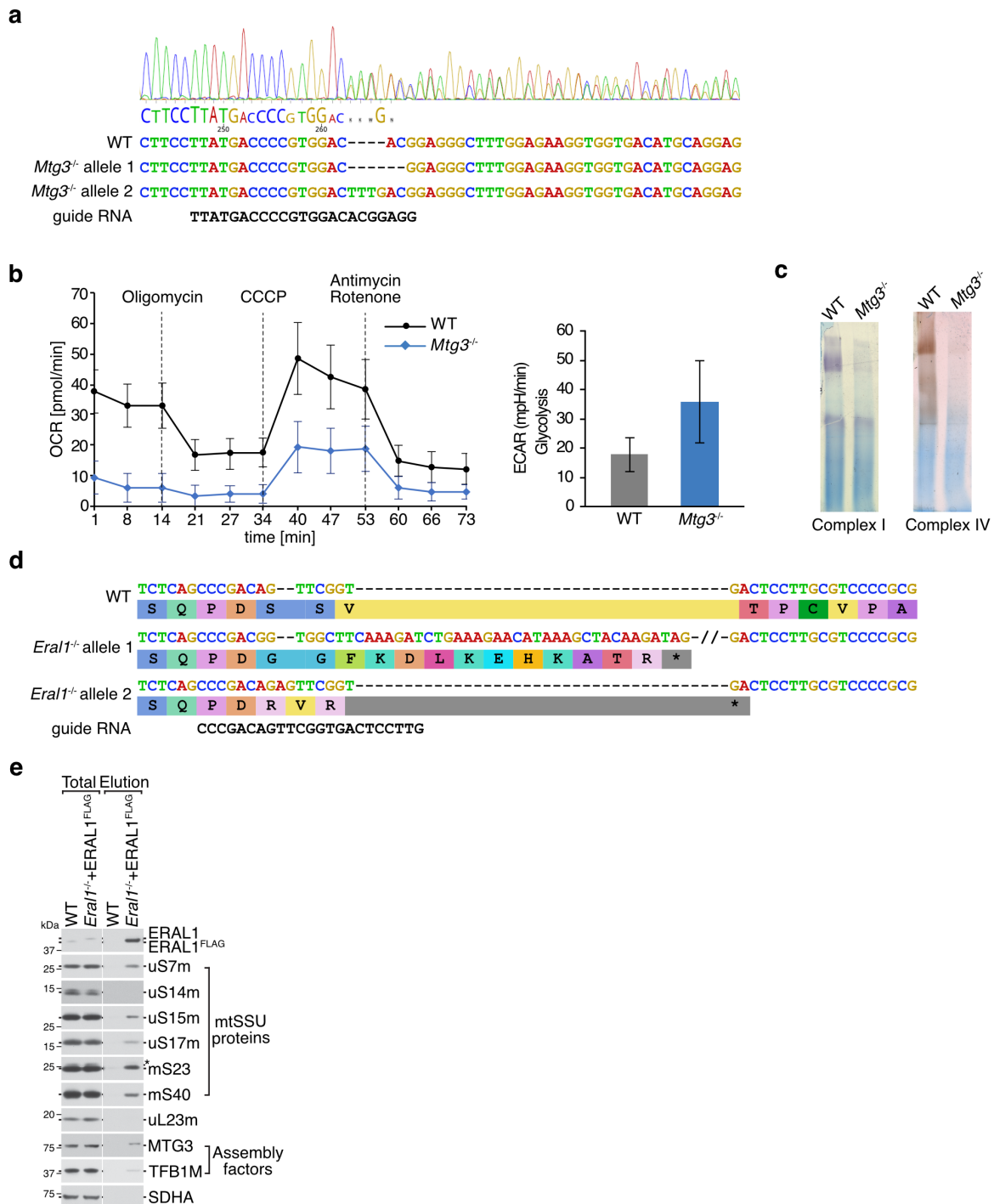


Supplementary information

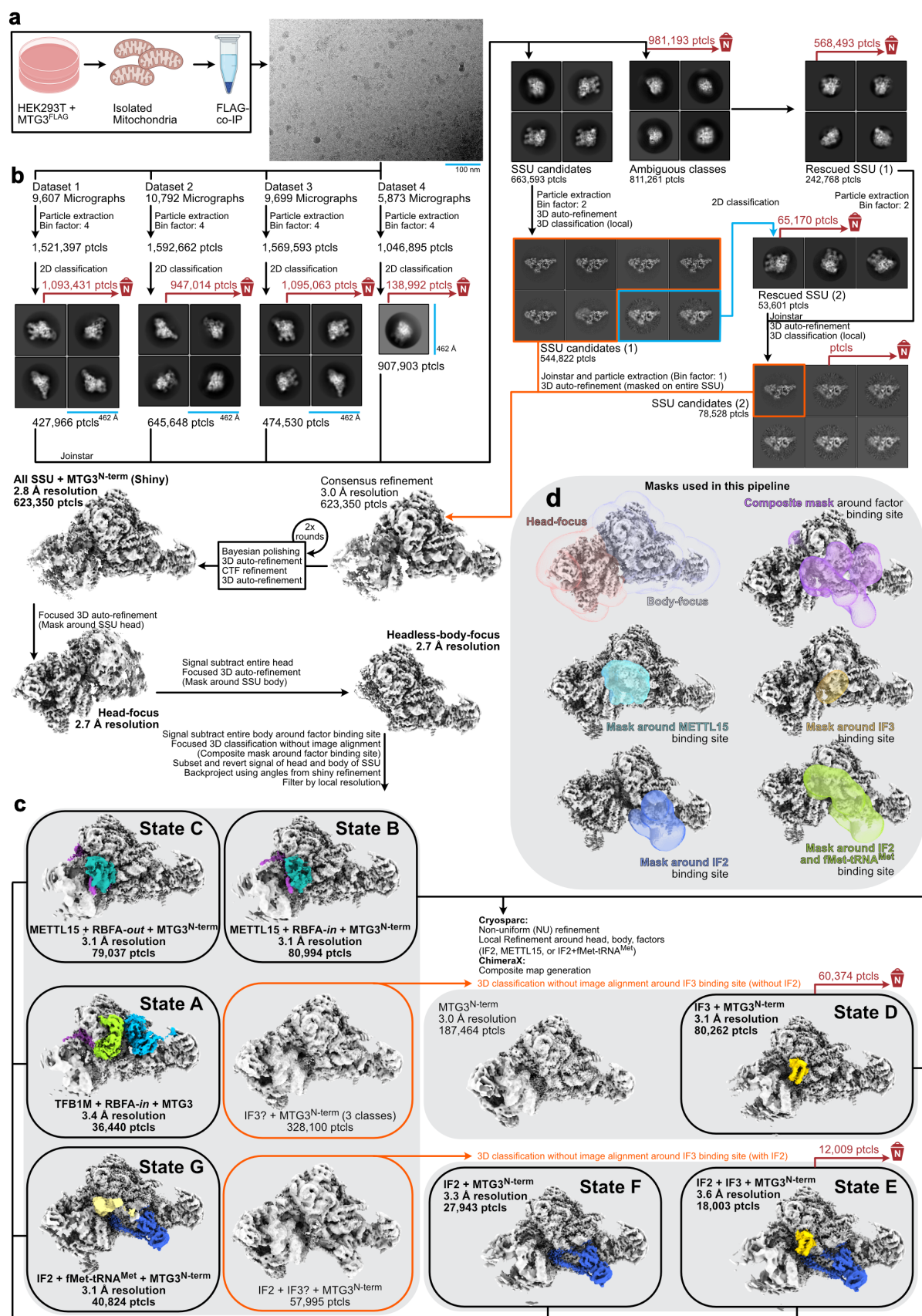
SUPPLEMENTARY FIGURES



Supplementary Fig. 1

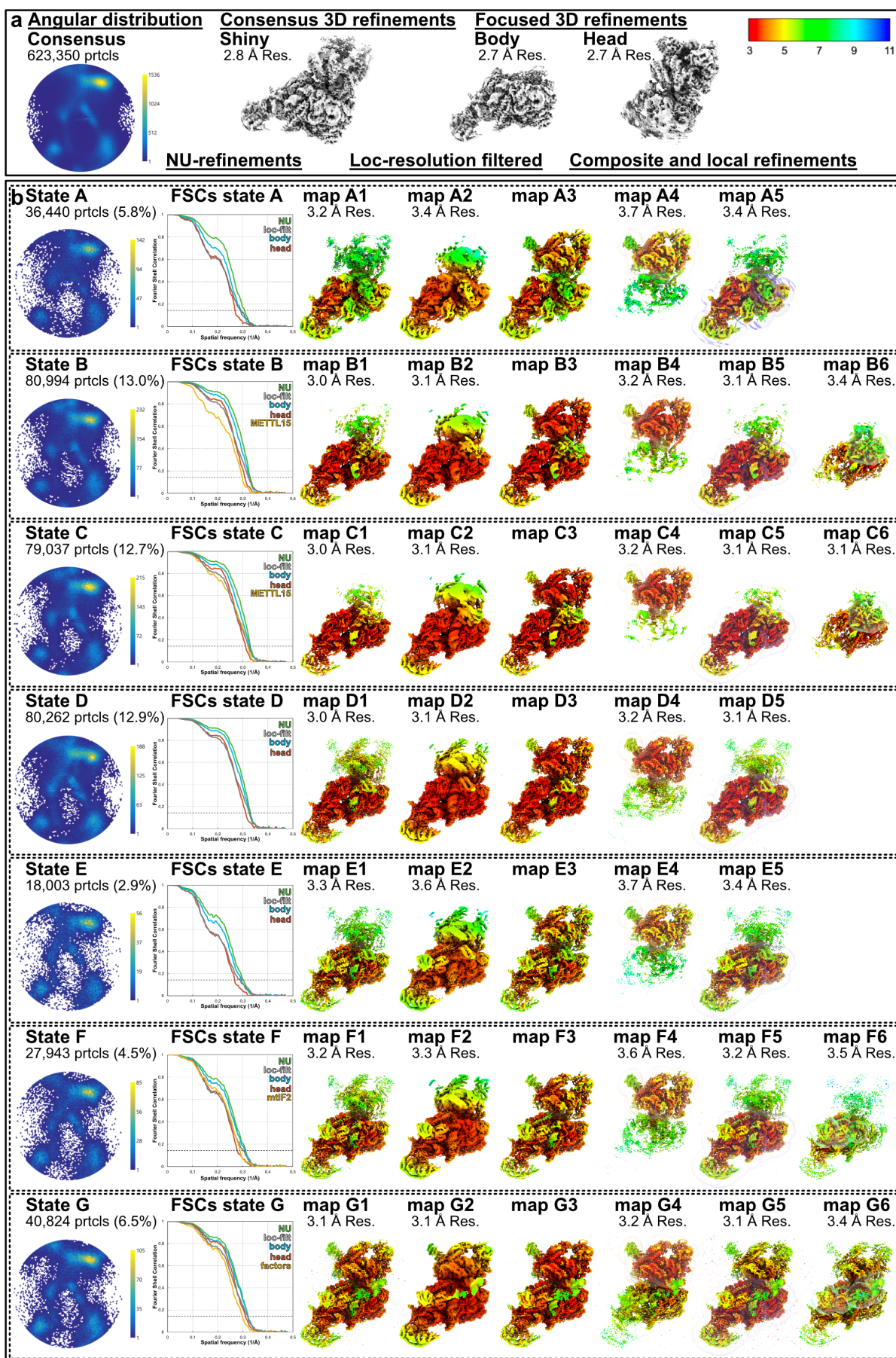
(a) Genomic sequencing of the *Mtg3^{-/-}* cell line. Genomic DNA was isolated from wild type and *Mtg3^{-/-}* cl.1 and the guide RNA target region amplified via PCR. PCR products were analyzed via TOPO cloning and subsequent sequencing. Chromatogram shows the target region with the corresponding sequence of the wild type cells and the two mutated alleles in the *Mtg3^{-/-}*. **(b)** Real time respiratory measurements. Left: Representative graph of the oxygen consumption rate (OCR) analyzed for wild

type and *Mtg3*^{-/-} cells. Reagents allowing the measurement of different metabolic conditions were added at indicated time points. Right: quantification of glycolysis activity. (mean ± SD, n= 16 technical replicates). **(c)** In gel activity analysis of complex I and complex IV. Solubilized mitochondria from wild type and *Mtg3*^{-/-} cells were loaded on a 3 %-10 % polyacrylamide gel and BN-PAGE was performed. Subsequently, gels were incubated in complex I or IV activity solutions, respectively. Similar results were obtained in n ≥ 3 biologically independent experiments. **(d)** Genomic sequencing of the *Eral1*^{-/-} cell line used for transfection with an ERAL1^{FLAG} construct for subsequent FLAG immunoprecipitation. Sequencing result was obtained as described in (a). The KO cell line was generated by using a guide RNA targeting the first exon of the *Eral1* gene. A two bp insertion and a 123 bp insertion in the two alleles lead to premature stop codons and truncated proteins. **(e)** Western blot analysis of mitoribosome complexes co-purified via ERAL1^{FLAG}. FLAG-immunoprecipitation was performed with lysed mitochondria from wild type cells and *Eral1*^{-/-} cells inducibly expressing ERAL1^{FLAG}. Samples (total = 3 %, eluate = 100 %) were subsequently analyzed via western blotting with antibodies as indicated. Similar results were obtained in n ≥ 3 biologically independent experiments.



Supplementary Fig. 2 Cryo-EM reconstruction pipeline. (a) Isolation of mitoribosomes and exemplary cryo-EM micrograph. (b) Cryo-EM processing workflow for structure determination. Exemplary aligned class averages from 2D and initial 3D classifications are shown. (c) Local resolution filtered maps of the final subsets. Additional densities for the factors are highlighted in the following

