactions with ATP were incubated for 10 minutes, to capture Cdt1 prior to ATP-hydrolysis dependent release. (**F**) Pre-RC induced

ATPase activity in the presence of Cdt1/MCM2-7, Cdt1-3A/MCM2-7 and Cdt1/MCM2-7-(6-5A). (**G**) ORC/Cdc6/MCM2-7 and pre-RC induced ATPase activity with MCM2-7-ΔC6. (**H**) Cdt1 addition to ORC/Cdc6 at equimolar concentration or 3 and 10 fold molar excess has no influence on ATPase activity. (**I**) Analysis of the pre-RC induced ATPase activity with Cdt1 and Cdt1 mutants. (**J**) Analysis of pre-RC assembly using ORC and Orc1-5. (**K**) Analysis of the pre-RC induced ATPase activity with ORC and Orc1-5. All ATPase data are represented as mean +/- SD. See also Figure S4 and S5.

Figure 6. The OCM represents a platform for MCM2-7 double-hexamer assembly

(**A**) Experimental outline for B. A two-step assay for pre-RC formation was used here, employing untagged MCM2-7 (first step) and MBP-tagged MCM2-7 (second step). An OCM was formed in the first step. Then, in the second step, the complex was washed and either buffer, Cdt1, MBP-MCM2-7 or Cdt1-MBP-MCM2-7 (MBP-Mcm7) were added back. Afterwards the samples were washed with low salt or high salt and analyzed by silver staining. (**B**) The OCM is a precursor of the MCM2-7 double-hexamer. Lanes 1 and 2 show a low and high salt wash of the OCM formed in the first step. Lanes 3-10 were performed as outlined in A. (**C**) Experimental outline for D. An OCM complex was formed, washed and incubated with Cdt1/MBP-MCM2-7 (MBP-Mcm3) or Cdt1/MCM2-7 (negative control). After a second incubation the samples were washed with high salt, released from DNA, added to anti-MBP beads and analyzed by western blot with an anti-Mcm3 antibody. (**D**) MCM2-7, as part of the OCM, can form a mixed tagged/untagged MCM2-7 double-hexamer. A load of purified proteins is shown, along with 20% IP input, 20% IP flow through (FT) and 100% of the IP. See also Figure S6.

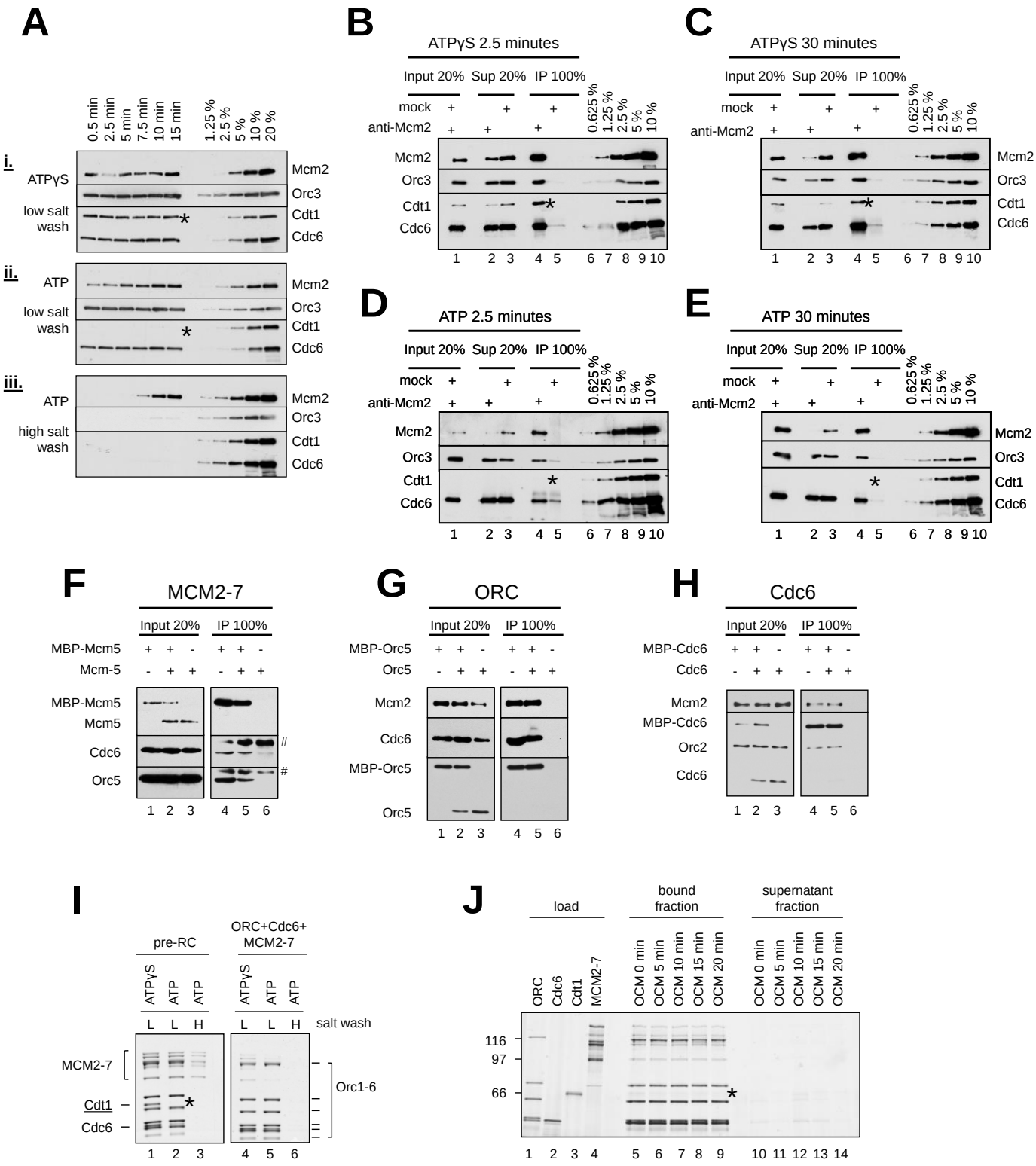
Figure 7. CDK regulates OCM establishment during pre-RC formation

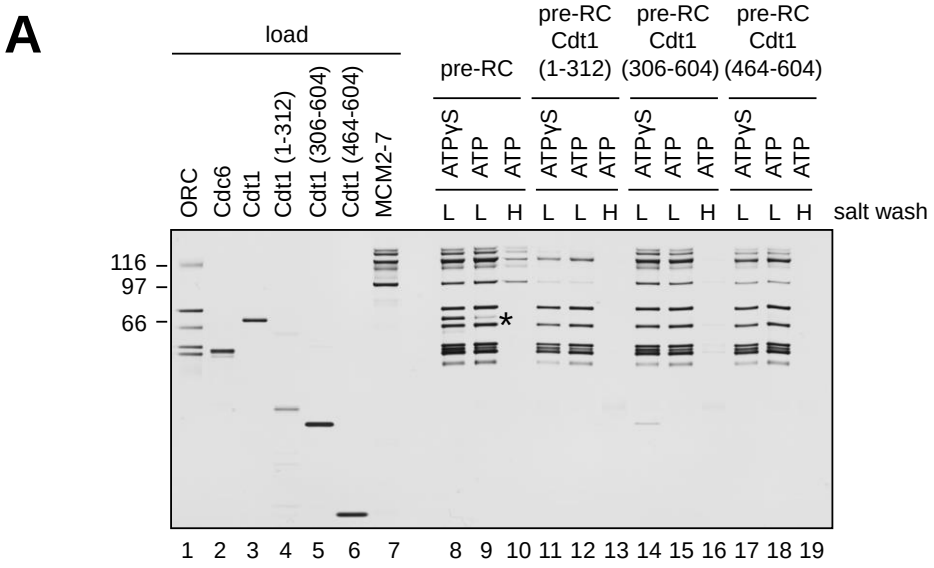
(**A**) Analysis of pre-RC assembly in the presence of CDK phosphorylated ORC. ORC was pre-phosphorylated using the indicated concentrations of CDK, washed and then incubated with Cdc6 and Cdt1/MCM2-7. A 30% load of CDK (40 nM), unphosphorylated ORC and CDK phosphorylated ORC is shown in lanes 1-5. The phosphorylated Orc2 and Orc6 proteins are marked with #. (**B**) Summary of the Cdt1 truncation mutant data. (**C**) Model of pre-RC formation. [1] ORC recognizes the replication origin and [2] recruits Cdc6 to form the ORC/Cdc6 complex. [3] Cdt1 binds MCM2-7 and alleviates an inhibitory activity in Mcm6 to promote ORC/Cdc6/Cdt1/MCM2-7 complex formation. Cdt1-3A, which carries a triple point mutation in its C-terminal Mcm6 binding domain, can bind MCM2-7, but cannot alleviate the inhibitory activity in Mcm6 and does not promote pre-RC formation. [4] A productive ORC/Cdc6 interaction with Cdt1/MCM2-7 triggers Orc1 and Cdc6 ATP hydrolysis, Cdt1 release and OCM formation. CDK blocks the establishment of the OCM and leads to

premature Cdt1/MCM2-7 release. [5] The OCM can recruit another Cdt1/MCM2-7 complex to form the MCM2-7 double-hexamer.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online.


