Supplemental Materials and Methods

Protein purification

The yeast strains and plasmids used are listed in Materials-Table 1 and Materials-Table 2 in this document.

LacI

To purify LacI, *Escherichia coli* expressing LacI-FLAG from the pBAD24 vector (Guzman et al. 1995) was cultured at 37°C in 1 L of M63 minimal medium containing 0.2% glycerol, 0.2% D-glucose, and 25 μg/ml of ampicillin. When the cell density reached approximately 1.0 OD_{600} (OD, optical density), cells were centrifuged at 8,000 × *g* for 5 min, resuspended in 2 L of M63 minimal medium containing 0.2% glycerol, 0.2% L-arabinose, and 25 μg/ml of ampicillin. Cells were cultured at 37°C for 2.5 h, harvested, and lysed using lysozyme and sonication in CSB buffer containing 200 mM Tris–HCl at pH7.5, 200 mM KCl, 10 mM $MgCl_2$, 5% glycerol, 0.3 mM dithiothreitol (DTT), 1 mM PMSF, 2 μg/ml of aprotinin, 20 μM leupeptin, and 2 μM Pepstatin A. The cell suspension was centrifuged at 12,000 × *g* for 10 min. The supernatant was collected and mixed with solid ammonium sulfate (35% saturation). The precipitate was collected, suspended in 0.2 M HP buffer containing 50 mM HEPES-KOH at pH 7.6, 0.2 M KCl, 0.02% NP-40, 10% glycerol, and 1 mM PMSF, dialyzed against the same buffer; and transferred to anti-FLAG M2 agarose beads (Sigma). The beads were washed with one column volume of 0.2 M HP buffer, one column volume of 0.35 M HP buffer containing 0.35 M KCl, and six column volumes of 0.5 M HP buffer containing 0.5 M KCl. LacI-Flag was eluted with 0.5 M HP buffer with 0.1 mg/ml of the 3×FLAG peptide. Further purification was performed using Resource Q (GE

Healthcare, Waukesha, WI) and Superdex 200 (GE Healthcare) consecutive column chromatography. Peak fractions were pooled and dialyzed against Strage^{LacI} buffer containing 25 mM HEPES–KOH at pH 7.5, 200 mM potassium acetate, 1 mM EDTA, 10% glycerol, 0.01% NP-40, and 1 mM DTT.

LexA

E. coli LexA was obtained from BioAcademia (Osaka, Japan).

ORC

ORC was purified from a yeast strain, yMYx01, that overexpresses the six ORC subunits under the control of the inducible GAL1-10 promoter. In the strain, Orc1 was tagged with 3×Flag at the C-terminus with a PreScission Protease site and a 5-glycine linker. The cell cycle was arrested at G_1 phase using the α factor, and cells were harvested, frozen in liquid nitrogen, and crushed in a freezer mill (SPEX CertiPrep 6850 Freezer/Mill). The cell powder was resuspended in lysis$^{SE+E+1T}$ buffer containing 50 mM HEPES-KOH at pH 7.6, 500 mM KCl, 2 mM EDTA, 0.1% Tween20, 1% Triton X-100, and 10% glycerol. Protease inhibitor cocktails purchased from Sigma (catalog number P8215; Protease Inhibitor Cocktail for use with fungal and yeast extracts) and Roche ("complete, EDTA free") were added. After sonication, an equal volume of Dilution Buffer containing 50 mM HEPES-KOH at pH 7.5, 5% glycerol, and 0.1% Tween20 was added to cell lysates, which were centrifuged at 18,000 × *g* for 15 min. The supernatant was collected and mixed with anti-FLAG M2 agarose beads (Sigma). After washing the beads with lysis$^{SE+E+0.02T}$ buffer (similar to lysis$^{SE+E+1T}$ buffer but containing 0.02% Triton X-100) and 0.3 M NaCl buffer containing 50 mM HEPES-NaOH at pH 7.5, 0.3 M NaCl, 10% glycerol, 0.1% NP-40, and 0.01% Tween 20, ORC was separated from the Flag tag bound to beads via PreScission Protease

treatment. Further purification was performed by successive column chromatography using Resource Q and Resource S (GE Healthcare). Peak fractions were pooled and dialyzed against Strage^{ORC} buffer containing 25 mM HEPES–KOH at pH 7.6, 300 mM potassium glutamate, 10% glycerol, 0.01% NP-40, and 1 mM DTT.

Cdc6

Cdc6 was purified as described previously (Hizume et al. 2017).

Mcm2-7–Cdt1

The Mcm2-7–Cdt1 complex was purified as described previously, with slight modifications (Hizume et al. 2017). Purification was performed using a yeast strain that overexpresses the six Mcm subunits and Cdt1 under the control of the inducible GAL1-10 promoter. In this strain, Mcm4 is linked to a C-terminal 3×Flag tag containing a PreScission Protease recognition protease site and a 5-glycine linker. This strain was cultured at 30°C, and synthesis of the Mcm2-7–Cdt1 complexes was induced by the addition of galactose (2% [w/v] final concentration). Two and a half hours after the addition of galactose, the yeast mating pheromone α factor (final concentration, 150 ng/ml) was added. The deletion of *BAR1* from this strain imparts hypersensitivity to the α factor. The cells were cultured at 30°C for 5 h in the presence of the α factor and then harvested. Cells were frozen in liquid nitrogen and crushed in a freezer mill (SPEX). The cell powder was suspended with Lysis^{MCM} buffer containing 25 mM HEPES–KOH at pH 7.6, 500 mM potassium glutamate, 5% glycerol, 0.1% Tween 20, 0.5% NP-40, 3 mM ATP, and the protease inhibitor cocktails from Sigma and Roche. The cell suspension was sonicated and centrifuged at 18,000 × *g* for 15 min. The supernatant was collected and mixed with an anti-

FLAG M2 affinity gel (Sigma-Aldrich). The beads were washed with $Wash^{MCM}$ buffer ($Lysis^{MCM}$ buffer with 5 mM ATP and 0.02% NP-40) and the gel filtration buffer containing 50 mM HEPES–KOH at pH 7.6, 100 mM potassium acetate, 5 mM magnesium acetate, 10% glycerol, 0.02% NP-40, 5 mM ATP, and 1 mM DTT. The Mcm2-7–Cdt1 complex was separated from the Flag tag bound to beads via PreScission Protease treatment. The eluted fraction was subjected to column chromatography using Superose 6 Increase 10/300 (GE Healthcare). Peak fractions were pooled and dialyzed against $Strage^{MCM–Cdt1}$ buffer containing 50 mM HEPES–KOH at pH 7.6, 100 mM potassium acetate, 5 mM magnesium acetate, 50% glycerol, 0.02% NP-40, and 0.1 mM DTT.

