

Supplementary Information

Distinct roles of MCM2-7 subunits in replication licensing in human cells

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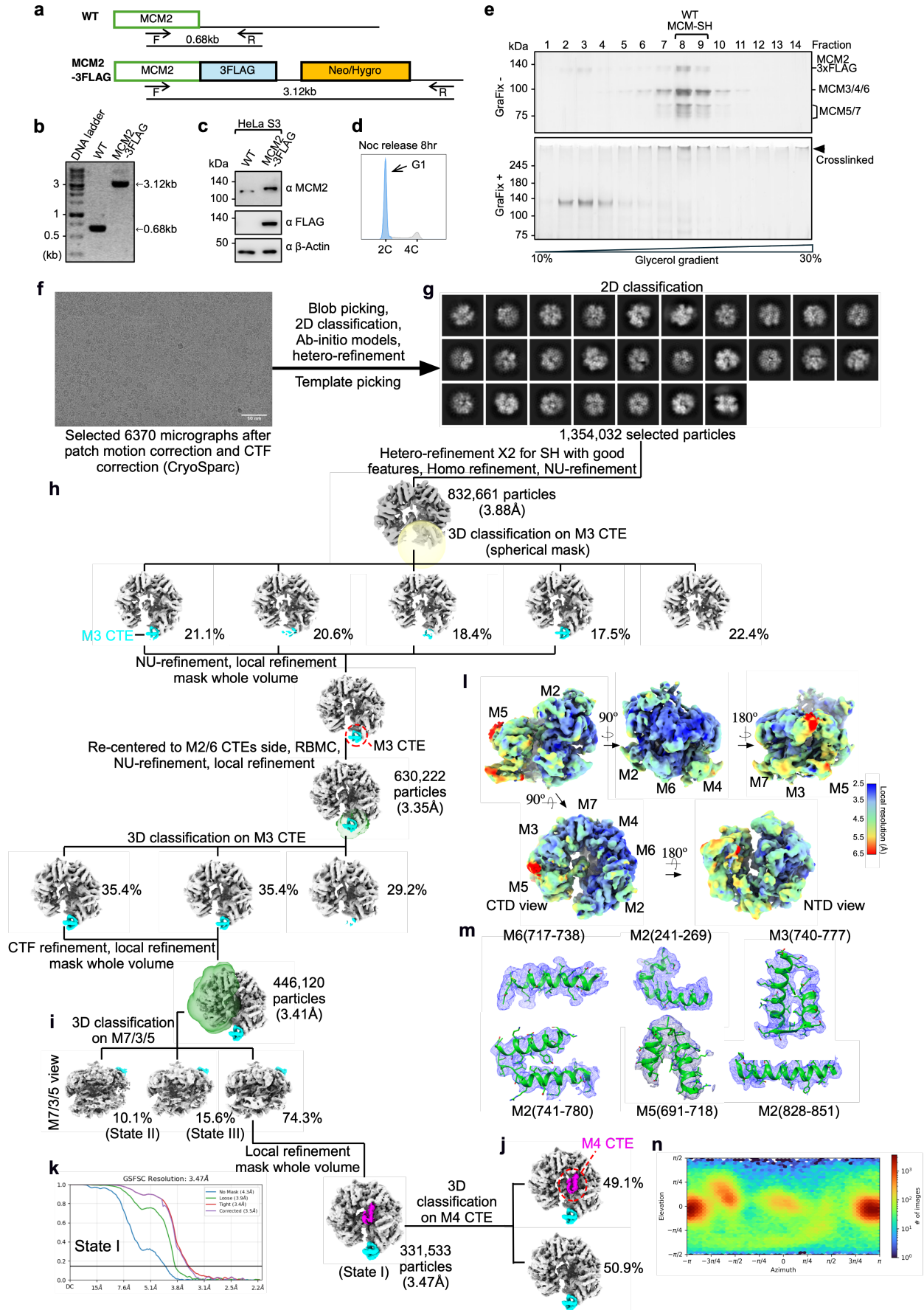
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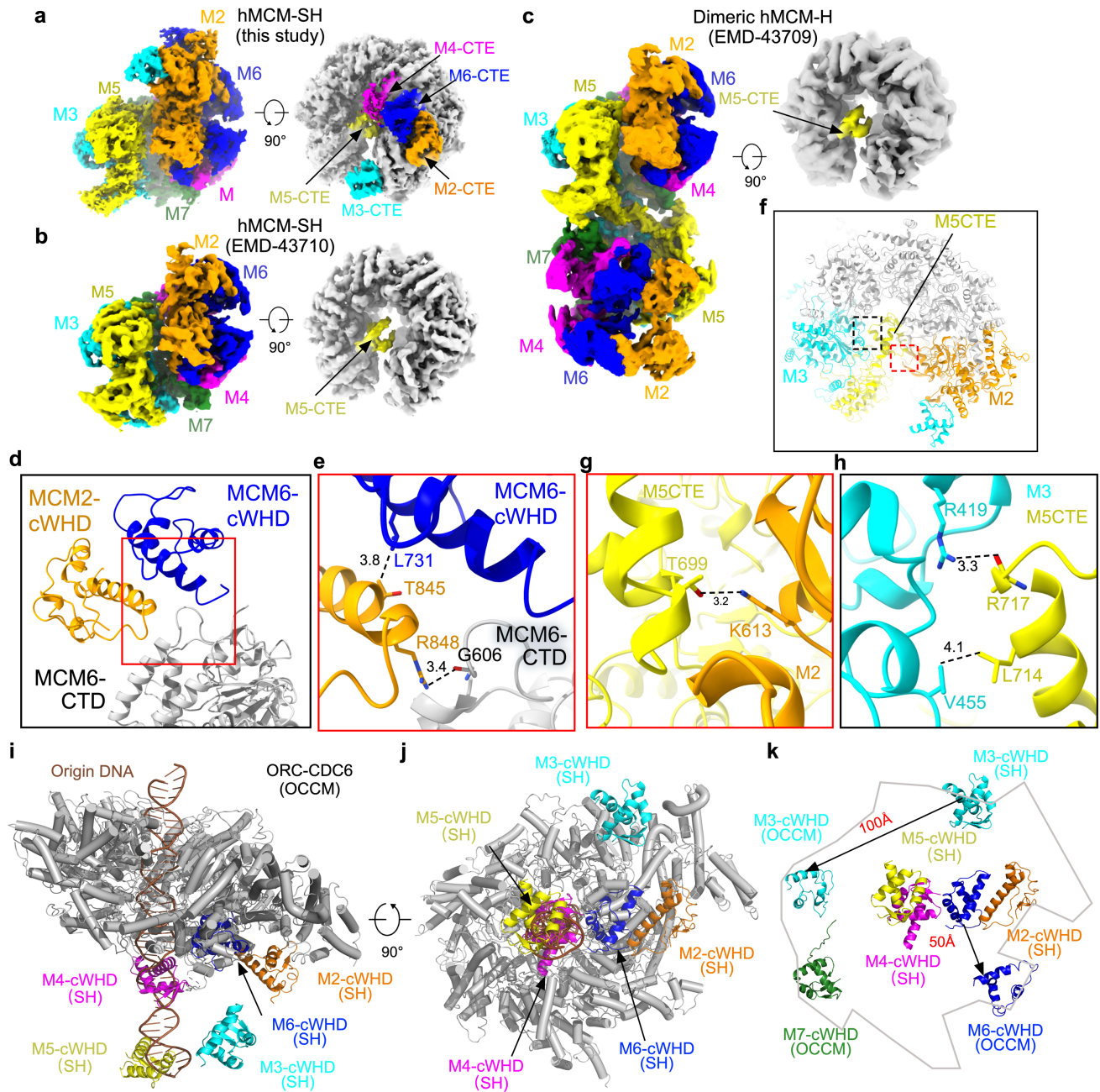
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Supplementary Figure 1 | Sample preparation and cryo-EM analysis of the endogenous hMCM2-7 SH.

