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Supplemental Information

**Structure of Human Mitochondrial
Translation Initiation Factor 3 Bound
to the Small Ribosomal Subunit**

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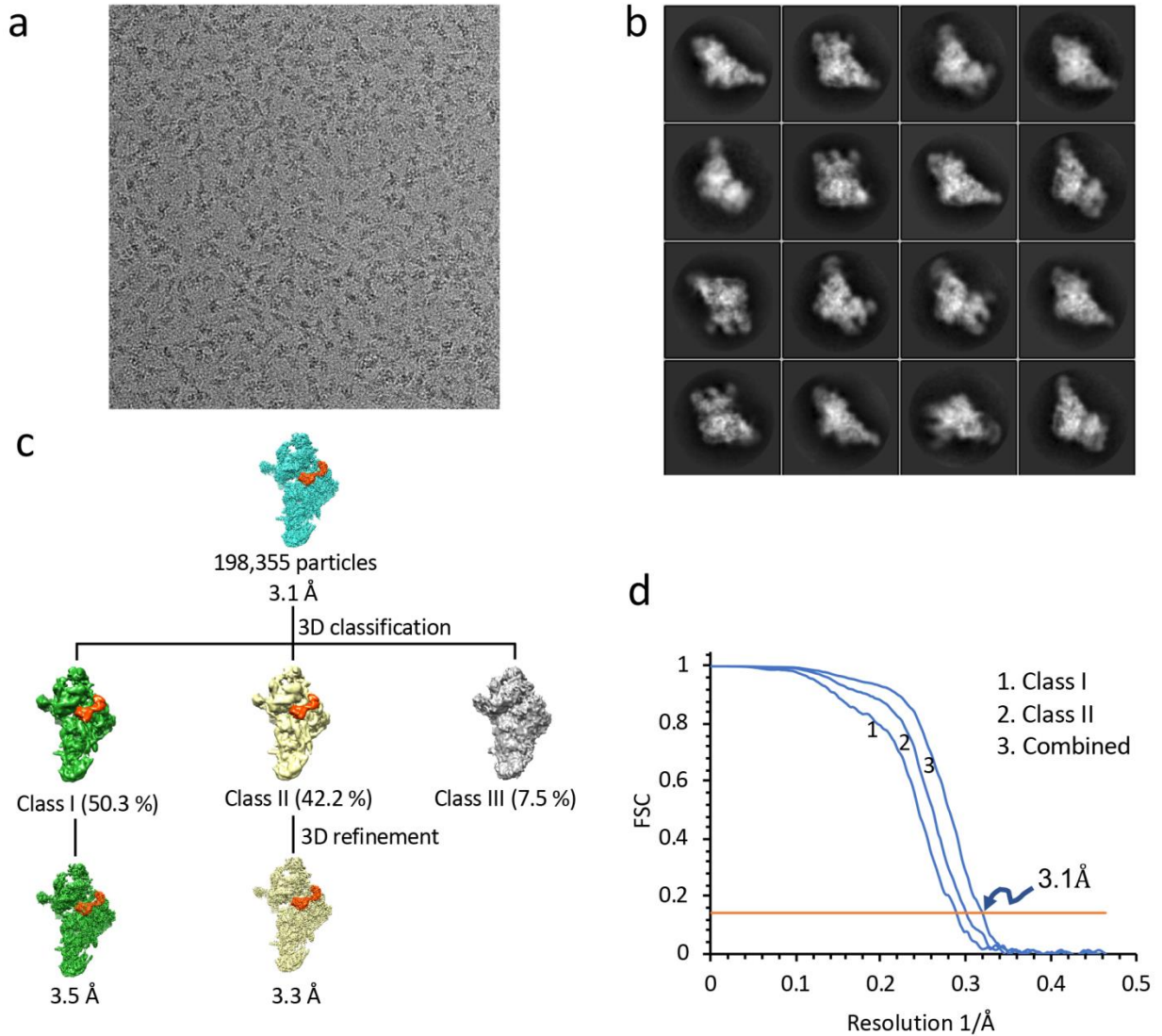