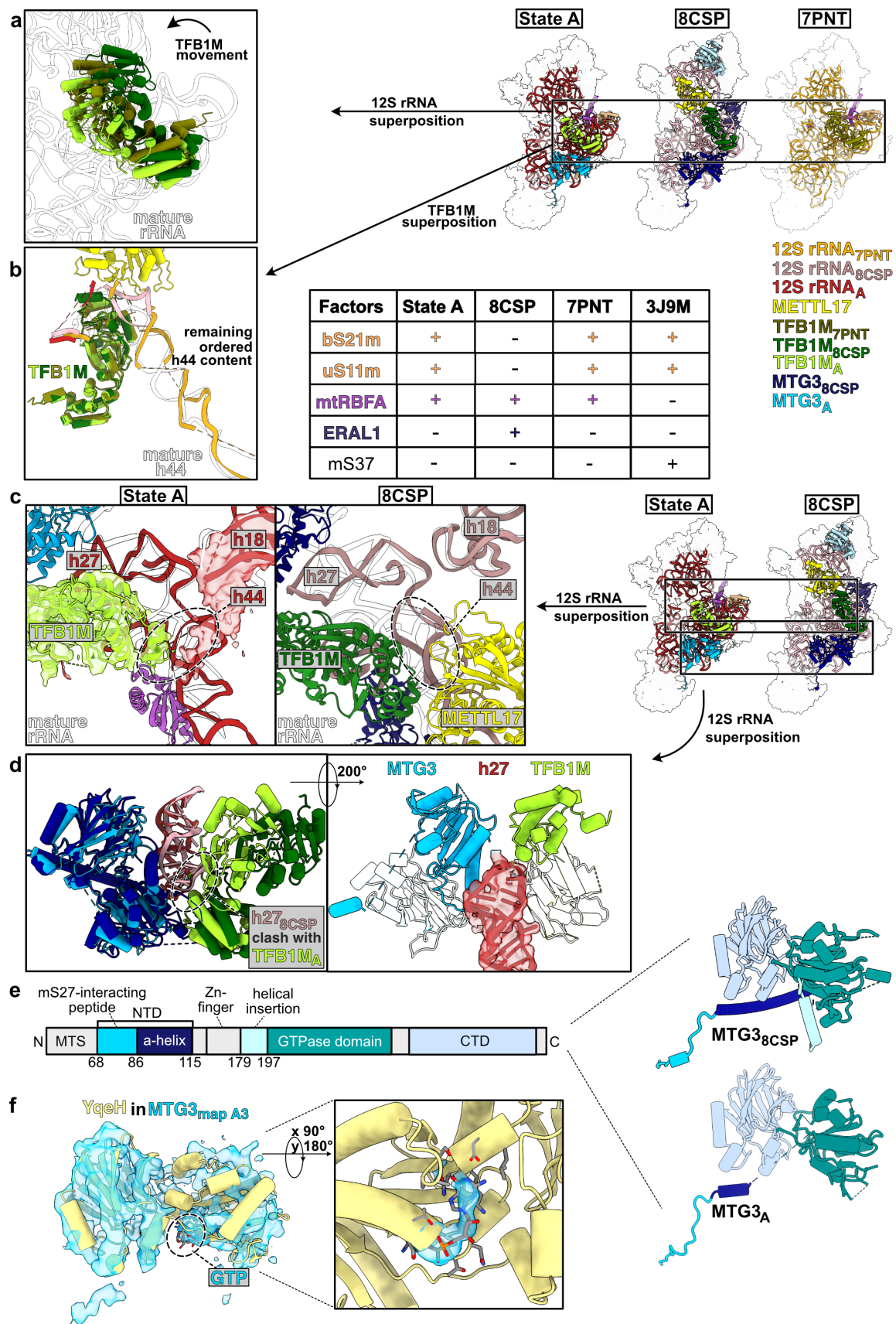
colors: MTG3: light blue, TFB1M: lime green, mtRBFA: indigo, METTL15: turquoise, mtIF3: yellow, mtIF2: blue, fMet-tRNA^{Met}: light yellow. **(d)** Local masks as utilized in the processing workflow. Created in BioRender. Finke, F. (2025) <https://BioRender.com/l09i305>.



Supplementary Fig. 3 Overall-refined maps for each state.

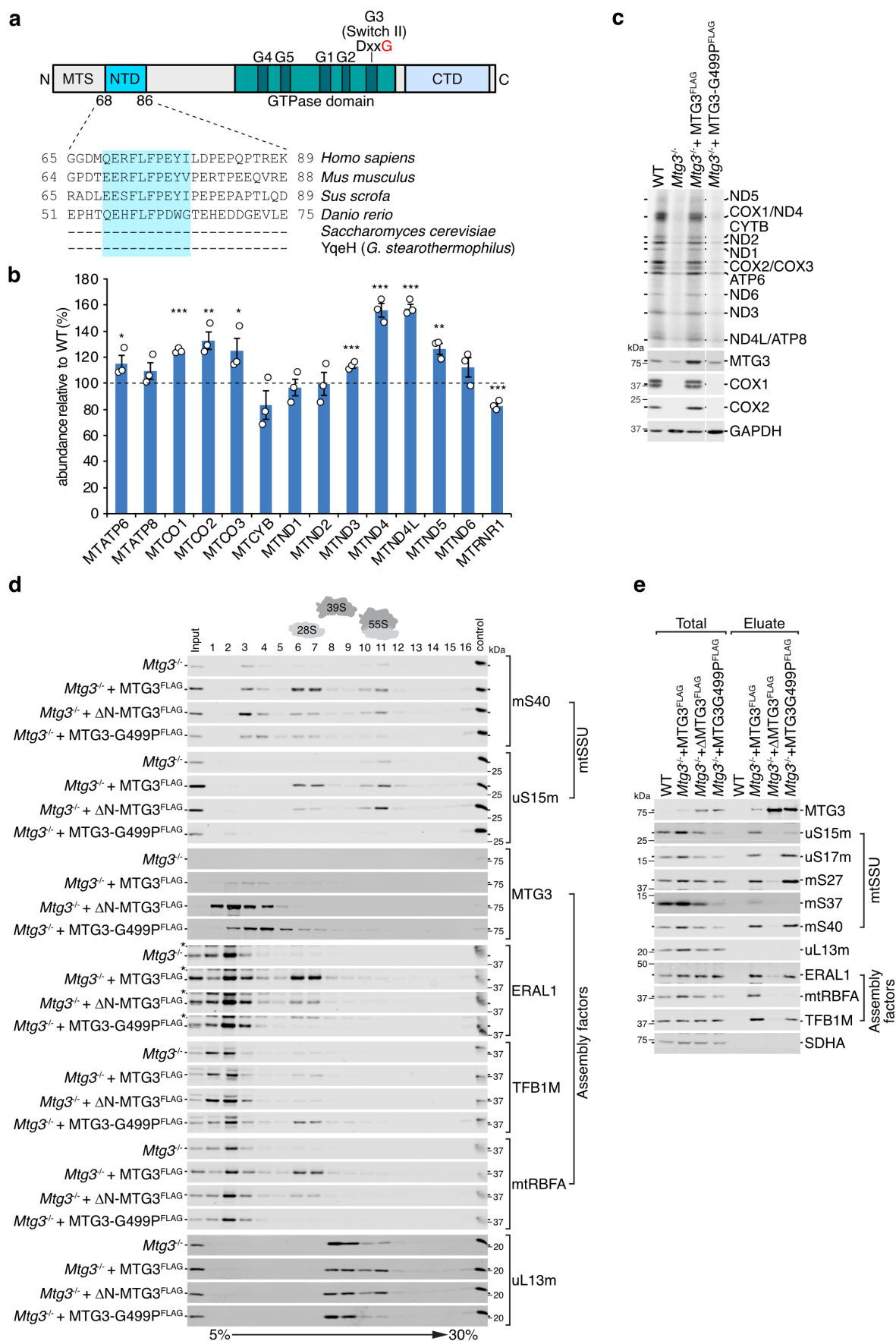
(a) Consensus refinement (unfocused, body-focused, head-focused) and angular distribution plot for all mtSSU particles. **(b)** Local resolution estimation of non-uniform refinements from cryoSPARC, local resolution filtered maps (B-Factor 0) from Relion of each state, as well as composite maps together with corresponding local refinements from cryoSPARC together with corresponding FSC-plots and angular distribution plots for each state.

