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Supplementary Information for

Mcm10 has potent strand annealing activity and prevents translocase-mediated fork regression

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Materials and Methods

Chemicals, DNAs and Proteins: Radioactive nucleotides were from Perkin Elmer and unlabeled nucleotides were from GE Healthcare. DNA oligonucleotides were from Integrated DNA Technologies. S.c. CMG and RPA were overexpressed and purified as previously described (13, 28). Protein concentrations were determined using the Bio-Rad Bradford Protein stain using BSA as a standard.

Purification of human SMARCAL1. The human SMARCAL1 gene containing a C-terminal 3X FLAG tag was placed behind a Gal1/10 promotor and integrated into the yeast strain OY001 (44, 48). Cells were grown in 1 L YPD from a 25 mL YPD starter culture, then split into 12L YP-glycerol. The YP-Glycerol cultures were grown to 9 x 10^6 cells/mL at 30°C and induced by addition of 20 g of galactose/L. After 6 h, cells were harvested by centrifugation, resuspended in a minimal volume of 20 mM HEPES pH 7.5, 3 mM EDTA, 300 mM KGlu, and protease inhibitors and frozen by dripping into liquid nitrogen. Purification of SMARCAL1 was performed by lysis of 12 L of frozen cells with a SPEX cryogenic grinding mill (6970 EFM). Ground cells were thawed and debris removed by centrifugation (19,000 r.p.m. in a SS-34 rotor for 1 h at 4 °C). The supernatant was batch bound in Buffer A (20 mM HEPES, pH 7.5, 10% glycerol, 1 mM EDTA, 1 mM DTT, 300 mM KGlu) with 1 ml anti-Flag M2 affinity beads (Sigma) for 1 hr at 8°C. The beads were washed 3 times with Buffer B (identical to Buffer A except for 750 mM KGlu rather than 300 mM). The beads were then packed into a column and the prep proceeded via FPLC. The column was equilibrated in Buffer A and washed until the A280 stabilized. Protein was eluted in Buffer A plus 0.15 mg/ml 3× Flag peptide (EZBiolab, Carmel, Indiana, USA) by pulsing four times with 2 mL per pulse followed by 20 min incubations between pulses. Peak fractions were pooled, passed through a 1ml MonoS column in Buffer A, then loaded onto a MonoQ column equilibrated in Buffer A and eluted with a 10 column volume gradient of 0.2-1.0 M NaCl in Buffer A. Peak fractions were aliquoted and stored frozen at −80 °C. Analysis of the MonoQ elution in SDS PAGE is shown in Fig. S6.

Mcm10 ssDNA binding assay: S.c. Mcm10 was purified as described (13). Mcm10 was examined for DNA binding using a ssDNA FITC 3'-labeled oligo and fluorescence anisotropy. Reactions were performed in 20 μL of 25mM Tris-Acetate pH 7.5, 5% glycerol, 40 μg/ml BSA, 5 mM DTT, 1mM EDTA, 25 mM K glutamate, and 1 nM 3' FITC labeled 60mer oligonucleotide (5' – TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGTGAGGGTTGGGAAGTGGAAGGATGGGCT- FITC - 3'). Titrations of Mcm10 were scanned on a Biotek Neo 2 384-well plate reader (Biotek Instruments, Inc.) and the binding curve was fit to a single-site binding equation using the Origin (OriginLab) software (Fig. S5). The analysis showed a K_d value of 50 nM compared to 120 nM for a 45mer oligo by full length *Xenopus laevis* Mcm10, which binds ssDNA with comparable affinity to dsDNA (38).

Helicase assays: DNA oligonucleotides used for helicase assays were 50duplexLAG and 50duplex LEAD or 160mer duplex LAG and 160mer duplex LEAD, as detailed in (13). In all assays the lagging strand oligo was ^{32}P-5' end-labeled and annealed to its respective LEAD oligo to form forked DNA as described (13, 28). Reactions contained 30 or 50 nM CMG (noted in figure legends), 60 nM Mcm10 (except for titrations of Mcm10 into assays), or 50 nM of reconstituted CMG-Mcm10 complex with 0.5 nM forked DNA substrate and 1 mM ATP in a 50 μl final volume of 20 mM Tris-Acetate pH 7.6, 5 mM DTT, 0.1 mM EDTA, 10 mM $MgSO_4$, 30 mM KCl, 40 μg/ml BSA. Reactions were mixed on ice and started by placing in a water bath at 30°C. In experiments containing a trap oligo, 25 nM unlabeled 50duplexLAG oligo was added 1' after initiating the reaction to prevent re-annealing of unwound radiolabeled DNA. At the indicated times, 12 μl aliquots were removed, stopped with 20 mM EDTA and 0.1% SDS (final concentrations), and flash frozen in liquid nitrogen to avoid any unwanted reactions while waiting for reactions to be analyzed by PAGE. Frozen reaction products were thawed quickly in 30°C water immediately prior to loading onto 10% native PAGE in TBE buffer. Gels were washed in distilled water, backed

with Whatman 3MM paper, wrapped in plastic and exposed to a phosphor screen that was scanned on a Typhoon 9400 laser imager (GE Healthcare). Scanned gels were analyzed using ImageQuant TL v2005 software.

