Structural basis of Mcm2–7 replicative helicase loading by ORC–Cdc6 and Cdt1

Zuanning Yuan1,7, Alberto Riera2,3,7, Lin Bai1,7, Jingchuan Sun1, Saikat Nandi4, Christos Spanos5, Zhuo Angel Chen5, Marta Barbon2,3, Juri Rappsilber5,6, Bruce Stillman4, Christian Speck2,3 & Huilin Li1

To initiate DNA replication, the origin recognition complex (ORC) and Cdc6 load an Mcm2–7 double hexamer onto DNA. Without ATP hydrolysis, ORC–Cdc6 recruits one Cdt1-bound Mcm2–7 hexamer, thus forming an ORC–Cdc6–Cdt1–Mcm2–7 (OCCM) helicase-loading intermediate. Here we report a 3.9-Å structure of Saccharomyces cerevisiae OCCM on DNA. Flexible Mcm2–7 winged-helix domains (WHDs) engage ORC–Cdc6. A three-domain Cdt1 configuration embraces Mcm2, Mcm4, and Mcm6, thus comprising nearly half of the hexamer. The Cdt1 C-terminal domain extends to the Mcm6 WHD, which binds the Orc4 WHD. DNA passes through the ORC–Cdc6 and Mcm2–7 rings. Origin DNA interaction is mediated by an α-helix within Orc4 and positively charged loops within Orc2 and Cdc6. The Mcm2–7 C-tier AAA+ ring is topologically closed by an Mcm5 loop that embraces Mcm2, but the N-tier-ring Mcm2-Mcm5 interface remains open. This structure suggests a loading mechanism of the first Cdt1-bound Mcm2–7 hexamer by ORC–Cdc6.

The S. cerevisiae ORC is an ATPase complex composed of Orc1–6 (refs. 1–3). The composition and architecture of ORC are conserved in all eukaryotes4. Low-resolution electron microscopy (EM) images showed that the six subunits are arranged into a crescent shape in the order Orc1, Orc4, Orc5, Orc3, Orc2, with Orc6 binding to Orc2/Orc3 (refs. 5, 6). This architecture was confirmed by a recent crystal structure of an inactive Drosophila ORC (DmORC)7. The DmORC core is a notched two-tiered ring composed of an N-tier ring of five AAA+ domains and a C-tier ring of five WHDs of Orc1–5. Because the DmORC1 AAA+ domain blocks the putative central DNA-binding channel in the autoinhibited conformation7, the configuration of an active ORC could not be determined. Replica gradients are bound by the budding yeast ORC throughout the cell-division cycle1, but they are ‘licensed’ during the G1 phase8. An early step is the binding of initiation factor Cdc6 to DNA-bound ORC to form the ORC–Cdc6–DNA complex9. EM studies have shown that Cdc6 closes a gap in the crescent-shaped ORC to form a ring9,10 and apparently activates a molecular switch in ORC, converting it from an origin-DNA binder to an active Mcm2–7 loader6,9. However, the physical nature of the molecular switch is currently unknown owing to the lack of a high-resolution structure of ORC–Cdc6 on DNA.

The next steps involve the sequential recruitment of two Cdt1-bound hexamers of Mcm2–7 onto the origin DNA by ORC–Cdc6 to form an Mcm2–7 double hexamer (DH) that composes part of the pre-replication complex (pre-RC)6,11. In vitro reactions with purified components have demonstrated that a high salt-stable Mcm2–7 DH is loaded on DNA in an ATP-dependent manner12,13. All hexamers within the DH are assembled in such a way that their respective Mcm3 and Mcm6 subunits face each other, as revealed by EM images of maltose-binding protein (MBP)-tagged DH as well as a 3.8-Å resolution cryo-EM structure14,15. The Mcm2–7 DH has a central channel that is wide enough to allow the passage of double-stranded DNA (dsDNA), in agreement with biochemical findings12,13. Because the two Mcm2–7 hexamers are twisted with respect to each other, it was speculated that within the interface between the two hexamers, an inflection point of the DNA path is created, possibly promoting melting of the dsDNA when the helicase becomes activated in S phase14. At the G1-to-S transition, the inactive DH is converted into an active replicative helicase that consists of an Mcm2–7 hexamer bound to Cdc45 and the four-subunit GINS complex, called the CMG16,17. Disruption of the Mcm2–7 DH and assembly of the CMG require activation by the Dbf4–Cdc7 protein kinase and cyclin-dependent kinase (Clb5–Cdc28), which phosphorylate some of the pre-RC components, including Mcm2–7 subunits and the accessory loading proteins Sld2 and Sld3 (refs. 1, 8, 18–26). Subsequently, primase and DNA polymerases load, along with many other replication factors, to form the replisome that executes DNA synthesis27,28.

The two Mcm2–7 hexamers are loaded on DNA sequentially15,29,30. In the presence of ATP/S, ORC–Cdc6 loads the first Mcm2–7 hexamer on DNA, thus forming the OCCM intermediate15,31. Then ATP hydrolysis is triggered, and Cdc6 and Cdt1 are released30,32,34. Single-molecule analysis suggests that a second Cdc6 protein is

1Cryo-EM Structural Biology Laboratory, Van Andel Research Institute, Grand Rapids, Michigan, USA. 2MRC London Institute of Medical Sciences (LMS), London, UK. 3DNA Replication Group, Institute of Clinical Sciences (ICS), Faculty of Medicine, Imperial College London, London, UK. 4Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA. 5Wellcome Trust Centre for Cell Biology, University of Edinburgh, Edinburgh, UK. 6Chair of Bioanalytics, Institute of Biotechnology, Technische Universität Berlin, Berlin, Germany. 7These authors contributed equally to this work. Correspondence should be addressed to J.R. (juri.rappsilber@ed.ac.uk), B.S. (stillman@cshl.edu), C. Speck (chris.speck@imperial.ac.uk) or H.L. (Huilin.Li@vai.org).

