Cryo-EM structure of a helicase loading intermediate containing ORC–Cdc6–Cdt1–MCM2-7 bound to DNA

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In eukaryotes, the Cdt1-bound replicative helicase core MCM2-7 is loaded onto DNA by the ORC–Cdc6 ATPase to form a prereplicative complex (pre-RC) with an MCM2-7 double hexamer encircling DNA. Using purified components in the presence of ATPγS, we have captured in vitro an intermediate in pre-RC assembly that contains a complex between the ORC–Cdc6 and Cdt1–MCM2-7 heteroheptamers called the OCCM. Cryo-EM studies of this 14-subunit complex reveal that the two separate heptameric complexes are engaged extensively, with the ORC–Cdc6 N-terminal AAA+ domains latching onto the C-terminal AAA+ motor domains of the MCM2-7 hexamer. The conformation of ORC–Cdc6 undergoes a concerted change into a right-handed spiral with helical symmetry that is identical to that of the DNA double helix. The resulting ORC–Cdc6 helicase loader shows a notable structural similarity to the replication factor C clamp loader, suggesting a conserved mechanism of action.

The replication of eukaryotic chromosomes is a multistep process that spans the G1 and S phases of the cell division cycle1,2. The yeast origin recognition complex (ORC), which comprises Orc proteins 1–6, constitutively binds to the replication origin DNA3. Replication licensing occurs upon mitotic exit or during the G1 phase, when cell division cycle 6 (Cdc6) and Cdc10-dependent transcript 1 (Cdt1) can interact with the ORC to load hexameric MCM2-7, which comprises minichromosome maintenance (Mcm) proteins 2–7 and is the core of the DNA helicase complex4. Details of how MCM2-7 is loaded onto the DNA have not yet been biochemically defined. The loaded MCM2-7 is initially inactive and becomes activated during early S phase, when many other factors promote unwinding of the duplex DNA and the replisome is assembled4.

Low-resolution EM structures of ORC from Saccharomyces cerevisiae (ScORC) and Drosophila melanogaster (DmORC) have been reported5,6. Both ScORC and DmORC were found to have a slightly twisted half-ring structure of length ~160 Å and width ~120 Å (ref. 7). The replication initiator Cdc6 was found to bind to one side of ScORC, thus completing an ORC–Cdc6 ring containing six proteins with predicted AAA+ architecture5,8–11. In vitro, the ScORC–Cdc6 complex was shown to load two MCM2-7 hexamers onto double-stranded DNA (dsDNA) in a Cdt1- and ATP-hydrolysis–dependent manner to form an inactive but stable double hexamer12,13 (Fig. 1a). During this reaction, Cdt1 was removed from the double hexamer. Thus the ORC–Cdc6 ring acts as an ATP-hydrolysis-dependent loading machine that enabled the ring-shaped MCM2-7 hexamers to encircle duplex DNA. The DNA-loaded MCM2-7 double hexamer is subsequently activated by Cdc45 and the GINS complex to form the CMG complex, shown to be a functional helicase14–17. EM structural studies revealed that Cdc45 and GINS together bridge a gap between Mcm2 and Mcm5 of the MCM2-7 ring18.

A cryo-EM-derived structure of ORC–Cdc6 bound to origin DNA demonstrated that ORC assumed a different structure upon recruiting Cdc6 to the DNA and that the length of DNA bound was almost double that bound by ORC alone5,11. On the basis of this structure and the two-claw DNA-binding mode of archaeal Orc1 and Cdc6 (Orc1/Cdc6) structure as revealed by X-ray crystallography19,20, we have proposed that ScORC–Cdc6 bends and topologically wraps the origin DNA11 and that this complex is primed to recruit the MCM2-7 hexamers. Similar wrapping of DmORC has been proposed21.

Bioinformatic analyses of DNA replication licensing factors and crystallography of archaeal homologs have shown that Orc proteins 1–5, Cdc6 and MCM2-7 belong to the AAA+ family of proteins5,20–24 (Supplementary Fig. 1). Orcs 1–5 and Cdc6 contain one or two C-terminal DNA-binding winged-helix domains (WHDs)5. Each Mcm protein is composed of an N-terminal zinc-binding domain (NTD) and an ATP-binding C-terminal AAA+ domain (CTD)25,26. The NTDs and CTDs of the MCM2-7 hexamer form a two-tiered ring structure18,24,27. Orc6 and Cdt1 are structurally different: Orc6 shares partial similarity with the transcription factor TFIIIB, and Cdt1 contains two WHDs but no ATP binding motif28,29.

A missing piece of information is how ORC–Cdc6 recruits and interacts with Cdt1–MCM2-7 before the formation of the MCM2-7 double hexamer (Fig. 1a). Using purified ScORC, Cdc6, Cdt1 and...
MCM2-7 proteins in the presence of origin DNA and ATP-γS, we have captured a 1.1-megadalton (1.1-MDa) complex that contains all 14 proteins essential for replication origin licensing. Cryo-EM and subunit mapping have resulted in a model for the architecture of a key intermediate in the replication-licensing process. The structure provides insights into the molecular mechanism of MCM2-7 recruitment and helicase loading.

