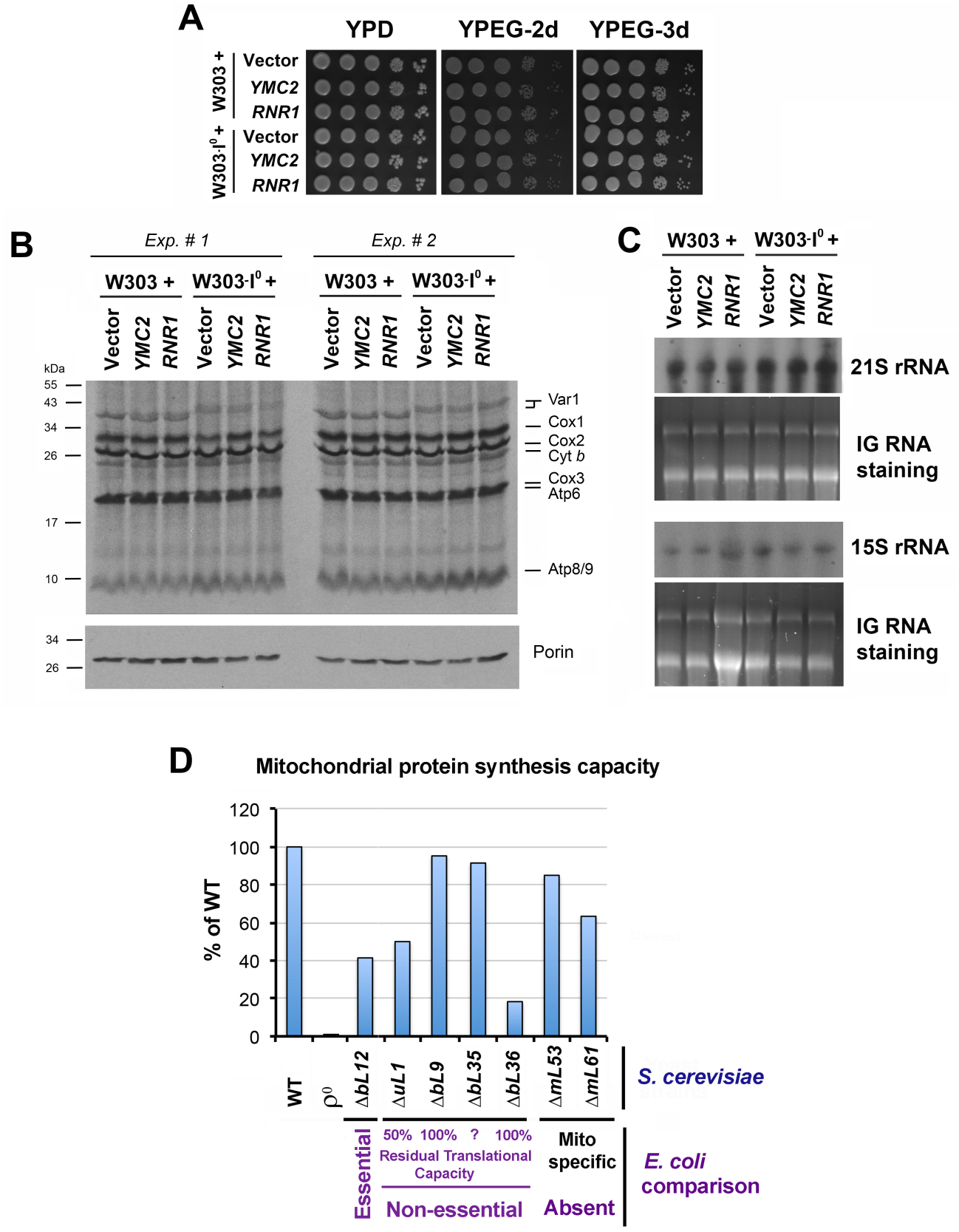