Received 2 December 2016; accepted 9 January 2017; published online 13 February 2017; doi:10.1038/nsmb.3372
recruited to the ORC, which functions to load a second Cdt1-bound Mcm2–7 to form an ORC–Cdc6–Mcm2–7–Mcm2–7 complex before Mcm2–7 DH formation\textsuperscript{15,30,35}. In vivo evidence suggests that ATP hydrolysis by Cdc6 causes the separation of the DH from ORC–Cdc6 (ref. 36). In this work, we describe a 3.9-Å-resolution cryo-EM structure of the 1.1-MDa 14-protein OCCM complex on DNA, as well as mass-spectrometry-based observations of interactions between individual proteins. The structure reveals how ORC–Cdc6 recognizes origin DNA and how this complex recruits the first Cdt1-bound Mcm2–7 hexamer, thereby illuminating a crucial step in eukaryotic DNA-replication initiation.

**RESULTS**

**Overall structure of the OCCM–DNA complex**

We prepared the OCCM complexes in the presence of ATP\textsubscript{γS} from purified proteins on a replication-origin-containing plasmid attached to magnetic beads. After DNase I treatment, the OCCM samples were released from the beads and directly processed for cryo-EM grid preparation. We derived a 3.9-Å-resolution cryo-EM 3D map of the OCCM from 304,288 particles that were selected from 7,500 raw electron micrographs, and nearly 1,000,000 raw particles that were recorded on a K2 camera in a Titan Krios microscope operated at a high tension of 300 kV (Fig. 1a–c, Supplementary Figs. 1–3, Table 1, Supplementary Video 1, and Online Methods). The 3D map had well-defined densities for the dsDNA and 13 subunits of the 14-protein complex (Fig. 1d,e). The only protein that was not well resolved was Orc6; its density was visible only at a lower display threshold. Nevertheless, a conserved C-terminal α-helix of Orc6 was resolved, which is important, as it is mutated in Meier–Gorlin syndrome\textsuperscript{37,38}. To better understand the architecture of the complex, and particularly of the flexible sections involving Orc6, we carried out cross-linking/mass spectrometry (CLMS) analysis, which confirmed the general architecture and also identified several interactions between Orc6 and Orc2/Mcm2 (Fig. 2a–c, Supplementary Fig. 4, and Supplementary Data Sets 1 and 2). We built an atomic model of the OCCM into the EM densities, with guidance from the published structures of Orc1 and Orc2, in agreement with a previous, lower-resolution EM study\textsuperscript{5} (refs. 40–42). The dsDNA was manually built into the EM density (Supplementary Fig. 5). Electron densities for eight nucleotides were observed at the Mcm2–Mcm6, Mm6–Mcm4, Mm4–Mcm7, Mm7–Mcm3, Cdc6–Orc1, Orc1–Orc4, Orc4–Orc5, and Orc5–Orc3 interfaces (Supplementary Fig. 6). No nucleotide densities were resolved between Mcm3 and Mcm5 or between Mcm5 and Mcm2 because of the reduced resolution and increased flexibility, as they were either at or near the DNA-loading gate.

In the top ORC–Cdc6 tier of the OCCM, Orc1–5 and Cdc6 formed a six-membered ring structure, with Cdc6 bridging the gap between Orc1 and Orc2, in agreement with a previous, lower-resolution EM study\textsuperscript{6} (Fig. 3). Interestingly, four WHDs of Mcm3, Mm4, Mm6, and Mm7 spiraled upward from the Mcm2–7 hexamer and engaged the ORC–Cdc6 ring, each interacting with ORC–Cdc6 subunits (Fig. 1d,e and Supplementary Fig. 7), and we observed an interaction between the Mcm5 WHD and Orc2 via CLMS (Fig. 2c). In the bottom Cdt1–Mcm2–7 tier, the six Mcm subunits formed a ring structure (order: Mcm2, Mcm6, Mm4, Mm7, Mm3, Mm5), in agreement with previous studies\textsuperscript{14,15,22,43,44}. At the interface between the Mcm2 AAA+ domain and the Mcm5 AAA+ domain, the domain-swapped α-helix of Mcm5 bound to the Mcm2 AAA+ domain, thereby topologically closing the DNA-loading gate (Fig. 1c,d and Supplementary Fig. 8).

However, the N-tier ring was still open at the Mcm2–5 interface. Hence Mcm2–7 was in a half-open, half-closed state. This structural feature explains why the Mcm2–7 ring in the OCCM is partially salt stable on DNA, but not as salt stable as the Mcm2–7 DH\textsuperscript{15,31,44}. The structure also explains why the DNA is intact in the C-tier AAA+ ring but invisible in the N-tier ring, as DNA in this region may be less constrained or be digested by the nucleases used to release the DNA-bound OCCM during sample preparation.

