PDE12 removes mitochondrial RNA poly(A) tails and controls translation in human mitochondria

Rorbach *et al*.

SUPPLEMENTARY DATA

SUPPLEMENTARY MATERIALS AND METHODS

Assessing mtDNA copy number by quantitative PCR

The relative copy number of mtDNA was determined by real-time PCR of the DNA encoding mitochondrial CytB using the nuclear, single-copy gene for amyloid precursor protein (APP) as a reference. Total cellular DNA was isolated using a DNeasy Blood and Tissue Kit (Qiagen). The reactions were performed as described above. Copy numbers of CytB and APP were determined by Taqman PCR on an ABI Prism 7600 using the standard curve (absolute quantification) method.

Ribonuclease protection assay (RPA)

In order to prepare an antisense RNA probe that anneals to the 3' end of the 12 S transcript with 3' - 20 nt oligo(T) extension, DNA corresponding to the 3' end of human mitochondrial 12 S rRNA was amplified by PCR using the following pair of primers: 5'-TAATACGACTCACTATAGGGAGACTTTTTTTTTTTTTTTTTTTTGTTCGTCCAAGTG 'CACTTTCCAG-3' and 5'-AAGTATACTTCAAAGGACATTAACTAAAACC-3'.

The DNA template was purified using a QIAquick PCR purification column (Qiagen) and radiolabelled antisense RNA probe synthesised using the MAXIscript *in vitro* transcription kit (Ambion, Huntington, Cambs., UK). In brief, 0.2 μg of template DNA was

added to a reaction mixture containing 1× transcription buffer, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 3.125 μM [^{32}P]UTP (800 Ci·mmol^{-1}) and 30 units of T7 enzyme mix in a final volume of 20 μl. The reaction was incubated at 37 °C for 10 min and template DNA was removed by the addition of 2 units of DNase I and a further 15 min incubation at 37 °C.

Total RNA for the nuclease protection assay was isolated from mitochondria of the control HEK293T cells and PDE12-expressing HEK293T cells following 4 days doxycycline induction using TRIzol reagent following the manufacturer's protocol (Invitrogen). All assays used the ribonuclease protection assay kit (RPA III, Ambion). In brief, 5 μg of mitochondrial RNA was mixed with 8 × 10^4 cpm probe. Two yeast RNA samples were also prepared as controls. RNAs were then co-precipitated by adding 0.5 M NH_4OAc and 2.5 volumes of ethanol and incubating at -20 °C for 15 min, followed by centrifugation at 10,000 × g for 15 min at 4 °C. Supernatants were aspirated and pellets resuspended in 10 μl of RPAIII hybridisation buffer. Samples were heated at 95 °C for 3 min followed by an overnight incubation at 42 °C. A 1:100 working dilution of RNase A/RNase T_1 in RNase digestion III buffer was prepared and 150 μl was added to each of the sample tubes and one of the yeast tubes. All reactions were incubated for 30 min at 37 °C before being terminated by the addition of 225 μl of RNase inactivation/precipitation solution. The samples were incubated at -20 °C for at least 20 min followed by centrifugation at 10,000 × g for 15 min at 4 °C. The supernatant was removed and RNA was dissolved in 10 μl of gel loading buffer. Samples were incubated for 3 min at 95 °C before being loaded onto a 6 % acrylamide/8 M urea gel and electrophoresed at 50 W. The gel was then dried and products visualised with a PhosphorImager system.

