Supplementary Information

Mechanism of DNA unwinding by hexameric MCM8-9 in complex with HROB

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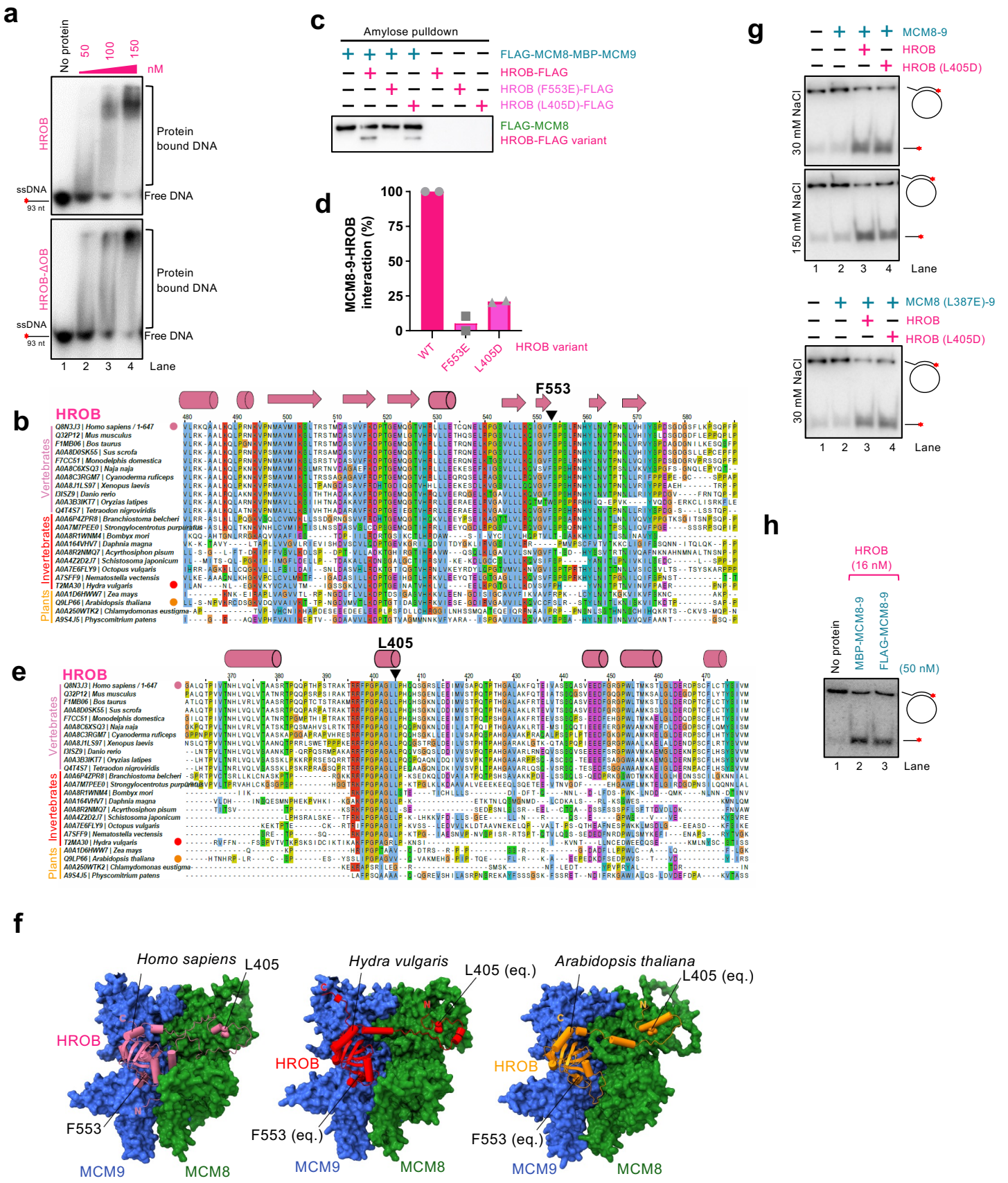
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Extended Data Figures ED Fig.1 to 6

