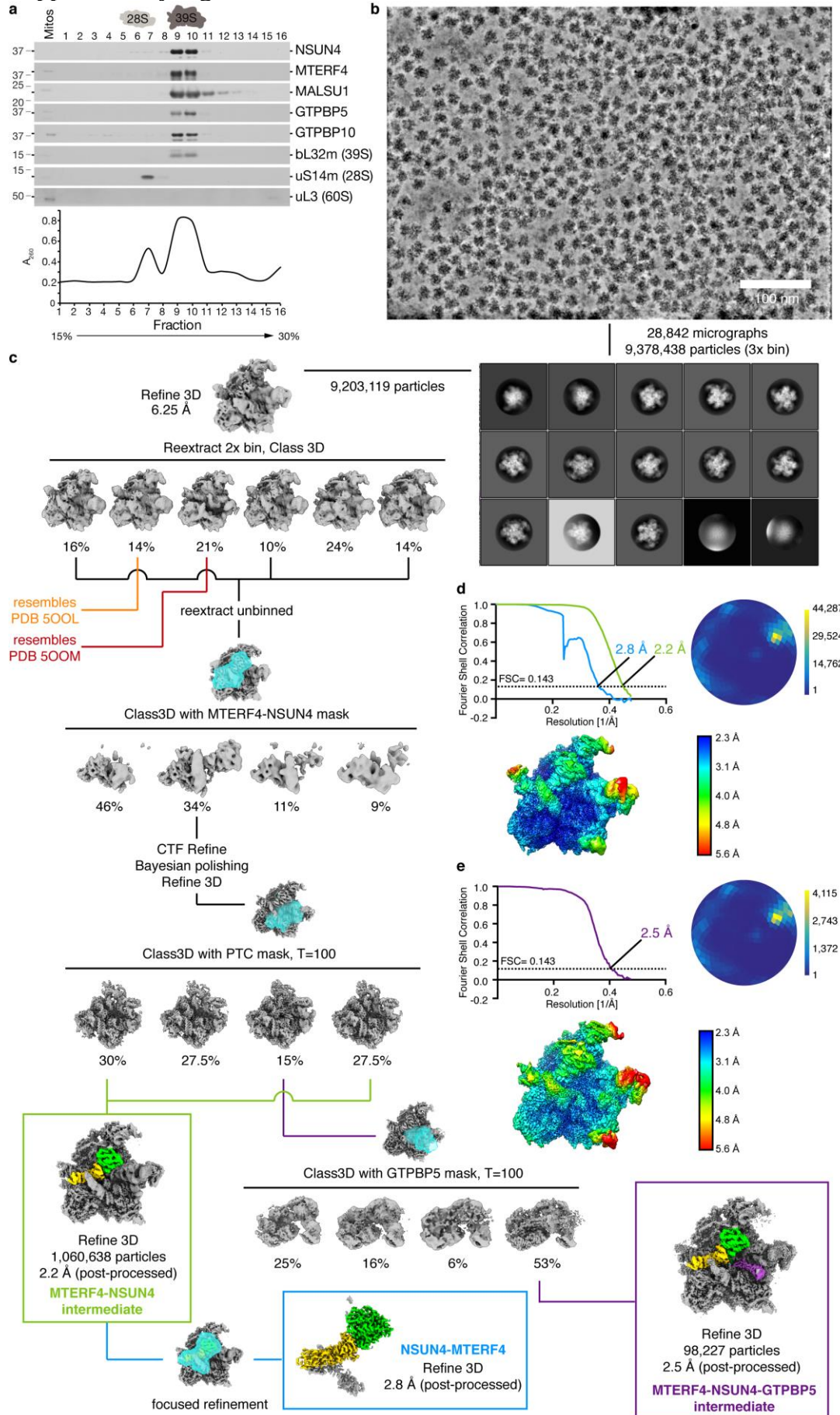


Structural basis of GTPase-mediated mitochondrial ribosome biogenesis and recycling

Hauke S. Hillen^{1,2,3,*}, Elena Lavdovskaia^{1,2}, Franziska Nadler¹, Elisa Hanitsch¹, Andreas Linden^{4,5}, Katherine E. Bohnsack⁶, Henning Urlaub^{4,5} and Ricarda Richter-Dennerlein^{1,2,*}

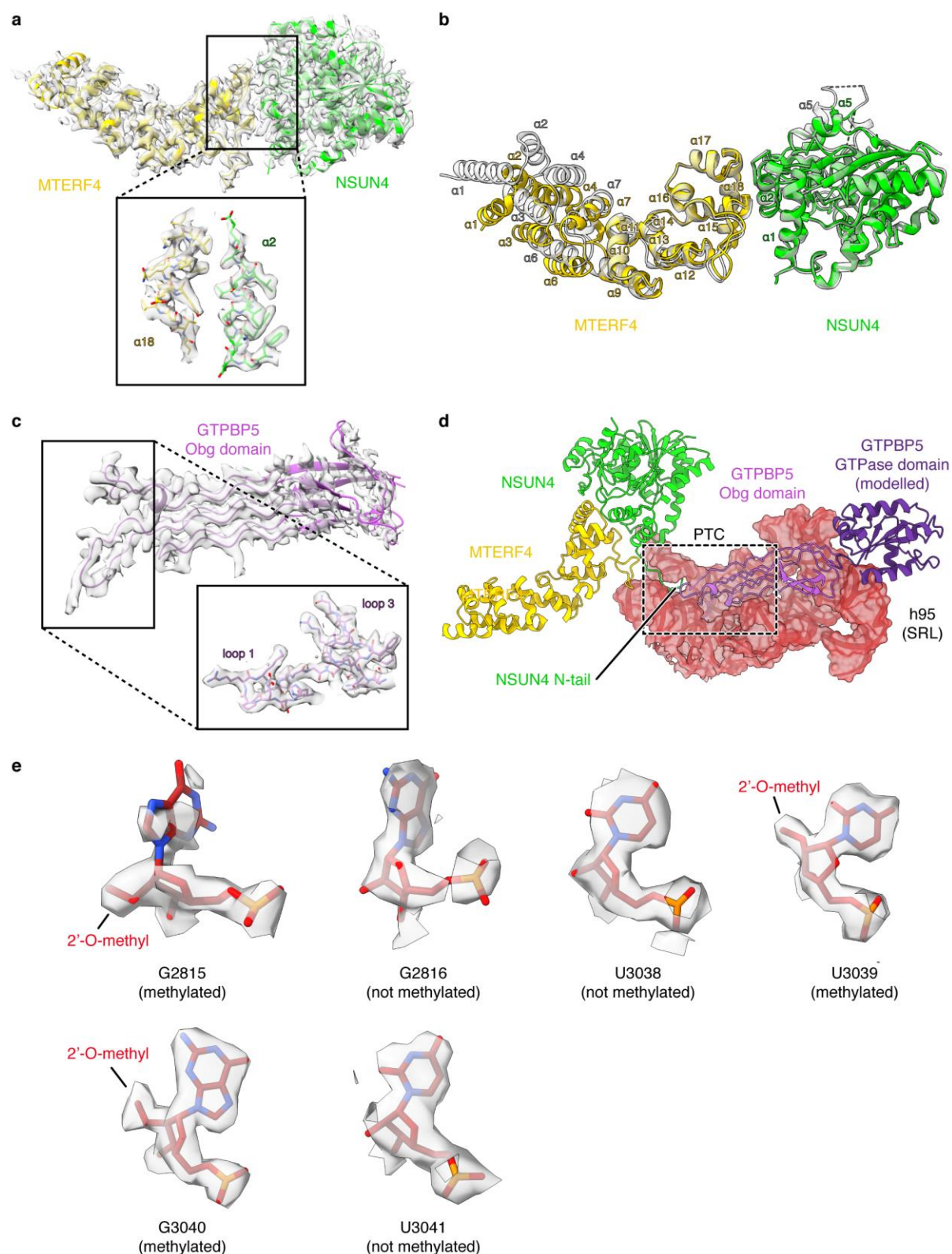
SUPPLEMENTARY DATA

Supplementary Figures



Supplementary Fig. 1 | Purification and Cryo EM data processing first dataset.