Sld2

To purify Sld2, *3×FLAG-SLD2,* which was engineered to express the fused protein connected by a 5-glycine linker–PreScission Protease site–5-glycine-alanine linker cassette located between the Sld2 N terminus and the Flag tag, was cloned on pESC-LEU and was expressed in the yeast strain BJ2168. The cells were frozen in liquid nitrogen and crushed in a freezer mill (SPEX). The cell powder was resuspended in $lysis^{SE+E+1T}$ buffer, and protease inhibitor cocktails purchased from Sigma and Roche were added. After sonication, cell lysates were centrifuged at 18,000 × *g* for 15 min, and the supernatant was collected and mixed with anti-FLAG M2 agarose beads (Sigma). After washing the beads with $lysis^{SE+E+0.1T}$ buffer (similar to $lysis^{SE+E+1T}$ buffer but containing 0.1% Triton X-100), 0.7 M NaCl buffer (similar to 0.3 M-NaCl buffer but containing 0.7 M NaCl), and 0.3 M-NaCl buffer, Sld2 was eluted using 200 μg/ml of the 3×FLAG peptide in 0.3 M NaCl buffer. After treatment with 18.5 μg/ml of PreScission Protease at 4°C overnight,

further purification was performed using HiTrap Heparin HP and HiTrap-Phenyl consecutive column chromatography (GE Healthcare).

GINS

To purify GINS, *3×FLAG-PSF1*, which was engineered to express the fused protein containing a 5-glycine linker and a PreScission Protease recognition site between the Psf1 N terminus and the 3×Flag tag, and the *SLD5* were cloned on pESC-LEU, *PSF2* and *PSF3* were cloned on pESC-URA, and they were expressed simultaneously in the yeast strain BJ2168. As for Sld2, cell extracts were prepared, and GINS was bound to anti-FLAG M2 agarose beads. The beads were washed with lysis$^{SE+E+0.1T}$ buffer and Cleavage Buffer containing 50 mM HEPES-NaOH at pH 7.5, 0.1 M NaCl, 1 mM EDTA, 5% glycerol, 0.1% Triton X-100, and 0.1% Tween 20. GINS was separated from the Flag tag bound to beads via PreScission Protease treatment. Further purification was performed using Resource Q column chromatography.

Cdc45

To purify Cdc45, *CDC45*, which was engineered to express the protein with an internal 2×Flag tag, as described previously (Yeeles et al. 2015), was cloned on pESC-URA and expressed in the yeast strain BJ2168. This strain was cultured at 30°C, and synthesis of Cdc45 was induced by the addition of galactose (2% [w/v] final concentration). Cells were cultured at 30°C for 7 h in the presence of galactose and then harvested. Cells were frozen in liquid nitrogen and crushed in a freezer mill (SPEX). The cell powder was suspended in H500 buffer containing 25 mM HEPES-KOH at pH 7.6, 10% glycerol, 1 mM EDTA, and 0.5 M potassium acetate, and the protease inhibitor cocktails from Sigma and Roche. The cell suspension was sonicated and centrifuged at 18,000 × *g* for 15 min. The supernatant was collected and mixed with an anti-FLAG M2 affinity

gel (Sigma-Aldrich). The beads were washed with H500 buffer, H300 buffer (similar to H500 buffer but containing 0.3 M potassium acetate), and P buffer containing 25 mM HEPES-KOH at pH 7.6, 5% glycerol, 0.5 mM DTT, and 0.15 M potassium acetate. One to 40^{th} volumes of 0.8 M K-Phosphate Buffer (similar to P buffer but containing 0.8 M potassium phosphate at pH 7.5) were added to adjust the concentration of potassium phosphate to 20 mM. Further purification was carried out using hydroxyapatite column chromatography.

Polε, Polε-pol2ΔN (ΔNPolε) and Pol2ΔN

To purify Polε, *DPB2*, *DPB4*, and *DPB3–5×FLAG*, which was engineered to express the fusion protein connected by a PreScission Protease recognition site located between the Dpb3 C-terminus and the 5×Flag tag, were cloned on pESC-URA (pSE3). pSE3 and pJL1 (Chilkova et al. 2003) bearing *POL2* were introduced to the yeast strain BJ2168 and four subunits of Polε were expressed simultaneously. The purification was carried out as described previously (Muramatsu et al. 2010).

To purify Polε-pol2ΔN complex (ΔNPolε), in which Pol2 lacks amino acids 176-1135, we deleted corresponding region of *POL2* on pJL1. The purification was carried out as Polε except the deletion strain of *pol2* (Materials-Table1) was used to exclude the wild-type Polε.

To purify Pol2ΔN, which lacks amino acids 176–1135 (Kesti et al. 1999; Dua et al. 1999), *POL2ΔN–HIS$_{10}$–FLAG,* which was engineered to express the fusion protein connected by a PreScission Protease recognition site located between the Pol2ΔN C terminus and the His_{10}Flag tag, was cloned on a pESC-LEU and expressed in the yeast strain BJ2168. As for Sld2, cell extracts were prepared, and Pol2ΔN was bound to anti-FLAG M2 agarose beads. The beads were washed with $lysis^{SE+E+0.02T}$ buffer and 0.3M $NaCl^{T}$ buffer (similar to 0.3M NaCl buffer but

containing 0.1% Triton X-100 instead of NP-40). Pol2ΔN was eluted using 150 μg/ml of the 3×FLAG peptide in 0.3M $NaCl^{T}$ buffer. Further purification was performed using HiTrap Heparin HP and Resource Q (GE Healthcare) consecutive column chromatography. Peak fractions were pooled and dialyzed against $Strage^{Pol2\Delta N}$ buffer (similar to $Strage^{Sld3-7}$ buffer but without Tween 20).