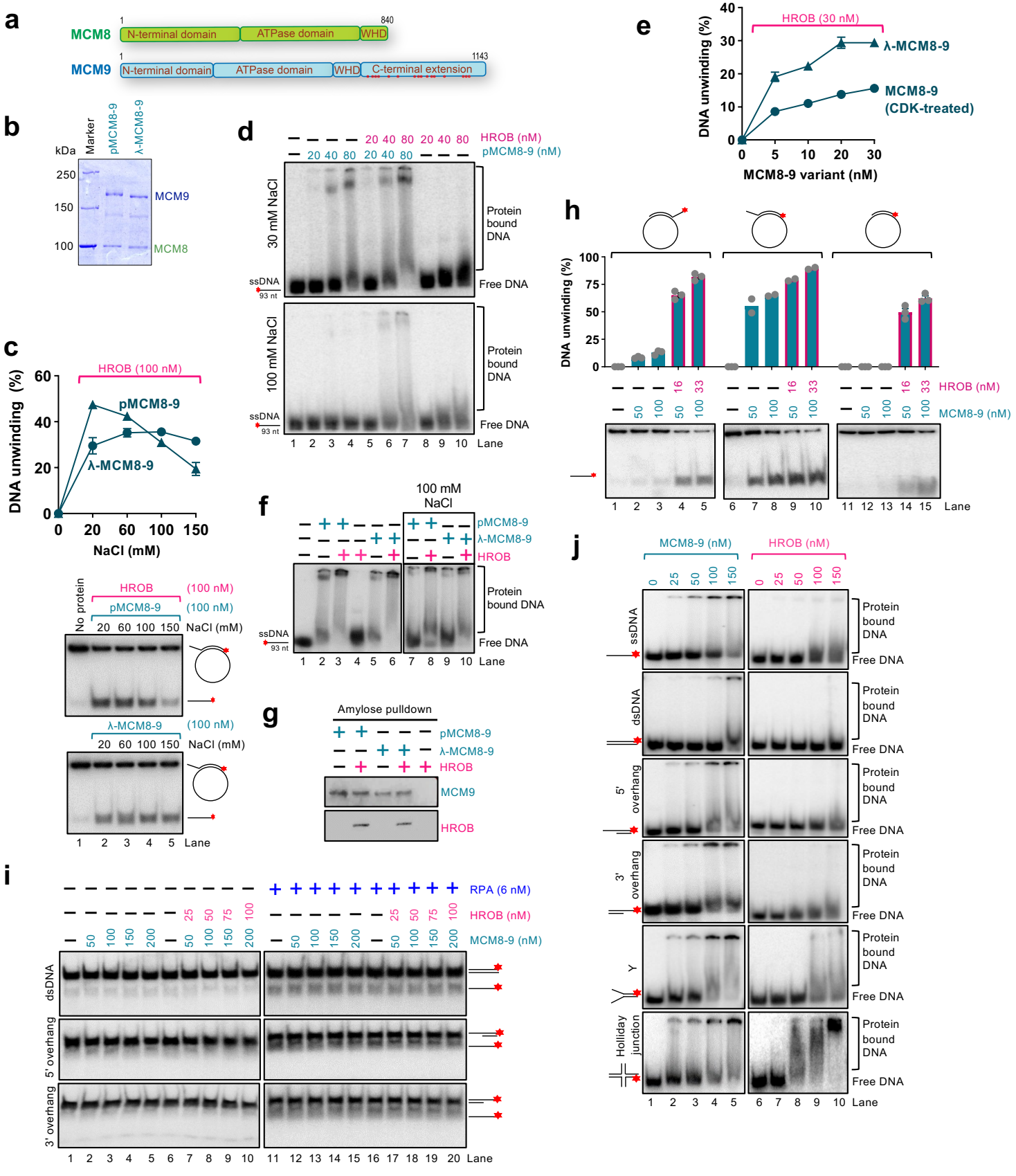
Extended Data Figure Legends

Supplementary Tables S1 to S4

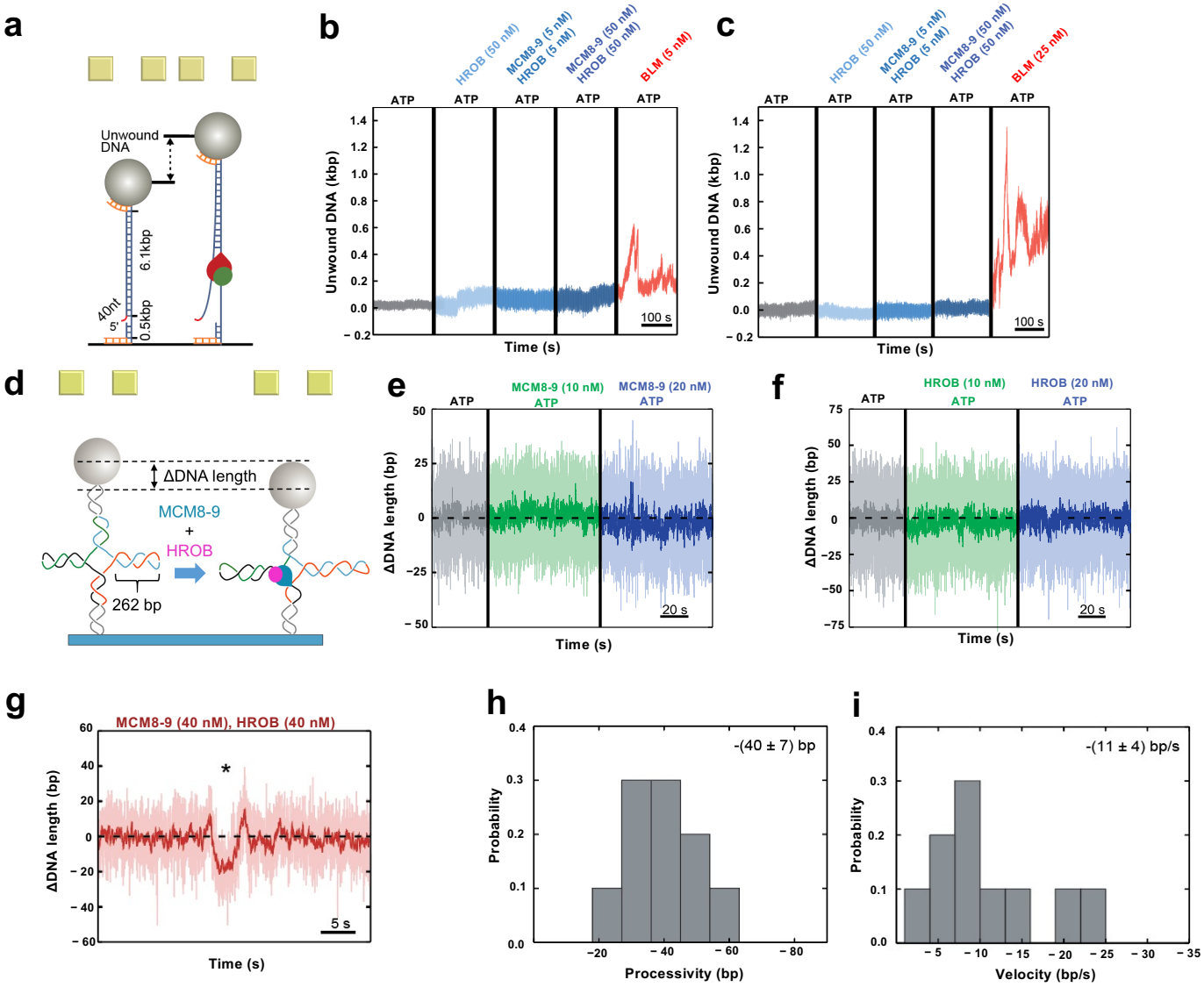
