

SUPPLEMENTARY INFORMATION for:

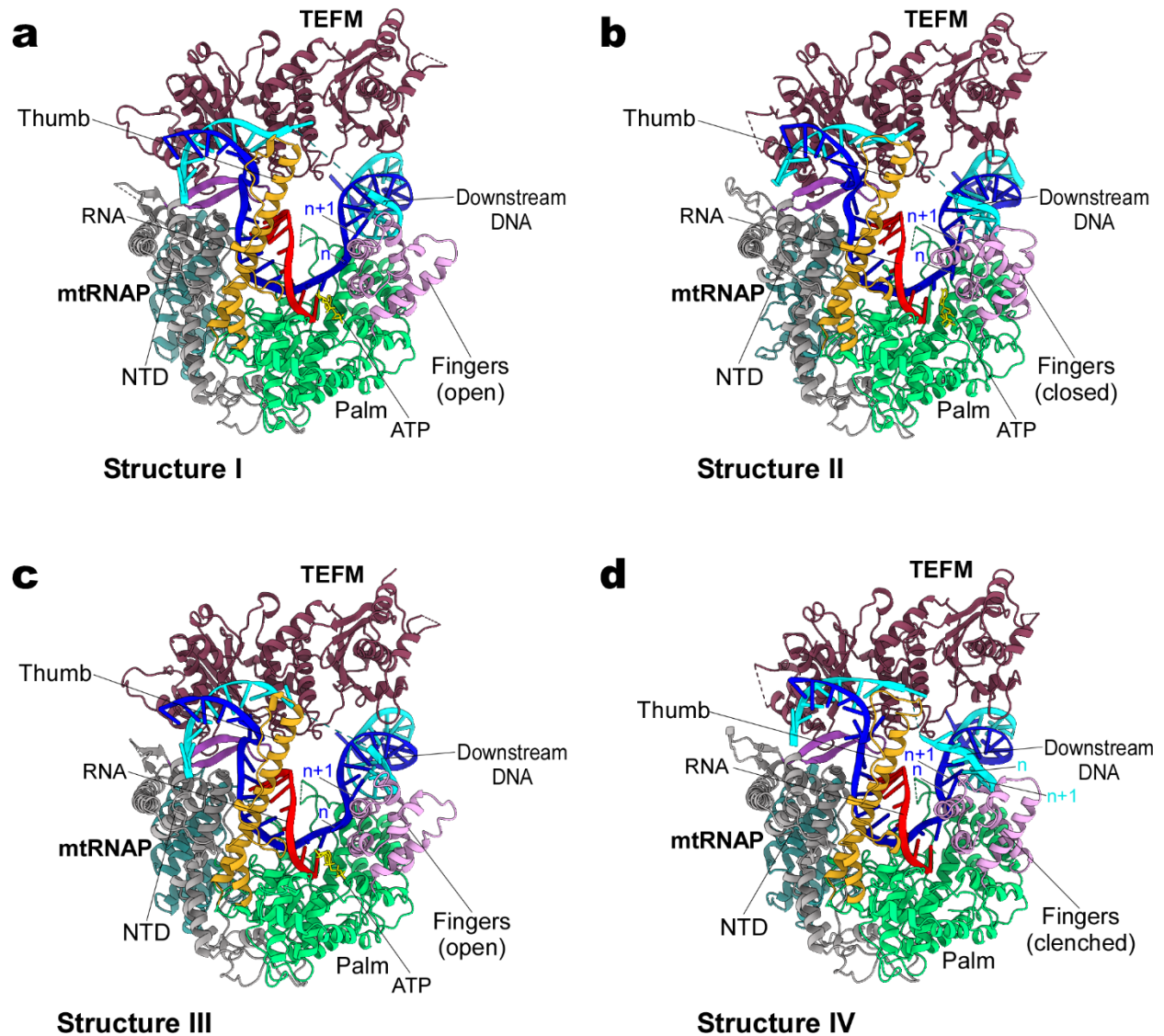
Structural Basis for Substrate Binding and Selection by Human Mitochondrial RNA Polymerase