C1486 from mature mtSSU is shown. **(a-b)** Depiction as followed: states A-C vs PDB: 7PO2; ¹⁹: surface: not shown vs white, 12S rRNA: red vs orange; MTG3: blue, METTL15: turquoise, mtRBFA-CTE: indigo.



Supplementary Fig. 5 Structural details of state A.

(a) Composition of the shoulder region in state A and resulting re-orientation of TFB1M compared to previously reported states (PDB: 7PNT; ¹⁹, PDB: 8CSP; ²⁰). **(b)** Comparison of resolved h44-h45 linker regions in the published states in **(a)** compared to state A in this study by superposition of TFB1M from all states. **(c)** Composition of h44-h45 linker region in state A (density from map A3) and the previously published state (PDB: 8CSP; ²⁰) **(d)** Close-up view of h27 in its mature conformation from our state A in density from map A3 (right) vs superposition with immature h27 from already known state (left) (PDB: 8CSP; ²⁰). **(e)** Schematic depiction of the primary structure of MTG3 and highlighted areas as depicted in published (PDB: 8CSP; ²⁰) and our models. **(f)** Superposition of YqeH (PDB: 3EC1; ²⁵) in MTG3 density from map A3 (state A) and close-up view of YqeH GTP-binding site containing GDP overlaid with density from map A3.



(a) Schematic representation of MTG3 domains and sequence alignment of the N-terminal region. MTS (mitochondrial targeting sequence), N-terminal domain, zink finger domain and GTPase domain are indicated as boxes. The location of G499P mutation in the G3 motif is indicated in red. Sequence alignment of the conserved N-terminal region with the 10 aa (transparent blue box) interacting with mS27 and displacing h44 is shown below. **(b)** Abundance of total mt-mRNAs is similar in the *Mtg3*^{-/-} + ΔN-MTG3^{FLAG} cell line in comparison to wild type MTG3^{FLAG}. Total mitochondrial lysates (‘input’ from sucrose gradients from (Fig. 5c)) were used to isolate RNA and perform NanoString analysis. Level of mt-mRNAs were normalized to 16S-rRNA and RNA abundance in the *Mtg3*^{-/-} + ΔN-MTG3^{FLAG} cell line was calculated relative to *Mtg3*^{-/-} + MTG3^{FLAG} cell line (n=3 biologically independent experiments shown as mean ± SEM; individual data points are shown as circles). Statistical analysis was performed as two-sample one-tailed Student’s t-test. Significance was defined as *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. **(c)** MTG3-G499P^{FLAG} mutant cannot restore mitochondrial translation. Translation of mtDNA-encoded proteins in wild type, *Mtg3*^{-/-} and *Mtg3*^{-/-} cell lines expressing full length (MTG3^{FLAG}) or mutated MTG3 (MTG3-G499P^{FLAG}), respectively, were analyzed using [³⁵S]Methionine *de novo* incorporation and visualized via autoradiography and western blotting. GAPDH was used as a loading control. Similar results were obtained in n ≥ 3 biologically independent experiments. **(d)** Comparison of monosome and mtSSU formation in MTG3 mutants. Mitoplasts (500 μg) were isolated from *Mtg3*^{-/-} and *Mtg3*^{-/-} cell lines expressing full length (MTG3^{FLAG}), ΔN-MTG3^{FLAG} or MTG3-G499P^{FLAG}, respectively. Mitoribosomal complexes were separated via sucrose density gradient centrifugation and collected fractions (1-16) were analyzed via western blotting with indicated antibodies against MRPs and assembly factors. Similar results were obtained in n ≥ 3 biologically independent experiments. **(e)** MTG3-G499P^{FLAG} can co-isolated early-stage mtSSU intermediates. FLAG-immunoprecipitation was performed with wild type cells and *Mtg3*^{-/-} cells expressing MTG3FLAG, ΔN-MTG3^{FLAG} or MTG3-G499P, respectively. Samples were analyzed using western blotting and antibodies as indicated (total = 3 %, eluate = 100 %). Similar results were obtained in n ≥ 3 biologically independent experiments.

SUPPLEMENTARY TABLES

Supplementary Table 1: Cryo-EM data collection for all states, and refinement and validation for state A-C.

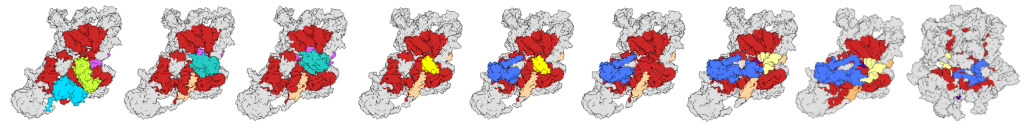
| Table 1. Cryo-EM data collection, refinement, and validation statistics | | | | | | |
|---|-------------------------------------|--------|---|--------|---|--------|
| Data collection and processing | | | | | | |
| Magnification | 81,000x | | Pixel size (Å) | | 1.05 | |
| Voltage (kV) | 300 | | Symmetry imposed | | C1 | |
| Electron exposure (e ⁻ /Å ²) | 40 | | Initial particle images (no.) | | 5,730,547 | |
| Defocus range (μm) | 0.6-1.6 | | | | | |
| | Map A1 | Map A2 | Map A3 | Map A4 | Map A5 | |
| EMDB ID | 51066 | 51053 | 51083 | 51064 | 51065 | |
| Final particle images (no.) | 36,440 | 36,440 | – | 36,440 | 36,440 | |
| Map resolution (Å) | 3.2 | 3.4 | – | 3.7 | 3.4 | |
| FSC threshold | 0.143 | 0.143 | – | 0.143 | 0.143 | |
| Map sharpening <i>B</i> factor (Å ²) | – | – | – | – | – | |
| | Map B1 | Map B2 | Map B3 | Map B4 | Map B5 | Map B6 |
| EMDB ID | 51063 | 51052 | 51084 | 51061 | 51062 | 51060 |
| Final particle images (no.) | 80,994 | 80,994 | – | 80,994 | 80,994 | 80,994 |
| Map resolution (Å) | 3.0 | 3.1 | – | 3.2 | 3.1 | 3.4 |
| FSC threshold | 0.143 | 0.143 | – | 0.143 | 0.143 | 0.143 |
| Map sharpening <i>B</i> factor (Å ²) | – | – | – | – | – | – |
| | Map C1 | Map C2 | Map C3 | Map C4 | Map C5 | Map C6 |
| EMDB ID | 51059 | 51051 | 51085 | 51057 | 51058 | 51056 |
| Final particle images (no.) | 79,037 | 79,037 | – | 79,037 | 79,037 | 79,037 |
| Map resolution (Å) | 3.0 | 3.1 | – | 3.2 | 3.1 | 3.1 |
| FSC threshold | 0.143 | 0.143 | – | 0.143 | 0.143 | 0.143 |
| Map sharpening <i>B</i> factor (Å ²) | – | – | – | – | – | – |
| Refinement | PDB: 9G5B | | PDB: 9G5C | | PDB: 9G5D | |
| Model resolution (Å) (A-C2) | 3.0 | | 2.8 | | 2.8 | |
| FSC threshold | 0.143 | | 0.143 | | 0.143 | |
| Model composition | | | | | | |
| Non-hydrogen atoms | 68,043 | | 67,559 | | 68,810 | |
| Protein residues | 6,226 | | 5,965 | | 6,049 | |
| Nucleotide residues | 817 | | 891 | | 918 | |
| Ligands | ATP: 1, ZN: 1, FES: 2, MG: 12 | | ATP: 1, ZN: 1, FES: 2, K: 4, MG: 23 | | ATP: 1, ZN: 1, FES: 2, NAD: 1, K: 1, MG: 10 | |
| Mean <i>B</i> factors (Å ²) (A-C2) | | | | | | |
| Protein | 95.82 | | 71.32 | | 73.64 | |
| Nucleotide | 121.73 | | 78.01 | | 79.32 | |
| Ligand | 112.03 | | 83.43 | | 87.23 | |
| RMSDs | | | | | | |
| Bond lengths (Å) | 0.004 | | 0.003 | | 0.004 | |
| Bond angles (°) | 0.810 | | 0.722 | | 0.727 | |
| Validation | | | | | | |
| MolProbity score | 1.46 | | 1.28 | | 1.27 | |
| Clashscore | 5.20 | | 5.30 | | 5.06 | |
| Poor rotamers (%) | 0.00 | | 0.00 | | 0.02 | |
| Ramachandran plot | | | | | | |
| Favored (%) | 96.89 | | 98.22 | | 98.14 | |
| Allowed (%) | 3.11 | | 1.78 | | 1.84 | |
| Disallowed (%) | 0.02 | | 0.00 | | 0.02 | |