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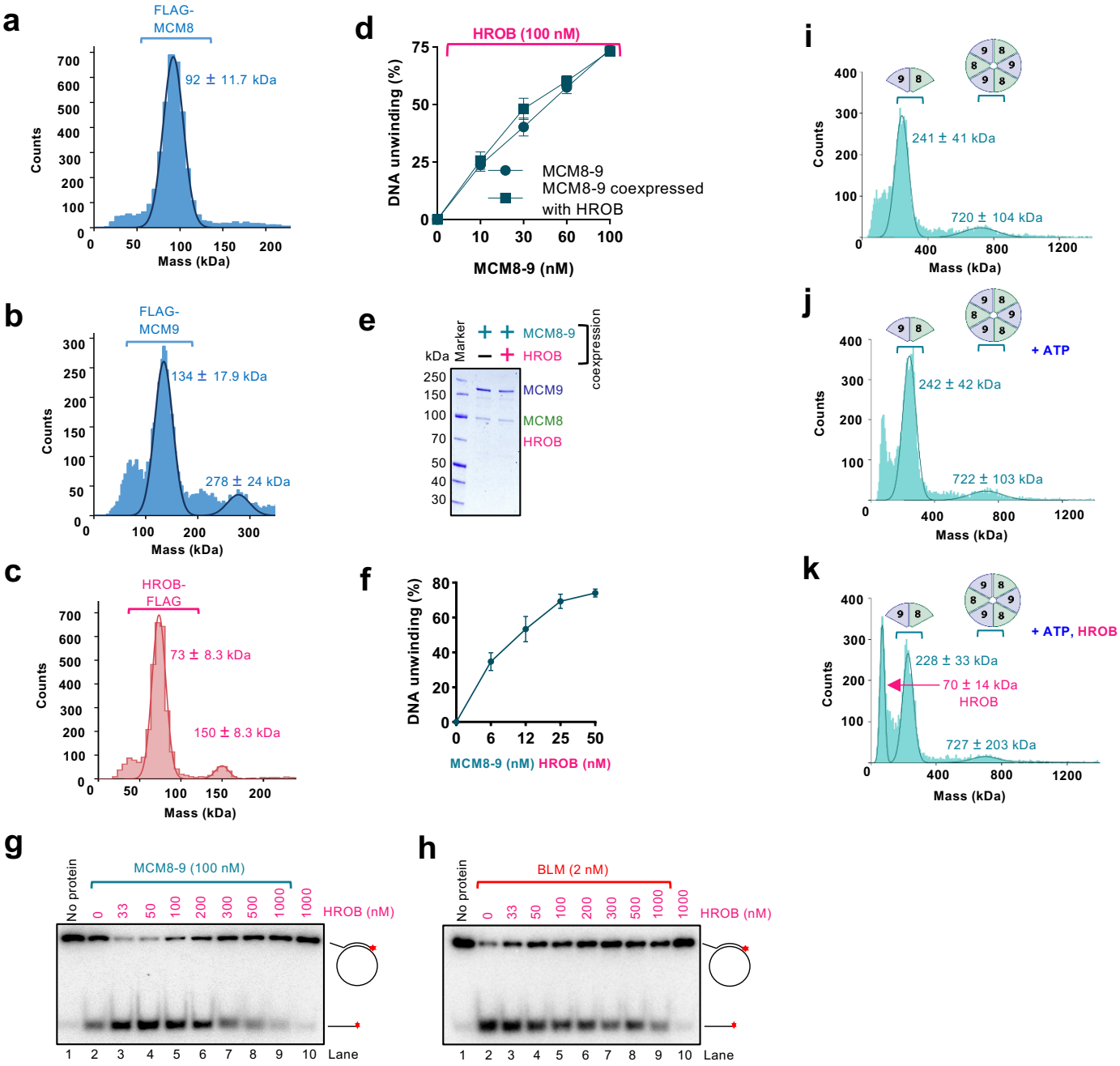
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