Supplementary information

Principles of mitoribosomal small subunit assembly in eukaryotes

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Principles of mitoribosomal small subunit assembly in eukaryotes

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TABLE OF CONTENTS

a METTL17 Genomic verification

Clone #: 1 2 3 4 5 6 7
Primer pair #: 1 2 1 2 1 2 1 2 1 2 1 2 1 2

kb
4 3
2
1.5
1
.5

kb
4 3
2
1.5
1
.5

Primer pair #: 1 2 1 2 1 2 1 2 1 2 1 2 1 2
Clone #: 8 9 10 11 12 13 14

b METTL17-GFP OXPHOS subunit expression western blot

Ladder WT METTL17-GFP

kDa
250
150
100
75
50
37
25
20
15
10

MT-CO1

Ladder WT METTL17-GFP

kDa
250
150
100
75
50
37
25
20
15
10

β-actin

Ladder WT METTL17-GFP

kDa
250
150
100
75
50
37
25
20
15
10

- Leftover β-actin bands

MT-ND1

c METTL17 Pulldown

Ladder 1 2 3 4 5 6 7 8 9 10 Ladder

kDa
250
150
100
75
50
37
25
20
15
10

kDa
250
150
100
75
50
37
25
20
15
10

1 - Insoluble mitochondrial extract
2 - Soluble mitochondrial extract
3 - anti-GFP beads flow through
4 - Wash 1
5 - Wash 2
6 - Wash 3
7 - 3C elution (fraction 1)
8 - 3C elution (fraction 2)
9 - 3C elution (fraction 3)
10 - anti-GFP beads (after elution)

d Rsm22 Pulldown

Ladder 1 2 3 4 5 6 Ladder

kDa
250
150
100
75
50
37
25
20
15
10

kDa
250
150
100
75
50
37
25
20
15
10

1 - anti-mCherry beads
2 - 3C elution
3 - anti-mCherry beads (after elution)
4 - anti-ALFA beads flow through
5 - TEV elution
6 - anti-ALFA beads (after elution)

e Mtg3 Pulldown   Ccm1 Pulldown

kDa Ladder 1 2 3 4   Ladder 5 6 7 8 Ladder kDa
250   250
150   150
100   100
75   75
50   50
37   37
25   25
20   20
15   15
10   10

1 - anti-GFP beads
2 - 3C elution (fraction 1)
3 - 3C elution (fraction 2)
4 - anti-GFP beads (after elution)
5 - anti-mCherry beads
6 - TEV elution (fraction 1)
7 - TEV elution (fraction 2)
8 - anti-mCherry beads (after elution)

f PET127 KO IP

kDa Ladder 1 2 3 4 5 6 Ladder   Ladder kDa
250   250
150   150
100   100
75   75
50   50
37   37
25   25
20   20
15   15
10   10

WT   PET127 KO

1 - TEV elution (replicate 1)
2 - TEV elution (replicate 2)
3 - TEV elution (replicate 3)
4 - TEV elution (replicate 1)
5 - TEV elution (replicate 2)
6 - TEV elution (replicate 3)

g PET127 KO Methylene Blue

Ladder 1 2

kb
9
6
5
4
3
2.5
2
1.5
1
0.5

1 - WT
2 - PET127 KO

h PET127 KO 5′ precursor probe

Ladder 1 2

kb
9
6
5
4
3
2.5
2
1.5
1
0.5

1 - WT
2 - PET127 KO

i PET127 KO 15S central probe

Ladder 1 2

kb
9
6
5
4
3
2.5
2
1.5
1
0.5

1 - WT
2 - PET127 KO

Supplementary Fig. 1. Uncropped gel images.

Uncropped gel images relating to: (**a**) Supplementary Fig. 2c, (**b**) Supplementary Fig. 2f, (**c**) Supplementary Fig. 2e, (**d**) Supplementary Fig. 20f, (**e**) Supplementary Fig. 20b,j, (**f**) Supplementary Fig. 27c, (**g-i**) Supplementary Fig. 27b. Blue dotted rectangles show approximate cropping dimensions used in related figures.

a

HEK293-F cells

Co-transfection

| LHA | METTL17 | tags | 3C | GFP | T2A | BtuF | RHA |
| LHA | METTL17 | tags | 3C | GFP | T2A | p24 | RHA |

Cas9   sgRNA

-/-   +/-   -/+   +/+   +/+   +/+

Labeled epitope-directed nanobodies

GFP+

BtuF-nanobody - Alexa647

Double-labeled Cells

Unlabeled or single-labeled cells

p24-nanobody - ApcCy7

+/-   -/+   +/+

Epitope 1 - BtuF
Epitope 2 - p24

PCR Verification

b

SSC Singlets

DAPI 405D-450-A

10.7%

GFP+

488C-515-A

GFP

GFP+

BtuF-nanobody - Alexa647 640C-670-A

0.6%

P2

640A-780-A

p24-nanobody - ApcCy7

c

Reaction 1

fw   rev

| Upstream DNA | LHA | METTL17 | tags | 3C | GFP | T2A | BtuF | RHA | 2.35 kb

METTL17 allele 1

Reaction 2

fw   rev

| Upstream DNA | LHA | METTL17 | tags | 3C | GFP | T2A | p24 | RHA | 2.25 kb

METTL17 allele 2

| Clone # | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Reaction | 1 2 | 1 2 | 1 2 | 1 2 | 1 2 | 1 2 | 1 2 |

kb
4
3
2
1.5
1
.5

e

kDa
250
150
100
75
50
37
25
20
15
10

NOA1
mS39
METTL17
ERAL1   MCAT
TFB1M
RBFA
mitoribosomal proteins
3C protease

f

kDa   WT   METTL17-GFP
37 -

MT-CO1

25 -

MT-ND1

β-actin

d

HEK293-F cells
METTL17-3C-GFP +/+

| METTL17 | tags | 3C | GFP |

Mitochondrial Isolation

anti-GFP nanobody coupled resin

3C protease

Purified mitoribosomal intermediates

Supplementary Fig. 2. Biallelic editing of human cell lines and human complex purification.

(**a**) Simplified scheme of genomic editing using SNEAK-PEEC targeting METTL17. See methods for details. (**b**) FACS sorting on cells transfected with repair templates and *Sp*Cas9 targeting METTL17. Inset percentages denote proportion of cells positive for GFP (upper panel), and proportion of GFP positive cells with signal for both cell surface display epitopes (bottom panel). Cells from P2 were sorted to generate single cell clonal populations. (**c**) PCR verification of single cell clones from FACS sorting. Primer annealing locations are shown in genomic context in the upper panel. Samples were run on a 1% Agarose-TAE gel with EtBr. Highlighted clone was used for subsequent experiments. (**d**) Purification scheme for METTL17-containing mitoribosomal intermediates. (**e**) Representative SDS-PAGE gel of eluted sample. Proteins were identified with LC-MS/MS (Supplementary Data S1). Immunoprecipitation experiments were repeated at least 3 times with similar results. (**f**) Western blot showing unchanged expression of mitochondrially encoded OXPHOS subunits MT-CO1 and MT-ND1 in METTL17-GFP tagged cell line versus WT cells. The western blot was performed 3 times with similar results. For gel source data, see Supplementary Fig. 1.

a

40 nm

b

430 Å

c

Test Subset
2,597 micrographs

Dataset 1
37,047 micrographs

Dataset 2
9,990 micrographs

MotionCorr2
GCTF1.18
crYOLO Particle Picking

MotionCorr2
GCTF1.18
crYOLO Particle Picking

MotionCorr2
GCTF1.18
crYOLO Particle Picking

8.64 Å/px 533,056 particles

7,597,700 particles

1,894,925 particles 1,894,925 particles 1,894,925 particles 1,894,925 particles 1,511,635 particles 8.64 Å/px

2D Classification
K=100 T=2

4.32 Å/px 65,174 particles

333,265 particles 1,376,127 particles 1,345,443 particles 1,361,064 particles 1,391,376 particles 971,563 particles 2.16 Å/px

Ab initio
Initial Model

7% 2%
9% 19%
61%

22% 20%
16% 18%
24%

24% 19%
22% 20%
15%

18% 27%
22% 19%
17%

19% 17%
24% 19%
19%

19% 16%
24% 17%
22%

3D Classification
w/ alignment
K=5 T=4
45 iterations

268,009 particles

587,994 particles 475,299 particles 441,304 particles 540,944 particles 384,642 particles

3D Refinement

Reference
4.36 Å

Merge & remove
duplicates
1,927,789 particles

Merge & remove
duplicates

Non-selected particles

4,015,406 particles 4.32 Å/px

1.08 Å/px 2,575,411 particles

Bayesian Polishing
CTF Refinement
3D Refinement

20% 15% 17% 33%
25% 21% 14% 16%
17% 19%

3D Classification
w/ alignment
K=5 T=4
45 iterations

2.45 Å

785,557 particles 397,807 particles

1,183,364 particles

Consensus

3D Classification w/o alignment
K=10 T=30
Body mask
35 iterations

10%
263,184 particles

0.7%

0.9%

19%
497,031 particles

0.5%

2.7%

10%
262,363 particles

28%

0.4%

27%
697,003 particles

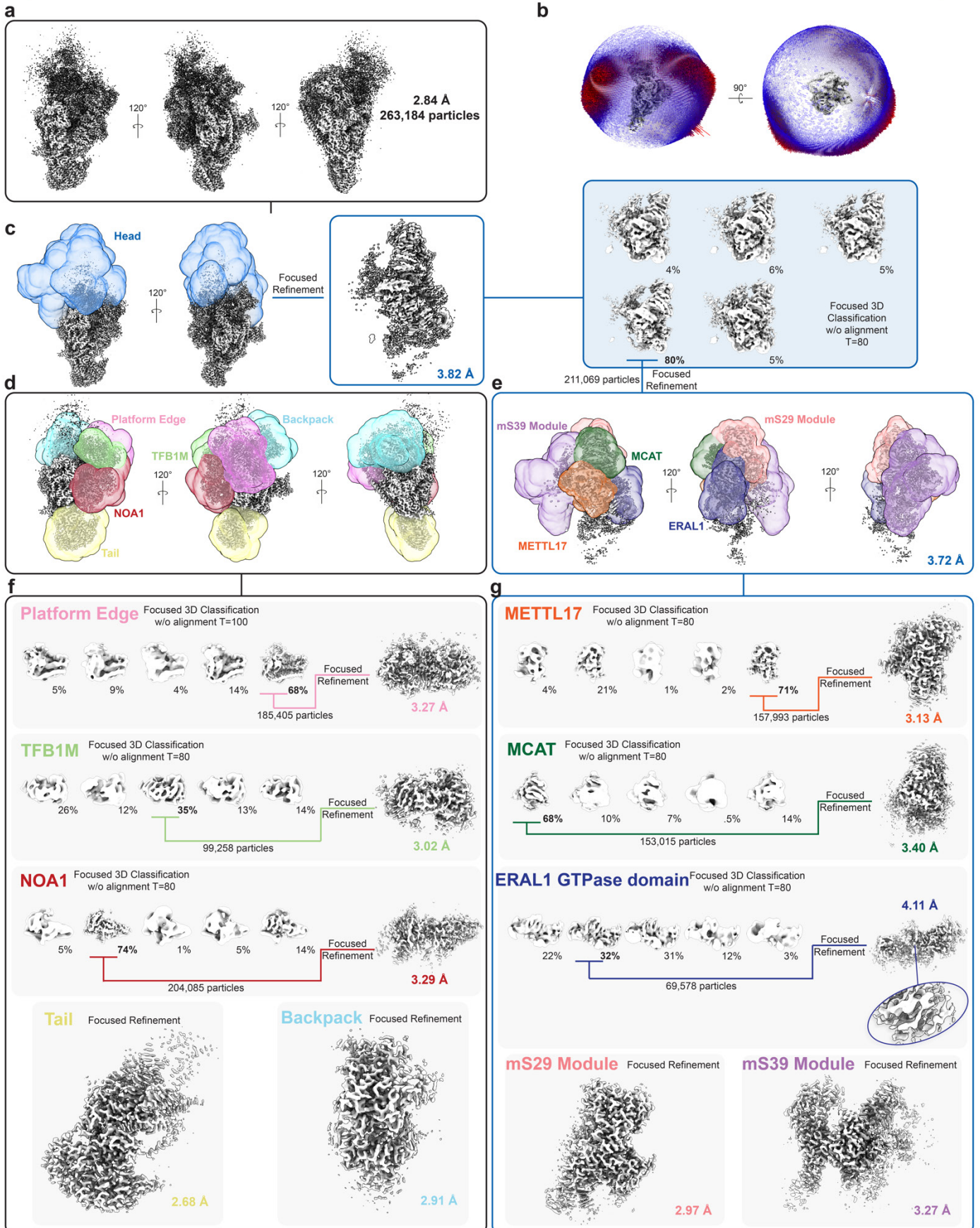State A

State B

State C

State D

Supplementary Fig. 3. Human METTL17 dataset cryo-EM data collection and analysis.

(**a**) Motion corrected cryo-EM micrograph representative of 47,037 total micrographs. (**b**) 15 most populated 2D class averages (8x binned, 8.64 Å/px). (**c**) Initial data processing workflow and identification of 4 discrete assembly states.

State A

a
120°   120°

2.84 Å
263,184 particles

b
90°

Focused 3D Classification w/o alignment T=80

4%   6%   5%

80%   5%

211,069 particles

Focused Refinement

c
Head

120°

Focused Refinement

3.82 Å

d
Platform Edge   Backpack

TFB1M

NOA1

Tail

120°   120°

e
mS39 Module   mS29 Module

MCAT

METTL17   ERAL1

120°   120°

3.72 Å

f
Platform Edge Focused 3D Classification w/o alignment T=100

5%   9%   4%   14%   **68%**

Focused Refinement

185,405 particles   3.27 Å

TFB1M Focused 3D Classification w/o alignment T=80

26%   12%   **35%**   13%   14%

Focused Refinement

99,258 particles   3.02 Å

NOA1 Focused 3D Classification w/o alignment T=80

5%   **74%**   1%   5%   14%

Focused Refinement

204,085 particles   3.29 Å

Tail Focused Refinement

2.68 Å

Backpack Focused Refinement

2.91 Å

g
METTL17 Focused 3D Classification w/o alignment T=80

4%   21%   1%   2%   **71%**

Focused Refinement

157,993 particles   3.13 Å

MCAT Focused 3D Classification w/o alignment T=80

68%   10%   7%   .5%   14%

Focused Refinement

153,015 particles   3.40 Å

ERAL1 GTPase domain Focused 3D Classification w/o alignment T=80

4.11 Å

22%   **32%**   31%   12%   3%

Focused Refinement

69,578 particles

mS29 Module Focused Refinement

2.97 Å

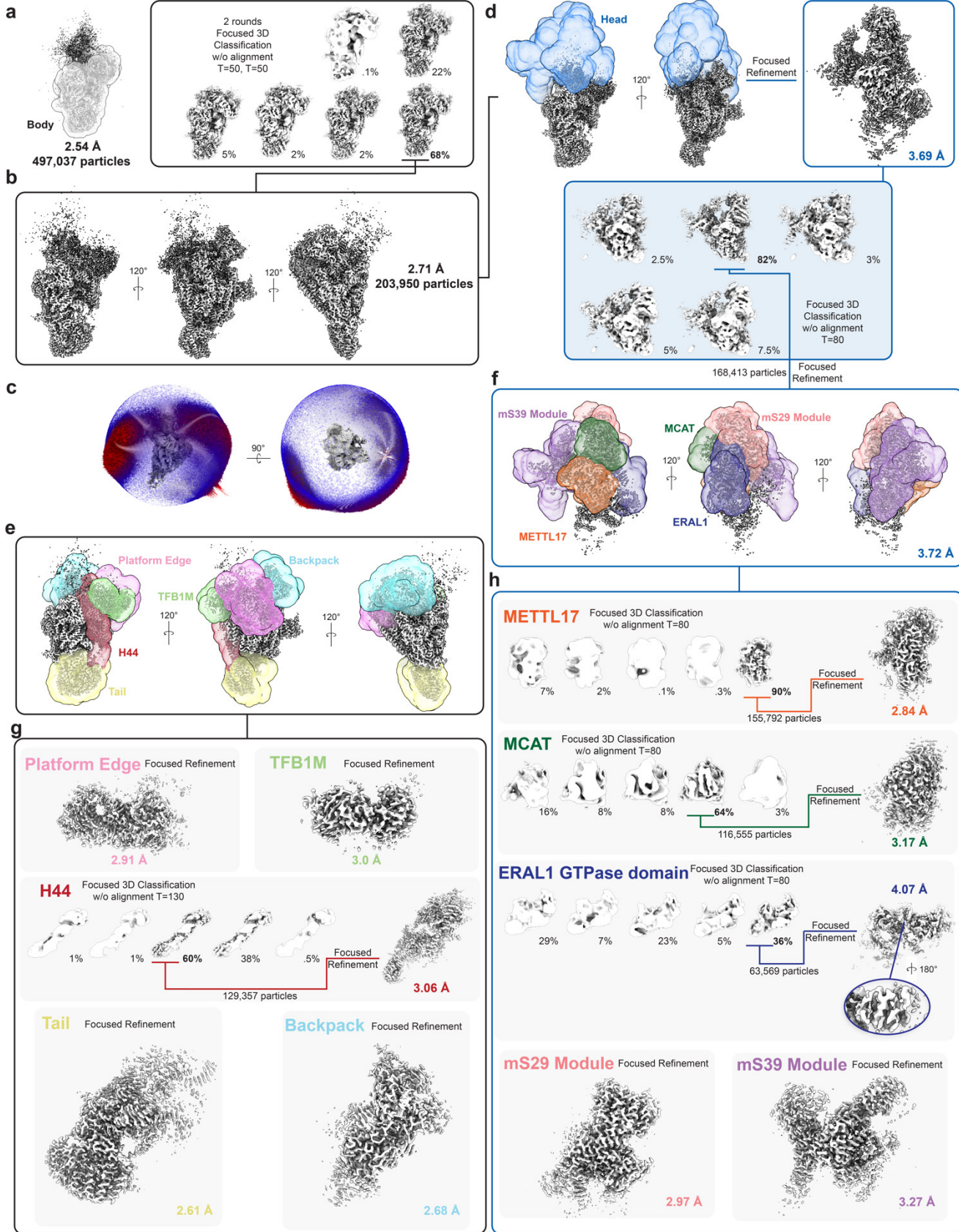**mS39 Module** Focused Refinement

3.27 Å

Supplementary Fig. 4. Cryo-EM processing of human State A.