RESULTS

Capturing a pre-RC intermediate by cryo-EM

The fleeting nature of the initial encounter between ORC–Cdc6 and Cdt1–MCM2-7 has so far prevented capture of a pre-RC intermediate in vitro. We wondered whether cryo-EM could visualize assembly intermediates before the formation of the MCM2-7 double hexamer. To slow the progression of the MCM2-7 loading reaction, we initially used several crippling factors, including (i) a short origin DNA (86-base-pair (86-bp) linear DNA containing autonomously replicating sequence 1 (ARS1)), which has been demonstrated to be too short to load the MCM2-7 double hexamer; (ii) ATP-γS, which is hydrolyzed very slowly compared with ATP; and (iii) ORC containing an ATP-hydrolysis mutation in the Orc4 subunit (ORC-4R)5,30.

Computational classification of the cryo-EM images of the heterogeneous reactants revealed two-dimensional (2D) class averages that suggested the formation of a three-tiered complex structure (Fig. 1b). On the basis of structural studies of ORC–Cdc6 and the MCM2-7 hexamer5,11,19,27,31, we conclude that the top, slanted tier is likely to be ORC–Cdc6 and that the bottom two tiers belong to Cdt1–MCM2-7. Assembly under these conditions hindered MCM2-7 double hexamer formation and resulted in the pre-RC intermediate that we have called ORC–Cdc6–Cdt1–MCM2-7 (OCCM).

In optimizing OCCM preparation for cryo-EM 3D reconstruction, we found that OCCM formed readily on an ARS1-containing plasmid (~3,000 bp) without the ORC-4R arginine finger mutation, as long as ATP-γS was present in the reaction. The improved protocol involves first attaching a biotinylated ARS1, which has been demonstrated to be too short to load the MCM2-7 double hexamer; (ii) ATP-γS, which is hydrolyzed very slowly compared with ATP; and (iii) ORC containing an ATP-hydrolysis mutation in the Orc4 subunit (ORC-4R)5,30.

Figure 1 In vitro assembly of the OCCM complex. (a) Model for Cdc6 recruitment to the replication origin readies the ORC for loading of MCM2-7. (b) Averaged cryo-EM images of the in vitro assembled OCCM. Boxes are scaled to 27 x 27 nm. The magnified view (right) shows the top area and lower region, tentatively assigned to ORC–Cdc6 and to Cdt1–MCM2-7, respectively. (c) Mcm2 immunoprecipitation (IP) identifies the OCCM components. Purified ORC, Cdc6, Cdt1, MCM2-7 (lanes 1–4) and origin DNA were used to assemble OCCM in the presence of ATP-γS. OCCM was cleaved from the plasmid DNA with DNase I and immunoprecipitated with an anti-Mcm2 antibody (lanes 5–7) or with anti-MBP control antibody (lanes 8–10). Asterisks mark nonspecific proteins from antibody-conjugated beads. FT, flow-through.

Figure 2 Cryo-EM of the eukaryotic OCCM complex. (a) A representative raw cryo-EM image of the purified OCCM complex embedded in vitreous ice. (b) Selected reference-free 2D class averages of the OCCM cryo-EM images (left) in comparison with their approximately corresponding re-projections from the 3D reconstruction (right). Box size, 34 x 34 nm. (c) Surface view of the cryo-EM 3D map of the OCCM complex rendered at the threshold that includes the expected molecular mass of 1.1 MDa. (d) Fourier shell correlation suggests that the 3D map of the OCCM complex has a resolution of 14 Å. (e) Tilt validation of the cryo-EM 3D map. Black dots represent predictions for each particle pair’s tilt axis and tilt angle based on the cryo-EM map. Most particle pairs cluster in a region (red circle) centered at the experimental tilt axis (90°) and tilt angle (10°).
Figure 3 Mapping the protein and DNA components of the OCCM. (a–f) 2D class averages (left) and 3D reconstruction (middle) of OCCM with MBP fused to the C terminus (CT) of Orc2 (Orc2-MBP) (a), the N terminus (NT) of Mcm2 (MBP-Mcm2) (b), the NT of Mcm3 (MBP-Mcm3) (c), the CT of Mcm5 (Mcm5-MBP) (d), the NT of Cdt1 (MBP-Cdt1) (e) or the NT of Mcm6 (MBP-Mcm6) (f). Left, two reference-free class averages (top) and the same images displayed at a higher contrast level (C = 0.3) (bottom). For 3D reconstructions (middle), the peripheral MBP density is shown in blue. Surface-rendering thresholds were lowered by ~20% to better visualize the small MBP density. Right, vertical (a,b) or horizontal (c-f) sections of the 3D map of the MBP-fused OCCM (column 1) in comparison to the corresponding section of that of the wild-type OCCM (column 2); bottom, higher contrast. Red arrows indicate the MBP density at the peripheral of OCCM. Quadrant size is 37 × 37 nm (left) and 34 × 34 nm (right). Fusion complexes were cleaved from plasmid DNA by DNase I (a–e) or AluI (f). (g) Reference-free class averages of wild-type OCCM with plasmid DNA digested by DNase I (top) or AluI (bottom). Blue arrows indicate dsDNA stub on the top ORC–Cdc6 region of OCCM. Box size, 31 × 31 nm. (h) MCM2-7 organization as mapped by the four MBP-fused Mcm subunits (red), viewed from the N-terminal end of MCM2-7. Scale bar, 20 nm (3D maps).

and observed co-precipitation of Mcm proteins 3–7, Cdt1 and ORC by an antibody to Mcm2 (anti-Mcm2) but not by an anti-MBP control antibody (Fig. 1c). This result demonstrates the integrity and composition of OCCM.

