Supporting Information

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SI Materials and Methods

Nucleotides and DNA. ATP was obtained from Sigma–Aldrich, and labeled dGTP and ATP were purchased from PerkinElmer. Oligonucleotides used in this study were synthesized commercially by Integrated DNA Technologies. Single-stranded M13mp18 was from Bayou Biolabs.

Antibodies. Antibodies and their sources were as follows: FLAG tag (Sigma–Aldrich); His_6 tag (GenScript); chromosome transmission fidelity 4 (Ctf4) (BioLegend); minichromosome maintenance (Mcm) subunits (Bethyl Laboratories); cell division cycle 45 (Cdc45) and GST (Santa Cruz Biotechnologies, Inc.); partner of Sld five (Psf)1 (Abgent); Histone H3 (Active Motif); and synthetically lethal with dpb11-1 5 (Sld)5, Psf3, and Psf2, which were prepared as described previously (1).

Construction of Vectors for Expression of Human Cdc45/Mcm2-7/GINS and Human Ctf4–Cdc45/Mcm2-7/GINS Complexes. Plasmids for baculovirus expression of His_6-$FLAG_2$ (HF)–tagged human Ctf4 (hCtf4) were described previously (2). For expression of N-terminally His_6 (H)-tagged hCtf4, the DNA fragment containing hCtf4 was excised from the pFastBacHtbFlag$_2$-Ctf4 (2) by BamHI and NotI, and subcloned into pFastBacHtb (Invitrogen). The hCtf4 cDNA was amplified from pFastBacHtb-Ctf4 with primers (BamHI-FLAG-Ctf4-5′: 5′-GCG GAT CCA TGG ATT ATA AAG ATG ACG ATG ACA AGA TGC CTG CCA CAC GGA AGC C-3′ and Ctf4-XhoI: 5′-GAG CCG CTC GAG TTA CTC CTG CTT AAA TGC-3′) and subcloned into BamHI and XhoI sites of pFastBac1 (Invitrogen) to express N-terminally FLAG (F)-tagged hCtf4. Human Cdc45 (hCdc45) cDNA was amplified from pET30-Cdc45 (kindly supplied by Joon-Kyu Lee, Seoul National University, Seoul, Korea) using primers (BamHI-Cdc45-5′: 5′-CGC GGA TCC AAC ATG TTC GTG TCC GAT TTC CGC-3′ and NotI-Cdc45-3′: 5′-AAG GAA AAA AGC GGC CGC CTA GGA CAG GAG GGA AAT AAG-3′) and subcloned into pIRESpuro-GST, the modified version of pIRESpuro2 (Clontech), which contained the N-terminal GST fusion tag. The Cdc45 cDNA fragment excised from pIRESpuro-GST-Cdc45 by BamHI and NotI was subcloned into pFastBac1 and pFastBacHtbFlag$_2$ to express untagged and N-terminally HF-tagged Cdc45, respectively. Plasmids for baculovirus expression of human Mcm2-7 (hMcm2-7) and human Go-Ichi-Nii-San (hGINS) subunits were described previously (1).

Expression and Purification of the Human Cdc45/Mcm2-7/GINS Complex from Insect Cells. The human Cdc45/Mcm2-7/GINS (hCMG) complex was purified as described previously with some modifications (1). Sf9 cells (2×10^6 cells per milliliter, 1.5 L), grown in suspension culture in Grace's medium supplemented with 10% FBS at 27 °C, were infected with 11 viruses expressing hMcm2-7, HF-hCdc45, and hGINS (GST-Sld5, Psf3, Psf2, and Psf1). After 60 h at 27 °C, cells were harvested by centrifugation at $650 \times g$ for 5 min at 4 °C, washed once with 50 mL of ice-cold PBS, and then frozen in dry ice and stored at −80 °C until used. The frozen pellet (20 mL) was thawed on ice and resuspended in 45 mL of hypotonic buffer [20 mM Hepes·NaOH (pH 7.5), 5 mM KCl, 1.5 mM $MgCl_2$, and protease inhibitors]. The cells were kept on ice for 10 min and then lysed by Dounce homogenization (pestle B, 30 strokes). The cell extract was adjusted to 0.42 M potassium acetate and centrifuged at $43,000 \times g$ for 30 min at 4 °C. The cleared lysate was mixed with 1.5 mL of anti-FLAG M2 Affinity Gel preequilibrated with FEQ buffer [20 mM Hepes·NaOH (pH 7.5), 0.42 M potassium acetate, 5 mM KCl, and 1.5 mM $MgCl_2$] and incubated overnight on a rocking platform. Following centrifugation at $290 \times g$ for 5 min at 4 °C, the beads were washed four times with 40 mL of FW buffer [20 mM Hepes·NaOH (pH 7.5), 0.42 M potassium acetate, 1 mM DTT, 1 mM EDTA, 0.01% Nonidet P-40, 10% (vol/vol) glycerol, and protease inhibitors]. Bound proteins were eluted two times with 7.5 mL of Q buffer [20 mM Hepes·NaOH (pH 7.5), 1 mM DTT, 1 mM EDTA, 0.01% Nonidet P-40, 10% glycerol, and protease inhibitors] supplemented with 0.15 M potassium acetate and 0.1 mg/mL 3× FLAG peptide (Sigma–Aldrich). The eluted fractions were combined and applied to a Q Sepharose Fast Flow column (1 mL, 1.5 × 1.5 cm; GE Healthcare Life Sciences) preequilibrated with Q buffer supplemented with 0.15 M potassium acetate. The column was washed once with 10 mL of FW buffer by gravity, and bound proteins were eluted with 6 mL of Q buffer containing 0.75 M potassium acetate by gravity. The eluted fraction was mixed with 0.25 mL of Glutathione Sepharose 4B beads (GE Healthcare Life Sciences) preequilibrated with Prescission buffer [50 mM Tris·HCl (pH 7.5), 0.15 M NaCl, 1 mM DTT, and 1 mM EDTA] overnight. The beads were then washed three times with 10 mL of Prescission buffer. The hCMG complex was eluted following cleavage of the GST tag from Sld5 by incubation of the beads with 0.38 mL of Prescission buffer plus 20 μL (40 units) of Prescission protease (GE Healthcare Life Sciences) for 6 h at 4 °C. The eluted fraction (0.38 mL) was layered on a 5-mL, 15–40% (vol/vol) glycerol gradient [25 mM Tris·HCl (pH 7.5), 0.05 M NaCl, 1 mM DTT, 1 mM EDTA, 0.01% Nonidet P-40, and protease inhibitors] and centrifuged at $260,000 \times g$ for 12 h at 4 °C. Fractions (150 μL) were collected from the bottom of the tube. This final step yielded 10–40 pmol of hCMG complex distributed between five fractions (concentration of the peak fraction: 25–100 fmol/μL). These fractions were frozen at −80 °C after addition of 1 mg/mL BSA.