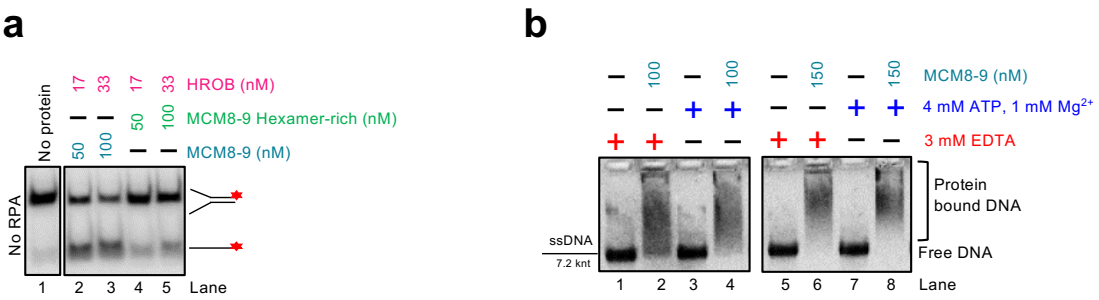
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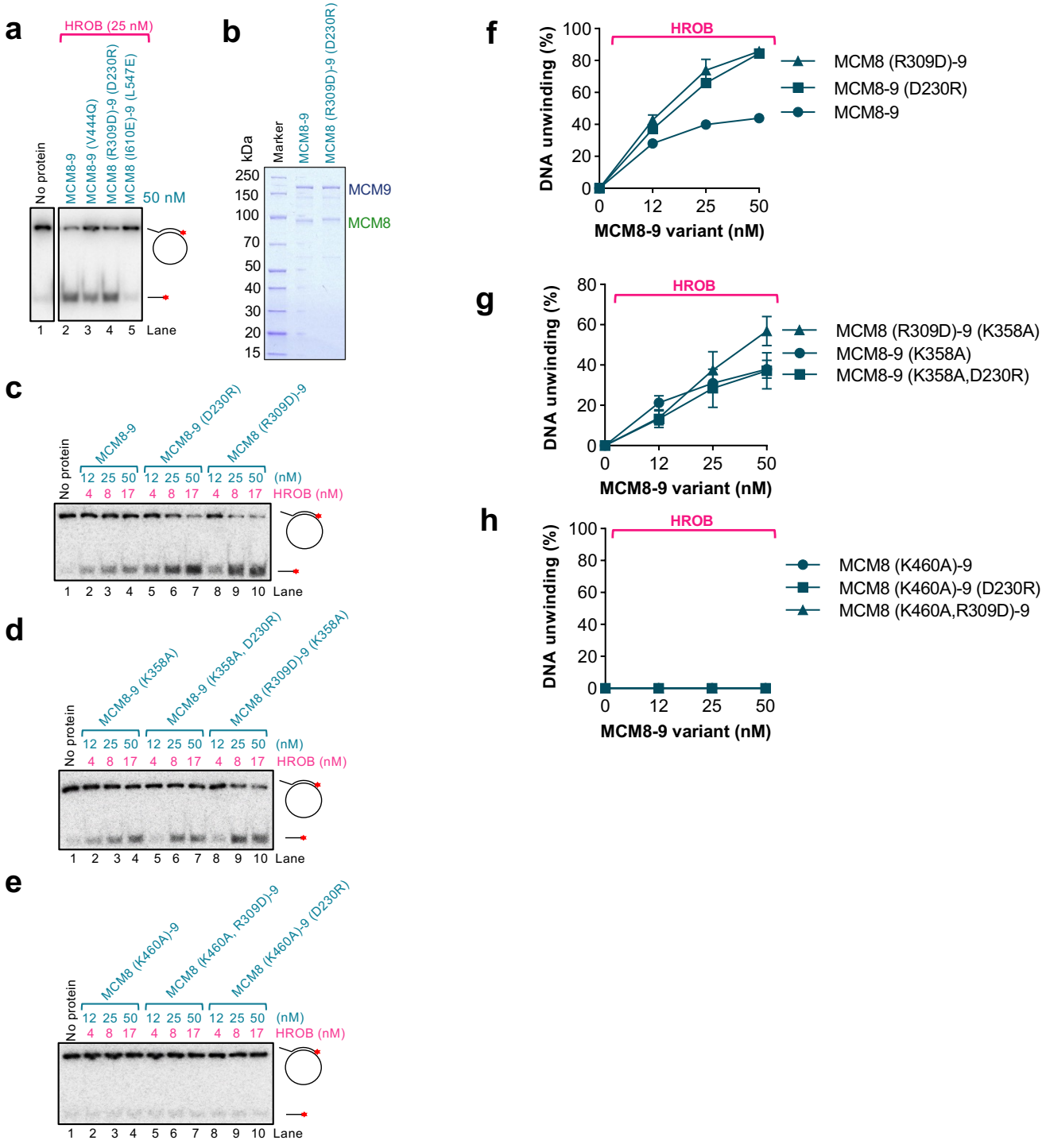
Extended Data Figure 4