Cryo-EM and 3D reconstruction of the OCCM complex
Because of their large mass (1.1 MDa), the OCCM particles embedded in vitreous ice have good contrast (Fig. 2a). We recorded >300 micrographs and selected >80,000 particle images (Fig. 2b,c and Supplementary Fig. 3a,b). The 3D map has an estimated resolution of 14 Å (Fig. 2d). We further validated the structure by the tilt-pair technique. We collected 142 pairs of untilted and 10°-tilted particle images, and we therefore interpreted this linear and partially flexible structure. The tilt geometry of each particle pair was then calculated and plotted as a dot in the polar coordinate system (Fig. 2e). Approximately 73% (47/64) of the plotted particles were found to cluster around the experimental tilt angle of 10° (9.65° average, with an r.m.s. deviation of 4.52°) and the vertical tilt axis (85.22° average, with an r.m.s. deviation of 14.02°). This result suggests that the cryo-EM structure and its associated handedness are correct.

Protein-subunit and DNA mapping of the OCCM structure
A strategy employing fusion of maltose binding protein (MBP) was initially developed for systematic subunit mapping within averaged 2D images of the yeast ORC. We systematically tested N-terminal, C-terminal or internal fusions of MBP to the OCCM proteins and examined their function in pre-RC assembly. We generated >10 stable MBP-fused OCCM complexes and found MBP densities in 2D reference-free class averages or 3D reconstructions of six of these complexes (Fig. 3a–f). All six MBP-fused complexes were functional: they formed a low-salt-stable MCM2-7 double hexamer (Supplementary Fig. 4a,b). MBP fused to Orc2 or Mcm2 is located to the right or at the bottom of OCCM, respectively, in the side view; it is visible in the 3D maps and their sections (Fig. 3a,b). However, MBP inserted in Mcm3, Mcm5, Mcm6 or Cdt1 is not visible in the 2D averages (Fig. 3c–f, left), indicating that the MBP is located in the front or back of OCCM in this view. Consistent with this assessment, we indeed observed MBP in the front or back of the MCM region in 3D reconstructions of these OCCM complexes (Fig. 3c–f, middle and right). MBP densities appear rather small owing to the small mass of MBP (38 kDa) relative to the 1.1-MDa OCCM and the fact that the fused MBP can move around results in reduced densities in 3D reconstructions. However, the small density at the periphery of each fusion complex can be assigned to MBP because we have shown that the fusion complex retains structural and functional integrity.

We had more success in observing the tag when MBP was inserted internally into an Mcm NTD or CTD. Insertion generates two linkages between MBP and Mcm, which probably results in a less flexible MBP and, thus, an improved detection rate.

DNase I is a sequence-nonspecific enzyme that cleaves essentially all accessible DNA. As expected, the cryo-EM 2D class averages did not show any densities protruding from OCCM treated with this enzyme (Fig. 3g, top). However, when we carried out DNA digestion using the DNA sequence-specific restriction enzyme AluI, we consistently observed a thin density protruding from the top of the ORC–Cdc6 region of the OCCM structure in the averaged cryo-EM images, and we therefore interpreted this linear and partially flexible density as DNA (Fig. 3g, bottom). Cryo-EM 3D reconstruction of the AluI-treated OCCM with MBP inserted in the NTD of Mcm6 revealed the MBP density at the bottom and the dsDNA stub at the top of the structure (Fig. 3f).

MBP insertions in the NTDs of the Mcm subunits were located near the bottom, whereas insertions in the CTD were located in the middle of the OCCM structure. This observation indicates that the MCM2-7 NTD faces outward and the AAA+–domain-containing CTD of MCM2-7 interacts with ORC–Cdc6 at the top of OCCM. This MCM2-7 orientation leaves the NTD free to interact with the NTD of
Figure 4 Segmented cryo-EM structure of the OCCM. (a–d) Side views of OCCM obtained by consecutive 90° rotations around a vertical axis. The blue asterisks (a,b) indicate the approximate location of the N-terminal peptide of Cdc6 in the OCCM structure. (e,f) Top and bottom views, rotated ±90° around the horizontal axis from the side view in b. (g) Bottom and back views of the OCCM map shown as semi-transparent surface and docked with homology crystal structure of the archaeal MCM NTD hexamer20 (PDB 1LTL). Surface-rendering threshold was set to enclose the expected 1.1-MDa mass of OCCM. Scale bar, 10 nm (a–g). (h) Ribbon presentation of the archaeal Orc1/Cdc6 crystal structure27 (PDB 2QBY). The N-terminal peptide meanders away from the N-terminal AAA+ domain and joins the middle helical domain (HD). (i) The segmented Cdc6 density (semi-transparent surface) and the rigid-body docked archaeal Orc1/Cdc6 structure27 (yellow ribbons) shown as salmon ribbons. M, Mcm; O, Orc; CT, C-terminal; NT, N-terminal.