- a** Schematic illustration of the strategy for endogenous C-terminal 3xFLAG tagging of MCM2 in HeLa S3 cells using CRISPR/Cas9.
- b** Genomic PCR validation of the edited HeLa S3 clones using primer sets as indicated in (a).
- c** Immunoblotting of MCM2-3FLAG proteins using anti-MCM2 and anti-FLAG antibodies.
- d** FACS profile of G1-synchronized cells used for hMCM2-7 SH purification.
- e** Glycerol gradient (10-30%) sedimentation of FLAG-IP eluates. Top: SDS-PAGE analysis of noncrosslinked fractions and visualized by silver staining. Bottom: GraFix-crosslinked fractions. Crosslinked fractions were pooled and processed for cryo sample preparation.
- f** Representative cryo-EM micrographs of hMCM2-7 SH sample. Scale bar: 50 nm.
- g** 2D class averages of hMCM2-7 SH particles.
- h-j** Cryo-EM data processing workflow.
- k** FSC curves (cutoff = 0.143) for MCM2-7 State I reconstruction.
- l** Color-coded local resolution map.
- m** Cryo-EM density (blue mesh) overlaid with atomic model for representative regions.
- n** Particle angular distribution for the refined hMCM2-7-SH (State I) map.



Supplementary Figure 2 | Structural rearrangement of MCM-CTEs during MCM loading.

a-c The structure of hMCM-SH determined in this study (**a**) is shown alongside previously reported structures of the monomeric (**b**, EMD-43710) and dimeric (**c**, EMD-43709) forms.

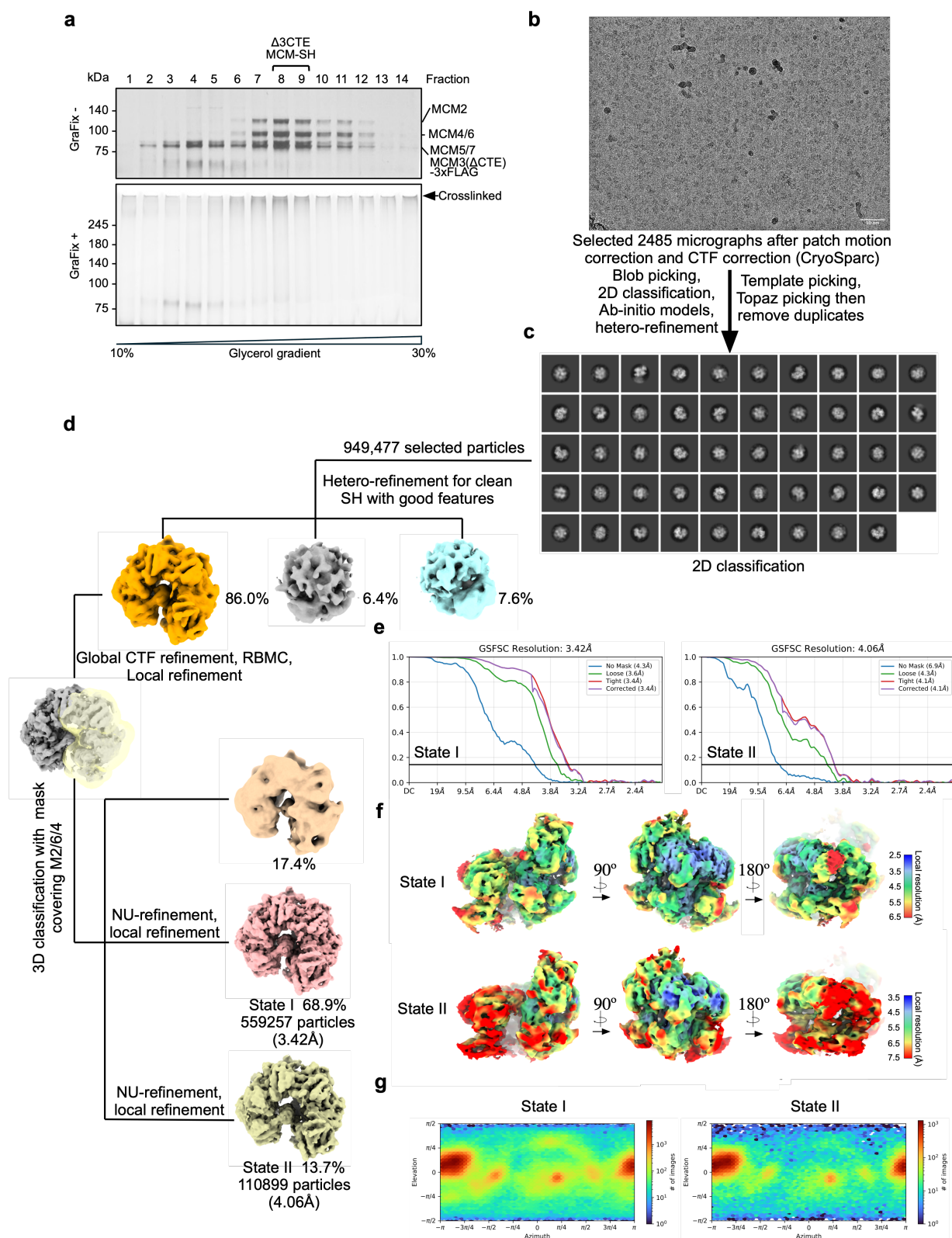
d Interaction interface between MCM2-cWHD (orange) and MCM6 (CTE: blue, CTD: gray).

e Magnified views of the boxed regions in (**d**), highlighting key interactions (dashed lines) with interatomic distances (Å) labeled.

f Binding of MCM5-cWHD (yellow) to the inner surfaces of MCM2-NTD (orange) and MCM3-CTD (cyan).