Supplementary Table 2: Cryo-EM data refinement and validation for state D-G.

| Table 2. Cryo-EM data refinement, and validation statistics | | | | | | |
|---|---|--|--|--------|--|--------|
| Data processing | | | | | | |
| | Map D1 | Map D2 | Map D3 | Map D4 | Map D5 | |
| EMDB ID | 51047 | 51046 | 52117 | 52063 | 52062 | |
| Final particle images (no.) | 80,262 | 80,262 | – | 80,262 | 80,262 | |
| Map resolution (Å) | 3.0 | 3.1 | – | 3.2 | 3.1 | |
| FSC threshold | 0.143 | 0.143 | – | 0.143 | 0.143 | |
| Map sharpening <i>B</i> factor (Å ²) | –60.8 | – | – | –60.4 | –56.0 | |
| | Map E1 | Map E2 | Map E3 | Map E4 | Map E5 | |
| EMDB ID | 51045 | 51043 | 52118 | 52065 | 52064 | |
| Final particle images (no.) | 18,003 | 18,003 | – | 18,003 | 18,003 | |
| Map resolution (Å) | 3.3 | 3.6 | – | 3.7 | 3.4 | |
| FSC threshold | 0.143 | 0.143 | – | 0.143 | 0.143 | |
| Map sharpening <i>B</i> factor (Å ²) | –36.8 | – | – | –41.1 | –35.8 | |
| | Map F1 | Map F2 | Map F3 | Map F4 | Map F5 | Map F6 |
| EMDB ID | 51055 | 51050 | 51086 | 51049 | 51054 | 51048 |
| Final particle images (no.) | 27,943 | 27,943 | – | 27,943 | 27,943 | 27,943 |
| Map resolution (Å) | 3.2 | 3.3 | – | 3.6 | 3.2 | 3.5 |
| FSC threshold | 0.143 | 0.143 | – | 0.143 | 0.143 | 0.143 |
| Map sharpening <i>B</i> factor (Å ²) | –43.6 | – | – | –47.3 | –42.1 | –65.1 |
| | Map G1 | Map G2 | Map G3 | Map G4 | Map G5 | Map G6 |
| EMDB ID | 51042 | 51039 | 52119 | 52067 | 52066 | 52068 |
| Final particle images (no.) | 40,824 | 40,824 | – | 40,824 | 40,824 | 40,824 |
| Map resolution (Å) | 3.1 | 3.1 | – | 3.2 | 3.1 | 3.4 |
| FSC threshold | 0.143 | 0.143 | – | 0.143 | 0.143 | 0.143 |
| Map sharpening <i>B</i> factor (Å ²) | –52.5 | – | – | –52.3 | –49.2 | –59.6 |
| Refinement | PDB: 9HFM | PDB: 9HFN | PDB: 9G5E | | PDB: 9HFO | |
| Model resolution (Å) (D-G2) | 2.8 | 2.9 | 2.9 | | 2.8 | |
| FSC threshold | 0.143 | 0.143 | 0.143 | | 0.143 | |
| Model composition | | | | | | |
| Non-hydrogen atoms | 66,106 | 69,310 | 68,649 | | 71,379 | |
| Protein residues | 5,756 | 6,169 | 6,088 | | 6,217 | |
| Nucleotide residues | 897 | 897 | 897 | | 979 | |
| Ligands | ATP: 1, ZN: 1, FES: 2, K: 1, MG: 44 | ATP: 1, GTP: 1 ZN: 1, FES: 2, K: 1, MG: 45 | ATP: 1, GTP: 1 ZN: 1, FES: 2, K: 1, MG: 45 | | ATP: 1, GTP: 1 ZN: 1, FES: 2, K: 1, MG: 45 | |
| Mean <i>B</i> factors (Å ²) (D-G2) | | | | | | |
| Protein | 146.73 | 103.71 | 102.61 | | 127.45 | |
| Nucleotide | 97.43 | 94.80 | 94.02 | | 77.39 | |
| Ligand | 69.12 | 44.69 | 44.24 | | 126.82 | |
| RMSDs | | | | | | |
| Bond lengths (Å) | 0.005 | 0.004 | 0.003 | | 0.004 | |
| Bond angles (°) | 0.808 | 0.811 | 0.798 | | 0.746 | |
| Validation | | | | | | |
| MolProbity score | 1.34 | 1.47 | 1.35 | | 1.26 | |
| Clashscore | 4.16 | 5.63 | 4.39 | | 4.96 | |
| Poor rotamers (%) | 0.00 | 0.00 | 0.00 | | 0.00 | |
| Ramachandran plot | | | | | | |
| Favored (%) | 97.27 | 97.07 | 97.30 | | 98.58 | |
| Allowed (%) | 2.70 | 2.93 | 2.68 | | 1.40 | |
| Disallowed (%) | 0.04 | 0.00 | 0.02 | | 0.02 | |

Supplementary Table 3: Events in the biogenesis pathway for each resolved mtSSU state in this study.