Annealing reactions: Oligonucleotides used in annealing reactions were: leading strand flap oligo (5'-ACCGGAGACCGAACGATCCTGTAATGTCCTAGCAAGCCAGAATTCGGCAGCGTCTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTTGAGGAAAGAATGTTGGTGAGGGTTGGGAAGTGGAAGGATGGGCTCGAGAGGTTTT TTTTTTTTTTTTTTTTTTTTTTTTT*T*T*T*T*T-3') and lagging strand flap oligo 5' -TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGACGCTGCCGA ATTCTGGCTTGCTAGGACATTACAGGATCGTTCG*G*T*C*T*C-3'), where asterisks indicate thiodiester linkages. Strand annealing reactions were performed using ^{32}P-5'- lagging strand flap oligo (10 nM final) and unlabeled leading strand flap oligo (20 nM final) in 30 mM Tris-Acetate pH 7.5, 1 mM DTT, 5 mM $MgSO_4$. The two oligos were mixed and immediately followed by addition of 60 nM Mcm10 and incubated at 30^{o}C. For experiments with CMG present, CMG was added either in place of or at the same time as Mcm10. Any changes to this procedure are noted in the figure legends. Aliquots were removed at the indicated times, added to an equal volume of 2x stop buffer (40mM EDTA, 2% SDS, 4% glycerol, 200 nM unlabeled lagging strand flap oligo) and flash frozen. Endpoint assays were terminated in the same manner at 5 min, unless indicated otherwise. Samples were analyzed in 8% native PAGE gels, and treated as described above for helicase assays. For experiments with RPA, the two oligos were individually incubated with saturating RPA (as determined by EMSA shown in Fig. 4a) for 5 min at 30^{o}C before combining the individually RPA coated oligos, and immediately adding Mcm10 to initiate the annealing reaction.

RPA gel shift assay. 5'-^{32}P end-labeled lagging strand flap oligo at 10 nM was incubated with 0-120 nM RPA, in annealing reaction buffer, for 5 min at 30^{o}C. Reactions were then analyzed in an 8% native PAGE gel to observe DNA bound to RPA based on a shift in gel mobility.

Chemical cross-linking with mass spectrometry readout. Cross-linking with mass spectrometry readout (CX-MS) was performed essentially as described (49, 50). CMG-Mcm10 (CMGM) complex was reconstituted and purified as previously described (13). CMGM was then cross-linked using disuccinimidyl suberate (DSS), in reactions containing ~12 µg CMGM, 1 mM DSS and 5% DMSO. The cross-linking reactions were incubated for 25 min at room temp with 1,200 rpm agitation in a thermomixer, then quenched with 50 mM (final concentration) NH_4CO_3. The crosslinked protein was then methanol precipitated by adding 9 volumes of 100% methanol and incubating at -80 ^{o}C overnight. The sample was then collected by centrifugation for 15 min at 14Krpm at 4 ^{o}C, washed with 100% methanol, collected by centrifugation again and air dried. The pellet was then resuspended in 1x SDS-PAGE loading buffer. Following reduction and alkylation of cysteines, the sample was separated by SDS-PAGE in two lanes of a 3-8% Tris-Acetate gradient mini-gel. Cross-linked products were digested and analyzed by mass spectrometry as described (51).

Fork Regression assays. Fork regression assays utilized three different forked structures: 1) lagging strand gapped DNA fork, 2) leading strand gapped fork and 3) no gap at the fork. These structures were formed using mixtures of 4 oligonucleotides from the following list of 6 oligo sequences derived from a study of SMARCAL1 (sequences from (33)).

