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# Prereplication-complex formation: a molecular double take?

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**In G1, two copies of the MCM2–7 helicase are recruited to each origin of replication. Whereas recruitment of the first MCM2–7 is likely to be analogous to the loading of sliding clamps around DNA, how the second MCM2–7 complex is recruited is highly contentious. Here, we argue that MCM2–7 loading involves specific modifications to the clamp-loading reaction and propose that the first and second MCM2–7 molecules are loaded via similar mechanisms.**

## Introduction

The initiation of chromosomal DNA replication begins with the origin recruitment of the replicative DNA helicase, which unwinds DNA at the replication fork[1]. In *Escherichia coli*, helicase recruitment involves several discrete steps (**Fig. 1**). First, the initiator protein DnaA binds to the origin of DNA replication, *oriC*, melts a short, AT-rich sequence and recruits a complex of DnaC and the DnaB helicase to the origin[2]. Subsequently, DnaC deposits DnaB onto each of the exposed single strands, after which the origin is unwound, and replication begins.

In eukaryotic cells, loading and activation of the helicase occur in two distinct steps[3]. In the G1 phase of the cell cycle, the replication factors origin recognition complex (ORC), Cdc6 and Cdt1 load two MCM2–7 helicases onto DNA, thus leading to the formation of a 'prereplication complex' (pre-RC). Pre-RC assembly is also referred to as 'licensing'. Within pre-RCs, the two MCM2–7 molecules encircle double-stranded DNA (dsDNA) as an inactive dimer. In S phase, MCM2–7 complexes are acted on by S-phase protein kinases and a large number of accessory factors, which together reconfigure MCM2–7 from a dsDNA-binding mode to a single-stranded DNA (ssDNA)-binding mode, thus rendering the helicase active for origin unwinding[4–6]. The separation of replication initiation into two temporally distinct steps, helicase loading and activation, allows these events to be differentially regulated in such a way as to prevent rereplication. Thus, in S phase, helicases are activated, but licensing is strictly prohibited, owing to a variety of overlapping mechanisms including Cdt1 proteolysis, inhibition of Cdt1 by Geminin and inhibition of ORC and Cdc6 by cyclin-dependent kinases (CDKs)[7]. As a result, MCM2–7 complexes that travel away from the origin during replication cannot be replaced for a second round of initiation. Although the mechanism of MCM2–7 activation in S phase remains mysterious, a model of pre-RC formation is starting to emerge, primarily from recent biochemical and structural studies in budding yeast. The work suggests that recruitment of the first MCM2–7 helicase onto DNA is analogous to the well-studied clamp-loading reaction of *E. coli*, although the parallels between these pathways remain vague. How the second MCM2–7 is recruited remains contentious. Here, we draw explicit comparisons between pre-RC formation and clamp loading and argue for a model in which the two MCM2–7 complexes are recruited by two rounds of essentially the same mechanism.