Karl Herbine, Ashok R. Nayak, and Dmitry Temiakov

Department of Biochemistry and Molecular Biology,

Thomas Jefferson University; 1020 Locust St, Philadelphia 19107, USA

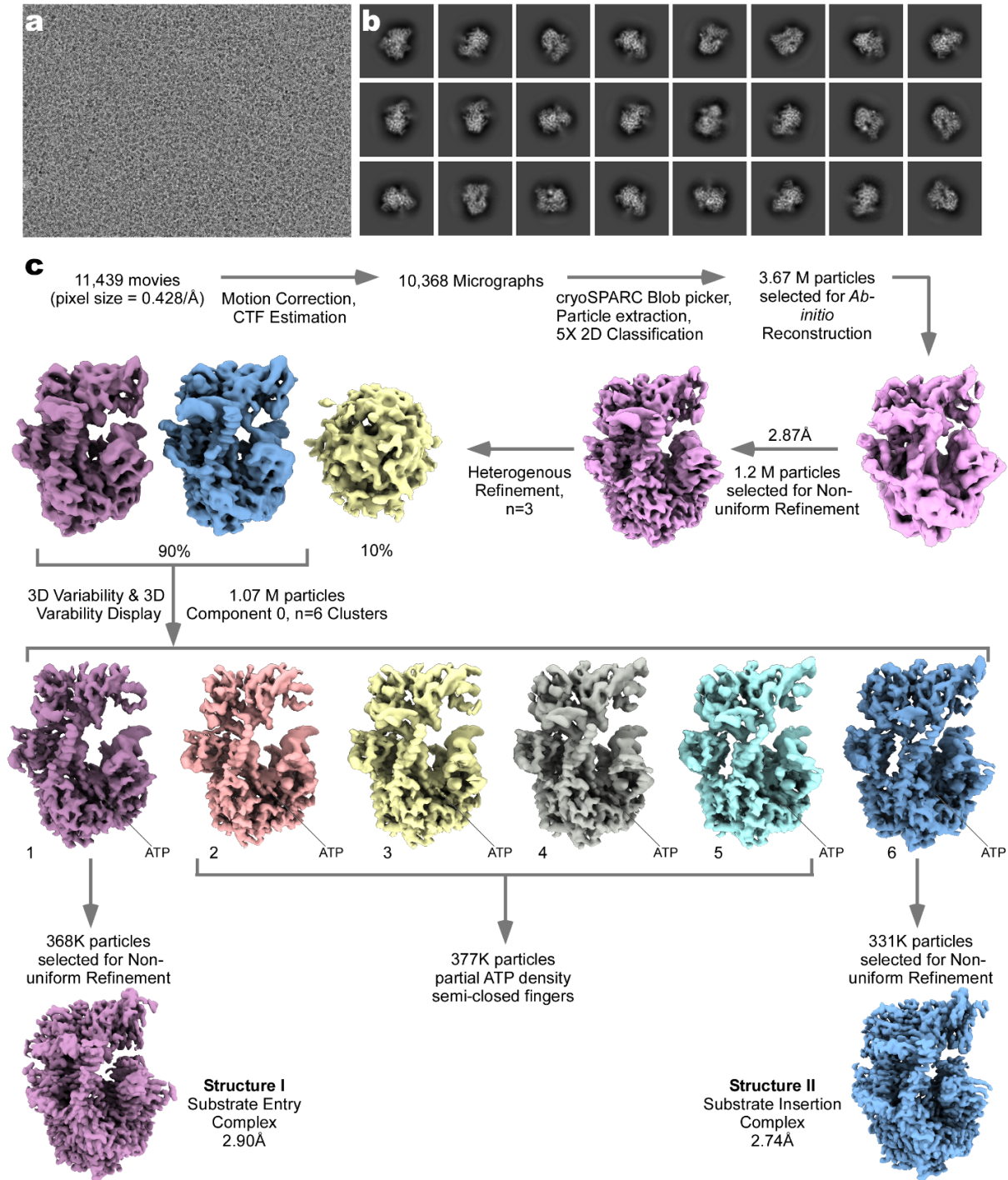
The file includes **SUPPLEMENTARY FIGURES 1-9** and **SUPPLEMENTARY TABLES 1,2**



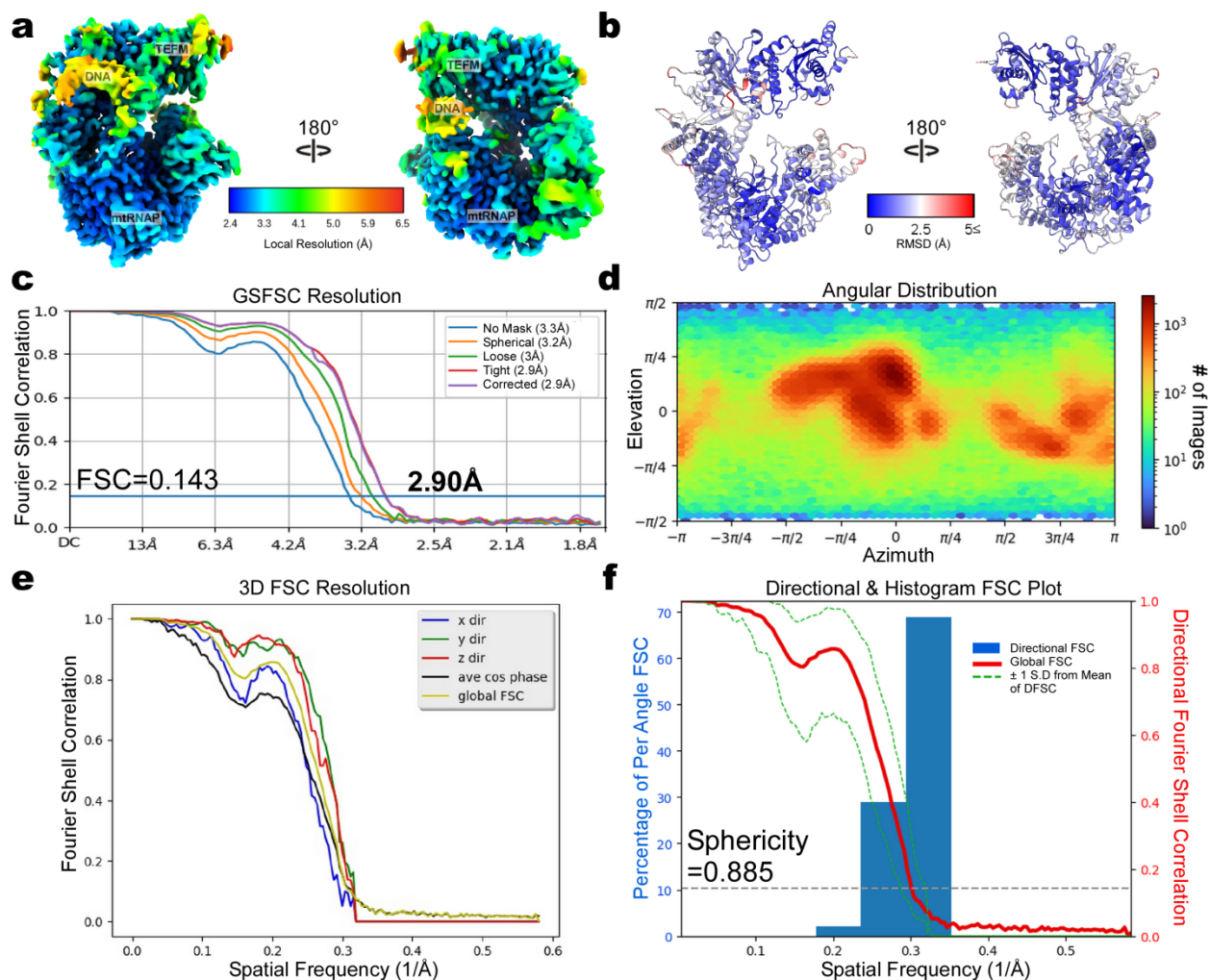
Supplementary Fig. 1 | Structure of Human mitochondrial transcription elongation complexes.

