SUPPORTING INFORMATION

Yeast mitochondrial protein Pet111p binds directly to two distinct targets in *COX2* mRNA, suggesting a mechanism of translational activation

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SUPPORTING INFORMATION EXPERIMENTAL PROCEDURES

Purification of RNA oligonucleotides

The RNA probes used in the EMSA and RNase footprinting experiments were 5'-labeled with T4 polynucleotide kinase (PNK) (New England Biolabs) in the presence of γ-[^{32}P]-ATP (3000 Ci/mmol, Perkin Elmer) and the reaction mixtures were run on 16 % polyacrylamide gels (19:1, acrylamide:bisacrylamide) in the presence of 7 M urea. The bands containing the target RNA were located on the gels by autoradiography, the corresponding gel fragments were excised, crushed into small pieces, and extracted by shaking in 0.3 ml of 20 mM Tris·HCl (pH 7.9) and 1 M NaCl for 1 h at room temperature. The RNA was recovered from the extracts using RNA Clean & Concentrator-5 columns (Zymo Research) as directed by the manufacturer's instruction manual, except that the concentration of ethanol was increased to 75 % in the binding step. The purified RNA was quantitated by UV/Vis spectrophotometry, incubated at 65 °C for 3 min, and then snap-cooled in ice.

Acquisition of PAR-CLIP data

The PAR-CLIP experiment was performed as described previously (1). Briefly, 4tU-labeled BY4741 yeast cells expressing C-terminally TAP-tagged Pet111p were UV-irradiated with an energy dose of 12 J/cm^2 at 365 nm. The cells were lysed by bead beating and IP was performed with rabbit IgG-conjugated protein G magnetic Dynabeads (Invitrogen). The cross-linked RNA was partially digested with RNase T1 (Thermo Scientific) and the IP was controlled by performing a western blot analysis of a part of the sample using the peroxidase anti-peroxidase antibody (Sigma). Protein-RNA crosslinking was controlled using radioactive labeling of the 5'-RNA end by γ-[^{32}P]-ATP (3000 Ci/mmol, Perkin Elmer) and T4 PNK (New England Biolabs). The protein-RNA complexes were separated by SDS-PAGE and visualized by a Typhoon FLA 9500 instrument (GE Healthcare). Following adapter ligation, RNA was recovered by digestion with proteinase K (New England Biolabs) and a subsequent acidic phenol/chloroform extraction. Reverse transcription was performed with SuperScript III RTase (Invitrogen) and was followed by PCR amplification using the NEXTflex barcode primer kit (Bio Scientific). The generated cDNA was purified, size selected, and quantified using TapeStation (Agilent Technologies). The resulting samples were sequenced on an Illumina HiSeq 2500 system.

Isolation of mitochondria

For the experiment shown in Figure 2A, mitochondria were isolated by following a previously published protocol (2) with slight modifications. Yeast cells carrying a BG1805 plasmid encoding tagged Pet111p were purchased from GE Healthcare Open Biosystems. The plasmid was isolated from the cells and DNA sequencing (GENEWIZ) revealed a 1634A>T substitution in the coding sequence leading to a K545M mutation in Pet111p. The mutation was corrected by site-directed mutagenesis using the QuikChange II kit (Agilent) and mutagenesis primers BG1805Pet111_fix and BG1805Pet111_fixc. BY4743 cells transformed with the corrected plasmid were grown at 30 °C to an OD^{600} of 9 in 3 L of SD-ura medium (yeast nitrogen base with ammonium sulfate and synthetic drop-out mixture -uracil, US Biological), 2 % galactose, and 5 μg/ml tetracycline. The cells were collected by centrifugation, washed with water, resuspended in a reduction buffer (100 mM Tris·SO_4 pH 9.4, 72 mM β-ME) at a density of 3 g wet cells per 10 ml buffer, and incubated for 30 min at 30 °C. The buffer was replaced with 20 mM K-phosphate (pH 7.4) and 1.2 M sorbitol, zymolyase 100T (Amsbio) was added at a ratio of 1.5 mg/g wet cells, and the suspension was incubated for 40 min at 30 °C to