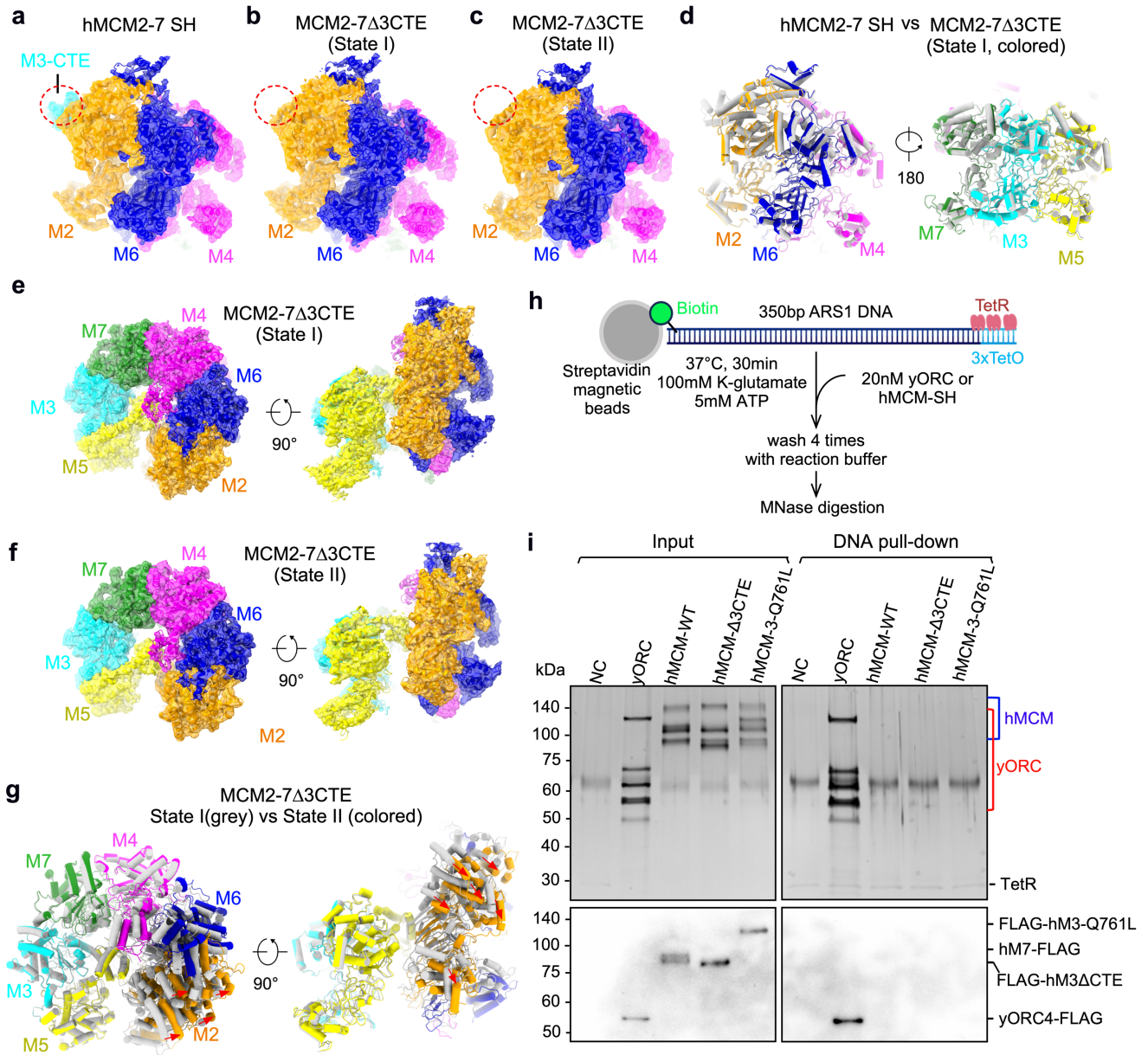
g-h Zoomed-in views of the boxed regions in (f). Detailed interactions of MCM5-cWHD with the presensor 1 β (PS1) hairpin of MCM2-CTD (g) and MCM3-CTD (h). Dashed lines indicate specific contacts, with interatomic distances ($\text{\AA}$) shown.

i-k Steric constraints imposed by CTEs for MCM loading. Structures of hMCM2-7 SH (this study) and the hOCCM (PDB 8S0E) were aligned via MCM2-CTD. Side (i) and bottom (j) views showing the WHDs of hMCM2-7 and the ORC-CDC6-DNA from hOCCM. **k** Top view of the aligned structures shown with ORC-CDC6 outline and the cWHDs from both SH and OCCM; arrows indicate cWHD repositioning during the SH-OCCM transition.



Supplementary Figure 3 | Sample preparation and cryo-EM analysis of hMCM2-7-Δ3CTE mutant SH

- a** Glycerol gradient (10-30%) sedimentation of FLAG-IP eluates from HeLa S3 cells expressing MCM3(Δ CTE)-3 $\times$ FLAG. Top: SDS-PAGE analysis of non-crosslinked fractions. Bottom: GraFix-crosslinked fractions. Pooled crosslinked fractions (8-9) were processed for cryo sample preparation.
- b** Representative cryo-EM micrographs of hMCM2-7 Δ 3CTE. Scale bar: 50 nm.
- c** 2D class averages of hMCM2-7 Δ 3CTE particles.
- d** Cryo-EM data processing workflow.
- e** FSC curves (cutoff = 0.143) for State I and State II of hMCM2-7 Δ 3CTE reconstructions.
- f** Color-coded local resolution maps.
- g** Particle angular distributions for the refined hMCM2-7 Δ 3CTE (State I and II) maps.



Supplementary Figure 4 | Structural comparison of hMCM2-7 SH and hMCM2-7Δ3CTE SH

a-c Sideview of segmented cryo-EM density maps superimposed with atomic models for hMCM2-7 (**a**), hMCM2-7Δ3CTE State I (**b**), and hMCM2-7Δ3CTE State II (**c**).