**ORC–Cdc6 forms a closed ring with a pseudo-six-fold symmetry**

In the OCCM structure, ORC–Cdc6 assembled into a complete ring encircling DNA with a pseudo-six-fold symmetry (Figs. 3a,b and 4). The N-terminal extension on Orc2 and an insertion in Orc3 give the ORC a helical shape\textsuperscript{44}. The four observed ATP\textsubscript{γS} molecules of ORC–Cdc6 define one circle on top, and the four observed nucleotides in Mcm2–7 define another circle below. The top circle is larger, off center, and tilted by 17° with respect to the lower circle (Fig. 4a,b). The six predicted AAA+ proteins, Orc1–5 and Cdc6, all had one AAA+ domain with an AAA-RecA fold (the RecA fold), an α-helical-lid domain (the lid), and a C-terminal α-helical WHD, and they were superimposable\textsuperscript{45,46} (Fig. 3c). There were variations on this general rule; for example, Orc2 lacked the α-helical lid, similar to DmOrc2, and Orc3 had an insertion consisting of a helical domain. Among the six initiator AAA+ subunits, Orc4 was unique in that it had one α-helix insertion and one insertion loop in the WHD. The six AAA-RecA folds and the six WHDs formed a two-tiered ring.
structure that surrounded the DNA within the central channel. In contrast, the six AAA-lid domains that each bridged the AAA-RecA-like domain and the WHD formed an outer brace that spiraled around the DNA-interacting domains. The bottom-tier ring of the WHDs was largely responsible for interacting with the Mcm CTDs below, as suggested in a previous study. The peripheral brace of the AAA lids also interacted with the Mcm subunit WHDs.

The crystal structure of the DmORC core revealed a conformation that is not compatible with DNA binding in S. cerevisiae ORC and DmORC. By aligning the two ORC structures, using the common Orc3--5 region as a reference, we found that the RecA fold of DmOrc1 and the WHD of DmOrc2 needed to move and flip by ~180° in order to match their respective yeast counterparts (Fig. 3e). These changes created a gap between Orc1 and Orc2 for DNA passage as well as for Cdc6 insertion between Orc1 and Orc2 after DNA binding by ORC (Fig. 3b, d and Supplementary Video 2). Because the Mcm2--7 hexamer had been loaded onto DNA by ORC--Cdc6, the conformation of ORC--Cdc6 in the OCCM structure is clearly in its active form, thus allowing DNA binding and both Cdc6--Orc1 and Orc1--Orc4 ATPase activities that are required for Mcm2--7 DH assembly or subsequent regulated initiation of DNA replication once per cell-division cycle.

### Cdt1 forms an extended three-domain structure

In the OCCM structure, Cdt1 exists in an unusually extended three-domain structure (Fig. 5a--c). The density of the Cdt1 NTD was relatively weak, which indicated a degree of flexibility. The Cdt1 NTD bound only to the Mcm2 CTD with an interface of ~600 Å². Surprisingly, the Cdt1 CTD was linked to the MHD by a long loop and was 60 Å away from the MHD, located between Mcm6 and Mcm4. In contrast, the Cdt1 MHD bound to both the NTD and the CTD of Mcm2 as well as to the NTD of Mcm6 with a larger interface of ~1,000 Å², which was also noted in the CLMS experiment (Fig. 2a). The Cdt1 CTD interacted extensively with all of the major domains of Mcm6, the Mcm4 NTD, and the Orc4 WHD insertion loop. Consistent with this observation, a previous NMR spectroscopy study showed interaction between the Mcm6 WHD and a short peptide in the CTD of Cdt1 (ref. 40). Importantly, the Cdt1 CTD formed an arch extending toward the Mcm6 WHD, which in turn interacted with the Orc4 WHD and the Orc5 AAA lid. The Mcm6 WHD in the Mcm2--7 hexamer before encountering ORC--Cdc6 is likely to be located in the middle of the ring between Mcm2 and Mcm6, because this is where the domain is found both in the active helicase CMG and in the inactive Mcm2--7 DH (Fig. 5d). Hence, the C-terminal arch of Cdt1 is probably responsible for displacing the Mcm6 WHD by 40 Å to the periphery, where the Mcm6 WHD is found in the OCCM structure. It is likely that this conformational change explains the inhibitory role of the Mcm6 WHD, which blocks OCCM formation in the absence of Cdt1 (ref. 32). Therefore, Cdt1 seems to have had a dual role in Mcm2--7 hexamer loading: it created the ORC--Cdc6-binding surface on the C-terminal surface of the Mcm2--7 hexamer by moving the obstructing Mcm6 WHD outward, and at the same time it formed an extended three-domain side brace that stabilized the Mcm2--Mcm6--Mcm4 half-ring, thereby potentially allowing the other half-ring (Mcm5--Mcm3--Mcm7) to move to (Fig. 1d, e). We suggest that these interactions underlie the essential roles of Cdt1 in Mcm2--7 loading on DNA, Cdt1 bound in the OCCM—particularly through its interaction with Mcm2 and the Mcm2--7 subunits that bind ATP (Mcm2, Mcm4, Mcm7, and Mcm3)—may keep the Mcm2--Mcm5 N-tier interface open and prevent Mcm2--7 ATP hydrolysis. ORC--Cdc6 ATPase activity, the next step after OCCM assembly, removes Cdt1 (ref. 30) and may promote Mcm2--7 ATPase activity to close the first Mcm2--7 hexamer.