The hCMG complex was also purified using a different protocol. Sf9 cells were infected, and lysates were prepared as above. The cleared lysates were mixed with 0.7 mL of Glutathione Sepharose 4B beads overnight. Following centrifugation at $290 \times g$ for 5 min at 4 °C, the beads were washed four times with 40 mL of FW buffer. Bound proteins were eluted three times with 3.5 mL of Q buffer supplemented with 0.15 M potassium acetate and 10 mM reduced glutathione. The eluted fractions were combined and applied to a 1-mL Q Sepharose Fast Flow column. The column was washed once with 10 mL of FW buffer by gravity, and bound proteins were eluted with 10 mL of Q buffer supplemented with 0.75 M potassium acetate by gravity. The eluted fraction was mixed with 0.15 mL of anti-FLAG M2 Affinity Gel overnight, and the beads were then washed three times with 10 mL of Prescission buffer. The hCMG complex was eluted by incubating the beads three times with 0.2 mL of Prescission buffer containing 0.2 mg/mL 3× FLAG peptide for 1 h. The eluates were combined and mixed with 20 units of Prescission protease and 0.1 mL of Glutathione Sepharose 4B beads for 4 h at 4 °C to remove the GST tag. After separation from the beads by centrifugation, the unbound fraction was layered on a glycerol gradient and centrifuged as described above. This procedure yielded comparable levels of hCMG with the same specific activity (helicase) as preparations described above.

Expression and Purification of the hCtf4–CMG Complex from Insect Cells. For purification of the hCtf4–CMG complex used for enzymatic assays, Sf9 cells (2×10^6 cells per milliliter, 1.5 L) were infected with 12 viruses expressing HF-hCtf4, hMcm2-7, hCdc45, and hGINS (GST-Sld5, Psf3, Psf2, and Psf1). After 54 h at 27 °C,

cells were harvested and the complex was isolated as described above for the hCMG complex. The level of hCtf4–CMG complex obtained was similar to that isolated as the hCMG complex.

To identify the dimeric structure of hCtf4 (shown in Fig. 3 and Fig. S3), Sf9 cells (2×10^6 cells per milliliter, 0.5 L) were infected with either 12 viruses [F-hCtf4, hMcm2-7, hCdc45, and hGINS (GST-Sld5, Psf3, Psf2, and Psf1)] or 13 viruses [H-hCtf4, F-hCtf4, hMcm2-7, hCdc45, and hGINS (GST-Sld5, Psf3, Psf2, and Psf1)], and the hCtf4–CMG complex was isolated as described above. The hCtf4 devoid of hCMG was isolated from the unbound fraction of Glutathione Sepharose 4B. The monomeric nature of the hCMG complex in the hCtf4–CMG complex was established as follows. Sf9 cells (2×10^6 cells per milliliter, 0.5 L) were co-infected with N-terminally H-tagged Sld5 virus (a generous gift from Danny Reinberg, New York University, New York) and the 12 hCtf4–CMG viruses [F-hCtf4, hMcm2-7, hCdc45, and hGINS (GST-Sld5, Psf3, Psf2, and Psf1)]. The hCtf4–CMG complex was isolated as described above. Free H-hGINS was isolated from the unbound fraction after incubation of the bead with anti-FLAG M2 Affinity Gel by binding to Ni-nitrilotriacetic acid (NTA) agarose beads, followed by elution and glycerol gradient sedimentation.

Expression and Purification of the hGINS Complex from Insect Cells. For the purification of the untagged hGINS complex, Sf9 cells (2×10^6 cells per milliliter, 1 L) were infected with four viruses expressing GST-Sld5, Psf3, Psf2, and Psf1. After 60 h, cells were harvested and the lysate was prepared as described above for purification of the hCMG complex. The cleared lysate was mixed with 1 mL of Glutathione Sepharose 4B beads overnight on a rocking platform at 4 °C. Following centrifugation at $290 \times g$ for 5 min 4 °C, the beads were washed four times with 40 mL of FW buffer. GST-hGINS was eluted five times with 0.9 mL of GE buffer [50 mM Tris·HCl (pH 8.0), 0.01 M reduced glutathione, and 0.15 M potassium acetate]. The first eluate was dialyzed against buffer D [20 mM Tris·HCl (pH 7.5), 0.15 M potassium acetate, 1 mM DTT, 1 mM EDTA, 10% (vol/vol) glycerol, and 0.01% Nonidet P-40] overnight at 4 °C. The dialyzed fraction (0.4 mL) was incubated with 40 units of Turbo3C (HRV3C) protease (A. G. Scientific, Inc.) overnight at 4 °C, and the cleaved GST protein and protease were removed by an additional incubation with 0.2 mL of Glutathione Sepharose 4B beads for 5 h, followed by centrifugation at $100 \times g$ for 3 min. The supernatant (containing untagged hGINS) was layered on a 5-mL 15–40% (vol/vol) glycerol gradient [25 mM Tris·HCl (pH 7.5), 0.05 M NaCl, 1 mM DTT, 1 mM EDTA, 0.01% Nonidet P-40, and protease inhibitors] and centrifuged at $260{,}000 \times g$ for 17 h at 4 °C. Fractions (150 μL) were collected from the bottom of the tube and frozen at −80 °C.