a,b, Cartoon ribbon representation of EC with the bound cognate substrate. Structures I (a) and II (b) are shown.

c,d, Cartoon ribbon representation of EC with the bound non-cognate substrate. Structure III (c) and Structure IV(d) are shown. Structures are aligned by their respective palm domains.

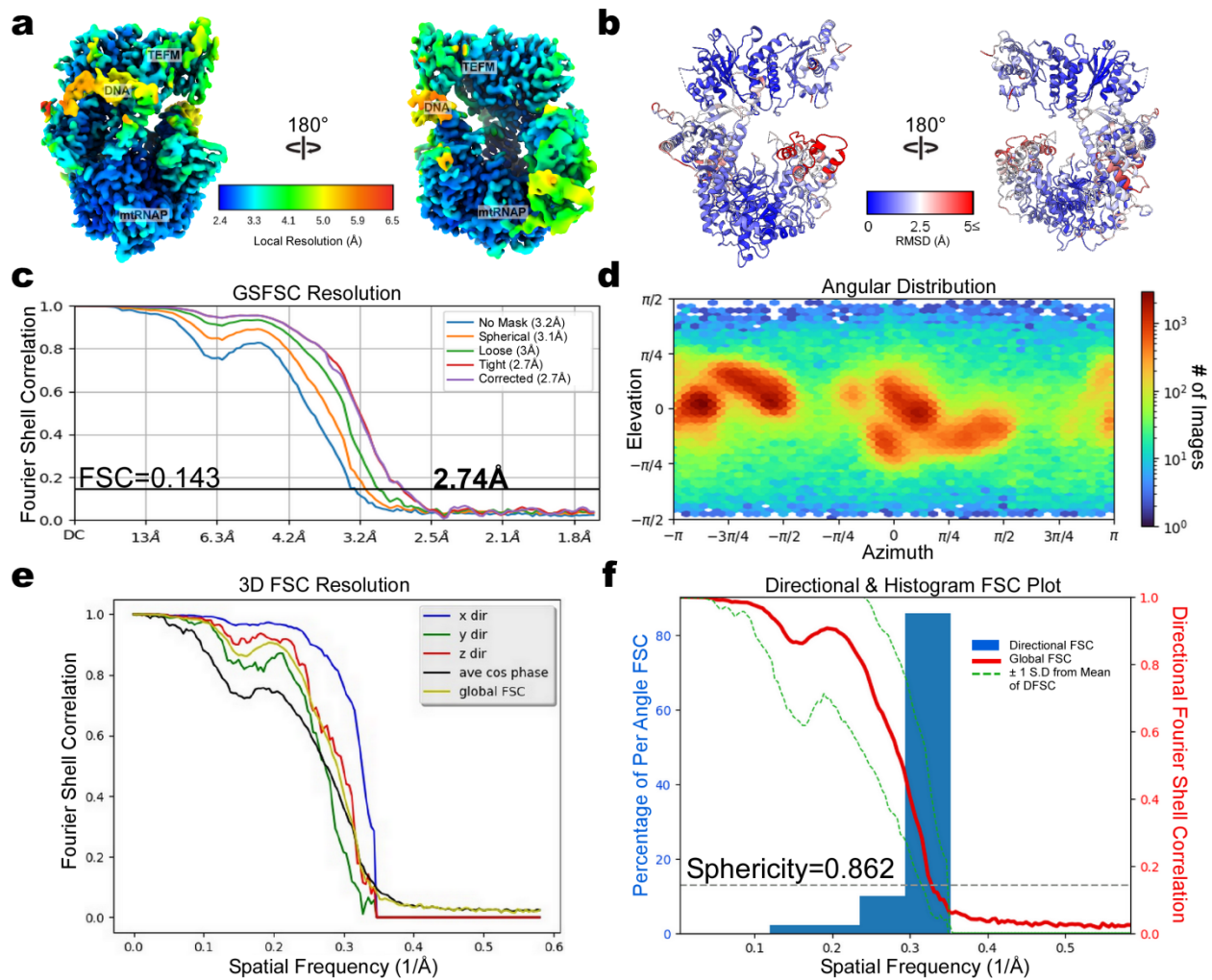


Supplementary Fig. 2 | Cryo-EM data processing workflow for human mitochondrial substrate-bound EC, complex ONE. **a**, Representative micrograph of EC-TEFM particles. **b**, Representative 2D class images of EC-TEFM particles processed in cryoSPARC. **c**, Flow chart of cryo-EM single particle analysis.