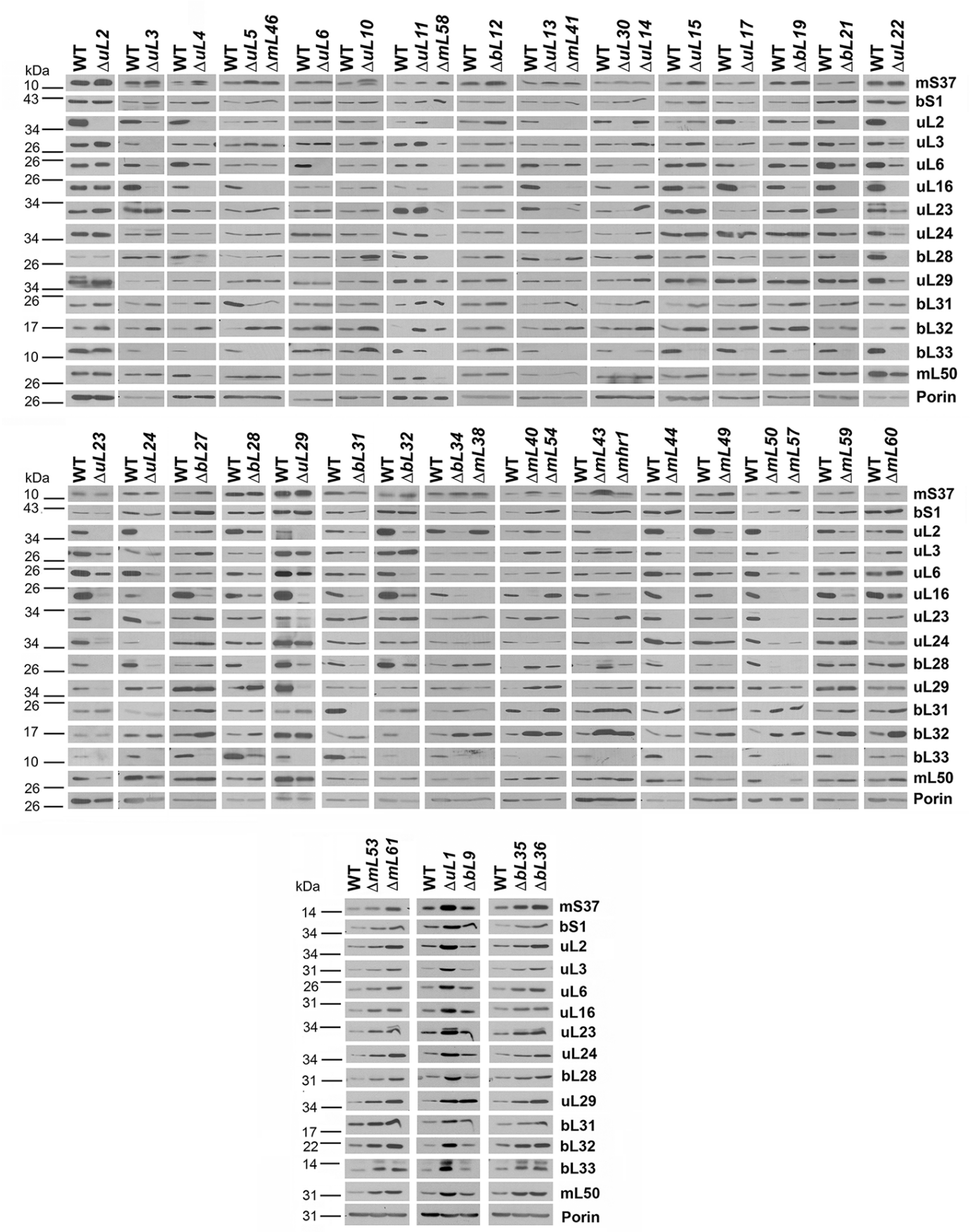
SUPPLEMENTAL FIGURES