A
0.5 min
2.5 min
5 min
7.5 min
10 min
15 min
1.25 %
2.5 %
5 %
10 %
20 %
i.
ATPγS
low salt wash
ii.
ATP
low salt wash
iii.
ATP
high salt wash
Mcm2
Orc3
Cdt1
Cdc6
B
ATPγS 2.5 minutes
Input 20%
Sup 20%
IP 100%
mock
anti-Mcm2
0.625 %
1.25 %
2.5 %
5 %
10 %
C
ATPγS 30 minutes
D
ATP 2.5 minutes
E
ATP 30 minutes
F
MCM2-7
MBP-Mcm5
Mcm-5
Mcm5
Orc5
G
ORC
MBP-Orc5
Orc5
H
Cdc6
MBP-Cdc6
Cdc6
Orc2
I
pre-RC
ORC+Cdc6+ MCM2-7
ATPγS
ATP
L
H
salt wash
MCM2-7
Cdt1
Cdc6
Orc1-6
J
load
bound fraction
supernatant fraction
ORC
Cdc6
Cdt1
MCM2-7
OCM 0 min
OCM 5 min
OCM 10 min
OCM 15 min
OCM 20 min
116
97
66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pre-RC Cdt1 (1-312)
pre-RC Cdt1 (306-604)
pre-RC Cdt1 (464-604)
ORC
Cdc6
Cdt1
Cdt1 (1-312)
Cdt1 (306-604)
Cdt1 (464-604)
MCM2-7
ATPγS
ATP
ATP
L L H
salt wash
116
97
66
*
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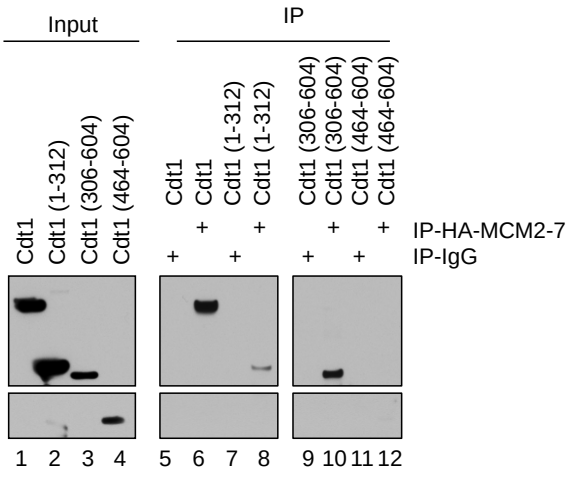
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Input
IP
Cdt1
Cdt1 (1-312)
Cdt1 (306-604)
Cdt1 (464-604)
IP-HA-MCM2-7
IP-IgG
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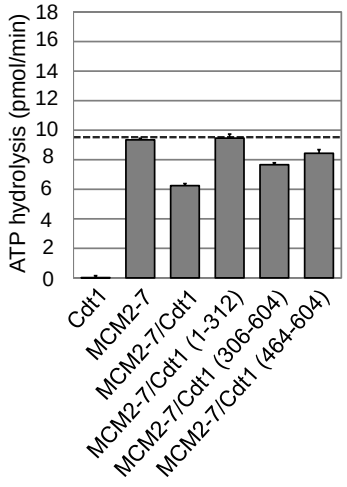
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ATP hydrolysis (pmol/min)
0
2
4
6
8
10
12
14
16
18
Cdt1
MCM2-7
MCM2-7/Cdt1
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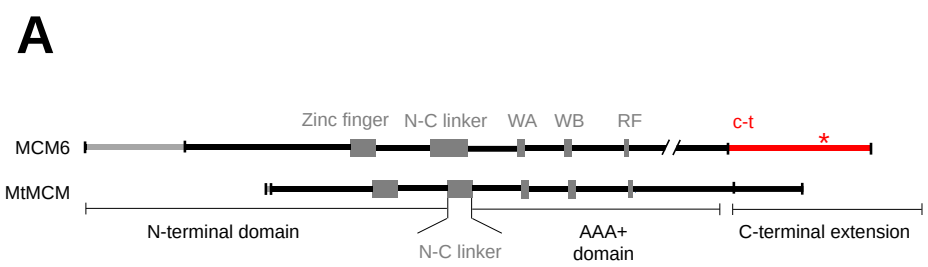
MCM2-7/Cdt1 (464-604)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Zinc finger
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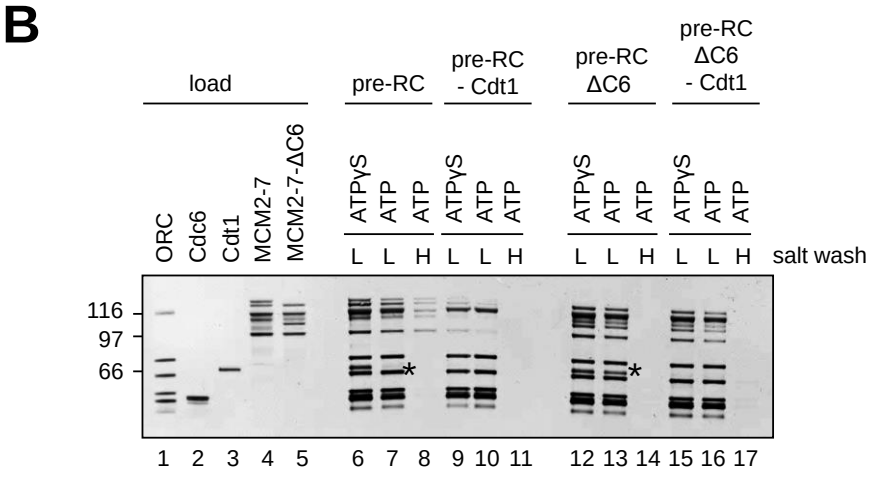
WA
WB
RF
c-t
MCM6
MtMCM
N-terminal domain
N-C linker
AAA+ domain
C-terminal extension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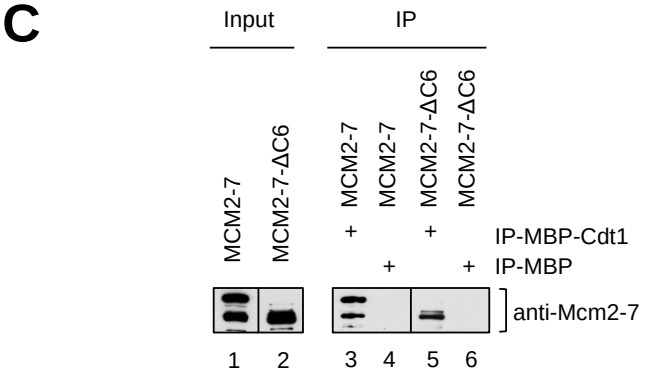
load
pre-RC
pre-RC - Cdt1
pre-RC ΔC6
pre-RC ΔC6 - Cdt1
ORC
Cdc6
Cdt1
MCM2-7
MCM2-7-ΔC6
ATPγS
ATP
L L H L L H L L H L L H
salt wash
116
97
66
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17