Matured helices and presence of certain assembly and translation initiation factors are marked with a plus symbol. States for comparison are taken from ¹⁹ (PDB: 7PO2) and ⁵³ (PDB: 6GAW).



| | State A | State B | State C | State D | State E | State F | State G | 7PO2 | 6GAW |
|----------------------------------|---------|---------|---------|---------|---------|---------|---------|------|------|
| matured? | | | | | | | | | |
| h18 | - | + | + | + | + | + | + | + | + |
| h24 | - | - | - | + | + | + | + | + | + |
| h45 | - | - | - | + | + | + | + | + | + |
| h44-h45 | - | - | - | + | + | + | + | + | + |
| h44 | - | - | - | - | - | - | - | + | + |
| present? | | | | | | | | | |
| MTG3 | + | - | - | - | - | - | - | - | - |
| MTG3^{N-term} | + | + | + | + | + | + | + | - | - |
| mS38 | - | + | + | + | + | + | + | + | + |
| RBFA^{(In)/(out)} | + | + | + | - | - | - | - | - | - |
| METTL15 | - | + | + | - | - | - | - | - | - |
| mS37 | - | - | - | + | + | + | + | + | + |
| mtlF3 | - | - | - | + | + | - | - | - | - |
| mtlF2 | - | - | - | - | + | + | + | + | + |
| fMet-tRNA^{Met} | - | - | - | - | - | - | + | + | + |
| mRNA^{AUG} | - | - | - | - | - | - | + | + | + |
| mtLSU | - | - | - | - | - | - | - | - | + |

Supplementary Table 4: Key Resource Table

| Reagent or resource | Supplier | Identifier |
|---|---------------------------|------------------------------|
| Antibodies | | |
| Rabbit polyclonal anti-C4orf14 (MTG3, 1:1000) | Invitrogen | PA5-57420 |
| Rabbit polyclonal anti-ERAL1 (1:2000) | ProteinTech | 11478-1-AP |
| Rabbit polyclonal anti-TFB1M (1:1000) | ProteinTech | 16604-1-AP |
| Rabbit polyclonal anti-RBFA (1:1000) | Novus Biologicals | NBP1-88471 |
| Rabbit polyclonal anti-NSUN4 (1:1000) | ProteinTech | 16320-1-AP |
| Rabbit polyclonal anti-mtIF2 (1:1000) | This study | PRAB5207 |
| Rabbit polyclonal anti-mtIF3 (1:1000) | ProteinTech | 14219-1-AP |
| Mouse monoclonal anti-Calnexin (1:500 000) | ProteinTech | 66903-1-Ig; 2A2C6 |
| Mouse monoclonal anti-SDHA (1:20 000) | Invitrogen | 459200; 2E3GC12FB2A E2 |
| Mouse monoclonal anti-GAPDH (1:100 000) | Santa Cruz | sc-32233 |
| Rabbit polyclonal anti-COX1 (1:2000) | ³⁹ | PRAB5121 |
| Mouse monoclonal anti-COX2 (1:1000) | Abcam | Ab110258; 12C4F12 |
| Rabbit polyclonal anti-ATP6 (1:5000) | ⁴² | PRAB5159 |
| Rabbit polyclonal anti-bS1m (1:1000) | ProteinTech | 16378-1-AP |
| Rabbit polyclonal anti-uS5m (1:5000) | Proteintech | 16428-1-AP |
| Rabbit polyclonal anti-uS7m (1:1000) | Sigma-Aldrich | HPA 023007 |
| Rabbit polyclonal anti-uS10m (1:1000) | ProteinTech | 16030-1-AP |
| Rabbit polyclonal anti-uS14m (1:1000) | ProteinTech | 16301-1-AP |
| Rabbit polyclonal anti-uS15m (1:5000) | ProteinTech | 17106-1-AP |
| Rabbit polyclonal anti-uS17m (1:1000) | ProteinTech | 18881-1-AP |
| Rabbit polyclonal anti-mS22 (1:5000) | ProteinTech | 10984-1-AP |
| Rabbit polyclonal anti-mS23 (1:10000) | ProteinTech | 18345-1-AP |
| Rabbit polyclonal anti-mS25 (1:5000) | ProteinTech | 15277-1-AP |
| Rabbit polyclonal anti-mS27 (1:10000) | ProteinTech | 17280-1-AP |
| Rabbit polyclonal anti-mS29 (1:1000) | ProteinTech | 10276-1-AP |
| Rabbit polyclonal anti-mS34 (1:5000) | ProteinTech | 15166-1-AP |
| Rabbit polyclonal anti-mS35 (1:5000) | ProteinTech | 16457-1-AP |
| Rabbit polyclonal anti-mS37 (1:1000) | ProteinTech | 11728-1-AP |
| Rabbit polyclonal anti-mS40 (1:5000) | ProteinTech | 16139-1-AP |
| Rabbit polyclonal anti-uL13m (1:5000) | Proteintech | 16241-1-AP |
| Rabbit polyclonal anti-uL23m (1:10 000) | ³⁹ | PRAB1716 |
| Rabbit polyclonal anti-POLRMT (1:1000) | ProteinTech | 17748-1-AP |
| Rabbit polyclonal anti-LRPPRC (1:2000) | ProteinTech | 21175-1-AP |
| Goat IgG anti-rabbit IgG (H+L)-HRPO (1:5000) | dianova | 111-035-144 |
| Goat IgG anti-mouse IgG (H+L)-HRPO (1:5000) | dianova | 115-035-146 |
| IRDye 800CW Donkey anti-mouse (1:10 000) | LI-COR | 926-32212 |
| IRDye 800CW Donkey anti-rabbit (1:10 000) | LI-COR | 926-32213 |
| Oligonucleotides | | |
| Primer: Generation of the FLAG-tagged version of MTG3 Forward: 5'- CTTCTGATATCCCACCATGCTGCCCCGCTCGCCTACCGT TC-3' | This study; Microsynth | |
| Primer: Generation of the FLAG-tagged version of MTG3 | This study; Microsynth | |

| | | |
|---|---------------------------|------------------------------|
| Reverse: 5’- CTCTCCCTCGAGCTACTTATCGTCGTCATCCTTGTAATC TACATTTATCTTTCCTTTCTTCTTCC-3’ | | |
| Primer: Generation of the FLAG-tagged version of mtlF3 Forward: 5’- CTTTCTGATATCCCACCATGGCTGCTCTTTTTCTAAAGA GGTT-3’ | This study; Microsynth | |
| Primer: Generation of the FLAG-tagged version of mtlF3 Reverse: 5’- CTCTCCCTCGAGCTACTTATCGTCGTCATCCTTGTAATC CTGATGCAGAACATTTGATTC-3’ | This study; Microsynth | |
| Primer: Generation of the FLAG-tagged mutant version of MTG3 (ΔN-MTG3) Forward: 5’- TTGGAGAAGGTGGTGACATGCTGGATCCGGAG-3’ | This study; Microsynth | |
| Primer: Generation of the FLAG-tagged mutant version of MTG3 (ΔN-MTG3) Reverse: 5’-GGTTGCGGCTCCGGATCCAGCATGTCACCA- 3’ | This study; Microsynth | |
| Primer: Generation of the FLAG-tagged mutant variant of MTG3 (G499P) Forward: 5’-CAC TGG TTT TAT GAC ACC CCT CCA ATT ACA AAA-3’ | This study; Microsynth | |
| Primer: Generation of the FLAG-tagged mutant variant of MTG3 (G499P) Reverse: 5’-ACA ATT TTC TTT TGT AAT TGG AGG GGT GTC ATA-3’ | This study; Microsynth | |
| Primer: Generation of the FLAG-tagged variant of ERAL1 Forward: 5’- CTTTCTGATATCCCACCATGGCTGCCCCCAGCTGGCG-3’ | This study; Microsynth | |
| Primer: Generation of the FLAG-tagged variant of ERAL1 Reverse: 5’- CTCTCCCTCGAGCTACTTATCGTCGTCATCCTTGTAATC CTTGAGGAGCTTCACAGAGAG-3’ | This study; Microsynth | |
| Guide RNA: targeting Exon 1 of MTG3 5’-TTATGACCCCGTGGACACGG-3’ | This study; IDT | |
| Guide RNA: targeting Exon 1 of ERAL1 5’- CCCGACAGTTCGGTGACTCCTTG-3’ | This study; IDT | |
| Probe for northern blot: targeting MTRNR1 (12S rRNA) 5’-TCGATTACAGAACAGGCTCCTCTAG-3’ | 27 | |
| Probe for northern blot: targeting MTRNR2 (16S rRNA) 5’- GTTTGGCTAAGGTTGTCTGGTAGTA-3’ | | |
| Probe for northern blot: targeting MTCO1 5’- GTCAGTTGCCAAAGCCTCCGATTATG-3’ | | |
| Probe for northern blot: targeting 18S-rRNA 5’- TTTACTTCTCTAGATAGTCAAGTTCGACC-3’ | | |
| NanoString sequences | | |
| CTTCATCAGGGTTTGCTGAAGATGGCGGTATATAGGCT GAGCAAGAGGTGCCTCAAGACCTAAGCGACAGCGTGA CCTTGTTTCA | IDT, 39, 42 | Mito_JCM__00 8.1:591_T001 |
| CTTTCTTAATTGGTGGCTGCTTTTAGGCCTACTATGGGT GTAAATTTTTCATCCTCTTCTTTTCTTGGTGTTGAGAA GATGCTC | | Mito_JCM__00 9.1:481_T002 |

