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The yeast model suggests the use of short peptides derived from mt LeuRS for the therapy of diseases due to mutations in several mt tRNAs

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ABSTRACT

We have previously established a yeast model of mitochondrial (mt) diseases. We showed that defective respiratory phenotypes due to point-mutations in mt $tRNA^{Leu(UUR)}$, $tRNA^{Ile}$ and $tRNA^{Val}$ could be relieved by overexpression of both cognate and non-cognate nuclearly encoded mt aminoacyl-tRNA synthetases (aaRS) LeuRS, IleRS and ValRS. More recently, we showed that the isolated carboxy-terminal domain (Cterm) of yeast mt LeuRS, and even short peptides derived from the human Cterm, have the same suppressing abilities as the whole enzymes.
In this work, we extend these results by investigating the activity of a number of mt aaRS from either class I or II towards a panel of mt tRNAs. The Cterm of both human and yeast mt LeuRS has the same spectrum of activity as mt aaRS belonging to class I and subclass a, which is the most extensive among the whole enzymes. Yeast Cterm is demonstrated to be endowed with mt targeting activity.
Importantly, peptide fragments β30_31 and β32_33, derived from the human Cterm, have even higher efficiency as well as wider spectrum of activity, thus opening new avenues for therapeutic intervention. Bind-shifting experiments show that the β30_31 peptide directly interacts with human mt $tRNA^{Leu(UUR)}$ and $tRNA^{Ile}$, suggesting that the rescuing activity of isolated peptide fragments is mediated by a chaperone-like mechanism.
Wide-range suppression appears to be idiosyncratic of LeuRS and its fragments, since it is not shared by Cterminal regions derived from human mt IleRS or ValRS, which are expected to have very different structures and interactions with tRNAs.

1. Introduction

AaRS are evolutionarily important enzymes on which the fidelity of genome decoding is based. All aaRS contain catalytic and anticodon recognition domains to catalyse the aminoacylation reactions specific for their cognate amino acids. Additionally, several aaRS have developed editing activities to hydrolyse mis-activated amino acids or mis-charged tRNAs and prevent insertion of incorrect amino acids during protein synthesis [1]. The canonical functions of aaRS, including aminoacylation and editing activities, are highly conserved throughout the three domains of life, i.e., archaea, bacteria and eukarya. Based on sequence, structure and functional features, including catalytic domain topology and tRNA esterification site, aaRS have been assigned to two different Classes, each divided into subclasses. Class I aaRS are characterized by two structural motifs – HIGH and KMSKS – and have a Rossmann fold dinucleotide-binding domain. This catalyses ATP dependent activation of amino acids and transfer of activated aminoacyl–adenylates to the 2′-OH of the CCA sequence at the tRNA 3′-end. Enzymes with a Class I catalytic domain include those active on amino acids Leu, Ile, Val, Met, Cys, Arg, Glu, Gln, Tyr, and Trp. Class II aaRS employ three degenerate motifs in an anti-parallel core of β strands to coordinate ATP binding, and aminoacylate the 3′-OH of their cognate tRNAs. The classification of aaRS is conserved in evolution, with only a few exceptions [2]. However, during evolution from prokaryotes to vertebrates, including mammals, certain aaRS have acquired other domains, unrelated to aminoacylation and generally localized at the amino- or carboxy-terminus [3]. The function of these additional domains include splicing of structured RNAs, translation control, transcription regulation, signal transduction, cell migration and others. In particular splicing of mt

Abbreviations: mt, mitochondrial; bp, base-pair; WT, wild type; MELAS, mitochondrial encephalomyophathy, lactic acidosis and stroke-like episodes; aa, amino acid(s); aaRS, aminoacyl-tRNA synthetase(s) (specificity is indicated by the name of the amino acid, abbreviated in the three-letter code); rho^+, mtDNA wild-type; rho°, mtDNA absent; MTS, Mitochondrial Targeting Sequence

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introns has been shown in *Saccharomyces cerevisiae* for mt LeuRS [4] and in *Neurospora crassa* for mt TyrRS [5]. This activity has been shown to be conserved in human mt LeuRS although human mt introns are not present [6].