To further investigate the interactions between Cdt1 and Mcm2--7, we expressed in baculoviruses each Mcm subunit and Cdt1 as Strep--Strep-SUMO-Cdt1 (SSS--Cdt1). Each Mcm subunit alone or all six in combination were expressed, and a pulldown assay with purified SSS-Cdt1 was performed (Supplementary Fig. 8). Cdt1 interacted with all six Mcm proteins when they were expressed together. Individually, Mcm2, Mcm6 (most strongly), and Mcm7 interact with Cdt1. The first two interactions were observed in the OCCM model. We did not see an interaction with Mcm4, which suggests that the Cdt1--CTD interaction with Mcm4 seen in the OCCM structure must depend on prior binding to Mcm2--Mcm6 in the Mcm2--7 hexamer. The interaction with Mcm7 is not present at this stage of OCCM, but it could be functional downstream, when ORC--Cdc6--Mcm2--7 recruits the second Cdt1--Mcm2--7 hexamer to form the DH (Fig. 1e). Indeed, a long Mcm7 α-helix projects down toward the incoming second Cdt1--Mcm2--7 complex (Fig. 1e).

By comparing the Mcm2--7 in the OCCM with that in the Mcm2--7 DH, we found that Mcm2--7 needed to undergo large conformational changes during the OCCM-to-DH transition, in particular within the entire Mcm2--7 NTD ring as well as in the CTDs of Mcm2 and Mcm5 (Fig. 6 and Supplementary Video 3). Specifically, the Mcm2--7 NTD ring needed to rotate by ~25° relative to the Mcm2--7 CTD to match the Mcm ring in the DH, and the CTDs of Mcm2 and Mcm5 had to rotate by ~5° and ~15°, respectively, to form the closed interface in the DH (Fig. 6b, c). Because the ATPase activity of Orc1 and Cdc6 is required during the loading reaction and, because Orc1 and Cdc6 appear in a conformation poised to hydrolyze ATP, it is possible.
Figure 2  CLMS analysis of the *S. cerevisiae* OCCM complex. (a) Linkage map showing the observed cross-linked residue pairs in the Mcm2–7–Cdt1 complex. (b) Linkage map showing the observed cross-linked residue pairs in the ORC–Cdc6 complex. (c) Linkage map showing the observed cross-linked residue pairs between the ORC–Cdc6 complex and the Mcm2–7–Cdt1 complex. Orc6, which was only partially resolved by cryo-EM, is in close proximity to Mcm2 and Cdt1. The WHD of Mcm5, which was only partially resolved by cryo-EM, is in close proximity to the N-terminal region of Orc2. Intramolecular cross-links are in color, and intermolecular cross-links are indicated by black dashed lines. Source data are provided in Supplementary Data Set 1.
Asymmetric interaction between ORC–Cdc6 and Cdt1–Mcm2–7 enables DNA insertion

Although both the Mcm2–7 hexamer and ORC–Cdc6 form ring-like structures with a pseudo-six-fold symmetry, the interaction between the two rings is asymmetrical owing to the ~17° tilt of the ORC–Cdc6 ring with respect to the Mcm2–7 ring (Figs. 1d and 4). As a consequence, the DNA is bent by ~20–25° at the interface. Furthermore, the tilt leads to a tight interface between Orc1–Or4–Or5 and Mcm4–Mcm6–Mcm2, and an apparent 'loose' interface between Orc3–Or4–Cdc6 and Mcm5–Mcm3–Mcm7. At the tight interface, the WHDs of Orc1 and Or4 insert into the gaps between the WHDs and the AAA-lid domains of Mcm4 and Mcm6; the Orc5 WHD interacts only with Mcm5 AAA-RecA-fold, because Mcm2 lacks a WHD (Fig. 1d,e and Supplementary Fig. 7). At the loose interface, the WHD of Mcm5 was not visible in the EM map, but the CLMS data identified it as the across-the-interface partner of Orc2. Interestingly, the WHDs of Mcm3 and Mcm7 reached upward more than 30 Å via their long loops to interact with Orc2 and Cdc6, respectively (Fig. 1f and Supplementary Fig. 7). Accordingly, we propose that the asymmetric interaction leaves half of the Mcm2–7 ring (Mcm5–Mcm3–Mcm7) only loosely tethered, such that the Mcm5–Mcm3–Mcm7 half-ring...
can move away from the tightly tethered Mcm4–Mcm6–Mcm2 half-ring to open up the Mcm2–Mcm5 gate for DNA insertion and then move back to close the gate. Cdt1 is the protein most likely to stabilize this conformation.