Figure. S1. Image processing of the 28S-IF3_{mt} complex, Related to Figure 1 and Transparent Methods. **(a)** A typical electron micrograph showing the bovine mitochondrial 28S subunit in complex with human IF3_{mt}. **(b)** Representative two-dimensional (2D) class averages used in three-dimensional (3D) reconstructions. **(c)** Flow-chart showing results of 3D classification and refinements. A total of 198,355 particles corresponding to the selected 2D averages were refined to 3.1 Å. To remove conformational heterogeneity, the particles were subjected to 3D classification that yielded three different classes. After discarding the poorly aligned particles (Class III), particles corresponding to the other two classes were independently refined, to 3.5 Å (Class I), and 3.3 Å (Class II) **(d)** Fourier-shell correlation (FSC) curves of the final two maps that were used in our analysis.

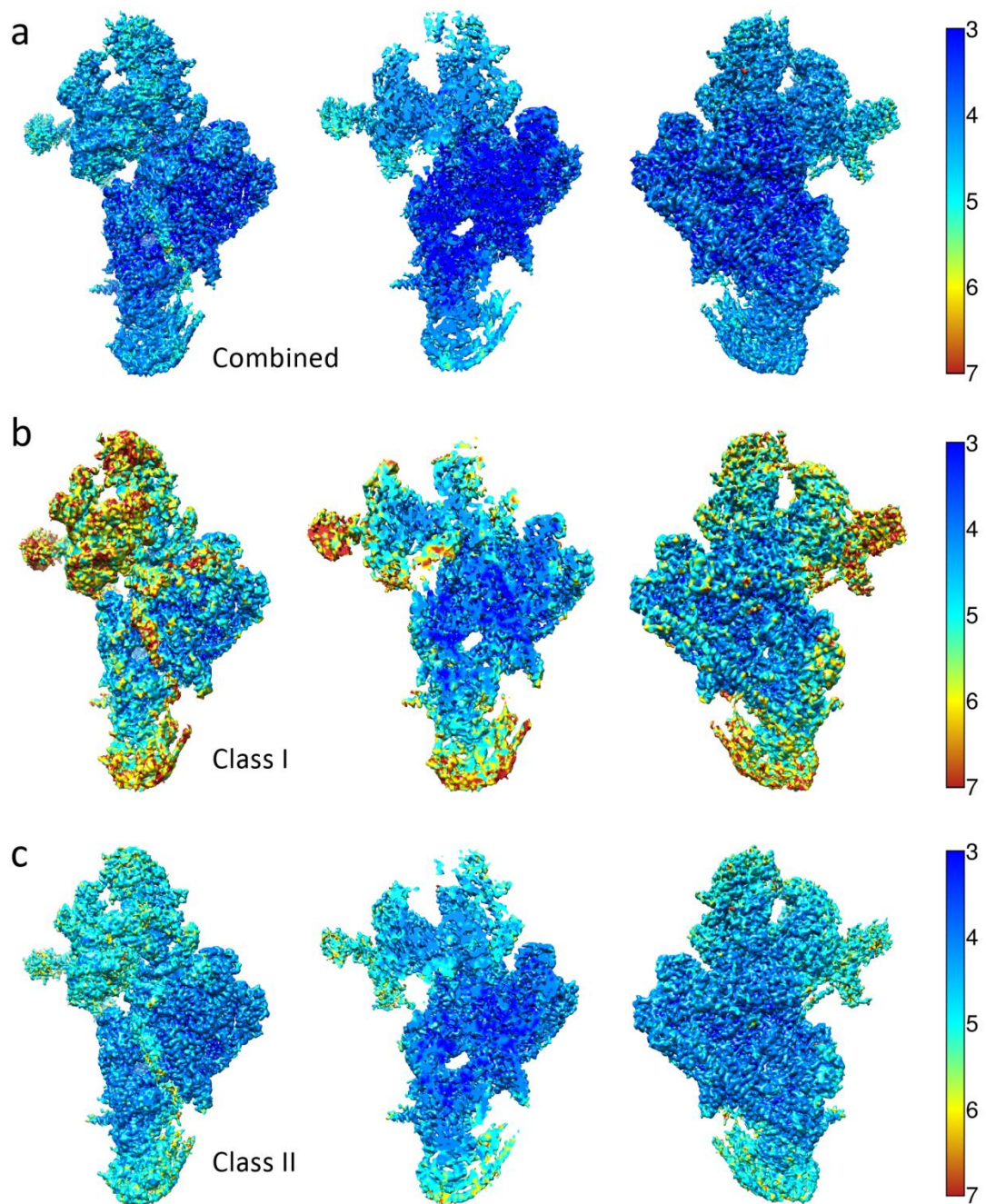