(**a**) Different views of the State A overall map. (**b**) Euler angle distribution of the overall refinement. (**c**) Focused refinement and classification of the head domain. (**d**) Masks used in focused classification and refinement for modules in the body domain. (**e**) Masks used in focused classification and refinement for modules in the head domain. (**f,g**) Workflows for focused 3D classification and refinements. Reported resolutions (FSC=0.143) from Relion post-processing are reported next to individual focused maps. Masks used are color coded as in panels d and e.
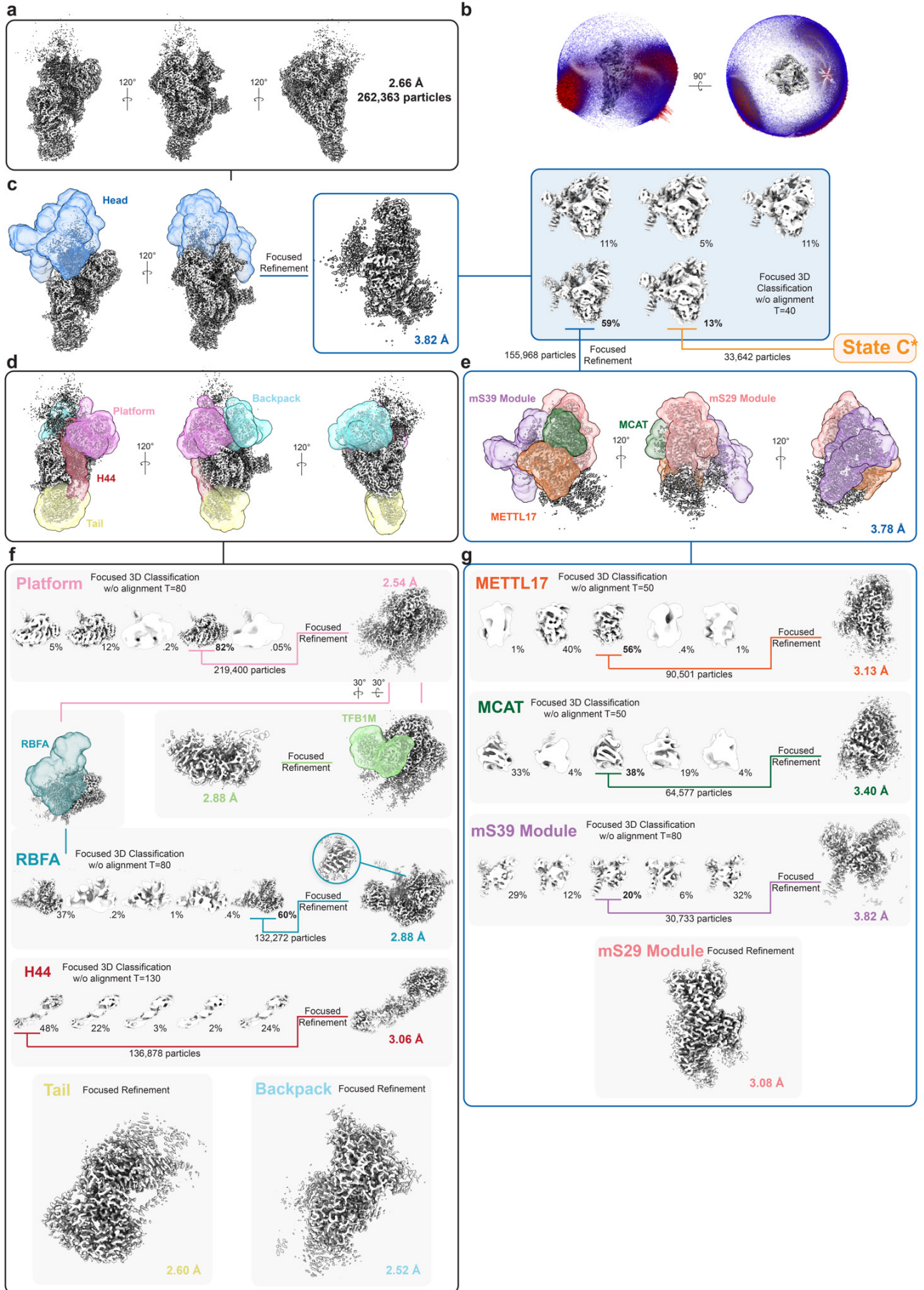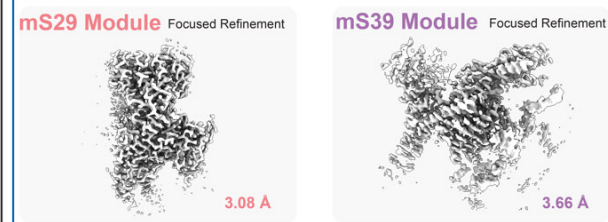
State B

a

Body
2.54 Å
497,037 particles

2 rounds
Focused 3D
Classification
w/o alignment
T=50, T=50

.1% 22%

5% 2% 2% **68%**

b

120° 120°

2.71 Å
203,950 particles

c

90°

d

Head

120°

Focused
Refinement

3.69 Å

2.5% **82%** 3%

5% 7.5%

Focused 3D
Classification
w/o alignment
T=80

168,413 particles Focused
Refinement

e

Platform Edge Backpack

TFB1M

H44

Tail

120° 120°

f

mS39 Module MCAT mS29 Module

120° 120°

METTL17 ERAL1

3.72 Å

g

Platform Edge Focused Refinement

2.91 Å

TFB1M Focused Refinement

3.0 Å

H44 Focused 3D Classification
w/o alignment T=130

1% 1% **60%** 38% .5% Focused
Refinement

129,357 particles 3.06 Å

Tail Focused Refinement

2.61 Å

Backpack Focused Refinement

2.68 Å

h

METTL17 Focused 3D Classification
w/o alignment T=80

7% 2% .1% .3% **90%** Focused
Refinement

155,792 particles 2.84 Å

MCAT Focused 3D Classification
w/o alignment T=80

16% 8% 8% **64%** 3% Focused
Refinement

116,555 particles **3.17 Å**

ERAL1 GTPase domain Focused 3D Classification
w/o alignment T=80

4.07 Å

29% 7% 23% 5% **36%** Focused
Refinement

63,569 particles 180°

mS29 Module Focused Refinement

2.97 Å

mS39 Module Focused Refinement

3.27 Å

Supplementary Fig. 5. Cryo-EM processing of human State B.

(**a**) Initial classification of particles with a body mask to clean the particle stack. (**b**) Different views of the State B overall map. (**c**) Euler angle distribution of the overall refinement. (**d**) Focused refinement and classification of the head domain. (**e**) Masks used in focused classification and refinement for modules in the body domain. (**f**) Masks used in focused classification and refinement for modules in the head domain. (**g,h**) Workflows for focused 3D classification and refinements. Reported resolutions (FSC=0.143) from Relion post-processing are reported next to individual focused maps. Masks used are color coded as in panels e and f.
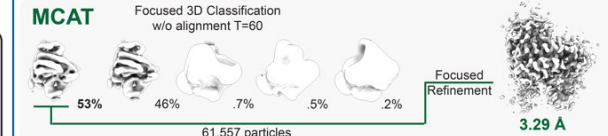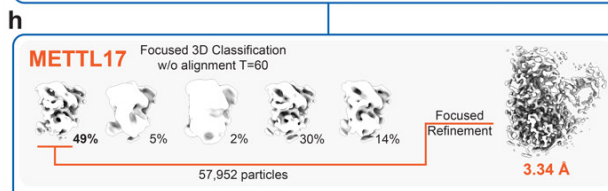
State C
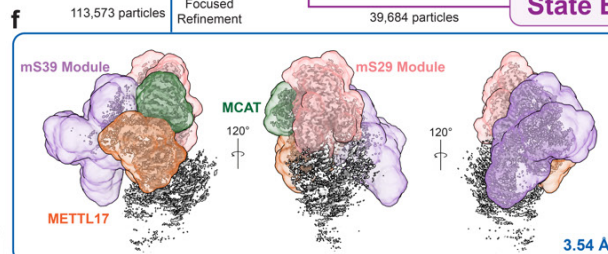
a

2.66 Å
262,363 particles

b

c

Head

Focused Refinement

3.82 Å

11%   5%   11%

Focused 3D
Classification
w/o alignment
T=40

59%   **13%**

155,968 particles   Focused
Refinement

33,642 particles   **State C***

d

Platform

Backpack

H44

Tail

e

mS39 Module   mS29 Module

MCAT

METTL17

3.78 Å

f

Platform   Focused 3D Classification
w/o alignment T=80   2.54 Å

5%   12%   .2%   **82%**   .05%   Focused
Refinement

219,400 particles

30°   30°

RBFA

TFB1M

Focused
Refinement

2.88 Å

RBFA   Focused 3D Classification
w/o alignment T=80

37%   .2%   1%   .4%   **60%**   Focused
Refinement

132,272 particles   2.88 Å

H44   Focused 3D Classification
w/o alignment T=130

48%   22%   3%   2%   24%   Focused
Refinement

136,878 particles   3.06 Å

Tail   Focused Refinement

2.60 Å

Backpack   Focused Refinement

2.52 Å

g

METTL17   Focused 3D Classification
w/o alignment T=50

1%   40%   **56%**   .4%   1%   Focused
Refinement

90,501 particles   3.13 Å

MCAT   Focused 3D Classification
w/o alignment T=50

33%   4%   **38%**   19%   4%   Focused
Refinement

64,577 particles   3.40 Å

mS39 Module   Focused 3D Classification
w/o alignment T=80

29%   12%   **20%**   6%   32%   Focused
Refinement

30,733 particles   3.82 Å

mS29 Module   Focused Refinement

3.08 Å

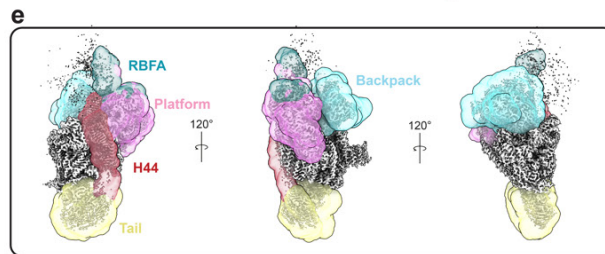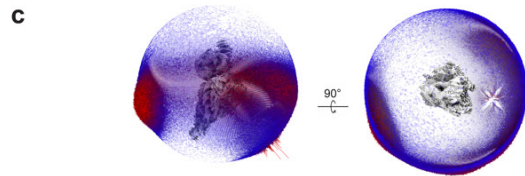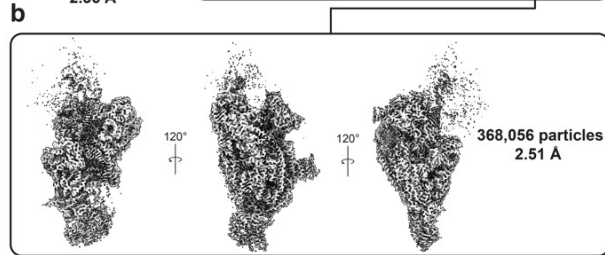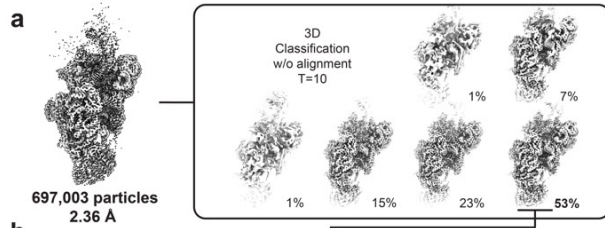**Supplementary Fig. 6. Cryo-EM processing of human State C.**

(**a**) Different views of the State C overall map. (**b**) Euler angle distribution of the overall refinement. (**c**) Focused refinement and classification of the head domain. Particles identified as State C* were further processed independently (Supplementary Fig. 9). (**d**) Masks used in focused classification and refinement for modules in the body domain. (**e**) Masks used in focused classification and refinement for modules in the head domain. (**f,g**) Workflows for focused 3D classification and refinements. Reported resolutions (FSC=0.143) from Relion post-processing are reported next to individual focused maps. Masks used are color coded as in panels d and e.
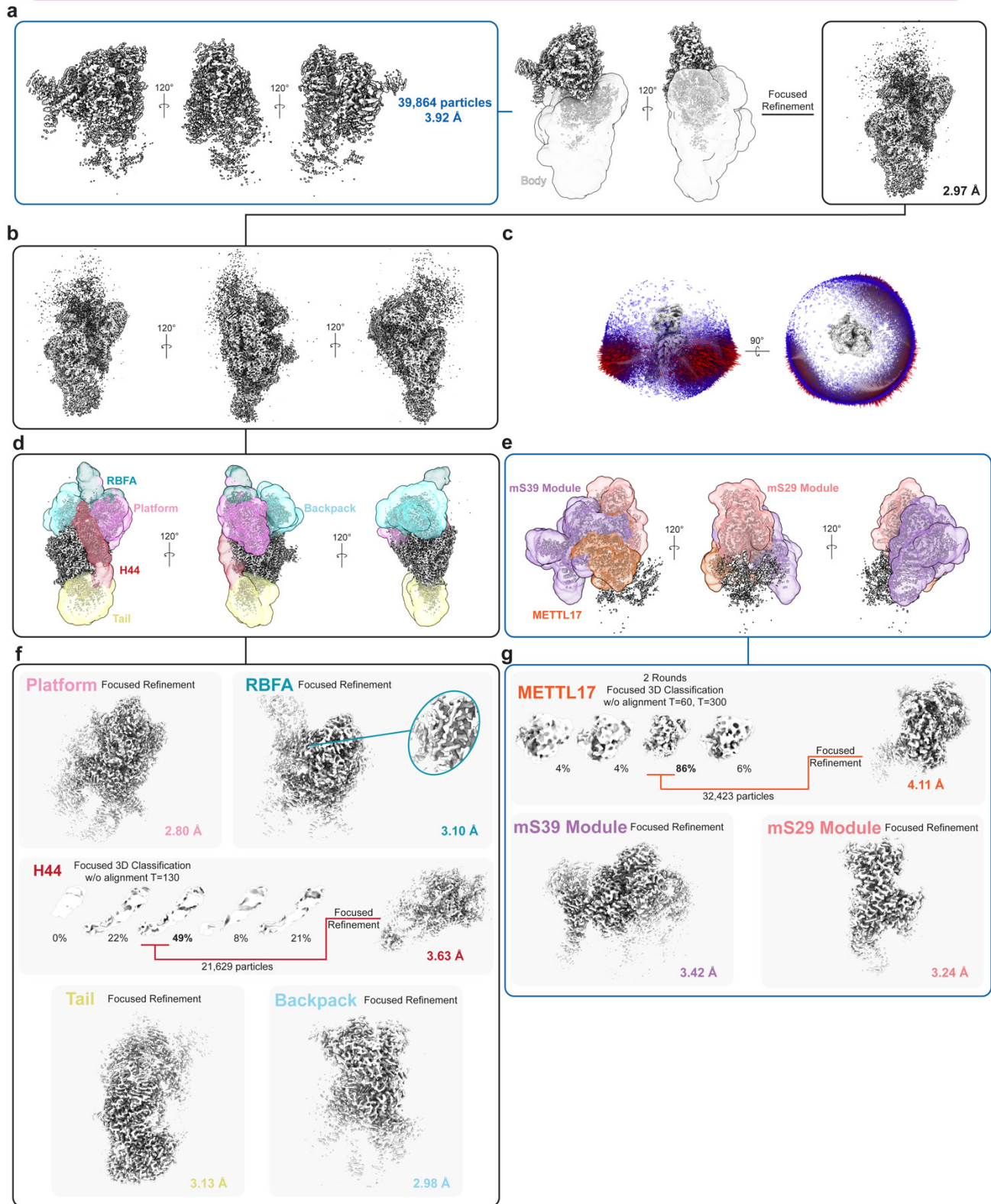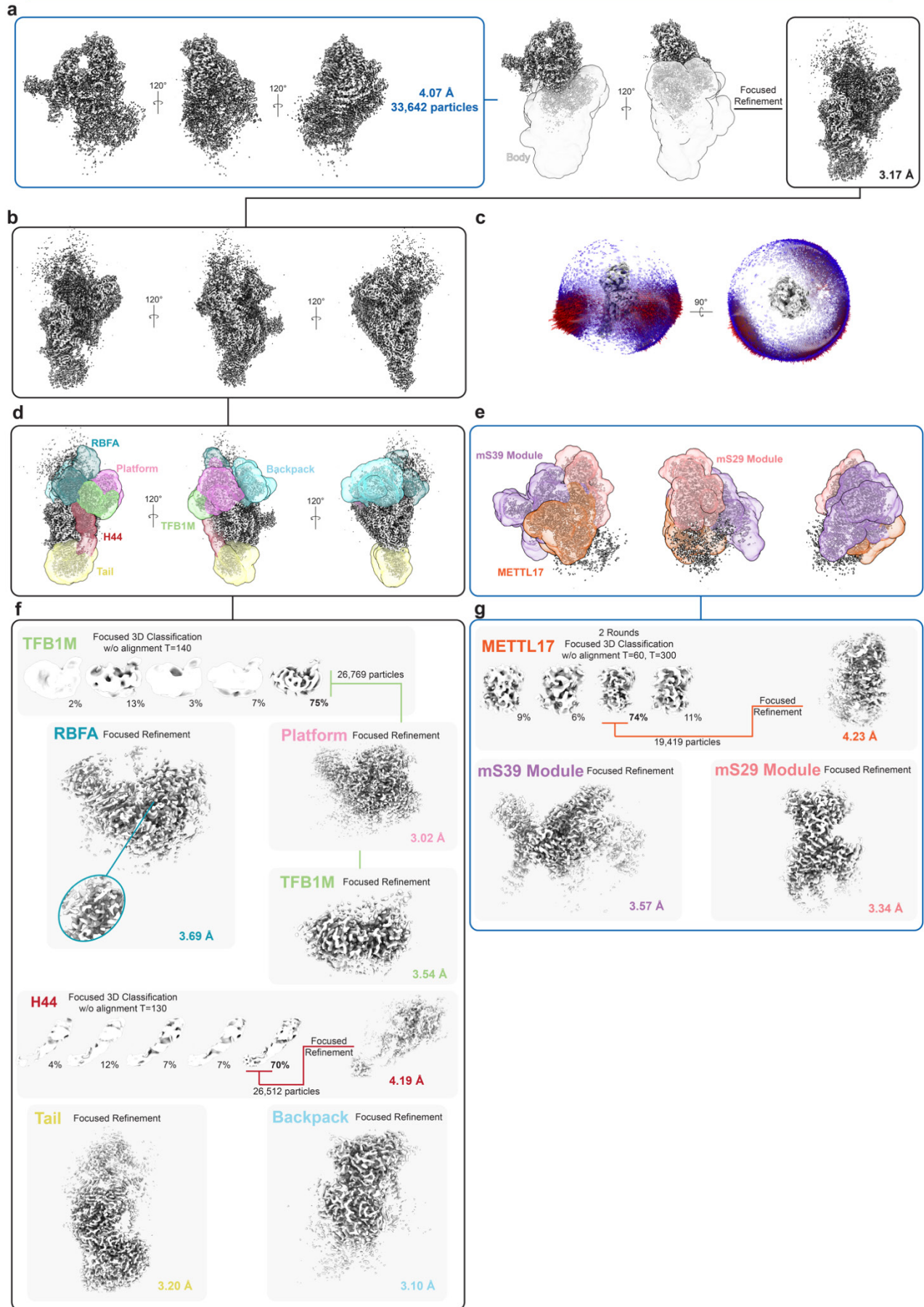
State D