Extended Data Figure 5



Extended Data Figure 6



Extended Data Figure Legends

ED Fig. 1. Interaction of HROB with MCM8-9.

a. Electrophoretic mobility shift assay with human HROB or HROB-ΔOB and 93 nt-long ssDNA. The red asterisk indicates the position of the radioactive label. Shown is a representative experiment.
b. Multiple sequence alignment of HROB showing evolutionary conservation of F553 and the surrounding region.
c. MBP-tagged MCM8-9 was immobilized on amylose resin and incubated with HROB or variants at 100 mM NaCl. Bound proteins were eluted and visualized by western blotting with anti-FLAG antibody, detecting FLAG-tagged MCM8 and FLAG-tagged HROB.
d. Quantification of assays such as shown in panel c; Data points show range; n = 2. The values were normalized to wild type HROB (lane 2).
e. Multiple sequence alignment of HROB showing the conservation of L405 and the surrounding region.
f. A cartoon showing a conservation of the interaction of HROB with MCM8-9 in various species, modelled by AlphaFold2.
g. DNA unwinding with HROB (L405D) (25 nM) and MCM8-9 (50 nM) to test for the impact of disrupting HROB interaction with MCM8, carried out at indicated NaCl concentrations using circular M13-based ssDNA. The red asterisk indicates the position of the radioactive label. Shown is a representative experiment.
h. A comparison of DNA unwinding by FLAG-MCM9 and untagged MCM8, or MBP-MCM9 and FLAG-MCM8, purified as a complex, in the presence of HROB using circular M13-based ssDNA. The red asterisk indicates the position of the radioactive label. The MBP and FLAG tags were not cleaved from the corresponding proteins. Shown is a representative experiment.

ED Fig. 2. Regulation of MCM8-9 by phosphorylation and DNA binding and unwinding specificity of MCM8-9.

a. A schematic representation of the main domains in human MCM8 and MCM9. WHD, winged-helix domain. The red dots represent approximate positions of 16 putative consensus CDK phosphorylation sites present in the unique C terminal tail of MCM9 (T673, S703, S707, S711, S762, S802, T879, S883, S890, S915, S942, S952, T977, T1064, S1073 and S1088).
b. MCM8-9 heterodimer variants prepared with phosphatase inhibitors (phosphorylated MCM8-9, pMCM8-9), or without phosphatase inhibitors and treated with λ phosphatase during purification

(dephosphorylated MCM8-9, λ-MCM8-9). The phosphorylated form of MCM9 shows a mobility shift in gel electrophoresis.

c. DNA unwinding assays to compare the activities of phosphorylated (pMCM8-9) and dephosphorylated (λ-MCM8-9) MCM8-9 variants (both 100 nM), with HROB, and its dependence on NaCl concentration using circular M13-based ssDNA. The red asterisk indicates the position of the radioactive label. Top, quantification; error bars, SEM; n = 3; bottom, a representative experiment.

d. Electrophoretic mobility shift assay with pMCM8-9 in the presence or absence of HROB using a 93 nt long ssDNA at no added and 100 mM NaCl with 3 mM EDTA. The red asterisk indicates the position of the radioactive label. Panel shows representative experiments.

e. Quantification of DNA unwinding experiments by human MCM8-9, either phosphorylated with model CDK, CDK2-CycA, MCM8-9 (CDK), or dephosphorylated, λ-MCM8-9, at 150 mM NaCl using circular M13-based ssDNA. Error bars, SEM; n = 3.

f. Electrophoretic mobility shift assays with pMCM8-9 or λ-MCM8-9 (both 100 nM), in the presence or absence of HROB (100 nM) with or without added salt (100 mM NaCl) and 93 nt-long ssDNA with 3 mM EDTA. Panel shows a representative experiment.

g. Physical interaction assay of pMCM8-9 or λ-MCM8-9 and HROB at 100 mM NaCl. The MBP-tagged MCM8-9 variant was immobilized on amylose resin, HROB was subsequently added, bound proteins were eluted, and subjected to Western blotting. MCM9 was detected by anti-MCM9 and HROB by anti-FLAG antibodies.