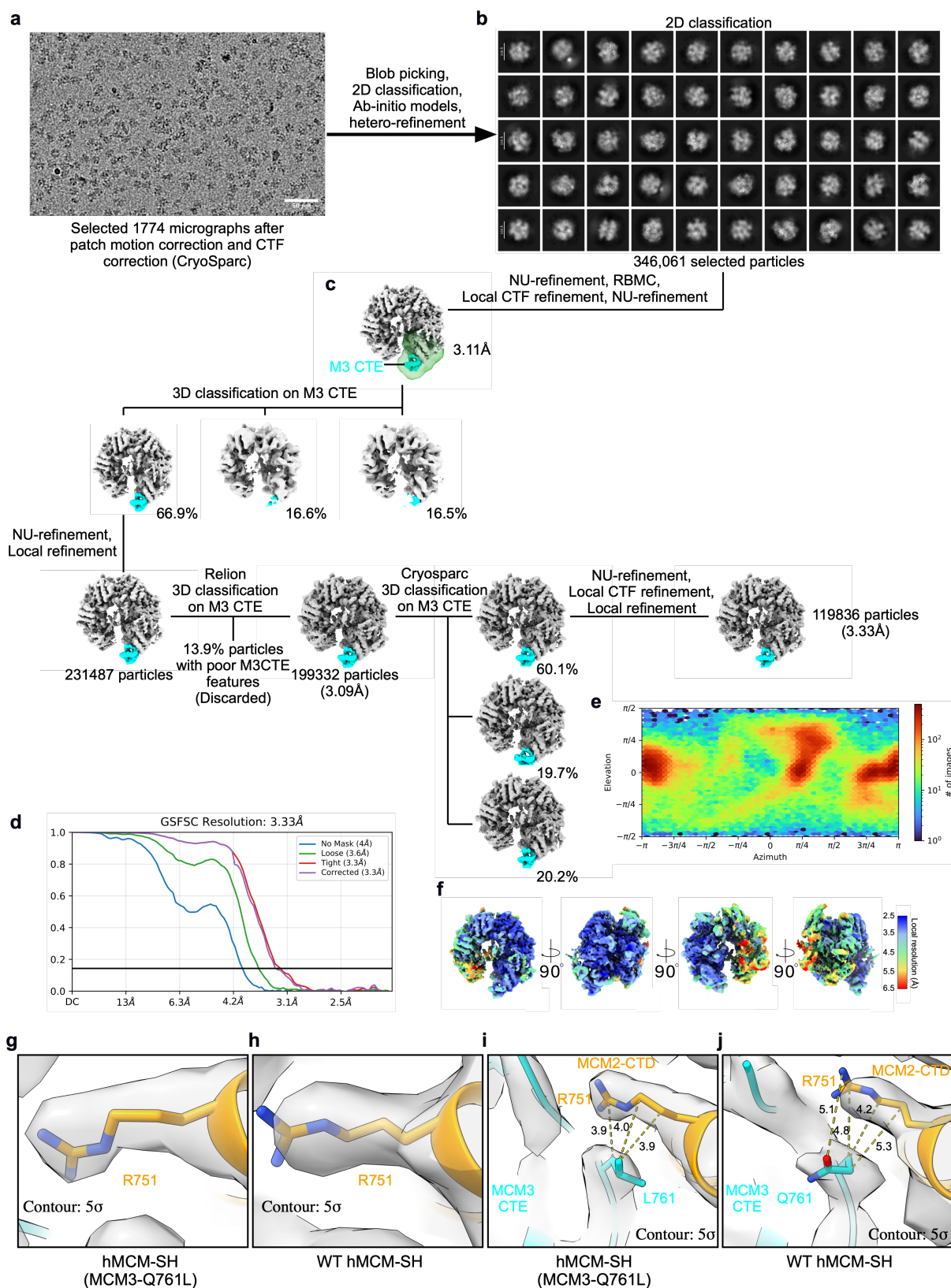
d Superimposition of hMCM2-7 SH (light grey) on hMCM2-7Δ3CTE state I (colored) using MCM5 as a reference for alignment.

e-f Rotational views of hMCM2-7Δ3CTE State I (**e**) and State II (**f**) from indicated perspectives.

g Structural comparison between hMCM2-7Δ3CTE State I (grey) and State II (colored), highlighting conformational differences at the M2/6/4 side. Black arrows indicate obvious rearrangements between these two states.

h Schematic illustration of the in vitro experimental setup for analyzing protein-DNA interaction and the workflow of the binding assay. Biotinylated ARS1 DNA (350 bp) containing a 3×TetO sequence at one end was immobilized on streptavidin-coated magnetic beads. The free end was blocked by TetR binding to prevent protein complexes from sliding off the DNA.

i Purified yORC (positive control), hMCM-WT, hMCM-Δ3CTE, and hMCM-Q761L were incubated with the immobilized DNA in the presence of 5 mM ATP. Following extensive washing, bound proteins were eluted by MNase digestion, analyzed by SDS-PAGE, and visualized by silver staining (upper panels) and immunoblotting (lower panels) as indicated.



Supplementary Figure 5 | Cryo-EM analysis of the hMCM2-7 SH containing MCM3-Q761L mutation.

a Representative cryo-EM micrographs of the mutant hMCM2-7 SH (MCM3-Q761L). Scale bar: 50 nm.

b 2D class averages of hMCM2-7 (MCM3-Q761L) particles.

c Cryo-EM data processing workflow.