C
Input
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MCM2-7
MCM2-7-ΔC6
IP-MBP-Cdt1
IP-MBP
anti-Mcm2-7
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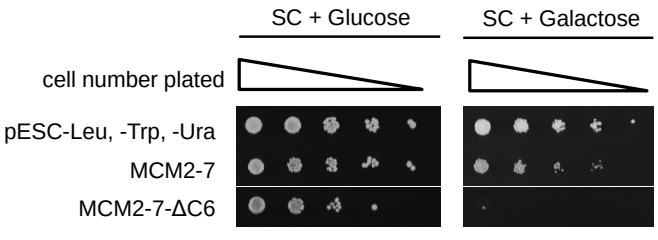
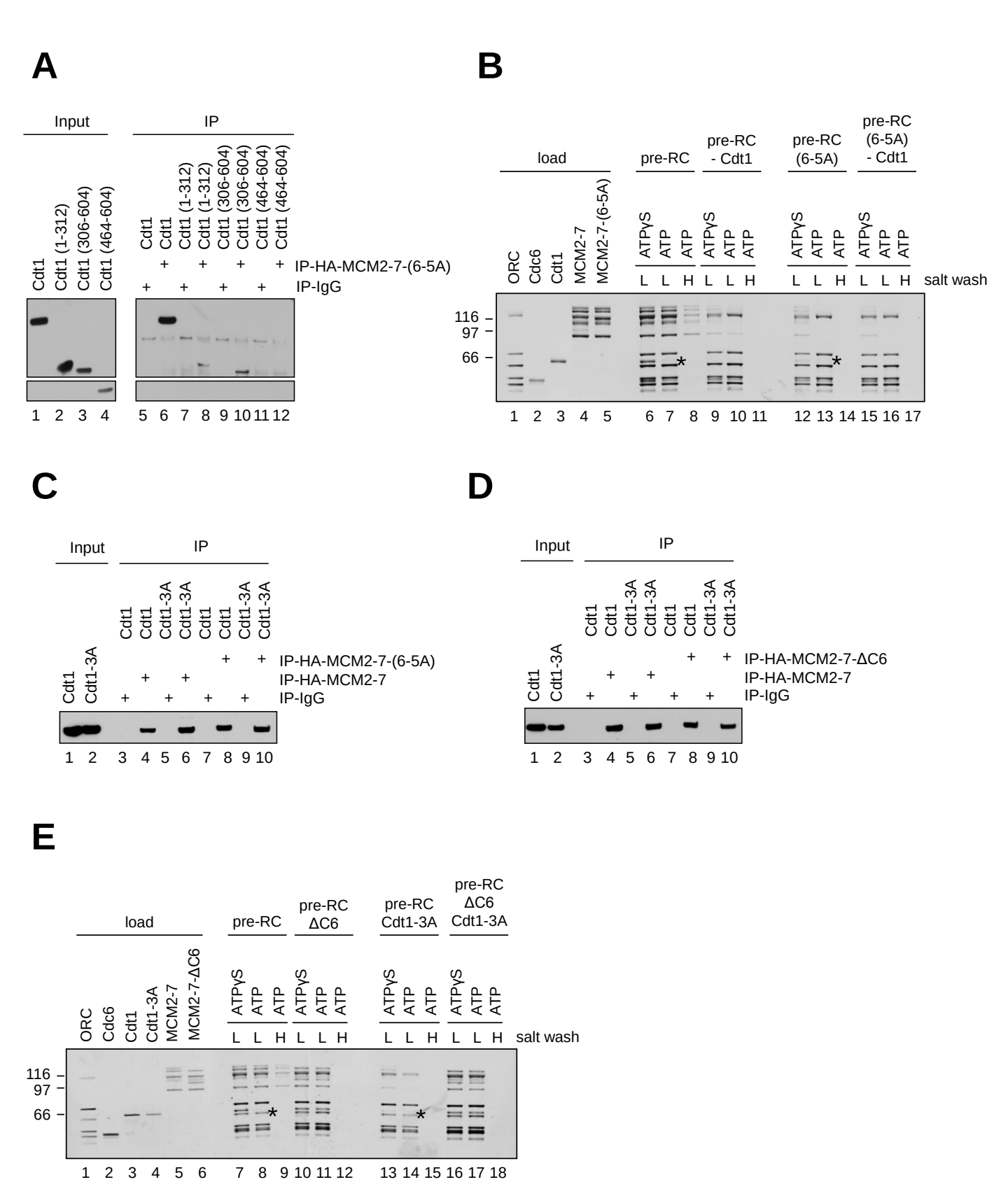
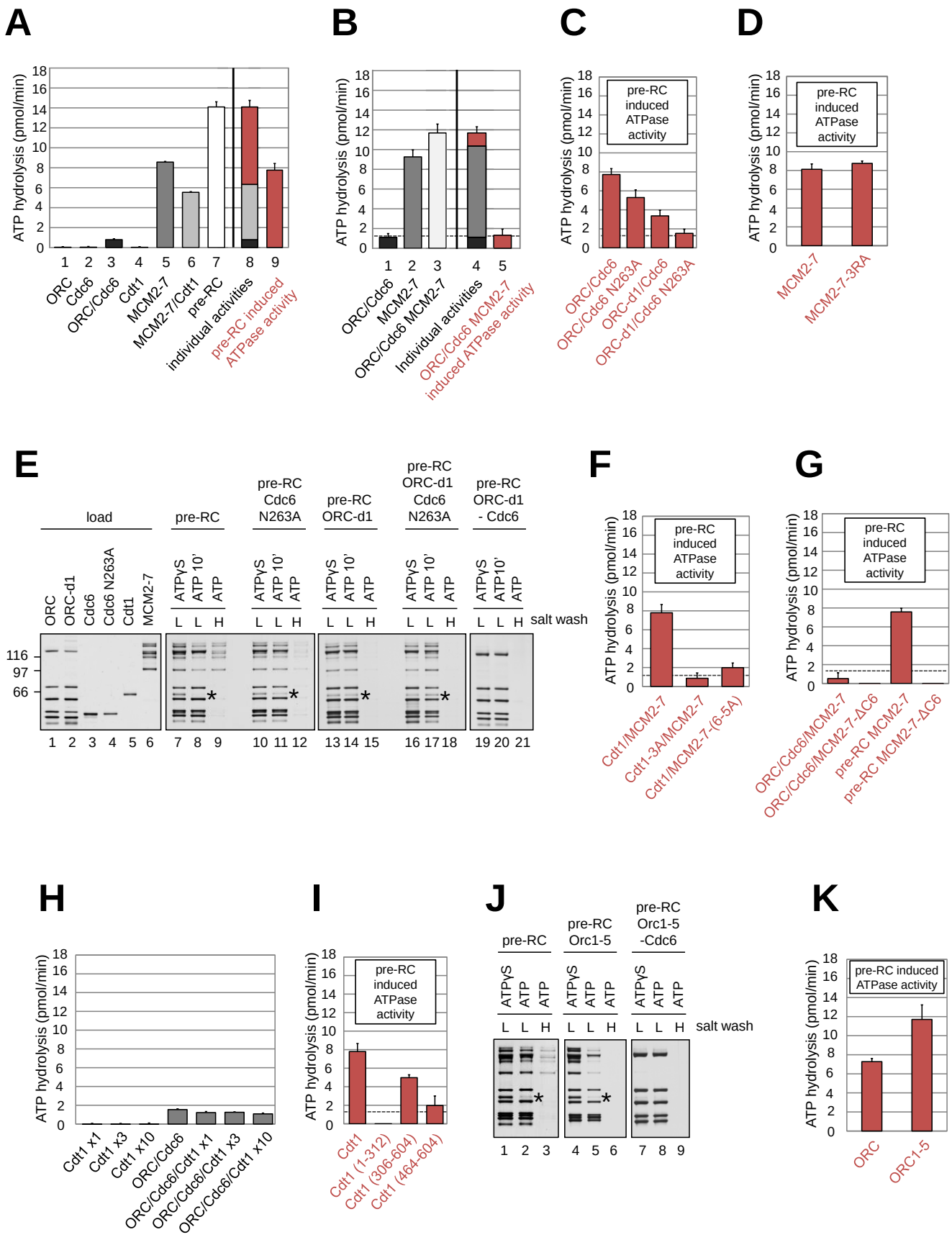

D
SC + Glucose
SC + Galactose
cell number plated
pESC-Leu, -Trp, -Ura
MCM2-7
MCM2-7-ΔC6


A
Input
IP
Cdt1
Cdt1 (1-312)
Cdt1 (306-604)
Cdt1 (464-604)
IP-HA-MCM2-7-(6-5A)
IP-IgG
1 2 3 4 5 6 7 8 9 10 11 12
B
load
pre-RC
pre-RC - Cdt1
pre-RC (6-5A)
pre-RC (6-5A) - Cdt1
ORC
Cdc6
Cdt1
MCM2-7
MCM2-7-(6-5A)
ATPγS
ATP
L L H
salt wash
116
97
66
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
C
Input
IP
Cdt1
Cdt1-3A
IP-HA-MCM2-7-(6-5A)
IP-HA-MCM2-7
IP-IgG
1 2 3 4 5 6 7 8 9 10
D
Input
IP
Cdt1
Cdt1-3A
IP-HA-MCM2-7-ΔC6
IP-HA-MCM2-7
IP-IgG
1 2 3 4 5 6 7 8 9 10
E
load
pre-RC
pre-RC ΔC6
pre-RC Cdt1-3A
pre-RC ΔC6 Cdt1-3A
ORC
Cdc6
Cdt1
Cdt1-3A
MCM2-7
MCM2-7-ΔC6
ATPγS
ATP
L L H
salt wash
116
97
66
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18