The architecture of the OCCM complex

To arrive at the subunit-assigned structure of OCCM (Fig. 4a–f) we first subjected the cryo-EM 3D map to semi-automatic segmentation39. The subunit densities in the top ORC–Cdc6 region were well defined, and the protein subunits could be segmented with little ambiguity. We mapped the location of Orc2 directly (Fig. 3a) and assigned the remaining proteins on the basis of the previously determined arrangement: Orc1-Orc4-Orc5-Orc2-Orc3, with Orc6 binding to Orc2 and Cdc6 bridging the gap between Orc1 and Orc3 (ref. 11) (Fig. 4e). There was an additional density in the center of the top region, and we conclude that this density belongs to the bound dsDNA that is protected by OCCM from DNase I digestion (Fig. 3). In the bottom tier of the MCM2-7 region, the hexameric NTD crystal structure of an archaeal MCM can be fit40 (Fig. 4g). It is important to note that the N terminus of each of the known archaeal Orc1/Cdc6 structures is located away from the N-terminal AAA+ domain and extends to and is part of the middle helical domain20,41 (Fig. 4h,i). The density identified as Cdc6 fits nicely to the rigid-body docked archaeal homolog Orc1/Cdc6 crystal structure42, with the N terminus in the middle of the structure (Fig. 4i). On the basis of the docking, we estimated the position of the Cdc6 N terminus in the OCCM structure (Fig. 4a,b). Notably, the Cdc6 C-terminal WHD is oriented upward, in contact with the central DNA density, and the Cdc6 N-terminal AAA+ domain points down, reaching toward an AAA+ CTD of MCM2-7 (Fig. 4a–f). Given the similar architecture and packing of the ORC–Cdc6 subunits containing AAA+ (refs. 5,6), we expect that their N termini are located in the outer middle regions and that their C-terminal WHDs contact central DNA. We note that a centrally located Orc2 WHD is not in conflict with the peripheral location of the MBP fused to the C terminus of Orc2 (Fig. 3a) because there is a linker between the predicted WHD and the MBP.

In the Cdt1–MCM2-7 region of the OCCM structure, segmentation subunit densities for Mcm3, Mcm4, Mcm5 and Mcm7 was nearly automatic and unambiguous (Fig. 4a–f). However, the densities belonging to Cdt1, Mcm2 and Mcm6 were intertwined. Therefore, their boundaries are less certain and more subject to interpretation, although the relative locations are defined. After all seven proteins in the Cdt1–MCM2-7 region were assigned, one segmented density remained unaccounted for. This density was in the central region of MCM2-7 and best viewed from the bottom (Fig. 4f). We have not established the identity of the density, but we speculate that it is from eukaryotic cell–specific N-terminal extensions of some Mcm subunits and perhaps contains DNA emerging from the bottom end of the OCCM structure. In the well-defined Mcm3 density (Fig. 4b), the crystal structure of the nearly full-length archaeal MCM can be fitted by rigid-body docking, with the NTD pointing down and the AAA+ domain–containing CTD facing the ORC–Cdc6 above27 (Fig. 4j).

The physical proximity between the individual subunits in the heptameric ORC–Cdc6 and subunits of the heptameric Cdt1–MCM2-7 can be summarized as follows: Orc1 is near Mcm4 and Mcm; Orc2 is near Mcm2 and Mcm6; Orc3 is near Mcm2 and Mcm5; Orc4 is near Mcm4; Orc5 is near Mcm4 and Mcm6; Orc6 is near Mcm2; and Cdc6 is near Mcm3 and Mcm7 (Fig. 4a–f). Although the putative Orc6 density is on the same side as Cdt1 in the OCCM, they are not touching. Earlier studies suggested that interaction between Orc6 and Cdt1 was crucial for MCM2-7 loading onto origin DNA by ORC–Cdc6 (ref. 42), but recent studies have suggested otherwise43,44.

We previously found that N- and C-terminal MBP fusions to Orc1-5 support ORC–Cdc6 complex formation34. Here, we observed efficient
OCCM and double-hexamer formation with all N-terminal ORC-MBP fusions (Supplementary Fig. 5a). This is in agreement with our OCCM model predicting the surface localization of the N termini of the ORC subunits. However, fusion of MBP to the C terminus of Orc1 or Orc4 reduced recruitment of MCM2-7 (low salt) and blocked double-hexamer formation (high salt) but did not affect ORC association with DNA. It is possible that MBP fusion to the C terminus of Orc1 or Orc4 interferes with the establishment of the correct DNA path, which in turn blocks MCM2-7 recruitment; however, MBP attachment to the C terminus of Orc2 did not interfere with the loading function, indicating that not all Orc C termini function in the same way.

Cdc6 is central to pre-RC formation. The OCCM model predicts the proximity of Cdc6 and Mcm3. We examined their potential interaction by independent immunoprecipitation. Mcm3 interacted efficiently with MBP-fused Cdc6 (MBP-Cdc6) but not with MBP, and all other Mcm subunits bound Cdc6 only weakly (Supplementary Fig. 5b). Mcm4 showed nonspecific interaction with MBP, rendering the result for Mcm4 inconclusive. Furthermore, the data indicated that the C terminus of Mcm3 is orientated toward ORC–Cdc6. An analysis of the MCM2-7 C termini shows that Mcm3 contains a long C-terminal extension that is not present in other MCM subunits (Supplementary Fig. 1b). We reasoned that this extension could be important for the interaction between Cdc6 and MCM2-7. We generated an MCM2-7 mutant lacking amino acids aa 740–971 (MCM2-7–ΔC3). Notably, MCM2-7–ΔC3 had a weaker interaction than wild-type MCM2-7 with MBP-Cdc6 (Supplementary Fig. 5c). MCM2-7–ΔC3 interacted with Cdt1 (Supplementary Fig. 5d) but did not associate with ORC–Cdc6 in low-salt buffer and failed to load high-salt-resistant MCM2-7 onto DNA (Supplementary Fig. 5e). Our result is consistent with a recent report on the importance of Mcm3 CTD for MCM2-7 loading.