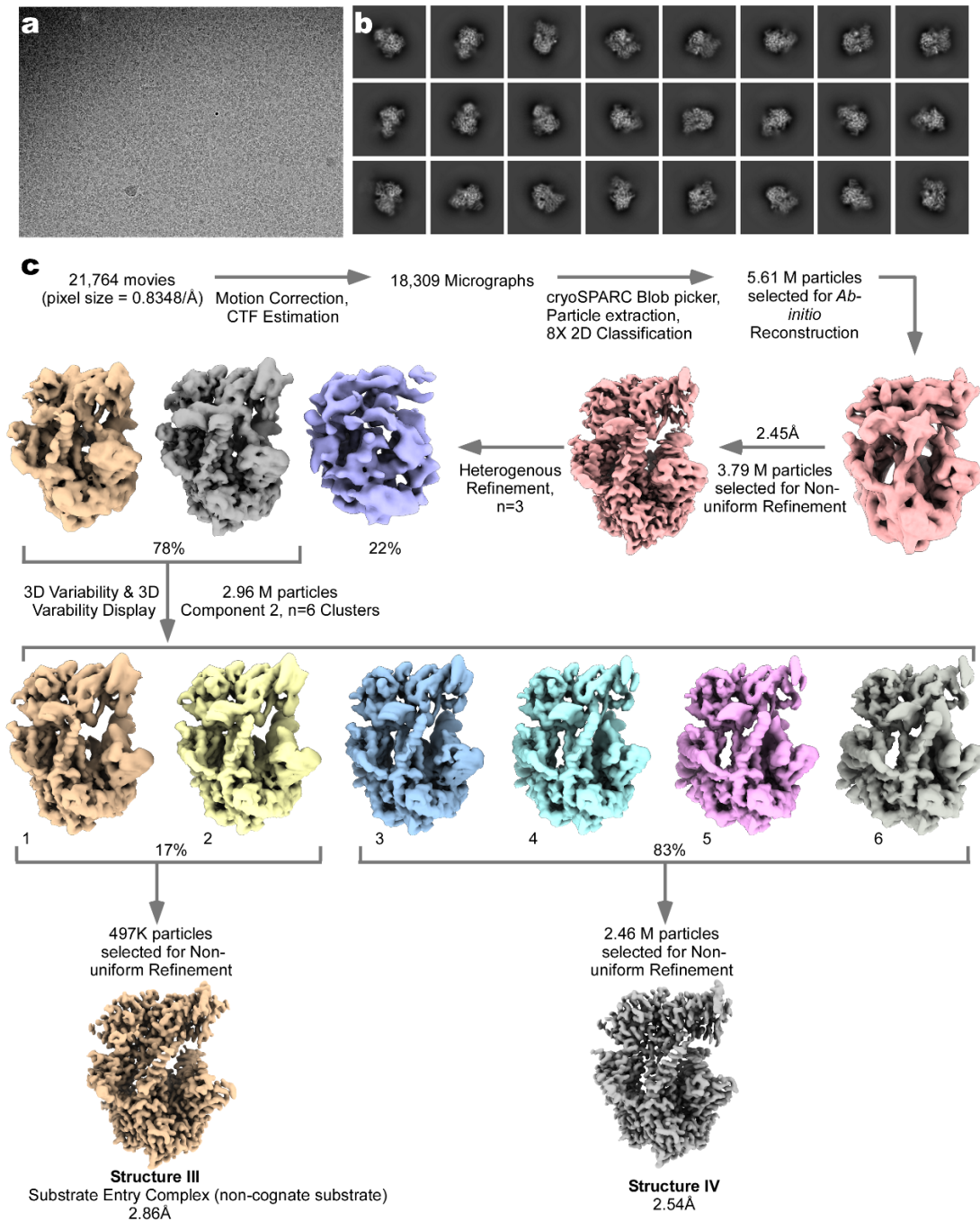
Supplementary Fig. 3 | Cryo-EM quality assessment of Structure I.

a, Local resolution estimation using cryoSPARC and surface colored in ChimeraX (step size 1, FSC threshold 0.143); **b**, Superposition of ES structure on model (5OLA.pdb) surface colored to represent C- α atoms Global RMSD. **c**, Gold Standard FSC curve from cryoSPARC (FSC threshold 0.143). **d**, Distribution of viewing angles and orientations for the EM density map. **e**, Directional FSC plots for the EM density map (FSC threshold 0.143). **f**, 3D FSC and sphericity assessment for the EM density map.