Figure. S2. Local resolution maps, Related to Figures 1, 2 and 3, and Transparent Methods. Local resolution for (a) the map obtained from full dataset, (b) Class I, and (c) Class II. Left panels show the local resolution maps as viewed from the subunit interface side, middle panels depict the core regions, after applying cutting planes, and right panels represent the view from the back side. Maps are color coded according to resolution bars shown on the extreme right.

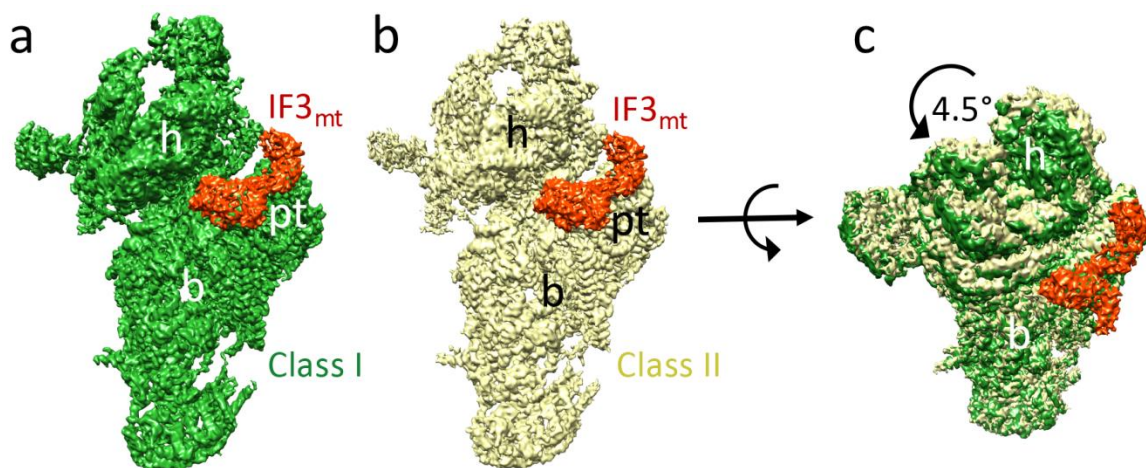


Figure. S3. Rotation of the 28S head relative to the platform region, Related to Figure 1. (a) Cryo-EM maps of class I (green) and (b) class II (yellow) with bound IF3_{mt} (orange). Landmarks of the 28S subunit: h, head, b, body, and pt, platform. (c) Superimposition of class I with class II reveals an overall ~ 4.5 ° rotation of the 28S head in Class I, away from the platform region.

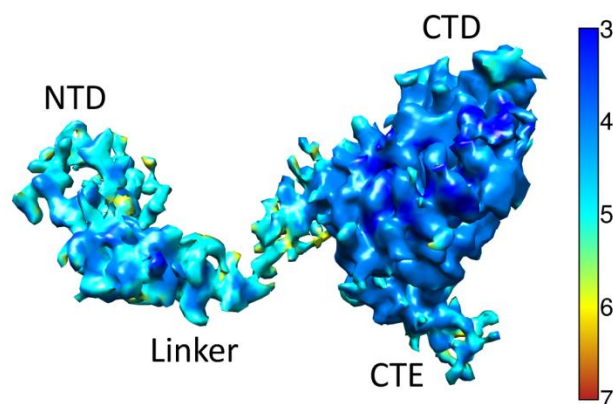


Figure. S4. Local resolution map of IF3_{mt}, Related to Figures 1 and 2. Local resolution for the density corresponding to IF3_{mt} extracted from the cryo-EM map of the 28S-IF3_{mt} Class II complex.