allow the formation of spheroplasts. Mitochondria were then extracted by breaking the spheroplasts with osmotic shock and homogenization followed by differential centrifugation as described (2). For the experiment in Figure 2B, BY4743 yeast cells were grown in YP medium in the presence of 3 % glycerol and 5 μg/ml tetracycline. The cells were converted to spheroplasts and mitochondria were isolated by differential centrifugation as described above. The mitochondrial pellet was then resuspended in 10 mM Hepes·NaOH pH 7.4, 250 mM sucrose, and 1 mM EDTA, and the suspension was fractionated on a discontinuous sucrose density gradient (60 %, 32 %, 23 %, and 15 % sucrose in 10 mM Hepes·NaOH pH 7.4, 1 mM EDTA) for 1 h (134,000×g, 4 °C) using a SW 41 Ti rotor (Beckman) as described (3). Purified mitochondria were collected from the 32% sucrose layer, resuspended in 5 times the volume of 20 mM Hepes·KOH (pH 7.4) and 0.6 M sorbitol, and the suspension was distributed into 1.5 ml tubes. The mitochondria were pelleted by centrifugation (20 min, 12,000×g, 4 °C), flash-frozen in liquid nitrogen, and stored at -80 °C.

Determination of the 5'-end of mature COX2

Approximately 100 μl of settled mitochondria isolated from WT yeast (see above) was resuspended in 0.9 ml of TRI Reagent® (Molecular Research Center), homogenized with 10 strokes in a teflon piston glass homogenizer, and the homogenate was incubated for 5 min at room temperature. Chloroform (0.2 ml) was then added, the mixture was vortexed for 15 s, incubated for 15 min at room temperature, and centrifuged for 15 min (12,000×g, 4 °C). The aqueous phase was separated and the RNA was precipitated with isopropanol. The pellet was dissolved in water and treated with RNase-free DNase I (New England Biolabs). The RNA was recovered from the solution using an RNA Clean & Concentrator-5 column (Zymo Research) and quantified by UV/Vis spectroscopy. 5'-[^{32}P]-labeled DNA primer *COX2*-96-76 (0.1 μM) was added to 3 μg of the purified RNA in 10 μl of water and hybridized by heating at 70 °C for 5 min followed by snap cooling in ice. To extend the primer, 10 μl of a solution containing 80 U ProtoScript® II RT (New England Biolabs), 8 U murine RNase inhibitor (New England Biolabs), 10 mM DTT, 0.5 mM each 2'-dNTP, and 2× ProtoScript® II RT buffer was added to the hybridization solution and the resulting mixture was incubated for 30 min at 42 °C. To generate DNA size markers, a DNA fragment spanning the positions from -7 to +109 relative to the *COX2* promoter start site (+1) was PCR-amplified by Phusion DNA polymerase (New England Biolabs) using plasmid pJJ10 (see below) as a template and amplification primers *COX2*-(-7)-15 and *COX2*-109-90. Due to the high A/T content of the template, the temperature during the annealing and extension steps was lowered to 45 °C and 52 °C, respectively, and the elongation time was extended to 2 min. The fragment was then purified by GeneJET PCR purification kit (Thermo Scientific) and used as a template (0.2 μM) to extend the 5'-[^{32}P]-*COX2*-96-76 primer (0.15 μM) with 1.5 U of Taq DNA polymerase (New England Biolabs) in the presence of 2',3'-ddNTPs. The extension reactions (50 μl) were carried out in a thermocycler in six cycles in the presence of 5 μM of each 2'-dNTP. Each cycle included a denaturation step (94 °C) and a prolonged annealing and elongation step (58 °C, 5 min). The concentrations of ddATP, ddCTP, and ddGTP present in the reactions were 400 μM, 300 μM, and 50 μM, respectively. The products of extension of the primer were resolved in a 6 % (19:1, acrylamide:bisacrylamide) gradient-thickness gel (0.4 to 1.2 mm) in the presence of 7 M urea and visualized by phosphor imaging with a Typhoon 9410 scanner (GE Healthcare).