A
B
C
D
ATP hydrolysis (pmol/min)
ORC
Cdc6
ORC/Cdc6
Cdt1
MCM2-7
MCM2-7/Cdt1
pre-RC
individual activities
pre-RC induced ATPase activity
ORC/Cdc6 MCM2-7 induced ATPase activity
pre-RC induced ATPase activity
ORC/Cdc6 N263A
ORC-d1/Cdc6
ORC-d1/Cdc6 N263A
MCM2-7-3RA
E
load
pre-RC
pre-RC Cdc6 N263A
pre-RC ORC-d1
pre-RC ORC-d1 Cdc6 N263A
pre-RC ORC-d1 - Cdc6
ORC-d1
Cdc6 N263A
ATPγS
ATP 10'
ATP
salt wash
116
97
66
F
G
Cdt1/MCM2-7
Cdt1-3A/MCM2-7
Cdt1/MCM2-7-(6-5A)
ORC/Cdc6/MCM2-7
ORC/Cdc6/MCM2-7-ΔC6
pre-RC MCM2-7
pre-RC MCM2-7-ΔC6
H
I
J
K
Cdt1 x1
Cdt1 x3
Cdt1 x10
ORC/Cdc6
ORC/Cdc6/Cdt1 x1
ORC/Cdc6/Cdt1 x3
ORC/Cdc6/Cdt1 x10
Cdt1
Cdt1 (1-312)
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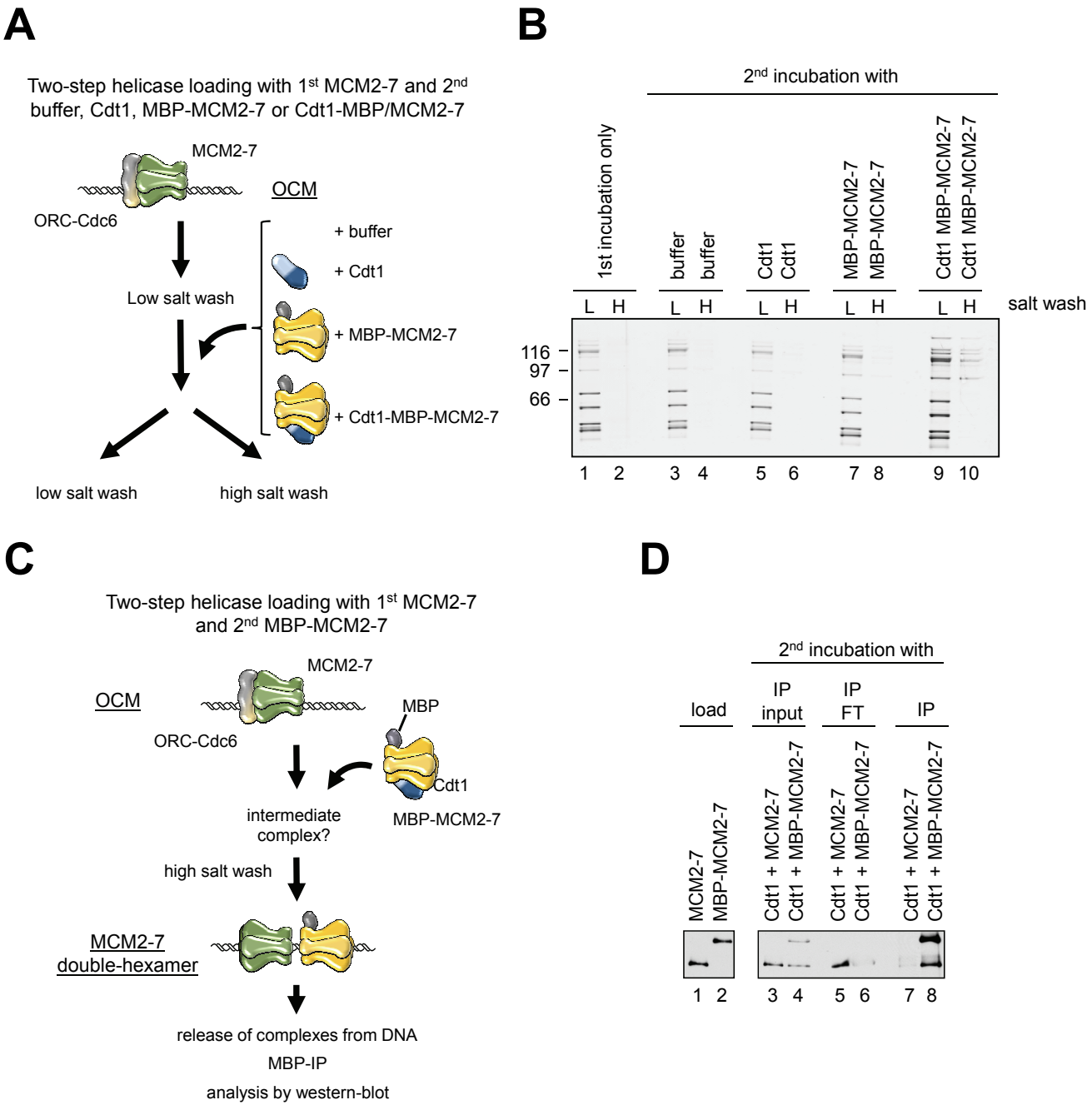
pre-RC Orc1-5 -Cdc6
ORC
ORC1-5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OCM
ORC-Cdc6
+ buffer
+ Cdt1
Low salt wash
+ MBP-MCM2-7
+ Cdt1-MBP-MCM2-7
low salt wash
high salt wash
B
2nd incubation with
1st incubation only
buffer
buffer
Cdt1
Cdt1
MBP-MCM2-7
MBP-MCM2-7
Cdt1 MBP-MCM2-7
Cdt1 MBP-MCM2-7
L H L H L H L H L H
salt wash
116
97
66
1 2 3 4 5 6 7 8 9 10
C
Two-step helicase loading with 1st MCM2-7 and 2nd MBP-MCM2-7
MCM2-7
OCM
MBP
ORC-Cdc6
Cdt1
intermediate complex?
MBP-MCM2-7
high salt wash
MCM2-7 double-hexamer
release of complexes from DNA
MBP-IP
analysis by western-blot
D
2nd incubation with
load
IP input
IP FT
IP
MCM2-7
MBP-MCM2-7
Cdt1 + MCM2-7
Cdt1 + MBP-MCM2-7
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Cdt1 + MCM2-7
Cdt1 + MBP-MCM2-7
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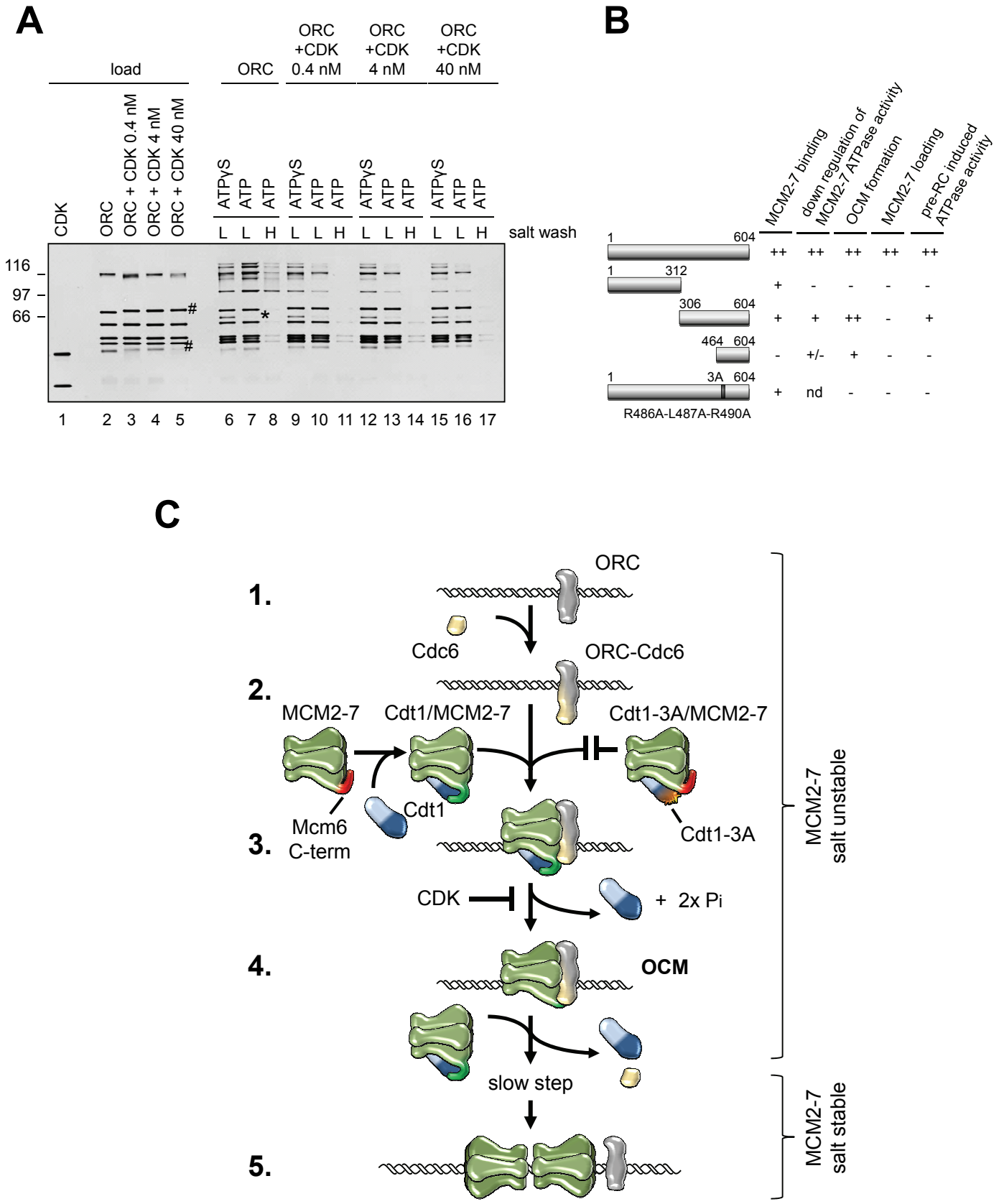

Fernández-Cid et al. Figure 7

Supplemental Information

An ORC/Cdc6/MCM2-7 complex is formed in a multistep reaction to serve as a platform for MCM double-hexamer formation

Alejandra Fernández-Cid, Alberto Riera, Silvia Tognetti, M. Carmen Herrera, Stefan Samel, Cecile Evrin, Christian Winkler, Emanuela Gardenal, Stefan Uhle and Christian Speck