For the purification of HF-hGINS, Sld5 cDNA was PCR-amplified and subcloned into pFastBacHtbFlag$_2$ to express N-terminally HF-tagged Sld5. High Five (Invitrogen) insect cells (1.8×10^7 cells per dish, 40 dishes) were grown as a monolayer in Grace's medium supplemented with 10% (vol/vol) FBS at 27 °C and infected with viruses expressing HF-Sld5, Psf3, Psf2, and Psf1. After 60 h at 27 °C, cells were harvested, washed once with ice-cold PBS, and resuspended in 30 mL of hypotonic buffer [20 mM Hepes·NaOH (pH 8.0), 5 mM KCl, 1.5 mM $MgCl_2$, and protease inhibitors]. The cells were kept on ice for 10 min and then lysed by Dounce homogenization (pestle B, 30 strokes). The cell extract was adjusted to 0.3 M potassium acetate and centrifuged at $43{,}000 \times g$ for 45 min at 4 °C. The cleared lysate was then mixed with 1.5 mL of Ni-NTA agarose beads (Qiagen) for 4 h at 4 °C and centrifuged at $290 \times g$ for 5 min, and the beads were then washed three times with 40 mL of NiW buffer [25 mM Hepes·NaOH (pH 7.5), 0.2 M potassium acetate, 10% (vol/vol) glycerol, 0.01 M imidazole, and protease inhibitors]. Bound proteins were eluted eight times with 1.5 mL of NiE buffer [25 mM Hepes·NaOH (pH 7.5), 0.2 M potassium acetate, 10% (vol/vol) glycerol, 0.2 M imidazole, and protease inhibitors], and the DTT level was adjusted to 1 mM (final) after elution. A fraction (0.05 mL) of the first eluate was subjected to glycerol gradient sedimentation analysis, as shown in Fig. S6*C*.

Expression and Purification of the hMcm2-7 Complex from Insect Cells. For the purification of F-tagged hMcm2-7, Mcm3 cDNA was PCR-amplified from pFastBac1-Mcm3 (1) using primers (EcoRI-Flag-SalI-Mcm3-1: 5′-CGG AAT TCA TGG ATT ATA AGG ATG ATG ACG ACA AGG TCG ACA TGG CGG GTA CCG TGG TGC TGG AC-3′ and SpeI-Mcm3-2: 5′-GCA CTA GTT CAG ATG AGG AAG ATG ATG C-3′) and subcloned into pFastBac1 to express N-terminally F-tagged Mcm3. Sf9 cells (2×10^6 cells per milliliter, 1 L) were infected with six viruses expressing Mcm2, F-Mcm3, Mcm4, Mcm5, Mcm6, and Mcm7. After 60 h, cells were harvested and a lysate was prepared as described above in the hCMG purification procedure. The cleared lysate was mixed with 1 mL of anti-FLAG M2 Affinity Gels overnight at 4 °C. Following centrifugation at $290 \times g$ for 5 min at 4 °C, the beads were washed four times with 40 mL of FW buffer. Bound proteins were eluted five times with 0.9 mL of Q buffer supplemented with 0.15 M potassium acetate and 0.2 mg/mL 3× FLAG peptide. The first eluate was dialyzed against buffer D overnight, and 0.4 mL of dialyzed fraction was then layered on a 5-mL 15–40% (vol/vol) glycerol gradient as described above and centrifuged at $260{,}000 \times g$ for 13.5 h at 4 °C. Fractions (150 μL) were collected from the bottom of the tube and frozen at −80 °C.

Expression and Purification of hCdc45 from Insect Cells. Sf9 cells (2×10^6 cells per milliliter, 1 L) were infected with a virus expressing HF-hCdc45 for 60 h at 27 °C. Cells were harvested, and the protein was purified as described for the isolation of the hMcm2-7 complex, with the exception that the 15–40% (vol/vol) glycerol gradient buffer contained 0.2 M NaCl rather than 0.05 M. Glycerol gradient sedimentation was carried out for 17 h at 4 °C.

Coexpression of hCtf4 with Individual Components of hCMG and Isolation from Insect Cells. To detect complex formation between hCtf4 and hCMG components, Sf9 cells (2×10^6 cells per milliliter, 0.5 L) were infected with viruses expressing HF-hCtf4 and untagged hCdc45, HF-hMcm2-7 (HF tag on Mcm5) and untagged hCtf4, and HF-hCtf4 with GST-hGINS (GST tag on Sld5) (Fig. S5 *A*–*C*). After 54 h at 27 °C, cells were harvested, washed with ice-cold PBS once, and then frozen in dry ice and stored at −80 °C until used. The frozen pellets (20 mL) were thawed on ice and resuspended in 15 mL of hypotonic buffer as described above and then lysed by Dounce homogenization (pestle B, 20 strokes). Cell extracts were adjusted to 0.15 M potassium acetate, sonicated, and centrifuged at $43{,}000 \times g$ for 30 min at 4 °C. Cleared lysates were mixed with 0.5 mL of anti-FLAG M2 Affinity Gels overnight and then washed four times with buffer containing 20 mM Hepes·NaOH (pH 7.5), 0.15 M potassium acetate, 1 mM DTT, 1 mM EDTA, 0.01% Nonidet P-40, 10% (vol/vol) glycerol, and protease inhibitors. Bound proteins were eluted three times with 1 mL of Q buffer supplemented with 0.15 M potassium acetate and 0.1 mg/mL 3× FLAG peptide. In experiments with extracts prepared from cells infected with HF-hCtf4 and untagged hCdc45 or with HF-hMcm2-7 and untagged hCtf4, the first eluate (0.4 mL) was subjected to glycerol gradient separation as described above and centrifuged for 12 h and 17 h, respectively. Anti-FLAG immunoprecipitates derived from eluates of cells coinfected with HF-hCtf4 and GST-GINS were combined and further purified using Glutathione Sepharose CL4B beads as described above, followed by glycerol gradient separation (16 h). To compare the efficiency of hCtf4–CMG and Ctf4–GINS complex formation, Sf9 cells (2×10^6 cells per milliliter, 1 L) were grown, divided into two

equal portions, and coinfected with either 12 viruses (including HF-hCtf4, hMcm2-7, hCdc45, and GST-hGINS) or five viruses (HF-hCtf4 and GST-hGINS) at the same time. After 54 h at 27 °C, cells were harvested and lysates were prepared as described above (420 mM potassium acetate, no sonication). Proteins were purified using anti-FLAG M2 Affinity Gel and Glutathione Sepharose 4B, followed by glycerol gradient sedimentation as described above.