Identification of tagged Pet111p by mass spectrometry

Following a partial purification on Ni beads and separation by PAGE, the 97 kDa band was excised from the gel and the protein was successively subjected to an in-gel reduction with tris(2-carboxyethyl)phosphine·HCl (2 mM), an alkylation with iodoacetamide (55 mM), and a digestion with mass spectrometry-grade trypsin (Promega) in 100 mM ammonium bicarbonate as previously described (4). The products of the digestion were extracted from the gel and analyzed by MALDI-TOF mass spectrometry on a Bruker Microflex LRF instrument operated in a positive-ion field reflectron mode and using α-cyano-4-hydroxycinnamic acid (10 mg/ml in 50 % acetonitrile and 0.1 % TFA) as the matrix. The spectrum was externally calibrated using the peptide calibration standard II (Bruker). The protein was identified as Pet111p by submitting the collected spectral data to Mascot search (Mascot score 188).

Expression and purification of rPet111p

A DNA fragment encoding His_6-tagged full-length Pet111p was PCR-amplified from the *S. cerevisiae* BY4743 genomic DNA using primers Pet111_Nco and Pet111_Xho. The amplicon was ligated into the pT7Blue vector (Novagen) to form plasmid pMA40, from which the gene was excised and ligated into pTrcHisA (Invitrogen) at the NcoI and XhoI sites. In the resulting plasmid, pMA41, the sequence coding for the 34 N-terminal amino acids of Pet111p was deleted by site-directed mutagenesis using the QuikChange II kit (Agilent Technologies) and mutagenesis primers Pet111_del34 and Pet111_del34c. The deletion yielded plasmid pGD1 expressing Δ34-Pet111p fused to N-terminal purification tag MAHHHHHH, which in this report is referenced to as rPet111p. The integrity of the target gene in pGD1 was confirmed by DNA sequencing (GENEWIZ). *E. coli* XJb(DE3) cells (Zymo Research) carrying pGD1 were grown at 37 °C in 6 L of LB medium supplemented with 3 mM L-arabinose, 1 mM $MgCl_2$, and 100 μg/ml ampicillin to an OD^{600} 0.8-0.9. The culture was then cooled down to 12 °C, the expression of rPet111p was induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside, and the cells were incubated at 12 °C for an additional 20 h. The cells were collected by centrifugation and resuspended in a lysis buffer containing 20 mM Hepes·NaOH pH 7.0, 300 mM NaCl, 5 mM β-ME, and 1 mM phenylmethanesulfonyl fluoride (PMSF) at a density of 1 g of wet cell pellet per 10 ml of buffer. To induce cell lysis, the suspension was frozen overnight at -80 °C and then allowed to thaw in the presence of fresh PMSF added to a concentration of 1 mM. The lysate was supplemented with 10 mM $MgCl_2$, bovine pancreatic DNase I (Roche) was added to a concentration of 2 μg/ml, and the mixture was stirred for 15 min at room temperature. The lysate was then cleared by centrifugation for 30 min (36,000×g, 4 °C), the concentration of NaCl in the supernatant was adjusted to 1.5 M, and imidazole was added to a concentration of 15 mM. The resulting solution was passed through a column packed with 2 ml of Ni-IDA agarose beads (Gold Biotechnology) equilibrated with wash buffer (20 mM Hepes·NaOH pH 7.0, 1.5 M NaCl, 15 mM imidazole). The beads were washed by passing 30 ml of the wash buffer through the column and the protein was released with 5 ml of elution buffer (20 mM Hepes·NaOH pH 7.0, 900 mM NaCl, 10 mM $MgCl_2$, 10 % glycerol, 225 mM imidazole, 5 mM β-ME). The eluate was diluted two-fold with a buffer containing 20 mM Hepes·NaOH pH 7.0, 10 % glycerol, and 5 mM β-ME to reduce the concentration of NaCl to 450 mM and loaded on a 8×7.5 TSKgel Heparin-5PW (Tosoh Bioscience) column. The protein was eluted from the column with a linear NaCl concentration gradient (450 mM to 1 M) in a buffer containing 20 mM Hepes·NaOH pH 7.0, 10 mM $MgCl_2$, 10 % glycerol, and 5 mM β-ME. Fractions containing rPet111p were combined, concentrated on an Ultracel®-50K centrifugal filter (Merck Millipore) to a volume of 2 ml, loaded on a HiLoad 16/600 Superdex 200 PG column, and the protein was eluted with gel filtration buffer (20 mM Hepes·NaOH pH 7.0, 600 mM NaCl, 10 mM $MgCl_2$, 10 % glycerol, 5 mM β-ME). Fractions containing pure rPet111p were pooled and concentrated on a centrifugal filter to increase the concentration of the protein to approximately 5 mg/ml. The concentrate was diluted two-fold with glycerol, distributed into small aliquots, and stored frozen at -80 °C. A typical yield of purified rPet111p was 5 mg.