Dpb11

Dpb11 was purified from the yeast strain yJY26 (Yeeles et al. 2015), which overexpresses Dpb11–3×Flag under the control of the inducible GAL promoter. In this strain, Dpb11 is linked to a C-terminal 3×Flag tag containing a PreScission Protease recognition protease site. As for Sld2, cell extracts were prepared and were bound to anti-FLAG M2 agarose beads. The beads were washed with $lysis^{SE+E+0.02T}$ buffer. Dpb11 was eluted using 150 μg/ml of the 3×FLAG peptide in $lysis^{SE+E+0.02T}$ buffer. Further purification was performed using HiTrap Heparin HP and HiTrap-SP-XL (GE Healthcare) consecutive column chromatography.

DDK

To purify the DDK complex, *3×FLAG–DBF4*, which was engineered to express the fusion protein containing a PreScission Protease recognition site located between the Dbf4 N terminus and the 3×Flag tag, and HIS_{10}*-CDC7* were cloned on pESC-URA and were expressed in the yeast strain BJ2168. As for Sld2, cell extracts were prepared, and DDK was bound to anti-FLAG M2 agarose beads. The beads were washed with $lysis^{SE+E+0.02T}$ buffer and 0.3M NaCl buffer. The DDK complex was eluted using 150 μg/ml of the 3×FLAG peptide in $lysis^{SE+E+0.02T}$ buffer.

Further purification was performed using HiTrap Heparin HP and Resource Q consecutive column chromatography. Peak fractions were pooled and dialyzed against Strage^{DDK} buffer containing 25 mM HEPES–KOH at pH 7.6, 200 mM potassium acetate, 10% glycerol, 0.02% NP-40, and 1 mM DTT.

Sld3–Sld7

To purify the Sld3–Sld7 complex, *SLD3–3×FLAG,* which was engineered to express the fused protein with a PreScission Protease recognition site located between the Sld3 C terminus and the 3×Flag tag, and *SLD7* were cloned on YEplac112, and were expressed in the yeast strain BJ2168. DNA sequences coding for Sld3 and Sld7 were codon optimized for expression in *S. cerevisiae*. As for Sld2, cell extracts were prepared, and Sld3–Sld7 was bound to anti-FLAG M2 agarose beads. The beads were washed with lysis$^{SE+E+0.02T}$ buffer and ATP buffer containing 25 mM HEPES-KOH at pH 7.6, 500 mM KCl, 10% glycerol, 0.02% NP-40, 1 mM DTT, 1 mM ATP, and 10 mM magnesium acetate, and with 0.3 M NaCl buffer. Sld3–Sld7 was separated from the Flag tag bound to beads via PreScission Protease treatment. Further purification was performed using HiTrap SP-XL and Superdex 200 10/300 consecutive column chromatography. Peak fractions were pooled and dialyzed against Strage^{Sld3-7} buffer containing 25 mM HEPES–KOH at pH 7.5, 200 mM potassium acetate, 2 mM magnesium acetate, 10% glycerol, 0.02% NP-40, 0.1% Tween 20, and 0.1 mM DTT.

Mcm10

To purify Mcm10, *E. coli* cells expressing His_6–Mcm10 with a PreScission Protease recognition site located between the Mcm10 N terminus and the His6 tag from the corresponding gene on pET15b vector were cultured at 37°C in LB medium. When the cell density reached

approximately 0.5 of OD_{600}, IPTG was added to the medium at a final concentration of 0.5 mM, and cells were cultured at 37°C for 2 h and then harvested. Cells were lysed via sonication in $Lysis^{Mcm10}$ buffer containing 50 mM HEPES-NaOH at pH 7.5, 10% glycerol, 0.2% Tween20, 0.01 mM AEBSF, and 0.01 mg/mL of lysozyme. The cell suspension was centrifuged at 18,000 × *g* for 15 min. The supernatant was collected, adjusted to 500 mM NaCl and 20 mM imidazole, and then transferred to Ni-NTA resin. The beads were washed with $Lysis^{Mcm10}$ buffer containing 50 mM imidazole and 500 mM NaCl. His_6–Mcm10 was eluted using $Lysis^{Mcm10}$ buffer with 500 mM imidazole and 500 mM NaCl. Mcm10 was separated from the His6 tag via treatment with PreScission Protease. Further purification was performed using Resource S column chromatography. Peak fractions were pooled and dialyzed against $Strage^{LacI}$ buffer.

RPA

To purify RPA, *E. coli* cells transformed with pSAS106 (Bastin-Shanower and Brill 2001) were cultured at 37°C in 1 L of LB medium. When the cell density reached approximately 0.5 of OD_{600}, IPTG was added into the medium at a final concentration of 0.4 mM, and the cells were cultured at 37°C for 2 h and then harvested. Cells were lysed via sonication in $Lysis^{RPA}$ buffer containing 25 mM HEPES-NaOH at pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.01% NP-40, and 1 mM AEBSF. The cell suspension was centrifuged at 18,000 × *g* for 15 min. The supernatant was collected and transferred to Affigel-Blue Gel (Bio-Rad). The beads were washed with Blue buffer containing 25 mM HEPES-NaOH at pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.01% NP-10, and 0.25 mM AEBSF. After further washing of the beads with Blue buffer containing 0.8 M NaCl and Blue buffer containing 0.5 M Sodium thiocyanate, RPA was eluted using Blue buffer containing 1.5 M Sodium thiocyanate. Further

purification was performed using Hydroxyapatite and Resource Q consecutive column chromatography. Peak fractions were pooled and dialyzed against Strage^{RPA} buffer containing 50 mM HEPES–NaOH at pH 7.5, 25 mM NaCl, 1 mM EDTA, 10% glycerol, 0.01% NP-40, and 1 mM DTT.