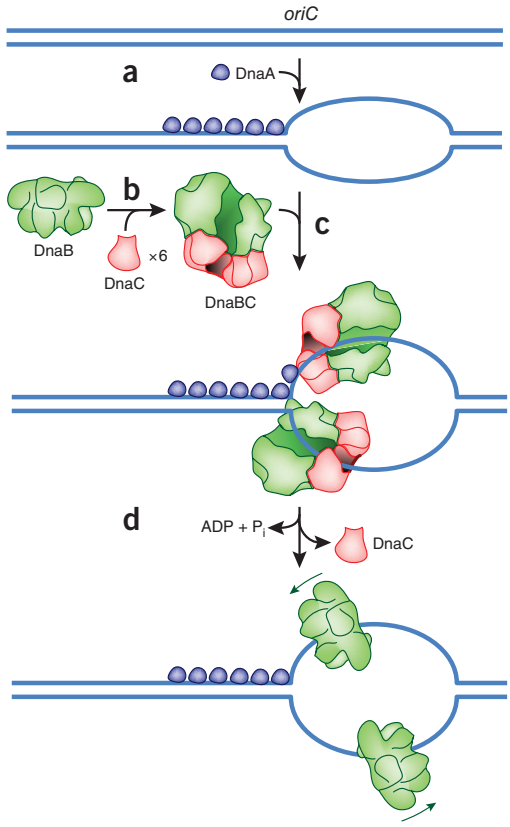


**Figure 1** The mechanism of helicase loading in bacteria. (**a**) The initiator DnaA binds to *oriC*, thus leading to DNA melting. (**b**) DnaB assembles with DnaC, thus leading to opening of the DnaB ring. (**c**) DnaA recruits the DnaB–DnaC complex to origins, where it assembles around ssDNA. (**d**) DNA-induced ATP hydrolysis promotes disassembly of DnaC, thus leaving DnaB encircling DNA. $P_i$, inorganic phosphate.

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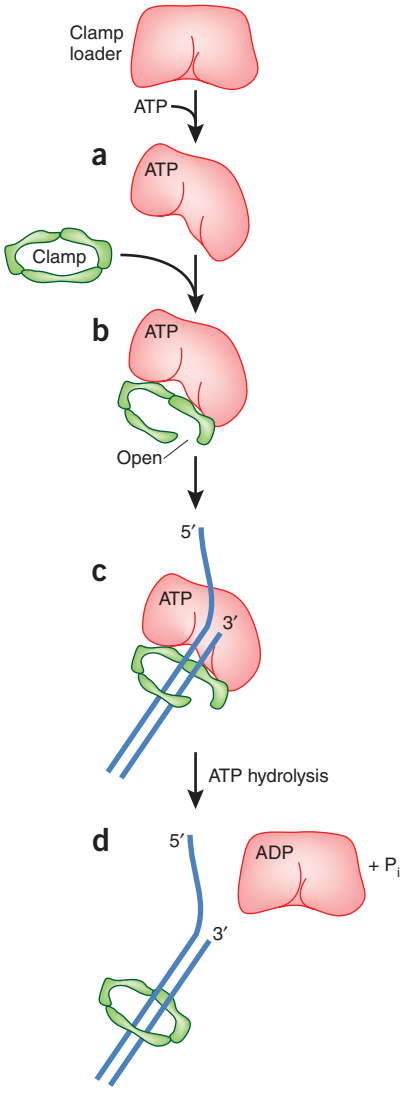


**Figure 2** The mechanism of clamp loading. Upon ATP binding, the clamp loader transitions from a planar conformation into a right-handed helical conformation. (**a**) The clamp loader binds to ATP and changes conformation. (**b**) The ATP-bound form of the clamp loader interacts with and forces open the clamp. (**c**) The complex of the clamp and clamp loader binds to a primer template. (**d**) Once bound to DNA, the clamp loader undergoes ATP hydrolysis, thus leading to dissociation from the clamp and leaving the clamp encircling the DNA.

## The clamp-loading paradigm

Sliding clamps are ring-shaped molecules that encircle DNA and confer processivity on DNA polymerases. Clamp loading is catalyzed by AAA+ ATPases called clamp loaders, whose five subunits are arranged in a ring[8]. Upon ATP binding, the clamp loader adopts a right-handed helical conformation and uses its AAA+ domains to make extensive contact with and thus stabilize the open form of the clamp (**Fig. 2a**,**b**). When primer-template DNA binds to the clamp–clamp loader complex (**Fig. 2c**), the loader hydrolyzes ATP (**Fig. 2d**). As a result, the clamp loader snaps back into a state that cannot bind the clamp. Dissociation of the clamp from the loader in the context of a primer template leaves the clamp encircling DNA.