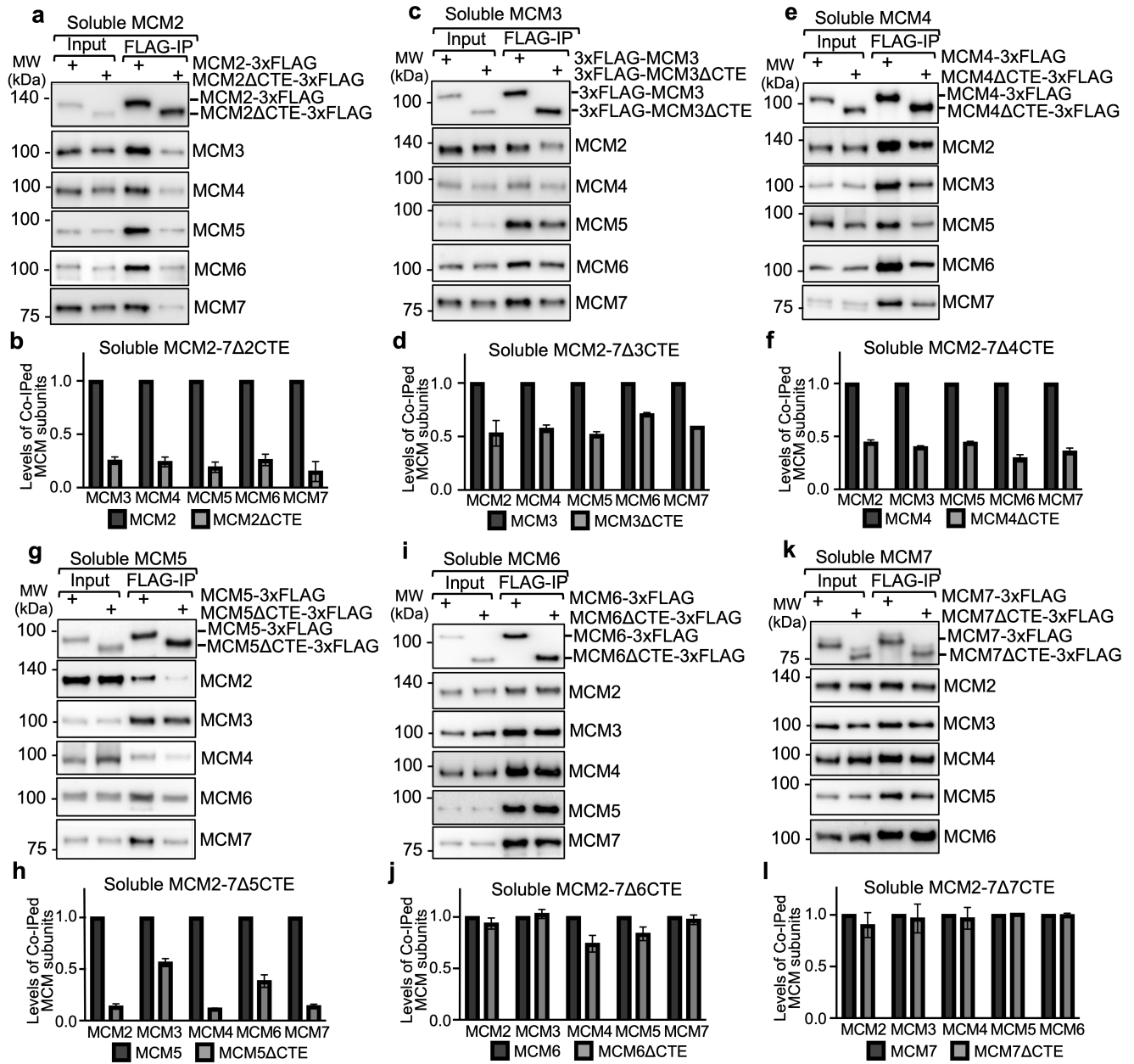
d FSC curves (cutoff = 0.143) for the final refined map.

e Particle angular distribution for the refined hMCM2-7 SH (MCM3-Q761L) map.

f Local resolution map of the final reconstruction, color-coded by resolution.

g-h Cryo-EM density overlaid with the atomic model for residue R651 of MCM2 in the mutant (**f**) and WT (**g**) complexes.

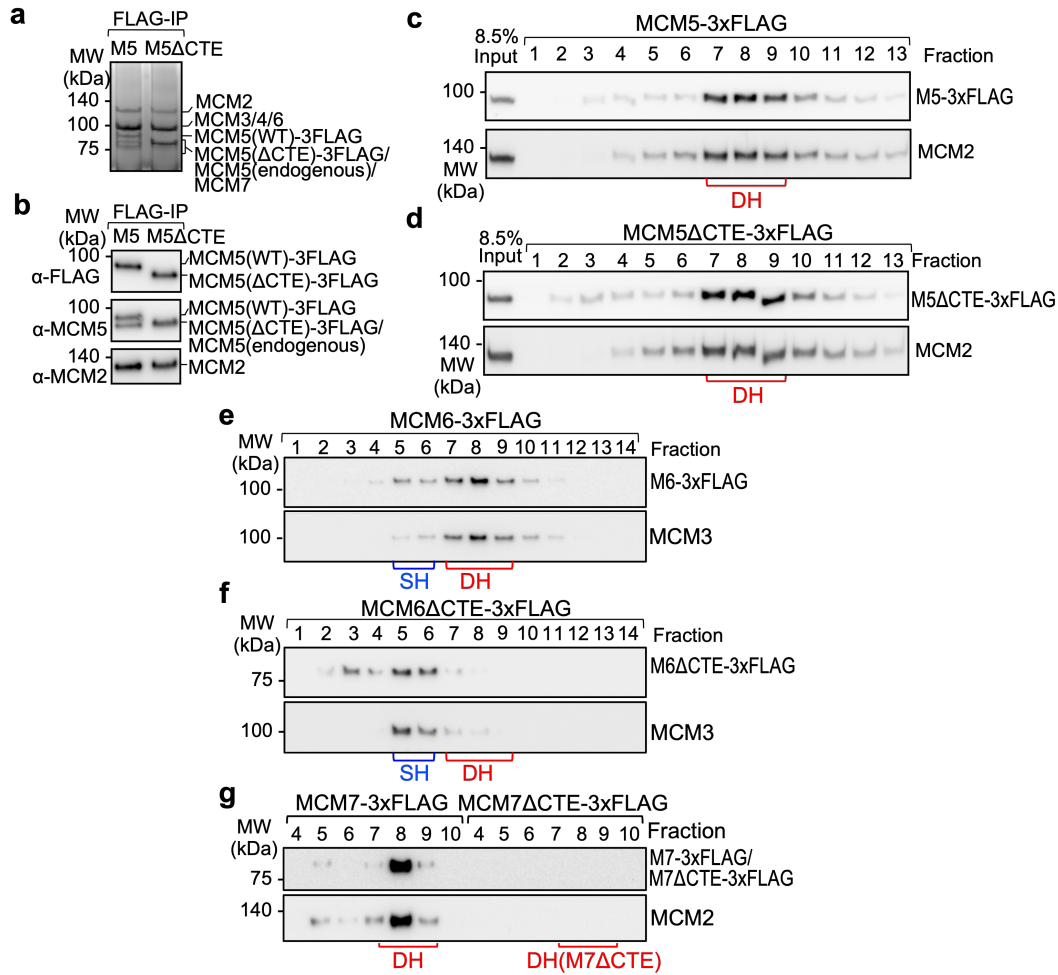
i-j Close-up view of the MCM3-MCM2 interface in the mutant (**h**) and WT (**i**) structures. Side chains of residues Q/L761 of MCM3 and R751 of MCM2 are shown, with measured interatomic distances (Å) indicated.



Supplementary Figure 6 | Systematic evaluation of CTEs in SH integrity.