ORC–Cdc6 interacts to form a right-handed spiral in the OCCM

Our previous determination of the DNA-bound ORC–Cdc6 structure in the absence of Cdt1–MCM2-7 (ref. 11) (Fig. 6a) and the current structure in the presence of Cdt1–MCM2-7 (Fig. 6b) provides an opportunity to examine how ORC–Cdc6 functions to recruit the MCM2-7 hexamer. Although the structures are both ring-shaped and have similar sizes, ORC–Cdc6 undergoes profound conformational

Figure 6 Upon recruitment of Cdt1–MCM2-7, ORC–Cdc6 undergoes concerted conformational change into a right-handed spiral structure. The ORC–Cdc6 structure before (a) and after (b) contacting Cdt1–MCM2-7. The question mark indicates the tentative assignment of Orc6. (c) Stereo side view of ORC–Cdc6 extracted from the OCCM structure. The dashed black line traces the right-handed helical rise, which occurs in the following order: Orc3, Orc2, Orc5, Orc4, Orc1, Cdc6. The vertical rise from lowest (Orc3) to highest (Cdc6) is 34 Å. The dashed red line represents the bound dsDNA. O, Orc; 1N, N-terminal domain of Orc1.
changes upon interaction with Cdt1–MCM2-7. First, ORC–Cdc6 alone is nearly flat but bends into a dome-like shape when it contacts Cdt1–MCM2-7 (Fig. 6a,b). This transformation is accomplished largely by movement of the individual C-shaped subunits—specifically, by the N-terminal AAA+ domains reaching down to interact with the CTDs of the MCM2-7 subunits (Fig. 4a–d). Furthermore, at one edge of ORC–Cdc6 Orc6, Orc3 and Cdc6 have undergone a series of concerted movements (Fig. 6a,b, middle). It seems that the mass tentatively assigned to Orc6 initiates the transition, because it has to move out of the way to allow MCM2-7 interaction with ORC–Cdc6; in the absence of Cdt1–MCM2-7, Orc6 occupies the site at which MCM2-7 will bind ORC–Cdc6 (Fig. 6a, middle). We suggest that upon encountering Cdt1–MCM2-7, Orc6 rotates ~60° downward, which would push Orc3 upward and away from Orc2. The upward movement of Orc3 narrows the gap between Orc1 and Orc3 that Cdc6 bridges, and this narrowing forces Cdc6 to rotate ~45° to fit into the gap (Fig. 6a,b, middle and right). Accompanying the Cdc6 rotation is the movement of the N-terminal domain of Orc1 out and away from the center (Fig. 6a,b, left). The movement of ORC–Cdc6 upon Cdt1–MCM2-7 binding also brings the Orc1 and Orc4 subunits into closer contact, potentially allowing Orc4 to activate the ATPase activity of Orc1, an activity necessary for pre-RC assembly.

Notably, in the presence of Cdt1–MCM2-7, ORC–Cdc6 forms a right-handed spiral structure (Fig. 6c and Supplementary Movies 1 and 2). Orc3 is at the lowest position in the spiral and is followed by Orc2, Orc5, Orc4 and Orc1; Cdc6 is at the highest position, between Orc1 and Orc3. The total vertical rise from Orc3 to Cdc6 is 34 Å, which translates to ~5.6 Å (34/6 Å) axial rise per protein subunit (Fig. 6c). The 34 Å rise in the ORC–Cdc6 structure is notable because it is equal to the helical pitch of the B-form dsDNA. Therefore, the six AAA+–domain-containing subunits in the ORC–Cdc6 heterohexamer form a spiral structure with a helical symmetry that matches exactly that of the dsDNA to which they bind. The symmetry match suggests that ORC–Cdc6 stably anchors DNA at the center of OCCM and that this anchoring may be important for MCM2-7 loading.

**MCM2-7 hexamer in the OCCM may be partially loaded**

There is a linear, nearly continuous density that passes through OCCM from the outside into the ORC–Cdc6 region and further into the MCM2-7 central chamber (Fig. 7). The central density is best viewed in consecutive horizontal sections from the top of the OCCM structure to the bottom (Fig. 7a,b, blue arrows) and in the 3D map when the front Orc3 and Mcm5 are removed (Fig. 7c). We have experimentally identified the top region of the linear density as dsDNA (Fig. 3f,g) and now suggest that the entire density may be dsDNA (Fig. 7c, dashed red lines). Our cryo-EM structure suggests that the first MCM2-7 may be partially loaded and encircles the dsDNA in the absence of extensive ATP hydrolysis before the second MCM2-7 hexamer is recruited, as we used nonhydrolyzable or weakly hydrolyzable ATP–γS in our preparation, and there is only one MCM2-7 hexamer in the OCCM. Because OCCM could be washed off of DNA by a high-salt buffer, we conclude that the loading was not completed.