A recent EM structure of the DnaB–DnaC complex illuminates how the ATPase DnaC loads the DnaB helicase onto ssDNA[9]. Whereas DnaB alone exists as a planar, closed ring[10], when complexed with DnaC it adopts a right-handed spiral with a crack in the ring ('lock-washer' conformation; **Fig. 1b**) that is proposed to act as a gate through which ssDNA can enter[9]. Thus, the effect of DnaC on DnaB is similar to that of clamp loaders on sliding clamps. However, there are also important differences. Unlike the pentameric clamp loaders, which form stable complexes on their own, DnaC assembles into a hexameric spiral only in the presence of DnaB[11] (**Fig. 1b**). In addition, DnaC binds DnaB independently of ATP; ATP hydrolysis is stimulated by ssDNA and promotes dissociation of DnaC after DnaB loading onto DNA[12] (**Fig. 1c**). Consistently with this, DnaC contacts DnaB through its N-terminal domains, not through its AAA+ ATPase folds[13]. In summary, the broad strategy of DnaB and clamp loading are similar, but the underlying molecular details differ considerably.

## Pre-RC assembly: loading of the first MCM2–7 complex

The mechanism of MCM2–7 loading is more complicated than that of DnaB loading, and the parallels with clamp loading remain vague. Below, we summarize the current understanding of pre-RC formation and make explicit comparisons with the clamp-loading reaction. We also discuss several models to explain how the first and second MCM2–7 complexes are loaded onto the origin.

**Binding of ORC and Cdc6 to DNA.** Pre-RC formation begins when ORC binds DNA (**Fig. 3**). In budding yeast, ORC binds in an ATP-dependent manner to an AT-rich motif called the ARS consensus sequence (ACS)[14]. In higher eukaryotes, ORC binding to DNA is much less specific[15,16], and the mechanism of origin recognition remains an active area of inquiry[17]. ORC comprises six subunits (Orc1–Orc6), five of which (Orc1–Orc5) exhibit homology to AAA+ ATPases while also containing winged-helix DNA-binding domains[18–20]. Orc1, Orc4 (in metazoans) and Orc5 contain functional ATP-binding sites, although the site in Orc5 is not essential for viability in yeast[21]. Orc4 contributes an essential catalytic arginine to complete the ATPase active site of Orc1 (ref. 22). Unlike Orc1–Orc5, Orc6 has no homology to ATPases but resembles the general transcription factor IIB[23,24]. Notably, the binding of ORC to DNA suppresses its intrinsic ATPase activity, perhaps to avoid futile hydrolysis until pre-RC formation is more advanced[21,25].

Cdc6 is another AAA+ protein with homology to Orc1. In G1, Cdc6 binds to the ORC–DNA complex (**Fig. 3**). The ATPase activity of Cdc6, which destabilizes the ORC-Cdc6 interaction, is inhibited by origin DNA[20]. Cryo-EM indicates that ORC, when bound to DNA, ATPγS and Cdc6, forms a crescent-shaped assembly whose subunits are arranged in the following order around the arc: Orc3, Orc2, Orc5, Orc4 and Orc1 (ref. 26). Cdc6 completes the arc by interacting with Orc3 and Orc1 to yield a ring-shaped assembly. Interestingly, all subunits that have been implicated in ATP binding or hydrolysis (Orc1, Orc4, Orc5 and Cdc6) reside on one side of the ring. Although DNA is not visible in the structure, the winged-helix and ATPase DNA-binding domains of Orc1–Orc5 are positioned toward the center of the crescent. This suggests that DNA is nestled within the center of the structure (**Fig. 3**). The structure also predicts that the ATPase domain of Orc1 and the arginine finger of Orc4 are too far apart to interact, implying that a conformational change is required to promote hydrolysis. In summary, the ORC–Cdc6 complex organizes six proteins containing AAA+ domains, three of which can hydrolyze ATP, into a circular structure, which is reminiscent of the RFC clamp-loading machine. The difference in the number of ATPase subunits in ORC–Cdc6 (six) versus the clamp loader (five) may be due to the different templates that each complex recognizes, i.e., dsDNA versus ssDNA-dsDNA junctions.