Interaction analysis of MCM subunits with CTE-truncation mutants. Anti-FLAG immunoprecipitation was performed using soluble cell extracts from HeLa S3 cells ectopically expressing MCM-WT or -ΔCTE subunits. Co-precipitating MCM subunits were detected by immunoblotting of the indicated subunits. Signal intensities were quantified and normalized to WT MCM (mean $\pm$ SD, $n=3$).

a-b MCM2-ΔCTE; **c-d** MCM3-ΔCTE; **e-f** MCM4-ΔCTE; **g-h** MCM5-ΔCTE; **i-j** MCM6-ΔCTE; **k-l** MCM7-ΔCTE;

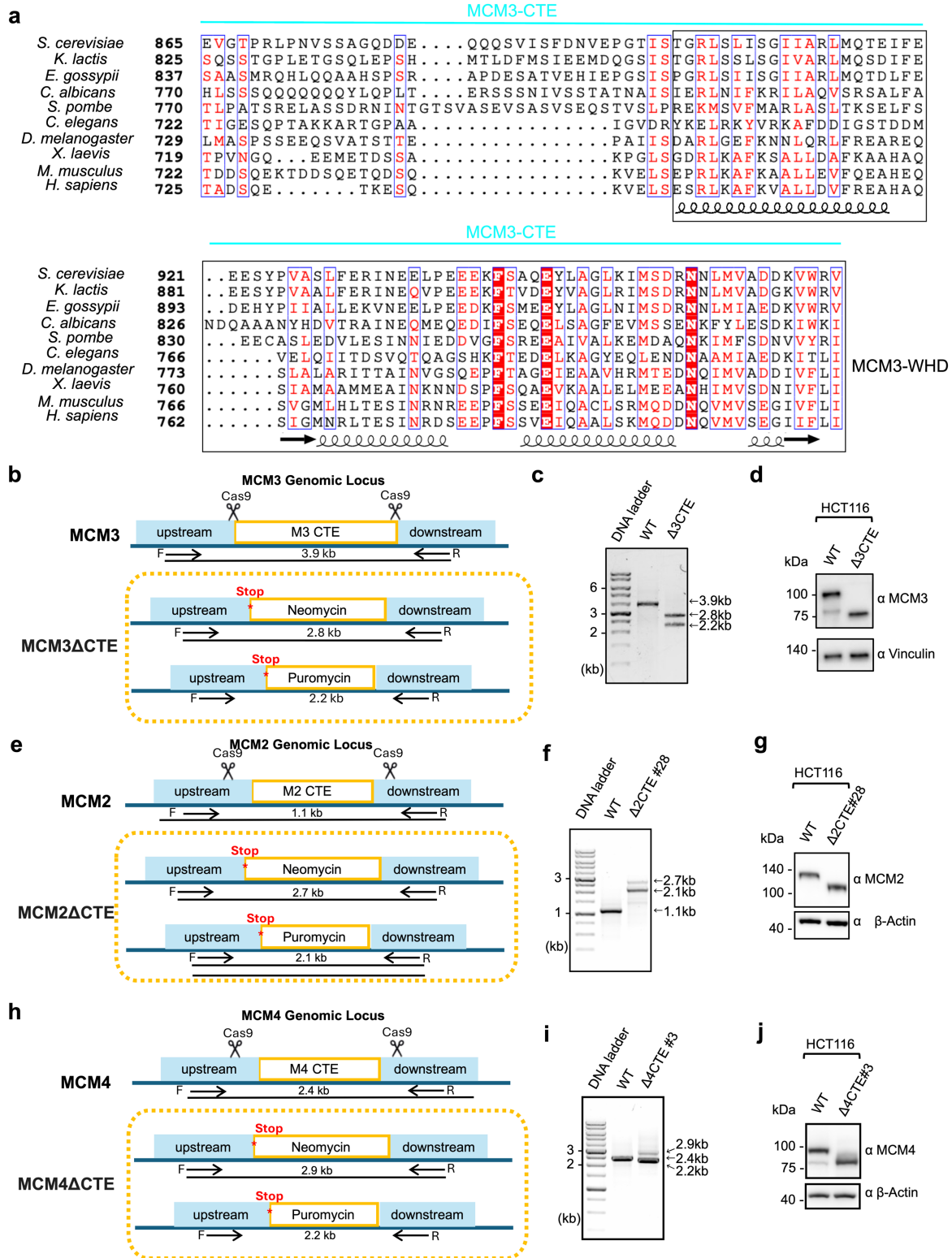


Supplementary Figure 7 | The effect of CTE truncation on pre-RC assembly in HeLa S3 cells overexpressing the relevant MCMΔCTE mutant.

a-b Analysis of affinity-purified complexes containing 3×FLAG-tagged WT MCM5 or MCM5ΔCTE- by silver staining (**a**) and western blotting (**b**). Immunoblot of the samples probed with the indicated antibodies.

c-d Glycerol gradient sedimentation of the immunopurified complexes. Eluates from (**a**) were fractionated on 20-40% glycerol gradient. Immunoblot analysis shows the distribution of WT MCM5 (**c**) or MCM5ΔCTE-containing complexes (**d**) across the gradient fractions.

e-g Glycerol gradient (20-40%) sedimentation analysis of chromatin-bound MCM2-7 complexes isolated from HeLa S3 expressing MCM6-3xFLAG (**e**), MCM6-ΔCTE-3xFLAG (**f**), MCM7-3xFLAG (**g**, left), or MCM6-ΔCTE-3xFLAG (**g**, right). Collected fractions were analyzed by SDS-PAGE and immunoblotting for the indicated proteins.



Supplementary Figure 8 | Construction of homozygous CTE-truncation cell lines

a Sequence alignment of MCM3-CTE across various species. The black box indicates the WHD region; coiled lines denote α -helices; arrows denote β -strands.

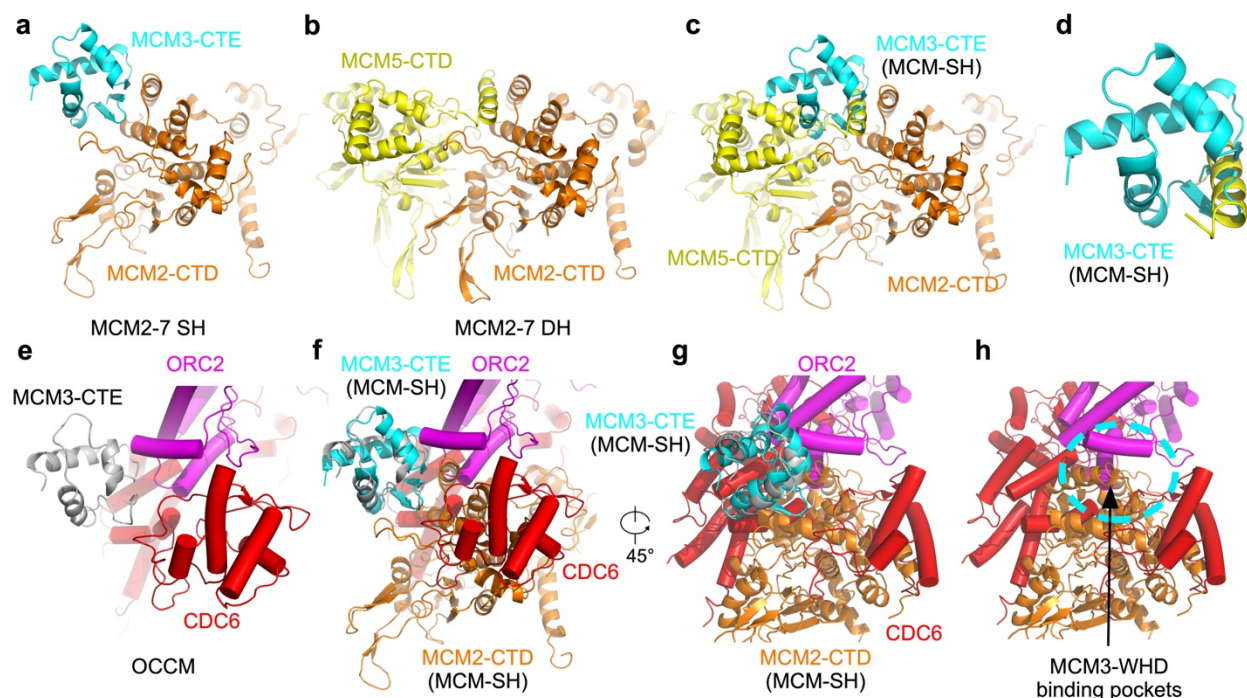
b Schematic of the CRISPR/Cas9 strategy for deleting the MCM3-CTE in HCT116 cells. The target sites and locations of genotyping primers (arrows) are shown.