**The ORC–Cdc6 spiral resembles the PCNA-loading RFC spiral**

There is a clear mismatch, of ~12°, between the central axis of the MCM2-7 hexamer and the helical axis of the ORC–Cdc6 (Fig. 7d). This mode of interaction between ORC–Cdc6 and Cdt1–MCM2-7 bears a strong similarity to the loading of the eukaryotic proliferating cell nuclear antigen (PCNA) DNA polymerase clamp by the ATPase complex replication factor C (RFC) (Fig. 7e). RFC is an ATP-dependent machine that loads PCNA onto primer-template DNA so that PCNA can then recruit and tether the DNA polymerase at the replication fork. In the crystal structure of the RFC–PCNA complex, the AAA+ domains of RFC-A, RFC-B, RFC-C, RFC-D and RFC-E form a spiral structure, and the gap between RFC-A and RFC-E is bridged by the extra domain (domain IV) of RFC-A. The helical axis of the RFC spiral tilts 9° away from the PCNA ring axis (Fig. 7e). Therefore, the six AAA+ subunits of ORC–Cdc6 form a spiral in the OCCM that resembles the PCNA ring-loading RFC spiral, which contains five AAA+ subunits.

**DISCUSSION**

Using cryo-EM, we have captured and characterized an intermediate in the loading onto DNA of the eukaryotic DNA helicase core complex by ORC–Cdc6 and Cdt1, thereby establishing the architecture of the OCCM complex. The cryo-EM structure reveals that several AAA+-like domains of ORC–Cdc6 unclench and latch onto the C-terminal AAA+ motor domains of the MCM2-7 hexamer, leading to the loading of MCM2-7 onto the dsDNA. During the loading process, ORC–Cdc6 transforms into a spiral structure.

The DNA gate in the MCM2-7 hexamer is widely believed to be located between Mcm2 and Mcm5 (refs. 18,38). In the OCCM, a gap exists between the NTDs of Mcm2 and Mcm5, but the CTDs are not separated (Figs. 4f and 5). Notably, Orc3 makes contact with both CTDs of Mcm2 and Mcm5 (Fig. 4b). Thus, it is possible that a slight descent of Orc3 would further separate Mcm2 and Mcm5 and fully open up the MCM2-7 ring.

The DNA in OCCM is nearly perpendicular to the spiral surface of ORC–Cdc6. In this DNA-binding model, we estimate that the
length of the DNA that can be protected by ORC–Cdc6 is <30 bp: the first AAA+ protein (Orc3) may protect up to 20 bp (refs. 19, 20), and the other five subunits in the spiral would cover another 10 bp in one DNA helical pitch (Fig. 6c). However, it has been well established that ORC alone has a DNase I footprint of 48 bp (ref. 3), and when Cdc6 binds ORC, the DNase I footprint extends to nearly 80 bp (ref. 53). Therefore, the DNA-binding mode in ORC–Cdc6 alone, before encountering Cdt1–MCM2-7, must be different from that seen in the OCCM structure, and the DNA in ORC–Cdc6 probably undergoes a profound transition when Cdt1–MCM2-7 is recruited. Indeed, the DNA-binding model proposed in our structure of ORC–Cdc6 alone is substantially different, with the origin DNA bent and wrapped around in the interior of the ORC–Cdc6 structure, in accordance with the DNA-binding model in the crystal structure of archaean Orc1 and in agreement with the extended DNase I footprint11.

We suggest that the DNA transition to the vertical position occurs along with the subunit rearrangement in ORC–Cdc6 that follows the interaction with Cdt1–MCM2-7 (Fig. 6). Clearly, DNA rearrangements in ORC–Cdc6 during helicase loading are important and require further investigation.

In the T4 bacteriophage clamp-loading system in the presence of DNA and ATP hydrolysis, the clamp ring opens and converts into a spiral shape whose helical symmetry matches that of the DNA as well as the clamp loader28. Furthermore, in the negative-stain EM structure of an archaean PCNA–RFC complex with DNA and ATP, the clamp ring is also open and resembles a washer24. In the case of ORC–Cdc6, six AAA+–containing components form a spiral encircling the dsDNA, the ORC–Cdc6 helical axis is tilted 12° away from the MCM2-7 ring axis and the MCM2-7 ring appears to be partially open. One important difference between these two ring-loading systems is that in RFC, the third and C-terminal helical domains form a tightly sealed collar that blocks DNA passage, whereas in ORC–Cdc6, the corresponding six WHDs from the six AAA+ proteins form an open collar that allows dsDNA to pass through (Figs. 3f,g, 6c and 7c). Another obvious difference is that MCM2-7 is an ATPase ring, whereas the PCNA ring is not, so their respective ring-opening mechanisms could be different. Despite these differences, it is notable that two structurally related ATP-dependent protein machines, ORC–Cdc6 and RFC, load ring-shaped proteins (MCM2-7 and PCNA, respectively) that are later involved in movement along DNA during replication fork progression.

A recent study also revealed an analogy between the bacterial DnaB helicase loading by DnaC and the PCNA loading by RFC25. The organization of the yeast OCCM observed here may provide insights into the mechanism of helicase loading for higher eukaryotes and Archaea, as structural information about the helicase loader in complex with the helicase has not yet been obtained.

METHODS
Methods and any associated references are available in the online version of the paper.

Accession code. The cryo-EM 3D density map of S. cerevisiae OCCM complex (ORC–Cdc6–Cdt1–MCM2-7 on dsDNA) has been deposited in the EM Data Bank under accession number EMD-5625.