**Cdt1–MCM2–7 docking.** In the next step of pre-RC formation, MCM2–7 and Cdt1 interact with ORC–Cdc6–DNA (**Fig. 3**). MCM2–7

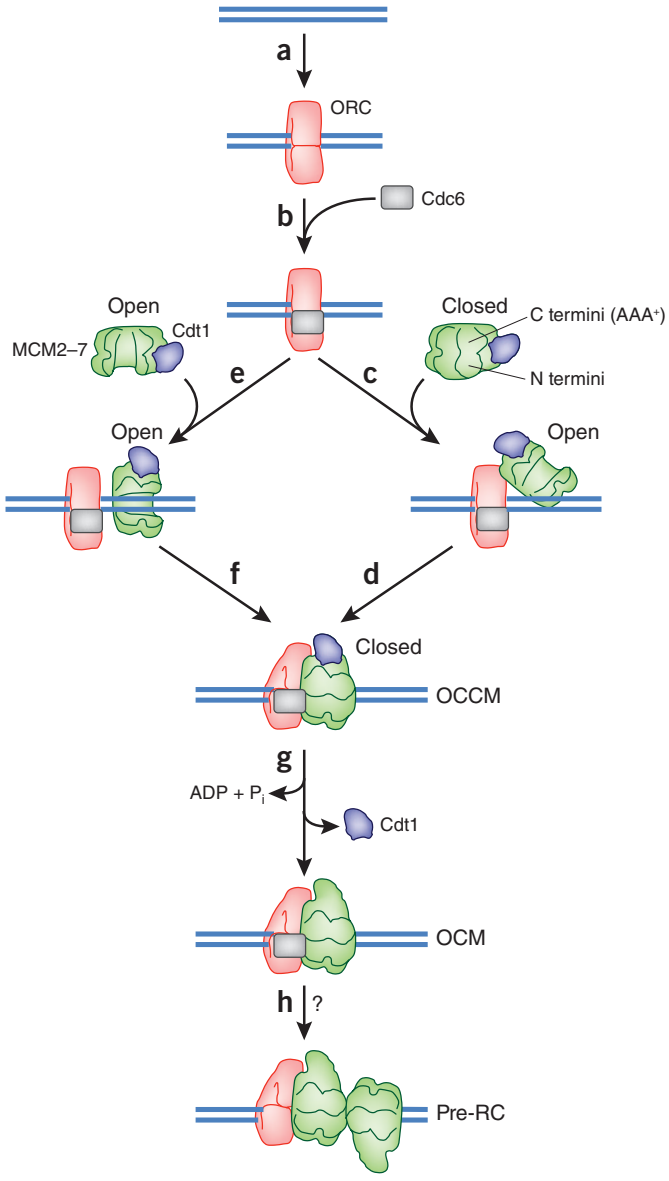


**Figure 3** Binding of the first MCM2–7 to the origin. (**a**,**b**) ORC binds to DNA (**a**) and recruits Cdc6 (**b**). (**c**–**h**) Two mechanisms for the recruitment of MCM2–7. Right, if MCM2–7–Cdt1 is in a closed conformation, it must form a partial contact with ORC–Cdc6 to open the MCM2–7 ring (**c**) so that DNA can enter the MCM2–7 ring, thus allowing MCM2–7 to stack coaxially with the ORC–Cdc6 complex for OCCM formation (**d**). Left, if MCM2–7–Cdt1 is normally open, it could accommodate DNA in its central channel (**e**) and then slide toward ORC–Cdc6 for OCCM formation (**f**). (**g**) ATP hydrolysis promotes dissociation of Cdt1 and conversion of OCCM to OCM. (**h**) A second MCM2–7 complex associates with OCM for MCM2–7 double-hexamer formation, the details of which are described in **Figure 4**.