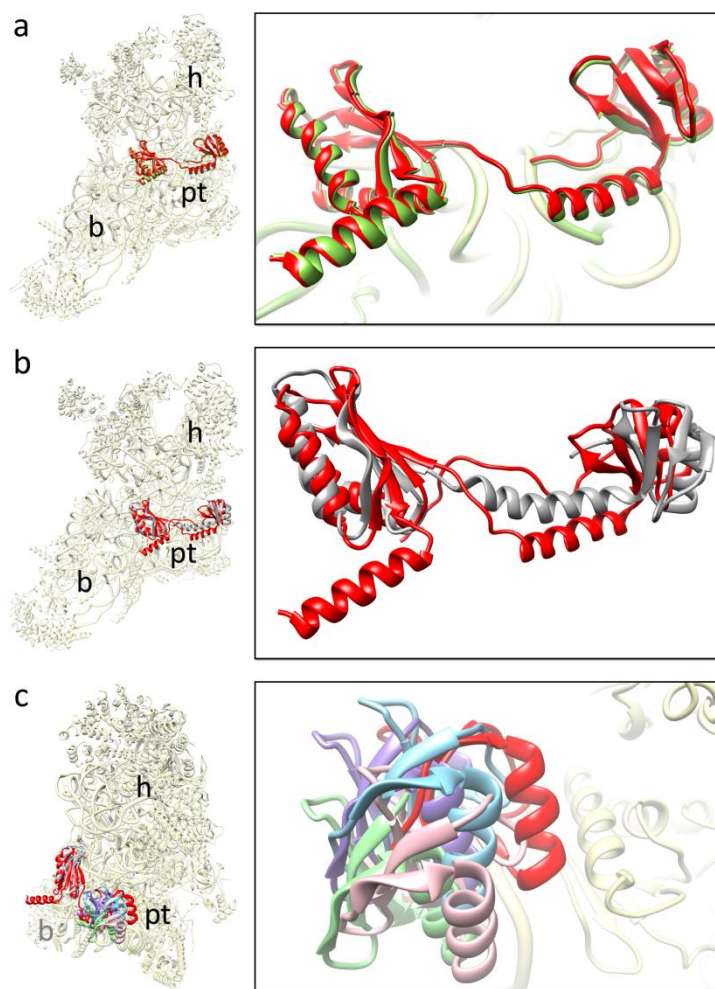


Figure. S5. Position of IF3_{mt} NTD and CTD on the 28S subunit, Related to Figures 1 and 2.

(a) The overall binding position of IF3_{mt} in class I (green) and class II (red) remained almost identical, though the conformation of the 28S head domain is significantly different between these classes (Fig. S3). (b) Structures of the human IF3_{mt} (red) and the bacterial IF3 (grey) (Hussain et al., 2016) superimposed. (c) The NTD of IF3_{mt} (red) is positioned uniquely on the 28S subunit, as compared to the positions of NTD of the bacterial factor on the 30S subunit captured in four conformational states (lighter shades of green, purple, pink and blue) (Hussain et al., 2016; Lopez-Alonso et al., 2017). Thumbnails to the left represent overlaid positions of the ligands relative to the overall orientation to the 28S subunit (yellow). Landmarks on the thumbnail: h, head, b, body, and pt, platform.