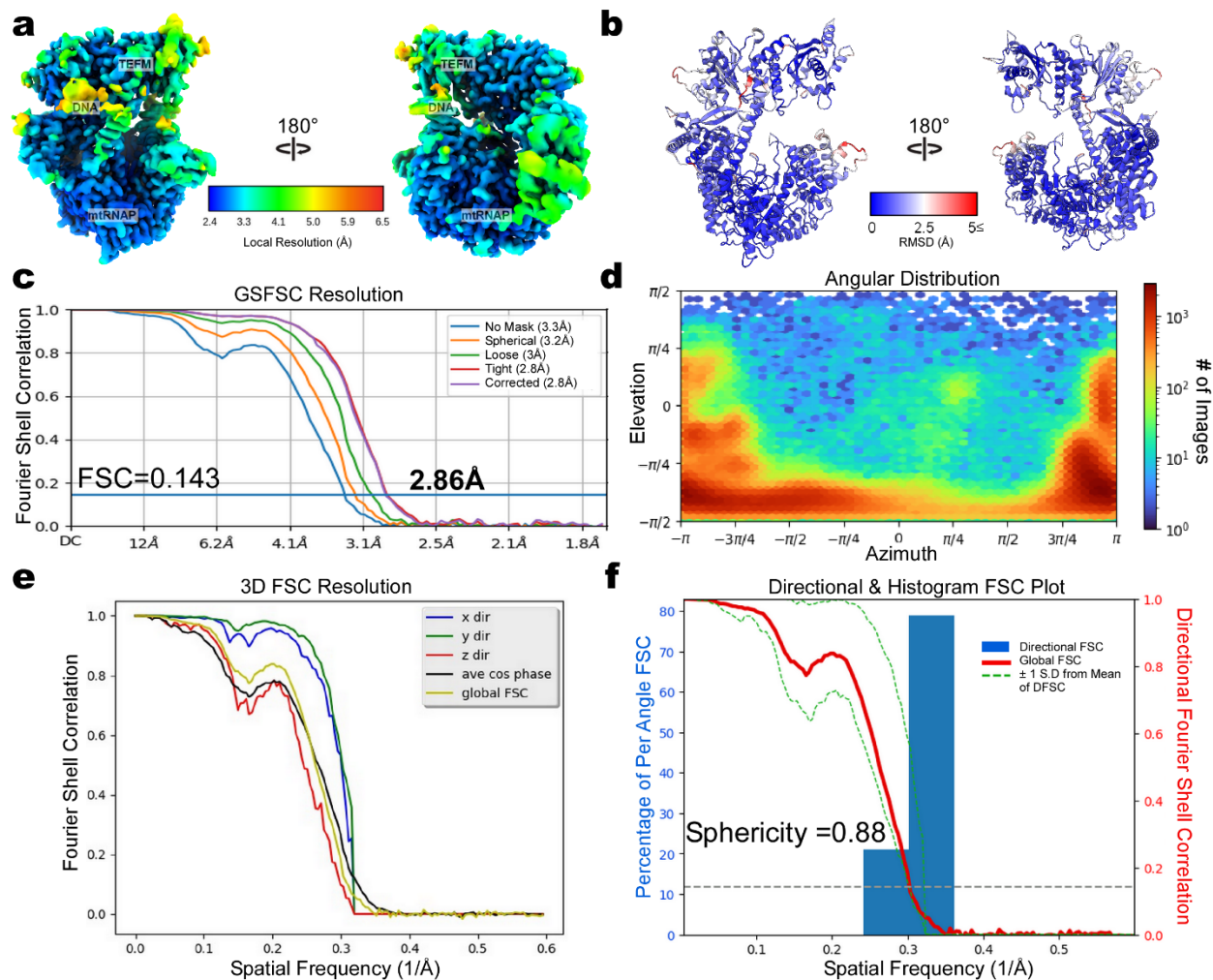
Supplementary Fig. 4 | Cryo-EM quality assessment of Structure II.

a, Local resolution estimation using cryoSPARC and surface colored in ChimeraX (step size 1, FSC threshold 0.143); **b**, Superposition of IS structure on model (5OLA.pdb) surface colored to represent C- α atoms Global RMSD. **c**, Gold Standard FSC curve from cryoSPARC (FSC threshold 0.143). **d**, Distribution of viewing angles and orientations for the EM density map. **e**, Directional FSC plots for the EM density map (FSC threshold 0.143). **f**, 3D FSC and sphericity assessment for the EM density map.