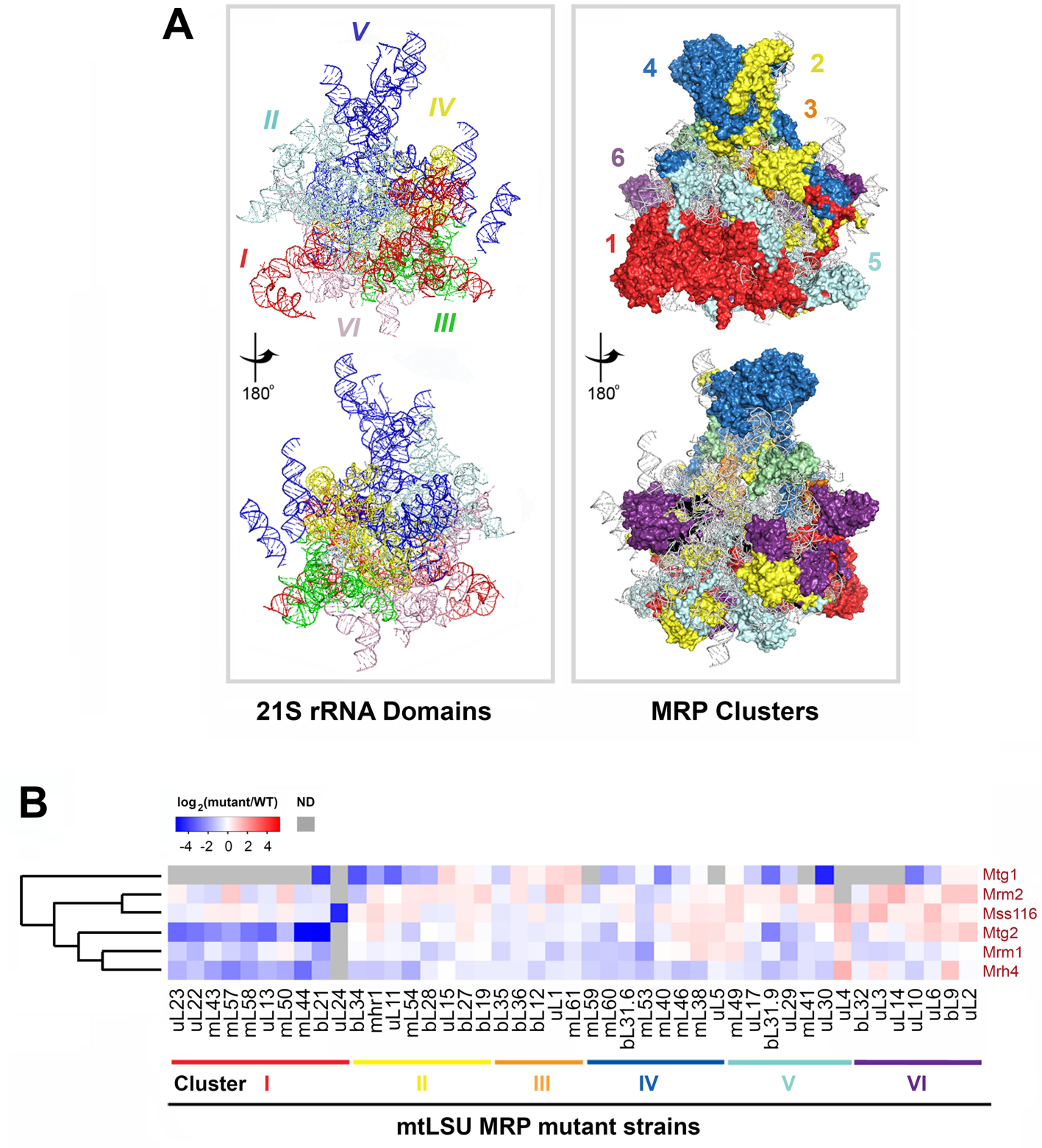
Figure S1. Related to Figure 1.