FR1: (122mer leading)
5'-CGTGACTTGATGTTAACCCTAACCCTAAGATATCGCGT**TA**TCAGAGTGTGAGGATACATGTA
GGCAATTGCCACGTGTCTATCAGCTGAAGTTGTTCGCGACGTGCGATCGTCGCTGCGACG-3'
FR2 (122mer lagging)
5'-CGTCGCAGCGACGATCGCACGTCGCGAACAACTTCAGCTGATAGACACGTGGCAATTGCC
TACATGTATCCTCACACTCTGA**AT**ACGCGATATCTTAGGGTTAGGGTTAACATCAAGTCACG-3'

FR 3: (82mer anneals to FR1)
5'-CGTCGCAGCGACGATCGCACGTCGCGAACAACTTCAGCTGATAGACACGTGGCAATTGCCT
ACATGTATCCTCACACTCTGA-3'
FR 4: (82mer anneals to FR2)
5'-TCAGAGTGTGAGGATACATGTAGGCAATTGCCACGTGTCTATCAGCTGAAGTTGTTCGCGA
CGTGCGATCGTCGCTGCGACG-3'
FR5: (52mer anneals to FR1)
5'-CGTCGCAGCGACGATCGCACGTCGCGAACAACTTCAGCTGATAGACACGTGG-3'
FR6: (52mer anneals to FR2)
5'-CCACGTGTCTATCAGCTGAAGTTGTTCGCGACGTGCGATCGTCGCTGCGACG-3'

Fork with a lagging strand gap: The substrate was constructed by annealing two pairs of oligos: FR2 was 5' end labeled with ^{32}P, and annealed to FR6 using a 1.5-fold excess of FR6 over FR2. Separately, FR1 was annealed to FR3 using a 1.5 molar excess of FR3. Oligo pairs were combined and heated to boiling then allowed to cool to room temp. The two reactions were then combined to make the forked substrate, using a 1.4-fold excess of the FR2/FR6 pair. This final annealing was performed by combining the two pre-annealed pairs and incubating at 30 °C for 20 min to form the FR (fork regression) substrate. The FR substrate was flash frozen in liquid nitrogen and stored until needed.

Fork with a leading strand gap: Same as for the fork containing a lagging strand gap, except the pairs were 5' end labeled FR2 with FR4 and FR1 with FR5.

Fork with no gap: The substrate was constructed by annealing two pairs of oligos: FR3 was 5' end labeled with ^{32}P, and annealed to FR1 using a 1.5-fold excess of FR3 over FR1. Separately, FR4 was annealed to FR3 using a 1.5 molar excess of FR4. Oligo pairs were combined and heated to boiling then allowed to cool to room temp. The two reactions were then combined to make the forked substrate, using a 1.4-fold excess of the FR2/FR4 pair. This final annealing was performed by combining the two pre-annealed pairs and incubating at 30 °C for 20 min to form the FR (fork regression) substrate. The FR substrate was flash frozen in liquid nitrogen and stored until needed.

Fork regression activity was assayed by incubating SMARCAL1 and/or Mcm10 with 0.5 nM FR substrate at 37°C in 20 mM Tris-Acetate pH 7.5, 4% glycerol, 0.1 mM EDTA, 40 μg/ml BSA, 5 mM DTT, 5 mM $MgSO_4$, 2 mM ATP. Reactions were stopped with 2x Stop buffer (40mM EDTA, 2% SDS, 4% glycerol), flash frozen in liquid nitrogen to prevent any unwanted reactions while waiting for analysis by PAGE, then warmed immediately before application to an 8% native-PAGE gel. The product of fork regression is observed as a faster migrating band on the gel that is formed when the ^{32}P-labeled "nascent" strands of the substrate are ejected together as a dsDNA product.

