Supplementary Materials and Methods

Yeast strains

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>ySDORC</td>
<td>\textit{MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100, bar1::Hyg pep4::KanMX, TRP1 Gal1-10 ORC5,ORC6, HIS3 Gal1-10 ORC3,ORC4, URA3 Gal1-10 CBP-ORC1,ORC2} (Frigola et al, 2013)</td>
</tr>
<tr>
<td>yJF38</td>
<td>\textit{MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100, bar1::Hyg pep4::KanMX, his3::HIS3pRS303/CDT1,GAL4, trp1::TRP1pRS304/MCM4, MCM5, leu2::LEU2pRS305/MCM6, MCM7, ura3::URA3pRS306/MCM2, FLAG-MCM3} (Frigola et al, 2013)</td>
</tr>
<tr>
<td>ySDK8</td>
<td>\textit{MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100, pep4::KanMX, trp1::TRP1pRS304/CDC7, CBP-DBF4}</td>
</tr>
<tr>
<td>yKO3</td>
<td>\textit{MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100, cdc7-4, pep4:: Hyg, his3::HIS3pRS303/SLD3-13MYC, trp1::TRP1pRS304/SLD2, leu2::LEU2pRS305/ SLD7, CDC45, ura3::URA3pRS306/ DPB11}</td>
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Plasmids used to express recombinant proteins in \textit{E. coli}

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Expressed proteins</th>
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<tr>
<td>pLD90</td>
<td>GST-Mcm2 (1-194aa)</td>
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<tr>
<td>pSM61</td>
<td>Sic1-His\textsubscript{6}</td>
</tr>
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Preparation of bead-coupled plasmid DNA templates

Plasmid DNA was purified from \textit{E. coli} using the QIAGEN Plasmid Plus Maxiprep kit (QIAGEN) according to the manufacturer’s instructions. The purified plasmids were then randomly biotinylated using the PHOTOPROBE (Long Arm) Biotin Kit (Vector Laboratories) according to manufacturer’s instructions.
Purification of pre-RC factors
Purifications of the pre-RC factors ORC, Cdc6 and MCM-Cdt1 complexes were performed as described previously (Frigola et al, 2013).