(a) Gradient fractions (1-16) upon ribosome isolation were analyzed by western blotting using indicated antibodies. uL3 (component of the cytosolic 60S large ribosomal subunit (LSU)) was used as a control to assess the level of contamination with cytosolic ribosomes. Absorbance was measured for each fraction at 260 nm. Fractions 8 and 9 were used for further analyses. The experiment was performed twice with reproducible outcome. Source data are provided as a Source Data file. **(b)** Example denoised micrograph calculated from two independently measured half sets of 40 frames each. Scale bar, 100 nm. **(c)** 2D class averages and Cryo-EM processing tree. **(d)** Fourier shell correlation (FSC) plot, Angular distribution plot and local resolution distribution for the MTERF4-NSUN4 large mitoribosomal subunit (mtLSU) intermediate. Scale for the angular distribution plot shows the number of particles assigned to a particular angular bin. Blue, a low number of particles; yellow, a high number of particles. **(e)** FSC plot, Angular distribution plot and local resolution distribution for the MTERF4-NSUN4-GTPBP5 mtLSU intermediate. Scale for the angular distribution plot shows the number of particles assigned to a particular angular bin. Blue, a low number of particles; yellow, a high number of particles.

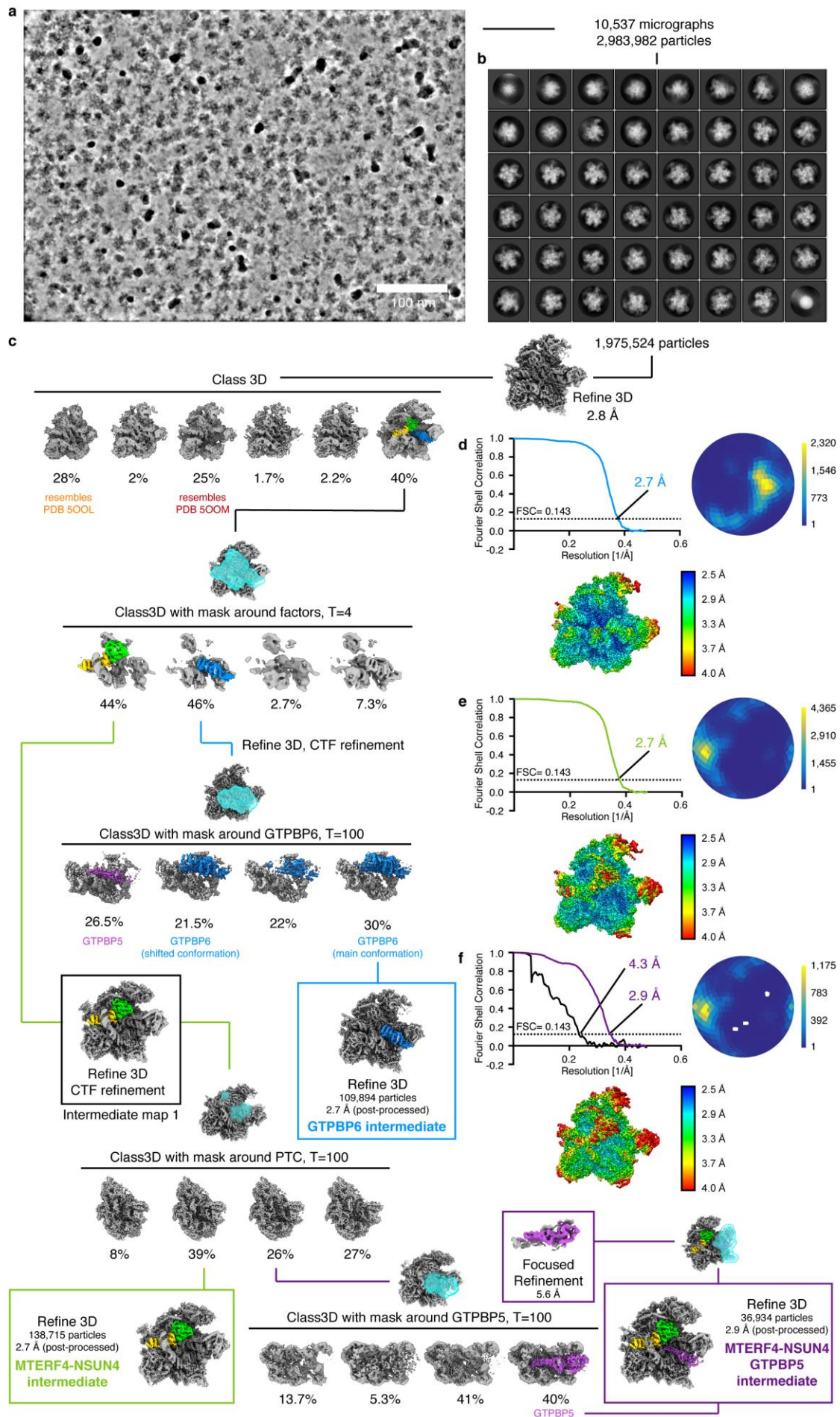


Supplementary Fig. 2 | Structural details of MTERF4, NSUN4 and ribosomal RNA.

(a) Density fit of MTERF4-NSUN4. The model of MTERF4-NSUN4 is shown as cartoon with coloring as in Fig. 1. The post-processed cryo-EM density from focused refinement is shown as transparent grey surface. The region at the interface between MTERF4 and NSUN4 is enlarged and shown as sticks. **(b)** Comparison between the MTERF4-NSUN4 complex bound to the large mitoribosomal subunit (mtLSU) and the previous crystal structure (PDB 4FP9)¹⁵. The two structures are shown superimposed as cartoon. The mtLSU-bound structure is colored

as in Fig. 1 and the free crystal structure is colored in grey and shown transparently. Secondary structure elements in MTERF4 and that adopt different conformations in NSUN4 are indicated. The helical repeats of MTERF4 form a widened curve when bound to the ribosome. **(c)** Density fit of GTPBP5. The model of GTPBP5 is shown as cartoon with coloring as in Fig. 2. The post-processed cryo-EM density is shown as transparent grey surface. The PTC-interacting loops described in Fig. 2 are shown enlarged as sticks. **(d)** Interaction of GTPBP5 with the mtLSU. Regions of the 16S rRNA interacting with MTERF4-NSUN4 are shown as cartoon and as transparent surface. MTERF4, NSUN4 and GTPBP5 are shown as cartoons. SRL: sarcin-ricin loop. **(e)** G2815, U3039 and G3040 are methylated. G2815, U3039 and G3040 are shown as sticks with the post-processed cryo-EM density of the MTERF4-NSUN4-GTPBP5 mtLSU intermediate (dataset 1) shown as transparent surface. Neighboring non-methylated bases are shown for comparison.