INVENTORY OF SUPPLEMENTAL INFORMATION

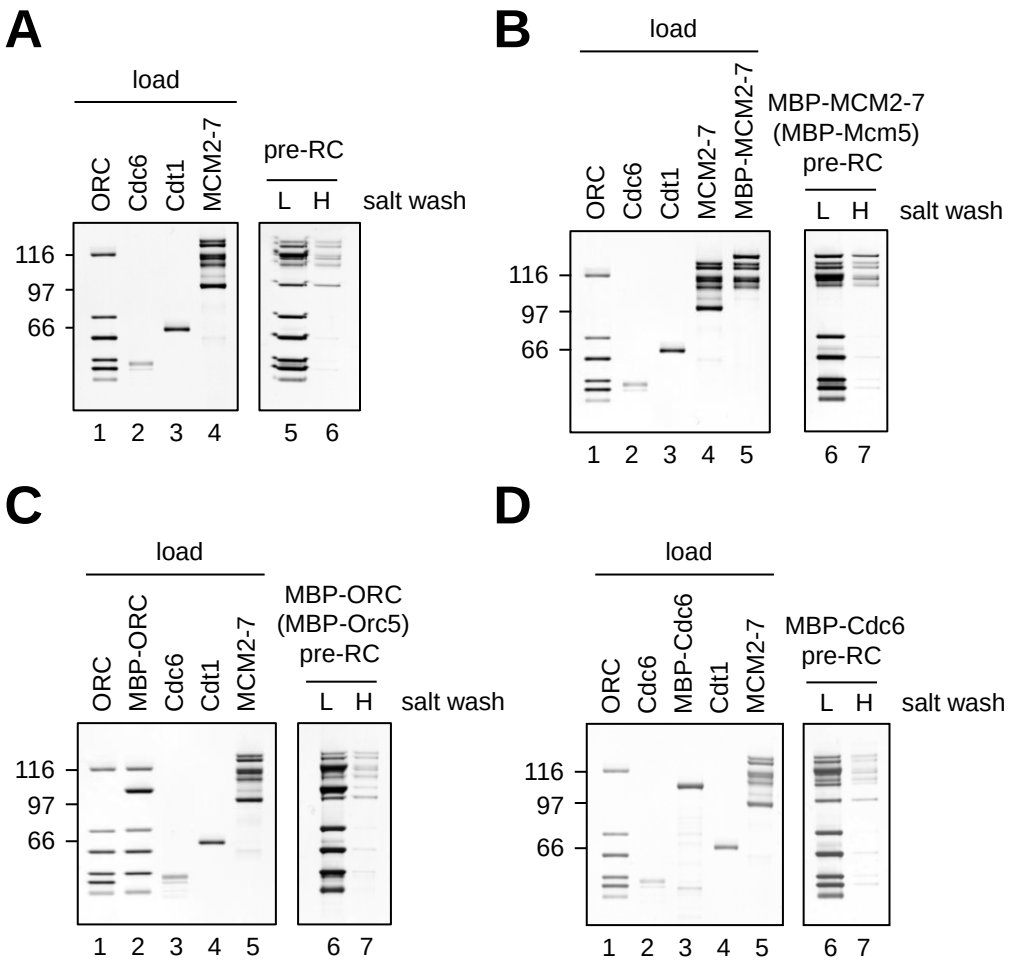

Figure S1 (related to main Figure 1). ATPase dependent formation of a stable OCM complex. Analysis of MBP-tagged ORC, Cdc6 and MCM2-7 for their ability to promote pre-RC assembly. The reactions were assembled, washed with low salt [L] or high salt [H] and analyzed by silver staining. This experiment verifies that (**A**) *wild type* pre-RC proteins, (**B**) MBP-MCM2-7 (MBP-Mcm5), (**C**) MBP-ORC (MBP-Orc5) and (**D**) MBP-Cdc6 are functional for salt stable MCM2-7 loading.

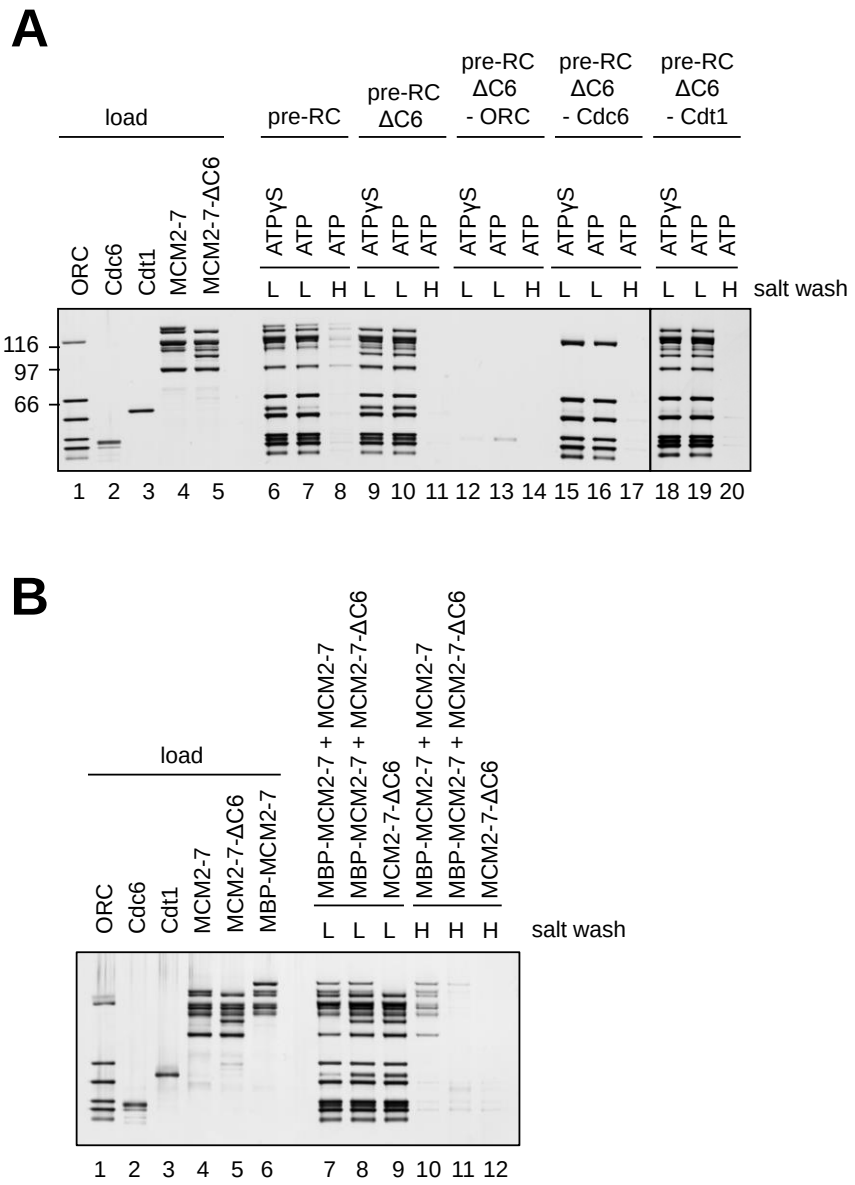

Figure S2 (related to main Figure 3). Cdt1 alleviates an inhibitory function in the Mcm6 C-terminus to promote pre-RC formation. (**A**) Analysis of pre-RC assembly in the presence and absence of ORC, Cdc6 and Cdt1 using MCM2-7-ΔC6.The reactions were assembled, washed with low salt [L] or high salt [H] and analyzed by silver staining. This experiment verifies that the DNA recruitment of MCM2-7-ΔC6 is dependent on ORC and Cdc6, but not Cdt1. (**B**) Analysis of MCM2-7-ΔC6 during pre-RC assembly. Analysis of pre-RC assembly using mixed populations of MBP-MCM2-7 and MCM2-7-ΔC6. The reactions were assembled, washed with low salt [L] or high salt [H] and analyzed by silver staining. This experiment shows that the addition of MBP-MCM2-7 (MBP fused to Mcm5) to MCM2-7-ΔC6 cannot support the salt-stable loading of MCM2-7-ΔC6 onto DNA.