Construction of plasmid pJJ10 carrying a COX2-related fragment of yeast mtDNA

Yeast BY4743 cells were grown in YP medium complemented with 2 % galactose and mitochondria were purified by sucrose gradient centrifugation as described above. mtDNA was isolated using a previously published protocol (5) with slight modifications. Briefly, 100 μl of settled mitochondria were lysed by gentle tumbling in 400 μl of a buffer containing 50 mM Tris·HCl (pH 7.8), 100 mM NaCl, 10 mM EDTA, and 1% SDS for 30 min at room temperature and the lysate was extracted with 0.5 ml of phenol/chloroform/isoamyl alcohol. The aqueous layer was extracted with an equal volume of chloroform, the DNA was precipitated with ethanol, dissolved in 10 mM Tris·HCl (pH 8.5), and quantitated by UV/Vis spectrophotometry. Using primers *COX2*-(-482)-(-461) and *COX2*-113-92 and the isolated mtDNA as a template, a fragment corresponding to the region from -482 to +113 relative to the *COX2* gene promoter start site was PCR-amplified by Phusion DNA polymerase (Thermo Fisher) utilizing a two-step annealing phase (55 ºC and 50 ºC) and an extended elongation phase (2 min) at a temperature of 60 ºC. The amplicon was purified using the GeneJET PCR purification kit (Thermo Scientific) and ligated into the pT7Blue vector (Novagen) in accordance with the manufacturer's protocol, resulting in plasmid pJJ10. To verify the accuracy of PCR

amplification, the sequence of the insert in the plasmid was determined (GENEWIZ) and found to match the corresponding sequence in the S288c yeast mtDNA (accession NC_001224).

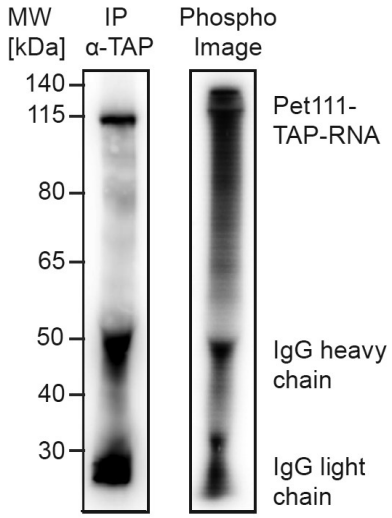

Figure S1. The image on the left shows a typical PAR-CLIP isolate obtained from UV-treated cells expressing a Pet111p-TAP fusion in the presence of 4tU. The immunoprecipitate was RNase-treated, resolved by SDS-PAGE, and the protein bands were visualized by western blotting. The apparent mobility of the upper band is consistent with the expected molecular weight of a Pet111p-TAP fusion. Shown on the right is a phosphor imager scan demonstrating the presence of photo cross-linked RNA in the same PAR-CLIP isolate, which was treated with PNK in the presence of γ-[^{32}P]-ATP and resolved by SDS-PAGE. The cluster of radioactive bands running at the top of the gel represents Pet111p-TAP cross-linked to RNA species of variable length.

