Supplementary information

CO2-sensitive tRNA modification associated with human mitochondrial disease

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tRNA	sequence	modification		m/z	
mt-tRNA ^{Asn}	UUAACUAAGp		z=-2	z=-3	z=-4
(RNase T ₁)		A37	1450.18	966.45	724.59
		t ⁶ A37	1522.70	1014.80	760.85
mt-tRNA ^{Ser(AGY)}	CUAACUCAUGp		z=-3	z=-4	z=-5
(RNase T ₁)		A37	1060.13	794.85	635.68
		t ⁶ A37	1108.48	831.11	664.68
mt-tRNA ^{lle}	AU <mark>A</mark> Gp		z=-1	z=-2	
(RNase T ₁)		A37	1326.18	662.59	
		t ⁶ A37	1471.22	735.11	
mt-tRNA ^{Thr}	UAAACCGp		z=-2	z=-3	
(RNase T ₁)		A37	1132.15	754.43	
		t ⁶ A37	1204.67	802.78	
mt-tRNA ^{Thr}	AGACp		z=-1	z=-2	
(RNase A)		A37	1325.20	662.09	
		t ⁶ A37	1470.23	734.61	
mt-tRNA ^{Thr}	UCUUGp		z=-1	z=-2	
(RNase T ₁)		C32	1585.17	792.08	
		m ³ C32	1599.18	799.09	
mt-tRNA ^{Lys}	CAUUAACCUUUUAAGp	A37, U34	z=-6	z=-7	
(RNase T ₁)		A, U	792.26	678.93	
		A, s ² U	794.92	681.22	
		A, $\tau m^5 U$	815.09	698.51	
		A, $\tau m^5 s^2 U$	817.76	700.79	
		t ⁶ A, U	816.43	699.65	
		t^6A, s^2U	819.09	701.94	
		t ⁶ A, τm ⁵ U	839.27	719.23	
		t^6A , τm^5s^2U	841.93	721.51	
ct-tRNA ^{Ile}	AUAACGp		z=-2	z=-3	
(RNase T ₁)		A37	979.63	652.75	
		t ⁶ A37	1052.15	701.10	

Supplementary Table 1. List of *m/z* values of RNA fragments for calculating modification frequencies.

Substrates	Km	k _{cat} (×10 ⁻³ , s ⁻¹)	$k_{cat}/K_m(s^{-1} \cdot mM^{-1})$
mt-tRNA ^{Thr}	$0.42\pm0.05~\mu M$	0.95 ± 0.028	2.26
ATP	$76\pm10\;\mu M$	0.87 ± 0.055	0.011
L-threonine	$39\pm3.7~\mu M$	1.08 ± 0.042	0.028
HCO ₃ -	$31\pm3.8\ mM$	1.54 ± 0.10	0.000050

The residues with modification are colored in red. An RNase used for digesting tRNA is shown in parenthesis.

Supplementary Table 2. Kinetic parameters of mitochondrial t⁶A37 formation

Substrates	Km	$k_{cat}(\times 10^{-2}, s^{-1})$
ATP	$170\pm220\;\mu M$	3.9 ± 2.23
L-threonine	$190\pm60~\mu M$	2.7 ± 0.29
HCO3 ⁻	$13\pm3.8\ mM$	2.5 ± 0.22

Supplementary Table 3. Kinetic parameters of NCA formation by YRDC

m	t-tRNAs	Asn (AAV)	Ile (AUV)	Lys	Ser (AGV)	Thr (ACN)	Total usage (%)				
((AAI)	(AUT)	(AAK)	(AUT)	(ACN)					
	ND1	4.1	7.2	2.2	0.9	11	25.4				
	ND2	5.8	8.9	3.5	1.4	12.4	32.0				
	ND3	3.5	7.8	2.6	0.9	6.1	20.9				
Ι	ND4	5.0	8.5	10.5	28.6						
	ND4L	6.1	7.1	0	0	5.1	18.3				
	ND5	5.5	8.9	3.5	15.7	10.8	44.4				
	ND6	2.3	6.9	1.1	2.9	1.7	14.9				
III	Cytb	3.9	10.3	2.4	1.1	7.9	25.6				
	COI	3.3	7.4	1.9	0.8	6.6	20.0				
IV	CO II	3.1	9.7	1.8	0.4	9.3	24.3				
	CO III	2.3	5.4	1.1	1.5	9.2	19.5				
V	ATPase6	4.8	12.8	2.6	1.3	11	32.5				
v	ATPase8	7.2	4.3	10.1	0	11.6	33.2				