| | | |
|--|--|-------------------------------|
| GTTCTTGTGTTGTGATAAGGGTGGAGAGGTTAAAGGAG CCACTTATTAGTCACAATTCTGCGGGTTAGCAGGAAGG TTAGGGAAC | | HSMT_ND1.1: 461_T003 |
| CCTGCTATGATGGATAAGATTGAGAGAGTGAGGAGAA GGCTTACGTTTAGCTGTTGAGATTATTGAGCTTCATCAT GACCAGAAG | | HSMT_ND2.1: 444_T004 |
| GCGGAGGTGAAATATGCTCGTGTGTCTACGTCTATTCC TACTGTAAATATCAAAGACGCCTATCTTCCAGTTTGAT CGGGAAACT | | HSMT_COX1.1: :873_T005 |
| TCGTCTGTTATGTAAAGGATGCGTAGGGATGGGAGGG CGATGAGGACTAGCGAACCTAACTCCTCGCTACATTCC TATTGTTTTT | | HSMT_COX2.1: :216_T006 |
| TATTTTTATGGGCTTTGGTGAGGGAGGTAGGTGGTAGT TTGTGTTTAATACCAATTTGGTTTTACTCCCCTCGATTA TGCGGAGT | | HSMT_ATP8.1: 82_T007 |
| GTGGGCTAGGGCATTTTTAATCTTAGAGCGAAAGCCTA TAATCACTGTGCCTTTCGGGTTATATCTATCATTTACTT GACACCCT | | HSMT_ATP6.1: 331_T008 |
| ATGTTGAGCCGTAGATGCCGTCGGAAATGGTGAAGGG AGACTCGAAGTACCAACAGCCACTTTTTTCCAAATTT TGCAAGAGCC | | HSMT_COX3.1: :539_T009 |
| AAGGTAATAGCTACTAAGAAGAATTTTATGGAGAAAG GGACGCGGGCGGGCACC GTGTGGACGGCAACTCAGAG ATAACGCATAT | | HSMT_ND3.1: 135_T010 |
| TATTCCTTCTAGGCATAGTAGGGAGGATATGAGGTGTG AGCGATATACTACCTGGAGTTTATGTATTGCCAACGAG TTTGTCTTT | | HSMT_ND4L.1: :58_T011 |
| ACTGTGAGTGCGTTCGTAGTTTGAGTTTGCTAGGCAGA ATAGTAATGAGGCAGATAAGGTTGTTATTGTGGAGGAT GTTACTACA | | HSMT_ND4.1: 967_T012 |
| TGCGGTTTCGATGATGTGGTCTTTGGAGTAGAAACCTG TGAGGAAAGGTACTTCCTTCCTGTGTTCCAGCTACAAA CTTAGAAAC | | HSMT_ND5.1: 1147_T013 |
| CCTCAGGATACTCCTCAATAGCCATCGCTGTAGTATAT CCAAAGACAACCCATAAAATTGGTTTTGCCTTTCAGCA ATTCAACTT | | HSMT_ND6.1: 191_T014 |
| GAGGTCTGCGGCTAGGAGTCAATAAAGTGATTGGCTTA GTGGGCGAAATACTGGTCAAGACTTGCATGAGGACCC GCAAATTCCT | | HSMT_CYTB.1: :946_T015 |
| AGGGCAGGGACTTAATCAACGCAAGCTTATGACCCGC ACTTACTGGGAATCTTTCGTTGGGACGCTTGAAGCGCA AGTAGAAAAC | | NR_003286.2:1 640_T016 |
| TGCTTAGCTTCCGAGATCAGACGAGATCGGGCGCGTTC AGGGCCAGCAGACCTGCAATATCAAAGTTATAAGCGC GT | | NR_023379.1:8 _T017 |
| CGAAAGCCATGACCTCCGATCACTCTACACCTTGACCT AACGTCTTTACGTGGGTACTTGCGCTTACTTTGTAGC | | Mito_JCM_00 8.1:591_ProbeB |
| CGAAAGCCATGACCTCCGATCACTCTTCAGTTATATGT TTGGGATTTTTTAGGTAGTGGGTGTTGAGCTTGAACG | | Mito_JCM_00 9.1:481_ProbeB |
| CGAAAGCCATGACCTCCGATCACTCGATAAATCATATT ATGGCCAAGGGTCATGATGGCAGGAGTAATCAGAGGT | | HSMT_ND1.1: 461_ProbeB |
| CGAAAGCCATGACCTCCGATCACTCAGTATGCTAAGAT TTTGCCTAGCTGGGTTTGGTTTAATCCACCTCAACTG | | HSMT_ND2.1: 444_ProbeB |