a

697,003 particles
2.36 Å

3D Classification w/o alignment T=10

1%   7%
1%   15%   23%   **53%**

b

120°   120°

368,056 particles
2.51 Å

c

90°

d

Head

120°

Focused Refinement

3.54 Å

Focused 3D Classification w/o alignment T=40

13%   11%   34%
31%   **11%**

113,573 particles   Focused Refinement   39,684 particles

State E

f

mS39 Module   mS29 Module   MCAT

120°   120°

METTL17

3.54 Å

e

RBFA   Backpack
Platform
H44
Tail

120°   120°

g

Platform   Focused 3D Classification w/o alignment T=80

.01%   14%   **85%**   .04%   .3%

Focused Refinement

317,147 particles

2.29 Å

H44   Focused 3D Classification w/o alignment T=130

1%   1%   1%   41%   **55%**

Focused Refinement

210,829 particles

2.52 Å

RBFA   Focused Refinement

2.52 Å

Tail   Focused Refinement

2.48 Å

Backpack   Focused Refinement

2.38 Å

h

METTL17   Focused 3D Classification w/o alignment T=60

49%   5%   2%   30%   14%

Focused Refinement

57,952 particles   3.34 Å

MCAT   Focused 3D Classification w/o alignment T=60

53%   46%   .7%   .5%   .2%

Focused Refinement

61,557 particles   3.29 Å

mS29 Module   Focused Refinement

3.08 Å

mS39 Module   Focused Refinement

3.66 Å

Supplementary Fig. 7. Cryo-EM processing of human State D.

(**a**) Initial classification of particles without a mask to clean the particle stack. (**b**) Different views of the State D overall map. (**c**) Euler angle distribution of the overall refinement. (**d**) Focused refinement and classification of the head domain. Particles identified as State E were further processed independently (Supplementary Fig. 8). (**e**) Masks used in focused classification and refinement for modules in the body domain. (**f**) Masks used in focused classification and refinement for modules in the head domain. (**g,h**) Workflows for focused 3D classification and refinements. Reported resolutions (FSC=0.143) from Relion post-processing are reported next to individual focused maps. Masks used are color coded as in panels e and f.
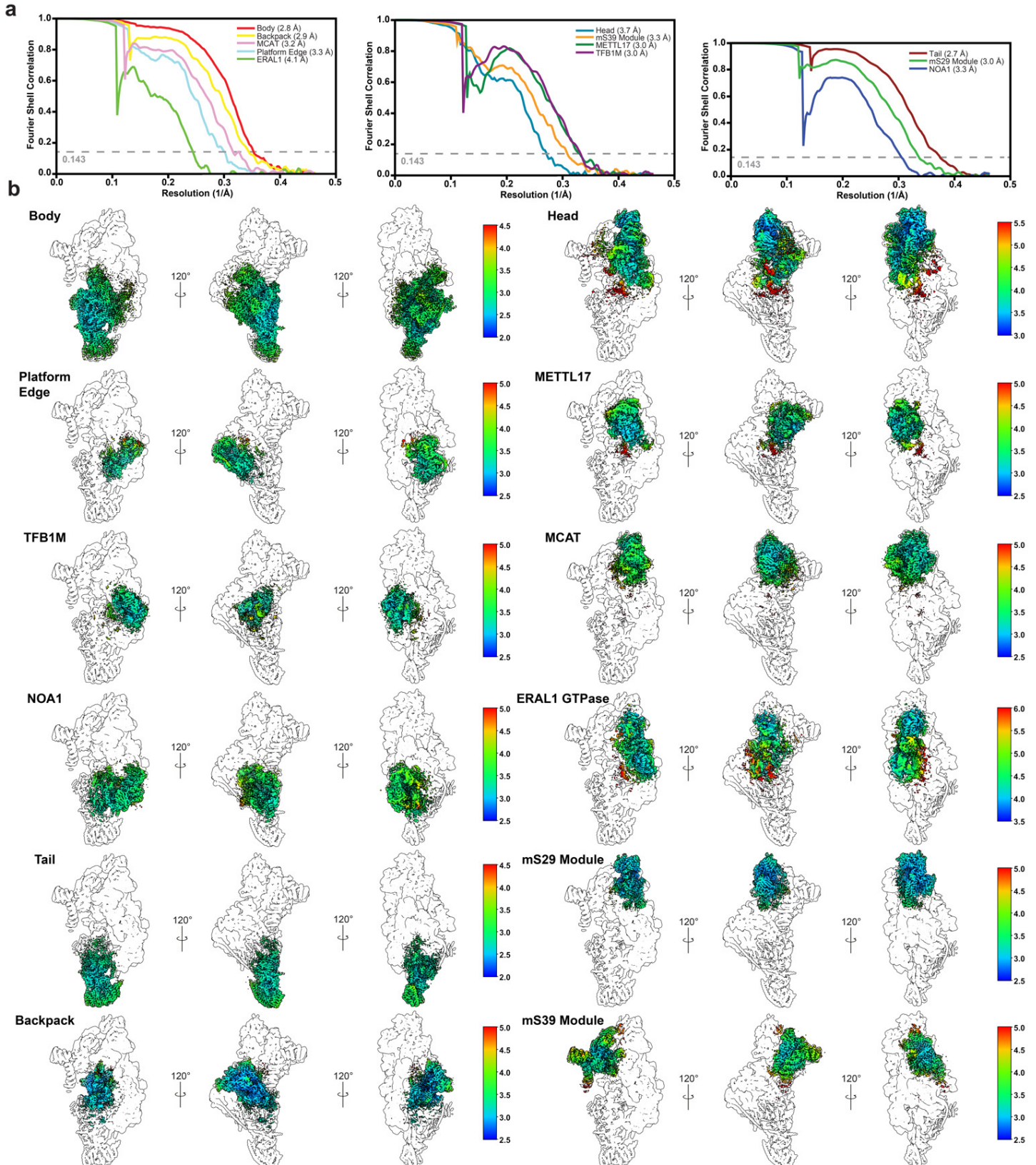
State E

a

39,864 particles
3.92 Å

Body

Focused
Refinement

2.97 Å

b

120°

120°

c

90°

d

RBFA

Platform

Backpack

H44

Tail

120°

120°

e

mS39 Module

mS29 Module

120°

120°

METTL17

f

Platform Focused Refinement

RBFA Focused Refinement

2.80 Å

3.10 Å

H44 Focused 3D Classification
w/o alignment T=130

0%   22%   **49%**   8%   21%

Focused
Refinement

21,629 particles

3.63 Å

Tail Focused Refinement

Backpack Focused Refinement

3.13 Å

2.98 Å

g

METTL17
2 Rounds
Focused 3D Classification
w/o alignment T=60, T=300

4%   4%   **86%**   6%

Focused
Refinement

32,423 particles

4.11 Å

mS39 Module Focused Refinement

mS29 Module Focused Refinement

3.42 Å

3.24 Å

Supplementary Fig. 8. Cryo-EM processing of human State E.

(**a**) Different views of the State E head map. Particles were then refined using a mask on the body. (**b**) Different views of the body domain. (**c**) Euler angle distribution of the overall (body) refinement. (**d**) Masks used in focused classification and refinement for modules in the body domain. (**e**) Masks used in focused classification and refinement for modules in the head domain. (**f,g**) Workflows for focused 3D classification and refinements. Reported resolutions (FSC=0.143) from Relion post-processing are reported next to individual focused maps. Masks used are color coded as in panels d and e.
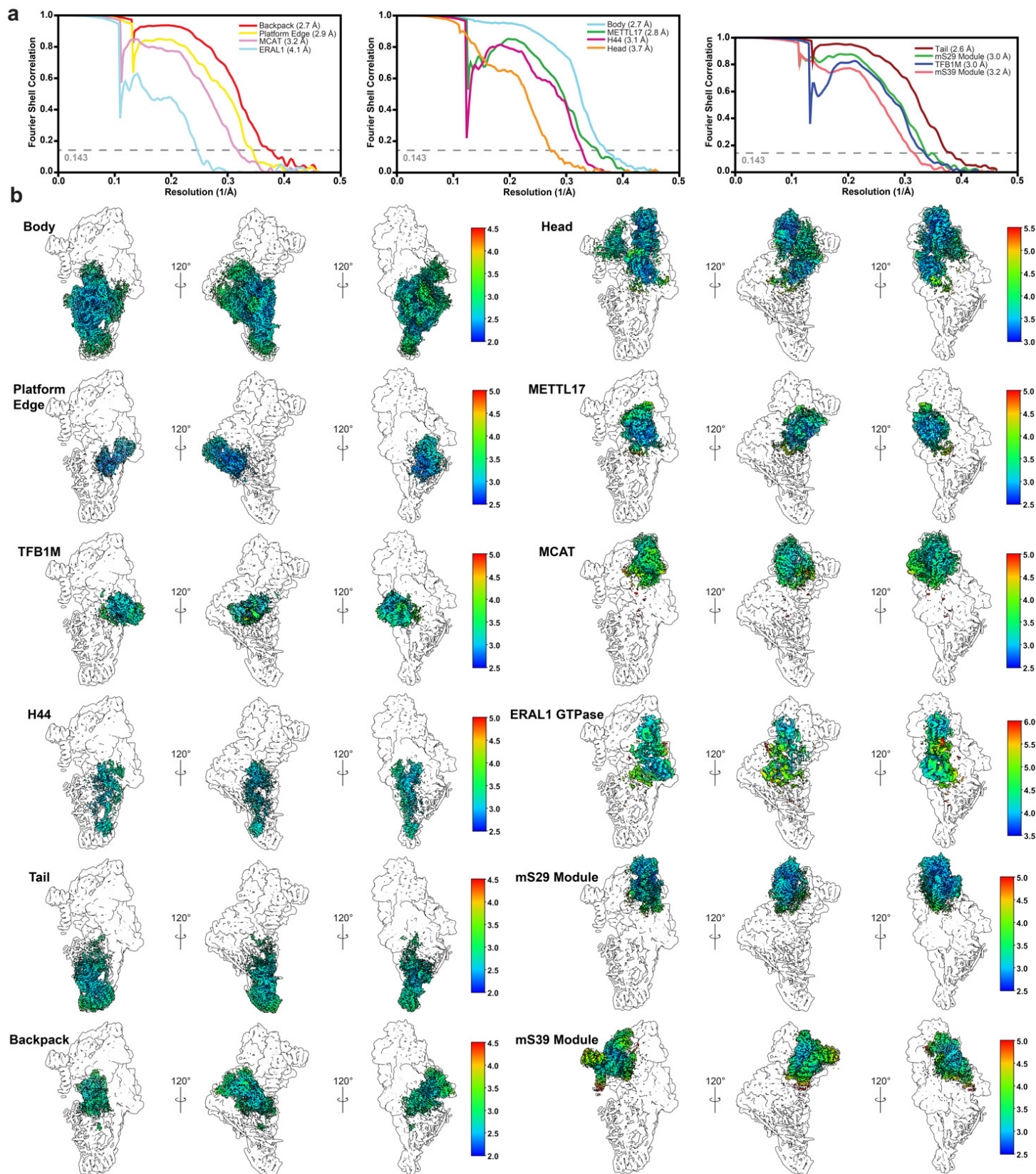
State C*

a

120° 120°

4.07 Å
33,642 particles

120°

Body

Focused Refinement

3.17 Å

b

120° 120°

c

90°

d

RBFA
Platform
Backpack
120° 120°
TFB1M
H44
Tail

e

mS39 Module
mS29 Module
METTL17

f

TFB1M Focused 3D Classification w/o alignment T=140

2% 13% 3% 7% **75%**

26,769 particles

RBFA Focused Refinement

3.69 Å

Platform Focused Refinement

3.02 Å

TFB1M Focused Refinement

3.54 Å

H44 Focused 3D Classification w/o alignment T=130

4% 12% 7% 7% **70%**

Focused Refinement

26,512 particles

4.19 Å

Tail Focused Refinement

3.20 Å

Backpack Focused Refinement

3.10 Å

g

METTL17 2 Rounds Focused 3D Classification w/o alignment T=60, T=300

9% 6% **74%** 11%

Focused Refinement

19,419 particles

4.23 Å

mS39 Module Focused Refinement

3.57 Å

mS29 Module Focused Refinement

3.34 Å

Supplementary Fig. 9. Cryo-EM processing of human State C*.

(**a**) Different views of the State C* head map. Particles were then refined using a mask on the body. (**b**) Different views of the body domain. (**c**) Euler angle distribution of the overall (body) refinement. (**d**) Masks used in focused classification and refinement for modules in the body domain. (**e**) Masks used in focused classification and refinement for modules in the head domain. (**f,g**) Workflows for focused 3D classification and refinements. Reported resolutions (FSC=0.143) from Relion post-processing are reported next to individual focused maps. Masks used are color coded as in panels d and e.

State A

a

b

Supplementary Fig. 10. Local resolution analysis of human State A.