Supplemental Figures

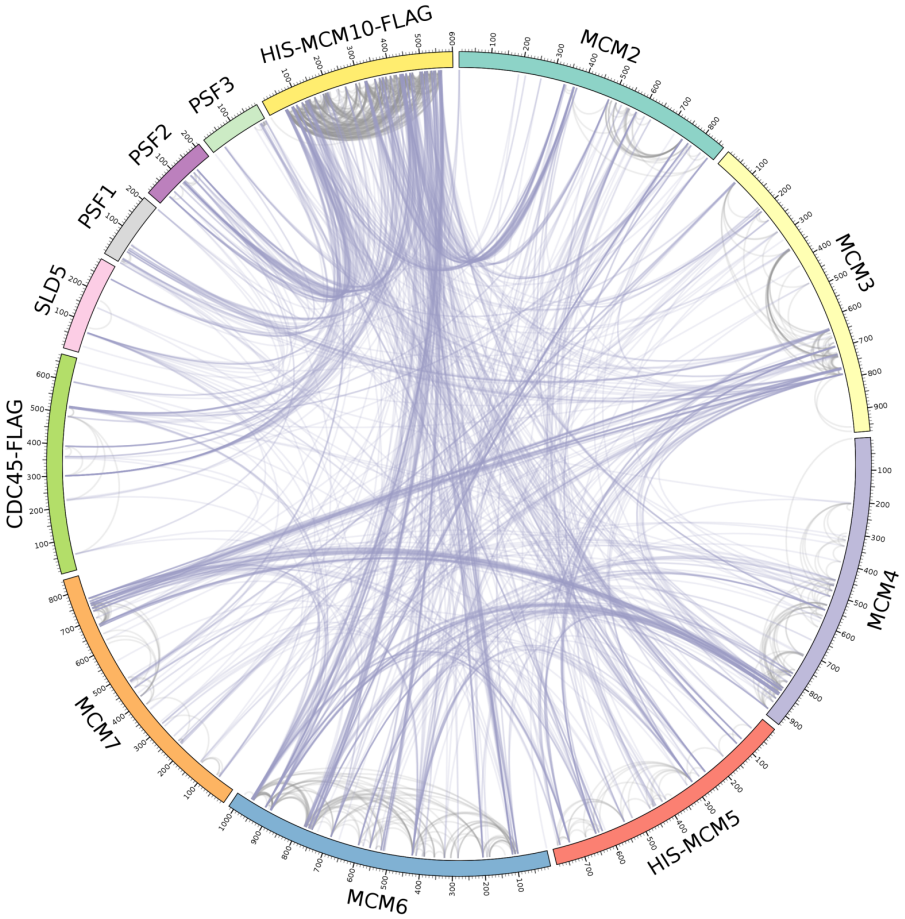

Figure S1. Overall schematic view of CX-MS cross-links within CMGM complex. Both intermolecular and intramolecular cross-links within CMGM complex are illustrated. For detailed cross-link data, see Dataset S1 and Movie S1.

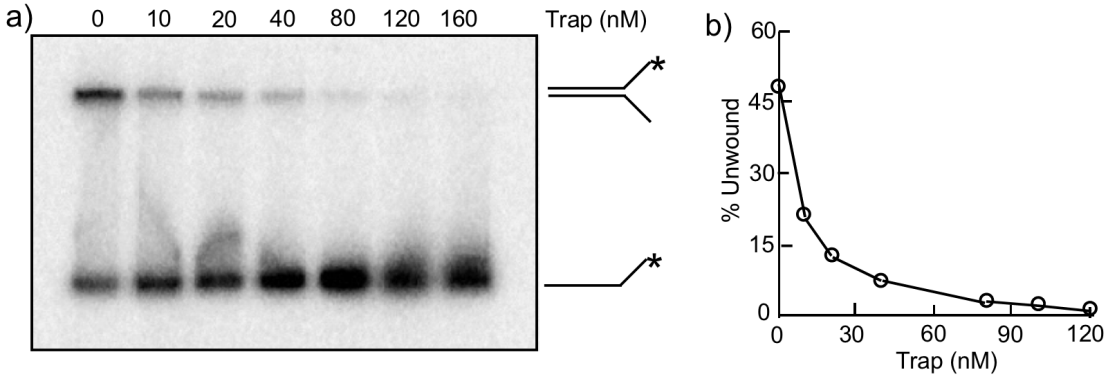

Figure S2. Effectiveness of a trap oligo to quench Mcm10 annealing reactions. Trap oligo (unlabeled lagging strand flap oligo) was titrated into annealing reactions containing 60nM Mcm10, 10 nM ^{32}P-5' flap lagging oligo, 15nM leading strand flap oligo at 5 min endpoints to determine the amount of trap required to quench annealing. a) Native PAGE analysis of annealing at different concentrations of trap oligo. The 5 min time was chosen to ensure that annealing was not fully complete in the absence of trap oligo. b) Quantification of the PAGE.

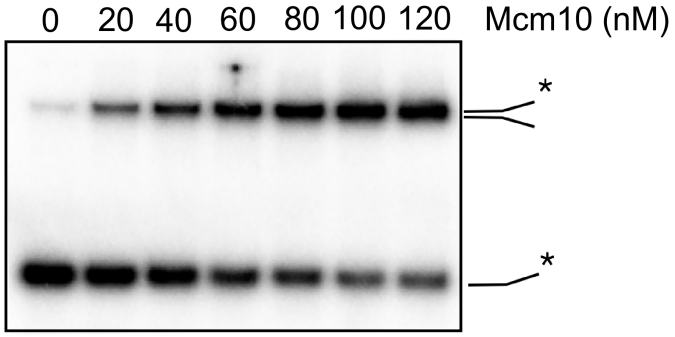

Figure S3. Concentration dependence of Mcm10 mediated annealing. Titration of Mcm10 into DNA annealing assays, incubated at 30^{o}C for 5 min.