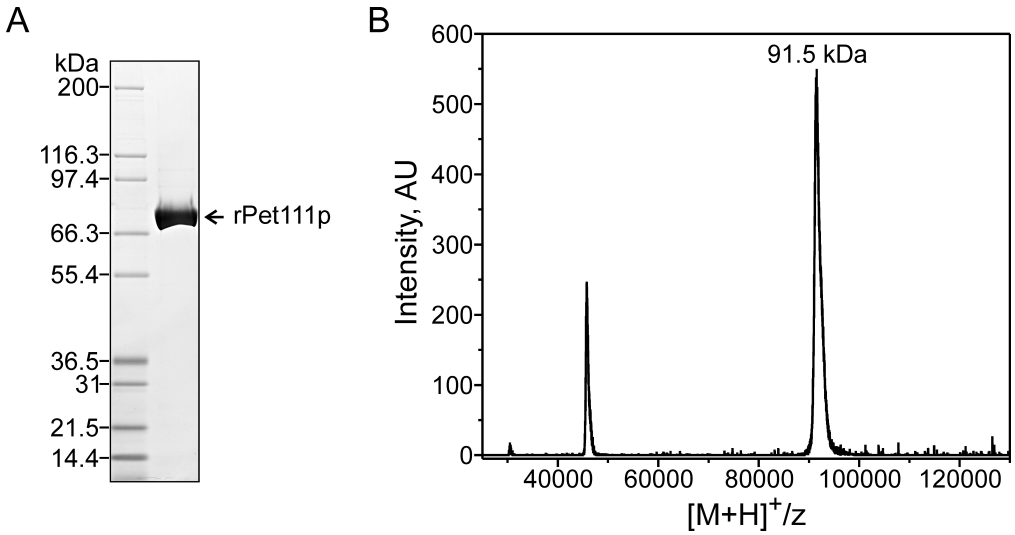

Figure S2. (A) An image of a coomassie-stained LDS 4-12 % polyacrylamide gel showing the purity of a typical preparation of rPet111p. (B) A MALDI-TOF mass spectrum of purified rPet111p showing that the observed molecular weight of the protein is in agreement with the calculated value of 91.3 kDa. The spectrum was taken on a Bruker Microflex LRF spectrometer in the positive-ion linear mode using sinapinic acid as the MALDI matrix. The spectrum was externally calibrated using bovine serum albumin as a standard.

Table S1. Sequences of the oligonucleotides used in the study.

Oligonucleotide	Sequence (5' to 3')
DNA oligonucleotides	
Pet111_Nco	CCATGGCACATCATCACCATCATCATTTACAACGGAGATTTATATCCTC
Pet111_Xho	CTCGAGTTATTACTCCTCCTCCTTTTTATTCTCTTC
Pet111_del34	GCACATCATCACCATCATCATTCAACTGAGTTGATCAAAAAAAAGC
Pet111_del34c	GCTTTTTTTTGATCAACTCAGTTGAATGATGATGGTGATGATGTGC
BG1805Pet111_fix	GGTGATTTCGAATAGTTTGAAGGAGGGCATTGCGCC
BG1805Pet111_fixc	GGCGCAATGCCCTCCTTCAAACTATTCGAAATCACC
COX2-96-76	CATAATGAATGTTGTTAATTG
COX2-(-7)-15	TAAAAGTAGTATTAACATATTA
COX2-109-90	TTGGTACATCATTCATAATG
COX2-(-482)-(-461)	GACCCCGAAGGAGTATAAATAA
COX2-113-92	GGTGTTGGTACATCATTCATAA
3' adapter	5rApp-TGGAATTCTCGGGTGCCAAGG-3ddC
RT primer	CCTTGGCACCCGAGAATTCCA
RNA oligonucleotides	
COX2-1-54	AGUAUUAACAUAUUAUAAAUAGACAAAAGAGUCUAAAGGUUAAGAUUUAUUAAA
NSC	AAUAAUUAUAAUAAUAUUCUUAAAUAUAAUAAAGAUAUAGAUUUAUAUUCUAUU
COX2-1-30	AGUAUUAACAUAUUAUAAAUAGACAAAAGA
COX2-23-54	CAAAAGAGUCUAAAGGUUAAGAUUUAUUAAA
COX2-58-95	UUAGAUUUAUUAAGAUUACAAUUAACAACAUUCAUUAU
5' adapter	5InvddT-GUUCAGAGUUCUACAGUCCGACGAUCNNNNN

SUPPORTING INFORMATION REFERENCES

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