CDK

CDK was purified from the yeast strain yAE37 (Yeeles et al. 2015), which overexpresses Cdc28, CBP–Clb5, and Cks1 under the control of the inducible GAL promoter. In this strain, Clb5 is linked to an N-terminal CBP tag. This strain was cultured at 30°C, and synthesis of the Cdc28–Clb5–Cks1 complex was induced by the addition of galactose (2% [w/v] final concentration). Galactose and 50 mg/L of benomyl were added and the cells were cultured at 30°C for 3 or 4 h and then harvested. As for Sld2, cell extracts were prepared using Lysis^{CDK} buffer containing 40 mM HEPES-KOH at pH 7.6, 300 mM potassium acetate, 10% glycerol, and 0.02% NP-40, and CDK was bound to Calmodulin Sepharose 4B beads (GE Healthcare) in the presence of 2 mM $CaCl_2$. The beads were washed with Lysis^{CDK} buffer containing 2 mM $CaCl_2$ and TEV protease buffer containing 40 mM HEPES-KOH at pH 7.6, 150 mM potassium acetate, 10% glycerol, 1 mM DTT, 0.02% NP-40, and 2 mM $CaCl_2$. CDK was separated from the CBP tag bound to beads via TEV protease treatment. Further purification was performed using Hi Trap Q-HP and Superdex 200 10/300 consecutive column chromatography with a buffer containing 50 mM HEPES-KOH at pH 7.6, 300 mM potassium acetate, 0.02% NP-40, 10% glycerol, and 0.1 mM DTT.

Polδ

To purify Polδ, *3×FLAG–POL3,* which was engineered to express the protein with a 5-glycine linker and a PreScission Protease recognition site located between the Pol3 N terminus and the 3×Flag tag, was cloned on pESC-LEU and *POL31* and *POL32* were cloned on pESC-URA. They were expressed in the yeast strain BJ2168. As for Sld2, cell extracts were prepared, and Polδ was bound to anti-FLAG M2 agarose beads. The beads were washed with lysis$^{SE+E+0.1T}$ buffer and 0.25 M NaCl buffer containing 50 mM HEPES-NaOH at pH 7.5, 0.25 M NaCl, 10% glycerol, and 0.02% NP-40. Polδ was separated from the Flag tag bound to beads via PreScission Protease treatment. Further purification was performed using HiTrap Heparin HP, Resource S, and Resource Q consecutive column chromatography. Peak fractions were pooled and dialyzed against Strage^{Polδ} buffer containing 25 mM HEPES–KOH at pH 7.6, 200 mM potassium acetate, 2 mM magnesium acetate, 10% glycerol, 0.02% NP-40, and 0.1 mM DTT.

Polα

Polα was purified from the yeast strain yJY23 (Yeeles et al. 2015), which overexpresses Pol1, Pol12, CBP-Pri1, and Pri2 under the control of the inducible GAL promoter. In this strain, Pri1 is linked to an N-terminal CBP tag. This strain was cultured at 30°C, and synthesis of Polα was induced by the addition of galactose (2% [w/v] final concentration). As for Sld2, cell extracts were prepared using Lysis^{Pola} buffer containing 25 mM HEPES-KOH at pH 7.6, 0.4 M NaCl, 10% glycerol, 1 mM DTT, 0.02% NP-40, and 1% Triton X-100. Protease inhibitor cocktails purchased from Sigma and Roche were added. The concentration of NaCl was decreased to 0.3 M via dilution with Dilution^{Pola} Buffer containing 25 mM HEPES-KOH at pH 7.6, 10% glycerol, 1 mM DTT, and 0.02% NP-40. Polα was bound to Calmodulin Sepharose 4B beads in the

presence of 2 mM $CaCl_2$. The beads were washed with $Wash^{Pola}$ buffer containing 25 mM HEPES-KOH at pH 7.6, 0.3 M NaCl, 2 mM $CaCl_2$, 10% glycerol, 1 mM DTT, and 0.02% NP-40, and Polα was eluted using $Elute^{Pola}$ buffer containing 25 mM HEPES-KOH at pH 7.6, 0.3 M NaCl, 10% glycerol, 1 mM DTT, 0.02% NP-40, 2 mM EGTA and 2 mM EDTA. Further purification was performed using HiTrap Heparin HP, Resource Q, and Superose 6 Increase (GE Healthcare) consecutive column chromatography. Peak fractions were pooled and dialyzed against $Strage^{Polα}$ buffer containing 25 mM HEPES–KOH at pH 7.6, 200 mM potassium acetate, 1 mM EDTA, 10% glycerol, 0.01% NP-40, and 1 mM DTT.

PCNA

To purify PCNA, *E. coli* cells transformed with pBL228 (Ayyagari et al. 1995) were cultured at 37°C in 1 L of LB medium. When the cell density reached approximately 0.5 of OD_{600}, IPTG was added to the medium at a final concentration of 0.5 mM, and cells were cultured at 37°C for 2 h and then harvested. Cells were lysed via sonication in $Lysis^{PCNA}$ buffer containing 25 mM HEPES-NaOH at pH 7.5, 0.3 M NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 0.5% Triton X-100, 1 mM PMSF, 1 mM AEBSF, and 0.5 mg/mL of lysozyme. The cell suspension was centrifuged at 18,000 × *g* for 15 min. The supernatant was collected and mixed with 1/25 volumes of 5 M NaCl and 1/33 volumes of 10% Polimin P, to reach a final concentration of 0.5 M NaCl and 0.3% PoliminP. After centrifugation at 18,000 × *g* for 15 min, the precipitate was washed with $Lysis^{PCNA}$ buffer lacking lysozyme and Triton X-100. PCNA was precipitated by centrifugation at 18,000 × *g* for 15 min and resuspended in 1 M NaCl buffer containing 50 mM HEPES-NaOH at pH 7.5, 1 M NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, and 1 mM AEBSF. The soluble fraction was collected after centrifugation at 18,000 × *g* for 15 min and was then

mixed with solid ammonium sulfate (70% saturation). The precipitate was collected and suspended in AKTA buffer containing 50 mM HEPES-KOH at pH 7.6, 0.1% Tween20, 0.01% Triton X-100, 2 mM $MgCl_2$, 0.1 mM EDTA, 5% glycerol, and 0.1 mM DTT, and dialyzed against the same buffer containing 0.1 M NaCl. Further purification was performed using Resource Q column chromatography.