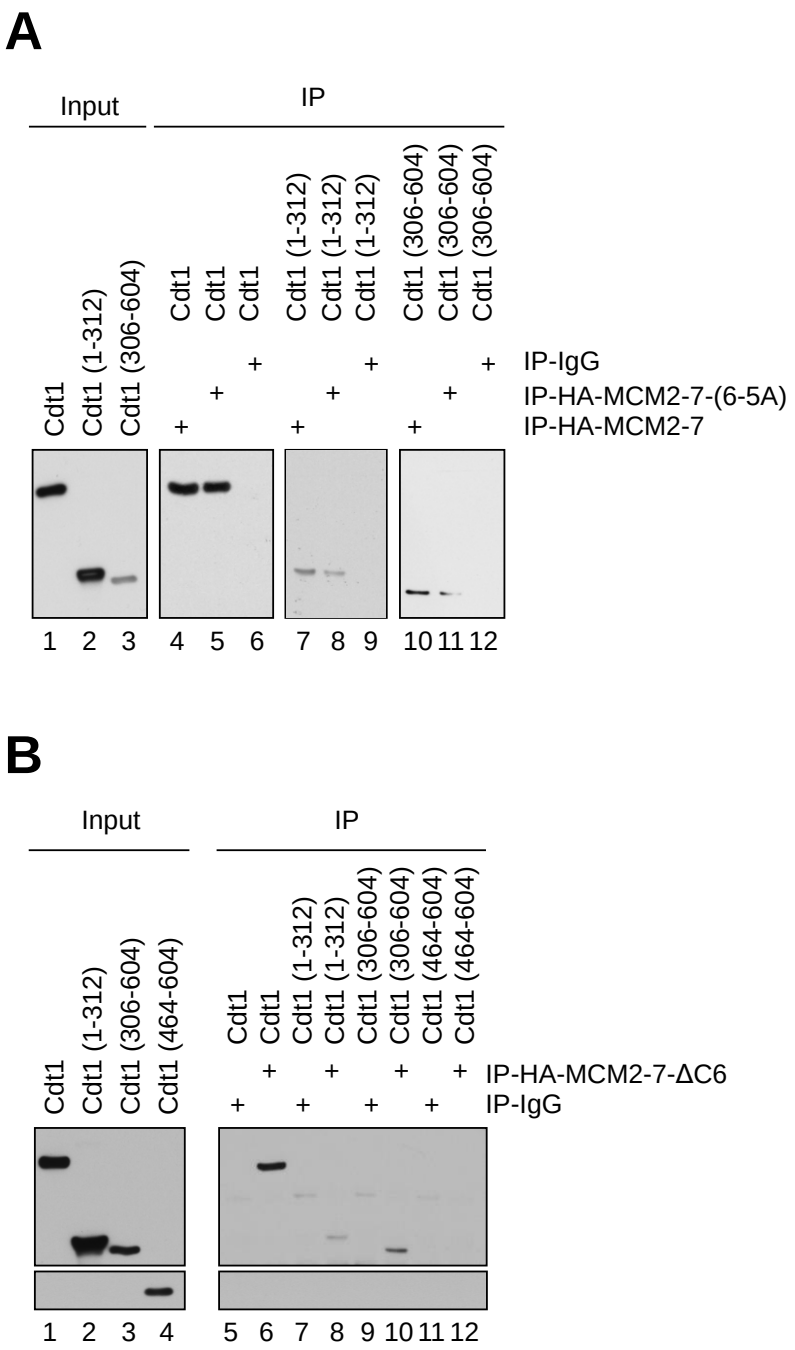

Figure S3 (related to main Figure 4). A specific Cdt1-Mcm6 interaction is required to alleviate an inhibitory function in Mcm6. (**A**) Interaction analysis of MCM2-7 and MCM2-7-(6-5A) with Cdt1. Anti-HA beads, anti-HA beads coupled with MCM2-7 or MCM2-7-(6-5A) were incubated with purified Cdt1, Cdt1 (1-312) or Cdt1 (306-604), washed and analyzed by western blot. A 14% input is shown along with 100% of the IP. (**B**) Interaction analysis of MCM2-7-ΔC6 with Cdt1. Anti-HA beads or anti-HA beads coupled with MCM2-7-ΔC6 were incubated with purified Cdt1, Cdt1 (1-312), Cdt1 (306-604) or Cdt1 (464-604), washed and analyzed by western blot. A 14% input is shown along with 100% of the IP.

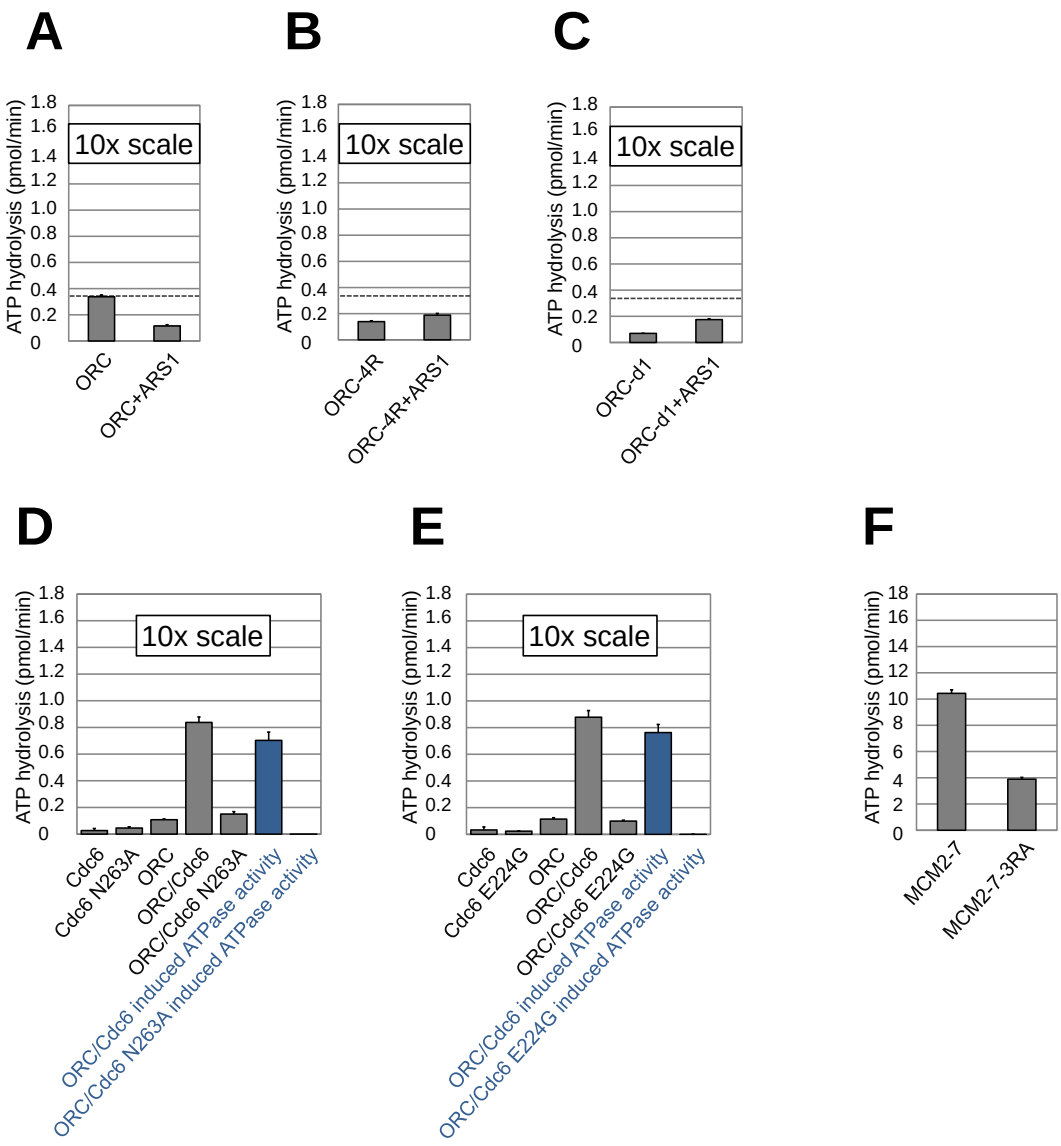

Figure S4 (related to main Figure 5). Cdt1 and MCM2-7 are both required to induce ATP-hydrolysis during pre-RC formation. Basic characterization of the ATP-hydrolysis defects of ORC-4R, ORC-d1, Cdc6 N263A, Cdc6 E224G and MCM2-7-3RA. These experiments confirm the general ATPase defects of the respective mutants relative to the wild-type proteins. Note than the scale for A-E has been adjusted to allow the evaluation of the ATPase defects and is different than the scale in F or in the main Figure 5. (**A**) Analysis of the ORC ATPase activity in the absence and presence of origin DNA. As expected, we observed that the ORC ATPase was down-regulated in the presence of DNA (Klemm et al., 1997). (**B**) Analysis of the ORC-4R ATPase activity in the absence and presence of origin DNA. As expected, we observed that the ORC-4R ATPase activity was weaker than the ORC ATPase activity (the dashed line in A-C indicates the ORC ATPase activity level observed in A) (Bowers et al., 2004). Note that the ORC-4R ATPase activity is increased at

elevated ATP concentrations (Speck and Stillman, 2007) and the ATPase activity we observed is therefore higher than the ORC-4R ATPase activity observed in before (Bowers et al., 2004). (**C**) Analysis of the ORC-d1 ATPase activity in the absence and presence of origin DNA. As expected, we observed that the ORC-d1 ATPase was weaker than the ORC ATPase activity (Klemm and Bell, 2001). (**D**) Analysis of the ORC/Cdc6 and ORC/Cdc6 N263A induced ATPase activity in the presence of origin DNA The ORC/Cdc6 and ORC/Cdc6 N263A induced ATPase activity is shown in blue. The ORC/Cdc6 induced ATPase activity is calculated by subtracting the individual ATPase activities of ORC and Cdc6 from the ATPase activity observed with ORC/Cdc6. As expected, our data show that Cdc6 N263A does not produce ORC/Cdc6 induced ATPase activity (Speck and Stillman, 2007). (**E**) Analysis of the ORC/Cdc6 and ORC/Cdc6 E224G induced ATPase activity in the presence of origin DNA. The ORC/Cdc6 and ORC/Cdc6 E224G induced ATPase activity is shown in blue. The ORC/Cdc6 induced ATPase activity is calculated by subtracting the individual ATPase activities of ORC and Cdc6 from the ATPase activity observed with ORC/Cdc6. As expected, our data show that Cdc6 E224G does not produce ORC/Cdc6 induced ATPase activity (Randell et al., 2006; Speck and Stillman, 2007). (**F**) Analysis of the MCM2-7 and MCM2-7-3RA ATPase activity. As expected, this analysis shows that MCM2-7-3RA has significantly weaker ATPase activity than MCM2-7 (Bochman et al., 2008). The ATPase data are represented as mean +/- SD.