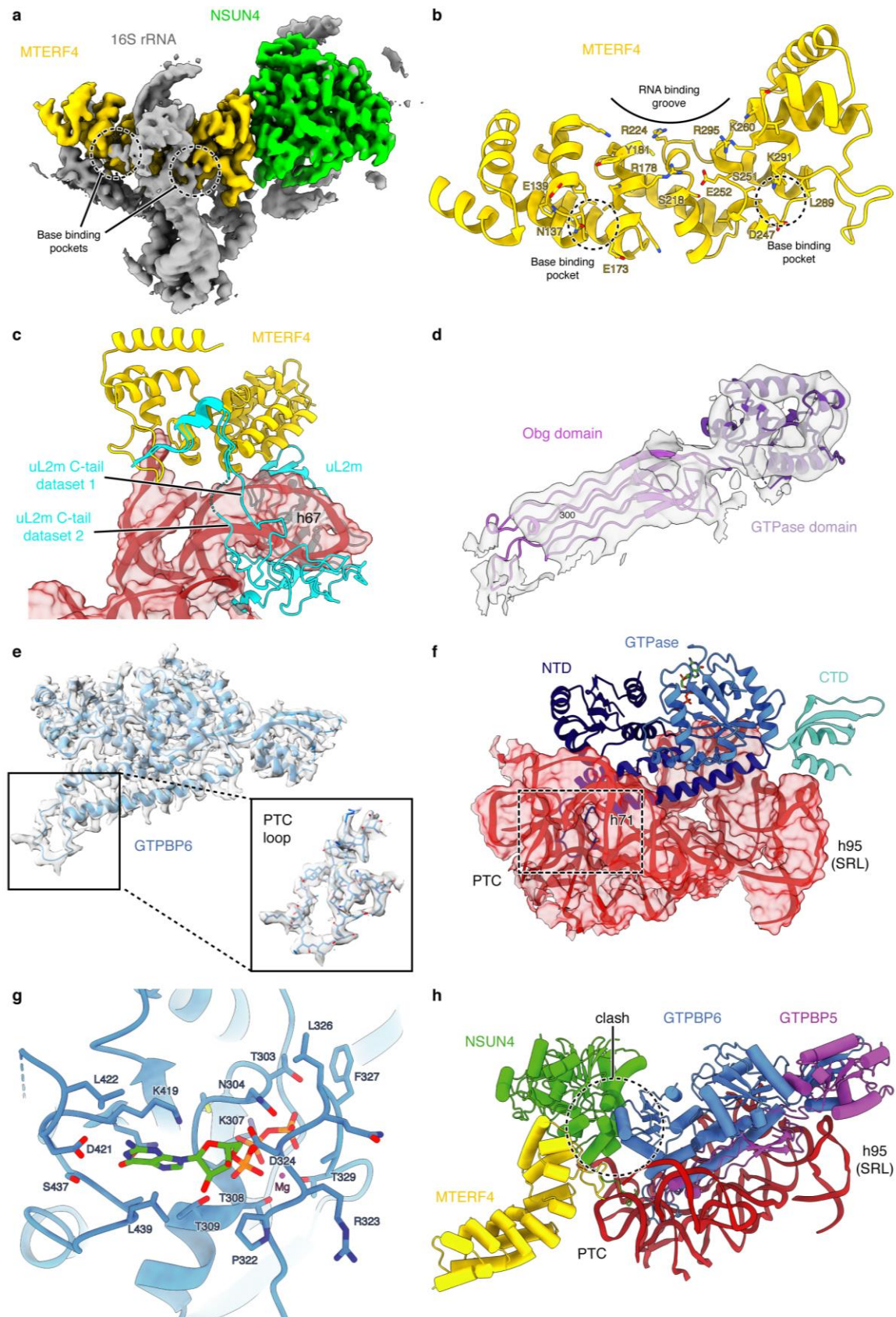
Structural basis of GTPase-mediated mitochondrial ribosome biogenesis and recycling



Supplementary Fig. 3 | Cryo-EM data processing second dataset.

(a) Example denoised micrograph calculated from two independently measured half sets of 40 frames each. Scale bar, 100 nm. **(b)** 2D class averages. **(c)** Cryo-EM processing tree. **(d)** Fourier shell correlation (FSC) plot, Angular distribution plot and local resolution distribution for the GTPBP6-bound large mitoribosomal subunit (mtLSU) intermediate. Scale for the angular distribution plot shows the number of particles assigned to a particular angular bin. Blue, a low number of particles; yellow, a high number of particles. **(e)** Fourier shell correlation (FSC) plot, Angular distribution plot and local resolution distribution for the MTERF4-NSUN4 mtLSU intermediate. Scale for the angular distribution plot shows the number of particles assigned to a particular angular bin. Blue, a low number of particles; yellow, a high number of particles. **(f)** FSC plot, Angular distribution plot and local resolution distribution for the MTERF4-NSUN4-GTPBP5 mtLSU intermediate. Scale for the angular distribution plot shows the number of particles assigned to a particular angular bin. Blue, a low number of particles; yellow, a high number of particles.

Structural basis of GTPase-mediated mitochondrial ribosome biogenesis and recycling

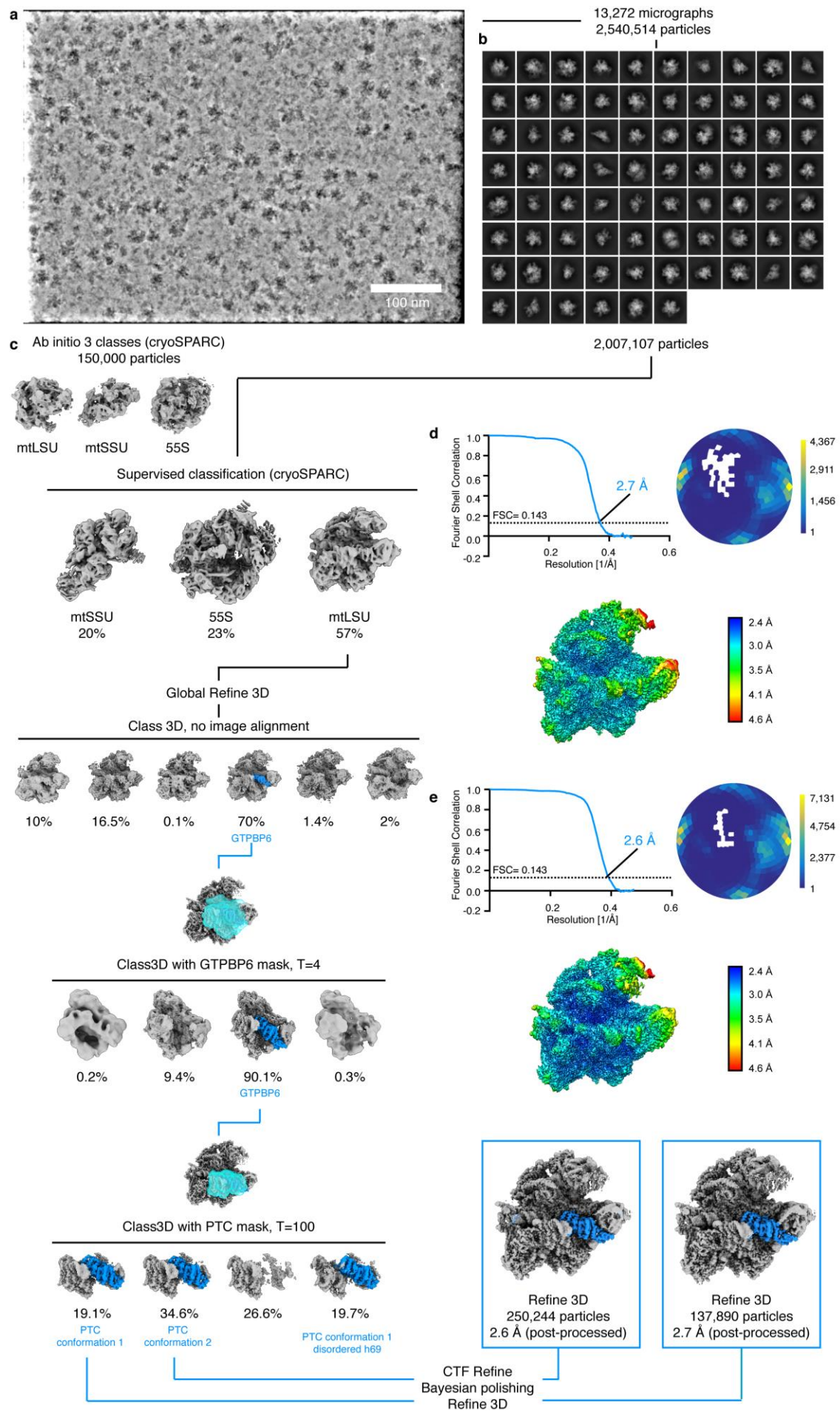


Supplementary Fig. 4 | Structural details of MTERF4, NSUN4, GTPBP5 and GTPBP6 in the second dataset.