RFC

To purify RFC, a yeast strain transformed with pBL420 (Gerik et al. 1997) carrying insertions of the genes of all five subunits of RFC was cultured and harvested. The cells were frozen in liquid nitrogen and crushed in a freezer mill (SPEX). The cell powder was resuspended in $Lysis^{RFC}$ buffer containing 50 mM HEPES-NaOH, 0.5 M NaCl, 3 mM DTT, 2 mM EDTA, and 5% glycerol. To the cell lysates, 1/50 volumes of a phosphatase inhibitor cocktail (Nakarai, Kyoto, Japan), 1/100 volumes of a Roche protease inhibitor cocktail, and 1/20 volumes of 10% Triton X-100 were added, followed by sonication and centrifugation at 18,000 × *g* for 15 min. The supernatant was collected and mixed with SP-Sepharose HP beads (GE Healthcare). The beads were washed with 0.1 M NaCl buffer containing 50 mM HEPES-NaOH at pH 7.5, 0.1 M NaCl, 3 mM DTT, 2 mM EDTA, 0.1% Triton X-100, and 5% glycerol, and with B-0 buffer containing 50 mM HEPES-NaOH at pH 7.5, 3 mM DTT, 0.5 mM EDTA, 1 mM ATP, 8 mM magnesium acetate, 0.01% Triton X-100, and 10% glycerol. RFC was eluted using B-500 buffer (similar to B-0 buffer but containing 500 mM NaCl), and 1/50 volumes of a phosphatase inhibitor cocktail and 2/3 volumes of B-0 buffer were added. RFC was bound to PCNA beads (purified PCNA was bound to Affi-Gel 10 (GE Healthcare), as described previously (Gerik et al. 1997)), and the beads were washed with B-300 and B-400 buffers (similar to B-0 buffer but containing 300 and 400

mM NaCl, respectively). RFC was eluted using C-400 buffer containing 50 mM HEPES-NaOH at pH 7.5, 400 mM NaCl, 3 mM DTT, 2 mM EDTA, 0.01% Triton X-100, and 10% glycerol. Further purification was performed using Resource S column chromatography.

Top2

Top2 was purified from the yeast strain yAE46 (Yeeles et al. 2015), which overexpresses Top2 under the control of the inducible GAL promoter. In this strain, Top2 is linked to an N-terminal CBP tag. This strain was cultured at 30°C, and synthesis of Top2 was induced by the addition of galactose (2% [w/v] final concentration). As for Sld2, cell extracts were prepared using $lysis^{Top2}$ buffer containing 25 mM HEPES-KOH at pH 7.6, 300 mM NaCl, 10% glycerol, 1 mM DTT, 0.02% NP-40, and 1% Triton X-100. Protease inhibitor cocktails purchased from Sigma and Roche were added. Top2 was bound to Calmodulin Sepharose 4B beads in the presence of 2 mM $CaCl_2$. The beads were washed with $lysis^{Top2+C}$ buffer (similar to $lysis^{Top2}$ buffer but without Triton X-100) and with $lysis^{Top2-C}$ buffer (similar to $lysis^{Top2+C}$ buffer but without $CaCl_2$), and Polα was eluted using $lysis^{Top2+C}$ buffer (similar to $lysis^{Top2-C}$ buffer but containing 2 mM EGTA and 2 mM EDTA). Further purification was performed using HiTrap Heparin HP, Resource Q, and Superdex 200 (GE Healthcare) consecutive column chromatography. Peak fractions were pooled and dialyzed against $Strage^{Top2}$ buffer containing 25 mM HEPES–KOH at pH 7.6, 200 mM potassium acetate, 10% glycerol, 0.01% NP-40, and 1 mM DTT.

Fob1

To purify Fob1, *GST–FOB1,* which was engineered to express the protein with a thrombin recognition site located between the Fob1 N terminus and the GST tag, was cloned on pEG(KT) (Pierce and Wendland 2009) (pHZM78) and was expressed in the yeast strain BJ2168. The cells

were disrupted by glass beads in lysis^{Fob1} buffer containing 50 mM HEPES-KOH at pH 7.6, 0.8 M NaCl, 0.1% Triton X-100, 5% glycerol, 1 mM PMSF, 10 μg/ml of Aprotinin, 100 μM leupeptin, and 10 μM Pepstatin A. The cell lysates were centrifuged at 15,000 × *g* for 30 min. The supernatant was collected and mixed with Glutathione Sepharose 4B beads (GE Healthcare). After washing the beads with lysis^{Fob1} buffer and PBS, Fob1 was separated from the GST tag bound to beads via thrombin treatment. Further purification was performed using Resource Q (GE Healthcare) column chromatography.

Tof1–Csm3

To purify Tof1–Csm3, *6×HIS–CSM3,* which was engineered to express the protein with a TEV protease recognition site located between the Csm3 N terminus and the 6×His tag was cloned on pEG(KT) (pHZM76) and *FLAG-TOF1,* which was engineered to express the protein with a PreScission Protease recognition site located between the Tof1 N terminus and the Flag tag, was cloned on YEplac112 (pHZM77). They were expressed simultaneously in the yeast strain BJ2168. The cells were disrupted by glass beads in lysis$^{SE+E+1T}$ buffer. Protease inhibitors, 1 mM PMSF, 10 μg/ml of aprotinin, 100 μM leupeptin, and 10 μM Pepstatin A were added. The cell lysates were centrifuged at 15,000 × *g* for 15 min. The supernatant was collected and mixed with anti-FLAG M2 agarose beads (Sigma). After washing the beads with lysis$^{SE+E+0.02T}$ buffer, Tof1–Csm3 was eluted using Lysis$^{SE+E+FLAG}$ buffer (similar to Lysis^{SE+E} buffer but containing 0.25 μg/ml of the 3×FLAG peptide). Further purification was performed using Resource Q (GE Healthcare) column chromatography.