It has been shown that the overexpression of genes coding for mt LeuRS, ValRS or IleRS, all belonging to Class I and subclass a, were able to suppress the pathological phenotypes associated with mutated mt tRNAs both in human and in yeast cells [7–13]. Further, we have observed that human mt aaRS were able to suppress the defective phenotypes of yeast mt tRNA mutants even more efficiently than the orthologous yeast enzymes [7]. Moreover we have demonstrated that the suppression activity does not require the catalytic activity of the enzyme [14]. The three aforementioned aaRS bind very similar amino acids, which only differ for the presence or position of a methyl group. It has been reported that this similarity can result in mis-adenylation, followed by mis-acylation by aaRS. Such mistakes are then corrected by the editing domain that is present in mt LeuRS, IleRS and ValRS [15].

More recently, two novel aspects of the suppressing ability of aaRS have been observed, namely the possibility to rescue the defective phenotypes of mutants having substitutions in mt $tRNA^{Leu(UUR)}$, $tRNA^{Ile}$ and $tRNA^{Val}$ by overexpressing i) non-cognate mt LeuRS, IleRS or ValRS (cross-suppression activity); ii) a 67 aa Carboxy-terminal sequence derived from mt LeuRS (Cterm suppression activity). The same properties were demonstrated to be present in human mt aaRS sequences in studies using cybrids, thus opening the way to new therapeutic perspectives. To this end, it is important to note that the Cterm peptide, even though lacking a canonical N-terminal mt targeting sequence (MTS), enters the mitochondria of human cybrids and interacts with mt tRNAs in vitro [9]. Subsequently, we have shown that two isolated peptides derived from the human mt LeuRS Cterm, named β30_31 (15 amino acids) and β32_33 (16 amino acids), which are in contact with the cognate $tRNA^{Leu}$ in known three-dimensional (3D) structures, are endowed with suppressing activity comparable to the whole Cterm towards mt $tRNA^{Leu(UUR)}$, $tRNA^{Val}$ and $tRNA^{Ile}$ mutants [7]. In view of potential therapeutic applications, some relevant issues need to be addressed. In this work, we aim at establishing: i) the extent of cross-suppression among different mt aaRS; ii) whether the Cterminal regions of human mt IleRS and ValRS are endowed with suppressing and cross-suppressing abilities analogous to the Cterm region of LeuRS; iii) whether the short β30_31 and β32_33 peptides derived from human mt LeuRS Cterm are able to rescue the defective phenotypes due to mutations in different mt tRNAs, as well as the previously studied mt $tRNA^{Leu(UUR)}$, $tRNA^{Ile}$ and $tRNA^{Val}$. Additionally, to investigate the molecular mechanism of the suppressing activity we tested the interaction between human mt tRNAs (leu, ile and gly) and the β30_31 and β32_33 peptides; demonstrated that the yeast mt LeuRS Cterm is endowed with mt targeting activity; and analysed the available 3D structures of aaRS–tRNA complexes to highlight the details of molecular recognition.

2. Material and methods

2.1. Strains, media and growth conditions

S. cerevisiae strains are the WT MCC123 *MATa*, *ade2-1*, *ura3-52*, *leu2*, *kar1-1* rho^+ [16] and the isogenic mt tRNA mutants listed in Tables 1 and 2, which were originated by cytoduction crosses as previously described [14]. Mutants are named with the three-letter code name of the amino acid indicating the tRNA gene and the base substitution. The original LeuA14G, LeuC25T, ValC25T and IleT32C mutants were obtained by biolistic transformation in order to obtain substitutions equivalent to pathological mutations in the yeast model [14,17,18]. The procedure is described in Rohou et al. [19] and details can be found in Feuermann et al. 2003 [17], supplementary information. The original mutants GlyG30T, PheC2T, PheC62T, AspC61T and the GlnC6T, and the HisG51A mutants were obtained by $MnCl_2$ random mutagenesis by M. Bolotin Fukuhara and A. Tzagoloff, respectively, as previously described [19–22].

The TUF1 null strain (MCC123ΔTUF1) was obtained as previously described [23]. Strains were grown in YP complete medium (1% yeast extract and 1% peptone from Difco) containing 3% glycerol, or 2% glucose. Minimal medium was 0.7% yeast nitrogen base (Difco), 5% ammonium sulphate and 2% glucose, supplemented with the necessary auxotrophic requirements. For solid plates 1.5% agar (Difco) was added to the above media. The glycerol growth capability of WT, mutant and transformant cells was investigated by serial dilutions from concentrated suspensions (5×10^6 cell/ml) prepared from fresh single colony spotted onto a unique plate.