SUPPLEMENTARY TABLES

Supplementary Table S1

Short name	Uniprot Acc. No.	Database name	Full name	Region used for alignment
ANGEL1	Q9UNK9	ANGE1_HUMAN	Protein angel homolog 1 (670 residues)	245-663
ANGEL2	Q5VTE6	ANGE2_HUMAN	Protein angel homolog 2 (544 residues)	167-540
NOC	Q9UK39	NOCT_HUMAN	Nocturnin (431 residues)	143-421
PDE12	**Q6L8Q7**	**PDE12_HUMAN**	**2',5'-phosphodiesterase 12 EC=3.1.4.- (609 residues)**	295-606
CNOT6	Q9ULM6	CNOT6_HUMAN	CCR4-NOT transcription complex subunit 6 EC=3.1.-.- (557 residues)	189-538
CNO6L	Q96LI5	CNO6L_HUMAN	CCR4-NOT transcription complex subunit 6-like EC=3.1.-.- (555 residues)	189-536
DNAS1	P24855	DNAS1_HUMAN	Deoxyribonuclease-1 EC=3.1.21.1 (282 residues)	23-281
DNSL1	P49184	DNSL1_HUMAN	Deoxyribonuclease-1-like 1 EC=3.1.21.- (302 residues)	19-272
DNSL2	Q92874	DNSL2_HUMAN	Deoxyribonuclease-1-like 2 EC=3.1.21.- (299 residues)	22-295
DNSL3	Q13609	DNSL3_HUMAN	Deoxyribonuclease gamma EC=3.1.21.- (305 residues)	21-281
I5P1	Q14642	I5P1_HUMAN	Type I inositol-1,4,5-trisphosphate 5-phosphatase EC=3.1.3.56 (412 residues)	12-392
INP5E	Q9NRR6	INP5E_HUMAN	72 kDa inositol polyphosphate 5-phosphatase EC=3.1.3.36 (644 residues)	301-591
SHIP2	O15357	SHIP2_HUMAN	Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 2 EC=3.1.3.n1 (1258 residues)	426-725
SHIP1	Q92835	SHIP1_HUMAN	Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 EC=3.1.3.n1 (1189 residues)	406-708
INP5K	Q9BT40	INP5K_HUMAN	Inositol polyphosphate 5-phosphatase K EC=3.1.3.56 (448 residues)	16-318
PI5PA	Q15735	PI5PA_HUMAN	Phosphatidylinositol 4,5-bisphosphate 5-phosphatase A EC=3.1.3.56 (1006 residues)	425-728
I5P2	P32019	I5P2_HUMAN	Type II inositol-1,4,5-trisphosphate 5-phosphatase EC=3.1.3.36 (993 residues)	347-636
OCRL	Q01968	OCRL_HUMAN	Inositol polyphosphate 5-phosphatase OCRL-1 EC=3.1.3.36 (901 residues)	242-531
SYNJ2	O15056	SYNJ2_HUMAN	Synaptojanin-2 EC=3.1.3.36 (1496 residues)	532-862
SYNJ1	O43426	SYNJ1_HUMAN	Synaptojanin-1 EC=3.1.3.36 (1573 residues)	535-866
NSMA	O60906	NSMA_HUMAN	Sphingomyelin phosphodiesterase 2 EC=3.1.4.12 (423 residues)	9-279
NSMA2	Q9NY59	NSMA2_HUMAN	Sphingomyelin phosphodiesterase 3 EC=3.1.4.12 (655 residues)	327-646
APEX1	P27695	APEX1_HUMAN	DNA-(apurinic or apyrimidinic site) lyase EC=4.2.99.18 (318 residues)	62-316
APEX2	Q9UBZ4	APEX2_HUMAN	DNA-(apurinic or apyrimidinic site) lyase 2 EC=4.2.99.18 (518 residues)	2-311
EEPD1	Q7L9B9	EEPD1_HUMAN	Endonuclease/exonuclease/phosphatase family domain-containing protein 1 (569 residues)	261-538
A0AR24	A0AR24	A0AR24_HUMAN	Putative exonuclease (204 residues)	8-201
TTRAP	O95551	TTRAP_HUMAN	TRAF and TNF receptor-associated protein (362 residues)	114-358

Supplementary Table S1

Uniprot accession numbers and names, and corresponding short names (column 1) used in the phylogenetic tree in Figure 1A. The region containing the EEP domain (PF03372) used in the aligment is also indicated for each entry.