consists of six related AAA+ ATPase proteins, Mcm2–Mcm7, that form a two-tiered ring in which the larger tier is composed of the C-terminal AAA+ domains, and the smaller tier contains the N-terminal domains (**Fig. 3**)[27–30]. The MCM2–7 ring appears to contain a dynamic 'gate' between the Mcm2 and Mcm5 subunits that allows MCM2–7 to engage with closed circular DNA[27,31]. Even though ATP induces closure of the gate, Cdt1 and Cdc6 may allow gate opening during licensing. In budding yeast, MCM2–7 and Cdt1 form a stable complex that docks onto the ORC–Cdc6–DNA complex as a unit[32]. Cdt1 appears to facilitate recruitment of MCM2–7 to ORC–Cdc6 by inducing a conformational change within MCM2–7 that relieves an autoinhibitory activity of Mcm6 (refs. 33,34). Cryo-EM shows that Cdt1 interacts primarily with Mcm2 but also with Mcm5 and Mcm6, consistent with it regulating the MCM2–7 gate[35]. The initial recruitment of MCM2–7 to ORC–Cdc6–DNA involves an interaction between the extreme C terminus of Mcm3 and Cdc6, but prolonged binding also requires Cdt1 (refs. 33,36). The resulting ORC–Cdc6–Cdt1–MCM2–7 complex has been called OCCM (**Fig. 3**). Cdt1 dissociates rapidly from the OCCM, thus yielding the OCM, which contains a single copy each of ORC, Cdc6 and MCM2–7. Finally, in a slow step, a second MCM2–7 complex is recruited, thus yielding a salt-resistant, head-to-head MCM2–7 double hexamer that is considered to be competent for replication initiation[30,33,37–40].

The role of ATP in pre-RC formation is an ongoing area of intense investigation. Although the presence of ATP is sufficient to support MCM2–7 recruitment into the OCCM, its hydrolysis is essential for the ejection of Cdt1 from the OCCM and its conversion to OCM as well as the formation of the salt-stable double hexamer[22,33,36,40–42]. Which pre-RC components must hydrolyze ATP during pre-RC formation remains an open question. In an extract-based system, it has been shown that ATP hydrolysis by Cdc6 is important for MCM2–7 loading[40], whereas ATP hydrolysis by Orc1 is required only for repeated rounds of MCM2–7 loading (as seen by a mutation in the Orc4 arginine finger)[22] and perhaps serves to reactivate ORC after each round of loading[41]. In reconstituted pre-RC assembly reactions, ATP hydrolysis by Cdc6 appears to be less critical, because certain hydrolysis-deficient mutants support MCM2–7 loading, albeit at reduced levels[33]. It will be interesting to explore whether extracts contain inhibitory activities that accentuate the need for Cdc6 hydrolysis. ATP hydrolysis by MCM2–7 is not required for pre-RC assembly in *Xenopus* egg extracts[43], but its role in yeast is still under investigation. More work is required to determine the precise role of ATP hydrolysis in pre-RC formation.

Interestingly, when pre-RC assembly proceeds abnormally, for example in the absence of Cdt1 or Orc6, or when ORC is phosphorylated by CDK (which blocks pre-RC assembly *in vivo*), ATP hydrolysis disengages MCM2–7 from ORC[33,36,44]. Therefore, when critical licensing factors are absent or during cell-cycle stages in which licensing is not allowed, MCM is actively removed from the origin.

A recent cryo-EM structure of the OCCM reveals that Cdt1–MCM2–7 stacks on top of ORC–Cdc6 in a coaxial arrangement (**Fig. 3**)[35]. The AAA+ domains of each subassembly face each other, thus leaving the N-terminal domains of MCM2–7 available to interact with a second MCM2–7 hexamer. Upon binding to Cdt1–MCM2–7, ORC–Cdc6 undergoes substantial conformational changes[26]. Specifically, the AAA+ domains reach out to interact with MCM2–7, and ORC–Cdc6 forms a right-handed spiral whose pitch matches that of B-form DNA, thus suggesting that DNA might settle within the spiral (**Fig. 3**). Strikingly, a central channel that traverses the entire length of the OCCM contains electron density. If this density represents dsDNA, then the MCM2–7 complex within OCCM already encircles DNA (**Fig. 3**).