Purification of Dbf4-dependent kinase
For DDK purification from baculovirus-infected insect cells, approximately $6 \times 10^6$ Hi5 cells (Invitrogen) were plated per 150 mm dish containing 25 mL of Grace’s medium (2.4 $\times$ 10$^5$ cells/mL, >97% viability). The cells were allowed to settle at 27°C for 3 days and were then infected with the baculovirus stocks (Dbf4 and Cdc7 respectively) followed by incubation at 27°C for 3 days. Infected High Five (Hi5) cells were harvested by centrifugation and washed in PBS/1 g/L MgCl$_2$. Next, the cell pellet was resuspended in Homo-buffer (25 mM Heps-KOH pH 7.6, 15 mM KCl, 2 mM Mg(OAc)$_2$, 0.1 mM EDTA)/2 mM β-mercaptoethanol/protease inhibitors and dounce-homogenized using a B-pestle for 12 times. Nuclei were then separated from the resultant cytoplasmic extract fraction by centrifugation. Nuclei were resuspended in NuSus buffer (25 mM Heps-KOH pH 7.6, 0.1 M KCl, 2 mM Mg(OAc)$_2$, 0.1 mM EDTA)/2 mM β-mercaptoethanol/protease inhibitors, supplemented with 0.1 volumes of saturated (NH$_4$)$_2$SO$_4$. This mixture was rotated for 30 min at 4 °C. The suspension was centrifuged for 30 min at 40,000 RPM at 4 °C in a Ti 45 rotor (~186,000 g). The resultant supernatant nuclear extract was precipitated with 0.3 g/mL (NH$_4$)$_2$SO$_4$. The nuclear extract precipitate was then resuspended in Buffer K (25 mM Heps-KOH pH 7.6, 0.1 mM EGTA, 0.1 mM EDTA, 0.02% NP-40, 10% Glycerol)/0.4 M NaCl/10 mM imidazole and subjected to Ni$^{2+}$-chelate (HisTrap) (GE Healthcare) chromatography in the same buffer using a gradient of 0.04-1 M imidazole to elute bound protein. DDK-containing fractions were pooled, concentrated using an Amicon Ultra, 10,000 MWCO (Millipore), treated with lambda phosphatase (NEB) for 1 h with rotation at 4 °C and fractionated by gel-filtration chromatography through a 120 mL Superdex 200 HiLoad 16/60 PG column (GE Healthcare) equilibrated in Buffer K/0.4 M NaCl/2 mM β-mercaptoethanol. DDK-containing peak fractions were pooled, dialyzed against Buffer K/0.1 M NaCl/2 mM β-mercaptoethanol and concentrated over a 1 mL MonoQ 5/50 GL column (GE Healthcare) using an elution gradient of 0.1–0.5 M NaCl over 20 CVs. Peak fractions containing DDK were pooled, dialysed against Buffer K/0.1 M K-Glutamate/2 mM β-mercaptoethanol and stored in aliquots at -80 °C.
For DDK purification from *Saccharomyces cerevisiae*, 2 L of *Saccharomyces cerevisiae* cells (ySDK8) were grown in YP-Raffinose at 30 °C to a cell density of 2 x 10⁷ cells per mL. Protein expression was induced by adding 2% galactose for 7-8 h at 30°C. Cells were harvested, washed with ice-cold Wash buffer I (25 mM Hepes-KOH pH 7.6, 1 M Sorbitol), then washed with Buffer A (25 mM Hepes-KOH pH 7.6, 0.05% NP-40, 10% Glycerol)/0.4 M NaCl. The cell pellet was then resuspended in 0.5 volumes of Buffer A/0.4 M NaCl/2 mM β-mercaptoethanol/protease inhibitors (Roche) and frozen drop-wise in liquid nitrogen. Frozen drops of cells were crushed using a freezer mill (SPEX CertiPrep 6850 Freezer/Mill) with 6 cycles of 2 min crushing at a rate of 15. Frozen cell powder was thawed completely on ice, resuspended in 1 volume of buffer A/0.4 M NaCl/2 mM β-mercaptoethanol /protease inhibitors, and the concentration of KCl was adjusted to 0.5 M. The suspension was centrifuged for 1 h at 50,000 RPM at 4 °C using a Ti 70 rotor (~257,000 g). The clear phase was recovered, and subjected to Calmodulin affinity purification by adding 2 mM CaCl₂ and 1.5 mL of packed beads of Calmodulin affinity resin (Stratagene). After 3 h rotation at 4°C, beads were collected, washed with 10 CVs (Column Volumes) of Buffer K/0.4 M NaCl/2 mM CaCl₂/2 mM β-mercaptoethanol. Elution was performed with Buffer K/0.4 M NaCl/ 2 mM EGTA/1 mM EDTA/2 mM β-mercaptoethanol. Peak fractions were pooled, treated with lambda phosphatase (NEB) for 1 h with rotation at 4 °C and subjected to fractionation over a Superdex 200 10/300 GL column (GE Healthcare) pre-equilibrated in Buffer K/0.4 M NaCl/2 mM β-mercaptoethanol. Fractions containing DDK were pooled and dialysed against Buffer K/0.2 M K-Glutamate and stored in aliquots at -80 °C.

**Purification of GST-Mcm2 (1-194aa) fragment**

1 L of *E. coli* cells transformed with the GST-Mcm2 (1-194aa) expression vectors were grown at 37 °C to a density of OD₆₀₀ = 0.5. Cells were chilled on ice for 30 min, and then IPTG was added to 1 mM. Induction was carried out overnight at 15 °C. Cells were harvested, washed once with PBS and stored at -80 °C. Next morning, the cell pellet was resuspended in 50 mL of Buffer G (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.05% NP-40, 10% Glycerol)/1 M NaCl /2 mM β-mercaptoethanol/0.2 mM PMSF/protease inhibitors (Roche). Cells were kept on ice and sonicated 5 x 30 s at 10 microns using a sonicator Soniprep 150 (Sanyo). Lysate was centrifuged for 1 h at
45,000 RPM at 4 °C using a Ti 45 rotor (~235,400 g). The soluble phase was collected and incubated with 1 mL packed Glutathione sepharose 4 fast flow beads (GE Healthcare) at 4 °C with rotation for 1 h. Beads were washed with 10 CVs of buffer G/0.3 M NaCl/1 mM EGTA/1 mM EDTA/2 mM β-mercaptoethanol. Elution was performed with buffer G/0.3 M NaCl/1 mM EGTA/1 mM EDTA/2 mM β-mercaptoethanol/10 mM reduced Glutathione. Peak fractions were pooled, concentrated using an Amicon Ultra, 10,000 MWCO (Millipore) and subjected to fractionation over a Superdex 200 10/300 GL column (GE Healthcare) pre-equilibrated in Buffer G/0.3 M NaCl/2 mM β-mercaptoethanol. Peak fractions were pooled and concentrated over a 1 mL MonoQ 5/50 GL column (GE Healthcare) using an elution gradient of 0.1–0.5 M KCl over 20 CVs. Peak fractions containing GST-Mcm2 (1-194aa) were pooled, dialysed against Buffer G/0.1 M K-Glutamate and stored in aliquots at -80 °C.