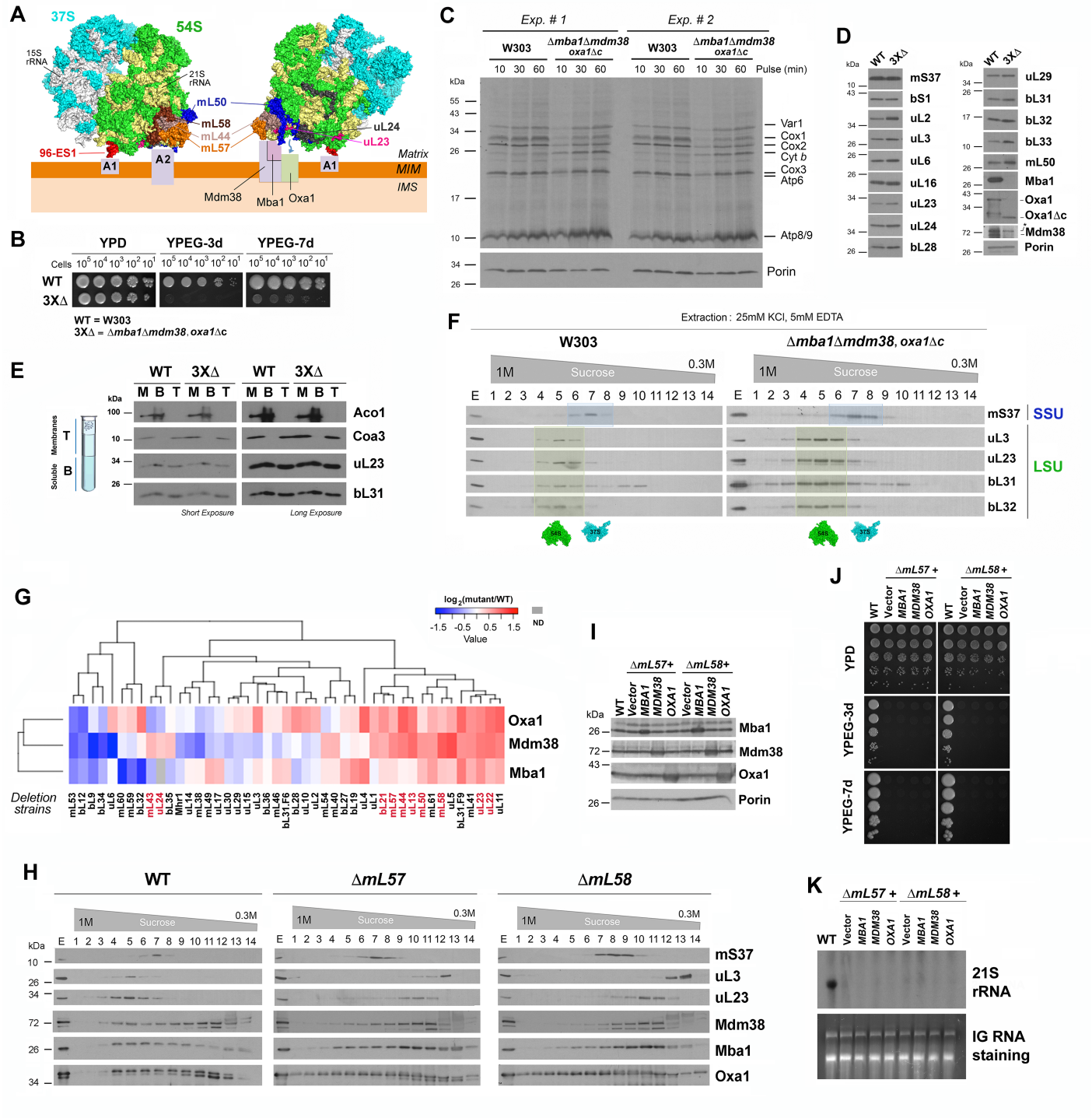
Supplemental Figure S2. Related to Figure 2.