In the clamp-loading paradigm, the clamp loader stacks with the clamp, thus imposing on it an open conformation that allows subsequent engagement with primer-template DNA (**Fig. 2**)[8]. The stacking of Cdt1–MCM2–7 with ORC–Cdc6 is reminiscent of this interaction. However, in pre-RC assembly, the clamp loader (ORC–Cdc6) is thought to bind DNA *before* interacting with the clamp (Cdt1–MCM2–7) (comparison of **Figs. 2b** and **3c**,**e**). Because this order of events departs from the canonical clamp-loading paradigm, it should be explicitly tested, for example by covalent tethering of ORC to DNA before addition of Cdc6 and Cdt1–MCM2–7 and determination of whether OCCM assembly still proceeds in the absence of a free ORC pool. If this sequence of

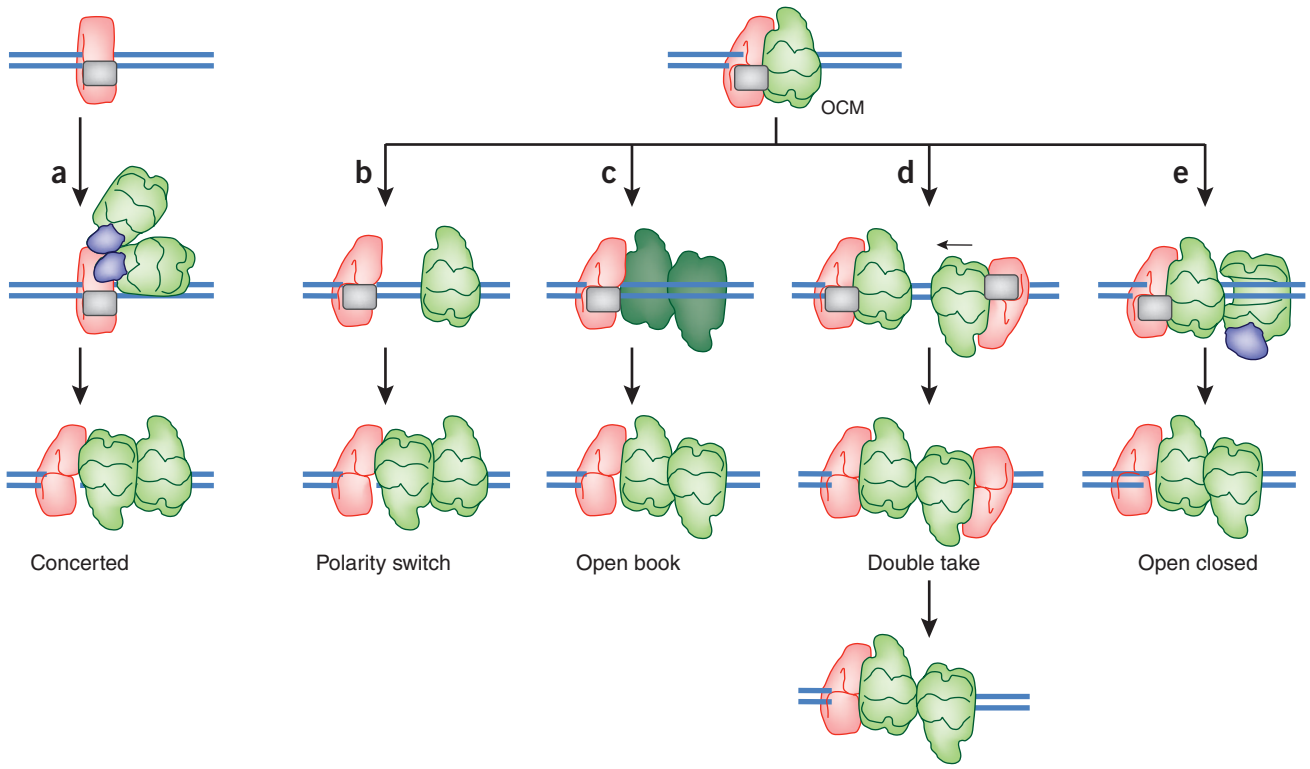


**Figure 4** Possible mechanisms for binding of the second MCM2–7 to the origin. (**a**–**e**) Five possible models for double-hexamer formation. The first model (**a**), in which two MCM2–7 complexes are loaded simultaneously, does not involve the OCM intermediate. The other four models (**b**–**e**) involve conversion of the OCM to a double hexamer. Complexes are depicted as in **Figure 3**. See main text for details.





events is correct, it raises a topological problem, because DNA might interfere with the ability of ORC to stack with MCM2–7. We predict two solutions. First, an initial contact between ORC–Cdc6–DNA and Cdt1–MCM2–7 that is not blocked by DNA would open the MCM2–7 ring (**Fig. 3c**), thus allowing entry of DNA into the MCM2–7 channel and subsequent full engagement of the ORC and MCM modules (**Fig. 3d**), as seen in the OCCM structure[35]. In a second possibility, the Cdt1–MCM2–7 module would be constitutively open. Thus, Cdt1–MCM2–7 would dock freely onto DNA adjacent to the ORC–Cdc6–DNA complex (**Fig. 3e**). Merging of the ORC–Cdc6 and Cdt1–MCM2–7 modules on DNA would induce closure of MCM2–7, thus yielding the OCCM (**Fig. 3f**). Subsequent ATP hydrolysis would eject Cdt1, thus making the reaction irreversible (**Fig. 3g**). This 'open-closed' model represents a notable departure from canonical