Supplementary Table 4. Usage of codons read by tRNAs bearing t⁶A37 in human mitochondria

Usage (%) of codons read by five mt-tRNAs bearing t⁶A37 in all 13 proteins encoded by human mtDNA.

a yrdc

Homo sapiens Mus musculus Danio rerio Drosophila melanogaster Saccharomyces cerevisiae Escherichia coli	MSPAR MSTAR	Ř Č Ř G P C A G	MRA MRA		V V A A S A A G	VG MG M	20 LSI LSI YL(E G F D G F G R H		GS- SSC	R SR S	30 SGR GCR MR SKA		R P R P Q T D T	PS PE SL KI	P A P A Y R L K	PA PA MC VN	A P L P K E A P L	GA GA LK HH SI	R L R L T R T S I F	5 L R I L R I V L I S R I S P I		3 S C E S E S S N H Q A H I D	BAN EPN FS SE GS	/QA /EA SDD ELR SLP	AS AS L I TP TI	PE FLH VC MN	R A Q P A V P E N N	GW GW GD TE LQ	TE TE A A R D		RASC	A V A V C A V C A R A A I	A E A A A A A A A A A A A A A A A A A A	L R L R L L L R L N	79 A A A G T E
Homo sapiens Mus musculus Danio rerio Drosophila melanogaster Saccharomyces cerevisiae Escherichia coli	G A V V A G A V V A G Q V V A G Q V I A D E T V A E R V I A	V P T C V P T C L P T C F P T C Y P T C	TLY TLY TVY TVY			A S A S A Q A N A L P D	CSS SSS NE ND SE	A A L E A I T A I N S \ T A \	- R / - S (- R - Q (/ L S / M	A V Y C V Y C V Y Q L Y S I Y R L L				E A E G D E G D E D S D	K P K P K P K P K G		V C I C I C T H L I	L G V G V H V S A A	R V R V E I N I S I N Y	A D A D D A D Q E Q		2 Y (2 Y (2 Y (2 F (2 F (2 K)	CRV CQV CKV GQA VFN I DE	/R· /R· /S· AAH NQF	 		TS	 L F	 D N	 I P	- V - V - L - V - S - V	P R K D S D Y R Q F	:GL (EL)RL)EL (EL (ET	L K L E L R T S I F	DL DL RL SL	
Homo sapiens Mus musculus Danio rerio Drosophila melanogaster Saccharomyces cerevisiae Escherichia coli	P G P V T P G P V T	L V M E L V M E L V L E I V I E I L L P F V F P	R S E R S E R S A R T S V P S A P A	SEI	EL EL QL SA	N N S N S N S P	K D I G D I R F I K L R W I			TPL TRL TKL TSK QP1 FDS	. V (P P P P P V T		A F P F P F P L	MQ ML MR AR VV		A Q Q C A Q Q A C A Q C A Q C A Q C A Q C A Q C A Q C A Q C A Q C A C C A Q C A C C A C C A C C A C C A C C A C C A C C A C C A C C A C	MF MC VW LS AY	E G G T E G D Q D E T K	- P - P - P - P - P	_ A L _ A L _ A L _ A L _ A L		6 A N 6 A N 6 A N 6 A N 6 A N	L L S S S S S S S	SC SC SC SF GL	A S A S S S S S S P S P S P P	S L S L T V S L P T C F	N V S V Q V L A T V	E E E E E E E E E E E E E E E E E E E	FCFF		WP WP WP WP WP C	QL HL QL DK	S L S L G A F P	>>>
Homo sapiens Mus musculus Danio rerio Drosophila melanogaster Saccharomyces cerevisiae Escherichia coli	D G G Q D G G P V D G G P F D A G R L D G G V P G	G D G G D S E D - G L 1 A C K V	Q S P Q S P - E - E 1	E C F E C F E C F E R F E S T V F G G F		 G L	C N 1	- S - S - S - S - S - S - S - S - S - S		V D L V D L V D L I D L I R P I R C	S	V P G V P G V C G T P G G F 1 L T G	KF KF RY YYE	G I G I E I E L	I R R R R K R	999999 999999	 G E -	 A W	 S L	C A C A C A C A C K	L E S L E I L S J L K I V E I		TAI TSI VQI LSL KTV			Y O Y O G I K V		PS EC FC	H A Q G P D M M M K	SY SC HR YR	L S R H Y	SP	' S A	ĸν	V L	L
Saccharomyces cerevisiae	VPHCE	GDGI	LKG	G V D F	RME	RL	KR	LIE	т	ELK	(A)	NSN	нк	кі	A I	LΤ	SL	κL	RD	S D	LQS	S K I	IFN	I E F	P D F	s s	кт	FI	ΙE	RL	GC) S G	3 E E	١Q	ŧΤΝ	L
Saccharomyces cerevisiae	FAALR	KVDE	NDK	VDL	. I F	VE	GII	NEE	G	EGL		VMN		RK	A A	A N	ΝС	١Q	F																	