a	R. norvegicus	GVDLIGTMHRADVIRLMDKQDLRLVQRNTTSEPPPEYQLMTGAQIHQERLRLRLEQEKAKPKT	150
	S. scrofa	GNDLIGHMHRANVIRLMAERDLRLVRRDPGAEPPEYQLLTGAQIHQERLRLREAGRAEPK-	152
	B. taurus	GNDLIGHMHRANVIRLMAERDLRLVVRDASAEPPEYQLLTGAQIHQERLRLREAEAAAPK-	147
	H. sapiens	GNDLIGNMHRANVIRLMDERDLRLVQRNTSTEPPEYQLMTGLQILQERQRLREMEKANPK-	152
	E. caballus	GNDLIGNMHRANVIRLMDERDLRLVRRNPGTEPPPEYQLMTGIQIHQERLRLREASGARPT-	153
	O. cuniculus	GNDLIGNMHRAEVIIRLMDERDLRLVQRDAHAEPPEYQLMTGLQIHQERLRLREMEKAQPK-	179
b	R. norvegicus	AGPTVTKELIFSSNIGQHDLDTKSKQIQQWIEKKYHVQVTIKKRRDAEQPGSEMDEIFNQ	210
	S. scrofa	PGPTLTKELTFFSSNIGQHDLDTKSKQIQQWIEKKYKVQITIKKGRNAEPEPNKMEELCNQ	212
	B. taurus	PGPTLTKELTFFSSNIGQHDLDTKSKQIQQWIEKKYKVQITVKKGRSADEPEDKMEEMCNR	207
	H. sapiens	TGPTLRKELILSSNIGQHDLDTKTKQIQQWIKKKHLVQITIKKGRNVDSVENEMEEIFHQ	212
	E. caballus	PGPTLTKELTFFSSNIGQHDLDTKSKQIQQWIEKKYKVQITIKKGRNAEPEPNKIEEIFNH	213
	O. cuniculus	AGPILTKELTFFSSNIGQHDLDTKNKQIQQWIEKKYQVQITIKKGRNADEPENKTEELFNQ	239
c	H. sapiens	TSTEPPEYQLMTGLQILQERQRLREMEKANPKTGPTLRKELILSSNIGQHDLDTKTKQIQ	180
	C. tetani	PTGKPPVCKIMNYGKFLYEQTKKDKE--AKKKQKVINVKELIRLSATIEEHDIGIKANNAR	110
	S. aureus	PNAKPPVARIMDYGKFKFEQQKKEKE--MKKKQKIVNVKELIRLSPTIEEHDVQTKLKNR	122
	L. monocytogenes	PTAKPPVARIMDYGKFRFEQQKDKKE--ARKNQKVIKVEVRLSPTIDEHDFDTKLRNAR	107
	E. coli	PNAEPPVCRIMDYGKFLYEKSKSKE--QKKKQKVIQVKEIKFRPGTDEGDIYQVKLRSLI	115
	S. typhimurium	PNAEPPVCRIMDYGKFLYEKSKSKE--QKKKQKVIQVKEIKFRPGTDEGDIYQVKLRSLI	115
	T. thermophilus	PNADPPVARIMDYKWKRYEQQMAEKE--ARKKAKRTEVKS IKFRVKIDEHDYQTKLGH IK	106
M. smegmatis	PNARPPVCKIMDYGKYKYETAQKARE--SRKNQQQTVVKEQKLRPKLDDHDYETKKGHVI	119	
d	H. sapiens	LRAFSKNEEKAYKETQETQERDTLNKDHGNDKESNVLHQ	278
	C. tetani	A-PRK-----	173
	S. aureus	A-PTAEK-----	186
	L. monocytogenes	A-PLHEK-----	171
	E. coli	A-PKKKQ-----	180
	S. typhimurium	A-PKKKQ-----	180
	T. thermophilus	A-PVKVSA-----	171
M. smegmatis	A-PHRGAKTRAKAAEAERPGGP-----APDEDAS----	206	

Figure. S6. Sequence alignment of mitochondrial IF3 homologues, Related to Figures 2, 3, and 6. Sequence alignment of select segments of (a, b) human IF3_{mt} with other mammalian homologues, and (c, d) with bacterial homologues.