(a) Improved density for the 16 rRNA region wrapping over MTERF4. A region of the cryo-EM reconstruction (Intermediate map 1, Supplementary Fig. 3) of MTERF4-NSUN4

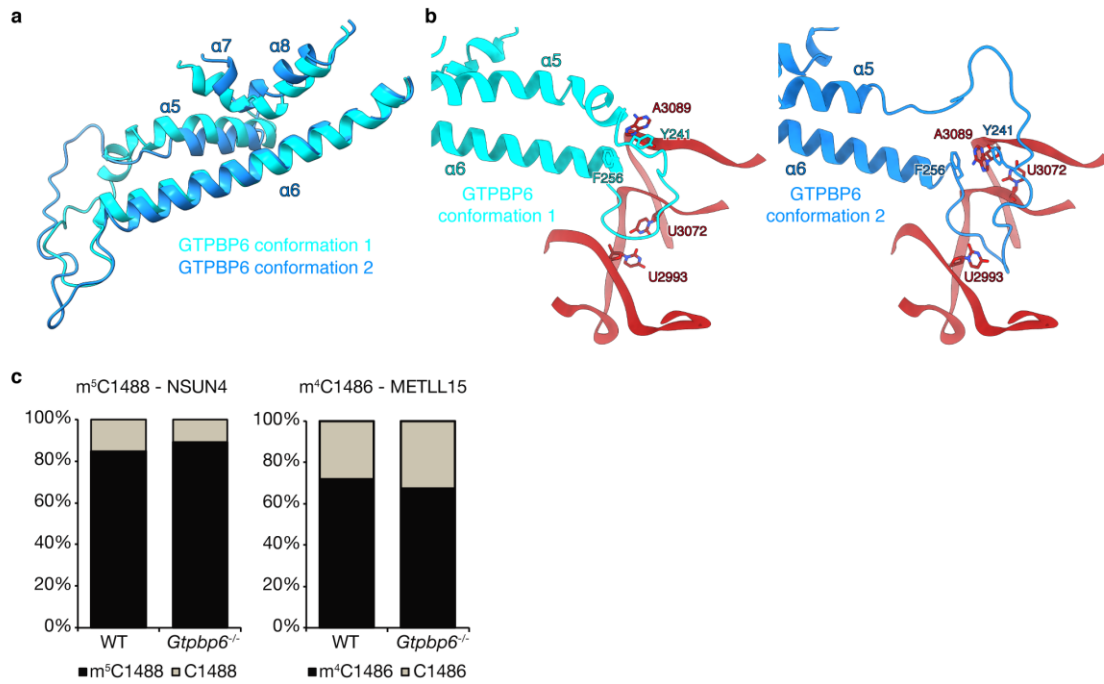
containing particles from dataset 2 is shown as surface and colored as follows: NSUN4: lime green, MTERF4: yellow, 16S rRNA: grey. The region of the rRNA wrapping above MTERF4 adopts a helical fold and may form base-mediated interactions with MTERF4 (indicated). **(b)** Close-up view of the RNA-binding groove of MTERF4. MTERF4 is shown as cartoon in yellow and residues close to the RNA density observed in (a) are shown as sticks. The potential base-binding pockets are indicated. **(c)** The C-tail of uL2m occupies different paths in the MTERF4-NSUN4 mtLSU intermediate from dataset 1 and dataset 2. MTERF4 and uL2m are shown as cartoons and the 16S rRNA elements interacting with them are shown as cartoon and transparent surface. Coloring as in Fig. 1. **(d)** Improved density for the GTPBP5 GTPase domain in dataset 2. GTPBP5 is shown as cartoon and colored as in Fig. 2. The cryo-EM density obtained from focused refinement of the GTPBP5-containing particle set in dataset 2 is shown as transparent grey surface. **(e)** Density fit of GTPBP6. GTPBP6 is shown as cartoon and colored in blue. The post-processed cryo-EM density of the GTPBP6-bound mtLSU intermediate structure is shown as transparent grey surface. The PTC loop described in Fig. 3 is shown enlarged as sticks. **(f)** Interaction of GTPBP6 with the mtLSU. Regions of the 16S rRNA interacting with GTPBP6 are shown as cartoon and as transparent surface. GTPBP6 is shown as cartoon. **(g)** GTP binding pocket of GTPBP6. The GTP binding site of GTPBP6 is shown as cartoon and colored as in Fig. 3. Residues within 4 Å of GTP are shown as sticks. GTP is shown in green as sticks. **(h)** GTPBP6 occupies the same binding site as GTPBP5 on the mtLSU and would clash with NSUN4. The structure of the MTERF4-NSUN4-GTPBP5 intermediate and the GTPBP6 intermediate were superimposed with their 16S rRNA. The peptidyl transferase center (PTC) and factors are shown as cartoons and colored as in Fig. 1-3. SRL: sarcin-ricin loop.

Structural basis of GTPase-mediated mitochondrial ribosome biogenesis and recycling



Supplementary Fig. 5 | Cryo-EM data processing third dataset.

(a) Example denoised micrograph calculated from two independently measured half sets of 40 frames each. Scale bar, 100 nm. **(b)** 2D class averages. **(c)** Cryo-EM processing tree. **(d)** Fourier shell correlation (FSC) plot, Angular distribution plot and local resolution distribution for the GTPBP6-bound split large mitoribosomal subunit (mtLSU) with peptidyl transferase center (PTC) conformation 1. Scale for the angular distribution plot shows the number of particles assigned to a particular angular bin. Blue, a low number of particles; yellow, a high number of particles. **(e)** FSC plot, Angular distribution plot and local resolution distribution for the GTPBP6-bound split mtLSU with PTC conformation 2. Scale for the angular distribution plot shows the number of particles assigned to a particular angular bin. Blue, a low number of particles; yellow, a high number of particles.



Supplementary Fig. 6 | GTPBP6 conformations.

(a) The PTC-binding loop of GTPBP6 can adopt two conformations. Superimposition of GTPBP6 in the PTC conformation 1 and PTC conformation 2 structures observed after ribosome splitting (dataset 3). GTPBP6 is shown as cartoon. GTPBP6 conformation 1, which is also observed during mtLSU biogenesis, is shown in cyan and GTPBP6 conformation 2 in maroon. **(b)** Close-up of PTC interactions in GTPBP6 PTC conformation 1 and 2. Depiction as in a, with the region of the 16S rRNA differing between the two states shown as cartoon. Bases that adopt different conformations as well as interacting GTPBP6 residues are shown as sticks. **(c)** Methylation of 12S rRNA is not affected in GTPBP6-deficient cells. Total RNA from wild type (WT) or *Gtpbp6*^{-/-} was treated with bisulfite, reverse transcribed and a region of the 16S rRNA containing m⁵C1488 and m⁴C1486 was amplified, cloned and sequenced. The relative proportions of unconverted cytosine reflecting m⁵C/m⁴C (black) and thymine reflecting converted, unmodified cytosine (grey) are shown. Data derived from sequencing of 46 individual clones per sample are presented. Source data are provided as a Source Data file.

Supplementary Tables**Supplementary Table 1 | Cryo-EM data collection and refinement for dataset 1.**

	MTERF4-NSUN4 intermediate (PDB: 7OF0)	MTERF4-NSUN4- GTPBP5-intermediate (PDB: 7OF7)
Data collection and processing		
Magnification	81,000	
Voltage (kV)	300	
Electron exposure (e ⁻ /Å ²)	36	
Defocus range (μm)	0.3 – 2.8	
Pixel size (Å)	1.05	
Symmetry imposed	C1	
Initial particle images (no.)	9,378,438	
Final particle images (no.)	1,060,638	98,227
Map resolution (Å)	2.2	2.5
FSC threshold	0.143	0.143
Map sharpening B factor (Å ²)	-50	-39.5
Map resolution range (Å)	5.6 – 2.2	5.6 – 2.2
Refinement		
Model resolution (Å)	2.3	2.7
FSC threshold	0.5	0.5
Model composition		
Non-hydrogen atoms	99,476	101,032
Protein/Nucleotide residues	8,446 / 1,432	8,655 / 1,432
Ligands	ZN: 3, MG: 61	ZN:3, MG:74
B factors (Å ²)		
Protein/Nucleotide	107.39 / 97.44	94.53 / 71.94
Ligand	62.66	46.80
R.m.s. deviations		
Bond lengths (Å)	0.006	0.006
Bond angles (°)	0.859	0.874
Validation		
MolProbity score	1.54	1.52
Clashscore	7.28	5.58
Poor rotamers (%)	0.00	0.00
Ramachandran plot		
Favored (%)	97.26	96.61
Allowed (%)	2.74	3.39
Disallowed (%)	0.00	0.00

Supplementary Table 2 | Cryo-EM data collection and refinement for dataset 2.