b OSGEPL1

Homo sapiens Mus musculus Danio rerio Drosophila melanogaster Saccharomyces cerevisiae Escherichia coli	MLÍ MLM	L T L R M	K T R T I S	A G I A G I	° VFI AII GT(FK PK M GR	P S P P F Q F L	K R K S S C L R L D	K V K V L P F N Y	20 Y E G A R I	F	L R L R S G I Q R	S F R F W S A N R A	N S R V	F H F H F G N R	P P F S C G F I	TL TL TS I F QL	F S T R R	LHCRLG	K I K L R L S Y K M R	-40 VL VL VL VL	G G G G A G		TS TS TS TS TS			50 A A G A G A G A G A G I C C I	A V A V A V A V A V A V A V A V A V A V		E E T R F	I G I G I G I S I S I S	60 N V N V R V R V G L	L G L G I A A A L A			H S H S E S L Y S	 N L	ĸ		70 EQVTELK	H L H L H L S I H A	KK	TG TG TG YG YG	77-0000000
Homo sapiens Mus musculus Danio rerio Drosophila melanogaster Saccharonyces cerevisiae Escherichia coli	V P V P P P V V P	P A P V P R T K E L		Q L Q L D L I H R D	R R R Q V	ENNRR	IQ IS IE TV	R I R V S A P L P L				S A R S A R A E I E K	S G S A C S A C S S S G		SP TP EP KP - R	S D E C C C				TTT TTT VT V Y	I K V K R G A G	P P P P P		A L A L D G V G	S S S S S S S S	G V G V G V S L V S L V	GGGGGG	S F S F D Y R F D F T V	S L S L S L S L F G F	QL QL KF CL SL		G Q R Q R R V A I F A I		****		P P P C P	>>	H H H H		AAAGG			R R R R R R R R R R	
Homo sapiens Mus musculus Danio rerio Drosophila melanogaster Saccharonyces cerevisiae Escherichia coli	TN - TN - LH - EH - GTN E	K - P - G K D N	- V - V - L V P P P	EFFFGF	PF				666666			L A L A L V F V L I	L V L V A L S S V	QQKNRT	G V G V G I G P G I G I	S D D S D C R D C C			GGGGCG	K S K S Q T D T E S			P A P V A		M L T L A F S L A F	DKKKKK DDKKKK	V A V A I A I G C G T A	RRRREL			K K R N K G	H P H P H P T M	E C E C E C E C F A	S G R I R I	T M T L L W E M - Y	SG SG SG SG S G - G	GGGGK GGGFK G			H L R L H A D K N			- G - G - G A S Q D - G	NNDDFT
Homo sapiens Mus musculus Danio rerio Drosophila melanogaster Saccharomyces cerevisiae Escherichia coli	R F H R F H Q L A P L A A L K A G R	F D F T F H F E F V	K N F F P M P F P	PPP SP SP SP SP SP SP SP	H N Q N G L A L K N T	H A Q N Q Q S D R	 A S	 K R	K N Y D R N P G			S F F F F F F F F	T G A G A G S G		QH QH RT KN KT	V T Q T Q T A L F A				K K K K K K K L D N	E E E E G G		G G R I Q	IE IE IE TP QE T-	KG KG PD LP	Q Q Q F G V 	LS LS IS ER	SA SA CV E F - R	A C A C G C R S A C) A) A) A) A) A		TV AV GV AF	Q H Q H Q H Q H Q H Q H Q H Q H Q H Q H	TI TN SN AN	MA VA VS VF VV	C H H H H H H H H H H H H H H H H H H H				H H H Q K K R R R R H F			FC FC FC S-	KKKL · ·
Homo sapiens Mus musculus Danio rerio Drosophila melanogaster Saccharomyces cerevisiae Escherichia coli	Q Q S L P H	R D K G Q P 	LL LL LF EK -D	P Q I S P E G D F K I Q T (N N A Q N I Q N I T P I N V I G F I	A V P T R R R R	L V L I V L I V V F V	AS VS MS MA	666666		SASASA	F L E D Q R	Y Y Y A T L	R R R R R R	R A Q I A N T K			- T / A - T - A / E (N A D A G T K	 L N	 S T	T C T C T C T C T C T C T C T C T C T C		T L T L R S N F E V	L C L C F Y F Y	P P P P P S P P A R	PR SK R D E		T T S S T			M M M M M	AW AW AW GW AY	N N N H A A		EEEEEV	LLLWF		GL GL GL GL GL GL GL GL GL GL GL GL GL G	G I G V G I E A L V T A	L F L S L S 1 . S 1 . S L S		I E V E S E Y D L D	GDEY-
Homo sapiens Mus musculus Danio rerio Drosophila melanogaster Saccharomyces cerevisiae Escherichia coli	IRY IRY VSY DSI ICP VSV		K K K K K K K K K K K K K K K K K K K				s- s- s- s- s- s- A-	- K - R - S E E D G	EVV EVV AVR	G E A E A Z T	A	S A A A	K V K V K C	P	Q L R L K L Q L	K N K L Q P		ŤN: /	s																									