Rrm3

To purify Rrm3, *FLAG–RRM3,* which was engineered to express the protein with a PreScission Protease recognition site located between the Rrm3 N terminus and the Flag tag, was cloned on YEplac112 (pHZM83) and was expressed in the yeast strain BJ2168. Cultivated cells were frozen in liquid nitrogen, ground with an automill "TK-AM5" (Tokken, Kashiwa, Japan), and suspended in lysis$^{SE+E+1T}$ buffer with 1 mM PMSF, 10 μg/ml of aprotinin, 100 μM leupeptin, and 10 μM Pepstatin A. The cell lysates were centrifuged at 16,000 × *g* for 30 min. The supernatant was collected and mixed with anti-FLAG M2 agarose beads (Sigma). After washing the beads with lysis$^{SE+E+0.02T}$ buffer, Rrm3 was separated from the Flag tag bound to beads via PreScission Protease treatment. Further purification was performed using Resource Q (GE Healthcare) column chromatography. The helicase activity of Rrm3 was confirmed as described previously (Ivessa et al. 2002), using forked DNA substrates having 48 bp dsDNA and 75 base ssDNA regions.

CMG

CMG was purified from the yeast strain OY163 (Georgescu et al. 2014), which overexpresses all subunits of the CMG complex under the control of the inducible GAL promoter. In this strain, Mcm5 is linked to an N-terminal 3×Flag tag, and Sld5 is linked to an N-terminal GST tag containing a PreScission Protease recognition protease site. This strain was cultured at 30°C, and synthesis of CMG was induced by the addition of galactose (2% [w/v] final concentration). As for Sld2, cell extracts were prepared using Lysis^{CMG} buffer containing 20 mM HEPES-KOH at pH 7.6, 350 mM KCl, 1 mM EDTA, 10% glycerol, 4 mM $MgCl_2$, 0.5% NP-40, and 3 mM ATP. The Complete EDTA-free protease inhibitor cocktail (Roche) was added. CMG was bound to

anti-FLAG M2 agarose beads. The beads were washed with Wash^{CMG} buffer (similar to Lysis^{CMG} buffer but containing 0.01% NP-40) and Wash^{CMG-A} buffer (similar to Wash^{CMG} buffer but without ATP). CMG was eluted with Wash^{CMG-A} buffer containing 150 μg/ml of the 3×FLAG peptide and bound to Glutathione Sepharose 4B beads. The beads were washed with Wash^{CMG} buffer, 0.5 M K-OAC buffer containing 50 mM HEPES-KOH at pH 7.5, 0.5 M potassium acetate, 10% glycerol, 1 mM DTT, and 0.01% NP-40, and again with Wash^{CMG} buffer. CMG was separated from the GST tag bound to beads via PreScission Protease treatment. Further purification was performed using Hi TrapQ-XL and Superose 6 Increase 10/300 consecutive column chromatography. Peak fractions were pooled and dialyzed against Strage^{CMG} buffer (similar to 0.5M K-OAC buffer but containing 0.4 M potassium acetate).

Dpb2

To purify Dpb2, *DPB2–3×FLAG,* which was engineered to express the protein with a PreScission Protease recognition site located between the Dpb2 C terminus and the 3×Flag tag, was cloned on a pESC-URA vector and was expressed in the yeast strain BJ2168. As for Sld2, cell extracts were prepared, and Dpb2 was bound to anti-FLAG M2 agarose beads. The beads were washed with lysis$^{SE+E+0.1T}$ buffer and 0.3 M NaCl^{T} buffer, followed by elution of Dpb2 using 150 μg/ml of the 3×FLAG peptide in 0.3M NaCl^{T} buffer. Further purification was performed using HiTrap Heparin HP and HiTrap Q consecutive column chromatography. Peak fractions were pooled and dialyzed against Strage$^{Pol2\Delta N}$ buffer.

Dpb3–Dpb4

To purify the Dpb3–Dpb4 complex, the *DPB2* gene was removed from pJL6 (Chilkova et al. 2003), which was based on the pRS426GAL vector, and the DNA fragment encoding a 5×Flag tag with a PreScission Protease recognition site was inserted at 3' end of *DPB3* to produce the Dpb3-5Flag protein. The resulting plasmid, pRS426GAL bearing *DPB3–5×FLAG* and *DPB4*, was named pSE17. The yeast strain BJ2168 transformed with pSE17 was cultured, and as for Dpb2, cell extracts were prepared, and Dpb3–Dpb4 was bound to anti-FLAG M2 agarose beads. The beads were washed with lysis$^{SE+E+0.1T}$ buffer, and Dpb3–Dpb4 was eluted using 150 μg/ml of the 3×FLAG peptide in 0.3M NaCl^{T} buffer. Further purification was performed using HiTrap Heparin HP, HiTrap Q, and Resource Q consecutive column chromatography. Peak fractions were pooled and dialyzed against Strage$^{Pol2\Delta N}$ buffer.

In vitro replication assay

For the MCM loading reaction, 2 nM DNA (plasmid) was mixed with 11 nM ORC, 23 nM Cdc6, and 50 nM MCM–Cdt1 in a buffer containing 25 mM HEPES-KOH at pH 7.6, 100 mM K-glutamate, 10 mM magnesium acetate, 0.01% NP-40, 100 μg/ml of BSA, 1 mM DTT, and 5 mM ATP. After 20 min of incubation at 30°C, DDK was added directly to the reaction to a final concentration of 26 nM and incubation was continued at 30°C for 20 min. When the reaction was performed in the presence of LacI, Fob1, Tof1–Csm3, or Rrm3, these proteins were added into the reaction, and incubation was continued at 30°C for 20 min. For the firing reaction, the following proteins and solutions were added directly to the reaction: 25 nM Sld3–Sld7, 50 nM Sld2, 30 nM Dpb11, 210 nM GINS, 40 nM Cdc45, 20 nM Polε, 5 nM Mcm10, 100 nM RPA, 3.4