**Protein-DNA interactions in the OCCM**

We modeled 39 bp of DNA in the OCCM density map; 24 bp were encircled by the ORC–Cdc6 complex, and the remaining 15 bp were encircled by the C-tier ring of Mcm2–7 (Fig. 7a and Supplementary Fig. 9). There was no apparent DNA density inside the N-tier ring of Mcm2–7. The N-tier ring was open at the Mcm2-Mcm5 interface, possibly because the dsDNA there was digested by the DNase I nuclease used to cleave the loading intermediate off of the plasmid DNA. In the top ORC–Cdc6 region, Orc1, Orc3, and Orc5 had light direct interaction with DNA. DNA was held in place by interactions with the initiator-specific motif (ISM) in the AAA+ domains of Orc2, a unique Orc4-specific insertion α-helix, and by the ISM and the WHDs of Cdc6 (Fig. 7a–e). These four binding components spiraled around the DNA just like RFC clamp loaders spiral around DNA44,49,50, although the overall ORC–Cdc6 ring itself appears flat because the Orc2 and Orc3 subunits break the helical path. Archaeal AAA+ replication initiators also interact with DNA via their respective ISMs39,51. The archaeal WHD is known to bind DNA with both the helix-turn-helix motif and the β-hairpin wing loop51. However, in yeast ORC–Cdc6, we found that only the β-hairpin wing loops of the WHDs of Cdc6 and Orc4 bound to DNA; their respective helix-turn-helix motifs did not bind to DNA and instead were engaged in subunit-subunit interactions. This different DNA-binding mode was caused by an ~90° rotation of the Orc4 and Cdc6 WHDs away from the central DNA channel (Supplementary Fig. 10). We demonstrated previously that ORC–Cdc6 causes a nuclease-protected footprint on the origin DNA that extends to 70–78 bp, larger than the 44–50-bp footprint of ORC alone5. Even allowing for limited nuclease access to the DNA near edges of the ORC–Cdc6 complex, which accounts for 10 bp (ref. 52), the amount of DNA found to interact with ORC–Cdc6 by nuclease footprinting was more than twice that found to interact with ORC–Cdc6 in the OCCM structure. There are positively charged patches on the front side (Orc2, Orc3, and Orc6) and the bottom surface of ORC–Cdc6 proximal to Mcm2–7

---

**Figure 5** Extensive interactions between Cdt1 and the Mcm hexamer. (a) OCCM structure, with Cdt1 electron density shown as blue mesh. The CTD of Cdt1 is located between Mcm6 and Mcm4, more than 60 Å away from the NTD and MHD of Cdt1. (b) Zoomed-in view of the Cdt1 NTD and MHD, showing their interactions with Mcm2 and Mcm6. (c) Zoomed-in view showing the Cdt1 CTD interacting with the Mcm6 WHD in OCCM. The solid red oval marks the Mcm6 WHD in OCCM, and the dashed red oval indicates the position of the Mcm6 WHD in CMG helicase. The blue arrow shows the displacement of the Mcm6 WHD in OCCM due to interaction with the Cdt1 CTD. Such displacement forms an unobstructed Mcm2–7 C-terminal face for binding with ORC–Cdc6. M, Mcm.

**Figure 6** Mcm2–7 conformational changes between OCCM and the DH. (a) Comparison of top CTD (left) and bottom NTD (right) views of the Mcm2–7 structure in the DH (gray subunits) versus in OCCM (colored subunits). The two structures were aligned using the CTDs of Mcm4, Mcm6, and Mcm7 as references. Changes in the CTD ring are focused on Mcm2–Mcm5–Mcm3. The NTD ring rotated en bloc by about 25°. The black and red hexagons outline Mcm2–7 in the DH and in OCCM, respectively. (b) Side views of Mcm2–7 (with Mcm2–5 in front) within OCCM (left) and within the DH (right). Transitioning from OCCM to the DH, each CTD AAA+ domain and NTD of Mcm2 and Mcm5 undergoes a combination of rotation and translation, with the indicated rotation and translation. The Mcm5 NTD needs to rotate by as much as 50° to close the DNA entry gate. (c) A schematic showing how the gate between Mcm2 and Mcm5 can be opened for DNA insertion in OCCM (left) and how the gate is closed in the DH (right). M, Mcm.
H2I, helix-2-insert
Mcm2, Mcm6, Mcm4, and Mcm7. PS1, presensor 1 of Cdc6, make the DNA visible. The five areas framed by red dashed rectangles are parts of Orc1 and Cdc6 and all of Mcm3 and Mcm5, have been omitted to OCCM–DNA structure in side view. Subunits in front of DNA, including in the DNA-replication field has been the question of how ORC must occur in Mcm2–7 in order for the active helicase to form, such strand in the CMG helicase, because extensive conformation changes it is unclear whether this strand functions as the leading or the lagging DNA54. Interestingly, most of these hairpin loops, except for the Mcm4 –hairpin loops, in particular the well-conserved KA motifs (A. Tocilj, K. On, Z. Yuan, J. Sun, E. Elkayam, H. Li, B. Stillman and L. Joshua-Tor, submitted manuscript), and it may help to explain the sequence specificity of ORC that is characteristic of origin binding in Saccharomyces spp. Cdc6 is particularly important for DNA binding with two principal DNA-binding sites, the ISM and the WHD, which explains why Cdc6 enhances the ORC's specificity for DNA.10

The most prominent feature of Mcm2–7 loading by ORC–Cdc6 is the extensive use of WHDs of these replication proteins. There were six resolved WHDs in ORC–Cdc6 that formed a larger winged-helix ring in the upper tier of the OCCM, and five cryo-EM- and CLMS-resolved WHDs in Mcm2–7 that formed a second slightly smaller winged-helix ring on the lower Mcm2–7 tier of the OCCM. This appears to be an evolutionarily conserved interaction, as it was reported recently for archaeal MCM and Orc1 (ref. 55). However, here we observed that the WHD-mediated correct stacking of the two rings mediated much of the recruitment mechanism. The position of the WHD of the Mcm proteins varies widely: it can sit right above the AAA+ domain, or move away from the main body of the protein, or even move to the side of the AAA+ domain in the case of Mcm5, as seen in the active helicase (Supplementary Fig. 7). The CTD of Cdt1 has a special role in displacing the Mcm6 WHD to create the ORC–Cdc6 binding surface; we showed previously that the WHD of Mcm6 is inhibitory and blocks OCCM formation in the absence of Cdt1 (ref. 32). The attachment of the WHDs by flexible linker provides these domains with manifold potential to interact with ORC–Cdc6, but after helicase activation they could also be important interaction partners with other proteins at the DNA replication fork.