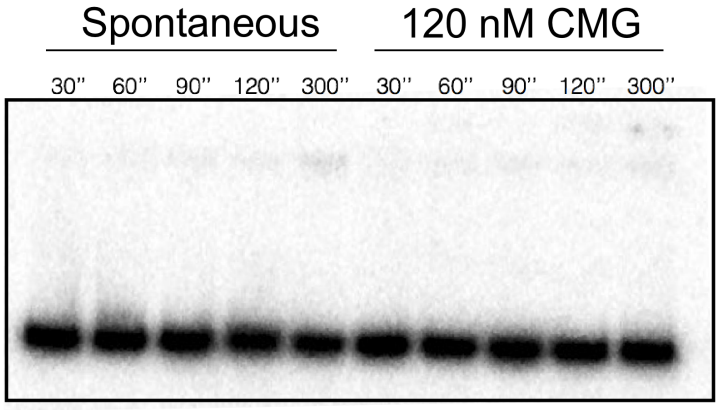

Figure S4. CMG does not have annealing activity. Complementary strands of the replication fork substrate were mixed together, then incubated either alone (spontaneous) or with 120 nM CMG. The results show no enhancement by CMG of spontaneous annealing.

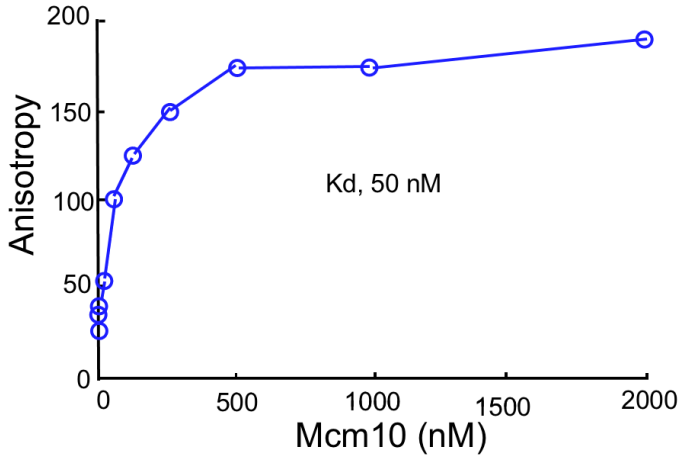

Figure S5. K_d determination of S.c. Mcm10 binding to ssDNA. Fluoresence anisotropy assays of Mcm10 affinity to a ssDNA 60mer oligo is described in Methods.

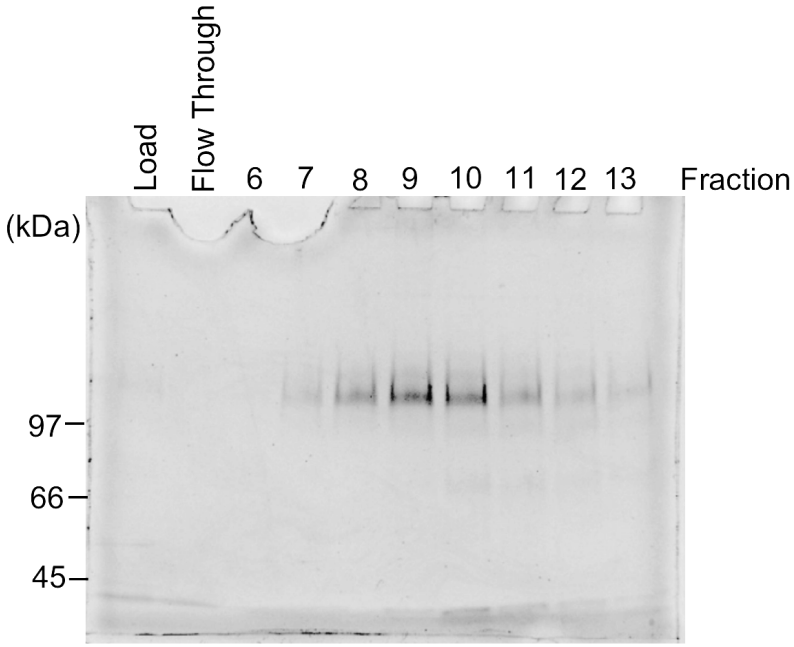

Figure S6. SDS PAGE of purified recombinant SMARCAL1. SDS-PAGE of fractions eluting from the MonoQ column.