Supplementary Table S2

Mitochondrial transcript	Number of clones
CO2	
Control untransfected HEK293T (11 clones)	
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGCC –poly(A)	**7**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGC –poly(A)	**3**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGCC –A32	**1**
Uninduced PDE12 WT (23 clones)	
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGCC –poly(A)	**16**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGC –poly(A)	**6**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGCC –A38	**1**
Induced PDE12 WT 2 days (33 clones)	
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGCC –poly(A)	**15**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGC –poly(A)	**2**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGCC –A39	**1**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGC –A39	**2**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGC –A38	**2**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGCC –A36	**2**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGC –A36	**1**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGC –A35	**1**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGC –A34	**1**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGCC –A29	**2**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGC–A5	**1**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGCC –A4	**1**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGCC –A1	**1**
…ACCCTATAGCACCCCCTCTACCCCCTCT–A0	**1**
Induced PDE12 WT 4 days (26 clones)	
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGCC –poly(A)	**10**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGC–poly(A)	**2**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGCC –A39	**1**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGCC–A36	**1**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGC–A36	**1**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGC–A35	**1**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGCC–A34	**1**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGCC–A33	**1**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGCC –A31	**1**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGC –A27	**1**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGCC –A26	**1**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGC –A8	**1**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGCC –A4	**2**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGC –A0	**1**
…ACCCTATAGCACCCCCTCTACCCCCTCT–A0	**1**
Induced PDE12 E351A 4days (10 clones)	
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGCC –poly(A)	**8**
…ACCCTATAGCACCCCCTCTACCCCCTCTAGACAGC –poly(A)	**2**
ND1	
Control untransfected HEK293T (9 clones)	
…TACAATCTCCAGCATTCCCCCTCAAACCTA–poly(A)	**8**
…TACAATCTCCAGCATTCCCCCTCAAACCTA–A33	**1**
Uninduced PDE12 WT (23 clones)	
…TACAATCTCCAGCATTCCCCCTCAAACCTA–poly(A)	**19**
…TACAATCTCCAGCATTCCCCCTCAAACCTA–A34	**1**
…TACAATCTCCAGCATTCCCCCTCAAACCTA–A33	**1**
…TACAATCTCCAGCATTCCCCCTCAAACCTA–A26	**1**
…TACAATCTCCAGCATTCCCCCTCAAACCTA–A5	**1**
Induced PDE12 WT 2 days (21 clones)	
…TACAATCTCCAGCATTCCCCCTCAAACCTA–A9	**1**
…TACAATCTCCAGCATTCCCCCTCAAACCTA–A4	**1**
…TACAATCTCCAGCATTCCCCCTCAAACCTA–A3	**2**
…TACAATCTCCAGCATTCCCCCTCAAACCTA–A0	**1**
…TACAATCTCCAGCATTCCCCCTCA–A0	**8**
…TACAATCTCCAGCATTCCCCCT–A38	**1**
…TACAATCTCCAGCATTCCCCCT–A35	**2**
…TACAATCTCCAGCATTCCCCCT–A33	**1**
…TACAATCTCCAGCATTCCCCCT–A2	**3**
Induced PDE12 WT 4 days (17 clones)	
…TACAATCTCCAGCATTCCCCCTCAAACCTA–A32	**1**
…TACAATCTCCAGCATTCCCCCTCAAACCTA–A28	**1**
…TACAATCTCCAGCATTCCCCCTCAAACCTA–A7	**1**
…TACAATCTCCAGCATTCCCCCTCAAACC–A0	**1**
…TACAATCTCCAGCATTCCCCCTCAA–A0	**1**
…TACAATCTCCAGCATTCCCCCTCA–A0	**5**
…TACAATCTCCAGCATTCCCCCT–A39	**2**
…TACAATCTCCAGCATTCCCCCT–A38	**2**
…TACAATCTCCAGCATTCCCCCT–A36	**1**
…TACAATCTCCAGCATTCCCCCT–A31	**1**
…TACAATCTCCAGCATTCCCCCT–A9	**1**
Induced PDE12 E351A 4days (10 clones)	
TACAATCTCCAGCATTCCCCCTCAAACCTA–poly(A)	**9**
TACAATCTCCAGCATTCCCCCTCAAACCTA–A38	**1**

Supplementary Table S2

cRT-PCR sequence analysis of mitochondrial transcripts in controls and cells overexpressing PDE12. Partial or complete stop codons are indicated in blue. In the case of ND1 one adenine must be added to the 3' of the mRNA to create the TAA stop codon. Poly(A) indicates an extension of 40 or more adenosine residues.