	GTPBP6 intermediate (PDB: 7OF2)	MTERF4- NSUN4 intermediate (PDB: 7OF3)	MTERF4- NSUN4- GTPBP5- intermediate (PDB: 7OF5)
Data collection and processing			
Magnification		81,000	
Voltage (kV)		300	
Electron exposure (e-/Å ²)		37	
Defocus range (µm)		0.3 – 2.1	
Pixel size (Å)		1.05	
Symmetry imposed		C1	
Initial particle images (no.)		2,983,982	
Final particle images (no.)	109,894	138,715	36,934
Map resolution (Å)	2.7	2.7	2.9
FSC threshold	0.143	0.143	0.143
Map sharpening <i>B</i> factor (Å ²)	-52	-58	-53
Map resolution range (Å)	4.0 – 2.5	4.0 – 2.5	4.0 – 2.5
Refinement			
Model resolution (Å)	2.8	2.9	3.0
FSC threshold	0.5	0.5	0.5
Model composition			
Non-hydrogen atoms	101,195	101,226	102,785
Protein/Nucleotide residues	8,560 / 1,466	8,627 / 1,442	8,829 / 1,445
Ligands	GTP: 3, ZN: 3, MG: 85	GTP: 2, ZN: 3, MG: 58	GTP: 2, ZN: 3, MG: 70
<i>B</i> factors (Å ²)			
Protein/Nucleotide	84.56 / 65.75	94.62 / 76.45	102.28 / 84.03
Ligand	59.47	69.93	72.24
R.m.s. deviations			
Bond lengths (Å)	0.004	0.04	0.004
Bond angles (°)	0.815	0.804	0.812
Validation			
MolProbity score	1.39	1.50	1.45
Clashscore	4.30	5.64	4.93
Poor rotamers (%)	0.01	0.00	0.00
Ramachandran plot			
Favored (%)	96.93	96.81	96.83
Allowed (%)	3.07	3.18	3.17
Disallowed (%)	0.00	0.01	0.00

Supplementary Table 3 | Cryo-EM data collection and refinement for dataset 3.

	Mature mtLSU with GTPBP6 – PTC state 1 (PDB: 7OF4)	Mature mtLSU with GTPBP6 – PTC state 2 (PDB: 7OF6)
Data collection and processing		
Magnification	81,000	
Voltage (kV)	30	
Electron exposure (e-/Å ²)	40	
Defocus range (μm)	0.2 – 2.7	
Pixel size (Å)	1.05	
Symmetry imposed	C1	
Initial particle images (no.)	2,540,514	
Final particle images (no.)	137,890	250,244
Map resolution (Å)	2.7	2.6
FSC threshold	0.143	0.143
Map sharpening <i>B</i> factor (Å ²)	-57	-66
Map resolution range (Å)	4.6 – 2.4	4.6 – 2.4
Refinement		
Model resolution (Å)	2.8	2.7
FSC threshold	0.5	0.5
Model composition		
Non-hydrogen atoms	100,322	100,321
Protein/Nucleotide residues	8,320 / 1,519	8,320 / 1,519
Ligands	GTP: 3, ZN: 3, MG: 89	GTP:3, ZN: 3, MG: 88
<i>B</i> factors (Å ²)		
Protein/Nucleotide	92.56 / 73.81	86.71 / 69.37
Ligand	70.84	65.35
R.m.s. deviations		
Bond lengths (Å)	0.005	0.003
Bond angles (°)	0.818	0.805
Validation		
MolProbity score	1.45	1.38
Clashscore	4.92	4.18
Poor rotamers (%)	0.00	0.01
Ramachandran plot		
Favored (%)	96.83	96.99
Allowed (%)	3.17	3.01
Disallowed (%)	0.00	0.00

Supplementary Table 4 | Primers

Primer	Sequence
12S-m ⁵ C841_RT	5'-TTTAATTAAATATCCTTTAAAATATAC-3'
12S forward	5'-TTTAATTAAATATCCTTTAAAATATAC-3'
12S reverse	5'-AATAGGGTTTTGAAGTGTGTATATA-3'
GTPBP6 LIC for	5'-TACTTCCAATCCAATGCACccgggaatctggaggggcc-3'
GTPBP6 LIC rev	5'-TTATCCACTTCCAATGTTATTAtcctggaaagagcttccggaatttgcc-3'

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

Hillen and colleagues use cryo-EM to illuminate the poorly understood process of GTPase-mediated mt-LSU assembly and peptidyltransferase center (PTC) maturation. Using a HEK293 cell line deficient in GTPBP6, a late-stage assembly factor, they isolate native mt-LSU intermediates and show how six biogenesis factors (GTPBP5, the MTERF4:NSUN4 complex, and the MALSU1:LOR8F8:mt-ACP module) bind premature mt-LSU and contribute to the folding of mt-rRNA. They then reconstitute mt-LSU assembly in vitro by complementing the biogenesis intermediates with recombinant GTPBP6. They propose a sequential model where binding of GTPBP6 triggers the release of MTERF4:NSUN4 and GTPBP5 from the assembling mt-LSU and remodels mt-rRNA to form a nearly mature PTC. Finally, the authors explore the involvement of GTPBP6 in the dissociation of mitoribosomes. They demonstrate that GTPBP6 can bind the mature mt-LSU and rearrange elements that contribute to inter-subunit bridges, providing a putative mechanism for GTPBP6-mediated ribosome recycling.

This is a well-executed and well-written structural study, and no further experiments are necessary before publication. However, I would recommend the authors take advantage of the more generous word count of Nature Communications to expand the introduction and discussion. The introduction should cover what is already known about the assembly of the mammalian mt-LSU, including identification of assembly factors and the current status of our structural understanding. The discussion should try and link the study with the late stages of the assembly of the bacterial ribosome. Which factors and processes are conserved, and which are specific to mammalian mitochondria? The discussion should also address what is known about GTPBP7 and GTPBP10, and why they were not have been observed in the cryo-EM maps despite their presence in the mass spectrometry data.

I commend the authors for uploading their proteomics data to ProteomXchange but also encourage them to upload their micrographs to EMPIAR, as they will be beneficial to the community working on ribosome biogenesis and the development of new cryo-EM methods.

Minor

1. Culture volumes should be provided in the Methods.
2. Abbreviations should be defined in the figure legend. For example, "MRP" in Fig. 1.
3. In general, the figures are excellent but the pink of GTPBP6 and the red of the mt-rRNA were hard for me to distinguish in many of the figures where they overlap.
4. Please include an mt-rRNA secondary structure diagram to show how the rRNA/PTC changes during the maturation process.
5. Please check the .cif files associated with dataset 3. They display abnormally in Coot v.0.9.3.
6. Please carefully check the rRNA for all atomic models. For example, nucleotides 2910-2911 are incorrectly positioned in the dataset 1 maps.
7. Given the high resolution of the maps, is it possible to map all post-transcriptional and post-translational modifications that have taken place in the mt-LSU? Where modifications are present, they should be included in the atomic model.
8. Please check the positions of the modeled Mg ions. Not all have density, for example Mg 3345 in the MTERF4-NSUN4 model.
9. To aid structural comparison by users, it would help if all maps and models are aligned before deposition.

Reviewer #2 (Remarks to the Author):

In this manuscript Hillen et. al. present two novel human mitoribosome assembly intermediates

isolated from a GTPBP6 deficient cell line. GTPBP6 is a dual function enzyme with both ATPase and GTPase activities and its GTPase activity is required for mitoribosome biogenesis progression and ribosome splitting.

Deletion of GTPBP6 resulted in the accumulation of assembly intermediates containing the MTERF4-NSUN4 complex and GTPBP5, both involved in peptidyl transferase center (PTC) maturation. In addition, the authors show that further addition of GTPBP6 to isolated intermediates progresses biogenesis by folding the PTC to a near mature stage. Lastly, the authors propose a mechanism of GTPBP6 ribosome recycling by examining its binding site on mature mitoribosomes using cryoEM. In the opinion of this reviewer, this manuscript should be published in Nature Communications provided that the following issues are addressed.