clamp loading, because the clamp loader closes rather than opens the clamp. In possible agreement with this idea, MCM2–7 can adopt an open lock-washer conformation[27,45]. However, in the presence of ATP it appears to be closed[27,31]. The key question is whether Cdt1–MCM2–7 bound to ATP contains an opening that can admit DNA, although the lack of reported DNA binding by MCM2–7–Cdt1 seems to disfavor this possibility. On balance, the pathway depicted in **Figure 3c**,**d** appears more likely.

## Loading of the second MCM2–7 complex

Over time, a second MCM2–7 complex associates with the OCM, thus generating a salt-resistant MCM2–7 double hexamer in a process accompanied by Cdc6 dissociation[22,33]. Highly divergent models have been discussed to explain how the second MCM2–7 helicase is loaded[46,47].

**Single-ORC models.** Three models are based on the premise that a single ORC, stably bound at the ACS, recruits both MCM2–7 hexamers. In the 'concerted' model, ORC would lead to the simultaneous loading of two MCM2–7 complexes (**Fig. 4a**). Specifically, two molecules of Cdt1 are proposed to interact with two independent Cdt1-binding domains within Orc6 (refs. 46,48). However, concerted loading appears to disagree with the recent observation that binding of the first and second MCM2–7 complexes can be temporally uncoupled[33]. In the 'polarity-switch' model, ORC would recruit two MCM2–7 complexes sequentially (**Fig. 4b**). After loading of the first MCM2–7 complex as part of the OCM, the first MCM2–7 would slide away from ORC. ORC would then recruit a second MCM2–7 in the opposite orientation, thus enabling formation of a head-to-head MCM2–7 dimer. How such a polarity switch would take place is difficult to conceive. In a third model (**Fig. 4c**), the first MCM2–7 complex, once loaded into the OCM, would fold open like a book[47]. Each half complex of three subunits would then recruit the appropriate missing subunits, thus yielding two complete, head-to-head hexamers. Although fantastically inventive, this 'open-book' model invokes conformational changes that are unlikely to be energetically favorable.