Preparation of Substrates for DNA Helicase Assays. The sequences of oligonucleotides used are listed in Table S1. M13 DNA helicase substrates were prepared as follows: M13 oligonucleotide no. 2 (1 pmol) was annealed to M13 (1 pmol) in the presence of 0.15 M NaCl by heating for 3 min at 100 °C, followed by slow cooling to 25 °C. Annealed oligonucleotides were 3′ end-labeled using Klenow (New England Biolabs) and [α-^{32}P]dGTP. Unannealed oligonucleotides and unincorporated nucleotides were removed by Sepharose CL-4B column chromatography (GE Healthcare Life Sciences). Oligonucleotide substrates used in Fig. S1*A* was prepared as follows: Two oligonuleotides (nos. 1 and 3, 10 pmol each) were annealed to each other, labeled as described above, and purified by 10% (wt/vol) PAGE separation.

ATPase Assays. Reactions (20 μL) containing 25 mM Hepes·NaOH (pH 7.5), 5 or 50 mM NaCl, 10 mM magnesium acetate, 1 mM DTT, 0.1 mg/mL BSA, 0.1 mM unlabeled ATP, and 66 nM [α-^{32}P] ATP (3,000 Ci/mmol) were incubated at 37 °C for 30 min. Aliquots (1 μL) were spotted onto a polyethylenimine-cellulose TLC plate (Merck) and developed in 0.5 M LiCl/1.0 M formic acid, and products were analyzed using a PhosphorImager (Fujifilm).

In Vitro Complex Formation Between hCtf4 and hCMG. hCMG (5 pmol) and untagged hCtf4 (15 pmol, as a dimer) were mixed together in 200 μL of solution containing 20 mM Tris·HCl (pH 7.5), 1 mM DTT, 0.01% Nonidet-P40, 50 mM NaCl, and protease inhibitors overnight at 4 °C. Proteins were captured by the addition of 50 μL of anti-FLAG M2 Affinity Gel and a further incubation for 3 h. Beads were washed three times with 0.3 mL of binding buffer, and bound proteins were eluted in 0.3 mL of buffer containing 20 mM Hepes·NaOH (pH 7.5), 150 mM potassium acetate, 1 mM EDTA, 0.01% Nonidet-P40, 50 mM NaCl, 10% (vol/vol) glycerol, and 0.1 mg/mL 3× FLAG peptide for 1 h. Eluted proteins were sedimented in glycerol gradients as described for the isolation of the hCMG complex.

In Vitro Protein–Protein Binding Assay. To detect in vitro interactions between hCtf4 and hCMG or components of the hCMG complex as shown in Fig. 5, proteins (amount indicated in legend for Fig. 5) were mixed in 50 μL of IP buffer [20 mM Hepes·NaOH (pH 7.5), 1 mM DTT, 0.01% Nonidet-P40, 5% (vol/vol) glycerol, 0.1 mg/mL BSA, and protease inhibitors] containing 50 mM NaCl on a rocking platform for 2 h at 4 °C. Specific antibodies were added, and the mixtures were incubated; after 1 h, antibody–protein complexes were captured with 50 μL of IP buffer containing 10 μL of Protein A agarose beads (Millipore), followed by a 1-h incubation. Beads were collected and washed three times with 0.5 mL of IP buffer containing 100 mM NaCl and lacking BSA. Bound proteins were eluted with 20 μL of 1× SDS loading buffer [40 mM Tris·HCl (pH 6.8), 2% (wt/vol) SDS, 2 mM β-mercaptoethanol, 4% (vol/vol) glycerol, and 0.01% bromophenol blue]. In the experiments shown in Fig. 4*A*, 150 mM NaCl was included in the binding and wash buffers.

hCMG and hCtf4 Interaction and Association with Chromatin. HeLa cells were cultured in DMEM supplemented with 10% (vol/vol) FBS and antibiotics, and they were maintained at 37 °C in a humidified incubator containing 5% CO_2. Chromatin fractionation experiments were performed as described (3) with modifications. Briefly, cells lysed in buffer containing 50 mM Hepes·NaOH (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.5% Tween-20, 1 mM ATP, and protease and phosphatase inhibitors were incubated at 4 °C for 10 min, and the chromatin and soluble fractions were separated by centrifugation at 15,000 × *g* for 10 min. Chromatin pellets were resuspended in the above buffer, sonicated, and treated with benzonase (final concentration of 1.25 U/μL; Novagen) at 4 °C for 2 h. Immunoprecipitation experiments were carried out by incubating 500 μg of soluble or chromatin fractions with 200 ng of Sld5, Ctf4, or nonspecific GST polyclonal antibodies at 4 °C for 4 h, followed by the addition of 20 μL of protein A agarose (Upstate Biotechnology) at 4 °C for 1 h. After centrifugation, beads were washed three times with the above buffer and bound proteins were eluted with SDS loading buffer, followed by SDS/PAGE separation on 4–20% (wt/vol) gels. Immunoblot analysis was performed with rabbit polyclonal antibodies against Ctf4, Mcm2, Cdc45, Sld5, and Histone H3 as indicated.