Major points:

1. In the ribosome assembly field intermediates originating from cells in which a key assembly factor was depleted should be treated with caution and the authors should acknowledge that some of their observations may be due to the absence of GTPBP6.
2. Based on the local resolution analyses in the supplement, it is clear that the well-ordered solvent exposed region majorly contributes to the high overall resolution. To visualize the quality of the map around each of the assembly factors, the authors should prepare supplementary density figures highlighting local resolution in each area.
3. For figures in which high levels of detail are shown (Fig. 2, 3, 4c; supplementary figure 4g) corresponding density figures should be provided.
4. For their reconstitution experiment the authors used GTPBP6 together with particles obtained from a GTPBP6^{-/-} cell line. In particular both ATP and GTP were added, presumably to occupy what the authors previously characterized as ATPase and GTPase activities. However, as ATP is absent from any of the presented structures, the authors should elaborate on the rationale of this experiment as well as the obtained results.
5. As mentioned above (point 1), depletion experiments can accumulate “off-pathway” particles. In addition, to clarify the proposed model (extended data figure 5), the authors should indicate which of the intermediates are based on their obtained structures, and which correspond to models.
6. As three studies have now appeared on biorxiv that describe human large subunit assembly intermediates, the authors may want to consider their data in light of the other data to put their findings into a broader context and to unify an assembly pathway.

Minor points:

1. Can the authors explain their choice of the initial assembly intermediate (extended data fig. 5)? Without clarity on rRNA structure in the figure, the starting model seems closer to a previously visualised & later stage intermediate (Brown et. al 2017). Data obtained from Trypanosoma intermediates further suggests that the MALSU1 complex arrives with Mtg2 (GTPBP5).
2. Line 137: “sequential sequence” is a tautology.
3. For Figure 2a-c the comparison with the mature mtLSU could be helpful.
4. It is currently unclear which reconstructions (i.e. from which of the two datasets) were used for Figure 2.

Reviewer #3 (Remarks to the Author):

Many thanks for asking me to look at these two papers from the labs of Rorbach and Richter-Dennerlein. It was a pleasure. I'd really appreciate it if I could review the two of them together rather than try and dissect them or just repeat some of my comments for each of them. Further, I think it is

really useful to review them together and commend you on asking reviewers to look at both together. I have absolutely no expertise in structural biology but I think I can comment more generally. Both pieces of work were of very high quality and address the pathway of mitoribosome biogenesis in human cell lines, particularly the maturation of the large subunit. The approach was similar in that Cipullo isolates partial complexes in the GTPBP5 KO cell line and Hellen in the GTPBP6 KO line. In addition, Cipullo use a GTPBP5 tagged IP and Hellen use an in vitro approach of adding back purified GTPBP6 to their partial complexes. There is substantial similarity between what is found by cryoEM with the GTPBP5 IP and the GTPBP6 KO but there are differences. For instance, in the IP Cipullo finds GTPBP7, MRM2 and intriguingly mt-EF-Tu. These are all not present in the cryoEM from the GTPBP6 KO. Why is this ? Whilst intriguing, I am a little worried that Cipullo claim from these images that mtEF-Tu is involved in assembly of the mitoribosome. I think this is speculation and would need more supporting evidence from whole cell studies. There is strong evidence that GTPBP6 acts downstream of GTPBP5 and the in vitro studies, where purified GTPBP6 is added back and images of a more mature mtLSU with GTPBP6 bound and GTPBP5 + NSUN/MTERF4 absent does make one think that perhaps indeed GTPBP6 has somehow displaced this proteins. However, it is surely possible that GTPBP6 has bound to some intermediates in the preps that have lost these other components naturally and GTPBP6 has bound the free sites. I would be more convinced if a similar GTPBP6 IP had been performed from whole cells. However, it is entirely possible that GTPBP6 has indeed displaced these components.

Overall, I am impressed by the quality of both sets of data. There are some weaknesses and if the manuscripts can be toned down a little to indicate where weaknesses of their data interpretation may lie then I would be supportive of publication. One thing that intrigues me is that in the Cipullo paper there is no mention of GTPBP6. It is a ghost! Have Cipullo never come across this protein ? Surely they must at least speculate that on the basis of their images it is very likely that other assembly factors need to function further downstream of GTPBP5 to fully mature the LSU and that GTPBP6 could be one ? Then together these two manuscripts read very well and the GTPBP6 manuscript follows neatly on from the GTPBP5 work. Finally, I liked the final assembly figure of Cipullo and was not massively wowed by the video in Heller. Could I ask that a similar figure be included to help the reader in Heller et al. ?

Response to the reviewers

Reviewer #1 (Remarks to the Author):

Hillen and colleagues use cryo-EM to illuminate the poorly understood process of GTPase-mediated mt-LSU assembly and peptidyltransferase center (PTC) maturation. Using a HEK293 cell line deficient in GTPBP6, a late-stage assembly factor, they isolate native mt-LSU intermediates and show how six biogenesis factors (GTPBP5, the MTERF4:NSUN4 complex, and the MALSU1:L0R8F8:mt-ACP module) bind premature mt-LSU and contribute to the folding of mt-rRNA. They then reconstitute mt-LSU assembly in vitro by complementing the biogenesis intermediates with recombinant GTPBP6. They propose a sequential model where binding of GTPBP6 triggers the release of MTERF4:NSUN4 and GTPBP5 from the assembling mt-LSU and remodels mt-rRNA to form a nearly mature PTC. Finally, the authors explore the involvement of GTPBP6 in the dissociation of mitoribosomes. They demonstrate that GTPBP6 can bind the mature mt-LSU and rearrange elements that contribute to inter-subunit bridges, providing a putative mechanism for GTPBP6-mediated ribosome recycling.

This is a well-executed and well-written structural study, and no further experiments are necessary before publication. However, I would recommend the authors take advantage of the more generous word count of Nature Communications to expand the introduction and discussion. The introduction should cover what is already known about the assembly of the mammalian mt-LSU, including identification of assembly factors and the current status of our structural understanding. The discussion should try and link the study with the late stages of the assembly of the bacterial ribosome. Which factors and processes are conserved, and which are specific to mammalian mitochondria? The discussion should also address what is known about GTPBP7 and GTPBP10, and why they were not have been observed in the cryo-EM maps despite their presence in the mass spectrometry data.

We thank the reviewer for the kind words. We have extended the introduction and discussion as suggested.

I commend the authors for uploading their proteomics data to ProteomXchange but also encourage them to upload their micrographs to EMPIAR, as they will be beneficial to the community working on ribosome biogenesis and the development of new cryo-EM methods.

We agree with the reviewer and plan to do so after publication.

Minor

1. Culture volumes should be provided in the Methods.

We provided the information accordingly.

2. Abbreviations should be defined in the figure legend. For example, “MRP” in Fig. 1.

We defined abbreviations in the figure legends.

3. In general, the figures are excellent but the pink of GTPBP6 and the red of the mt-rRNA were hard for me to distinguish in many of the figures where they overlap.

To address this, we have modified Figure 2 and colored the three conserved loops of GTPBP5 in cyan throughout the figure to enhance contrast to the RNA. We have additionally simplified this figure by changing the depiction slightly, and omitting bases and residues not relevant to the discussion.

4. Please include an mt-rRNA secondary structure diagram to show how the rRNA/PTC changes during the maturation process.

We thank the reviewer for the suggestion and have included a simplified secondary structure diagram of the mt-rRNA in Figure 1. This shows the secondary structure of the mature mt-rRNA with the regions that undergo changes during the described maturation steps in red.

5. Please check the .cif files associated with dataset 3. They display abnormally in Coot v.0.9.3.

We thank the reviewer for pointing this out, the issue should be fixed now.

6. Please carefully check the rRNA for all atomic models. For example, nucleotides 2910-2911 are incorrectly positioned in the dataset 1 maps.

We have carefully re-evaluated all models and maps prior to final submission to the PDB.

7. Given the high resolution of the maps, is it possible to map all post-transcriptional and post-translational modifications that have taken place in the mt-LSU? Where modifications are present, they should be included in the atomic model.

We have modeled the described 2'-O-methylations of the 16S rRNA in all models except for those representing the MTERF4-NSUN4-bound state prior to GTPBP5 binding. In this state, the modified residues are partially mobile and thus have less clear density. As we do not know with certainty whether this state is prior or after methylation, we refrained from modeling these modification. To our knowledge, the only other known modifications are m¹A2617 and PsiU3067. We do not observe density for the former, and would likely not be able to distinguish the latter from unmodified U.