The Mcm2–7 DH structure, although obtained in the absence of DNA, showed that six H2I hairpin loops are arranged in an approximately helical trajectory that was suggested to facilitate DNA translocation and unwinding. However, in the OCCM, only three H2I hairpin loops of Mcm2–Mcm4–Mcm6–Mcm7 contact DNA. In addition, the three PS1 hairpin loops of Mcm4, Mcm6, and Mcm7 contact DNA with their respective presensor 1 (PS1) β-hairpin loops, in particular the well-conserved KA motifs (Fig. 7f and Supplementary Fig. 9). In contrast, the same H2I and PS1 within the Mcm2–7 DH are involved in intersubunit interactions14. In the apo form of the active CMG helicase, the PS1 loops face the central DNA channel23, and in a recent cryo-EM structure of CMG at sub-nanometer resolution, the PS1 loops were found to interact with a six single-stranded DNA44. Interestingly, most of these hairpin loops, except for the Mcm4 H2I loop, interacted with the same strand of the duplex DNA (Fig. 7f). It is unclear whether this strand functions as the leading or the lagging strand in the CMG helicase, because extensive conformation changes must occur in Mcm2–7 in order for the active helicase to form, such that Mcm-DNA interaction in the CMG may be very different.

DISCUSSION
Since the discovery of ORC more than two decades ago2, a key issue in the DNA-replication field has been the question of how ORC recognizes dsDNA and cooperates with Cdc6 to load the Mcm2–7 hexamers onto DNA. The current atomic model of the OCCM provides the first high-resolution structure of ORC–Cdc6 bound to origin DNA. Overall, the ORC–Cdc6 structure is flat, but within the structure the subunits that bind ATP, Orc1, Orc4, Orc5, and Cdc6 form a right-handed spiral around the dsDNA, similar to the spiral of AAA+ subunits around primer-template DNA in RFC clamp loaders49,50. The human ORC has an almost identical structure (A. Tocilj, K. On, Z. Yuan, J. Sun, E. Elkayam, H. Li, B. Stillman and L. Joshua-Tor, submitted manuscript). Orc4 has an unusual insertion α-helix that appears to contact the major groove of the dsDNA. This helix is unique to budding yeast Orc4 subunits, being absent in the DmORC and human ORC4 structures7 (A. Tocilj, K. On, Z. Yuan, J. Sun, E. Elkayam, H. Li, B. Stillman and L. Joshua-Tor, submitted manuscript), and it may help to explain the sequence specificity of ORC that is characteristic of origin binding in Saccharomyces spp.
AAA+ complex loads a ring-shaped, multisubunit complex of proteins to dsDNA to promote DNA replication. We suggest that ORC–Cdc6 ATPase promotes complete Mcm2–7 ring closure just as ATP hydrolysis by the clamp loader RFC locks the PCNA DNA polymerase clamp onto dsDNA.

**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

**ACKNOWLEDGMENTS**

Cryo-EM data were collected on a FEI Titan Krios at HHMI Janelia Farm. We also collected a cryo-EM data set on an FEI Tecnai F20 equipped with a K2 detector at NRAMM at the Scripps Research Institute, which is supported by NIH grant P41 GM103310. We thank Z. Yu, C. Hong, and R. Huang at HHMI, and C. Porter and B. Carragher at Scripps for help with data collection. H.L. dedicates this work to his loving memory of his son Paul J. Li. This work was funded by the US National Institutes of Health (grant GM111742 to H.L., and grant GM45436 to B.S.); the Biotechnology and Biological Sciences Research Council UK (grant P56061 to C. Speck), and the Wellcome Trust (Investigator Award P56628 to C. Speck, Senior Research Fellowship 103139 to J.R., Centre core grant 092076 to J.R., and instrument grant 108504 to J.R.).

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Bell, S.P. & Dutta, A. DNA replication in eukaryotic cells. *Annu. Rev. Biochem.* 71, 331–374 (2002).
2. Bell, S.P. & Stillman, B. ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. *Nature* 357, 128–134 (1992).
3. Stillman, B. Origin recognition and the chromosome cycle. *FEBS Lett.* 579, 877–884 (2005).
4. Li, H. & Stillman, B. The origin recognition complex: a biochemical and structural view. *Subcell. Biochem.* 62, 37–58 (2012).
5. Chen, Z. et al. The architecture of the DNA replication origin recognition complex in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* 105, 10326–10331 (2008).
6. Sun, J. et al. Cdc6-induced conformational changes in ORC bound to origin DNA revealed by cryo-electron microscopy. *Structure* 20, 534–544 (2012).
7. Bleichert, F., Botchan, M.R. & Berger, J.M. Crystal structure of the eukaryotic origin recognition complex. *Nature* 519, 321–326 (2015).
8. Bell, S.P. & Labib, K. Chromosome duplication in *Saccharomyces cerevisiae*. *Genetics* 203, 1027–1067 (2016).
9. Speck, C., Chen, Z., Li, H. & Stillman, B. ATP-dependent cooperative binding of ORC and Cdc6 to origin DNA. *Nat. Struct. Mol. Biol.* 12, 965–971 (2005).
10. Speck, C. & Stillman, B. Cdc6 ATPase activity regulates ORC × Cdc6 stability and the selection of specific DNA sequences as origins of DNA replication. *J. Biol. Chem.* 282, 11705–11714 (2007).
11. Cocker, J.H., Piatti, S., Santocanale, C., Nasmyth, K. & Diffley, J.F. An essential role for the Cdc6 protein in forming the pre-replicative complexes of budding yeast. *Nature* 379, 180–182 (1996).
12. Evrin, C. et al. A double-hexameric MCM2-7 complex is loaded onto origin DNA during licensing of eukaryotic DNA replication. *Proc. Natl. Acad. Sci. USA* 106, 20240–20245 (2009).
13. Remus, D. et al. Concerted loading of Mcm2-7 double hexamers around DNA during DNA replication origin licensing. *Cell* 139, 719–730 (2009).
14. Li, N. et al. Structure of the eukaryotic MCM complex at 3.8 Å. *Nature* 524, 186–191 (2015).
15. Sun, J. et al. Structural and mechanistic insights into Mcm2-7 double-hexamer assembly and function. *Genes Dev.* 28, 2291–2303 (2014).
16. Moyer, S.E., Lewis, P.W. & Botchan, M.R. Isolation of the Cdc45/Mcm2-7/GINS (CMG) complex, a candidate for the eukaryotic DNA replication fork helicase. *Proc. Natl. Acad. Sci. USA* 103, 10236–10241 (2006).
49. O’Donnell, M. & Kuriyan, J. Clamp loaders and replication initiation. *Curr. Opin. Struct. Biol.* **16**, 35–41 (2006).