2.2. Plasmids and cloning

Standard protocols [24] were used for *Escherichia coli* and yeast transformations as well as plasmid preparations.

The vectors containing mt aaRS sequences are:

pNAM2 (kindly provided by Prof. C. Herbert) in which the yeast mt LeuRS gene with its own promoter is cloned into the multi-copy vector pFL44S [25];

pCtermNAM2 in which the sequence of the Carboxy-terminal domain of *NAM2* gene (from aa 829 to 894) is cloned in multi-copy

Table 1
Suppression capability of Class I and Class II aaRS on mt tRNA mutants aminoacylated by Class I mt aaRS.

			Glycerol growth of transformant strains							
			Class I aaRS							**Class II aaRS**
tRNA Mutant	tRNA defect	Glycerol growth 28 °C 37 °C	**LeuRS** Sc (*NAM2*) Ia	Hs (*LARS2*) Ia	**ValRS** Sc (*VAS1*) Ia	Hs (*VARS2*) Ia	**IleRS** (*ISM1*) Ia	**GluRS** (*MSE1*) or Cyt **GlnRS** (*GLN4*) Ib	**TyrRS** (*MSY1*) Ic	**GlyRS, HisRS, AspRS, LysRS, PheRS**
VAL C25T [14]	Low amount of $tRNA^{Val}$	+/− +/−	+ [29] +	+ [29] +	+ [14,29] +	+ [29] +	+ +	+/− +/−	+ +	+/− +/−
LEU A14G [17]	Aminoacylation defect	− −	+ [38] +	+ +	+ +	+ +	+ +	− −	− −	− −
LEU C25T [17]	No tRNAs	− −	+ [14,29,38] +	+ [29] +	+ [29] +	+ [29] +	+ +	− −	− −	− −
ILE T32C [18]	No tRNAs	− −	+ [18] +	+ +	+ [18] +	+ +	+ [18] +	− −	− −	− −
GLN C6T [40]	Structural and aminoacylation defects	+/− −	+ +	+ −	+/− −	+/− −	+/− −	+/− −	+/− −	+/− −

+ indicates growth similar to wild type; − indicates absence of growth; +/− indicates partial growth.

Table 2
Suppression capability of Class I and Class II aaRS on mt tRNA mutants normally aminoacylated by class II mt aaRS.

			Glycerol growth of transformant strains												
			Class I aaRS							**Class II aaRS**					
tRNA Mutant	tRNA defect	Glycerol growth 28 °C 37 °C	**LeuRS** Sc (*NAM2*) Ia	Hs (*LARS2*) Ia	**ValRS** Sc (*VAS1*) Ia	Hs (*VARS2*) Ia	**IleRS** (*ISM1*) Ia	**GluRS** (*MSE1*) or Cyt **GlnRS** (*GLN4*) Ib	**TyrRS** (*MSY1*) Ic	**GlyRS** (*GRS1*) IIa	**HisRS** (*HTS1*) IIa	**AspRS** (*MSD1*) IIb	**LysRS** (*MSK1*) IIb	**PheRS** (*MSF1*) IIc
ASP C61T [21]	3' end processing	− −	− [38] −	+ −	− −	+/− −	+ −	− −	− −	− −	+ −	+ [27] +	+ −	+ −
GLY G30T [19]	No tRNAs	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −
HIS G51A [40]	Structural defect	+/− −	+/− −	+ −	+/− +/−	+ −	+ −	+/− −	+ +	+ +	+ [38] +	+/− −	+ +	+ +
PHE C2T [20]	Ternary complex formation	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −	+ −
PHE C62T [20]	3′ end processing	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −	− −

+ indicates growth similar to wild type; − indicates absence of growth; +/− indicates partial growth.

vector pYES2.1/V5-His-TOPO (Invitrogen, Life Technologies) under the inducible Gal1 promoter [7];
pVAS1 in which the yeast gene coding for the mt ValRS was cloned into pCM262 by the "gap repair" technique [14];
pHTS1 (kindly provided by Prof. G. Fink), in which the yeast *HTS1* gene with its own promoter, has been cloned in multi-copy vector YEp24 [26];
pISM1 in which the entire *ISM1* gene coding for yeast mt IleRS is cloned into multi-copy vector pYES2.1/V5-His-TOPO (Invitrogen, Life Technologies) under the inducible Gal1 promoter [18].