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1

Multiple protein sequence alignment of the catalytic domain of confirmed and putative human deadenylases of the EEP family. The EEP domains of human proteins of the "Deadenylases" subgroup as indicated in Figure 1A of the main text were aligned using CustalW2. The boundaries of the sequence segment used in the alignment are indicated. The secondary structure alignment is based on the resolved structure of the human CNOT6L and is indicated above the alignment (helix, H, red font and strand, E, blue font). Active site residues involved in the binding of Mg^{2+} are indicated in yellow.

Supplementary Figure S2

(A) Mitochondrial localisation of PDE12.Strep2.Flag

The HEK293T cells containing Flp-In-integrated PDE12.Flag.Strep2 (lanes 1-4) or the E351A mutant (lanes 5-8) were induced for 2 days with 50 μg/ml of doxycycline and fractionated into unbroken cells and cell debris (D, lanes 2 and 6), cytosol (C, lanes 3 and 7) and mitochondria (M, lanes 4 and 8). 'T' denotes the total cell lysate. The protein fractions were analysed by Western blotting using anti-FLAG antibodies. The location of PDE12.Strep2.Flag was compared with that of the following marker proteins: UNG1 (mitochondrial matrix), UNG2 (nucleus) and GAPDH (cytosol).

(B) Purification of PDE12.Strep2.Flag

PDE12.Flag.Strep2 and the E351A mutant were purified as described in **Materials and Methods**, 22 μg of each protein were analysed by SDS-PAGE and visualised by Coomassie staining. MW – Molecular weight marker.

Supplementary Figure S3

Effect of PDE12 overexpression on lactic fermentation.

The levels of medium acidification (yellow colour) was analysed for the HEK293T cells containing Flp-In-integrated PDE12.Flag.Strep2 (top) or the E351A mutant (bottom) in a 8-day time course. The cells were seeded at 5 x 10^4 cells/well of a 6-well plate and induced with 50 μg/ml of doxycycline.

Supplementary Figure S4

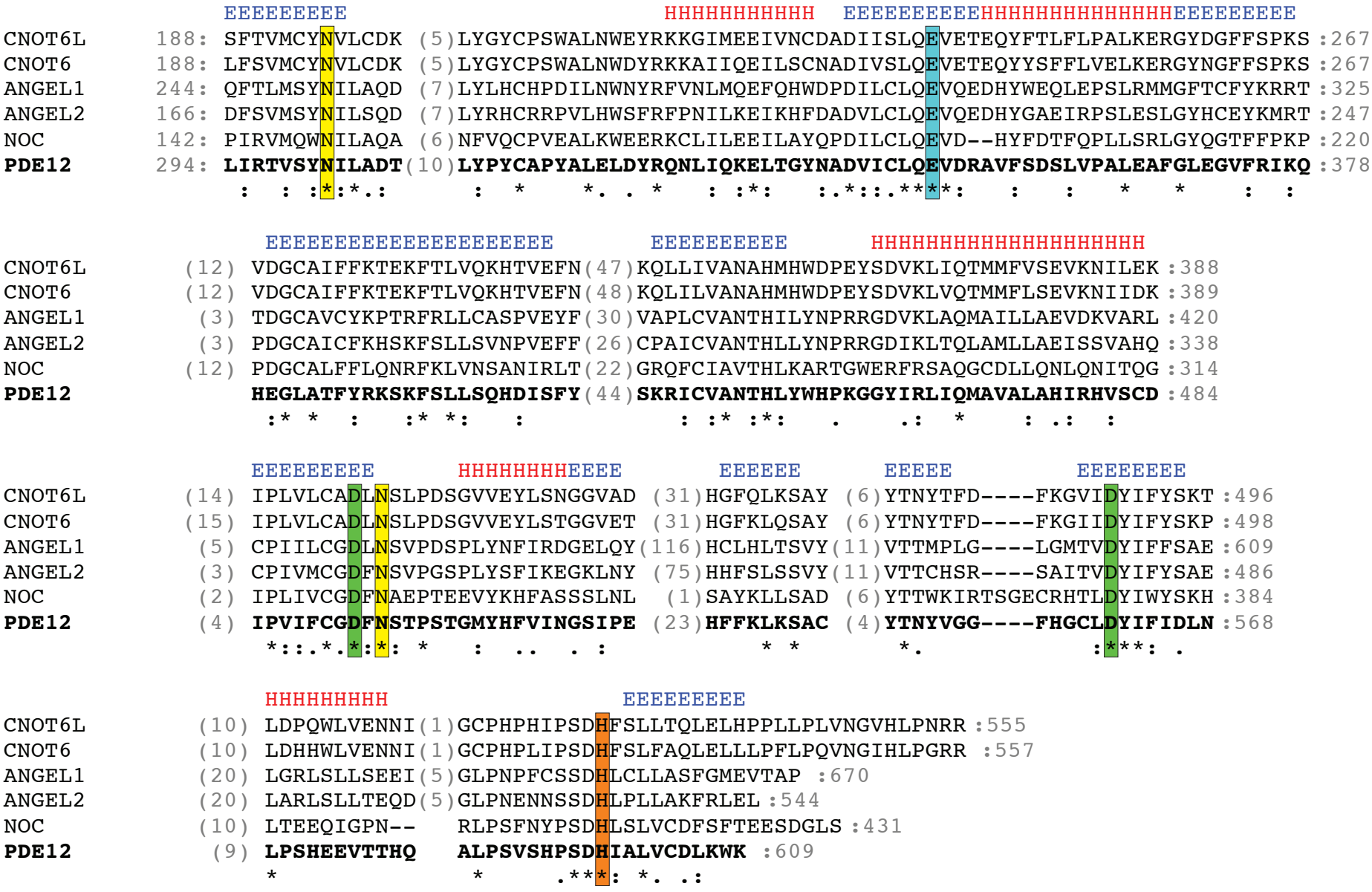
Mapping the 3` end of 12S rRNA by RNase Protection Assay (RPA).