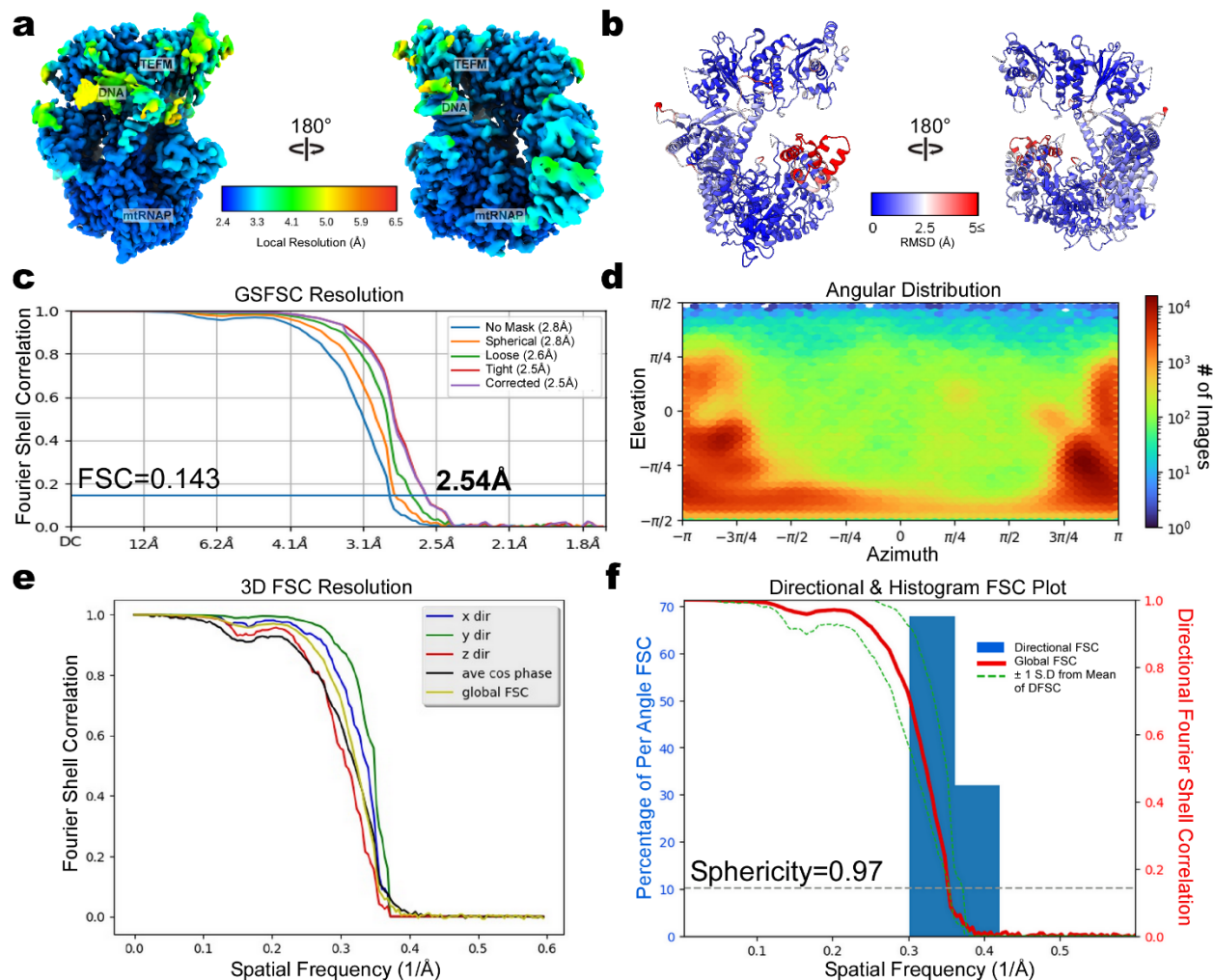
Supplementary Fig. 5 | Cryo-EM data processing workflow for human mitochondrial substrate-bound EC, Complex TWO.

a, Representative micrograph of EC-TEFM particles. **b**, Representative 2D class images of EC-TEFM particles processed in cryoSPARC. **c**, Flow chart of cryo-EM single particle analysis.



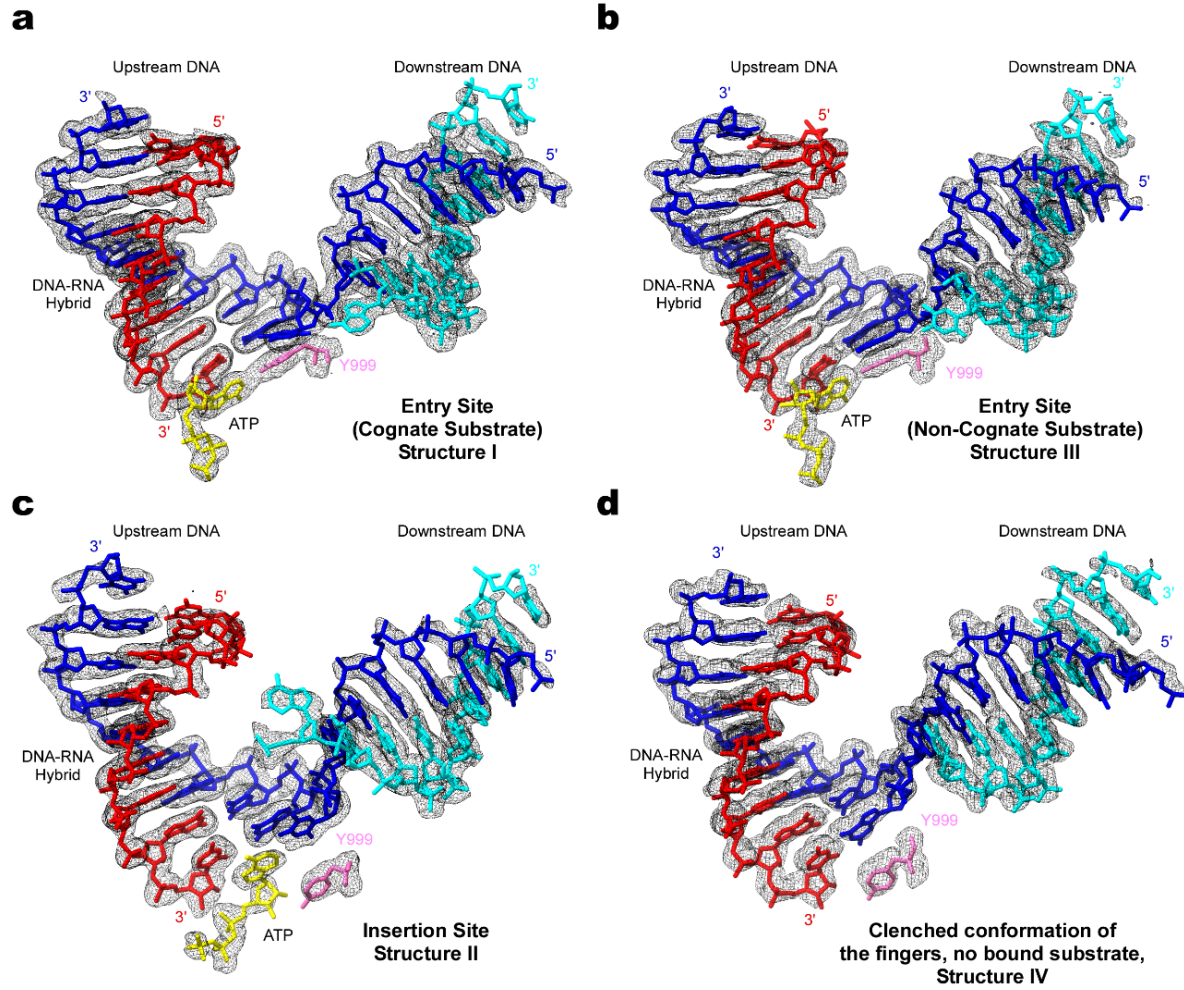
Supplementary Fig. 6 | Cryo-EM quality assessment of Structure III.