a	H.sapiens	UUUAGAUCACCCUCUCCCAAUAAGCUAAAACUCACCUGAGUUGUAAAAACUCCAGUU	359
	B.taurus	UAAAG---CACCAUACCAAUAAGGUUAAAUUCUAACUAAGCUGUAAAAAGCAUGAUU	352
	H.sapiens	CAAACUGGGAUUAAGAUACCCCACUAUGC UUA GCCCUAAACCUC AACAGUAAAUCAACAA	478
	B.taurus	CAAACUGGGAUUAAGAUACCCCACUAUGC UUA GCCCUAAACA CAGAUAAUUA CAUAAACAA	471
	H.sapiens	ACCCUACGCAUUUAUUAUAGAGGAGACAAGUCGUACAUGGUAAGUGUACUGGAAAAGUGC	942
	B.taurus	CGCACUAGCU---ACAUGAGAGGAGACAAGUCGUACAAGGUAAGCAUACUGGAAAAGUGU	943
b	H.sapiens	HTKFSI YP PIPGEESSLRWAGKK FEE IP IAH IKASHNN TQI QVVSASNEPLAFASCGT EG	116
	B.taurus	RSSFSI YP PIPGQESSLRWAGKK FEE IP IAH IKASYNN TQI HVVSAAHQPLARASCGT EG	119
	H.sapiens	ERNAKKGTGIAAQTAGIAAARA KQKGV IHI RVVVKGLGPGRLSAMHGLIMGGLEV IS IT	176
	B.taurus	ERNAKKGTGIAAQTAGIAAARA KATGKGV THVRVVKGLGPGRLSAI KGLTMGGLEV IS IT	179
c	H.sapiens	DTQNEGKKTCKNK TAF SNVGRKI SQRVI HLF DE KGN DLGNMHRANV IRLMDERDLRLVQR	119
	B.taurus	-TQDEM TKKKKNE TAF SSVGRKI NER I I HVL DE QGN DLGHMHRANV IRLMAERDLRLVKR	114
	H.sapiens	NTS TEPAE YQLMTGLQ ILQERQR L EME KAN PKTGP TLRKELI LSSNIGQHLLDTKTKCI	179
	B.taurus	DASAEP PQYQLLTGAQ IHQERLR L EAE RAA PKPGPTLRKELTFSSNIGQHLLDTKSKCI	174
	H.sapiens	QQWIKK KHLVQITIKKGNVDVSENE ME EIFHQ ILQTMPGIATFSSRPQAVQGKALMCV	239
	B.taurus	QQWIEK KYKVQITVKKGSADEPEDKME EMCNR IVQTMGSIATFSSRPQPIRGKAVMCV	234

Figure. S7. Alignment of interacting segments of the 28S subunit components and IF3_{mt} in human and bovine, Related to Figure 5. Select segments of (a) the 12S rRNA (b) MRP uS11 and (c) IF3_{mt}.

Table S1. Data Collection, Refinement and Model Validation, Related to Figures 1, 2, and 3, and Transparent Methods.

Description	28S-IF3 _{mt} (Class I)	28S-IF3 _{mt} (Class II)
Data collection and Refinement		
Microscope	FEI Titan Krios	
Voltage (kV)	300	
Pixel size (Å)	1.07	1.07
Defocus range (µm)	-1 to -3	-1 to -3
Average e ⁻ dose per image (e ⁻ /Å ²)	70.0	70.0
Software	RELION /cryoSPARC	RELION /cryoSPARC
Particles (initial)	198,355	198,355
Particles (final)	99,178	83,508
Symmetry	C1	C1
FSC-threshold	0.143	0.143
Resolution (Å)	3.48	3.32
Map-sharpening <i>B</i> factor (Å ²) overall	87.0	97.9
RMS deviations		
Bonds lengths (Å)	0.001	0.001
Bonds angles (°)	0.5	0.37
Molprobrity clashscore	1.73 (88 th)	1.70 (89 th)
Clashscore, all atoms	3.23 (97 th)	2.92 (98 th)
Rotamer outliers (%)	0.06	0.04
Ramachandran plot		
Favored (%)	87.34	87.21
Outliers (%)	1.46	1.81
RNA		
Correct sugar puckers (%)	99.79	99.26
Angle outliers (%)	0.00	0.00
Bond outliers (%)	0.00	0.00
Good backbone conformations (%)	75.53	77.1
Model composition		
RNA bases	952	952
Protein residues	5,482	5,482

Transparent Methods

Preparation of the 28S-IF3_{mt} Complex: The mature form of human IF3_{mt} was expressed and purified as described previously (Koc and Spremulli, 2002). Bovine mitochondrial 28S ribosomal subunits were prepared as described previously (Spremulli, 2007). Complexes containing IF3_{mt} bound to 28S subunits were assembled in reaction mixtures (20 μ L) containing 1 μ M 28S subunits, 10 μ M IF3_{mt}, 50 mM Tris-HCl, pH 7.6, 40 mM KCl, 7.5 mM MgCl₂, 10% glycerol, 2 mM dithiothreitol and 0.1 mM spermine. Reaction mixtures were incubated for 20 min at 25 °C, divided into 5 μ L aliquots, fast-frozen in a dry ice isopropanol bath and stored at -70 °C. The amount of IF3_{mt} bound to the 28S subunits was measured using a quantitative immuno dot blot using antibodies to bovine IF3_{mt} as described previously (Haque and Spremulli, 2008). This analysis indicated that the complexes contained greater than 0.9 mol IF3_{mt}/mol 28S subunits. For cryo-EM analysis, the 28S-IF3_{mt} complex was diluted to 100 nM with buffer containing 10 mM Tris-HCl, pH 7.6, 20 mM MgCl₂, 40 mM KCl, 1 mM DTT, 0.1 mM spermine and 5% glycerol, and then incubated for five min at 37°C prior to loading on the grids.