**Purification of Sic1**

1 L of *E. coli* cells transformed with the Sic1-His$_6$ expression vectors were grown at 25 °C to a density of OD$_{600}$ = 0.6. Cells were chilled on ice for 20 min, and then IPTG was added to 1 mM. Induction was carried out overnight at 15 °C. Cells were harvested, washed once with PBS and stored at -80 °C. Next morning, the cell pellet was resuspended in 50 mL of Buffer S (25 mM Heps-KOH pH 7.6, 0.02% NP-40, 10% Glycerol)/0.3 M NaCl/2 mM β-mercaptoethanol/0.2 mM PMSF/protease inhibitors (Roche). Cells were kept on ice and sonicated 5 x 30 s at 10 microns using a sonicator Soniprep 150 (Sanyo). Lysate was centrifuged for 1 h at 50,000 RPM at 4 °C using a Ti 70 rotor (~257,000 g). The soluble phase was collected and was added 10 mM imidazole. This lysate was then subjected to Ni$^{2+}$-chelate (HisTrap) (GE Healthcare) chromatography in the same buffer using a gradient of 0.04-1 M imidazole to elute bound protein. Peak fractions were pooled and subjected to fractionation over a Superdex 200 10/300 GL column (GE Healthcare) pre-equilibrated in Buffer S/0.3 M KCl/2 mM β-mercaptoethanol. Peak fractions were pooled and concentrated over a 1 mL MonoQ 5/50 GL column (GE Healthcare) using an elution gradient of 0.1–1.0 M KCl over 20 CVs. Peak fractions containing Sic1-His$_6$ were pooled, concentrated using an Amicon Ultra, 10,000 MWCO (Millipore) and dialysed against Buffer S/0.2 M K-Glutamate and stored in aliquots at -80 °C.
Antibodies for immunoblotting analysis

αMcm2 (yN-19, sc-6680, Santa Cruz), αMcm4 (yC-19, sc-6685, Santa Cruz), αOrc6 (SB49). Antibodies against Psf1 was a kind gift of the Labib laboratory (Gambus et al, 2006). Antibodies against Sld7 was a kind gift of the Zegerman laboratory. Polyclonal antibodies against C-terminus of Mcm6 (20147) were raised against peptides containing Mcm6 C-terminal residues 997-1017. Polyclonal antibodies against Cdc45 (JDI46) were raised against recombinant Cdc45. Polyclonal antibodies against Dpb11 (BPF19) were raised against GST-Dpb11 (555-746aa). Polyclonal antibodies against Sld2 (BPF4) were raised against His-T7-Sld2. Polyclonal antibodies against Sld3 (BPF9) were raised against His-Stag-Sld3 (401-529aa).

Electron microscopy

DNA-bound Mcm2-7 complexes were released from the beads by EcoR1 digestion as previously described (Remus et al, 2009), applied directly onto carbon-coated copper grids and stained with 2 % uranyl acetate. Images were collected with a Tecnai TF20 electron microscope (FEI) operating at 200 kV on a 4k x 4k CCD detector (TVIPS) at a nominal magnification of x 50,000 and an under focus level of ~ 900 nm. Images were binned by a factor of two, resulting in a calibrated pixel size of 3.47 Å at specimen level. Low dose settings were used exposing with ~100 e/Å².

Single particle analysis

Particle selection was carried out using Boxer, part of EMAN2 (Tang et al, 2007) and subsequent processing was performed with Imagic (van Heel et al, 1996) and SPIDER (Frank et al, 1996). Three-dimensional reconstructions were calculated with a locally developed Fourier space program (da Fonseca et al, 2012). Reference free class averages for both untreated and DDK treated Mcm-2-7 were obtained using the refine2d program from EMAN. Three-dimensional analysis of untreated Mcm2-7 was initiated by aligning and projection matching reference free classes to our earlier reconstruction of the Mcm2-7 double hexamer using the C2 symmetry of the system. The final map was obtained by further iterative refinement involving alignment and projection matching of 2,807 side view molecular images to the previous map(s). A similar procedure was used for the 3D analysis of the 267 side view molecular images of DDK treated Mcm2-7. The final maps were compared as rendered surfaces. Further comparison made use of cylindrically averaged maps calculated by artificially imposing a high rotational symmetry during the three-dimensional reconstruction.
References