In the same vector we cloned the yeast mt TyrRS and GlyRS genes (pMSY1 and pGRS1 respectively). The oligonucleotides for the PCR reactions were: TyrRS+: (5′-GGTAGACATTGTGAATCATG-3′) and TyRS-: (5′-CATGTACTTTATATACCTCCC-3′); GRS1+ (5′-GTTAAGATTAAAAG AATGAGTG-3′) and GRS1− (5′-CATTTATTTAGTCAGTTTCAGC-3′).

pMSD1 in which the ClaI–KpnI fragment containing the gene coding for yeast mt AspRS and its promoter is subcloned from the YEp13 vector [27] in the SmaI site of pELAC181plasmid;

pMSK1 in which the gene coding for yeast mt LysRS was cloned in Yep13 was kindly provided by Prof A Tzagoloff;

pMSF1 in which the a subunit of yeast mt PheRS is cloned in YEp351 [20];

pGLN4 in which the gene coding for yeast cytosolic GlnRS is cloned in the multi-copy vector pRS416 was kindly provided by Prof B. Krett (we tested the suppression activity of this gene because the presence of cytoplasmic GlnRS in the organelle and its involvement in mt Gln-tRNA synthesis had been suggested by Rinehart et al. [28]. Moreover, no evidence is available about transamidation of $tRNA^{Glu}$ to form $tRNA^{Gln}$ in *S. cerevisiae* mitochondria). We also tested the suppressing effect of pMSE1 in which the gene coding for yeast mt GluRS is cloned by PCR into multi-copy vector pYES2.1/V5-His-TOPO (Invitrogen, Life Technologies) under the inducible Gal1 promoter. The oligonucleotides for the PCR reactions were MSE 5′+ (5′-GAAAGGATCGTTCATAAAG-3′) and MSE pst- (5′-GCTGCAGCGTATATGACGTATTTACATG-3′).

pLARS2 and pVARS2 in which the human gene coding for mt LeuRS and ValRS, respectively, is cloned into pYES2 transcription vector under the inducible Gal1 promoter [29];

pCtermLARS2, pβ30_31 and pβ32_33 are plasmids expressing fragments of *LARS2* gene (aa 837–903, 841–855, and 886–901, respectively), cloned in the pYES2.1/V5-His-TOPO (Invitrogen, Life Technologies) vector under the inducible Gal1 promoter [7].

In the same vector we cloned the human sequence coding for the mt IleRS variant from aa 880 to aa 945 (pCtermIARS2) by amplifying the insert from pTUNEIARS2 [7] with CtermIARS2+ (5′-ATTTTCCTGAGCCA GTAAAG-3′) and CtermIARS2− (5′-ATGAGTGCGTGTGCAATGCGAG-3′) oligonucleotides.

pCtermVARS2 is the pYES2.1/V5-His-TOPO (Invitrogen, Life Technologies) vector expressing the mt ValRS variant from aa 908 to 974, cloned by amplifying the insert with CtermVARS2+ (5′ATGGAGGTCG TGCAGGTGC-3′) and VARS2− (5′TTAAGCCTGGGCCCAGCCG-3′) oligonucleotides, from the pVARS2 vector.

pETUF1 is the multicopy pFL61 plasmid bearing the *TUF1* gene with its own MTS and promoter [23]. The pEΔMTSTUF1 plasmid was obtained by amplifying the pETUF1 plasmid with ATG TUF10+ (5′-ATGGCA GCTGCTTTTGATCG-3′) and TUF4- (5-CCAGTTGCATCAATAAGTC-3′) primers and cloning the DNA fragment into the pYES2.1/V5-His-TOPO (Invitrogen, Life Technologies) vector. For the pECterm-ΔMTSTUF1 plasmid we used a PCR fusion strategy whereby the Cterm domain from pNAM2 (see above) was amplified using the following primers: NamΔCtermEcoR1+ (5′-CGGAATTCCGATGAAATTCAAAAAGTTTCA AAT-3′) and CtermNAMTUF11- (5′-GGAACGATCAAAAGCAGCTGCCTT GTGGAATAAGAAACTAATCAC-3′). The other fusion fragment was obtained from pETUF1 (see above) by amplifying the gene sequence with opportune ligation primers: CtermTUF11+ GTGATTAGTTTCTTAT TCCACAAGGCAGCTGCTTTTGATCGTTCC and TUF4- (see above). These last two PCR products were ligated by PCR amplification with NAM2CtermEcoRI+ and TUF4− described above and cloned into the pYES2.1/V5-His-TOPO (Invitrogen, Life Technologies).