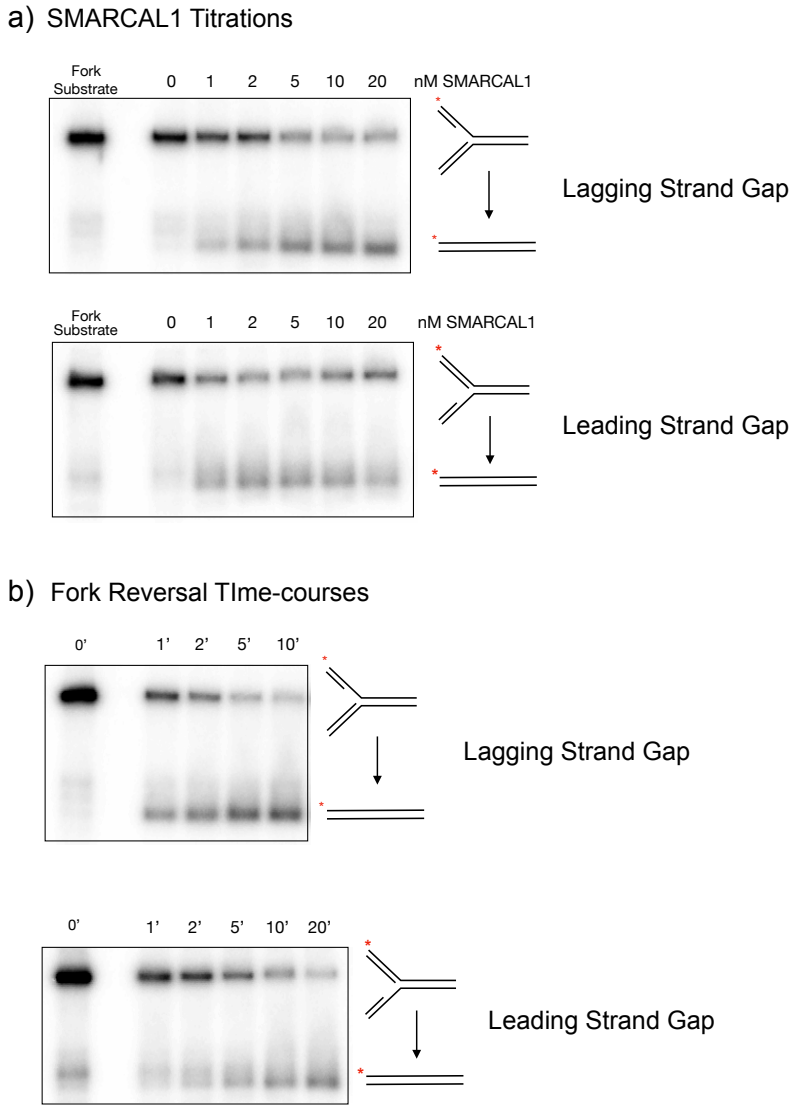

Figure S7. Testing SMARCAL1 activity in fork regression assays. a) Titrations of SMARCAL1 into fork reversal assays with either leading or lagging strand gap substrates. **b)** Time courses for 2 nM SMARCAL1 with the lagging strand gap substrate and 5 nM SMARCAL1 with the leading strand gap substrate.