(**a**) Solvent-corrected FSC curves for each focused refinement determined at FSC=0.143 via post-processing in Relion. (**b**) Local resolution estimation (calculated in Relion) for each focused map in context with the rest of the reconstruction.
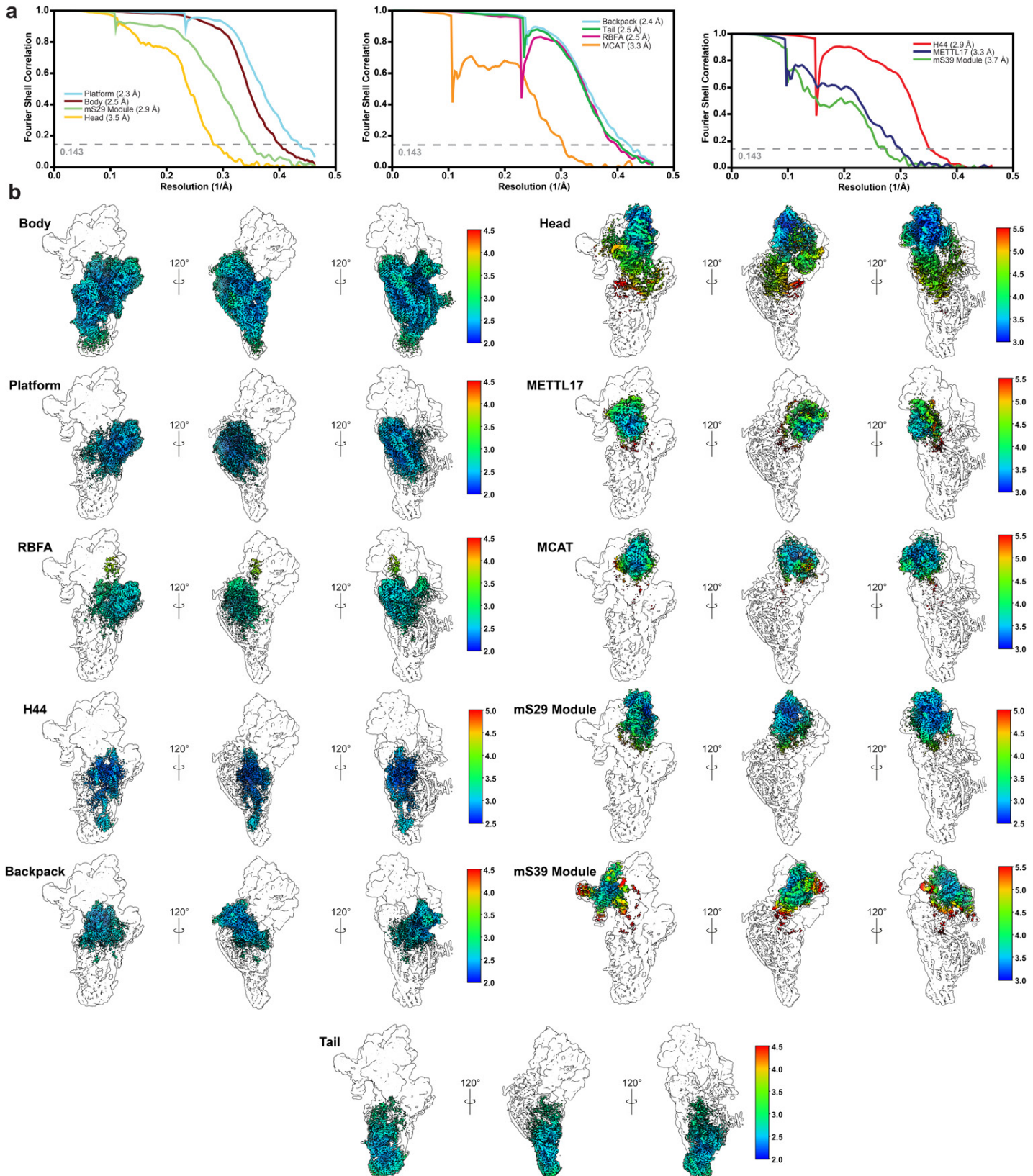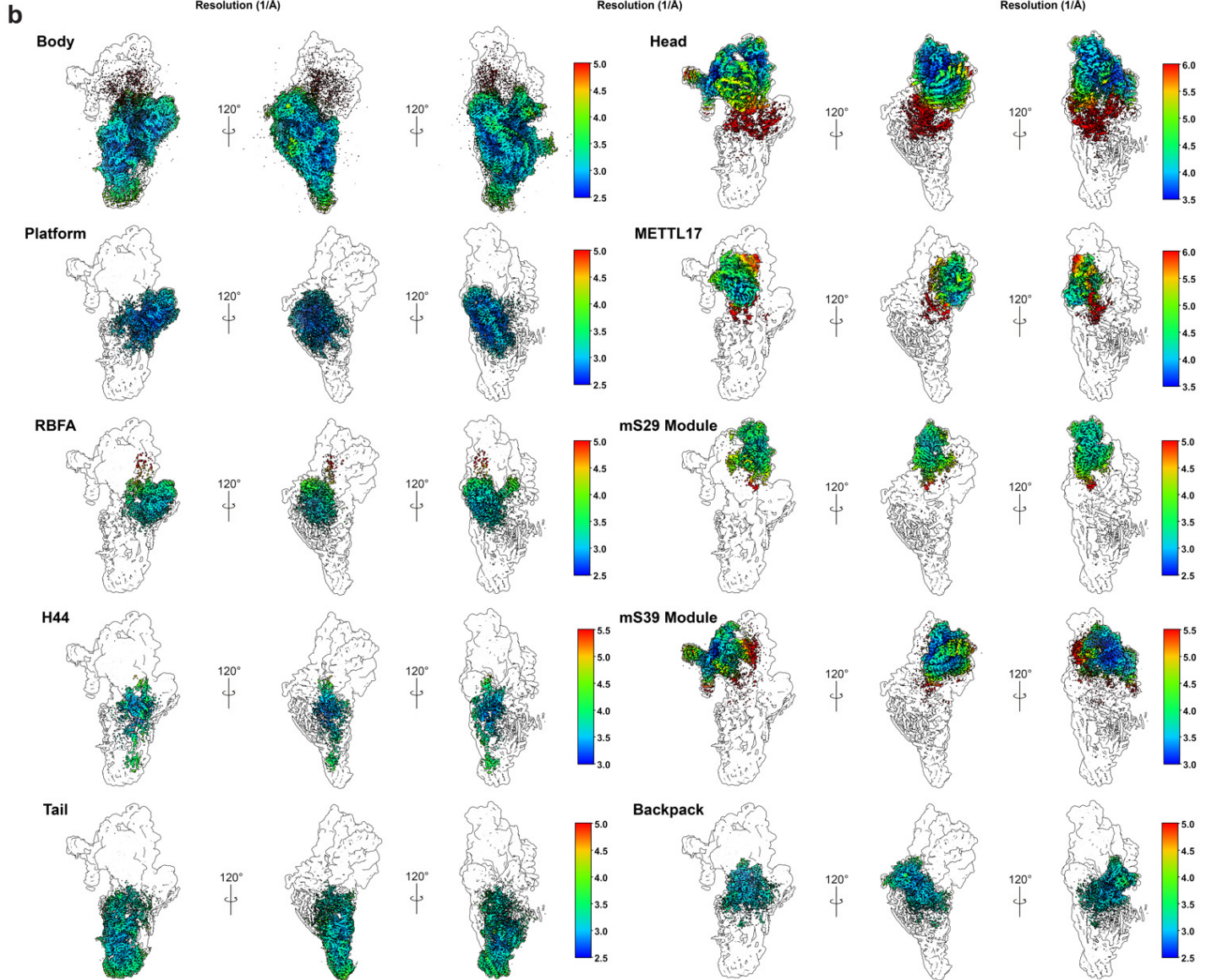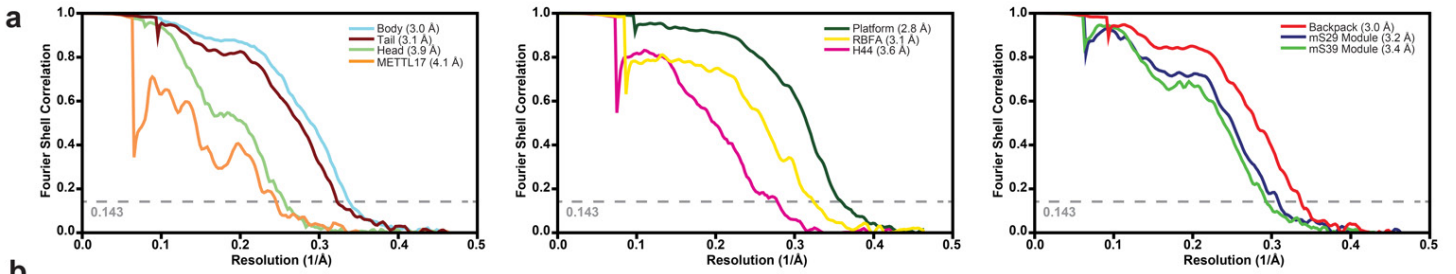
State B

a

b

Supplementary Fig. 11. Local resolution analysis of human State B.

(**a**) Solvent-corrected FSC curves for each focused refinement determined at FSC=0.143 via post-processing in Relion. (**b**) Local resolution estimation (calculated in Relion) for each focused map in context with the rest of the reconstruction.

State C

Supplementary Fig. 12. Local resolution analysis of human State C.

(**a**) Solvent-corrected FSC curves for each focused refinement determined at FSC=0.143 via post-processing in Relion. (**b**) Local resolution estimation (calculated in Relion) for each focused map in context with the rest of the reconstruction.
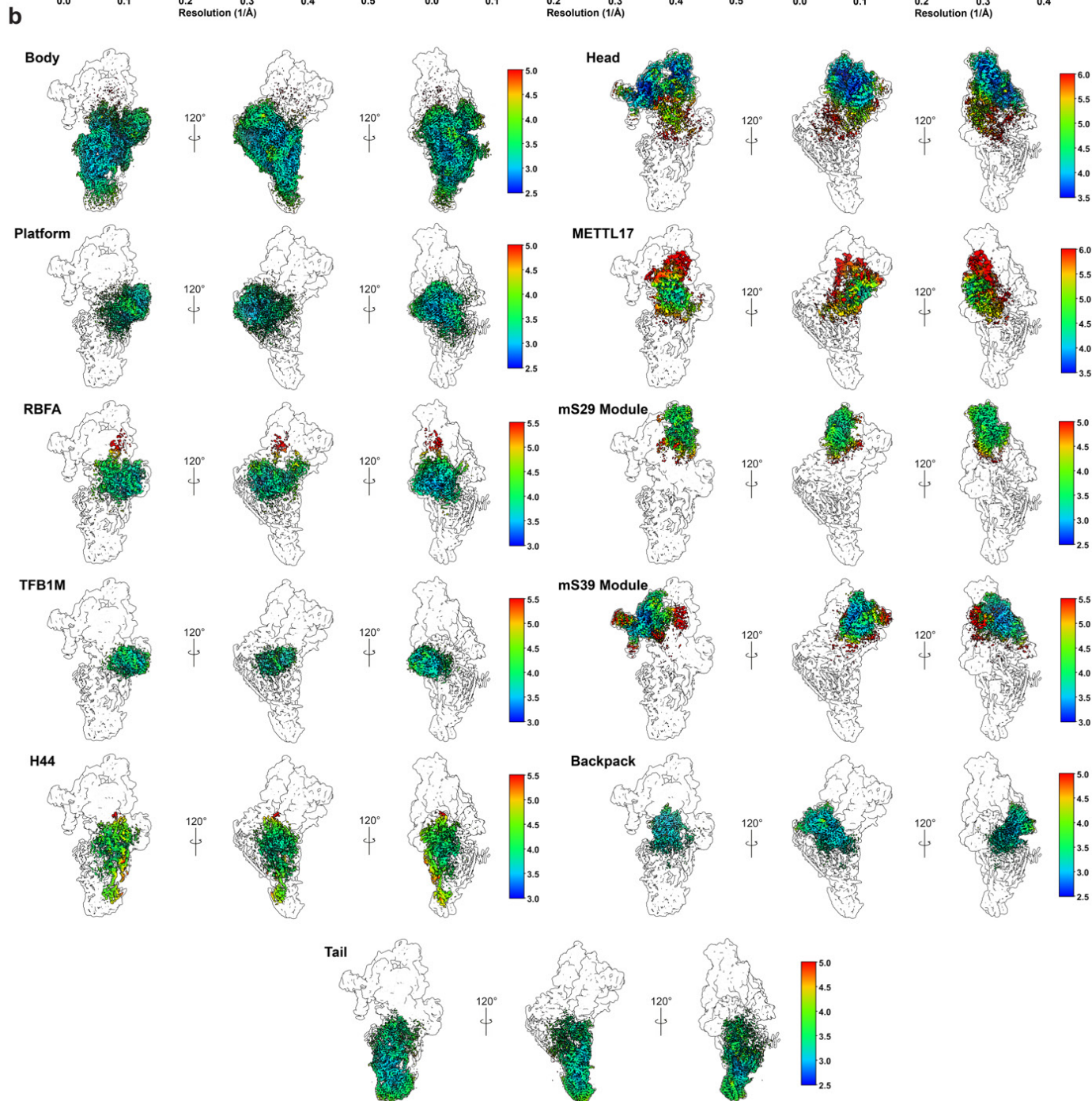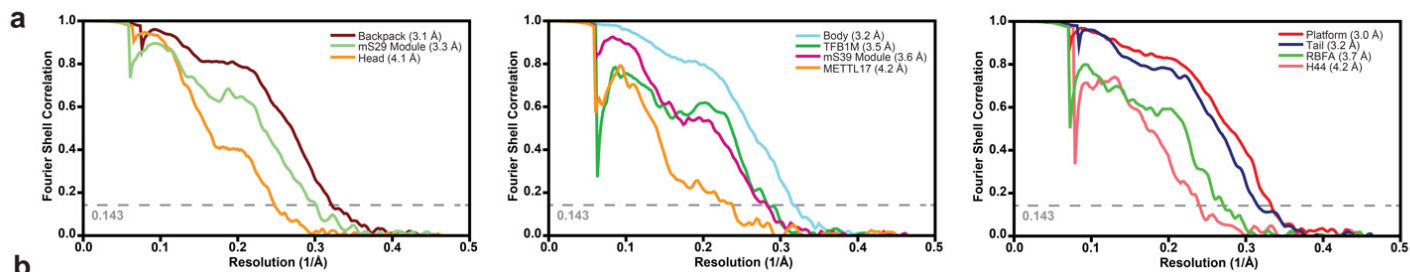
State D

a

Left panel (Fourier Shell Correlation vs Resolution (1/Å)):
- Platform (2.3 Å)
- Body (2.5 Å)
- mS29 Module (2.9 Å)
- Head (3.5 Å)
- 0.143

Middle panel:
- Backpack (2.4 Å)
- Tail (2.5 Å)
- RBFA (2.5 Å)
- MCAT (3.3 Å)
- 0.143

Right panel:
- H44 (2.9 Å)
- METTL17 (3.3 Å)
- mS39 Module (3.7 Å)
- 0.143

b

Body · Platform · RBFA · H44 · Backpack

Head · METTL17 · MCAT · mS29 Module · mS39 Module

Tail

Supplementary Fig. 13. Local resolution analysis of human State D.

(**a**) Solvent-corrected FSC curves for each focused refinement determined at FSC=0.143 via post-processing in Relion. (**b**) Local resolution estimation (calculated in Relion) for each focused map in context with the rest of the reconstruction.
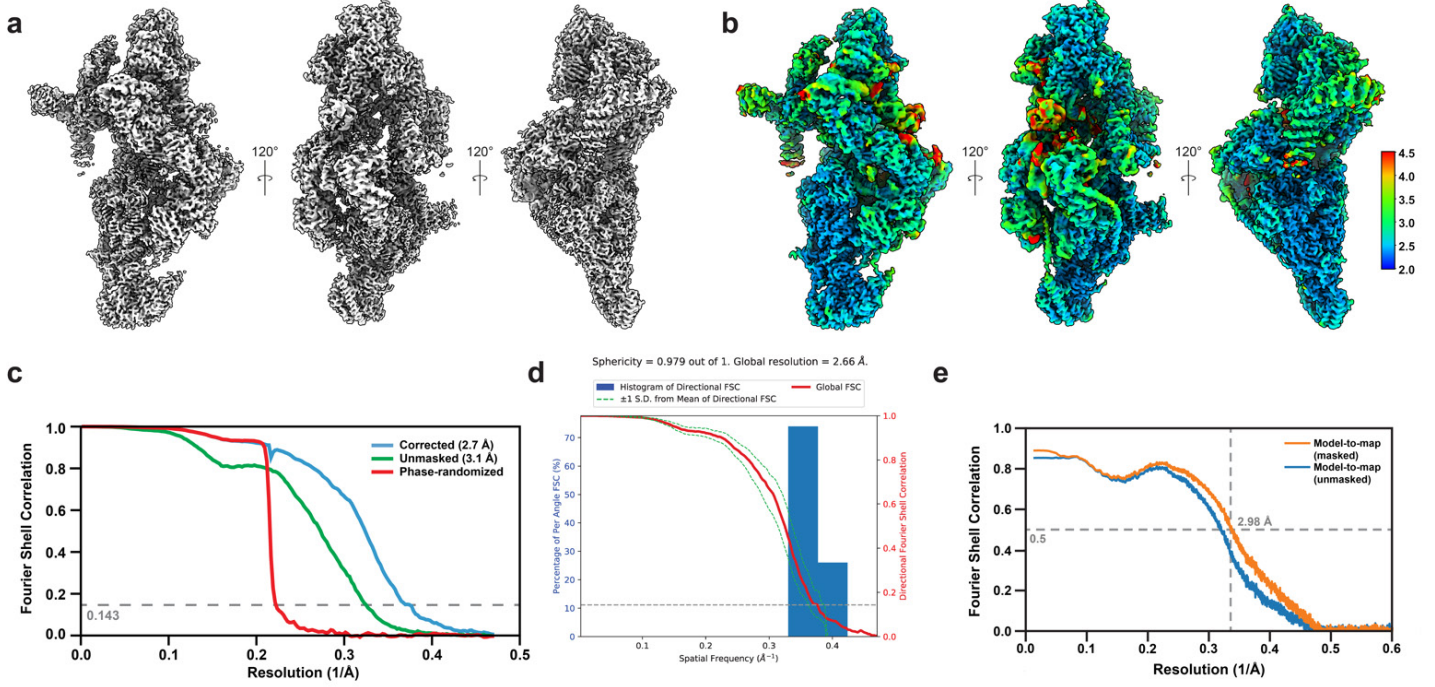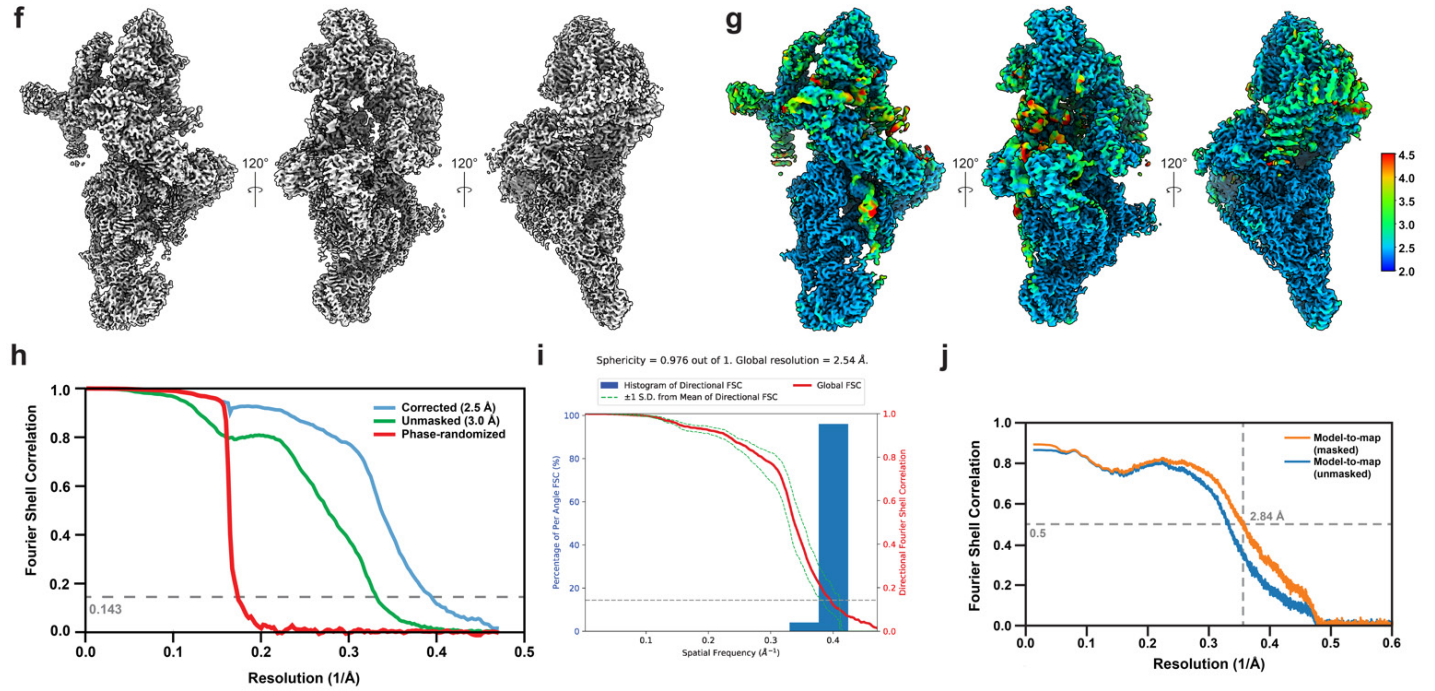
State E

Supplementary Fig. 14. Local resolution analysis of human State E.

(**a**) Solvent-corrected FSC curves for each focused refinement determined at FSC=0.143 via post-processing in Relion. (**b**) Local resolution estimation (calculated in Relion) for each focused map in context with the rest of the reconstruction.

State C*

a

b

Body

Head

Platform

METTL17

RBFA

mS29 Module

TFB1M

mS39 Module

H44

Backpack

Tail

Supplementary Fig. 15. Local resolution analysis of human State C*.