h. DNA unwinding by pMCM8-9 with and without HROB, using M13-based substrates with 5', 3' or no overhang (all substrates contained 37 bp of duplex DNA, overhang length 40 nt). Top, quantification; error bars, SEM; n = 3; data points in lanes 6-10 show range, n = 2; bottom, a representative experiment.

i. DNA unwinding by pMCM8-9 without or with HROB and RPA, using oligonucleotide-based DNA substrates with 1 mM ATP, 5 mM magnesium acetate and 15 mM NaCl. Representative assays are shown. The red asterisk indicates the position of the radioactive label.

j. Electrophoretic mobility shift assays with pMCM8-9 or HROB, using various oligonucleotide-based DNA substrates with 3 mM EDTA. Representative assays are shown. The red asterisk indicates the position of the radioactive label.

ED Fig. 3. Magnetic tweezer experiments with the MCM8-9 helicase and HROB.

a. A schematic representation of an overhanged DNA construct, the unwinding of which would lead to a change in bead position.

b. Representative time trajectories of DNA length changes at room temperature under the following conditions: no protein (grey), 50 nM HROB (light blue), 5 nM MCM8-9:HROB (1:1) (blue) and 50 nM MCM8-9:HROB (1:1) (dark blue). Additionally, 5 nM BLM (red) was used as a positive control. Each measurement was performed at least 3 times.
c. Experiments as in panel b, but at 37 °C. BLM concentration was 25 nM.
d. A schematic representation of the magnetic tweezer setup and the investigated Holliday junction construct with 262 bp in each arm. When the protein ensemble is added, MCM8-9 with the cofactor HROB can translocate in the vertical direction, causing the arms of the Holliday junction to extent further, leading to a net-decrease in DNA length.
e. Processing of the Holliday junction construct by MCM8-9 alone without HROB. No protein activity was detected.
f. Processing of the Holliday junction construct by HROB alone. No protein activity was detected.
g. An example trace of a net-downward event (highlighted by *) at a concentration of 40 nM for MCM8-9:HROB (1:1).
a. Probability distribution of DNA unwinding processivity by MCM8-9 with HROB, with a mean of –(40 ± 7) bp, of events leading to DNA shortening, n = 10.
b. Probability distribution of DNA unwinding velocity by MCM8-9 with HROB, with a mean of –(11 ± 4) bp sec^{-1}, of events leading to DNA shortening, n = 10.

ED Fig. 4. Analysis of MCM8-9 oligomerization.

a. Measured molecular weight distribution of human FLAG-MCM8 (theoretical value 95 kDa including tag) using mass photometry. Error, SD.
b. Measured molecular weight distribution of human FLAG-MCM9 (theoretical value 128 kDa including tag) using mass photometry. Error, SD.
c. Measured molecular weight distribution of human HROB-FLAG (theoretical value 71 kDa including tag) using mass photometry. Error, SD.
d. Helicase assays with MCM8-9 complex expressed as a heterodimer or co-expressed with HROB. MCM8-9 was 100 nM. As MCM8-9 did not co-purify with HROB, all reactions were supplemented with 100 nM HROB. Error bars, SEM; n = 3.
e. Purified recombinant MCM8-9 complex expressed together with HROB, as used in panel d. HROB could not be co-purified with MCM8-9.
f. Quantification of DNA unwinding assays using equimolar concentrations of MCM8-9 and HROB, as indicated, using M13-based circular ssDNA substrate. Error bars, SEM; n = 3.

g. Representative DNA unwinding assays using human MCM8-9 in the presence of various HROB concentrations using M13-based circular ssDNA substrate. A representative experiment is shown.
h. Representative DNA unwinding assays using human BLM in the presence of various HROB concentrations using M13-based circular ssDNA substrate. A representative experiment is shown.
i. Measured molecular weight distribution of human FLAG-MCM8-MBP-MCM9 (MCM8-9, theoretical value 266 kDa for the heterodimer, 798 kDa for hexamer, including tags) using mass photometry. Errors, SD.
j. Experiment as in panel i, but in a buffer additionally containing 2 mM ATP and 1 mM magnesium acetate.
k. Experiment as in panel j, but together with HROB.

ED Fig. 5. DNA binding and unwinding by MCM8-9

a. Representative DNA unwinding assays with standard and hexamer-rich MCM8-9 preparations in the presence HROB, using Y-structured DNA substrate. The red asterisk indicates the position of the radioactive label.
b. Representative electrophoretic mobility shift assays with human MCM8-9 without or with ATP, using M13-based linear ssDNA (7.2 knt).

ED Fig. 6. Analysis of MCM8-9 mutants disrupting the N-terminal part of interface II.

a. Representative DNA unwinding assays with MCM8-9 variants (50 nM) mutated in interface II at 25 mM NaCl in the presence of HROB (25 nM) using M13-based circular ssDNA substrate.

b. Wild type MCM8-9 and N-terminal interface II mutant MCM8 (R309D)-MCM9 (D230R) used in this study.

c-e. MCM8-9 variants with disrupted N-terminal part of interface II, in combination with ATPase Walker A mutations, as indicated, at 25 mM NaCl in the presence of HROB at indicate concentrations using M13-based circular ssDNA substrate. Panel shows a representative experiment.

f-h. Quantification of assays such as shown in panels c-e. HROB was used at 1/3 concentration of MCM8-9 (nM). Error bars, SEM; n = 3.