50. Kelch, B.A., Makino, D.L., O’Donnell, M. & Kuriyan, J. How a DNA polymerase clamp loader opens a sliding clamp. *Science* **334**, 1675–1680 (2011).

51. Dueber, E.L.C., Corn, J.E., Bell, S.D. & Berger, J.M. Replication origin recognition and deformation by a heterodimeric archaeal Orc1 complex. *Science* **317**, 1210–1213 (2007).

52. Suck, D. & Oefner, C. Structure of DNase I at 2.0 Å resolution suggests a mechanism for binding to and cutting DNA. *Nature* **321**, 620–625 (1986).

53. Yuan, Z. et al. Structure of the eukaryotic replicative CMG helicase suggests a pumpjack motion for translocation. *Nat. Struct. Mol. Biol.* **23**, 217–224 (2016).

54. Abid Ali, F. et al. Cryo-EM structures of the eukaryotic replicative helicase bound to a translocation substrate. *Nat. Commun.* **7**, 10708 (2016).

55. Samson, R.Y., Abeyrathne, P.D. & Bell, S.D. Mechanism of archaeal MCM helicase recruitment to DNA replication origins. *Mol. Cell* **61**, 287–296 (2016).
Sample preparation and electron microscopy. The S. cerevisiae loading intermediate OCM complex was assembled in vitro with purified ORC, Cdc6, Cdt1, and Mcm2–7 on plasmid DNA containing the ARS1 sequence in the presence of ATP8, and isolated via a previously described 48 magnetic bead pull-down approach, with minor modifications. Twenty-four pre-RC reactions containing 40 nM ORC, 80 nM Cdc6, 40 nM Cdt1, 40 nM Mcm2–7 and 6 nM pUC19-ARSI beads in 50 μl of buffer A (50 mM HEPES-KOH, pH 7.5, 100 mM potassium glutamate, 10 mM magnesium acetate, 50 μM zinc acetate, 3 mM ATPγS, 5 mM DTT, 0.1% Triton X-100 and 5% glycerol) were incubated for 15 min at 24°C. After three washes with buffer B (50 mM HEPES-KOH, pH 7.5, 100 mM K acetate, 3 mM ATPγS) the complex was eluted with 1 U of DNease I in buffer B and 1 mM CaCl₂.

To prepare cryo-EM grids, we pooled all the elutions together and concentrated the sample to about 0.9 mg/ml in buffer B with a Microcon centrifugal filter unit (YM-100 membrane). Before preparing the EM grid, we checked the sample for homogeneity by negative-stain EM. We then applied 3 μl of OCM complex sample at a final concentration of 0.9 mg/ml to glow-discharged C-flat 1.2/1/3 holey carbon grids, incubated the grids for 10 s at 6°C and 95% humidity, blotted the grids for 3 s, and plunged the grids into liquid ethane using an FEI Vitrobot IV. We loaded the grids into an FEI Titan Krios electron microscope operated at 300 kV high tension and collected images semi-automatically with SerialEM under low-dose mode at a magnification of 29,000× and a pixel size of 1.0 Å per pixel. A Gatan K2 summit direct electron detector was used under super-resolution mode for image recording with an under-focus range from 1.5 to 3.5 μm. The dose rate was 10 electrons per Å² per second, and the total exposure time was 5 s. The total dose was divided into a 25-frame movie, and each frame was exposed for 0.2 s.

We used 240 nM pUC19-ARSI beads to assemble the OCM complex as previously described 22. The complex was cross-linked in the presence of the beads with BS3 1:8:100 (molar protein-cross-linker ratio) for 2 h at 4°C. Then the reaction was quenched with 50 μl of saturated ammonium bicarbonate for 45 min at 4°C and subsequently transferred into digestion buffer (50 mM ammonium bicarbonate, 8 M urea). Next the reaction was subjected to reduction with DTT and alkylation with iodoacetamide. We added 3 μg of LysC (with an estimated 1:50 enzyme-to-protein ratio) and incubated it at room temperature for 4 h. The digestion buffer was then diluted with 50 mM ammonium bicarbonate to a final urea concentration of 2 M. We added 3 μg of trypsin (at an estimated 1:50 enzyme-to-protein ratio) and incubated it for 16 h at room temperature. After digestion, the supernatant was collected and acetified using 200 μl of 10% trifluoroacetic acid. We added 3 μg of Tryptic Phosphopeptide Cleavage Enzyme cocktail (Promega, Madison, WI) to the peptide mixture for each mass spectrometric acquisition. We carried out LC-MS/MS analysis on an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermofisher Scientific), applying a ‘high-high’ acquisition strategy 71.