Supplemental Figure S3. Related to Figure 3.



Supplemental Figure S4. Related to Figure 4.



SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure S1. Effect of genetic background on the phenotype of yeast mutant strains and quantification of the overall protein synthesis capacity in the non-essential yeast mutant strains. Related to Fig. 1.

A-C. Overexpression of *YMC2* or *RNR1* has no effect on cell growth, rRNA accumulation or mitochondrial translation:

A. Serial dilution growth test of wild type cells carrying intron-containing mtDNA (W303) or intron-less mtDNA (W303I^0), with or without overexpression of *YMC2* or *RNR1*.

B. Metabolic labeling of newly synthesized mitochondrial translation products with ^{35}S-methionine in the presence of cycloheximide to inhibit cytoplasmic proteins synthesis. Immunoblotting with an anti-Porin antibody was used as a loading control.

C. Steady-state levels of 21S rRNA and 15S rRNA analyzed by northern blotting using total cellular RNA. In gel (IG) RNA staining image of the cellular ribosomal RNAs was used as a loading control.

D. Quantification of the overall protein synthesis capacity in the non-essential yeast mutant strains. Following radiolabel incorporation following 15min pulse with ^{35}S-methionine, the proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane and exposed to X-ray film (**Fig 1C**). To quantify the incorporation of radiolabel into newly synthesized proteins, the images were digitalized, and densitometric analyses of overall signal per line was performed using the histogram function of the Adobe Photoshop program. The results from two independent experiments did not differ by more than 5%. In the bottom part of the figure, the results are compared with what is known for mutants of the homolog proteins in *E. coli*.

Supplemental Figure S2. Steady-state levels of mitoribosome proteins in WT and MRP mutant strains. Related to Fig. 2.

Immunoblot analyses of the steady-state levels of the indicated MRP proteins in WT and a selection of MRP mutant strains. Porin was used as loading control.

Figure S3. Mapping of protein clusters on the 21S rRNA structure and presence of assembly factors in mitoribosome subassemblies. Related to Fig. 3.

A. Structural tertiary domains of 21S rRNA and location of the protein clusters identified. Six domains of 21S rRNA were mapped to the mtLSU structure (PDB-3J6B (Amunts et al., 2014)). The RNA domains are numbered in Roman numerals and the protein clusters in Arabic numbers.

B. Hierarchical cluster analyses of levels of assembly factors in assembly intermediates accumulated in the mutant strains. Following analysis by mass spectrometry of the mtLSU intermediates that accumulate in the mtLSU mutant strains (**Fig. 3**), the abundance in each intermediate of five known 54S assembly factors was

considered in a hierarchical cluster analysis. These assembly factors are the 21S rRNA methyltransferases Mrm1 and Mrm2, the DEAD-box helicases Mss116 and Mrh4, and the GTPases Mtg1 and Mtg2. ND, not detected.

Supplemental Figure S4. Mba1, Mdm38 and Oxa1 are not essential for mitoribosome biogenesis. Related to Fig. 4.

A-F. Mba1, Mdm38 and Oxa1 are not essential for the anchoring of yeast mitoribosomes to membrane.

A. Two contact or anchoring sites (A1 and A2) between yeast mitoribosomes and the inner membrane where recently identified by cryoelectron tomography (Pfeffer et al., 2015) are depicted here, using a modified cartoon of the yeast mitoribosome structure (PDB 5mrc; (Desai et al., 2017)). The two distinct membrane contact sites are formed by the 21S rRNA expansion segment 96-ES1, labeled in red (A1) and the inner membrane protein Mba1 (A2). Because Mba1 has been shown to interact with Mdm38 and Oxa1, the three proteins are presented in contact, for simplification. mtSSU (37S) proteins are colored in cyan. Most proteins in the mtLSU (54S) are labeled in green color, except those that form part of the membrane facing protuberance, our Cluster 1 proteins, which are color-coded. A blue arrow indicates the tunnel exit opening. MIM, mitochondrial inner membrane; IMS, intermembrane space.