1. Kang YH, Galal WC, Farina A, Tappin I, Hurwitz J (2012) Properties of the human Cdc45/Mcm2-7/GINS helicase complex and its action with DNA polymerase epsilon in rolling circle DNA synthesis. *Proc Natl Acad Sci USA* 109(16):6042–6047.
2. Bermudez VP, Farina A, Tappin I, Hurwitz J (2010) Influence of the human cohesion establishment factor Ctf4/AND-1 on DNA replication. *J Biol Chem* 285(13):9493–9505.
3. Im JS, et al. (2009) Assembly of the Cdc45-Mcm2-7-GINS complex in human cells requires the Ctf4/And-1, RecQL4, and Mcm10 proteins. *Proc Natl Acad Sci USA* 106(37): 15628–15632.

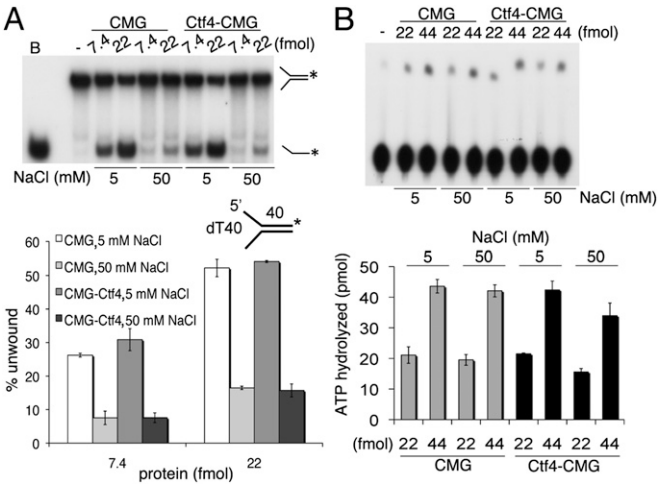

Fig. S1. Comparison of the ATPase and helicase activities of hCMG and hCtf4–CMG using oligonucleotide substrates. (*A*) Helicase activities of the hCMG and hCtf4–CMG complexes using fork-structured oligonucleotides were measured with the indicated protein levels in the presence of 5 or 50 mM NaCl. (*Lower*) Substrate unwound (%) is shown in the graph. The structure of the substrate used, containing a 40-mer duplex region and both 5′ and 3′ dT_{40} tails, is shown at the upper right corner of the graph. (*B*) ATPase activities of hCMG and hCtf4–CMG were measured with the indicated protein levels in the presence of 5 or 50 mM NaCl. (*Lower*) ATP hydrolyzed (%) is shown in the graph.

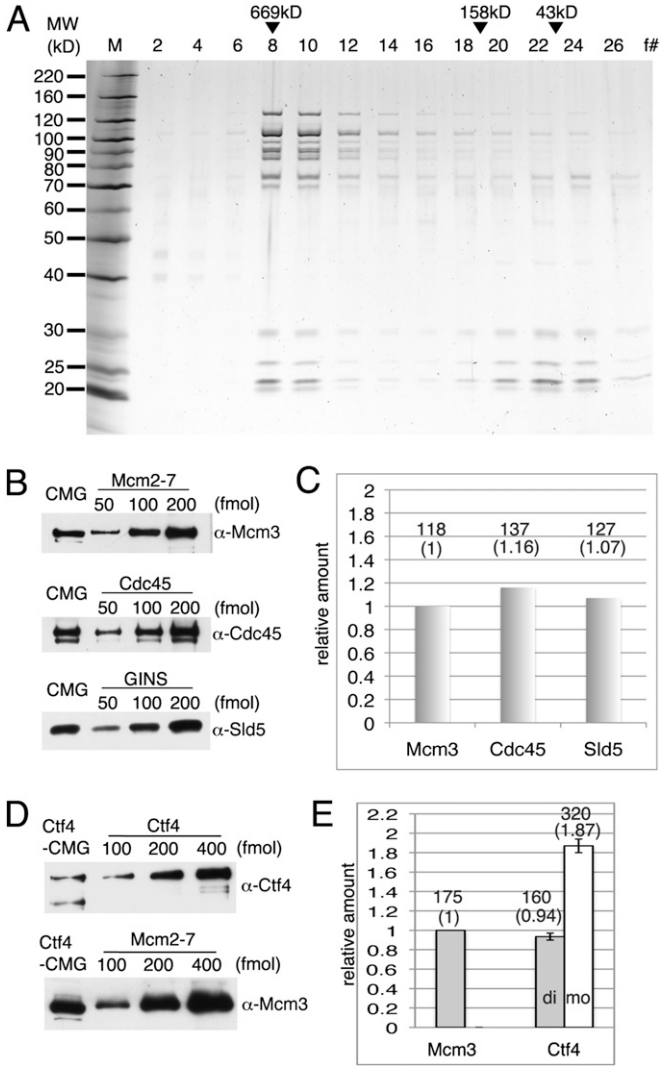

Fig. S2. Purification of the hCMG complex and stoichiometry of the hCMG and hCtf4–CMG complexes. (*A*) Glycerol gradient fractions (5 μL) were separated on a 4–20% gel and silver-stained. (*B*) Fraction of the hCMG complex was loaded side by side with various levels of hMcm2-7, hCdc45, and hGINS (50, 100, and 200 fmol). Western blot analyses were performed against hCdc45 and one of the subunits of hMcm2-7 (Mcm3) or hGINS (Sld5). (*C*) Based on standard curves obtained from densitometric analyses of Mcm3, Cdc45, and Sld5 bands, the levels of Mcm3, Cdc45, and Sld5 in hCMG were calculated and the relative amounts are shown in the graph. The absolute concentration (femtomoles) of each subunit and relative levels, in parentheses, are noted above each column. (*D*) Fraction of hCtf4–CMG (glycerol gradient fraction no. 7 in Fig. 1*C*) used for helicase assays was loaded side by side with various levels of hCtf4 and hMcm2-7 (100, 200, and 400 fmol; hCtf4 as a dimer). Western blot analyses were performed against hCtf4 and Mcm3. We noted some cleavage of Ctf4 in the hCtf4–CMG complex during storage of the proteins (faster migrating band). (*E*) Levels of Ctf4 (including the cleaved product) and Mcm3 in hCtf4–CMG were calculated from *D*, and the relative amounts are shown in the graph [both as dimeric (di) and monomeric (mo)]. The absolute level (femtomoles) of each subunit and its ratio, in parentheses, are denoted above the each column.