(A) Schematic representation of the 12S rRNA specific RPA.

(B) Electrophoretic analysis of the protected probe. RNA isolated from purified mitochondria of parental HEK293T cells (H, lanes 2 and 6), HEK293T cells overexpressing PDE12 for 4 days (PDE12, lanes 3 and 7) or total RNA isolated from yeast (Y, lanes 5 and 9) were incubated with an antisense RNA probes that anneal to the 3` end of 12S or 16S rRNA and treated with RNase A/RNase T1 as described in **Materials and Methods**. After RNase digestion, protected probes were analysed in a 5% polyacrylamide urea gel. 'L' denotes size ladder (lane 1).

(C) Northern blot analyses of mitochondrial tRNAs in the total RNA isolated from the HEK293T cells (H) and cells overexpressing PDE12 for 0, 2 or 4 days. The RNA samples were resolved in 4 % polyacrylamide urea gel.

(D) Quantification of steady-state levels of the mitochondrial tRNAs in cells overexpressing PDE12 for 4 days analysed by Northern blots. The values of the relative RNA level (mt-tRNA/ND3 mRNA) were obtained by quantifying PhosphoImager scans of blots in the ImageQuant software. $n = 3$ for tRNASer-AGY, $n = 2$ for remaining tRNAs, error bars = 1 SD.