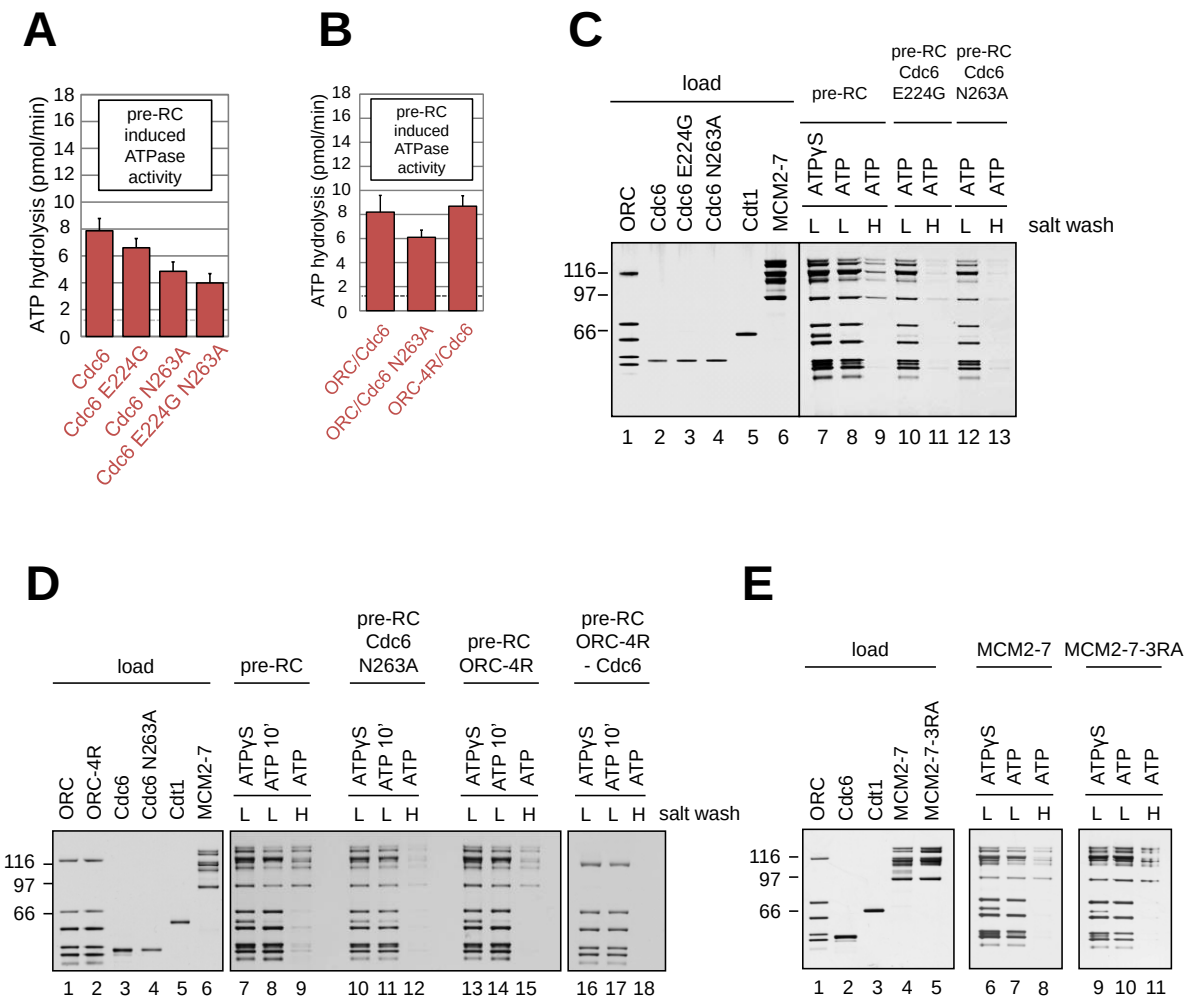

Figure S5 (related to main Figure 5). Cdt1 and MCM2-7 are both required to induce ATP-hydrolysis during pre-RC formation. (**A**) Investigation of the pre-RC induced ATPase activity with Cdc6 E224G, Cdc6 N263A and Cdc6 E224G N263A. (**B**) Investigation of the pre-RC induced ATPase activity with ORC-4R and Cdc6 N263A. The ATPase data are represented as mean +/- SD. (**C**) Analysis of pre-RC assembly with Cdc6 E224G and Cdc6 N263A.The reactions were assembled, washed with low salt [L] or high salt [H] and analyzed by silver staining. (**D**) Analysis of pre-RC assembly with ORC-4R and Cdc6 N263A. The reactions were assembled, washed with low salt [L] or high salt [H] and analyzed by silver staining. These experiments (B and D) confirm that ORC-4R supports pre-RC induced ATP-hydrolysis and MCM2-7 loading. (**E**) Analysis of pre-RC assembly with MCM2-7-3RA.The reactions were assembled, washed with low salt [L] or high salt [H] and analyzed by silver staining. This experiment confirms that an MCM2-7-3RA ATPase mutant supports MCM2-7 loading.

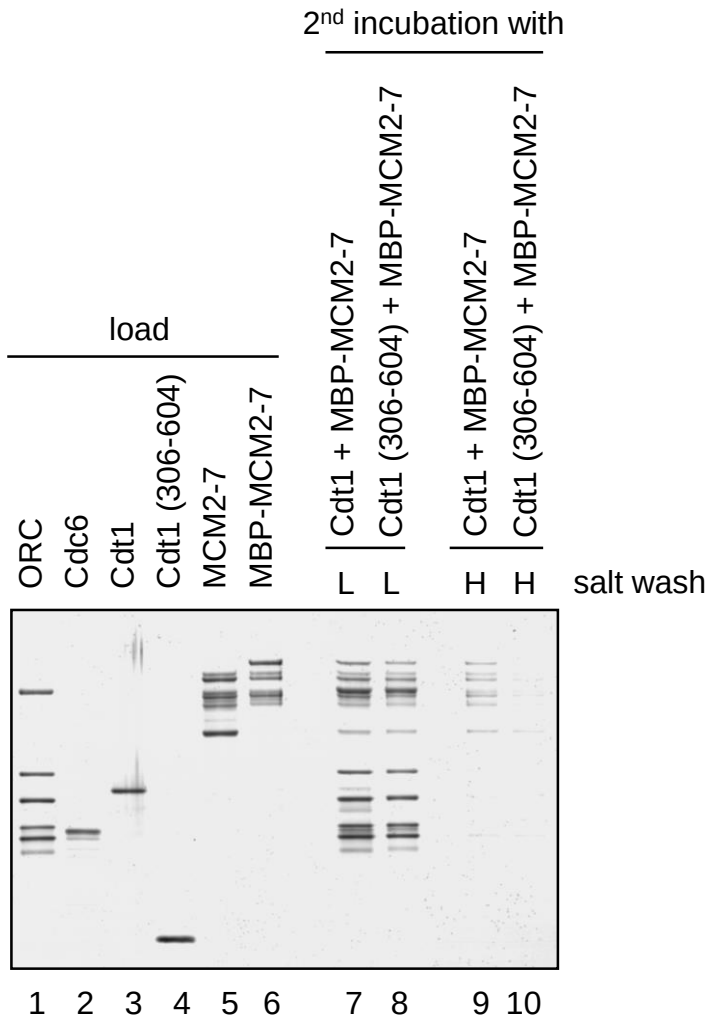

Figure S6 (related to main Figure 6). The OCM represents a platform for MCM2-7 double-hexamer assembly. Analysis of pre-RC assembly with Cdt1 (306-604). An OCM complex was formed with Cdt1/MCM2-7 for 4 minutes, washed and incubated with Cdt1/MBP-MCM2-7 or Cdt1 (306-604)/MBP-MCM2-7. Then the reactions were washed with low salt [L] or high salt [H] and analyzed by silver staining. This experiment shows that the addition of Cdt1 (306-604)/MBP-MCM2-7 to an OCM complex does not support the salt-stable loading of MBP-MCM2-7 (MBP fused to Mcm5) onto DNA.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cloning of MCM2-7 and Cdt1 mutants

Mcm6-ΔC6 (1-838), Mcm6-5A (E945A, D947A, L951A, E953A and Y954A) and MCM2-7-3RA (Mcm3 R542A) were created by site directed mutagenesis of pCS15 (pESC-Trp Mcm4-Mcm6) or pCS232 (pESC-URA HA-MCM3-MCM5) yielding pCS496, pCS520 and pCS551, respectively.

Cdt1-3A (R486A, L487A and R490A) was created by site directed mutagenesis of pCS178 yielding pCS521.

MBP-Mcm3, MBP-Mcm5 and MBP-Mcm7 were created in two steps: First a restriction site was inserted after aa111 (Mcm3), aa591 (Mcm5) and aa30 (Mcm7) by site directed mutagenesis employing the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene). Afterwards, MBP coding sequence was amplified from pCS280 (Evrin et al., 2013) (pESC-LEU-MBP-MCM2/MCM7) with primers MBP-20A_linker (FWD taattagcgatcgcaTATAGAACTTTGACTGTTTTGAAAATC) and MBP-20A_linker (REV tattatgcgatcgcTATAGAACTTTGACTGTTTTGTTTAG), which encode the flexible linkers (N-term: YRTLTVL) and (C-term: QNSQSSI) at both ends of MBP. The resulting product was inserted in the restriction site generating pCS444 (pESC-URA MBP-Mcm3/Mcm5), pCS470 (pESC-URA Mcm3/Mcm5-MBP) and pCS479 (pESC-LEU MBP-MCM2/MBP-MCM7).

The Cdt1 truncation mutants were generated by PCR (oligo sequence available on request) using the BamHI and NotI restriction sites and cloned into pGEX6P-1 vector, resulting in pCS189 (1-312), pCS179 (306-604) and pCS185 (464-604).