Cryo-Electron Microscopy and Image Processing: Home-made carbon was coated as a continuous layer (~ 50 Å thick) onto Quantifoil holey copper 1.2/1.3 grids, which were then glow-discharged for 30 s on a plasma sterilizer. After loading 4 μ L of the sample to the grids, they were incubated for 15 s at 4°C and 100% humidity and then blotted for 4 s before flash-freezing into the liquid ethane using a Vitrobot (FEI). Data was acquired on a Titan Krios electron microscope equipped with a Gatan K2 summit direct-electron detecting camera at 300 KV. A defocus range of -1.0 to -3.0 μ m was used at a calibrated magnification of 22,500 X, yielding a pixel size of 1.07 pixels Å on the object scale. A dose rate of 7 electrons per pixel per s and an exposure time of 10 s resulted in a total dose of 70 eÅ⁻². Out of the 2,435 micrographs that were collected, 2,095 were selected after determining their contrast transfer function (CTF) using CTFFIND3 (Rohou and Grigorieff, 2015). The data was then processed in Relion 2.0 (Scheres, 2012) where a total of 320,824 particles were picked using its autopick function. All the downstream steps including 2D classification, 3D

classification and 3D refinement were performed using CryoSPARC (Punjani et al., 2017). After reference-free 2D classification, 198,355 good particles were selected, and refined to a resolution of 3.1 Å. The map showed strong IF3_{mt} density, but the 28S head region appeared to contain heterogeneity. Therefore, the dataset was subjected to an initial reference-based heterogeneous 3D classification (Fig. S1). 3D classification yielded two major classes that turned out to be 28S-IF3_{mt} complexes that showed conformational differences in their head regions and a minor class that contained mostly deformed ribosomal particles that could not be processed further. 3D refinement was performed on the two major classes that yielded a final resolution of 3.5 Å for Class I (99,178 particles) and 3.3 Å for Class II (83,508 particles) (Figs. S1 and S2).

Model Building: Coordinates from the published bovine 28S subunit (Kaushal et al., 2014; PDB ID: 3JD5) were docked independently as rigid bodies into the corresponding cryo-EM density maps of Class I and Class II using Chimera 1.11 (Pettersen et al., 2004). The models were subsequently refined and validated in PHENIX (Adams et al., 2010) using the real-space refinement function. Initial homology models for human IF3_{mt} were obtained from the Robetta server (Kim et al., 2004). The homology models were then placed independently as rigid bodies into the region of density map corresponding to the IF3_{mt} using Chimera 1.11 (Pettersen et al., 2004), and the model that showed optimal fitting were selected. Portions of the homology model that could not be explained by the cryo-EM density corresponding to IF3_{mt} were modelled in Chimera, based on the recognizable secondary structural elements (SSEs) and bulky side-chains of the amino acids in the cryo-EM map. The mito-specific C-terminal extension (CTE) was built *de novo* in Chimera 1.11 (Pettersen et al., 2004), guided by SSEs and position of the side-chains, and Coot (Emsley et al., 2010). Low resolution in the region of the cryo-EM map corresponding to the mito-specific N-terminal extension (NTE) restricted us from modelling the complete NTE, but the carbon backbone. The final model was refined for optimal fitting into the density map and further validated in PHENIX (Adams et al., 2010). The statistics of EM reconstructions and molecular modeling is provided in Table S1.

Data Availability: Both cryo-EM maps of the 28S subunit of the mammalian (*Bos taurus*) mitochondrial ribosome bound to human IF3 have been deposited in the Electron Microscopy and PDB Data Bank (wwPDB.org) under accession codes EMD-9362 and PDB ID 6NF8 for Complex I and under accession codes EMD-9358 and PDB ID 6NEQ for Complex II.

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