| | | |
|---|-------------------------|-----------------------------|
| CGAAAGCCATGACCTCCGATCACTCCGAGTCAGCTAAA TACTTTGACGCCGGTGGGGATAGCGATGATTATGGTA | | HSMT_COX1.1 :873 ProbeB |
| CGAAAGCCATGACCTCCGATCACTCAGTACCATTGGTG GCCAATTGATTGATGGTAAGGGAGGGATCGTTGACC | | HSMT_COX2.1 :216 ProbeB |
| CGAAAGCCATGACCTCCGATCACTCAGCGAACAGATTT TCGTTCAATTTTGGTTCTCAGGGTTTGTATAATTTT | | HSMT_ATP8.1: 82 ProbeB |
| CGAAAGCCATGACCTCCGATCACTCATAATAACTAGTA TGGGGATAAGGGGTGTAGGTGTGCCTTGTGGTAAGAA | | HSMT_ATP6.1: 331 ProbeB |
| CGAAAGCCATGACCTCCGATCACTCAGTTGAGCCAATA ATGACGTGAAGTCCGTGGAAGCCTGTGGCTACAAAAA | | HSMT_COX3.1 :539 ProbeB |
| CGAAAGCCATGACCTCCGATCACTCGGGCTCATGGTAG GGGTAAAAGGAGGGCAATTTCTAGATCAAATAATAAG | | HSMT_ND3.1: 135 ProbeB |
| CGAAAGCCATGACCTCCGATCACTCGAGTGGGTGTTGA GGGTTATGAGAGTAGCTATAATGAACAGCGATAGTAT | | HSMT_ND4L.1 :58 ProbeB |
| CGAAAGCCATGACCTCCGATCACTCGCTATTAGTGGGA GTAGAGTTTGAAGTCCTTGAGAGAGGATTATGATGCG | | HSMT_ND4.1: 967 ProbeB |
| CGAAAGCCATGACCTCCGATCACTCGTAGCGATGAGA GTAATAGATAGGGCTCAGGCGTTTGTGTATGATATGTT | | HSMT_ND5.1: 1147 ProbeB |
| CGAAAGCCATGACCTCCGATCACTCCGCTAACCCCACT AAAACACTCACCAAGACCTCAACCCCTGACCCCATG | | HSMT_ND6.1: 191 ProbeB |
| CGAAAGCCATGACCTCCGATCACTCATGGTAAAAGGG TAGCTTACTGGTTGTCCTCCGATTCAGGTTAGAATGAG | | HSMT_CYTB.1 :946 ProbeB |
| CGAAAGCCATGACCTCCGATCACTCGCCTCACTAAACC ATCCAATCGGTAGTAGCGACGGGCGGTGTGTACAA | | NR_003286.2:1 640 ProbeB |
| CGAAAGCCATGACCTCCGATCACTCCGGTCTCCCATCC AAGTACTAACCGAGCCCGACCC | | NR_023379.1:8 ProbeB |
| Experimental models: cell lines | | |
| HEK293-Flp-In T-Rex | ThermoFisher Scientific | R78007 |
| HEK293-Flp-In T-Rex- <i>Mtg3</i> ^{-/-} | This study | |
| HEK293-Flp-In T-Rex-MTG3 ^{FLAG} | This study | |
| HEK293-Flp-In T-Rex- <i>Mtg3</i> ^{-/-} +MTG3 ^{FLAG} | This study | |
| HEK293-Flp-In T-Rex- <i>Mtg3</i> ^{-/-} + ΔN-MTG3 ^{FLAG} | This study | |
| HEK293-Flp-In T-Rex- <i>Mtg3</i> ^{-/-} + MTG3-G499P ^{FLAG} | This study | |
| HEK293-Flp-In T-Rex-mtIF3 ^{FLAG} | This study | |
| HEK293-Flp-In T-Rex- <i>Erall</i> ^{-/-} +ERAL1 ^{FLAG} | This study | |
| Recombinant DNA | | |
| pOG44 Flp-Recombinase Expression Vector | ThermoFisher Scientific | V600520 |
| pcDNA5/FRT/TO | ThermoFisher Scientific | V6520-20 |
| Chemicals | | |
| T4 Polynucleotide Kinase (T4 PNK) | ThermoFisher Scientific | EK0031 |
| QuikChange Lightning Site-Directed Mutagenesis Kit | Agilent Technologies | 210519-5 |
| TOPO TA Cloning® Kit | ThermoFisher Scientific | 45-0030 |
| OneShot® TOP10 Chemically Competent Cells | ThermoFisher Scientific | C404004 |
| L-[³⁵ S]methionine | Hartmann Analytic | SCM-01 |

| | | |
|---|------------------------------|-------------|
| Adenosine 5'-triphosphate, [γ - ^{32}P] | Hartmann-Analytic | SRP-501 |
| Emetine dihydrochloride hydrate | Sigma-Aldrich | 219282 |
| Lipofectamine 3000 | Invitrogen | L3000-015 |
| Alt-R® CRISPR-Cas9 tracrRNA, ATTO™ 550 | Integrated DNA technologies | 1075927 |
| Alt-R® S.p. Cas9 Nuclease V3 | Integrated DNA technologies | 1081058 |
| TRIzol® Reagent | Ambion | 15596018 |
| Anti-FLAG M2 Affinity Gel | Sigma-Aldrich | A2220 |
| FLAG-peptide | Sigma-Aldrich | F3290-4MG |
| Glutaraldehyde | Electron microscopy sciences | 16220 |
| Deposited data | | |
| mtSSU state A, map A1 | This study | EMD-51066 |
| mtSSU state A, map A2 | This study | EMD-51053 |
| mtSSU state A, map A3 | This study | EMD-51083 |
| mtSSU state A, map A4 | This study | EMD-51064 |
| mtSSU state A, map A5 | This study | EMD-51065 |
| mtSSU state B, map B1 | This study | EMD-51063 |
| mtSSU state B, map B2 | This study | EMD-51052 |
| mtSSU state A, map B3 | This study | EMD-51084 |
| mtSSU state A, map B4 | This study | EMD-51061 |
| mtSSU state A, map B5 | This study | EMD-51062 |
| mtSSU state A, map B6 | This study | EMD-51060 |
| mtSSU state C, map C1 | This study | EMD-51059 |
| mtSSU state C, map C2 | This study | EMD-51051 |
| mtSSU state C, map C3 | This study | EMD-51085 |
| mtSSU state C, map C4 | This study | EMD-51057 |
| mtSSU state C, map C5 | This study | EMD-51058 |
| mtSSU state C, map C6 | This study | EMD-51056 |
| mtSSU state D, map D1 | This study | EMD-51047 |
| mtSSU state D, map D2 | This study | EMD-51046 |
| mtSSU state E, map E1 | This study | EMD-51045 |
| mtSSU state E, map E2 | This study | EMD-51043 |
| mtSSU state F, map F1 | This study | EMD-51055 |
| mtSSU state F, map F2 | This study | EMD-51050 |
| mtSSU state F, map F3 | This study | EMD-51086 |
| mtSSU state F, map F4 | This study | EMD-51049 |
| mtSSU state F, map F5 | This study | EMD-51054 |
| mtSSU state F, map F6 | This study | EMD-51048 |
| mtSSU state G, map G1 | This study | EMD-51042 |
| mtSSU state G, map G2 | This study | EMD-51039 |
| mtSSU state A model | This study | PDB: 9G5B |
| mtSSU state B model | This study | PDB: 9G5C |
| mtSSU state C model | This study | PDB: 9G5D |
| mtSSU state F model | This study | PDB: 9G5E |
| comparable MTG3-TFB1M-bound-mtSSU model | ²⁰ | PDB: 8CSP |
| comparable TFB1M-mtRBFA(in)-bound-mtSSU model | ¹⁹ | PDB: 7PNT |
| comparable METTL15-mtRBFA(out)-bound-mtSSU model | ¹⁹ | PDB: 7PNX-Z |
| comparable mtIF3-bound-mtSSU model; starting model | ¹⁹ | PDB: 7PO1 |
| comparable mtIF2-mtIF3-bound-mtSSU model | ²⁶ | PDB: 6RW5 |

| | | |
|--------------------------------------|------------|-----------|
| comparable IC-mtSSU model | 19 | PDB: 7PO2 |
| Comparable mature mitoribosome model | 2 | PDB: 3J9M |
| Software and Algorithm | | |
| Fiji ImageJ | 43 | |
| nSolver software | NanoString | v.4.0.70 |
| SerialEM | 44 | v.4.0 |
| Warp | 45 | v1.0.9 |
| RELION | 46 | v3.1.0 |
| cryoSPARC | 47 | v4.4.1 |
| UCSF ChimeraX | 48, 49 | v1.5 |
| COOT | 50 | v0.9.8.1 |
| Phenix real space refine | 51 | v1.20.1 |
| ISOLDE | 52 | |