nM CDK, 5 nM Polα, 20 nM RFC, 20 nM PCNA, 10 nM Top2, 10 nM Polδ, 0.2 mM each NTP, 80 μM dATP, dCTP, and dGTP, 60 μM dTTP, and 20 μM Biotin-16-dUTP (Sigma-Aldrich). After incubation at 30°C for 20 min, the reaction was terminated by the addition of 1/5 volume of Gel Loading Buffer containing 0.3 N NaOH, 6 mM EDTA, 36% glycerol, and Orange G dye. The products were separated on 1% alkaline agarose gels in 0.05 N NaOH and 1 mM EDTA for 75 min at 75 V. DNA was transferred from the gels to Hybond N+ membranes according to the manufacturer's instructions. After the sequential treatment of the membranes with 2% ECL Advance Blocking Reagent in TBST and with 0.1 μg/ml of IR-Dye 680 RD streptavidin (LI-COR) in TBST, the membrane was scanned on an Odyssey infrared imaging system (LI-COR).

AFM imaging of the DNA product of the in vitro replication assay

After the replication reaction, instead of the Gel Loading Buffer, an equal amount of 1 M Replication Assay High Salt Wash Buffer containing 45 mM HEPES-KOH, 5 mM magnesium acetate, 0.02% NP-40, 1 mM EDTA, 1 mM EGTA, and 1 M NaCl was added for the termination of the reaction. DNA fragments longer than ~400 bp were separated on a gel-filtration spin column containing Sepharose CL-4B (GE Healthcare) equilibrated with 0.5 M Replication Assay High Salt Wash Buffer (as 1 M Replication Assay High Salt Wash Buffer, but with 0.5 M NaCl), as described previously (Mizuguchi and Wu 1999). From the eluted fraction containing DNA, the DNA–protein complex, or both, DNA was purified by phenol extraction and 2-propanol precipitation. DNA was suspended in CutSmart Buffer (New England Biolabs, MA) and treated with the restriction enzyme *Sca*I-HF (New England Biolabs) at 37°C for 60 min. DNA was purified by phenol extraction and 2-propanol precipitation, and suspended in HE buffer

containing 10 mM HEPES-KOH and 1 mM EDTA. DNA was fixed with HMgG Buffer containing 5 mM HPEPS-KOH at pH 7.6, 8 mM $MgCl_2$, and 0.1% glutaraldehyde for 30 min at room temperature and dropped onto a freshly cleaved mica surface. After incubation for 10 min at room temperature, the mica was washed with water and dried under a stream of nitrogen. AFM was performed using a Nanoscope IIIa (Bruker AXS, Karlsruhe, Germany) in air, using the tapping mode with a silicon nitride cantilever (OMCL-AC160TS-R3; Olympus, Tokyo, Japan). The scanning frequency was 1.8–3 Hz, and images were captured in height mode (512 × 512 pixels). The images obtained were plane fitted and flattened using the software supplied with the imaging module.

Forked DNA substrate for CMG helicase assay used in Supplemental Fig. S5 and S6

For the "w/o LacO" (or "w/ LacO") substrate in Fig. 3A and Supplemental Fig. S4B, pHZM84 (or pHZM 87) (the sequences of these plasmids are shown in Supplemental Table S1) was digested with *Pst*I-HF and *Sph*I (or *Pst*I-HF and *Eco*RV-HF). To assemble the short-forked DNA, oligonucleotide "5tx72_PstI_2" (Supplemental Table S1) was annealed to oligonucleotide "3Tx75_BstEII" (Supplemental Table S1). A 40-fold molar excess of the short-forked DNA was mixed with *Pst*I-*Sph*I-digested pHZM84 (or *Pst*I-*Eco*RV-digested pHZM87) and then ligated overnight at room temperature with T4 DNA ligase (Toyobo, Osaka, Japan). The ligated product was purified by phenol extraction and 2-propanol precipitation, digested with *Eco*RI (or *Not*I), and separated on a 1.0% agarose gel. The band corresponding to the ligated product with a length of 1.4 kb (or 1.1 kb) was cut out of the gel, and the DNA was eluted from the gel slice using a Gel Extraction Kit (Qiagen, Hilden, Germany). To assemble the short IR-labeled DNA,

oligonucleotide “IR700M13-47” was annealed to oligonucleotide “Pho_27comp_EcoRI” (or “Pho_27_comp_NotI”). A 40-fold molar excess of the short IR-labeled DNA was mixed with the forked DNA purified from the agarose gel, then ligated overnight at room temperature with T4 DNA ligase. DNA was purified by phenol extraction and 2-propanol precipitation, followed by application to a gel-filtration spin column containing Sepharose CL-4B (GE Healthcare) equilibrated with HE Buffer.

For the “w/ RFB^{FW}”, “w/ RFB^{RV}”, or “w/o RFB” substrates in Supplemental Fig. S5, infrared labeled fragment containing nucleosome positioning sequence “601” and RFB^{FW}, “601” and RFB^{RV}, or “601” without RFB were amplified by polymerase chain reaction (PCR) using pHZM69, pHZM68, or pHZM67 (the sequences of these plasmids are shown in Supplemental Table S1) as a template and a primer set of “IR700 M13-RV-M” and “pUC MCS PstIplusBstEII” (Supplemental Table S1), and digested with *Bst*EII-HF. To assemble the short-forked DNA, oligonucleotide “5Tx75_BstEII” (Supplemental Table S1) was annealed to oligonucleotide “3Tx75_BstEII” (Supplemental Table S1). A 4-fold molar excess of the short-forked DNA was mixed with *Bst*EII-digested PCR fragment using pHZM68, pHZM69, or pHZM67 and then ligated overnight at room temperature with T4 DNA ligase (Toyobo, Osaka, Japan). The ligated product was purified by phenol extraction and 2-propanol precipitation, and separated on a 5–20% acrylamide gel in the Tris-Glycine buffer containing 25 mM Tris and 192 mM Glycine. The band corresponding to the ligated product was cut out of the gel, and the DNA was eluted from the gel slices with a Bio-Rad Electro-Eluter. DNA was purified by phenol extraction and 2-propanol precipitation, and resuspended in HE buffer containing 10 mM HEPES-KOH at pH 7.6 and 1 mM EDTA.