(**a**) Solvent-corrected FSC curves for each focused refinement determined at FSC=0.143 via post-processing in Relion. (**b**) Local resolution estimation (calculated in Relion) for each focused map in context with the rest of the reconstruction.
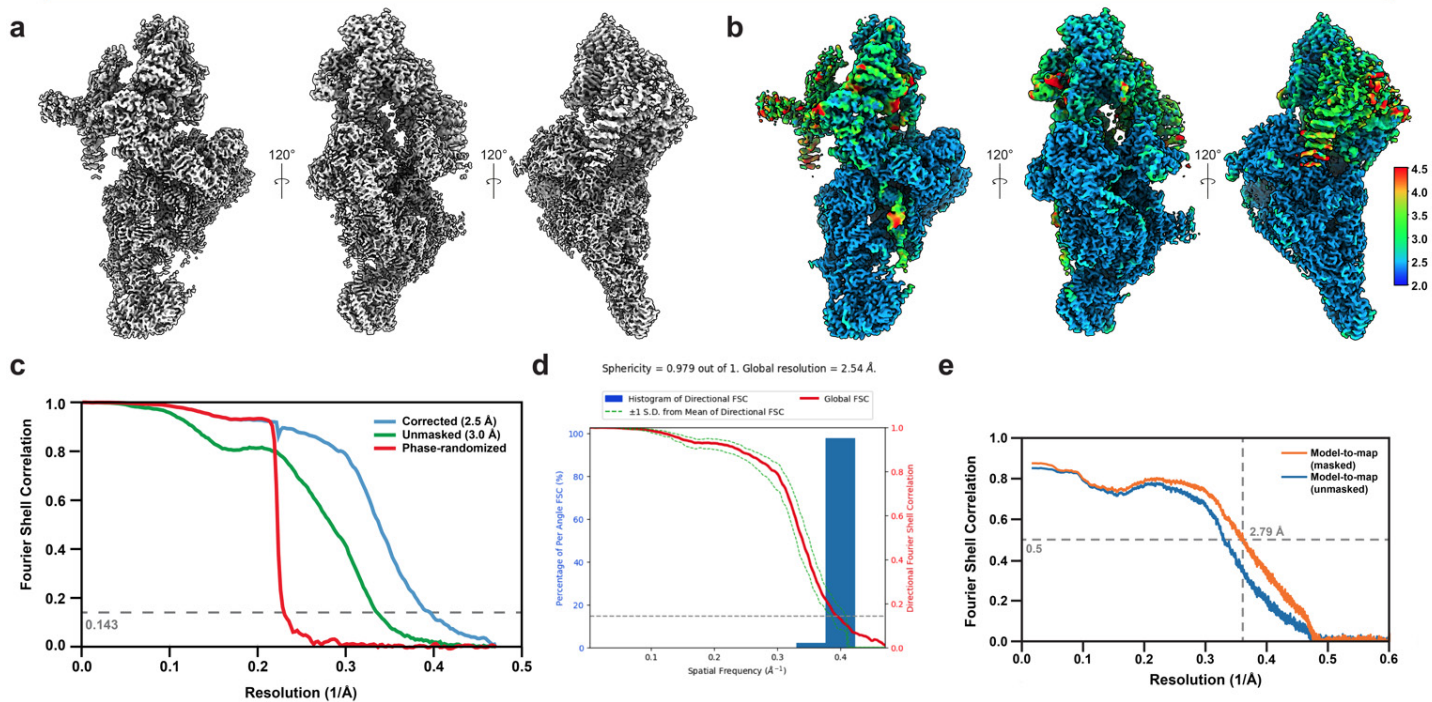
State A

a

b

c

Sphericity = 0.979 out of 1. Global resolution = 2.66 Å.

d

e

State B

f

g

h

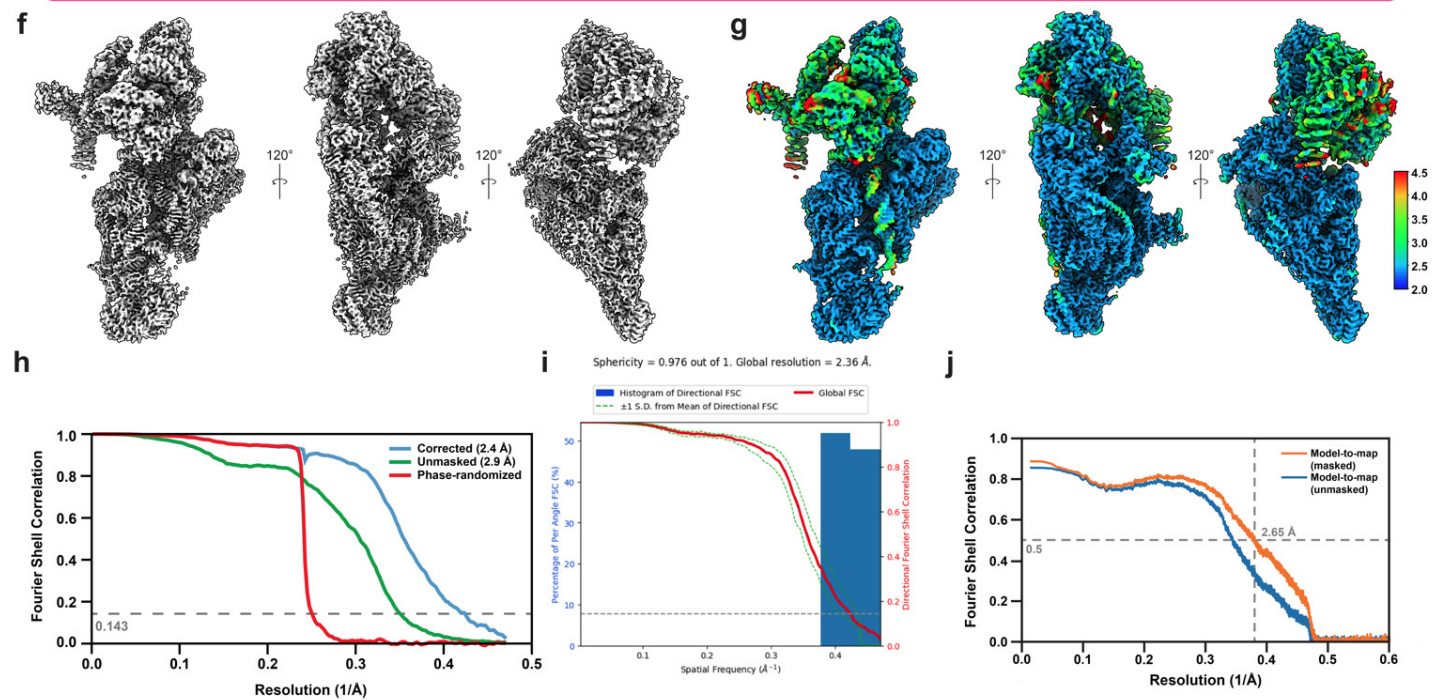Sphericity = 0.976 out of 1. Global resolution = 2.54 Å.

i

j

Supplementary Fig. 16. Validation of composite maps for human States A and B.

(**a**) Different views of the composite map of State A. (**b**) Different views of the composite map of State A filtered and colored according to local resolution as calculated by cryoSPARC. (**c**) FSC curves for the State A composite map as calculated in Relion. Reported resolutions were determined at FSC=0.143. (**d**) 3DFSC for the State A composite map calculated using the Remote 3DFSC Processing Server[1]. (**e**) Map-to-model correlation between the State A composite map and the State A model. (**f**) Different views of the composite map of State B. (**g**) Different views of the composite map of State B filtered and colored according to local resolution as calculated by cryoSPARC. (**h**) FSC curves for the State B composite map as calculated in Relion. Reported resolutions were determined at FSC=0.143. (**i**) 3DFSC for the State B composite map calculated using the Remote 3DFSC Processing Server[1]. (**j**) Map-to-model correlation between the State B composite map and the State B model.
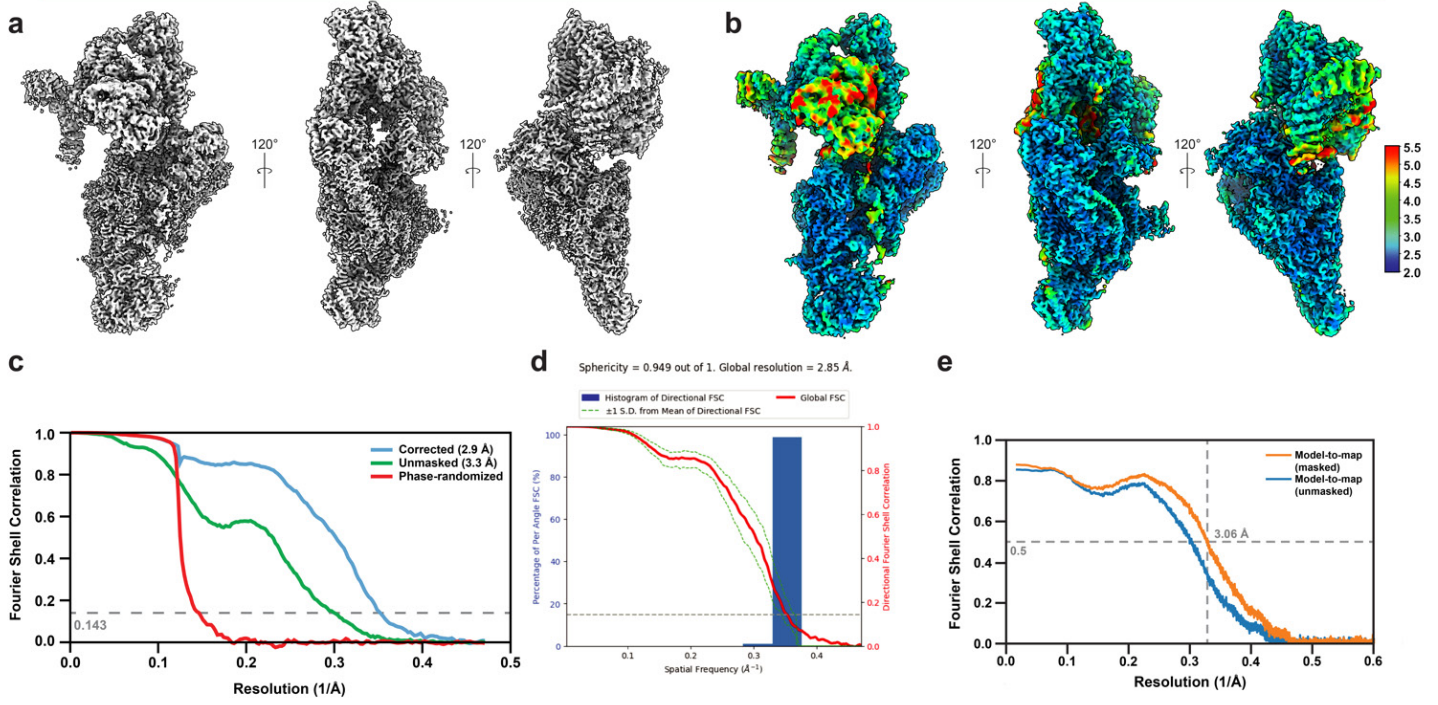
State C

a

b

c
Fourier Shell Correlation vs Resolution (1/Å)
- Corrected (2.5 Å)
- Unmasked (3.0 Å)
- Phase-randomized
0.143

d
Sphericity = 0.979 out of 1. Global resolution = 2.54 Å.
- Histogram of Directional FSC
- Global FSC
- ±1 S.D. from Mean of Directional FSC

e
Fourier Shell Correlation vs Resolution (1/Å)
- Model-to-map (masked)
- Model-to-map (unmasked)
0.5   2.79 Å

State D

f

g

h
Fourier Shell Correlation vs Resolution (1/Å)
- Corrected (2.4 Å)
- Unmasked (2.9 Å)
- Phase-randomized
0.143

i
Sphericity = 0.976 out of 1. Global resolution = 2.36 Å.
- Histogram of Directional FSC
- Global FSC
- ±1 S.D. from Mean of Directional FSC

j
Fourier Shell Correlation vs Resolution (1/Å)
- Model-to-map (masked)
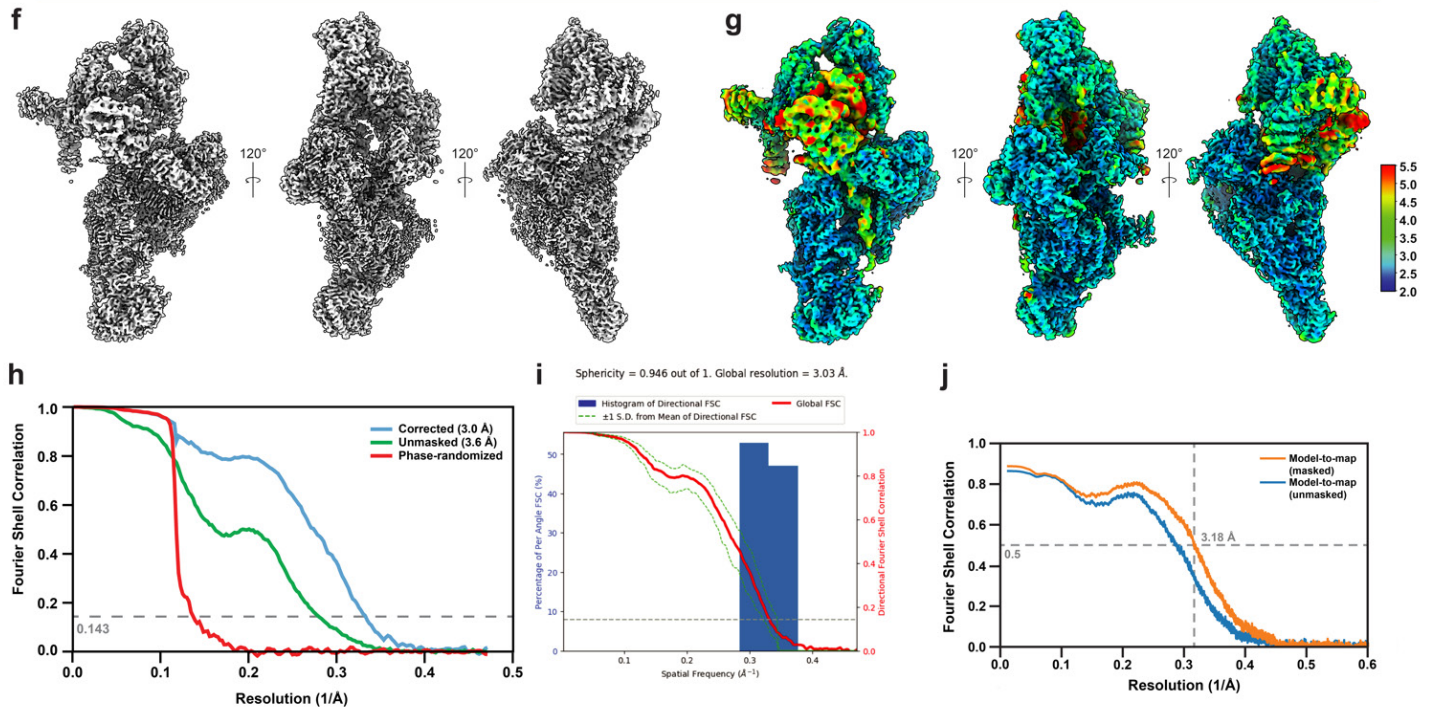- Model-to-map (unmasked)
0.5   2.65 Å

Supplementary Fig. 17. Validation of composite maps for human States C and D.