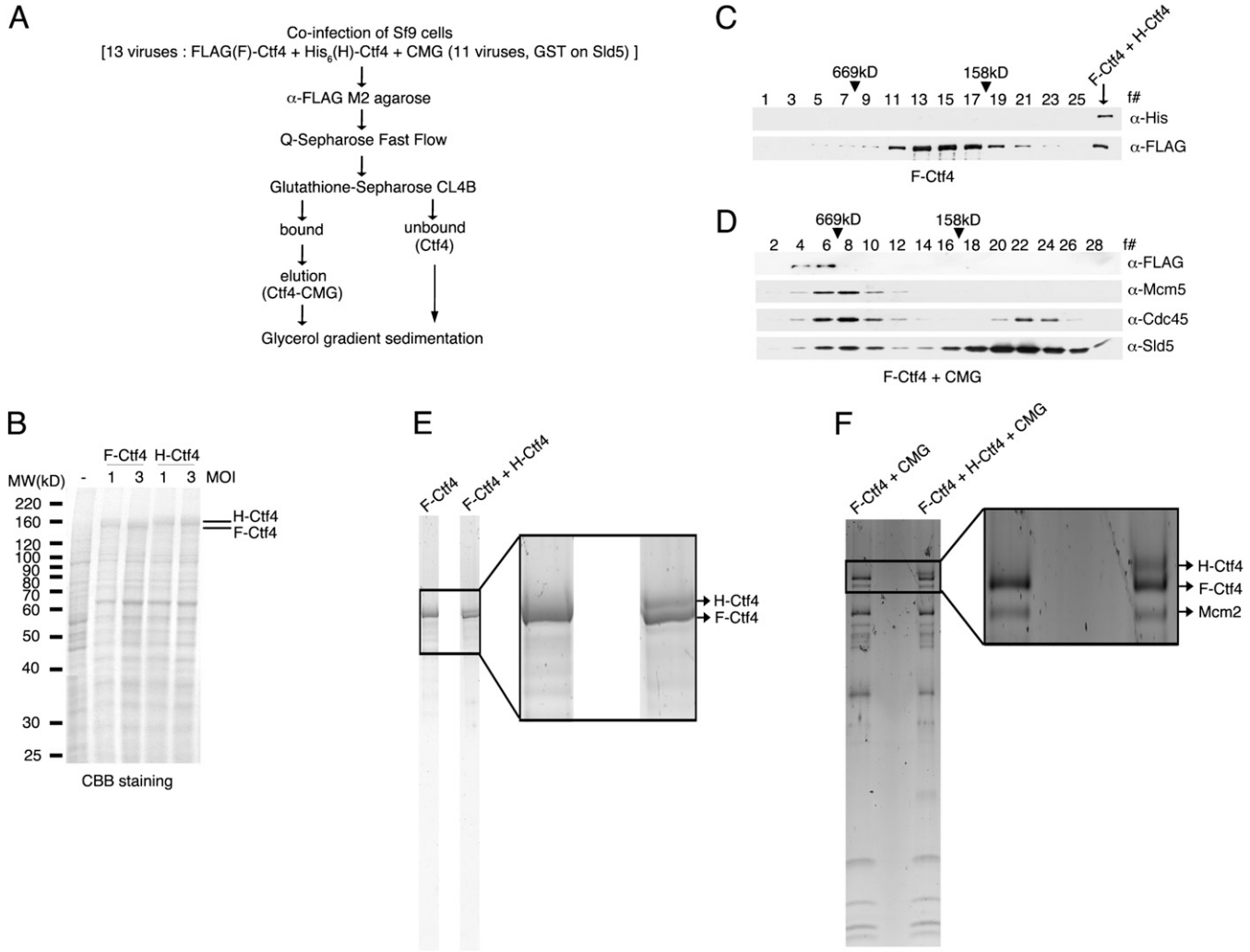

Fig. S3. Purification of the hCtf4–CMG complex and free hCtf4 from Sf9 cells infected with viruses expressing F-hCtf4, H-hCtf4, and the hCMG complex. (*A*) Flow chart of the steps used in the isolation of the hCtf4–CMG and free hCtf4. (*B*) Analysis of the expression of H-hCtf4 and F-hCtf4 proteins. Sf9 cells (1×10^6 cells in wells) were infected with F-Ctf4– or H-Ctf4–expressing viruses (multiplicity of infection of 1 and 3, respectively). After 54 h, cells were harvested, re-suspended in 0.3 mL of 1× SDS loading buffer, sonicated, and boiled. Aliquots (8 μL) were separated on a 10% (wt/vol) SDS/PAGE gel and Coomassie brilliant blue (CBB)-stained. (*C* and *D*) hCtf4 and hCtf4–CMG were also purified from cells infected with F-hCtf4– and hCMG-expressing viruses. Following glycerol gradient sedimentation, fractions containing hCtf4, shown in *C,* and fractions containing Ctf4–CMG, shown in *D,* were detected by Western blot analysis with the indicated antibodies. The hCtf4, purified by glycerol gradient centrifugation from cells infected with F-hCtf4-, H-hCtf4–, and hCMG-expressing viruses, was loaded in the last lane as a control (α-His and α-FLAG) in *C*. Glycerol gradient peak fractions of either hCtf4 or hCtf4–CMG, isolated from cells infected with F-hCtf4–, H-hCtf4–, and hCMG-expressing viruses, were loaded onto a 4–20% (wt/vol) acrylamide gradient gel side by side with either hCtf4 (*E*) or the hCtf4–CMG complex (*F*) purified from cells infected with F-hCtf4– and hCMG-expressing viruses, respectively. The regions containing hCtf4 bands were cropped and enlarged.