Supplementary Figure 1. Sequence alignment of YRDC and OSGEPL1.

(a) Sequence alignment of YRDC homologs from *Homo sapiens* (NP_078916.3), *Mus musculus* (NP_705794.2), *Danio rerio* (NP_001077336.1), *Drosophila melanogaster* (NP_001027444.1), *Saccharomyces cerevisiae* (NP_011346.1), and *Escherichia coli*

(NP_417741.1). Black and grey boxes represent identical and similar amino acids, respectively. Multiple cleavage sites within the long and the short isoforms of YRDC expressed in HeLa cells are indicated by white and black arrowheads, respectively. (b) Sequence alignment of OSGEPL1 homologs in *Homo sapiens* (NP_071748.2), *Mus musculus* (NP_001272768.1), *Danio rerio* (NP_001005301.1), *Drosophila melanogaster* (NP_001245765.1), *Saccharomyces cerevisiae* (NP_010179.1), and *Escherichia coli* (NP_417536.1). Black and grey boxes represent identical and similar amino acids, respectively. The cleavage site in the MTS of OSGEPL1 expressed in HeLa cells is indicated by a black arrowhead.



Supplementary Figure 2. Multiple cleavage sites in the MTS of YRDC-FLAG expressed in HeLa cells.

(a) Peptide sequence of the MTS of YRDC with multiple cleavage sites in the long (white arrowheads) and the short (black arrowhead) isoforms. Point mutations A15F and S17F are indicated.

(b) Mass chromatograms of N-terminal tryptic peptides of YRDC. Divalent cations of proton-adducts of N-terminal peptides starting from positions 13–18 and 52 (from top to bottom panels) are indicated.

(c) CID spectra of each N-terminal tryptic peptide derived from the long isoforms of YRDC. Product ions are assigned on the peptide sequence.



Supplementary Figure 3. ESI detection efficiency of RNA fragments with varying t⁶A frequency.

Native human mt-tRNA^{IIe} isolated from WT HEK293T cells and its unmodified one isolated from *OSGEPL1* KO cells (KO#2) were mixed with different mixing ratios, digested by RNase T₁ and subjected to LC/MS. The peak area ratio of the A37-containing fragments with and without t⁶A was linearly regressed on the mixing ratio (slope=0.95, r²=0.9913). Actual t⁶A frequency of the native mt-tRNA^{IIe} was calculated to be 95% from the slope. Then, the mixing ratios were transformed to t⁶A frequencies on the x-axis to generate a plot of intensity ratio (%) against t⁶A frequency (%) (slope=1, r²=0.9913).