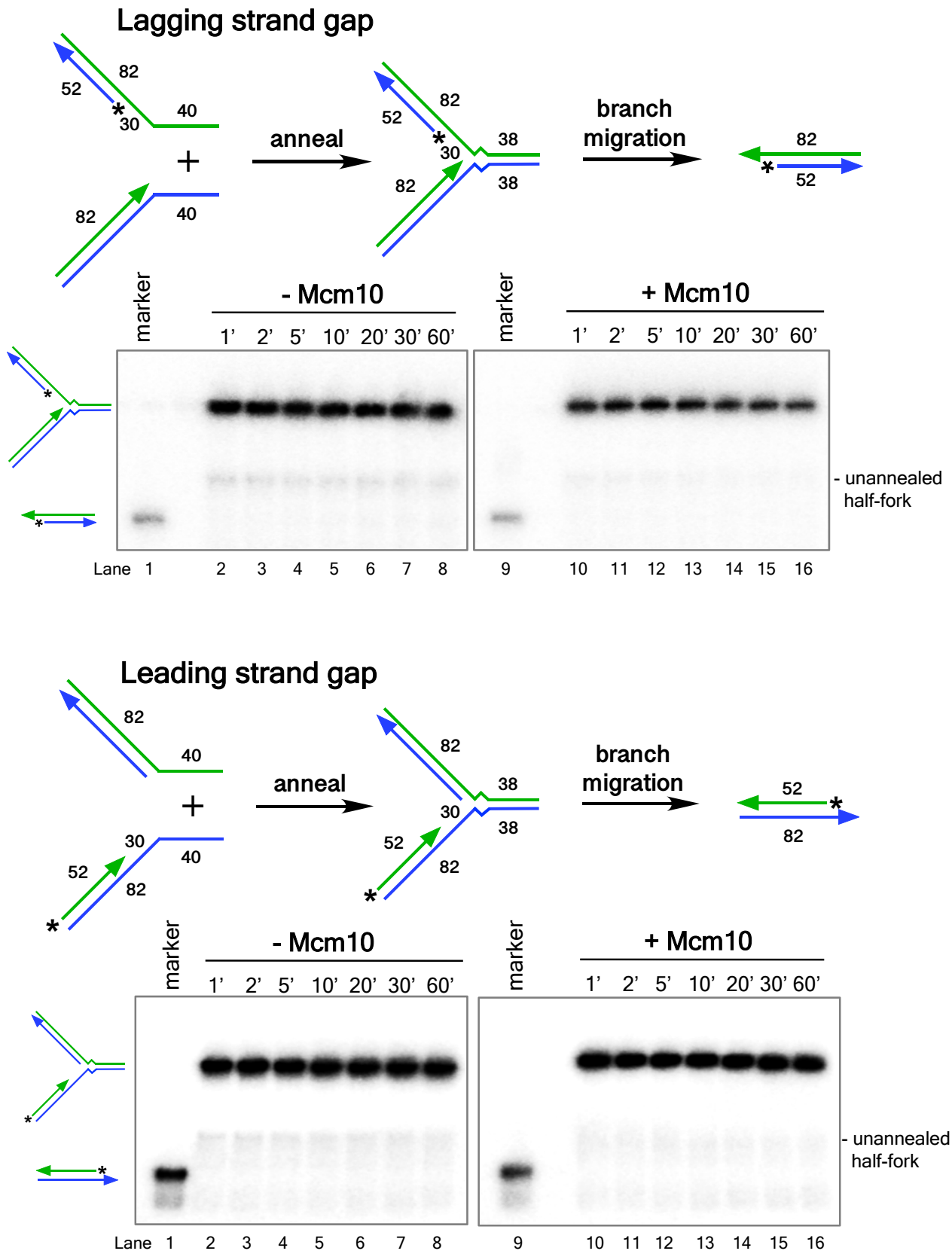

Figure S8. Mcm10 does not reanneal the products of fork regression to reform forked DNAs. Fork reversal assays for both leading and lagging strand gap substrates, both spontaneous and with Mcm10.