a, Local resolution estimation using cryoSPARC and surface colored in ChimeraX (step size 1, FSC threshold 0.143); **b**, Superposition of ES structure on model (5OLA.pdb) surface colored to represent C-α atoms Global RMSD. **c**, Gold Standard FSC curve from cryoSPARC (FSC threshold 0.143). **d**, Distribution of viewing angles and orientations for the EM density map. **e**, Directional FSC plots for the EM density map (FSC threshold 0.143). **f**, 3D FSC and sphericity assessment for the EM density map.



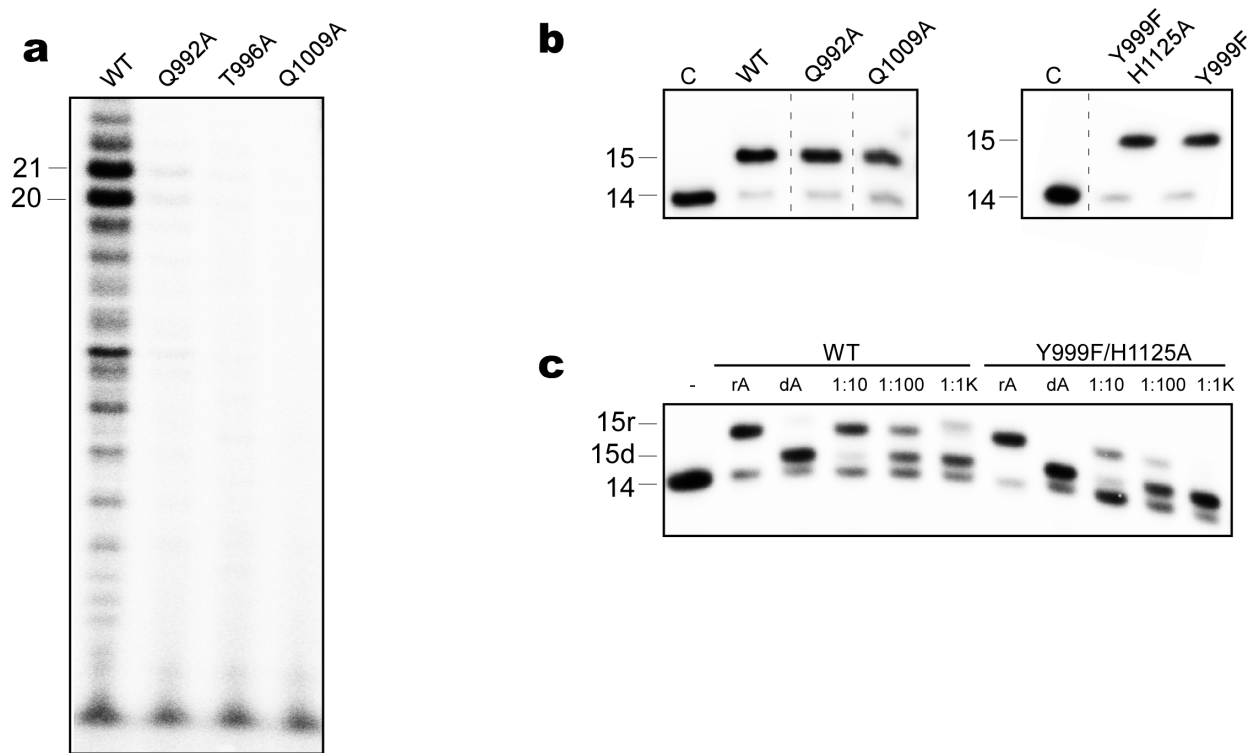
Supplementary Fig. 7 | Cryo-EM quality assessment of Structure IV.

a, Local resolution estimation using cryoSPARC and surface colored in ChimeraX (step size 1, FSC threshold 0.143); **b**, Superposition of ES structure on model (5OLA.pdb) surface colored to represent C- α atoms Global RMSD. **c**, Gold Standard FSC curve from cryoSPARC (FSC threshold 0.143). **d**, Distribution of viewing angles and orientations for the EM density map. **e**, Directional FSC plots for the EM density map (FSC threshold 0.143). **f**, 3D FSC and sphericity assessment for the EM density map.