Supplementary Figure 4. Lack of t⁶A37 in five mt-tRNAs from *OSGEPL1* KO cells. Extracted ion chromatograms (XIC) generated by integration of multiply-charged negative ions of A37-containing fragments of human mt-tRNAs for Thr, Ile, Asn, Ser (AGY) and Lys harboring A37 (upper panels) or t⁶A37 (lower panels) (Supplementary Table 1) isolated from WT HEK293T (left panels), KO#1 (middle panels), and KO#2 (right panels). N.D., not detected. Intensity fractions (%) of modified or unmodified fragments are indicated.



Supplementary Figure 5. Hypomodification of m³C32 in mt-tRNA^{Thr} from *OSGEPL1* KO cells and cells from a patient with MERRF-like symptoms harboring the A15923G mutation.

(a) XICs generated by integration of multiply-charged negative ions of C32-containing fragments of human mt-tRNA^{Thr} bearing C32 (upper panels) and m³C32 (lower panels) (Supplementary Table 1) isolated from WT HEK293T (left panels), *OSGEPL1* KO#1 (middle panels), and *OSGEPL1* KO#2 (right panels). Intensity fractions (%) of modified or unmodified fragments are indicated.

(b) XICs generated by integration of multiply-charged negative ions of C32-containing fragments of human mt-tRNA^{Thr} bearing C32 (upper panels) and m³C32 (lower panels) (Supplementary Table 1) isolated from the fibroblasts (left panels) and myoblasts (right panels) of a patient with the A15923G mutation.



CBB staining

Supplementary Figure 6. Loading controls for protein analyses.

(a) CBB staining of mitochondrial fractions from WT HEK293T, *OSGEPL1* KO#1, and *OSGEPL1* KO#2 used for western blotting (shown in Fig. 4e).
(b) CBB staining of total proteins from WT HEK293T, *OSGEPL1* KO#1, and *OSGEPL1* KO#2 used for the pulse-labeling experiment (shown in Fig. 4f).



Supplementary Figure 7. Northern blotting of five mt-tRNAs in WT HEK293T and *OSGEPL1* KO cells.

U6 snRNA was detected as a loading control.



Supplementary Figure 8. *In vitro* reconstitution of t⁶A37 on mt-tRNAs.

(a) *In vitro* formation of t⁶A37 on mt-tRNAs for Asn and Lys. XICs generated by integration of multiply-charged negative ions of A37-containing fragments of mt-tRNAs for Asn (left panels) and Lys (right panels) bearing A37 (top) and t⁶A37 (bottom) (Supplementary Table 1). Intensity fractions (%) of modified or unmodified fragments are indicated.

(b) *In vitro* formation of t⁶A37 on mt-tRNA^{Ser(AGY)} transcript (left panels) or native mt-tRNA^{Ser(AGY)} isolated from *OSGEPL1* KO#1 cells (right panels). XICs generated by integration of multiply-charged negative ions of A37-containing fragments mt-tRNA^{Ser(AGY)} bearing A37 (top) and t⁶A37 (bottom) (Supplementary Table 1). Intensity fractions (%) of modified or unmodified fragments are indicated.

(c) *In vitro* formation of t⁶A37 on five mt-tRNAs mediated by *E. coli* t⁶A37-modifying enzymes (YrdC, YgjD, YeaZ, and YjeE). XICs generated by integration of multiply-charged negative ions of A37-containing fragments of respective tRNAs bearing A37 (top) and t⁶A37 (bottom) (Supplementary Table 1). Intensity fractions (%) of modified or unmodified fragments are indicated.



Supplementary Figure 9. t⁶A37-forming activity of YRDC isoforms. *In vitro* formation of t⁶A37 on mt-tRNA^{Asn} with YRDC isoforms with different N-termini: full length (black circle), 17-279 a.a. (gray circle) and 52-279 a.a. (white circle).