B. Serial dilution growth test of WT (W303) and a triple mutant strain Δ*mba1*Δ*mdm38*,*oxa1*Δ*c* (3XΔ) obtained from Dr. J. Herrmann, that was not characterized previously. The triple deletion strain lacks Mdm38, Mba1, and the C-terminal 71 residues of Oxa1 ($Oxa1^{1–331}$ or *oxa1*Δ*C*) and was constructed as reported (Bauerschmitt et al., 2010).

C. Metabolic labeling of newly synthesized mitochondrial translation products in the indicated strains, following 10, 30 and 60 min pulses with ^{35}S-methionine in the presence of cycloheximide to inhibit cytoplasmic protein synthesis. Immunoblotting for Porin was used as a loading control. Two experiments are presented.

D. Immunoblot analyses of the steady-state levels of the indicated mitoribosome proteins, Mba1, Mdm38 and Oxa1 in WT and Δ*mba1*Δ*mdm38*,*oxa1*Δ*c* (3XΔ) strain. Porin was used as loading control. *unspecific band.

E. Mitochondria of the indicated strains were fractionated into membrane (half top of the gradient –T) and soluble (half bottom of the gradient -B) proteins by sonication with freeze-thawing and flotation. M: total mitochondria extract. Proteins of these fractions were analyzed by immunoblotting. Markers of soluble (Aco1) and integral membrane proteins (Coa3) were used as controls. Two exposures of the films are presented.

F. Sucrose gradient sedimentation analyses of mt-SSU and mt-LSU proteins in mitochondrial extracts from the indicated strains, prepared in the presence of 25 mM KCl, 5mM EDTA and 0.8% Triton X-100. Blue and green transparent colors mark the fractions where the mtSSU and mtLSU sediments, respectively.

G-K. Overexpression of the mitoribosome membrane anchoring proteins Mba1, Mdm38 or Oxa1 does not restore 21S rRNA stability of mitoribosome assembly in the Δ*mL57* and Δ*mL58* strains.

G. Hierarchical cluster analyses of levels of Mba1, Mdm38 and Oxa1 in assembly intermediates accumulated in the mutant strains, performed as in Fig. 3 and S4. ND, not detected.

H. Sucrose gradient sedimentation analyses of Mba1, Mdm38, Oxa1 and markers of the mt-SSU (mS37) and mt-LSU (uL3 and uL23), in mitochondrial extracts from the indicated strains. The extracts were prepared in the presence of 0.8% Triton X-100, 25mM KCl and 5mM EDTA to force the dissociation of mitoribosome subunits.

I. Immunoblot analyses of the steady-state levels of Mba1, Mdm38 and Oxa1 in the indicated strains overexpressing each of the proteins from episomal constructs. Porin was used as a loading control.

J. Serial dilutions growth test of WT ($W303I^0$), and mutant strains Δ*mL57* and Δ*mL58* carrying an empty vector or constructs for overexpression of *MBA1, MDM38,* or *OXA1*.

K. Steady-state levels of 21S rRNA analyzed by northern blotting using total cellular RNA. In gel (IG) RNA staining image of the cellular ribosomal RNAs was used as a loading control.

SUPPLEMENTAL TABLES

Supplemental Table 1. List of *S. cerevisiae* mitoribosomal LSU proteins. Related to Fig. 1. The recently proposed nomenclature, the old traditional nomenclature and the bacterial homolog are presented for comparison. Respiratory growth phenotypes and mtDNA retention after 20 generations for the MRP mutant strains are listed. Conserved proteins are in red, those common to other mitochondria in blue and proteins specific to yeast mitochondria in green.