(**a**) Different views of the composite map of State C. (**b**) Different views of the composite map of State C filtered and colored according to local resolution as calculated by cryoSPARC. (**c**) FSC curves for the State C composite map as calculated in Relion. Reported resolutions were determined at FSC=0.143. (**d**) 3DFSC for the State C composite map calculated using the Remote 3DFSC Processing Server[1]. (**e**) Map-to-model correlation between the State C composite map and the State C model. (**f**) Different views of the composite map of State D. (**g**) Different views of the composite map of State D filtered and colored according to local resolution as calculated by cryoSPARC. (**h**) FSC curves for the State D composite map as calculated in Relion. Reported resolutions were determined at FSC=0.143. (**i**) 3DFSC for the State D composite map calculated using the Remote 3DFSC Processing Server[1]. (**j**) Map-to-model correlation between the State D composite map and the State D model.
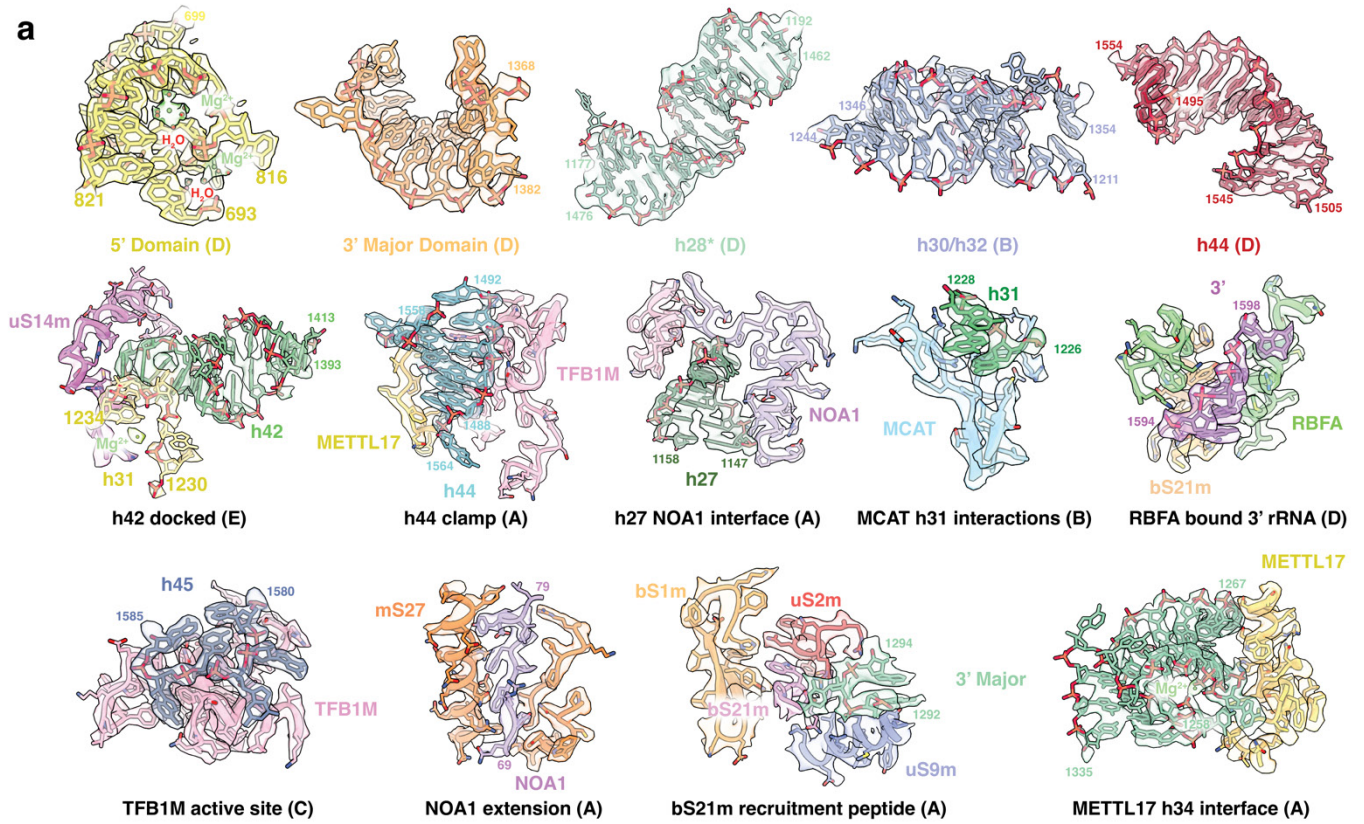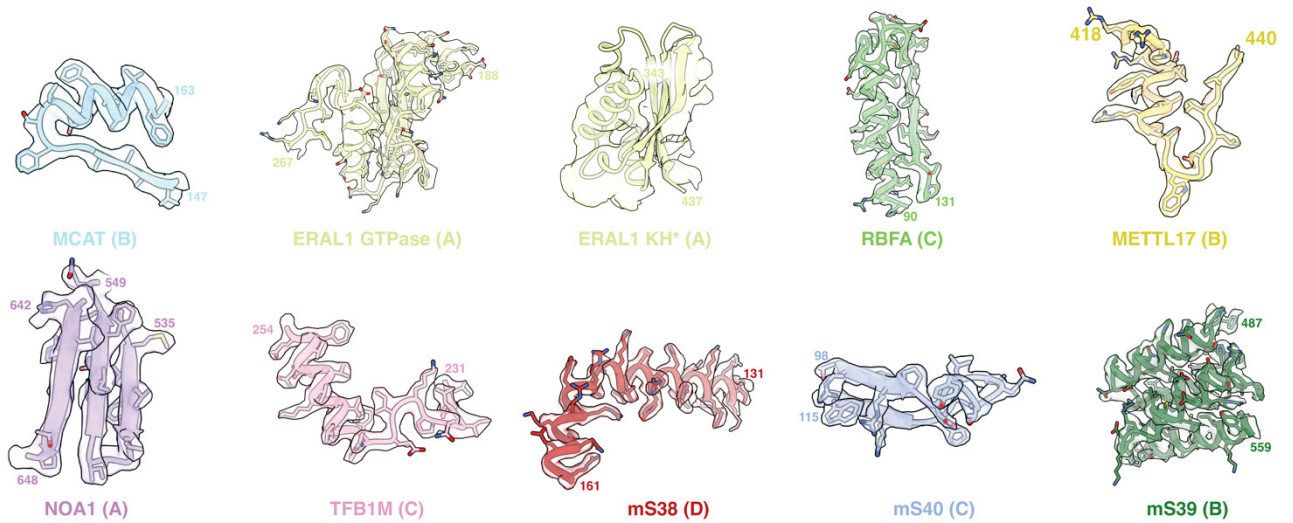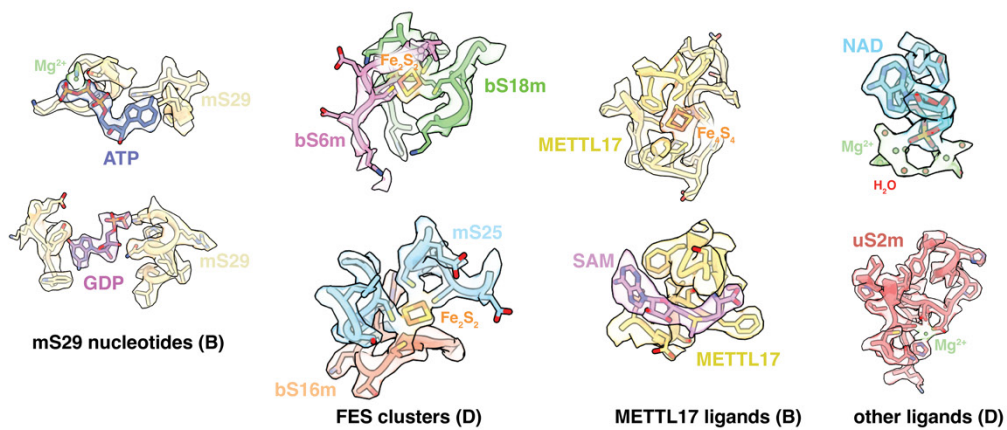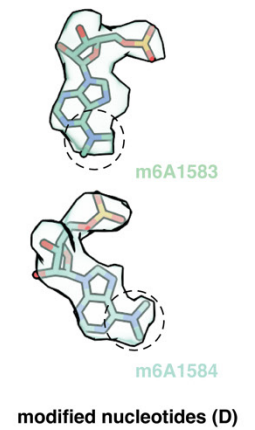
State E

a

b

c
Fourier Shell Correlation vs Resolution (1/Å)
- Corrected (2.9 Å)
- Unmasked (3.3 Å)
- Phase-randomized
- 0.143

d
Sphericity = 0.949 out of 1. Global resolution = 2.85 Å.
- Histogram of Directional FSC
- Global FSC
- ±1 S.D. from Mean of Directional FSC
Percentage of Per Angle FSC (%) / Directional Fourier Shell Correlation vs Spatial Frequency (Å⁻¹)

e
Fourier Shell Correlation vs Resolution (1/Å)
- Model-to-map (masked)
- Model-to-map (unmasked)
- 3.06 Å
- 0.5

State C*

f

g

h
Fourier Shell Correlation vs Resolution (1/Å)
- Corrected (3.0 Å)
- Unmasked (3.6 Å)
- Phase-randomized
- 0.143

i
Sphericity = 0.946 out of 1. Global resolution = 3.03 Å.
- Histogram of Directional FSC
- Global FSC
- ±1 S.D. from Mean of Directional FSC
Percentage of Per Angle FSC (%) / Directional Fourier Shell Correlation vs Spatial Frequency (Å⁻¹)

j
Fourier Shell Correlation vs Resolution (1/Å)
- Model-to-map (masked)
- Model-to-map (unmasked)
- 3.18 Å
- 0.5

Supplementary Fig. 18. Validation of composite maps for human States E and C*.

(**a**) Different views of the composite map of State E. (**b**) Different views of the composite map of State E filtered and colored according to local resolution as calculated by cryoSPARC. (**c**) FSC curves for the State E composite map as calculated in Relion. Reported resolutions were determined at FSC=0.143. (**d**) 3DFSC for the State E composite map calculated using the Remote 3DFSC Processing Server[1]. (**e**) Map-to-model correlation between the State E composite map and the State E model. (**f**) Different views of the composite map of State C*. (**g**) Different views of the composite map of State C* filtered and colored according to local resolution as calculated by cryoSPARC. (**h**) FSC curves for the State C* composite map as calculated in Relion. Reported resolutions were determined at FSC=0.143. (**i**) 3DFSC for the State C* composite map calculated using the Remote 3DFSC Processing Server[1]. (**j**) Map-to-model correlation between the State C* composite map and the State C* model.
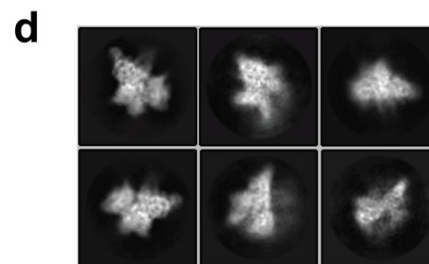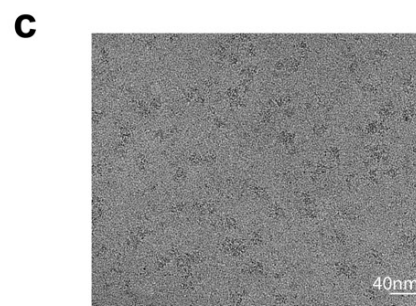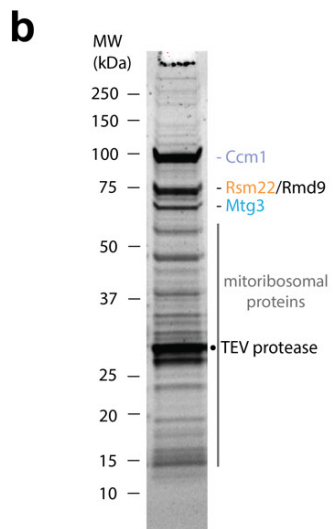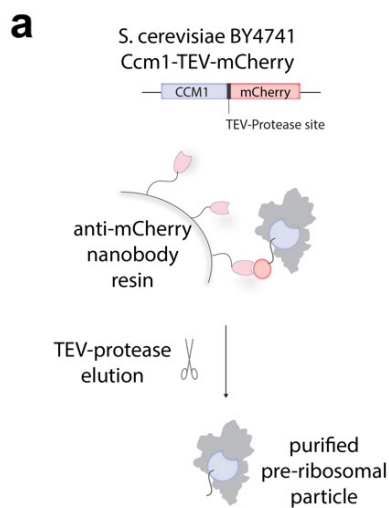
a

5' Domain (D) — 699, Mg²⁺, H₂O, 821, 816, 693

3' Major Domain (D) — 1368, 1382

h28* (D) — 1192, 1462, 1177, 1476

h30/h32 (B) — 1346, 1244, 1354, 1211

h44 (D) — 1554, 1495, 1545, 1505

h42 docked (E) — uS14m, 1234, 1413, 1393, h42, Mg²⁺, h31, 1230

h44 clamp (A) — 1492, 1558, TFB1M, METTL17, 1488, 1564, h44

h27 NOA1 interface (A) — 1228, h31, 1226, NOA1, 1158, 1147, h27

MCAT h31 interactions (B) — 1228, h31, 1226, MCAT

RBFA bound 3' rRNA (D) — 3', 1598, 1594, RBFA, bS21m

TFB1M active site (C) — h45, 1580, 1585, TFB1M

NOA1 extension (A) — mS27, 79, 69, NOA1

bS21m recruitment peptide (A) — bS1m, uS2m, 1294, bS21m, 1292, uS9m

METTL17 h34 interface (A) — METTL17, 1267, 3' Major, Mg²⁺, 1258, 1335

b

MCAT (B) — 163, 147

ERAL1 GTPase (A) — 188, 267

ERAL1 KH* (A) — 343, 437

RBFA (C) — 90, 131

METTL17 (B) — 418, 440

NOA1 (A) — 642, 549, 535, 648

TFB1M (C) — 254, 231

mS38 (D) — 131, 161

mS40 (C) — 98, 115

mS39 (B) — 487, 559

c

mS29 nucleotides (B) — Mg²⁺, mS29, ATP, GDP, mS29

FES clusters (D) — Fe₄S₄, bS18m, bS6m, mS25, Fe₂S₂, bS16m

METTL17 ligands (B) — METTL17, Fe₄S₄, SAM, METTL17

other ligands (D) — NAD, Mg²⁺, H₂O, uS2m, Mg²⁺

d

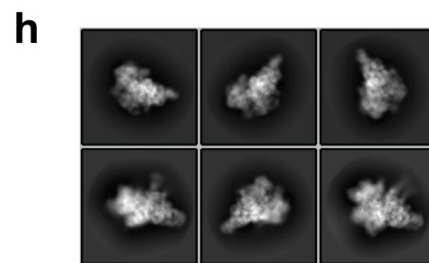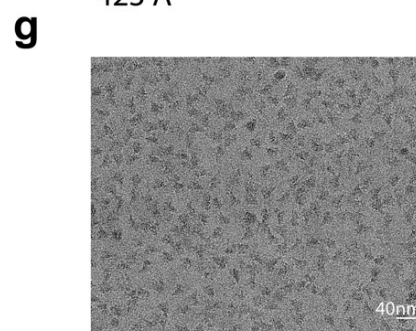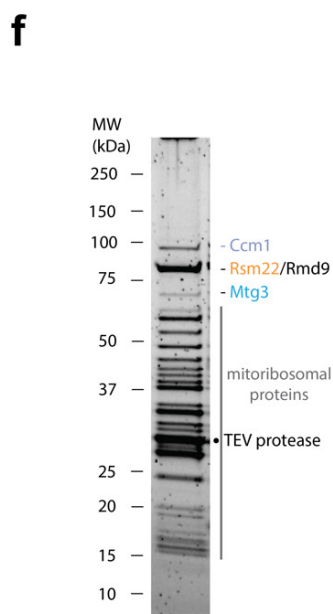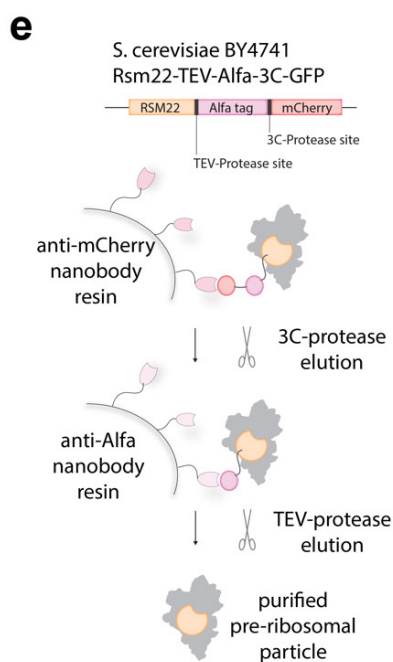modified nucleotides (D) — m6A1583, m6A1584

Supplementary Fig. 19. Representative cryo-EM densities and models for human structures.

Selections of representative densities of RNA and protein across all states, comprised of (**a**) rRNA and functionally relevant protein-RNA and protein-protein interfaces, (**b**) assembly factors and mitoribosomal 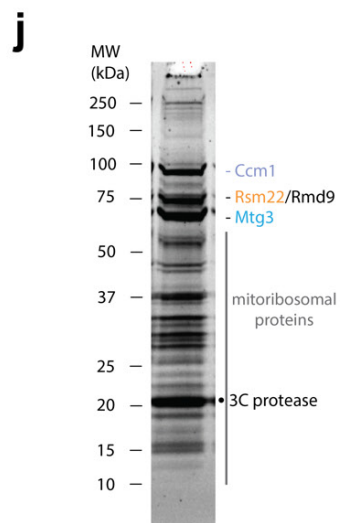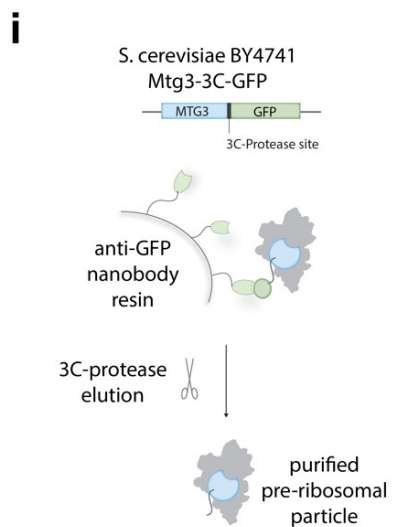proteins, (**c**) ligands, and (**d**) modified nucleotides. The states from which density and models are derived are shown in parentheses below each panel, and * denotes maps that have been filtered to local resolution.
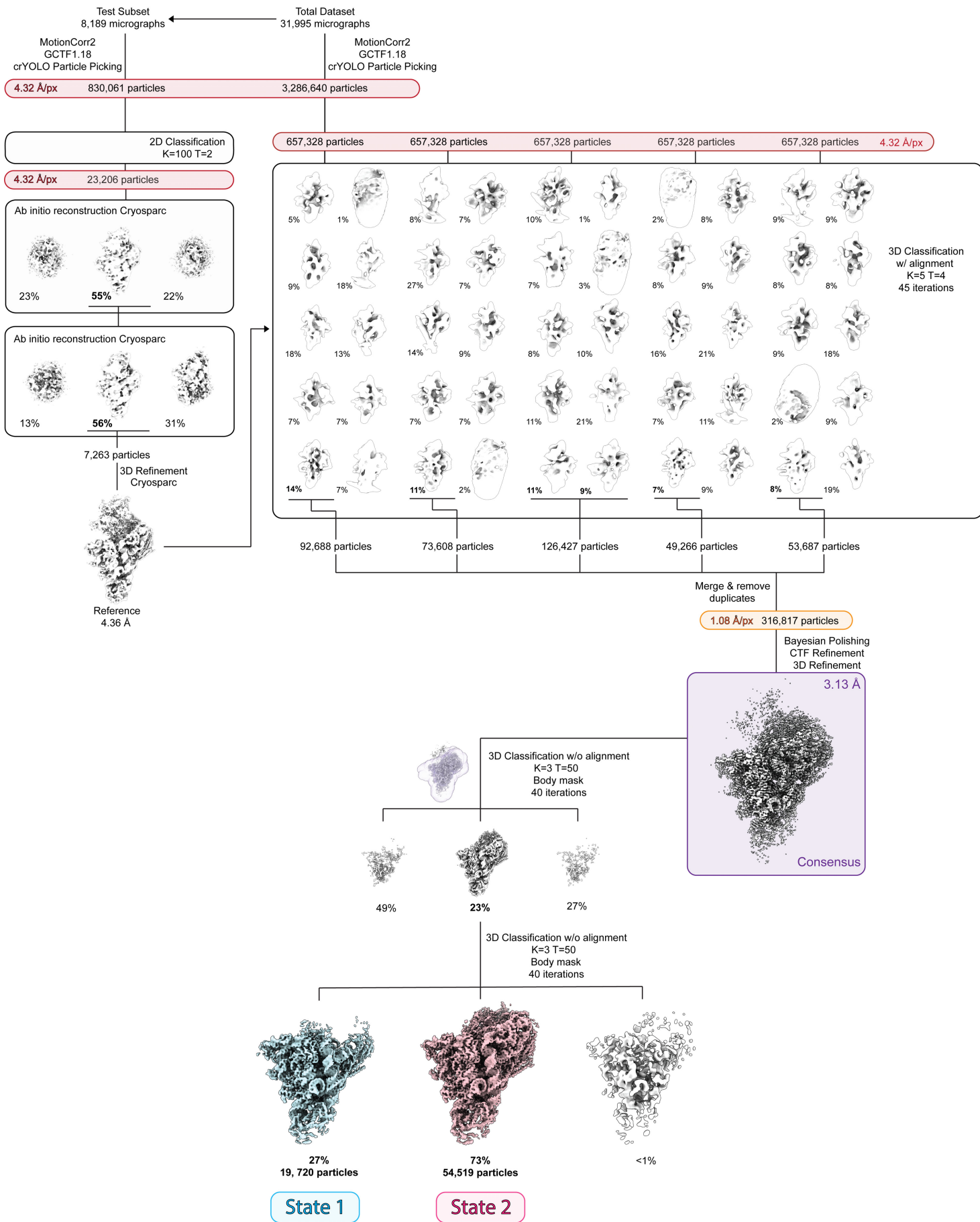
a