While the overall achieved resolution is comparably high and allows to distinguish between the presence or absence of known modifications in well-ordered regions, it is still very difficult to identify and model modifications that may be present but are not well characterized. Notably, we observed several instances of extra densities near RNA or protein residues that could potentially correspond to covalent modifications. In particular, the sample for dataset 1 may have undergone partial oxidation, as we observed densities near several solvent-exposed cysteine residues accompanied by slight rearrangements of protein backbone and RNA bases near these residues (see methods section). These extra densities likely correspond to covalent oxidation adducts, but we refrained from modeling these due to their unknown nature and lack of clear features.

8. Please check the positions of the modeled Mg ions. Not all have density, for example Mg 3345 in the MTERF4-NSUN4 model.

We have revised the Mg ions in all models.

9. To aid structural comparison by users, it would help if all maps and models are aligned before deposition.

We agree with the reviewer, and have thus superimposed all final maps and models that were submitted to the PDB.

Reviewer #2 (Remarks to the Author):

In this manuscript Hillen et. al. present two novel human mitoribosome assembly intermediates isolated from a GTPBP6 deficient cell line. GTPBP6 is a dual function enzyme with both ATPase and GTPase activities and its GTPase activity is required for mitoribosome biogenesis progression and ribosome splitting.

Deletion of GTPBP6 resulted in the accumulation of assembly intermediates containing the MTERF4-NSUN4 complex and GTPBP5, both involved in peptidyl transferase center (PTC) maturation. In addition, the authors show that further addition of GTPBP6 to isolated intermediates progresses biogenesis by folding the PTC to a near mature stage. Lastly, the authors propose a mechanism of GTPBP6 ribosome recycling by examining its binding site on mature mitoribosomes using cryoEM.

In the opinion of this reviewer, this manuscript should be published in Nature Communications provided that the following issues are addressed.

Major points:

1. In the ribosome assembly field intermediates originating from cells in which a key assembly factor was depleted should be treated with caution and the authors should acknowledge that some of their observations may be due to the absence of GTPBP6.

We agree with the reviewer and discussed this possibility in the revised manuscript.

2. Based on the local resolution analyses in the supplement, it is clear that the well-ordered solvent exposed region majorly contributes to the high overall resolution. To visualize the quality of the map around each of the assembly factors, the authors should prepare supplementary density figures highlighting local resolution in each area.

In the local resolution figures, blue coloring corresponds to high resolution while red corresponds to lower resolutions. Thus, the most rigid core, non-solvent exposed region displays highest resolution, while the solvent-exposed and more flexible regions display lower resolution, as is typical for single-particle cryo-EM maps. As suggested by the reviewer, we have added density figures for each of the factors we describe (Supplementary Fig. 2 and Supplementary Fig. 4)

3. For figures in which high levels of detail are shown (Fig. 2, 3, 4c; supplementary figure 4g) corresponding density figures should be provided.

We agree in principle with the reviewer. However, as the mentioned figures already contain a lot of information, we feel that adding density would disrupt the clarity and

the resulting figures would fail to convey the model-to-map fit appropriately. As an alternative, we have included close-up density figures for the PTC-interacting regions of GTPBP5 and GTPBP6 in Supplementary Fig. 2 and 4. We believe this can convey a good impression of the map quality in the regions relevant to our results without sacrificing clarity of our main figures, and hope the reviewer agrees with this assessment.

4. For their reconstitution experiment the authors used GTPBP6 together with particles obtained from a GTPBP6^{-/-} cell line. In particular both ATP and GTP were added, presumably to occupy what the authors previously characterized as ATPase and GTPase activities. However, as ATP is absent from any of the presented structures, the authors should elaborate on the rationale of this experiment as well as the obtained results.

We recently measured ribosome dissociation activity of GTPBP6 in a heterologous in vitro system (Lavdovskaia et al., 2020) showing that ribosome recycling mediated by GTPBP6 is dependent on GTP binding, but not on GTP hydrolysis. The addition of ATP or non-hydrolysable ATP analogs had no influence on this function. It has been suggested that the bacterial homolog HflX has ATP-dependent RNA-helicase activity to split heat-damaged ribosomes (Dey et al., 2018). Prior to the structural analyses we did not have any evidence whether ATP is required for the second function of GTPBP6 as a ribosome biogenesis factor. Thus, we collected cryo-EM datasets of the assembly intermediates purified from GTPBP6^{-/-} cells and treated with recombinant GTPBP6 in the presence of both ATP and a non-hydrolysable ATP analog. However, in neither case could we observe any bound nucleotide in the N-terminal domain. Thus, ATP is apparently also not required for its second function, and we decided to describe the sample for which we had the best cryo-EM dataset in this manuscript.

5. As mentioned above (point 1), depletion experiments can accumulate “off-pathway” particles. In addition, to clarify the proposed model (extended data figure 5), the authors should indicate which of the intermediates are based on their obtained structures, and which correspond to models.

We agree with the reviewer that this was not clear in our initial figure. We have decided to redesign the model figure and move it to the main figures (Figure 4). This new figure now includes clear indications of where the respective models are derived from.

6. As three studies have now appeared on biorxiv that describe human large subunit assembly intermediates, the authors may want to consider their data in light of the other data to put their findings into a broader context and to unify an assembly pathway.

We discussed our results in the context of the findings provided by Cipullo et al. (bioRxiv, doi.org/10.1101/2021.03.15.435084); Cheng et al. (bioRxiv, doi.org/10.1101/2021.03.17.435838); Lenarcic et al. (bioRxiv, doi.org/10.1101/2021.03.29.437532) and Chandrasekaran et al. (bioRxiv, doi.org/10.1101/2021.03.19.436169).

Minor points:

1. Can the authors explain their choice of the initial assembly intermediate (extended data fig. 5)? Without clarity on rRNA structure in the figure, the starting model seems closer to a previously visualised & later stage intermediate (Brown et. al 2017). Data obtained from Trypanosoma intermediates further suggests that the MALSU1 complex arrives with Mtg2 (GTPBP5).

Brown et al. (2017) described two assembly intermediates, one of which contains a highly unstructured interfacial rRNA (PDB 5OOM). We thus reasoned that this intermediate likely represents an earlier state than the ones observed in this study, and chose it as starting model. Note, however, that this model already contains the MALSU1 complex.

2.Line 137: "sequential sequence" is a tautology.

We have changed this accordingly.

3.For Figure 2a-c the comparison with the mature mtLSU could be helpful.

We thank the reviewer for the suggestion. We feel that including the comparison of the PTC to the mature state in this figure would disrupt the flow, because a further step is required (GTPBP6) which is depicted in the next figure. Instead, our revised model (Figure 4) contains close-up views of the PTC as it undergoes GTPase-mediated maturation, and this is also animated in the accompanying movie.

4.It is currently unclear which reconstructions (i.e. from which of the two datasets) were used for Figure 2.

Figure 2 was created using the models built from dataset 1, and we have now indicated this in the figure legend.

Reviewer #3 (Remarks to the Author):

Many thanks for asking me to look at these two papers from the labs of Rorbach and Richter-Dennerlein. It was a pleasure. I'd really appreciate it if I could review the two of them together rather than try and dissect them or just repeat some of my comments for each of them. Further, I think it is really useful to review them together and commend you on asking reviewers to look at both together. I have absolutely no expertise in structural biology but I think I can comment more generally. Both pieces of work were of very high quality and address the pathway of mitoribosome biogenesis in human cell lines, particularly the maturation of the large subunit. The approach was similar in that Cipullo isolates partial complexes in the GTPBP5 KO cell line and Hellen in the GTPBP6 KO line. In addition, Cipullo use a GTPBP5 tagged IP and Hellen use an in vitro approach of adding back purified GTPBP6 to their partial complexes. There is substantial similarity between what is found by cryoEM with the GTPBP5 IP and the GTPBP6 KO but there are differences. For instance, in the IP Cipullo finds GTPBP7, MRM2 and intriguingly mt-EF-Tu. These are all not present in the cryoEM from the GTPBP6 KO. Why is this ?