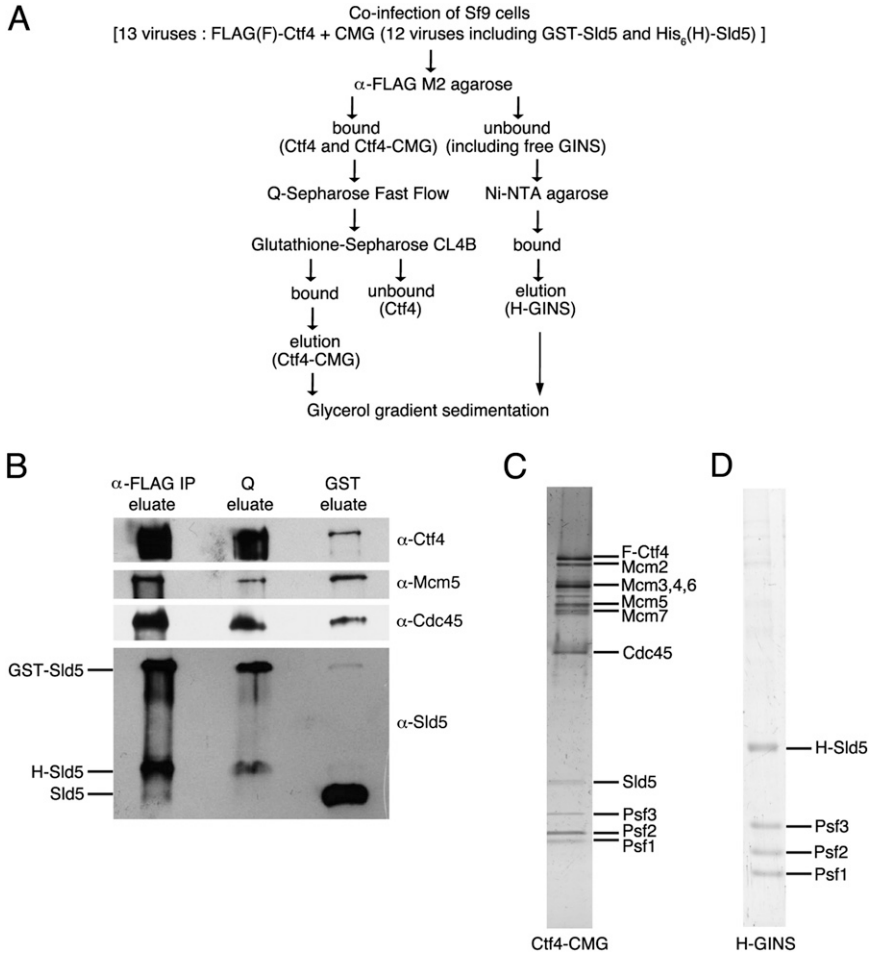

Fig. S4. Purification of the hCtf4–CMG complex from Sf9 cells infected with F-hCtf4 and hCMG expression viruses containing differently tagged Sld5 subunits (GST-Sld5 and H-Sld5). (*A*) Flow chart of the steps used in the isolation of hCtf4–CMG and H-GINS. (*B*) Western blot analyses were used to detect hCtf4 and hCMG components in fractions derived from the first three consecutive isolation steps. (*C*) Peak fraction of hCtf4–CMG, isolated by glycerol gradient sedimentation, was loaded onto a 4–20% (wt/vol) acrylamide gradient gel and silver-stained. (*D*) Peak fraction of H-GINS, isolated following glycerol gradient sedimentation, was loaded onto a 10% (wt/vol) gel and stained with CBB.