Supplementary Figure 10. Impairment of t⁶A37 in mt-tRNA^{Thr} with the A15923G mutation isolated from fibroblasts and myoblasts of a patient with MERRF-like symptoms.

(a) Detection of the A15923G mutation in mtDNA by *AccI* digestion. DNA fragments (1091 bp) amplified from mtDNAs from the patient's fibroblasts and myoblasts using a set of specific primers (Supplementary Table 5) were treated with (+) or without (-) *AccI* and the digestion products subjected to agarose gel electrophoresis. Digestion of the 1091 bp DNA containing the A15923G mutation yielded two fragments of 760 bp and 331 bp.

(b) Sequence chromatogram of mtDNAs from the patient's fibroblasts and myoblasts. Mutation rate of position 15923 was calculated from the A-to-G ratio of the peak height.
(c) XICs generated by integration of multiply-charged negative ions of A37-containing fragments of mt-tRNA^{Thr} with A37 (upper panels) and t⁶A37 (lower panels)
(Supplementary Table 1) isolated from WT (left panels) and patient fibroblasts harboring the A15923G mutation (right panels). Intensity fractions (%) of modified or unmodified fragments are indicated.

(d) XICs generated by integration of multiply-charged negative ions of A37-containing fragments of ct-tRNA^{Ile} (left panels) and mt-tRNA^{Ser(AGY)} (right panels) (Supplementary Table 1) isolated from the patient's fibroblasts. Intensity fractions (%) of modified or unmodified fragments are indicated. N.D., not detected.

(e) XICs generated by integration of multiply-charged negative ions of A37-containing fragments of ct-tRNA^{IIe} (left panels) and mt-tRNA^{Ser(AGY)} (right panels) (Supplementary Table 1) isolated from the patient's myoblasts. Intensity fractions (%) of modified or unmodified fragments are indicated. N.D., not detected.



Supplementary Figure 11. Growth curves for WT HEK293T cultured with or without CO_2/HCO_3 ⁻. HEK293T cells were cultured in DMEM medium under 5% CO₂ (black circles), non-bicarbonate DMEM with 44mM NaHCO₃ under 5% CO₂ (white circles), or non-bicarbonate DMEM under air (gray circles). Mean values \pm s.e.m. of four independent cultures are plotted.



Supplementary Figure 12. Hypomodification of t⁶A37 in mt-tRNA^{Lys} with different U34 modifications in HEK293T cells cultured in non-bicarbonate medium.

XICs generated by integration of multiply-charged negative ions of A37-containing fragments of mt-tRNA^{Lys} with s²U34 (upper panels), unmodified U34 (middle panels) and $\tau m^5 s^2 U34$ (lower panels) (Supplementary Table 1) isolated from HEK293T cells cultured with normal DMEM medium (44 mM NaHCO₃) in 5% CO₂ (left panels) and non-bicarbonate medium in air (right panels). t⁶A frequencies (%) are described as mean values ± s.d. of technical triplicate.



Supplementary Figure 13. Time-course of t⁶A37 formation *in vitro* in three mt-tRNAs.

(a) Rates of t⁶A37 formation on mt-tRNAs for Ile and Ser(AGY) were much slower than that on mt-tRNA^{Thr}. Right-hand graph is an expanded version of the left-hand graph, intended to emphasize the difference between mt-tRNA^{Ser(AGY)} and mt tRNA^{Ile}. Mean values \pm s.d. of biological triplicates are shown.

(b) Bicarbonate-sensitive t⁶A37 formation on mt-tRNA^{Ser(AGY)}. Time-course of t⁶A37 formation *in vitro* in each tRNA was carried out in different concentrations of bicarbonate (20, 30, or 40 mM). Mean values \pm s.d. of biological triplicates are shown.

a Solid tumor xenograft



Supplementary Figure 14. t⁶A status in tRNAs isolated from solid tumors and culture cells of HT29.

Mass chromatograms show A37-containing fragments of ct-tRNA^{Ile} (left panel), and mt-tRNA^{Ser(AGY)} (right panel) bearing A37 (blue) and t⁶A37 (black), isolated from subdermal HT-29 xenografts (a), and from *in vitro* culture of HT-29 (b).

Fig. 2b



Supplementary Figure 15. Full-size images for the blotting.

The cut region of each image in the indicated figure is red-boxed.