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CNOT6 188: LFSVMCYNVLCDK (5)LYGYCPSWALNWDYRKKAIIQEILSCNADIVSLQEVETEQYYSFFLVELKERGYNGFFSPKS :267
ANGEL1 244: QFTLMSYNILAQD (7)LYLHCHPDILNWNYRFVNLMQEFQHWDPDILCLQEVQEDHYWEQLEPSLRMMGFTCFYKRRT :325
ANGEL2 166: DFSVMSYNILSQD (7)LYRHCRRPVLHWSFRFPNILKEIKHFDADVLCLQEVQEDHYGAEIRPSLESLGYHCEYKMRT :247
NOC 142: PIRVMQWNILAQA (6)NFVQCPVEALKWEERKCLILEEILAYQPDILCLQEVD--HYFDTFQPLLSRLGYQGTFFPKP :220
PDE12 294: LIRTVSYNILADT(10)LYPYCAPYALELDYRQNLIQKELTGYNADVICLQEVDRAVFSDSLVPALEAFGLEGVFRIKQ :378
EEEEEEEEEEEEEEEEEEEE EEEEEEEEEE HHHHHHHHHHHHHHHHHHHH
CNOT6L (12) VDGCAIFFKTEKFTLVQKHTVEFN(47)KQLLIVANAHMHWDPEYSDVKLIQTMMFVSEVKNILEK :388
CNOT6 (12) VDGCAIFFKTEKFTLVQKHTVEFN(48)KQLILVANAHMHWDPEYSDVKLVQTMMFLSEVKNIIDK :389
ANGEL1 (3) TDGCAVCYKPTRFRLLCASPVEYF(30)VAPLCVANTHILYNPRRGDVKLAQMAILLAEVDKVARL :420
ANGEL2 (3) PDGCAICFKHSKFSLLSVNPVEFF(26)CPAICVANTHLLYNPRRGDIKLTQLAMLLAEISSVAHQ :338
NOC (12) PDGCALFFLQNRFKLVNSANIRLT(22)GRQFCIAVTHLKARTGWERFRSAQGCDLLQNLQNITQG :314