Table S1: Protein sequence of HROB-FLAG used in this study.

Protein	Protein Sequence
HROB-FLAG	MACSLQKLFAVEEEFEDEDFLSAVEDAENRFTGSLPVNAGRLRPVSSRPQETVQAQSSRL LLLHPTAPSEALGLPDLDLCLPASSTPSADSRPSCIGAAPLRPVSTSSSWIGNQRRVTVTEV LREPARPQSSALHPLLTFESQQQQVGGFEGPEQDEFDKVLASMELEEPGMELECGVSSEA IPILPAQQREGSVLAKKARVVDLSGSCQKGPVPAIHKAGIMSAQDESLDPVIQCRTPRPPL RPGAVGHLPVPTALTVPTQQLHWEVCPQRSPVQALQPLQAARGTIQSSPQNRFPCQPFQS PSSWLSGKAHLPRPRTPNSSCSTPSRTSSGLFPRIPLQPQAPVSSIGSPVGTPKGPQGALQTP IVTNHLVQLVTAASRTPQQPTHPSTRAKTRRFPGPAGILPHQQSGRSLEDIMVSAPQTPTH GALAKFQTEIVASSQASVEEDFGRGPWLTMKSTLGLDERDPSCFLCTYSIVMVLRKAALK QLPRNKVPNMAVMIKSLTRSTMDASVVFKDPTGEMQGTVHRLLLETCQNELKPGSVLLL KQIGVFSPSLRNHYLNVTPNNLVHIYSPDSGDGSFLKPSQPFPKDSGSFQHDVAAKPEEGF RTAQNLEAEASPEEELPEADDLDGLLSELPEDFFCGTSSGGTSDYKDDDDK

Table S2: List of oligonucleotides used for site-directed mutagenesis to produce recombinant proteins

Primer	Sequence (5'-3')
HROB-F553E Forward Primer	CTGAAGCAGATTGGAGTGGAGTCTCCTTCACTTCGAAATCAC
HROB-F553E Reverse Primer	GTGATTTCGAAGTGAAGGAGACTCCACTCCAATCTGCTTCAG
HROB-L405D Forward Primer	CCCTGGCCCAGCTGGGATCGACCCTCACCAGCAGAGTG
HROB-L405D Reverse Primer	CACTCTGCTGGTGAGGGTCGATCCCAGCTGGGCCAGGG
MCM8 R309D Forward Primer	CGTGAGGCTGGTCGTATCCCTGACACCATCGAGTGCGAGCTG
MCM8 R309D Reverse Primer	CAGCTCGCACTCGATGGTGTCAGGGATACGACCAGCCTCACG
MCM8 I610E Forward Primer	CTCCTGTCCGAGCACGTGGAGGCTATCCGTGCTGGTAAG
MCM8 I610E Reverse Primer	CTTACCAGCACGGATAGCCTCCACGTGCTCGGACAGGAG
MCM8 L387E Forward Primer	GGTATGCTGATGGAGTTCTCCGAGAAGGACCTGTACGCTATC
MCM8 L387E Reverse Primer	GATAGCGTACAGGTCCTTCTCGGAGAACTCCATCAGCATACC
MCM8 E314R Forward Primer	CCTCGTACCATCGAGTGCCGGCTGGTGCACGACTTGGTG
MCM8 E314R Reverse Primer	CACCAAGTCGTGCACCAGCCGGCACTCGATGGTACGAGG
MCM8 V547Q Forward Primer	CTGGCTAAGGCTGGTGTGCAGTGCTCCCTGCCTGCTCGC
MCM8 V547Q Reverse Primer	GCGAGCAGGCAGGGAGCACTGCACACCAGCCTTAGCCAG
MCM8 R697D Forward Primer	GACTTCTACCTGGAGCTGGACAAGCAGTCCCAGCGTCTG
MCM8 R697D Reverse Primer	CAGACGCTGGGACTGCTTGTCCAGCTCCAGGTAGAAGTC
MCM9 V444Q Forward Primer	GTGGCCAAAGCAGGACTGCAGTGCAAACTAAACACGCGAACC
MCM9 V444Q Reverse Primer	GGTTCGCGTGTTTAGTTTGCACTGCAGTCCTGCTTTGGCCAC
MCM9 L547E Forward Primer	GGGAACCAAGTACTTGAGCGCTACTATCAGATG
MCM9 L547E Reverse Primer	CATCTGATAGTAGCGCTCAAGTACTTGGTTCCC
MCM9 R256E Forward Primer	CCCTTTCAACAAGATGTAGAGTGTGAAGTGGAGATTGTG
MCM9 R256E Reverse Primer	CACAATCTCCACTTCACACTCTACATCTTGTTGAAAGGG
MCM9 D230R Forward Primer	CTGGAAGATGATCTGGTGCGCTCCTGCAAGAGTGGTGATG
MCM9 D230R Reverse Primer	CATCACCACTCTTGCAGGAGCGCACCAGATCATCTTCCAG
MCM9 L506E Forward Primer	GAATAATAAGCTCCTTCATCGAGGAGAACAAAGGGTATCCCTCC
MCM9 L506E Reverse Primer	GGAGGGATACCCTTTGTTCTCCTCGATGAAGGAGCTTATTATTC
MCM9 M45E Forward Primer	CCAGTGGTGGTAAATGCCGAGACGCTCTTTGAAACCAAC
MCM9 M45E Reverse Primer	GTTGGTTTCAAAGAGCGTCTCGGCATTTACCACCACTGG
MCM8 K460A Forward Primer	TTGGAGATCCAGGCCTAGGAGCTAGTCAAATGCTACAGGCAGC
MCM8 K460A Reverse Primer	GCTGCCTGTAGCATTTGACTAGCTCCTAGGCCTGGATCTCCAA
MCM9 K358A Forward Primer	TTGGGGATCCTGGCACAGGGGCTTCTCAGTTCCTCAAATATGC
MCM9 K358A Reverse Primer	GCATATTTGAGGAACTGAGAAGCCCCTGTGCCAGGATCCCCAA
MCM8 L387E Forward Primer	GGAGTTCTCAGAGAAAGACCTTTATGCC
MCM8 L387E Reverse Primer	ATCAACATTCCATGCTTAC
MCM9 M45E Forward Primer	GGTTAATGCCGAGACTCTGTTTGAGAC
MCM9 M45E Reverse Primer	ACAACTGGGTAATGAGCATC