Note: Supplementary information is available in the online version of the paper.

ACKNOWLEDGMENTS
We thank M. Smulczeski and S. Zhang for helping to manually select a large number of particles from raw cryo-EM micrographs and E. Gardenal and C. Winkler for the MCM2-7–Cdc6 interaction analysis. This work was supported by US National Institutes of Health grants GM45436 (to B.S.) and GM74985 (to H.L.) and the United Kingdom Medical Research Council (to C.S.). H.K. was supported by Postdoctoral Fellowships for Research Abroad from the Japan Society for the Promotion of Science and the Uehara Memorial Foundation.

AUTHOR CONTRIBUTIONS
J.S., C.E., S.A.S., A.E.-C., A.R. and H.K. performed the specimen preparation and biochemical work. J.S. collected the cryo-EM data, performed the cryo-EM reconstructions. J.S., B.S., C.S. and H.L. designed experiments and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Pre-RC assay. The pre-RC was performed as described12. ORC (40 nM), 80 nM Cdc6, 40 nM Cdt1, 40 nM MCM2-7 and 6 nM pUC19-ARS1 beads in 50 µl buffer A (50 mM HEPES-KOH (pH 7.5), 100 mM potassium glutonate, 10 mM magnesium acetate, 50 µM zinc acetate, 3 mM ATP, 5 mM DTT, 0.1% Triton X-100 and 5% glycerol) were incubated for 15 min at 24 °C. After three washes with buffer A or B (50 mM HEPES-KOH (pH 7.5), 1 mM EDTA, 500 mM NaCl, 5% glycerol, 0.1% Triton X-100 and 5 mM DTT) the complex was eluted with 1 µl of DNase I in buffer A and 5 mM CaCl2.

Pre-RC assembly for electron microscopy analyses. Pre-RC assembly was performed as described above12; however, ATPγ-S was used instead of ATP. The DNase I or Aln elution was performed in 5 µl buffer C (50 mM HEPES-KOH (pH 7.5), 100 mM potassium acetate, 5 mM magnesium acetate, 5 mM CaCl2). For the initial experiment (Fig. 1b) an ARS1 origin DNA fragment of 86 bp was used (TTTTTGACACTTGGCTGAGCCTTTGGAAAAACCAAGCATATAAGATCTAAACATAAAATCTGTAAAATAACAAGATGTAAAGATAA), and proteins and DNA were mixed at equimolar ratios.

Immunoprecipitation of OCCM. The pre-RC complexes were prepared as described (pre-RC assay) and then immunoprecipitated with anti-Mcm2 (no. 49) (ref. 56) and anti-MBP (E8032L; NEB) antibody coupled to protein G beads for 7.5 min at 24 °C, washed three times with buffer A and analyzed by western blot with anti-Mcm2 (no. 49) (1/2,000) (ref. 56) antibody.

MBP-Cdc6 and MBP-Cdt1 immunoprecipitation. MBP-Cdc6 (100 ng) and MBP-Cdt1 were immobilized on 2 µl anti-MBP antibody beads (NEB) in 50 µl buffer D (buffer A with 300 mM potassium glutonate, 0.25% Triton X-100, 0.5% NP-40, 0.1% BSA and 10% glycerol). A bacterial extract containing equal amounts of overexpressed GST–MCM2-7 in buffer D was incubated with MBP or MBP-Cdc6 beads for 10 min at 24 °C. Purified wild-type MCM2-7 or MCM2-7–ΔC3 in buffer D was incubated with MBP or MBP-Cdt1 beads for 10 min at 24 °C. The beads were washed three times afterward. The bound proteins were analyzed by western blotting with anti-GST antibody (3G10/1B3; Abcam) (1:500) or an anti–MCM2-7 antibody44 (ab77) (1:500).

Cloning of GST-MCM2-7. The individual MCM genes were amplified by PCR (primers available on request) and cloned via XmaI-NotI into pGEX6-P1 (GE Healthcare) resulting in pCS328 (Mcm5), pCS329 (Mcm4), pCS330 (Mcm6) and pCS333 (Mcm7).

Cloning of MBP-Mcm3, Mcm5-MBP, MBP-Mcm6 and Mcm3ΔC. Using site-directed mutagenesis a restriction site was inserted after aa 111 (Mcm3), aa 591 (Mcm5) and aa 124 (Mcm6). MBP was amplified from pMAL-c2X (NEB) with primers incorporating flexible linkers (sequences available on request) and inserted in the restriction sites, generating pCS444 (pESC-URA-MBP-Mcm3/Mcm5), pCS470 (pESC-URA-Mcm3/Mcm5-MBP) and pCS473 (pESC-TRP-Mcm4/Mbp-Mcp6) for the expression of MBP-Mcm3, Mcm5-MBP, Mbp-Mcm6, respectively. The plasmid for expression of MBP-Mcm2 pCS280 was described previously45. Residues 740–971 of Mcm3 were deleted to generate pCS493 (pESC-URA-MCM3ΔC740-971-MCM5) for the expression of MCM2-7–ΔC3.

Protein purification. The untagged ORC complex and the MBP-tagged ORC complexes24 were expressed and purified as described27. Cdc6 and Cdt1 were expressed in bacteria and purified as described12. Wild-type or mutant MCM2-7 was expressed in yeast and purified as described12.