c Genomic PCR validation of MCM3-CTE knockout clone. The primer pairs indicated in (**b**) were used to confirm homozygous deletion.

d Immunoblot analysis confirming the absence of full-length MCM3 protein and expression of the MCM3 Δ CTE variant in the engineered HCT116 clone.

e-g Generation and validation of the MCM2-CTE^{-/-} cell line. **e** CRISPR/Cas9 targeting strategy. **f** Genomic PCR analysis. **g** Immunoblot validation.

h-j Generation and validation of the MCM4-CTE^{-/-} cell line. **h** CRISPR/Cas9 targeting strategy. **i** Genomic PCR analysis. **j** Immunoblot validation.



Supplementary Figure 9 | Structural analysis of MCM3-CTE conformations in hMCM-SH, hOCCM, and hMCM-DH.

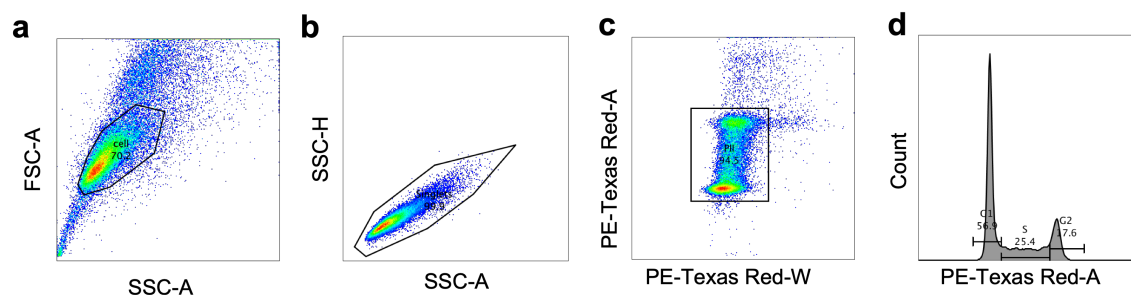
a MCM3-CTE (cyan) interaction with MCM2-CTD (orange) in hMCM-SH (this study).

b MCM5-CTD (yellow) binding to MCM2-CTD (orange) in hMCM-DH (PDB-7W1Y).

c, d Structural modelling of MCM3-CTE docking onto MCM2 surface in MCM-DH (c), highlighting a steric conflict between MCM3-CTE and MCM5-CTD.

e Interaction interface between MCM3-CTE (grey), ORC2 (magenta), and CDC6 (red) in the hOCCM structure (PDB-8S0E).

f-h Structural alignment of hMCM-SH and hOCCM via MCM3-CTE showing that MCM3-CTE (cyan) is capable of binding to both MCM2 (orange, from hMCM-SH) and ORC2-CDC6 complex (from OCCM). MCM3-CTE was not visualized to highlight MCM3-CTE binding pockets on both ORC-CDC6 (hOCCM) and MCM2 (hMCM-SH).



Supplementary Figure 10 | Gating strategy for cell cycle analysis in Figure 5m, illustrated with representative flow cytometry plots

a Cells were first selected from debris based on side scatter area (SSC-A) versus forward scatter area (FSC-A).

b Cell doublets were excluded by gating on single cells using SSC-A versus SSC-H.

c Cells with 2C-4C DNA content were selected based on PE-Texas Red-W versus PE-Texas Red-A.

d Cell cycle distribution was analyzed by plotting PE-Texas Red-A (DNA stained by propidium iodide) against cell count. Gates indicate the proportions of cells in G1, S, and G2/M phases.

Supplementary Table 1 | Cryo-EM data collection, refinement and validation statistics

	hMCM2-7 (EMDB-64408) (PDB 9UQ0)	hMCM2-7Δ3CTE (State I) (EMDB-65161) (PDB 9VLN)	hMCM2-7Δ3CTE (State II) (EMDB-65170) (PDB 9VLW)	hMCM2-7 (M3 Q761L) (EMDB-68728) (PDB 22VT)
Data collection and processing				
Magnification	81000	81000	81000	81000
Voltage (kV)	300	300	300	300
Electron exposure (e ⁻ /Å ²)	51	50	50	50
Defocus range (μm)	-1.3 to -2.5	-1.2 to -2.6	-1.2 to -2.6	-1.2 to -2.6
Pixel size (Å)	1.06	1.06	1.06	1.05
Symmetry imposed	C1	C1	C1	C1
Initial particle images (no.)	1354032	949477	949477	346185
Final particle images (no.)	331533	559257	110899	119836
Map resolution (Å)	3.5	3.4	4.1	3.3
FSC threshold	0.143	0.143	0.143	0.143
Map resolution range (Å)	2.3 to 7.1	2.3 to 6.6	2.3 to 8.2	2.3 to 5.6
Refinement				
Initial model used (PDB code)	7W1Y	7W1Y	7W1Y	7W1Y
Model resolution (Å)	3.47	3.42	4.06	3.33
FSC threshold	0.143	0.143	0.143	0.143
Model resolution range (Å)				
Map sharpening <i>B</i> factor (Å ²)	132.6	112.6	94.5	116.6
Model composition				
Non-hydrogen atoms	28580	28224	28224	27147
Protein residues	3756	3686	3686	3747
Ligands	12	11	11	12
<i>B</i> factors (Å ²)				