S. cerevisiae mtLSU proteins		Bacterial homolog	Null mutant phenotype	
New Name	Old name		Respiration (~ % of WT)	Percentage rho$^+$
uL1	Mrpl1	L1	67.92±5.73	100
uL2	Rml2	L2	0	79
uL3	Mrpl9	L3	0	70
uL4	Yml6	L4	0	67.80
uL5	Mrpl7	L5	0	87.70
uL6	Mrpl6	L6	0	72.30
bL12	Mnp1	L7/12	27.17±1.43	91.80
bL9	Mrpl50	L9	97.19±3.31	100
uL10	Mrpl11	L10	0	63.80
uL11	Mrpl19	L11	0	67.50
uL13	Mrpl23	L13	0	55.20
uL14	Mrpl38	L14	0	89.20
uL15	Mrpl10	L15	0	80
uL16	Mrpl16	L16		
uL17	Mrpl8	L17	0	45.50
bL19	Img1	L19	0	69.70
bL21	Mrpl49	L21	0	70.80
uL22	Mrpl22	L22	0	77.30
uL23	Mrp20	L23	0	91.10
uL24	Mrpl40	L24	0	72
bL27	Mrp7	L27	0	60
bL28	Mrpl24	L28	0	50
uL29	Mrpl4	L29	0	100
uL30	Mrpl33	L30	0	87.80
bL31	Mrpl36	L31	0	54.70
bL32	Mrpl32	L32	0	69.50
bL33	Mrpl39	L33		
bL34	Mrpl34	L3	0	51.20
bL35	Ynl122c	L35	96.65±2.28	100
bL36	Rtc6	L36	46.01±4.49	100
mL38	Mrpl35	-	0	65.30
mL40	Mrpl28	-	0	63
mL41	Mrpl27	-	0	61.70
mL43	Mrpl51	-	0	51.80
mL44	Mrpl3	-	0	50.50
mL46	Mrpl17	-	0	82.40
mL49	Img2	-	0	42.80
mL50	Mrpl13	-	0	71.10
mL53	Mrpl44	-	77.08±2.99	100
mL54	Mrpl37	-	0	48.90
mL57	Mrpl15	-	0	67.30
mL58	Mrpl20	-	0	64.60
mL59	Mrpl25	-	0	54
mL60	Mrpl31	-	0	81.80
mL61	Mrp49	-	34.52±1.20	100
Mhr1	Mhr1	-	0	47.40

Supplemental Table S2. Primers used for creation of MRP mutant strains. Related to Key Resources.