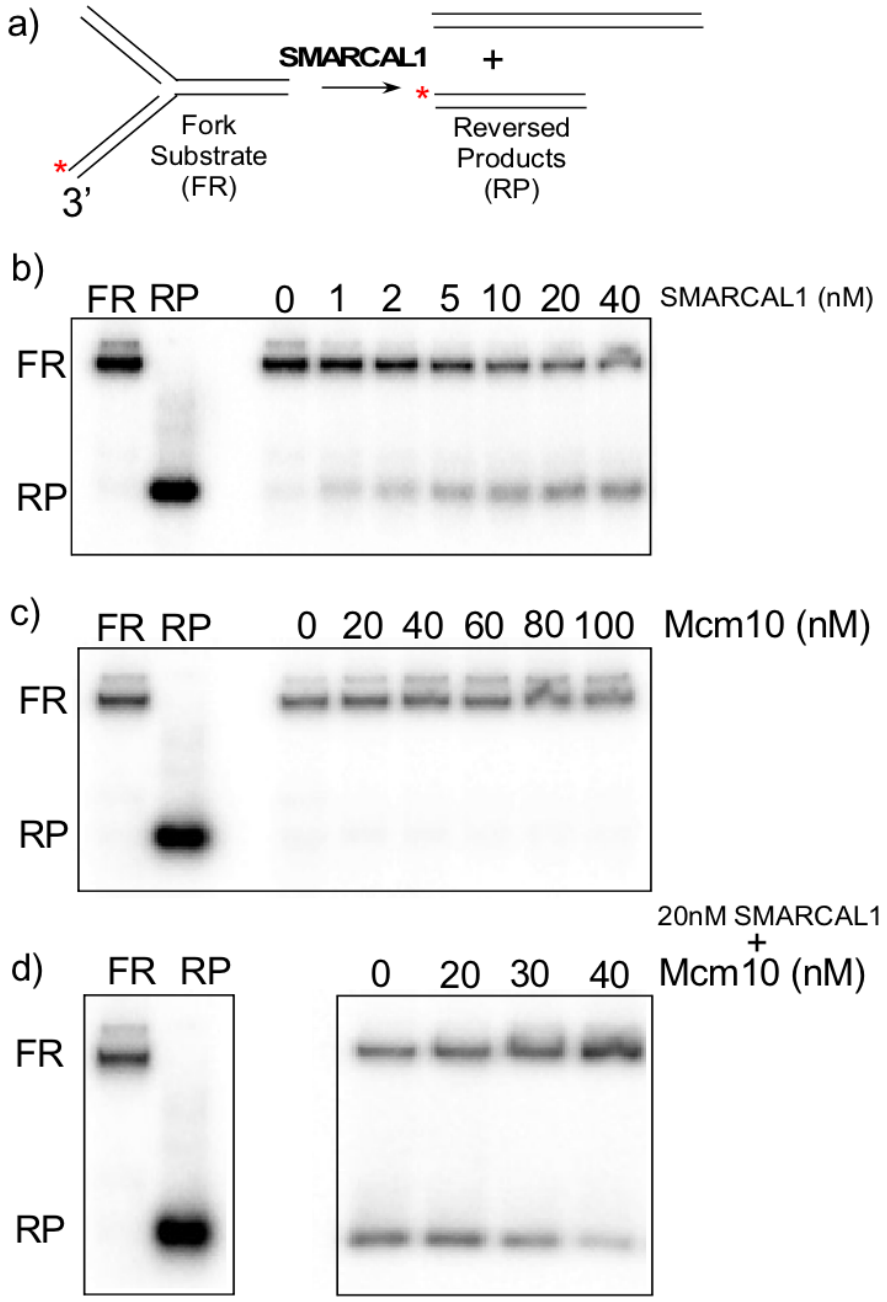

Figure S9. Mcm10 inhibits SMARCAL1 fork reversal of a DNA substrate lacking ssDNA gaps. a) Scheme of the fork regression assay. The two arms of the forked DNA are complementary, and substantial spontaneous regression is prevented by two mismatches at the forked junction. **b)** SMARCAL1 performs fork regression. Human SMARCAL1 was titrated into the fork regression assay. **c)** Mcm10 does not catalyze fork regression. **d)** A titration of Mcm10 into SMARCAL1 (20 nM) fork regression assays. Each assay is a 30' time point as described in Materials and Methods.

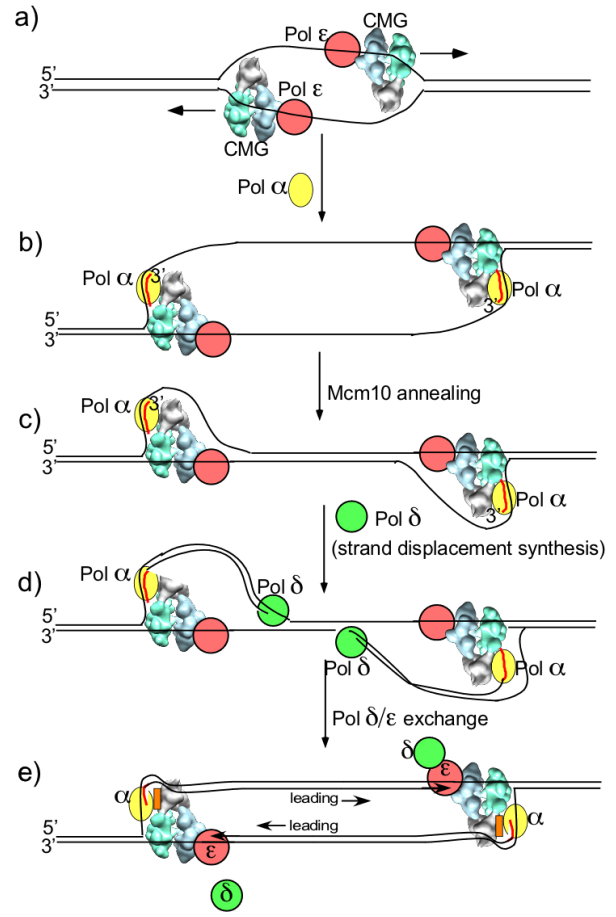

Figure S10. Mcm10 annealing may facilitate the Pol δ requirement for initial primer extension at some origins. a) CMGs (with bound Pol ε) encircle opposite strands while expanding the origin bubble (4, 5, 28). **b)** CMG stimulates Pol α-primase to prime the strand that is excluded from the CMG central channel (i.e. the lagging strand) (44). **c)** Mcm10 may reanneal the unwound ssDNA at the origin between the first primed sites. These first primers, on the lagging strands, may then be extended to form the leading strands by analogy to bacterial origin initiation (43). This is also consistent with the structure of the eukaryotic core replisome showing Pol α–primase is on the opposite side of CMG from Pol ε as illustrated above (4, 5, 52). **d)** Pol ε-PCNA cannot perform strand displacement synthesis, and thus Pol δ, demonstrated to perform strand displacement with RFC/PCNA, is required to proceed through the reannealed DNA. **e)** Upon Pol δ collision with CMG it hands-off the PCNA-3' terminus to Pol ε-CMG as observed *in vitro* (44,48), for leading strand synthesis. See text for additional details.

Dataset S1: Intermolecular and intramolecular cross-links within CMGM. Intermolecular cross-links between Mcm10 and CMG are listed first, then intramolecular cross-links for Mcm10, followed by cross-links within CMG of the CMGM complex.

Movie S1. Movie of Mcm10 cross-link positions (purple) on a surface representation of CMG (PDB 3JC7) from (35, 36). Horizontal rotation by 360° is followed by vertical rotation by 360°. Particular subunits of CMG that are most representative of cross-linked subunits are colored the same as in panel a of Figure 6.

References:

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