S. cerevisiae BY4741
Ccm1-TEV-mCherry

CCM1 | mCherry
TEV-Protease site

anti-mCherry
nanobody
resin

TEV-protease
elution

purified
pre-ribosomal
particle

b

MW
(kDa)
250 —
150 —
100 — - Ccm1
75 — - Rsm22/Rmd9
      - Mtg3
50 —
37 — mitoribosomal
      proteins
      • TEV protease
25 —
20 —
15 —
10 —

c

40nm

d

423 Å

e

S. cerevisiae BY4741
Rsm22-TEV-Alfa-3C-GFP

RSM22 | Alfa tag | mCherry
TEV-Protease site | 3C-Protease site

anti-mCherry
nanobody
resin

3C-protease
elution

anti-Alfa
nanobody
resin

TEV-protease
elution

purified
pre-ribosomal
particle

f

MW
(kDa)
250 —
150 —
100 — - Ccm1
75 — - Rsm22/Rmd9
      - Mtg3
50 —
37 — mitoribosomal
      proteins
      • TEV protease
25 —
20 —
15 —
10 —

g

40nm

h

423 Å

i

S. cerevisiae BY4741
Mtg3-3C-GFP

MTG3 | GFP
3C-Protease site

anti-GFP
nanobody
resin

3C-protease
elution

purified
pre-ribosomal
particle

j

MW
(kDa)
250 —
150 —
100 — - Ccm1
75 — - Rsm22/Rmd9
      - Mtg3
50 —
37 — mitoribosomal
      proteins
25 —
20 — • 3C protease
15 —
10 —

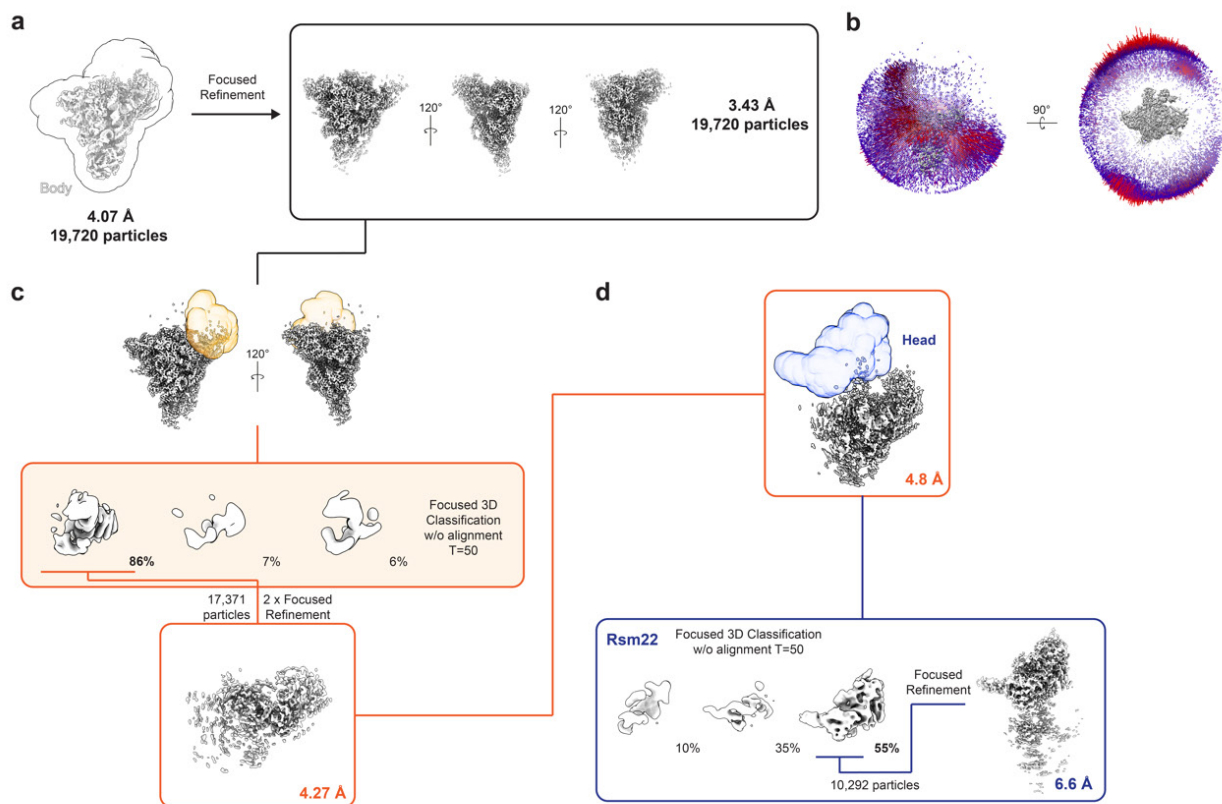**Supplementary Fig. 20. Purification of yeast mtSSU assembly intermediates.**

(**a**) Purification scheme for Ccm1-containing mitoribosomal intermediates. (**b**) Representative SDS-PAGE gel of eluted Ccm1 sample. Proteins were identified with LC-MS/MS (Supplementary Data S2). Immunoprecipitation experiments were repeated at least 3 times with similar results. (**c**) Motion corrected cryo-EM micrograph of Ccm1-bound particles representative of 31,995 total micrographs. (**d**) 6 representative 2D class averages for the Ccm1 dataset (4x binned, 4.32 Å/px). (**e**) Purification scheme for Rsm22-containing mitoribosomal intermediates. (**f**) Representative SDS-PAGE gel of eluted Rsm22 sample. Proteins were identified with LC-MS/MS (Supplementary Data S3). Immunoprecipitation experiments were repeated at least 3 times with similar results. (**g**) Motion corrected cryo-EM micrograph of Rsm22-bound particles representative of 14,111 total micrographs. (**h**) 6 representative 2D class averages for the Rsm22 dataset (8x binned, 8.64 Å/px). (**i**) Purification scheme for Mtg3-containing mitoribosomal intermediates. (**j**) Representative SDS-PAGE gel of eluted Mtg3 sample. Proteins were identified with LC-MS/MS (Supplementary Data S4). Immunoprecipitation experiments were repeated at least 3 times with similar results. For gel source data, see Supplementary Fig. 1.

Test Subset
8,189 micrographs

Total Dataset
31,995 micrographs

MotionCorr2
GCTF1.18
crYOLO Particle Picking

MotionCorr2
GCTF1.18
crYOLO Particle Picking

4.32 Å/px   830,061 particles

3,286,640 particles

2D Classification
K=100 T=2

4.32 Å/px   23,206 particles

Ab initio reconstruction Cryosparc

23%   **55%**   22%

Ab initio reconstruction Cryosparc

13%   **56%**   31%

7,263 particles
3D Refinement
Cryosparc

Reference
4.36 Å

657,328 particles   657,328 particles   657,328 particles   657,328 particles   657,328 particles   4.32 Å/px

5%   1%   8%   7%   10%   1%   2%   8%   9%   9%

9%   18%   27%   7%   7%   3%   8%   9%   8%   8%

3D Classification
w/ alignment
K=5 T=4
45 iterations

18%   13%   14%   9%   8%   10%   16%   21%   9%   18%

7%   7%   7%   11%   21%   7%   11%   2%   9%

14%   7%   **11%**   2%   **11%**   **9%**   **7%**   9%   **8%**   19%

92,688 particles   73,608 particles   126,427 particles   49,266 particles   53,687 particles

Merge & remove
duplicates

1.08 Å/px   316,817 particles

Bayesian Polishing
CTF Refinement
3D Refinement

3.13 Å

3D Classification w/o alignment
K=3 T=50
Body mask
40 iterations

Consensus

49%   **23%**   27%

3D Classification w/o alignment
K=3 T=50
Body mask
40 iterations

27%
19, 720 particles

73%
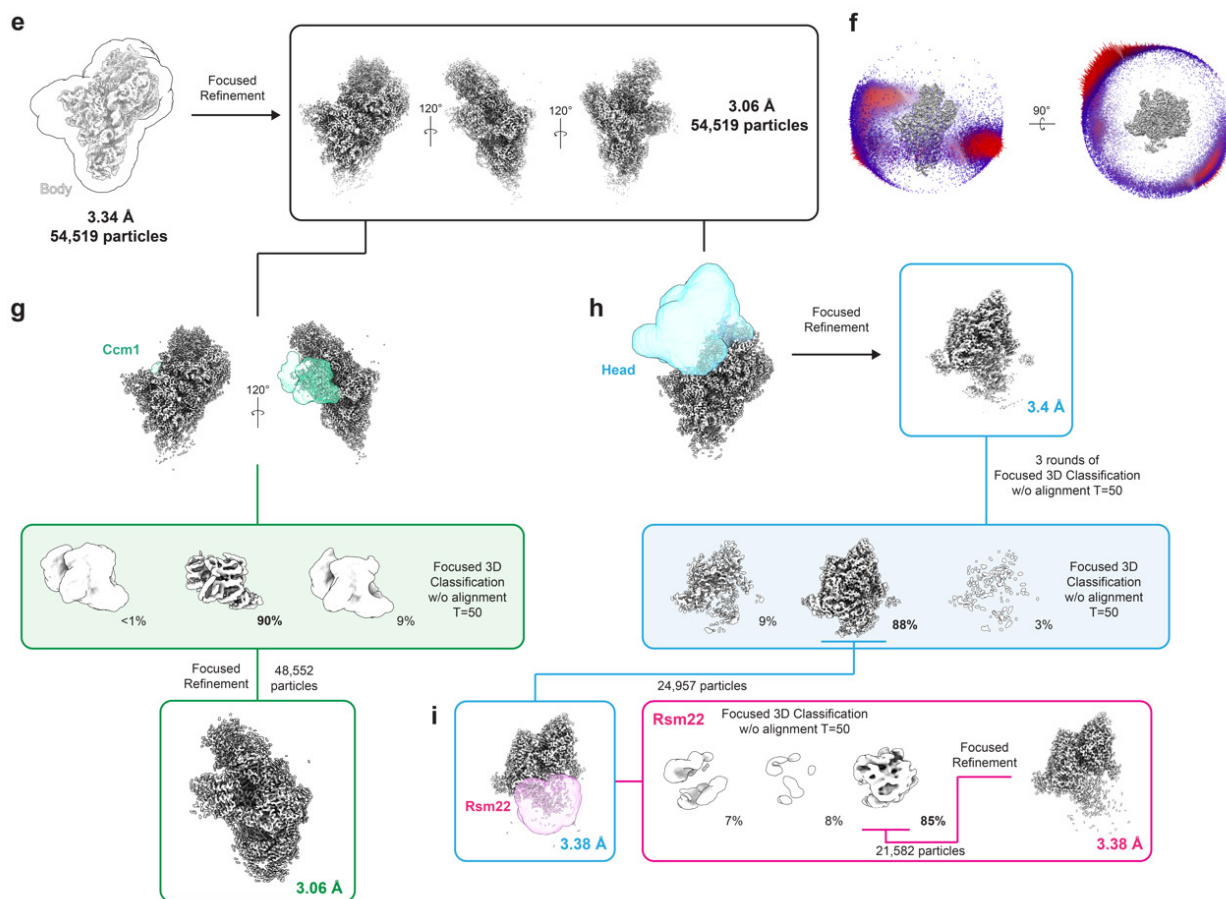**54,519 particles**

<1%

State 1   State 2

Supplementary Fig. 21. Initial cryo-EM processing of yeast Ccm1-containing particles.

Workflow for data processing and identification of two assembly states, States 1 and 2. Reported resolutions (FSC=0.143) from Relion post-processing are reported next to individual maps.
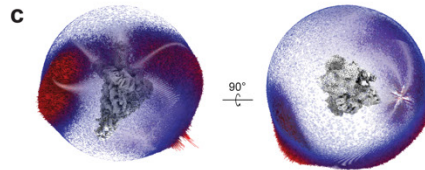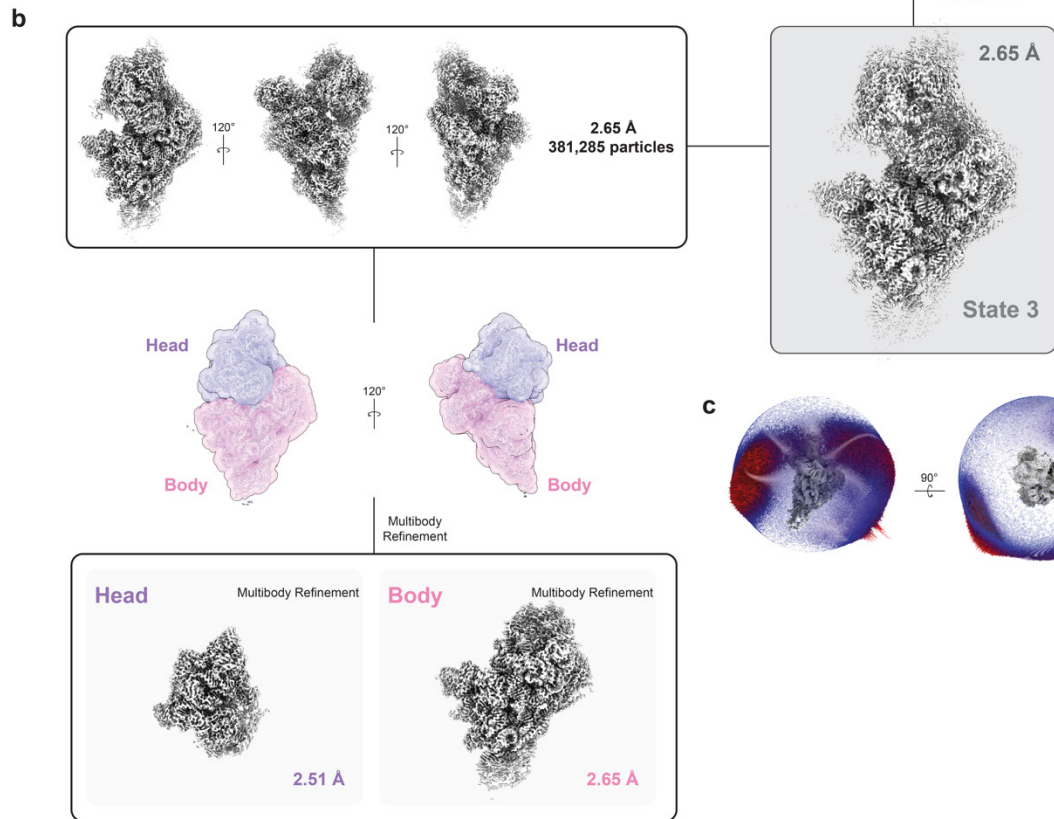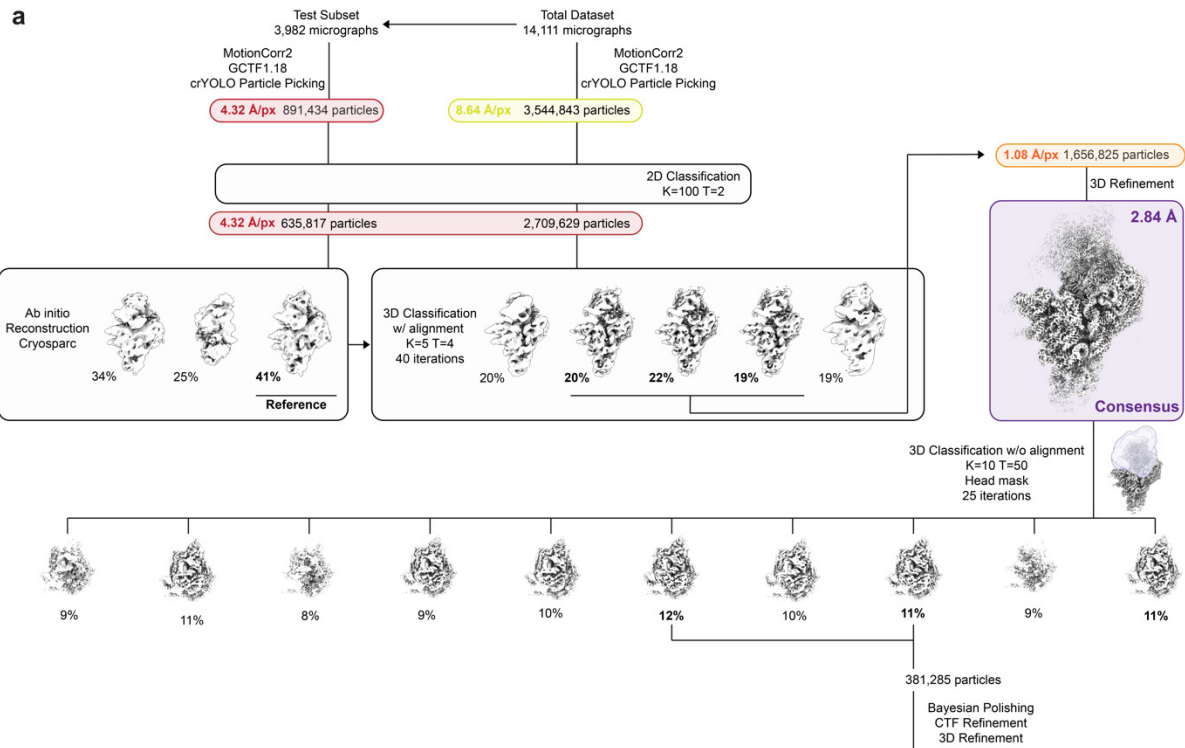
State 1