Gene	Forward (5'-3')	Reverse (5'-3')
uL1	CGACCCAGAATCACTTGC	GCTCTGCCAGCCTCAATC
uL2	CAGTGTTTGTTGTTTGTAAAATCAG	GTGAAACATTGGTTGCTGATC
uL3	CTATGAAACCTCGCTATAAGC	GCACTTTGAGGGACTTTTTG
uL4	CGTACCCAACAAACCTCG	CGGAGACGAGTTCTTTGC
uL5	GAATTGACGCATTACCCTC	GGACAAGGTTTGGCTCTG
uL6	CCTCCTACAGATGGCACTAAC	GTCCACCATACCCTGAAATTC
bL12	GAAGGAACAGGACAAGCAGCCCGTCAGCAGCAAACACAGTC AATTAAATAAGCGCCCAGTCGTACGCTGCAGGTCGAC (set 1)	CTCGAAGAGAAAACAGACTATTTTGATTAACACGATTGGTA GATTGCTACAGTTTTAAAAATCGATGAATTCGAGCTCG (set 1)
	GTCTTGAAGTCCAATAAAACAACTCAAACTAATGGCAAACAA GAACTCGGTAAGTTCTGGAAGGAACAGGACAAGCAG (set 2)	GGCAGGACGAAAGAAGAATAAAGAGTTAAACATATTTACA TGATATATTCCTTATTTTCTCGAAGAGAAAACAGAC (set 2)
bL9	CTCGGAATCCACCAGTTG	CTACGATGGGTTCACTCAAG
uL10	CCTGATTAACCATTGCCTAC	GGGAGATACTGGGTACTCTAAC
uL11	GTTAGAGGAGAAGACCATCATC	GCCACTTACGACGACGAG
uL13	GCACCTTACTGTAGAAGCACC	GGGTAAACAAGTAGGCAATAG
uL14	CGATTGACTTGGATAAGAGGTG	GGCATTATCTTTGGAAAGTAGC
uL15	GCCAGTCATTGGGTTATTG	GGTTAAGGCGTGCGACTG
uL17	CGCAAAGCCTATTGATGG	GTGAGTTCATCTGGGAGCG
bL19	GGGAGGCAAAGACAAGGAG	CCTGAAGTTGGGCACAATATC
bL21	CTTCCCAATGCTCTTCCTAC	CGATAACTGGCGTGCTTTC
uL22	GGGTTCATCTACTCAAGGTGG	CTTTGCCAGATGCTGTCACC
uL23	CCGAATTTACCCGTACACG	CCAAAGTCTCGCACAGCAG
uL24	GACCATAATAACCACCATTTG	GCCTGGCTAACGTTCTGG
bL27	GGCGTTCTATCTGCTCATTC	CGTCCGATCATTACGTGAG
bL28	GTGACGATGAAGTAGTAAAGGAGAG	GATTAATGACCAATGGAAATAGC
uL29	CCCTTTGTTTTCTCCTCCTC	GATTTTGGCACAGCATACG
uL30	GCACGAACCTTAGCGATATG	GCAGAACGGATTTACTGGATG
bL31	CTCAGAAGAGGGAATGGG	GTACGTGATAAGTATCGCAGC
bL32	CAAATGTGAGGCAAATAACG	CTACTTCTGATGCGGCTAAG
bL34	CACGGAAAGTGATAGTGAAGC	GGTACGGTGAGTGATGATATAAG
bL35	GGTCTGATCCAGTATTGGCTA	CATCACGCACAAATGGATTG
bL36	CATAGTCTGCCCTCATTCG	CATCAATCTGGTGTCAAGTCC
mL38	GATTATCATCGCCCGCTG	GAATACGCCAAGCTACACCC
mL40	GTCGTTTCATCGGCTTCG	GGGAAACAAACTTGTGGTAGTC
mL41	CAGGGTTGTTCGGTTTCTC	CAGGTTCCTCTGCTCGTG
mL43	GAATGCGATTGGAGAAGC	GTATTTCGGGATGAACAGG
mL44	GGCACATTCACATTCAGACG	CCATTGAGACTCTGCTTCATTAC
mL46	CAACAGCAGCAGCAACAAC	CTGCGGTAACAACACTAGACG
mL49	CTTTCTGTTGACCTCCCTGG	GGACCTGATTCTAACACCTCG
mL50	GCACTCCTTTCGTCTTCCC	CACAGTAACGCATGACCG
mL53	GTGCCTCTAAGTTTGCCG	CTAACTCGCAAGGCTTGTC
mL54	CGAGGTGATACTGACCGAAG	CTTTCTTGTGAGCGTCCTTC
mL57	CCATCTCATCTGTGTAGATC	CTGGATTTAGGCGAGCACTG
mL58	GGAGAATCTAGCTCATCTTC	CCATTAGCAGCAGCAATTGG
mL59	GGACTCTTTGCACGAGACG	CCACCGAGAACCATCATGTG
mL60	CCTTAGCACAATCTGCCATTGC	CCACTAACTGTCTTACCTC
mL61	GAACCACCAATCCAACAG	GGTAGGACTATTACTAGCACCAC
MHR1	CTACTTAAACGCCTTCAATCG	CAGTAAAGACGAAGACCCG