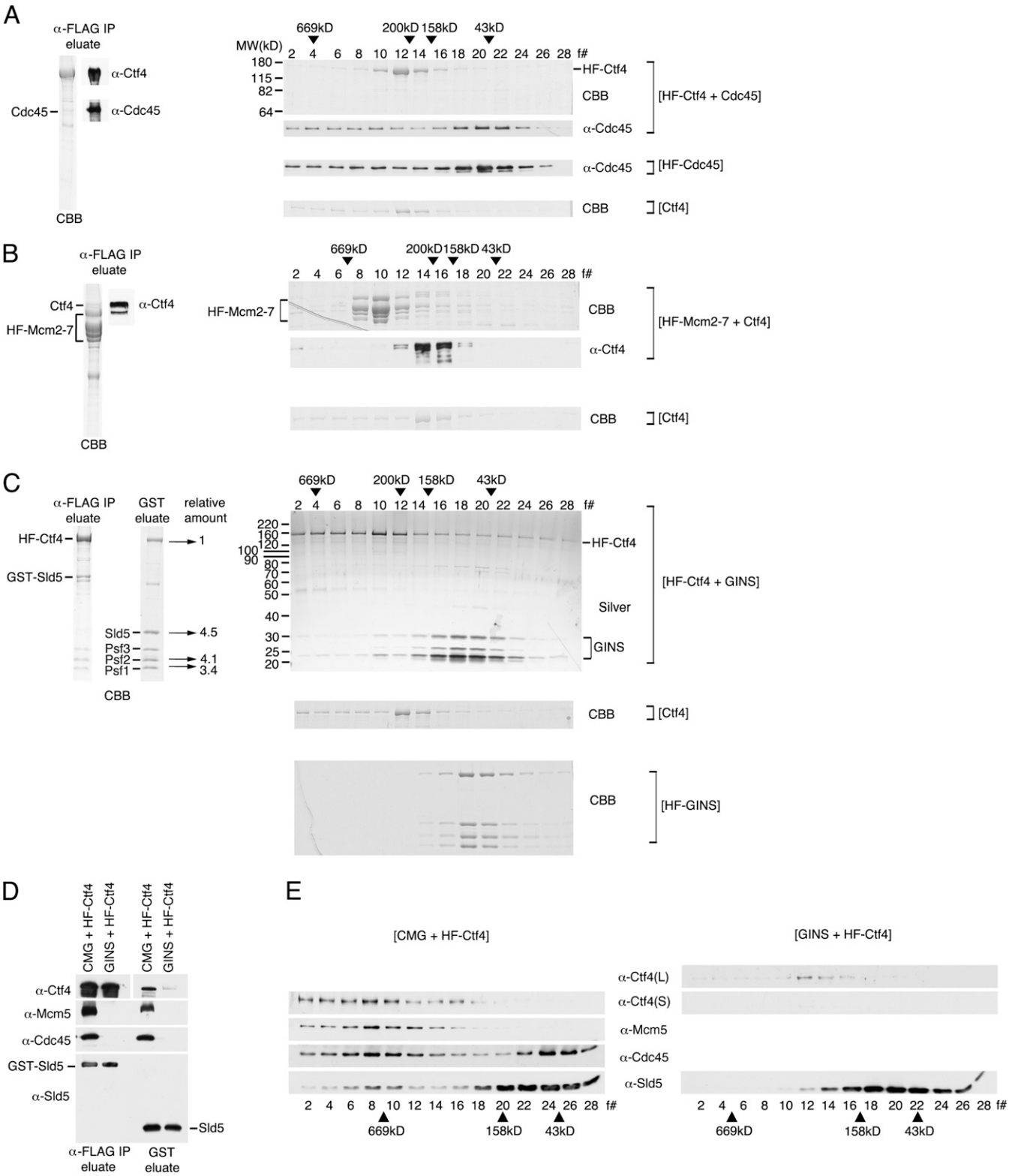

Fig. S5. Analysis of complexes formed following infections of cells with viruses expressing hCtf4 and hCMG components. (*A*) Sf9 cells were coinfected with viruses expressing HF-hCtf4 and untagged hCdc45. Complexes were purified by anti-FLAG immunoprecipitation (*Left*), followed by glycerol gradient separation (*Right*). (*Right*) Immunoprecipitation was also carried out with cell extracts derived from cells infected with a virus expressing HF-hCdc45 and followed by glycerol gradient separation as a control sedimentation as shown in the middle portion of the gel. Purified untagged hCtf4 was sedimented as a control as shown in the lower portion of the gel. (*B*) Sf9 cells were coinfected with HF-hMcm2-7 (HF tag on Mcm5) and untagged hCtf4-expressing viruses. Complexes were purified by anti-FLAG immunoprecipitation (*Left*), followed by glycerol gradient separation (*Right*). (*C*) Sf9 cells were coinfected with viruses expressing HF-Ctf4 and GST-GINS (GST tag – Sld5). Complexes were purified by consecutive anti-FLAG immunoprecipitation (*Left*) and GST affinity pull-down (*Center*), followed by glycerol gradient sedimentation (*Right*). (*Center*) Densitometric analysis was performed with eluates isolated from GST-binding beads, and the

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relative protein level (molar ratio) is indicated on the right side of the gel. (*Right*) Glycerol gradient fractions were separated by SDS/PAGE through a 4–20% (wt/vol) gradient gel and silver-stained as shown in the upper portion of the gel. HF-hGINS (HF-tagged Sld5) was used to demonstrate the sedimentation of free hGINS as shown in the lower portion of the gel. (*D*) Sf9 cells were infected with HF-hCtf4 virus and with either hCMG- or GINS-expressing viruses, and extracts were prepared (420 mM potassium acetate). The purification steps used were as described in *C*. Eluates obtained from anti-FLAG and GST affinity isolation steps were loaded onto a 10% (wt/vol) SDS/PAGE gel, and the level of immunoprecipitated proteins was compared by Western blot analysis against the indicated proteins. (*E*) Glycerol gradient fractions from each purification were resolved by a 10% (wt/vol) SDS/PAGE gel. The proteins in both gels were transferred side by side onto a single nitrocellulose membrane, and immunoblotting was performed against the indicated proteins. Both short (S) and long (L) exposures are shown for an α-Ctf4 Western blot.

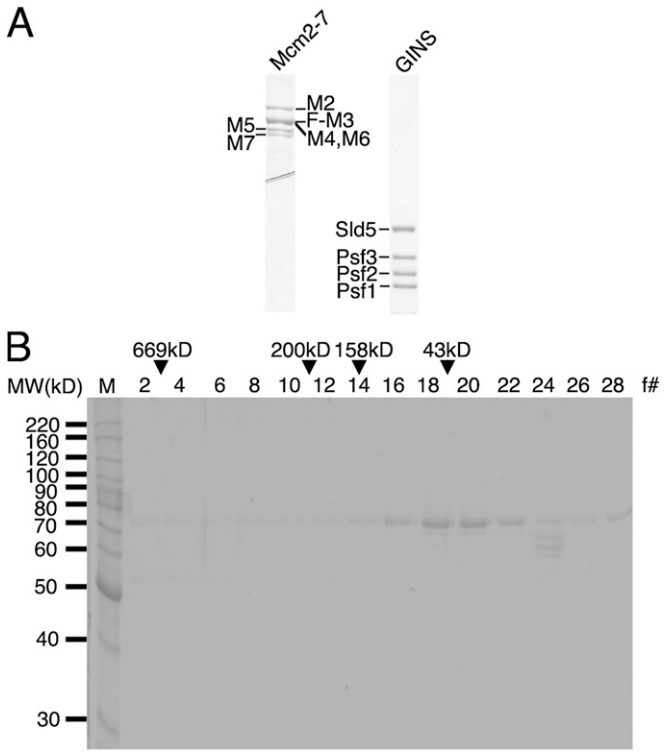

Fig. S6. (*A*) Purity of isolated hMcm2-7 and hGINS complexes. (*B*) Purity of isolated hCdc45. CBB-stained gel of glycerol gradient fractions. Each gradient fraction (10 μL) was loaded onto a 10% (wt/vol) gel.

Table S1. Oligonucleotides used in this study

No.	Name	Sequence (length)
1	M13-39–5′dT40	5′-$(T)_{40}$GATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGAC-3′ (79 nt)
2	M13-56–5′dT40	5′-$(T)_{40}$GGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGAC-3′ (96 nt)
3	5′C-anti–M13-39-3′dT40	5′-CGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATC$(T)_{40}$-3′ (80 nt)