a

Body
4.07 Å
19,720 particles

Focused Refinement →

120°   120°

3.43 Å
19,720 particles

b

90°

c

120°

Focused 3D Classification w/o alignment T=50

86%   7%   6%

17,371 particles   2 x Focused Refinement

4.27 Å

d

Head

4.8 Å

Rsm22   Focused 3D Classification w/o alignment T=50

10%   35%   **55%**   Focused Refinement

10,292 particles

6.6 Å

State 2

e

Body
3.34 Å
54,519 particles

Focused Refinement →

120°   120°

3.06 Å
54,519 particles

f

90°

g

Ccm1

120°

Focused 3D Classification w/o alignment T=50

<1%   **90%**   9%

Focused Refinement   48,552 particles

3.06 Å

h

Head

Focused Refinement →

3.4 Å

3 rounds of Focused 3D Classification w/o alignment T=50

Focused 3D Classification w/o alignment T=50

9%   **88%**   3%

24,957 particles

i

Rsm22

3.38 Å

Rsm22   Focused 3D Classification w/o alignment T=50

7%   8%   **85%**   Focused Refinement

21,582 particles

3.38 Å

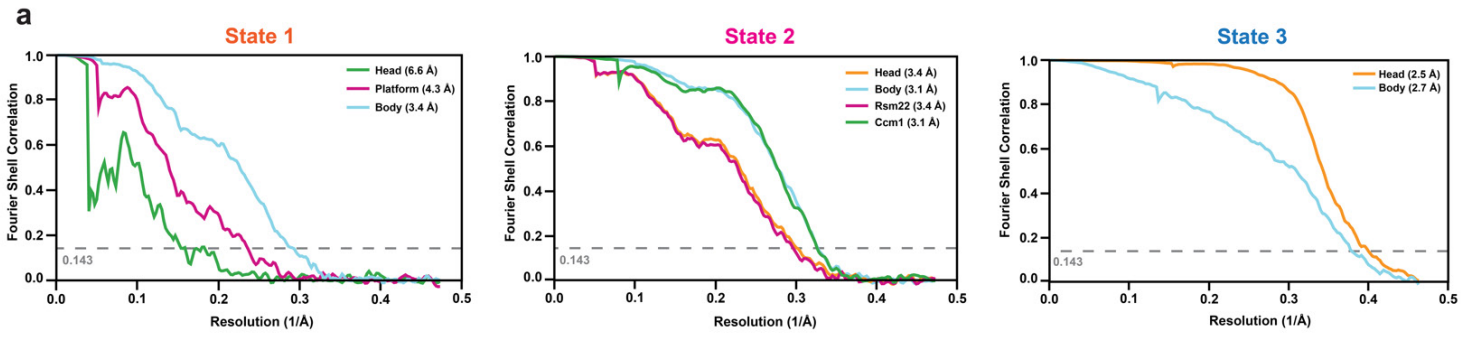**Supplementary Fig. 22. Cryo-EM processing of yeast States 1 and 2.**

(**a**) Particles identified as State 1 were refined using a mask on the body domain. (**b**) Euler angle distribution of the overall (body) refinement for State 1. (**c**) Particles were classified using a mask on the platform and subsequently refined. (**d**) Classification of these particles on the head domain and Rsm22. (**e**) Particles identified as State 2 were refined using a mask on the body domain. (**f**) Euler angle distribution of the overall (body) refinement for State 2. (**g**) Particles were classified using a mask on Ccm1 and subsequently refined using a larger mask encompassing most of the body. (**h**) Particles were refined with a mask on the head domain and further classified. (**i**) To improve fragmented density corresponding to Rsm22, this region was subjected to an additional round of masked classification and refinement. Reported resolutions (FSC=0.143) from Relion post-processing are reported next to individual focused maps. Masks used are color coded.
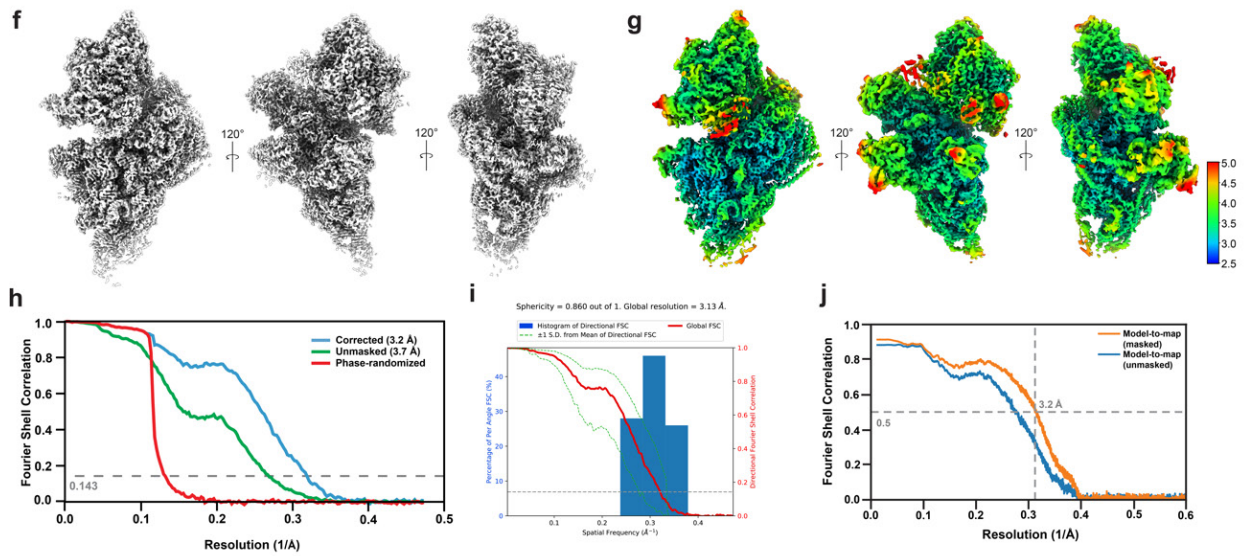
a

Test Subset
3,982 micrographs

← Total Dataset
14,111 micrographs

MotionCorr2
GCTF1.18
crYOLO Particle Picking

MotionCorr2
GCTF1.18
crYOLO Particle Picking

4.32 Å/px 891,434 particles

8.64 Å/px 3,544,843 particles

1.08 Å/px 1,656,825 particles

3D Refinement

2D Classification
K=100 T=2

4.32 Å/px 635,817 particles

2,709,629 particles

Ab initio
Reconstruction
Cryosparc

34%   25%   **41%**

Reference

3D Classification
w/ alignment
K=5 T=4
40 iterations

20% **20%** **22%** **19%** 19%

2.84 Å

Consensus

3D Classification w/o alignment
K=10 T=50
Head mask
25 iterations

9%   11%   8%   9%   10%   **12%**   10%   **11%**   9%   **11%**

381,285 particles

Bayesian Polishing
CTF Refinement
3D Refinement

b

120°   120°

2.65 Å
381,285 particles

2.65 Å

State 3

Head   Head

120°

Body   Body

Multibody
Refinement

Head Multibody Refinement   **Body** Multibody Refinement

2.51 Å   2.65 Å

c

90°

Supplementary Fig. 23. Cryo-EM processing of yeast Rsm22-containing particles (State 3).

(**a**) Workflow for initial data processing to identify particles corresponding to State 3, resulting in a 2.65 Å consensus map. (**b**) Three views of the consensus map. The resolution of the head domain was improved using multibody refinement. (**c**) Euler angle distribution of the overall (body) refinement for State 3. Reported resolutions (FSC=0.143) from Relion post-processing are reported next to individual focused maps. Masks used are color coded.

a

State 1

State 2

State 3

b

Body

Platform

Head

State 1

Body

Ccm1

Head

Rsm22

State 2

Body

Head

State 3

Supplementary Fig. 24. Local resolution analysis of yeast reconstructions.

(**a**) Solvent-corrected FSC curves for each focused refinement determined at FSC=0.143 via post-processing in Relion. (**b**) Local resolution estimation (calculated in Relion) for each focused map in context with the rest of the reconstruction.
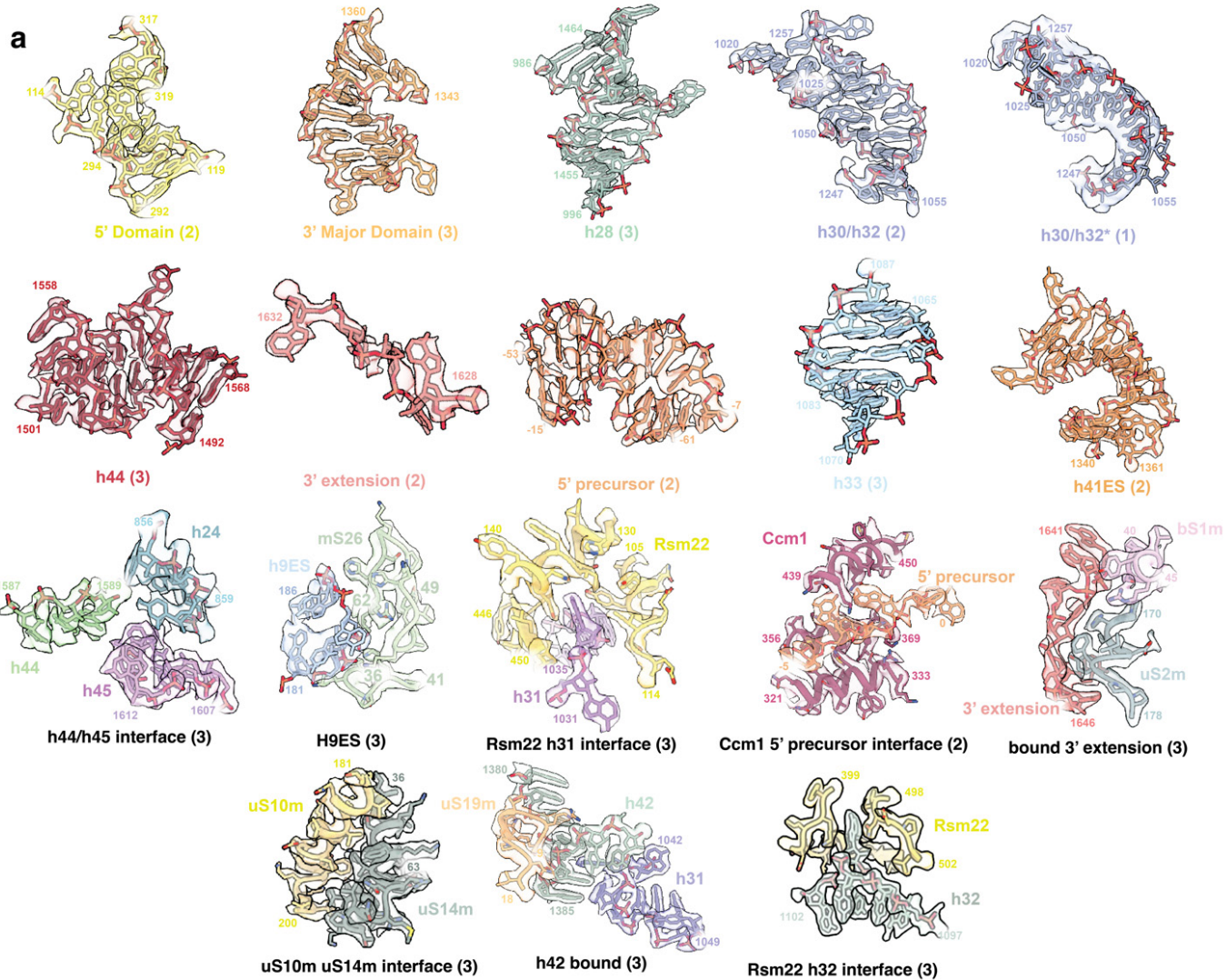
State 1

State 2

State 3

Supplementary Fig. 25. Validation of composite maps for yeast States 1-3.
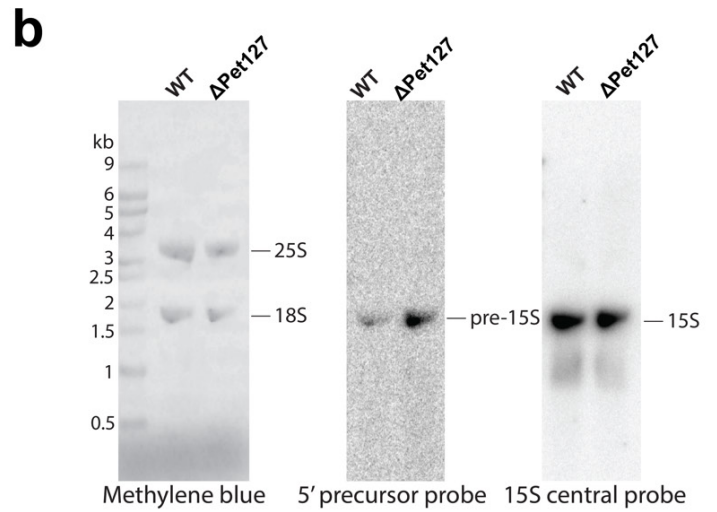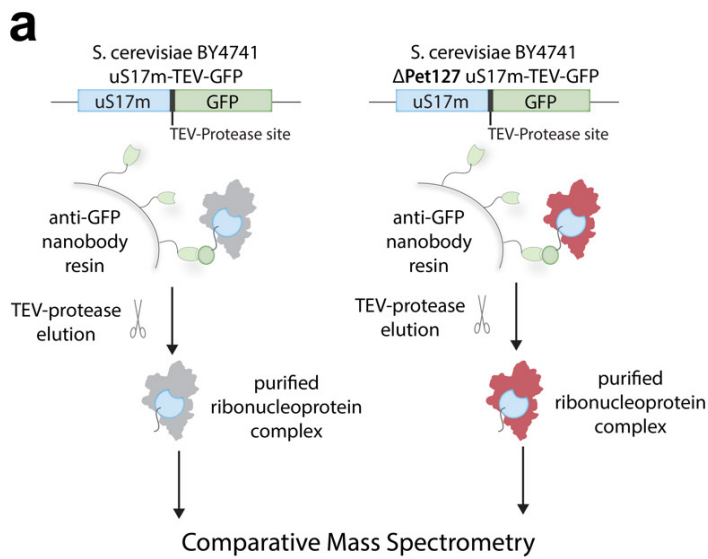
(**a**) Different views of the composite map of State 1. (**b**) Different views of the composite map of State 1 filtered and colored according to local resolution as calculated by cryoSPARC. (**c**) FSC curves for the State 1 composite map as calculated in Relion. Reported resolutions were determined at FSC=0.143. (**d**) 3DFSC for the State 1 composite map calculated using the Remote 3DFSC Processing Server[1]. (**e**) Map-to-model correlation between the State 1 composite map and the State 1 model. (**f**) Different views of the composite map of State 2. (**g**) Different views of the composite map of State 2 filtered and colored according to local resolution as calculated by cryoSPARC. (**h**) FSC curves for the State 2 composite map as calculated in Relion. Reported resolutions were determined at FSC=0.143. (**i**) 3DFSC for the State 2 composite map calculated using the Remote 3DFSC Processing Server[1]. (**j**) Map-to-model correlation between the State 2 composite map and the State 2 model. (**k**) Different views of the composite map of State 3. (**l**) Different views of the composite map of State 3 filtered and colored according to local resolution as calculated by cryoSPARC. (**m**) FSC curves for the State 3 composite map as calculated in Relion. Reported resolutions were determined at FSC=0.143. (**n**) 3DFSC for the State 3 composite map calculated using the Remote 3DFSC Processing Server[1]. (**o**) Map-to-model correlation between the State 3 composite map and the State 3 model.

a

5' Domain (2)

3' Major Domain (3)

h28 (3)

h30/h32 (2)

h30/h32* (1)

h44 (3)

3' extension (2)

5' precursor (2)

h33 (3)

h41ES (2)

h44/h45 interface (3)

H9ES (3)

Rsm22 h31 interface (3)

Ccm1 5' precursor interface (2)

bound 3' extension (3)

uS10m uS14m interface (3)

h42 bound (3)

Rsm22 h32 interface (3)

b

Rsm22 (2)

Rsm22* (1)

Rsm22 c-terminal extension* (1)

Ccm1 (2)

mS35 (2)

bS6m (3)

uS13m (3)

uS12m (1)

uS5m (1)

Unknown Protein (2)

c

mS29 nucleotide (3)

Rsm22 ligand (3)

Supplementary Fig. 26. Representative cryo-EM densities and models for yeast structures.

Selections of representative densities of RNA and protein across all states, comprised of (**a**) rRNA and functionally relevant protein-RNA and protein-protein interfaces, (**b**) assembly factors and mitoribosomal proteins, and (**c**) ligands. The states from which density and models are derived are shown in parenthesis below each panel, and * denotes maps that have been filtered to local resolution.

Supplementary Fig. 27 - Pet127 knockout results in accumulation of pre-15S rRNA and a decrease in bound assembly factors.

(**a**) Experimental scheme for comparative IP-MS/MS in WT and PET127 KO yeast cells.

(**b**) Northern blots of extracted RNA after immunoprecipitation of uS17m-bound complexes. Northern blotting experiments were performed twice with similar results. (**c**) SDS-PAGE gel of eluted complexes after uS17m pulldown in triplicate. Assembly factors identified by mass spectrometry that are significantly decreased in Pet127 KO samples are labeled. (**d**) Volcano plot for comparative IP-MS/MS of WT and PET127 KO cells using uS17m as bait. IP-MS/MS experiments were performed in triplicate for each condition (n=3 biologically independent experiments). A 2-sided student t-test was used to determine p-values for changes in protein abundance between WT and PET127 KO mtSSU purification experiments. Individual proteins are shown as circles, with blue circles indicating mtSSU proteins, orange circles indicating mtLSU proteins, green circles indicating proteins involved in translational activation, and purple circles indicating mtSSU assembly factors. Raw data is available in Supplementary Data S5. For gel source data, see Supplementary Fig. 1.

SUPPLEMENTARY REFERENCES

1.   Tan, Y. Z. *et al.* Addressing preferred specimen orientation in single-particle cryo-EM through tilting. *Nat. Methods* **14,** 793–796 (2017).