Supplementary Fig. 8 | Cryo-EM density map of nucleic acid scaffold in Structures I-IV.

a, b, c, d. Cryo-EM densities of the RNA, DNA, ATP, and Y999 residue in ECs represented by Structures I-IV.



Supplementary Fig 9 | Properties of mtRNAP variants.

a, Activity of mtRNAP variants in transcription initiation assay. **b**, Catalytic activity of mtRNAP mutants. **c**, rNTP/dNTP competition assay. The RNA primer (14 nt) was extended in the present of rNTP/dNTP mixtures, as indicated. The extended products differ in their mobility due to rAMP (15r) or dAMP (15d) incorporation.

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

Data Collection	Structure I: Entry Site (cognate NTP) (EMDB-42027; PDB 8U8U)	Structure II: Insertion Site (EMDB-42028; PDB 8U8V)
Electron Microscope	Titan Krios	
Voltage (kV)	300	
Detector	Gatan K3-Summit	
Camera mode	Super-resolution	
Magnification	105,000x	
Total electron dose (e ⁻ Å ⁻²)	60	
Defocus range (- μm)	0.5-2.25	
Pixel Size (Å)	0.428	
Grid type	300 mesh Holey gold Ultra-AuFoil 1.2/1.3	
Software	Latitude S	
Dose rate (e ⁻ s ⁻¹ / pixel)	12.93	
Frames (no.)	40	
Micrographs collected (no.)	11,439	
Micrographs used (no.)	10,368	
Image Processing		
SPA Software	CryoSPARC 3.2+	
Symmetry imposed	C1	
Initial particles picked (no.)	4,798,227	
Particles picked after 2D Classification (no.)	3,685,736	
Particles picked after initial 3D model (no.)	1,196,880	
Particles picked after het. refinement (no.)	1,077,192	
Particles picked after 3DVA (no.)	700,175	
Final particles picked (no.)	368,317	331,858
Map Resolution (Å)	2.90	2.74
FSC Threshold	0.143	0.143
Refinement		
Initial model used (PDB)	5OLA	5OLA
Map sharpening B factor (Å ²)	45.0	45.0

Model composition		
Nonhydrogen atoms	12344	11968
Protein residues	1398	1392
Nucleotides	61	61
Water	0	0
Ligands	APC:1	APC:1, MG:1
B factors ($\text{\AA}^2$) (min/max/mean)		
Protein	30.00/279.56/155.42	30.00/286.49/152.44
Nucleotide	20.00/319.98/212.12	20.00/319.98/206.70
Ligand	20.00/20.00/20.00	13.56/67.51/28.61
R.m.s. deviations		
Bond lengths ($\text{\AA}$) (# > 4σ)	0.003 (0)	0.004 (0)
Bond angles ($^\circ$) (# > 4σ)	0.611 (1)	0.709 (0)
Validation		
MolProbity score	1.09	1.44
Clash Score	3.03	3.94
Poor rotamers (%)	0.00	0.00
Ramachandran Plot		
Preferred (%)	98.27	96.16
Allowed (%)	1.73	3.84
Outliers (%)	0.00	0.00

Supplementary Table 2 | Cryo-EM data collection, refinement, and validation statistics

Data Collection	Structure III: Entry Site (non-cognate NTP) (EMDB-44449; PDB:9BDD)	Structure IV: Closed Complex (EMDB-44448; PDB 9BDC)
Electron Microscope	Titan Krios	
Voltage (kV)	300	
Detector	Gatan K3-Summit	
Camera mode	Counting	
Magnification	105,000x	
Total electron dose (e- Å ⁻²)	50.6	
Defocus range (- μm)	0.75-2.00	
Pixel Size (Å)	0.835	
Grid type	300 mesh Holey gold Ultra-AuFoil 1.2/1.3	
Software	Latitude S	
Dose rate (e- s ⁻¹ / pixel)	18.7	
Frames (no.)	40	
Micrographs collected (no.)	21,764	
Micrographs used (no.)	18,309	
Image Processing		
SPA Software	CryoSPARC 3.2+	
Symmetry imposed	C1	
Initial particles picked (no.)	7,998,459	
Particles picked after 2D Classification (no.)	5,612,490	
Particles picked after initial 3D model (no.)	3,789,265	
Particles picked after het. refinement (no.)	2,955,627	
Particles picked after 3DVA (no.)	2,955,627	
Final particles picked (no.)	497,782	2,457,845
Map Resolution (Å)	2.86	2.54
FSC Threshold	0.143	0.143
Refinement		
Initial model used (PDB)	5OLA	5OLA
Map sharpening B factor (Å ²)	45.0	45.0
Model composition		

Nonhydrogen atoms	12317	12026
Protein residues	1397	1392
Nucleotides	61	61
Water	0	0
Ligands	APC:1	0
B factors ($\text{\AA}^2$) (min/max/mean)		
Protein	30.00/279.56/155.73	30.00/286.49/151.41
Nucleotide	20.00/319.98/211.33	20.00/319.42/142.41
Ligand	20.00/20.00/20.00	--/--/--
R.m.s. deviations		
Bond lengths ($\text{\AA}$) (# > 4σ)	0.003 (0)	0.003 (0)
Bond angles ($^\circ$) (# > 4σ)	0.647 (2)	0.582 (0)
Validation		
MolProbity score	1.25	1.36
Clash Score	4.70	4.30
Poor rotamers (%)	0.00	0.00
Ramachandran Plot		
Preferred (%)	97.98	97.17
Allowed (%)	2.02	2.83
Outliers (%)	0.00	0.00