Table S3: Sequence of oligonucleotides used for substrate preparation in this study.

Name	Sequence (5'-3')
X12-3 HJ3	GAGATCTATCTGGTGCCTTCTGACAGTGAATGGGTAACGAATC GTAATAGTCTCTAGACAGCATGTCCTAGCAATGTAATCGTCTA TGACGTC
M13-5'-dT 40 overhang	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGTTTTCC CAGTCACGACGTTGTAAAACGACGGCCAGT
M13-37mer no overhang	GTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGT
M13-3'-dT 40 overhang	GTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
M13-5'-dT20overhang-30bp_c	TTTTTTTTTTTTTTTTTTTGTTTTCCCAGTCACGACGTTGTAAA ACGAC
M13 5' dT20overhang-60bp_c	TTTTTTTTTTTTTTTTTTTGTTTTCCCAGTCACGACGTTGTAAA ACGACGGCCAGTGCCAAGCTTGCATGCCTGCAGGT
M13 5' dT20overhang-90bp_c	TTTTTTTTTTTTTTTTTTTGTTTTCCCAGTCACGACGTTGTAAA ACGACGGCCAGTGCCAAGCTTGCATGCCTGCAGGTCGACTCTA GAGGATCCCCGGGTACCGAGCT
PC1253	TGGGTCAACGTGGGCAAAGATGTCCTAGCAATGTAATCGTCTA TGACGTT
PC1254	TGCCGAATTCTACCAGTGCCAGTGATGGACATCTTTGCCCACG TTGA CCC
PC1255	GTCGGATCCTCTAGACAGCTCCATGATCACTGGCACTGGTAGA ATTC GGC
PC1256	CAACGTCATAGACGATTACATTGCTACATGGAGCTGTCTAGAG GATC CGA
PC1253C	AACGTCATAGACGATTACATTGCTAGGACATCTTTGCCCACGT TGAC CCA
312	AACGTCATAGACGATTACATTGCTA
314	CATGGAGCTGTCTAGAGGATCCGAC
HJ-fragment 4229 bp Forward Primer	GGCGCCGCCTCAGCCGCAGGAAAGAACATGTGA
HJ-fragment 4229 bp Reverse Primer	TTATCGATACCGTCGACCTCGAGCAGCAACCGCA
HJ-fragment 5326 bp Forward Primer	GGCGCCGCCTCAGCCGCAGGAAAGAACATGTGA
HJ-fragment 5326 bp Reverse Primer	AAACAGGCTCTGCGGGCCCGGCGT
Handles Forward Primer	GACCGAGATAGGGTTGAGTG
Handles Reverse Primer	TTTGTGATGCTCGTCAGGGG

Table S4: List of oligonucleotides used for site-directed mutagenesis to produce plasmids for cellular assays in this study.

Primer	**Sequence (5'-3')**
MCM8 L387E Forward Primer	GGAGTTCTCAGAGAAAGACCTTTATGCC
MCM8 L387E Reverse Primer	ATCAACATTCCATGCTTAC
MCM9 M45E Forward Primer	GGTTAATGCCGAGACTCTGTTTGAGAC
MCM9 M45E Reverse Primer	ACAACTGGGTAATGAGCATC