Protein	145.93	82.10	82.10	93.64
Ligand	111.72	66.54	66.54	70.89
R.m.s. deviations				
Bond lengths (Å)	0.004	0.002	0.004	0.004
Bond angles (°)	0.601	0.557	0.965	0.601
Validation				
MolProbity score	1.71	1.79	1.92	1.64
Clashscore	7.01	8.17	11.33	5.50
Poor rotamers (%)	0.07	0.00	0.00	0.08
Ramachandran plot				
Favored (%)	95.38	94.99	94.94	95.13
Allowed (%)	4.56	5.01	5.06	4.84
Disallowed (%)	0.06	0.00	0.00	0.03

Supplementary Table 2 | The effects of CTE-truncation in human cells

	MCM2-7 SH integrity	Chromatin association	DH formation (incorporation into chromatin-bound DH)
MCM2 Δ CTE	reduced	reduced	supported
MCM3 Δ CTE	reduced	reduced	supported
MCM4 Δ CTE	reduced	reduced	supported
MCM5 Δ CTE	reduced	unaffected	supported
MCM6 Δ CTE	unaffected	Severely reduced	not supported
MCM7 Δ CTE	unaffected	Abolished	not supported

Note: The summary is based on the results from HeLa S3 overexpression cell lines

Supplementary Table 3 | List of cell lines used in this study

Cell line	Genotype	Source
HeLa S3 MCM2-3FLAG (endogenous)	MCM2-S-tag-3FLAG	This study
HeLa S3 MCM2-3FLAG (ectopic expression)	MCM2-3FLAG	This study
HeLa S3 MCM2 Δ CTE-3FLAG (ectopic expression)	MCM2 Δ CTE-3FLAG	This study
HeLa S3 MCM3-3FLAG (ectopic expression)	MCM3-3FLAG	This study
HeLa S3 MCM3 Δ CTE-3FLAG (ectopic expression)	MCM3 Δ CTE-3FLAG	This study
HeLa S3 MCM4-3FLAG (ectopic expression)	MCM4-3FLAG	This study
HeLa S3 MCM4 Δ CTE-3FLAG (ectopic expression)	MCM4 Δ CTE-3FLAG	This study
HeLa S3 MCM5-3FLAG (ectopic expression)	MCM5-3FLAG	This study
HeLa S3 MCM5 Δ CTE-3FLAG (ectopic expression)	MCM5 Δ CTE-3FLAG	This study
HeLa S3 MCM6-3FLAG (ectopic expression)	MCM6-3FLAG	This study
HeLa S3 MCM6 Δ CTE-3FLAG (ectopic expression)	MCM6 Δ CTE-3FLAG	This study
HeLa S3 MCM7-3FLAG (ectopic expression)	MCM7-3FLAG	This study
HeLa S3 MCM7 Δ CTE-3FLAG (ectopic expression)	MCM7 Δ CTE-3FLAG	This study
HCT 116 mAID-MCM3	CMV-OsTIR1(F74G), mAID-MCM3	Saito and Kanemaki, 2021
HCT 116 mAID-MCM3 Vector	CMV-OsTIR1(F74G), mAID-MCM3, Vector	This study
HCT 116 mAID-MCM3 3FLAG-MCM3	CMV-OsTIR1(F74G), mAID-MCM3, 3FLAG-MCM3	This study
HCT 116 mAID-MCM3 3FLAG-MCM3(Δ CTE)	CMV-OsTIR1(F74G), mAID-MCM3, 3FLAG-MCM3(Δ CTE)	This study
HCT 116 MCM2 Δ CTE	MCM2CTE-/-	This study
HCT 116 MCM3 Δ CTE	MCM3CTE-/-	This study
HCT 116 MCM4 Δ CTE	MCM4CTE-/-	This study
HCT 116 MCM5-mAID-3HA	Tet on-OsTIR1(F74A), MCM5-mAID-3HA	Li <i>et al.</i> , 2023
HCT 116 MCM5-mAID-3HA Vector	Tet on-OsTIR1(F74A), MCM5-mAID-3HA, Vector	This study
HCT 116 MCM5-mAID-3HA MCM5-3FLAG	Tet on-OsTIR1(F74A), MCM5-mAID-3HA, MCM5-3FLAG	This study
HCT 116 MCM5-mAID-3HA MCM5(Δ CTE)-3FLAG	Tet on-OsTIR1(F74A), MCM5-mAID-3HA, MCM5(Δ CTE)-3FLAG	This study

Supplementary Table 4 | List of plasmids used for construction of cell lines by CRISPR/Cas9 mediated genome editing

Cell line	Plasmid	Description
HeLa S3 MCM2-3FLAG (endogenous)	pX330-sgMCM2(C)	Expressing guide RNA targeting MCM2 C-terminus (sequence: GAGGCCCTATGCCATCCATA)
	pUC57-MCM2-S-tag-3FLAG-Neo	MCM2-S-tag-3FLAG-Neo donor
	pUC57-MCM2-S-tag-3FLAG-Hygro	MCM2-S-tag-3FLAG-Hygro donor
HCT 116 MCM3ΔCTE	pX330-sgMCM3(ΔCTE)	Expressing guide RNA targeting proximity of genomic region encoding amino acid 670 of MCM3 (sequence: ACCTAAGACCAAGGCAAGTG)
	pX330-sgMCM3(C)	Expressing guide RNA targeting MCM3 C-terminus (sequence: ACAACCCAAGTTCAGAGACG)
	pUC57-MCM3(ΔCTE)-Neo	MCM3(ΔCTE)-Neo donor
	pUC57-MCM3(ΔCTE)-Puro	MCM3(ΔCTE)-Puro donor
HCT 116 MCM2ΔCTE	pX330-sgMCM2(ΔCTE)	Expressing guide RNA targeting proximity of genomic region encoding amino acid 825 of MCM2 (sequence: TGACCTGGGTGCTGGCACGT)
	pX330-sgMCM2(C)	Expressing guide RNA targeting MCM2 C-terminus (sequence: GAGGCCCTATGCCATCCATA)
	pUC57-MCM2(ΔCTE)-Neo	MCM2(ΔCTE)-Neo donor
	pUC57-MCM2(ΔCTE)-Puro	MCM2(ΔCTE)-Puro donor
HCT 116 MCM4ΔCTE	pX330-sgMCM4(ΔCTE)	Expressing guide RNA targeting proximity of genomic region encoding amino acid 775 of MCM4 (sequence: ATGTCCACGATGCCAGTCCG)
	pX330-sgMCM4(C)	Expressing guide RNA targeting MCM4 C-terminus (sequence: GGTGTGGTCTGCATCTCAGT)
	pUC57-MCM4(ΔCTE)-Neo	MCM4(ΔCTE)-Neo donor
	pUC57-MCM4(ΔCTE)-Puro	MCM4(ΔCTE)-Puro donor