MBP-Cdt1 cloning, expression and purification

The coding sequence of Cdt1 was amplified by PCR (oligo sequence available on request) adding HindIII and XmnI restriction sites and cloned in the p2CX vector from New England Biolabs (NEB). The resulting plasmid (pCS134) was transformed in BL21 Codon Plus RIL *E. coli* competent cells. The cells were grown in Terrific Broth media supplemented with appropriate antibiotics at 37°C to an OD600 of 1.1. Protein expression was then induced by addition of 0.5 mM IPTG for 5h at 16°C. Cells were lysed by sonication and the fusion protein was bound to amylose resin (NEB) in buffer D [50 mM Tris-HCl pH 7.2, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1% Triton X-100] for 2h at 4°C. The MBP fusion protein was then eluted from the beads with buffer E [25 mM Tris-HCl pH 7.2, 25 mM Hepes-KOH

pH 6.0, 250 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1% Triton X-100, 15% glycerol, 10 mM maltose]. Peak fractions were pooled, diluted with 2 volumes of buffer F [25 mM Hepes-KOH pH 6.0, 5 mM $MgCl_2$, 1 mM DTT] and bound to SP Sepharose (GE Healthcare) for 2h at 4°C. MBP-Cdt1 was then eluted with buffer E without maltose. Peak fractions were pooled and stored at -80°C. One liter of bacteria culture yielded approximately 0.22 mg of pure MBP-Cdt1 with a concentration of 0.6 mg/ml.

Purification of ORC, Cdc6, Cdt1 and MCM2-7 proteins

ORC, MBP-ORC (MBP-Orc5), ORC-4R, ORC-d1 and Orc1-5 were expressed by using baculovirus-infected cells and purified as described (Klemm et al., 1997). Cdc6, Cdt1 and the respective mutants were expressed in bacteria and purified as described (Evrin et al., 2009; Speck et al., 2005). MCM2-7 *w*t and mutants were expressed in *Saccharomyces cerevisiae* and purified as described (Evrin et al., 2009).

HA-Cdc28-Clb5 expression and purification

Hi5 cells were grown on plates and infected with HA-Cdc28 (Reynard et al., 2000) and Clb5 (Duncker et al., 1999) baculoviruses at a MOI of 10. After a 44 h incubation, cells were harvested in a clinical centrifuge at 4°C, washed once with PBS, and resuspended in ice cold lysis buffer L-buffer [50 mM Hepes KOH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP40, 0.5 mM DTT, Complete Protease Inhibitor Cocktail without EDTA (Roche), 10 mM Sodium Fluoride, 1 mM Sodium Orthovanadate, 1 mM Sodium Pyrophosphate], lysed for 30 minutes at 4°C with rotation and clarified by centrifugation at 4°C (15K, 15 min, Sorvall SS34 rotor). The supernatant was incubated with HA-agarose resin for 2 h at 4°C. The beads were washed three times with L-buffer [100 mM NaCl, 0.02% NP40] and eluted three times with HA-peptide in L-buffer [100 mM NaCl, 0.02% NP40, 0.5 mg/ml HA peptide] for 1 h at 15°C. The three elutions were pooled, diluted with one volume of LD buffer [50 mM Hepes/KOH pH 6, 0.5 mM DTT] and bound to SP-Sepharose (GE Healthcare). The beads were washed three times with LW buffer [50 mM Hepes/KOH pH 6.5, 50 mM NaCl, 0.5 mM DTT] and eluted with LE buffer [50 mM Hepes/KOH pH 7.5, 200 mM NaCl, 0.5 mM DTT, 10 % Glycerol].

MBP-pulldown reactions

100 ng of MBP and MBP-Cdt1 were immobilized on anti-MBP antibody (NEB) beads (protein G - SIGMA) for 15 min at 24°C with mixing in 50 µl buffer MA [50 mM Hepes-KOH pH 7.5, 100 mM KGlu, 10 mM MgAc, 50 µM ZnAc, 3 mM ATP, 5 mM DTT, 0.1% Triton X-100, and 10% Glycerol] followed by three washes with 100 µl of the buffer MA. Purified *wt* MCM2-7 or

MCM2-7-ΔC6 (1 µg) were incubated with MBP or MBP-Cdt1 beads for 15 min with mixing at 24 °C in 200 µl buffer MA. Afterwards the beads were washed three times with 100 µl of buffer MA. The bound proteins were analyzed by SDS-PAGE and western blotting with an anti-MCM2-7 (ab77) antibody.

HA-pulldown reactions

1 µg of HA-MCM2-7 was immobilized on IgG control beads (anti-MBP (NEB) on protein G) or anti-HA (SIGMA) beads (protein G) for 15 min at 24°C with mixing in 200 µl buffer MB [50 mM Hepes-KOH pH 7.5, 300 mM KGlu, 10 mM MgAc, 50 µM ZnAc, 3 mM ATP, 5 mM DTT, 0.1% Triton X-100, 10% Glycerol and 0.1% BSA] followed by three washes with 200 µl of the buffer MB. 3.65 pmoles of purified Cdt1 (*wt* or mutant) were incubated with IgG control or HA-MCM2-7 beads for 10 minutes with mixing at 24 °C in 200 µl buffer MB. Afterwards the beads were washed three times with 200 µl of buffer MB. The bound proteins were analyzed by SDS-PAGE and western blotting with anti-Cdt1 N-terminal (CS1411) and anti-Cdt1 C-terminal (CS1415) antibodies.

ATPase assay

The ATPase assay was performed as described (Speck and Stillman, 2007) with minor modifications. We used a 150 bp DNA fragment containing the ARS1 sequence that was amplified with primer ARS150 FWD (CAAAATAGCAAATTTCGTCAAAAATGC) and primer ARS1 150 REV (TTTACATCTTGTTATTTTACAGATTTTATGTTTAGATC) from the plasmid pCS372 - (pUC19 ARS1). 2.5 pmol of *wt* or mutant ORC, Cdc6, Cdt1 and MCM2-7 were incubated for 30 min on ice in 12 µl of ATPase buffer [25 mM Hepes, pH 7.6, 100 mM KGlu, 5mM MgAc, 1mM DTT, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 10% glycerol] containing 2.5 pmol of DNA (when indicated) and 1 mM ATP. After the incubation, 5 µCi of [α-32P] ATP (3000 Ci/mmol) was added and the reaction was started by moving the tubes from ice into a 24°C water bath. 2 µl aliquots were collected at 15, 30, 45, and 60 min and stopped with 0.5 µl of stop solution (2% SDS, 50 mM EDTA). 1 µl of the samples were consequently spotted on TLC plates and developed. Error bars represent the standard deviation from at least three independent experiments.

Immunoprecipitation assay

One standard size pre-RC reaction was prepared as described above (pre-RC assay) employing untagged MCM2-7 (40 nM). After 2.5 minutes of incubation the beads were washed 3 times with buffer A, incubated for 12.5 minutes with buffer, Cdt1, MBP-tagged MCM2-7 or Cdt1-MBP-tagged MCM2-7. The beads were washed 2 times with buffer A plus

1 mM EDTA or buffer B [50 mM Hepes-KOH pH 7.5, 1 mM EDTA, 500 mM NaCl, 10% Glycerol, 0.1% Triton X-100, and 5 mM DTT] before DNA digestion with 1 U of DNase I in buffer A plus 5 mM $CaCl_2$ for 2 min at 24 °C. Reaction products were analyzed by SDS-PAGE and silver-staining or immunoprecipitated with anti-MBP (New England Biolabs) antibody coupled to protein G beads (SIGMA) for 7.5 min at 24 °C, washed two times with buffer A and analyzed by western blot.

MBP Immunoprecipitation assay

One standard size pre-RC reaction was prepared as described above (pre-RC assay) employing MBP-tagged protein (20 nM), MBP-tagged (20 nM) and untagged protein (20 nM) or only untagged protein (20 nM). After 4 minutes of incubation the beads were washed 2 times with buffer A, before DNA digestion with 0.03 U of DNase I in buffer A plus 5 mM $CaCl_2$ for 7 min at 24 °C. Reaction products were immunoprecipitated with anti-MBP (New England Biolabs) antibody coupled to protein G beads (Invitrogen) for 4 min at 24 °C, washed two times with buffer A and analyzed by western blot.

CDK – pre-RC assay

This method was performed as described (Chen and Bell, 2011) with minor modifications. The pre-RCs were formed as described above with minor modifications. In the first step ORC and DNA were incubated with buffer A and ATP for 5 minutes. Then, CDK was added and the reactions were incubated for 15 minutes at 27 °C. Afterwards, the reactions were washed two times with buffer A and twice with buffer A without ATP. Then 80 nM Cdc6, 40 nM Cdt1, 40 nM MCM2-7 in buffer A with ATP or ATPγS were added and the reactions continued as described for the standard pre-RC assay.

Yeast growth assay on plates

Yeast strain AS499 (*MATa. bar1Δ, leu2-3,-112, ura3-52, his3-Δ200, trp1-Δ-63, ade2-1 lys2-801, pep4*) was transformed with pESC-LEU, pESC-TRP and pESC-URA plasmids (YC41); pESC-LEU-MCM2-MCM7, pESC-TRP-MCM6-MCM4 and pESC-URA-HA_MCM3-MCM5 (YC119) or pESC-LEU-MCM2-MCM7, pESC-TRP-MCM6 (Mcm6 1-838)-MCM4 and pESC-URA-MCM3-MCM5 (YC393). The yeasts were grown overnight in selective medium at 30°C. A five-fold serial dilution starting from 10^6 cells were spotted on selective plates in the presence of either 2% glucose or galactose. Plates were incubated at 30°C for 4 days.

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