Cryo-electron microscopy. To prepare cryo-EM grids, we first evaporated a thin layer of carbon film (~2 nm) on a freshly cleaved mica in an Edwards vacuum evaporator, then floated the carbon film off the mica surface in deionized water and deposited film onto the lacey carbon-coated EM grids. The dried EM grids were glow discharged in the 100 mTorr argon atmosphere for 40 s. Sample vitrification was carried out in an FEI Vitrobot plunge-freezing device set to an offset for the blotting pad. Three microliters of the OCCM sample was pipetted onto the freshly glow-discharged EM grid; after 30 s, the grid was blotted for 5 s and plunged into the liquid ethane. The cryo-EM grids were transferred in liquid nitrogen into a Gatan 626 cryo-specimen holder. The specimen was maintained at below ~170 °C during data collection. Cryo-EM was performed in the JEM-2010F operated at an accelerating voltage of 200 kV. Cryo-EM images were recorded with an electron dose of 15 e−Å−2 s−1 × 500,000 magnification on a Gatan Ultra-Scan 4000 CCD camera (4,000 × 4,000 pixel), corresponding to 2.12 Å/pixel sampling at the specimen level.

2D image analyses. Computational image analyses and 3D reconstruction of the OCCM images followed procedures as outlined58. Briefly, we used EMAN for most of the image processing done in this study59. Raw particle images were selected semi-automatically with e2boxer.py in EMAN2 (ref. 59). The particle images were manually inspected, and ‘bad’ particles (those that were broken, contacting other particles or of low contrast) were rejected at this stage, leaving ~90,000 particles in the final wild-type OCCM data set. The contrast transfer function (CTF) was determined on whole micrographs and its effects corrected in the program ctfi. The raw particles were pooled, phase flipped, edge normalized and high-pass filtered (hp = 1). Reference-free 2D classification and averaging of the raw data set were carried out in reine2d.py: A large number of class averages (up to 500) were produced by running the program for nine cycles with at least 20 particles in each class. Careful inspection of the averaged images led us to conclude that the OCCM structure was homogeneous at the 1–2-nm resolution studied here.

3D reconstruction. We used the 2D class averages as input into the program e2initmodel.py to produce ten starting models. We then carefully inspected the consistency between the model re-projections and the original reference-free class averages, and selected three models for the full-scale refinement. We used their low-pass filtered versions (99-Å resolution) as the starting models to minimize initial model bias. Refinement was carried out in the EMAN1.8 with options dfilt and refine turned on. Refinement was carried out in a 144-CPU Dell Linux cluster. The resolution of the cryo-EM 3D map was estimated from Fourier shell correlation at the threshold of 0.5. Refinement with the three models resulted in essentially the same final 3D map at the stated 14-Å resolution. This result is probably due to the good contrast of the 1.1-MDa particle images and the distinct shape of the OCCM structure. 3D reconstruction of the MBP-fusion OCCM complexes followed essentially the same procedure, with the 40-Å low-pass filtered wild-type OCCM 3D map as the starting model for refinement. The number of particles used and the 3D map resolution for each fusion complex were 7,036 and 20 Å for OCCM with MBP fused to the C1 of Orc2 (OCCM_Orc2-MBP); 4,029 and 28 Å for OCCM with MBP fused to the NT of Cdt1 (OCCM_MBP-Cdt1); 2,079 and 20 Å for OCCM_MBP-Mcm2; 1,654 and 30 Å for OCCM_MBP-Mcm3 (negatively stained images); 6,491 and 26 Å for OCCM_Mcm5-MBP; and 6,036 and 22 Å for OCCM_MBP-Mcm6, respectively.

Cryo-electron microscopy 3D map validation. We followed the Henderson-Rosenthal tilt-pair technique as implemented in EMAN2 version 2.06 (ref. 33). The critical part of the technique was to identify cryo-EM grids that produced high particle contrast. Tilt image pairs were recorded by the CCD camera, with the 10° tilted images recorded first and then the untilted images, at the accumulative dose of 30 e−Å−2. The particle pairs were selected in e2RTBoxer.py. Then, in program e2protocutmanager.py, the CTF effects were corrected for the tilted and the untilted particles separately, and the program e2tiltvalidate.py was used to find the Euler angles for all particle images using the default parameters, as suggested in the EMAN2 documentation. The relative tilt axis and angle of each tilt pair was calculated and plotted in the polar coordinate system. We limited the out-of-plane tilt angle to 0.3°, which removed ~50% of the particle pairs. Clustering of the plotted dots around the experimental tilt geometry indicates the correctness of the 3D map and its associated handedness. We found that the ORC–Cdc6 structure in the OCCM was consistent with the mirrored version of the earlier ORC–Cdc6 map11. Therefore, the ORC–Cdc6 map shown in Figure 7a (EMDB EMD-5381) has been mirrored.
Density segmentation and crystal structure docking. The 3D density map was segmented with SEGGER\textsuperscript{39}. The homolog crystal structures were docked into 3D maps with the fit function in Chimera. Surface-rendered figures were also prepared in Chimera\textsuperscript{60}. Although crystal structures of some archaeal replication initiators are known, homology modeling and molecular dynamics flexible fitting were not carried out because eukaryotic replication initiators are larger and often contained several additional domains that have no known homolog structures (and therefore cannot be modeled confidently), and also because of the medium resolution of the OCCM 3D map.

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