We performed one round of coordinate refinement followed by a B-factor refinement. The refined model was then correlated with the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143. We correlated the 3D maps of the two half-maps (Half1 and Half2) in Fourier shell correlation, at the correlation cutoff value of 0.143.
were processed into peak lists with MaxQuant version 1.5.2.8 (ref. 72) with the default parameters, except that “FTMS top peaks per 100 Da” was set to 20 and “FTMS de-isotoping” was disabled. The peak lists were searched against the sequences as well as the reversed sequences (as decoys) of 14 OCCM subunits using Xi software (ERI, Edinburgh) for the identification of cross-linked peptides. The search parameters were as follows: MS accuracy, 0 p.p.m.; MS2 accuracy, 20 p.p.m.; enzyme, trypsin; specificity, fully tryptic; allowed number of missed cleavages, four; cross-linker, BS3; fixed modifications, carbamidomethylation on cysteine; variable modifications, oxidation on methionine, and modifications by BS3 that are hydrolyzed or amidated on the other end. The reaction specificity for BS3 was assumed to be for lysine, serine, threonine, tyrosine, and protein N termini. For acquisitions where collision-induced dissociation and higher-energy collisional dissociation fragmentations were applied (acquisitions 1, 3, 4, 5, 7, and 9), only b- and y-ions were considered for the fragment ion matches; for data acquired using combined fragmentation by electron-transfer dissociation supplemented with collision-induced dissociation or higher-energy collisional dissociation (acquisitions 2, 5, and 8), b-, y-, c-, and z-ions were considered for fragment ion matches. Two independent quality control approaches were applied for identified cross-linked peptide candidates. All cross-linked peptides with an estimated 5% false discovery rate at the residue-pair level were accepted for further structural interpretation. We also accepted cross-linked peptides identified with MS2 spectra that passed machine-learning-based auto-validation.

In vitro pulldown of Cdt1 with Mcm2–7 proteins. Baculoviruses expressing Mcm2–7 subunits (Mcm3 was tagged with hemagglutinin at the N terminus) and SSS–Cdt1 were prepared in the Profold-C1 expression vector (AB Vector, San Diego, California). We infected 2.5 × 107 Hi-Five insect cells at a multiplicity of infection with 20 of each Mcm subunit alone or in combination with SSS–Cdt1, and harvested them at 55 h post-infection. After the cells had been washed in cold PBS, they were placed for 10 min on ice in <1 ml of hypotonic buffer (25 mM Hepes-KOH, pH 7.5, 20 mM K glutamate, 1 mM Mg acetate, 1 mM DTT, 5 mM ATP, protease inhibitor (Roche, 1 tablet in 50 ml)). Cell extracts were prepared by Dounce B homogenization and centrifugal clarification (10,000 r.p.m. in a Sorvall SS34 rotor) at 4 °C, and then 100 µl of supernatant was incubated for 2 h on ice with 50 µl of Strep–Tactin sepharose (IBA) that had previously been washed in PBS and hypotonic buffer. Beads were washed (three times, 5 min) with IP buffer (25 mM Hepes-KOH, pH 7.5, 300 mM K glutamate, 10 mM Mg acetate, 0.04% NP-40, 1 mM DTT, 5 mM ATP, and protease inhibitor). Bead-bound proteins were run on an SDS-PAGE gel and stained with silver.

Data availability. The 3D cryo-EM map of OCCM at 3.9-Å resolution has been deposited at the EMD database with accession code EMD-8540. The corresponding atomic model was deposited at the RCSB PDB with accession code SUDB. Source data for Figure 2 and Supplementary Figure 4 are available with the paper online.

65. De Marco, V. Quaternary structure of the human Cdt1-Geminin complex regulates DNA replication licensing. J. Struct. Biol. 189, 114–122 (2015).
66. Afonine, P.V. Towards automated crystallographic structure refinement with phenix.refine. Acta Crystallogr. D Biol. Crystallogr. 68, 352–367 (2012).
67. Adams, P.D. et al. PHENIX: a comprehensive Python-based system for macromolecular crystallography. Acta Crystallogr. D Biol. Crystallogr. 66, 213–221 (2010).
68. Amunts, A. et al. Structure of the yeast mitochondrial large ribosomal subunit. Science 343, 1485–1489 (2014).
69. Wiedemann, C. et al. Structure and regulatory role of the C-terminal winged helix domain of the archaeal minichromosome maintenance complex. Nucleic Acids Res. 43, 2958–2967 (2015).
70. Rappsilber, J., Mann, M. & Ishihama, Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat. Protoc. 2, 1896–1906 (2007).
71. Chen, Z.A. et al. Architecture of the RNA polymerase II-TFIIF complex revealed by cross-linking and mass spectrometry. EMBO J. 29, 717–726 (2010).
72. Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. 26, 1367–1372 (2008).
NATURE STRUCTURAL & MOLECULAR BIOLOGY