PDE12 HEGLATFYRKSKFSLLSQHDISFY(44)SKRICVANTHLYWHPKGGYIRLIQMAVALAHIRHVSCD :484
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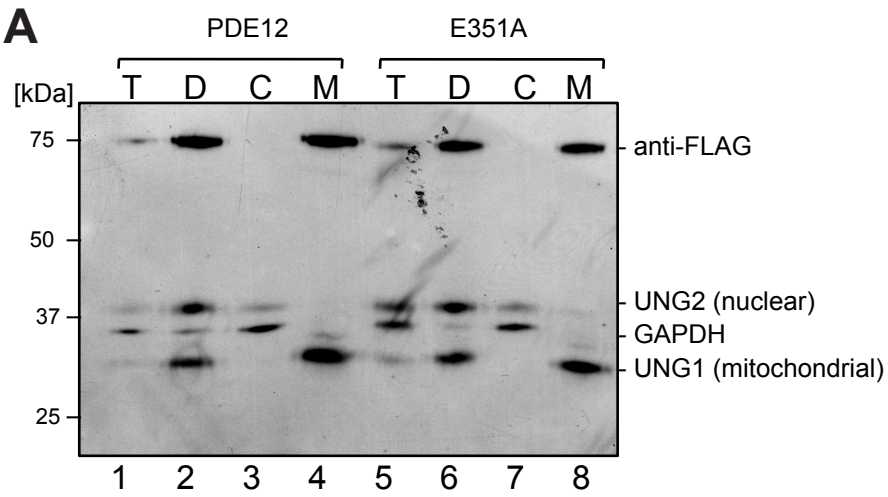
ANGEL1 (5) CPIILCGDLNSVPDSPLYNFIRDGELQY(116)HCLHLTSVY(11)VTTMPLG----LGMTVDYIFFSAE :609
ANGEL2 (3) CPIVMCGDFNSVPGSPLYSFIKEGKLNY (75)HHFSLSSVY(11)VTTCHSR----SAITVDYIFYSAE :486
NOC (2) IPLIVCGDFNAEPTEEVYKHFASSSLNL (1)SAYKLLSAD (6)YTTWKIRTSGECRHTLDYIWYSKH :384
PDE12 (4) IPVIFCGDFNSTPSTGMYHFVINGSIPE (23)HFFKLKSAC (4)YTNYVGG----FHGCLDYIFIDLN :568
HHHHHHHHH EEEEEEEEE
CNOT6L (10) LDPQWLVENNI(1)GCPHPHIPSDHFSLLTQLELHPPLLPLVNGVHLPNRR :555
CNOT6 (10) LDHHWLVENNI(1)GCPHPLIPSDHFSLFAQLELLLPFLPQVNGIHLPGRR :557
ANGEL1 (20) LGRLSLLSEEI(5)GLPNPFCSSDHLCLLASFGMEVTAP :670
ANGEL2 (20) LARLSLLTEQD(5)GLPNENNSSDHLPLLAKFRLEL :544
NOC (10) LTEEQIGPN-- RLPSFNYPSDHLSLVCDFSFTEESDGLS :431
PDE12 (9) LPSHEEVTTHQ ALPSVSHPSDHIALVCDLKWK :609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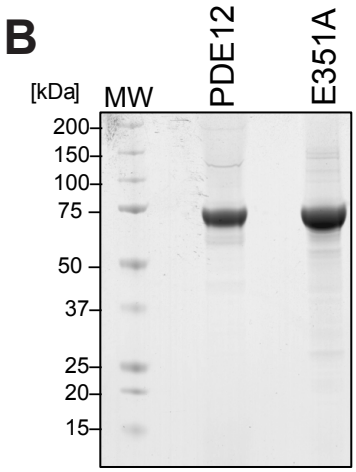
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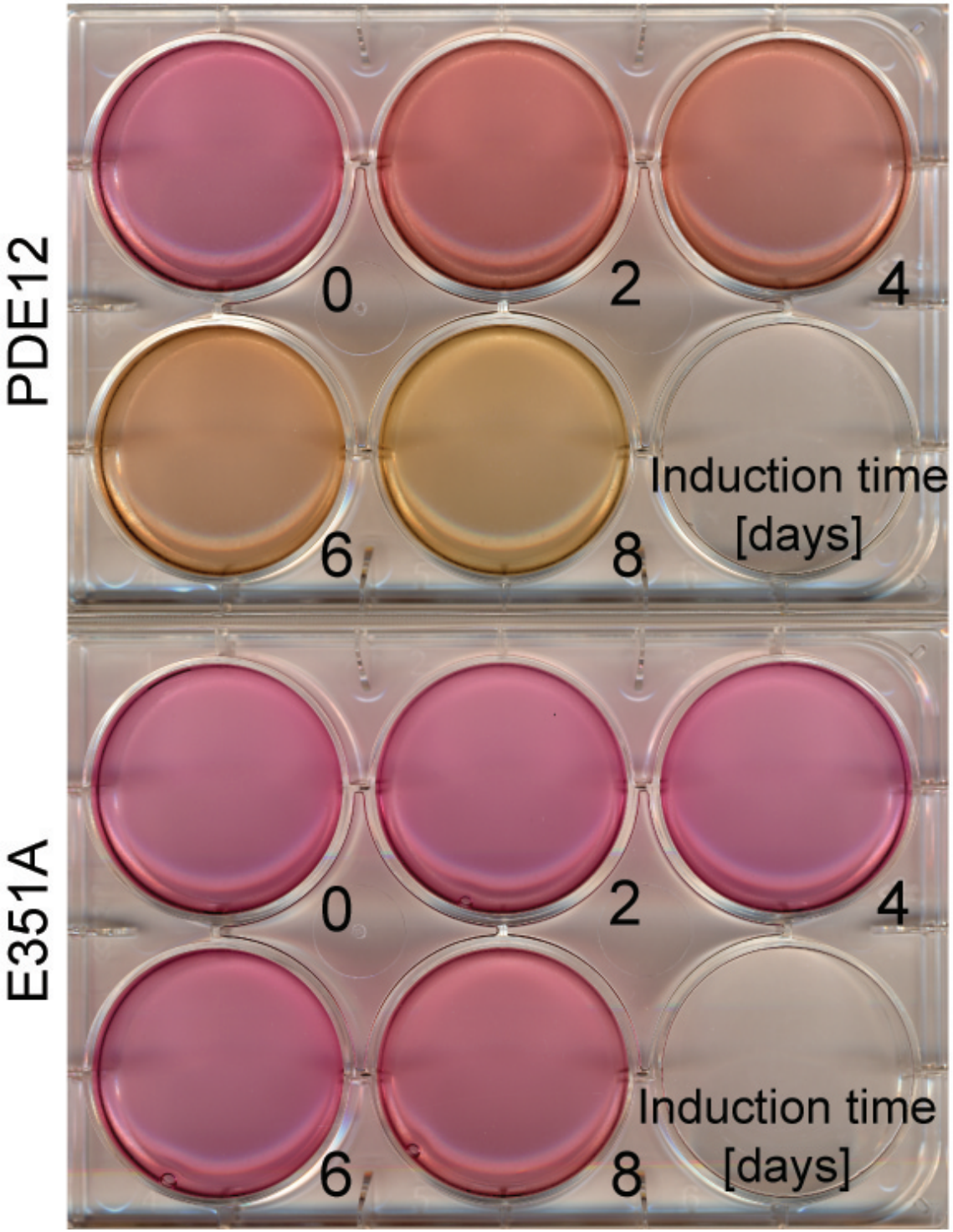
Suppl. Figure S3 - Rorbach et al.



Suppl. Figure S4 - Rorbach et al.