For the SOS-box containing substrates in Supplemental Fig. S6, infrared labeled fragment containing SOS-box was amplified by PCR using pBlue8LexAo_deletion (the sequence of the plasmid is shown in Supplemental Table S1) as a template and a primer set of "IR700M13-47" (Supplemental Table S1) and M13-RV-M, and digested with *Pst*I-HF. To assemble the short-forked DNA, oligonucleotide "5Tx72_PstI_2" (Supplemental Table S1) was annealed to oligonucleotide "3Tx75_BstEII" (Supplemental Table S1). A 4-fold molar excess of the short-forked DNA was mixed with *Pst*I-digested PCR fragment using pBlue8LexAo_deletion and then ligated overnight at room temperature with T4 DNA ligase (Toyobo, Osaka, Japan). The ligated product was purified by phenol extraction and 2-propanol precipitation, and separated on a 5–20% acrylamide gel in the Tris-Glycine buffer containing 25 mM Tris and 192 mM Glycine. The band corresponding to the ligated product was cut out of the gel, and the DNA was eluted from the gel slices with a Bio-Rad Electro-Eluter. DNA was purified by phenol extraction and 2-propanol precipitation, and resuspended in HE buffer containing 10 mM HEPES-KOH at pH 7.6 and 1 mM EDTA.

CMG helicase assay

The helicase assay was performed as flollows unless otherwise indicated. The reaction contained 0.2 nM IR-labeled forked DNA substrate, and 20 nM CMG was incubated at 20°C for 50 min in the buffer containing 20 mM Tris-Acetate at pH 7.9, 0.1 mM EDTA, 10 mM $MgSO_4$, 50 mM K-glutamate, 5 mM DTT, and 0.2 mM ATPγS. After the addition of LacI, the reaction was incubated at 20°C for 10 min, and ATP was added at a final concentration of 5 mM and incubated again at 20°C for 32 min. The reaction was stopped by the addition of 1/3 volume of MNStopDye containing 2.5% *N*-lauroyl sarcosine, 100 mM EDTA, 44% glycerol, 0.5 mg/mL

Proteinase K, and Orange G, and was flash frozen in liquid nitrogen. Reaction products were thawed quickly and separated on 5%–20% native PAGE in Tris–glycine buffer containing 25 mM Tris-base and 192 mM glycine (Fig. 3 and Supplemental Fig. S4 and S5) or in TBE buffer containing 89 mM Tris-boric acid (pH 8.3) and 2 mM EDTA (Supplemental Fig. S6). The gel was scanned directly using an Odyssey imaging system (LI-COR Biosciences, Lincoln, NE).

Materials-Table 1. Yeast Strains used for protein purification

Yeast strain	Protein expressed	Reference or source
yMYx01	Orc1–3×Flag, and Orc2–6	This work (adding Flag-tag to C-terminus of Orc1 gene inthe yeast strain, ySC15 (Tsakraklides and Bell 2010)
yMY287	Mcm2, Mcm3, Mcm4–5×FLAG, Mcm5–Mcm7, and Cdt1	(Hizume et al. 2017)
yJY26	Dpb11–3×Flag	(Yeeles et al. 2015)
yAE37	Cdc28, CBP–Clb5, and Cks1	(Yeeles et al. 2015)
yJY23	Pol1, Pol12, CBP–Pri1, and Pri2	(Yeeles et al. 2015)
yAE46	CBP–Top2	(Yeeles et al. 2015)
OY163	CMG (3×Flag–Mcm5, GST–Sld5)	(Georgescu et al. 2014)
BJ2168	General host for expression of proteins	Laboratory stock (Muramatsu et al. 2010)
BJ2168*Δpol2*	Pol2ΔN, Dpb2, Dpb3-5Flag, and Dpb4	This work (*Δpol2::LEU2* in BJ2168 background)

Materials-Table 2. Plasmids used for protein purification

Plasmid	Protein expressed from the insert	Reference or source	Original vector
pHZM30	LacI–Flag	this work	pBAD24 (Guzman et al. 1995)
pST351-1	His_6–Cdc6		(Hizume et al. 2017)
pSE14	3×FLAG–Sld2	this work	pESC-LEU
pSE5	3×FLAG–Psf1, and Sld5	this work	pESC-LEU
pSE7	Psf2, and Psf3	this work	pESC-URA
pSE54	Cdc45 internal 2×Flag	this work	pESC-URA
pSE3	Dpb2, Dpb3–5×Flag, and Dpb4	this work	pESC-URA
pJL1	Pol2	(Chilkova et al. 2003)	
pMYx01	3×Flag–Dbf4, and His_{10}–Cdc7	this work	pESC-URA
pST1394	Sld3–5×Flag, and Sld7	this work	YEplac112 (inserted GAL promoter)
pMYx02	His_6–Mcm10	this work	pET15b
pSAS106	Rfa1, Rfa2, and Rfa3	(Bastin-Shanower and Brill 2001)	
pMYx03	3×Flag–Pol3	this work	pESC-LEU
pMYx04	Pol31, and Pol32	this work	pESC-URA
pBL228	Pol30	(Ayyagari et al. 1995)	
pBL420	Rfc1, Rfc2, Rfc3, Rfc4, and Rfc5	(Gerik et al. 1997)	
pHZM78	GST–Fob1	this work	pEG(KT)
pHZM76	His_6–Csm3	this work	pEG(KT)
pHZM77	Flag–Tof1	this work	YEplac112 (inserted GAL promoter)
pHZM83	Flag–Rrm3	this work	YEplac112 (inserted GAL promoter)
pSE1	ΔNPol2– His_{10}–Flag	this work	pESC-LEU
pSE51	Dpb2–3×Flag	this work	pESC-URA
pSE17	Dpb3-5×Flag, and Dpb4	this work	pJL6 (Chilkova et al. 2003)

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