**The 'double-take' model.** In a fundamentally different, double-take model, pre-RC assembly would involve two rounds of identical MCM2–7 loading by two ORC–Cdc6 complexes. One ORC–Cdc6 module would load the first MCM2–7 complex. A second ORC would then bind on the other side of the first MCM2–7, in an orientation opposite to the first ORC, and load a second MCM2–7 complex (**Fig. 4d**). On the basis of work in *Xenopus* egg extracts, ORC does not have to bind a specific sequence to load MCM2–7 (ref. 16). In support of the model, MCM2–7 is flanked by two ORC molecules at some origins of replication[49,50]. This model is further consistent with observations that loading of the first and second MCM2–7 complexes involves the same domains of Cdt1 and MCM2–7 (refs. 36,51). The model suggests that the first MCM2–7

complex loaded fully encircles DNA, in agreement with the structure of the OCCM complex[35]. However, the first MCM2–7 becomes resistant to high salt only after it interacts with a second MCM2–7 molecule. Indeed, much of the stability of the double hexamer comes from protein-protein interactions of the N-terminal head domains, because these hold the complex together even after DNA has been removed[30,38].

Where would the second ORC molecule bind? Yeast origins coincide with a nucleosome-free region (NFR) that is ~125 bp long—leaving enough room to accommodate two ORC and two MCM2–7 molecules[26,38]. ORC binds the ACS at one end and MCM2–7 in the middle of the NFR[52]. Therefore, the second ORC would have to bind at the other end of the NFR, near the B3 element of the ARS1 replication origin[14]. The second ORC molecule might bind transiently and/or at a greater distance from the ACS and therefore would not be readily detectable via chromatin immunoprecipitation. The second MCM2–7, once bound, might slide along DNA[30,38] until meeting the first MCM2–7 in a head-to-head embrace. Single MCM2–7 complexes that do not find a partner would eventually dissociate. Cooperativity might promote double-hexamer formation. Thus, once bound, the first MCM2–7 helicase might help recruit a second ORC, preferably in an inverted orientation

**The open-closed model.** In a fifth possibility, ORC–Cdc6 might not be required for loading of the second MCM2–7 complex. This idea extends the open-closed mechanism (presented in **Fig. 3e**,**f**) in which Cdt1–MCM2–7 contains a constitutively open channel. After loading of the first MCM2–7 complex, a second Cdt1–MCM2–7 module would bind DNA and engage with the previously bound complex (**Fig. 4e**). This model seems to agree with the observation that the free pool of ORC–Cdc6 can be removed without affecting the second round of MCM2–7 loading[33], although ORC might dissociate from DNA and reassociate for recruitment of the second MCM2–7 complex. This possibility could be addressed by covalent attachment of ORC to ARS DNA and determination of whether the second MCM2–7 loads in the absence of free ORC. A potential drawback of the open-closed model is that at some frequency, MCM2–7 double hexamers might form independently of ORC–Cdc6, thus leading to rereplication.

## Final thoughts

The coaxial stacking of the Cdt1–MCM2–7 and ORC–Cdc6 AAA$^+$ ATPase modules in the OCCM strongly supports the long-standing notion that pre-RC assembly parallels clamp loading. However, unlike classical clamp loaders, ORC–Cdc6 recruits two MCM2–7 clamps instead of one, and it is likely to be bound to DNA *before* binding Cdt1–MCM2–7. Therefore, there must be differences between the two mechanisms. In the double-take model (**Fig. 4d**), which involves two rounds of a classic clamp-loading reaction, the only substantial innovation is an ORC–MCM2–7 intermediate that overcomes the interference by DNA (**Fig. 3c**). In contrast, the other models suggest radical departures from canonical clamp loading. Because evolution is inherently conservative, we favor the double-take model. Given the extremely rapid progress in the field, a more complete picture of pre-RC assembly is likely to emerge soon, including the role of ATP hydrolysis by the various pre-RC components.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.



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