We can of course only speculate on the reason for the absence of these factors in our reconstructions. We did observe GTPBP7 in our mass spectrometry results, however, despite extensive classification efforts, we did not observe a particle

population showing density corresponding to GTPBP7. Based on the recent studies by the Ban group (Lenarcic et al., bioRxiv, doi.org/10.1101/2021.03.29.437532), by Cipullo et al. and Chandrasekaran et al. that appeared simultaneously with our work, we speculate that GTPBP7-binding may be flexible and highly transient. This could explain the absence of structurally interpretable density for GTPBP7 in our datasets, as the population of GTPBP7-bound mtLSU may be very small in our datasets. One important difference between our approaches is obviously that purification via GTPBP5-FLAG enriches for intermediates with this factor bound. In the GTPBP6-deficient cells, GTPBP5-bound mtLSU presents just a small subclass of particles. Thus, it is not unexpected that factors that may bind transiently together with GTPBP5, such as MRM2, are not dominant in our dataset. The absence of MRM2 in MS and in the structure also matches with our results that we observe corresponding density for 2'-O-methylation at position U3039, which is in contrast to MRM2-bound mtLSU provided by Cipullo et al. and clearly indicates that our GTPBP5-bound state represents an intermediate downstream of MRM2-action. This suggests that MRM2 can dissociate after methylating U3039, whereas GTPBP5 can remain bound until GTPBP6 binds.

Whilst intriguing, I am a little worried that Cipullo claim from these images that mtEF-Tu is involved in assembly of the mitoribosome. I think this is speculation and would need more supporting evidence from whole cell studies. There is strong evidence that GTPBP6 acts downstream of GTPBP5 and the in vitro studies, where purified GTPBP6 is added back and images of a more mature mtLSU with GTPBP6 bound and GTPBP5 + NSUN/MTERF4 absent does make one think that perhaps indeed GTPBP6 has somehow displaced this proteins. However, it is surely possible that GTPBP6 has bound to some intermediates in the preps that have lost these other components naturally and GTPBP6 has bound the free sites. I would be more convinced if a similar GTPBP6 IP had been performed from whole cells. However, it is entirely possible that GTPBP6 has indeed displaced these components.

We thank the reviewer for his/her comment and agree that it is also possible that GTPBP6 binds to mtLSU intermediates with free sites. Unfortunately, a similar IP for GTPBP6 as performed for GTPBP5 would not yield adequate amounts of mtLSU complexes. The interaction of GTPBP6 with the mitochondrial ribosome seems to be rather transient (Lavdovskaia et al., 2020). In addition, we have to be very careful with the expression levels of GTPBP6 as elevated levels will lead to ribosome splitting and translation deficiency.

Although we do not have direct evidence that GTPBP6 releases GTPBP5 and MTERF4-NSUN4, it is clear that the binding of GTPBP6 and MTERF4-NSUN4-GTPBP5 is mutually exclusive. In addition, the number of particles containing MTERF4-NSUN4-GTPBP5 was reduced by approximately 50% comparing dataset 2 with dataset 1, which also supports the hypothesis that GTPBP6 replaces these assembly factors. We have, however, reflected this uncertainty in our conclusions accordingly.

Overall, I am impressed by the quality of both sets of data. There are some weaknesses and if the manuscripts can be toned down a little to indicate where weaknesses of their data interpretation may lie then I would be supportive of

publication. One thing that intrigues me is that in the Cipullo paper there is no mention of GTPBP6. It is a ghost! Have Cipullo never come across this protein ? Surely they must at least speculate that on the basis of their images it is very likely that other assembly factors need to function further downstream of GTPBP5 to fully mature the LSU and that GTPBP6 could be one ? Then together these two manuscripts read very well and the GTPBP6 manuscript follows neatly on from the GTPBP5 work. Finally, I liked the final assembly figure of Cipullo and was not massively wowed by the video in Heller. Could I ask that a similar figure be included to help the reader in Heller et al. ?

We thank the reviewer for his/her suggestion and included a new main figure (Fig. 4), which contains both a simplified cartoon representation of the model as well as more detailed views of the PTC rearrangements that occur.

Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- ☒ ☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- ☒ ☐ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☒ ☐ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- ☒ ☐ A description of all covariates tested
- ☒ ☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- ☒ ☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☒ ☐ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
- ☒ ☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☒ ☐ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection Serial EM 3.8 beta 8

Data analysis RELION 3.0 beta-2, UCSF Chimera 1.13, UCSF ChimeraX v1.2, COOT 0.9.5, Warp v1.0.7, PHENIX 1.18, MaxQuant 1.6.0.1

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research [guidelines for submitting code & software](#) for further information.

Data

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All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The electron density reconstructions and structure coordinates were deposited with the Electron Microscopy Database (EMDB) under accession codes EMD-12865, EMD-12872, EMD-12867, EMD-12868, EMD-12870, EMD-12869, EMD-12871 and with the Protein Data Bank (PDB) under accession codes 7OF0, 7OF7, 7OF2, 7OF3, 7OF5, 7OF4, 7OF6.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2018) partner repository with the dataset identifier PXD023502. Project Name: GTPase driven maturation of the human mitoribosomal peptidyl transferase center; Project accession: PXD023502.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No statistical methods were used to predetermine sample size. All biochemical experiments were replicated two or more times.
Data exclusions	No data were excluded from the analyses.
Replication	All attempts at replication were successful.
Randomization	Samples were not allocated to groups.
Blinding	Investigators were not blinded during data acquisition and analysis because it is not a common procedure for the methods employed.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

Methods

n/a	Involved in the study	n/a	Involved in the study
☐	☒ Antibodies	☒	☐ ChIP-seq
☐	☒ Eukaryotic cell lines	☒	☐ Flow cytometry
☒	☐ Palaeontology and archaeology	☒	☐ MRI-based neuroimaging
☒	☐ Animals and other organisms		
☒	☐ Human research participants		
☒	☐ Clinical data		
☒	☐ Dual use research of concern		

Antibodies

Antibodies used	Primary polyclonal anti-rabbit antibodies used in this study (dilution is indicated in brackets): anti-NSUN4 [1:1000] (Lavdovskaia et al., 2020; ProteinTech; #16320-1-AP), anti-MTERF4 [1:1000] (Lavdovskaia et al., 2020; Sigma Prestige; #HPA027097; RRID:AB_10603879), anti-MALSU1 [1:1000] (Lavdovskaia et al., 2018; Proteintech; #22838-1-AP; RRID:AB_11182483), anti-GTPBP5 [1:1000] (Sigma Prestige; #HPA047379; RRID:AB_10965845), anti-GTPBP10 [1:1000] (Lavdovskaia et al., 2018; Novusbio; #NBP1-85055; RRID:AB_11037644), anti-bL32m [1:1000] (Lavdovskaia et al., 2018; gift from Prof. P. Rehling; PRAB4957), anti-uS14m [1:1000] (Proteintech; #16301-1-AP; RRID:AB_2878240). Primary monoclonal anti-mouse antibody used in this study (dilution is indicated in brackets): anti-uL3 [1:500] (Proteintech; #66130-1-IG; RRID:AB_2881529).
Validation	Antibodies have been validated by ProteinTech (anti-uL3) and in recent studies: PMID: 30085210 (anti-GTPBP10, anti-MALSU1, anti-bL32m), PMID: 33264405 (anti-uS14m, anti-NSUN4, anti-MTERF4, anti-GTPBP5) PMID: 32652011 (anti-MTERF4, anti-GTPBP5).

Eukaryotic cell lines

Policy information about [cell lines](#)

Cell line source(s)	HEK293-Flp-In T-Rex - wild type (ThermoFisher Scientific, #R78007), HEK293-Flp-In T-Rex - Gtpbp6-/- (Generated by Elena Lavdovskaia, Research group Richter-Dennerlein, University Medical Center Göttingen; Lavdovskaia et al., 2020)
Authentication	HEK293-Flp-In T-Rex - wild type cell line was not authenticated. CRISPR/Cas9 mediated knockout of GTPBP6 was confirmed by sequencing (Lavdovskaia et al., 2020).
Mycoplasma contamination	Cells were systematically confirmed to be negative for the presence of Mycoplasma by GATC Biotech.

Commonly misidentified lines
(See [ICLAC](#) register)

No commonly misidentified cell lines were used.