For in vitro transcription the entire gene coding for WT human mt-$tRNA^{Leu(UUR)}$, $tRNA^{Ile}$ and $tRNA^{Gly}$ were inserted in the pGEM EASY vector (Promega). The inserts were generated by PCR from total WT human fibroblast DNA. The following oligonucleotide pairs containing the T7 promoter and terminator: Hleu+T7 (5′-TAATACGACTCACTATAG TTAAGATGGCAGAGCCCG-3′) and Hleu−T7 (5′-CAAAAAACCCCTCA AGACCCGTTTAGAGGCCCCAAGGGGTTATGCTATGTTAAGAAGAGGAA TTGAAC-3′);

Hile + T7 (5′-TAATACGACTCACTATAAGAAATATGTCTGATAA AAG-3′) and Hile−T7 (5′-CAAAAAACCCCTCAAGACCCGTTTAGAGGC CCCAAGGGGTTATGCTA TAGAAATAAGGGGGTTTAAGC-3′).

Hgly+T7 (5′-TAATACGACTCACTATAACTCTTTTAGTATAAATAG-3′) and Hgly−T7 (5′-CAAAAAACCCCTCAAGACCCGTTTAGAGGCCCCAAG GGGTTATGCTATACTCTTTTTTGAATGTTGT-3′);

Site directed mutagenesis was used to generate the pGEM EASY vector (Promega) and insert mutations T4277C and C3256T in

human mt $tRNA^{Ile}$ and $tRNA^{Leu(UUR)}$ respectively. Oligonucleotides pairs were: HileT4277C+ (5′-GAAATATGTCTGACAAAAGAGTTACT-3′) and HileT4277C-(5′-AGTAACTCTTTTGTCAGACATATTTC-3′); HleuC3256T + (5′-AGCCCGGTAATCGTATAAAACTTAAAAC-3′) and HleuC3256T− (5′-GTTTTAAGTTTTATACGATTACCGGGCT-3′)

2.3. Band shift experiments

Purified amplicons were used as template in the transcription reaction. We followed the HiScribe T7 in vitro transcription Kit (New England BioLabs, Inc.) instruction designed for the production of small RNAs (50–300 nucleotides final length) with minor modifications: [α-^{32}P]UTP RNA labelling was performed as described by Sambrook et al. [24]. After three hours of incubation at 42 °C the in vitro labelled reaction was precipitated with Na acetate 0.3 M, glycogen 0.5 μg/μl. The peptide NH_2-MAVLINNKACGKIPV-COOH coding for the β30_31 (15 amino acids) region of human mt LeuRS Cterm domain was commercially synthesized and HPLC purified by Primm Biotech service. As control we used [Lys^3]-Bombesin (15 amino acids in length, Sigma-Aldrich).

The binding reaction was performed as previously described by Ke et al. [30] using 4 mRad of the labelled reaction and different peptide concentrations.

The complexes were separated on a 16 × 20 × 0.4 cm non-denaturing Tris/Borate/EDTA (TBE) 15% polyacrylamide gel. Electrophoresis was performed at 200 V for 24 h at +4 °C.

A sample of commercial *E. coli* $tRNA^{Val}$ (Sigma-Aldrich) which has the same length as mt $tRNA^{Leu(UUR)}$ was loaded and visualized by Ethidium bromide staining as a reference.

2.4. Structure and sequence analysis

The 3D structures of tRNA molecules determined in complex with aaRS homologous to yeast or human enzymes listed in Tables 1 and 2 were downloaded from the Protein Data Bank (PDB, www.rcsb.org) [31]. When more than one aaRS–tRNA complex from the same species was available, only the structure with the best resolution was analysed (see Supplementary Table S1). Structure visualization and analyses were performed using the software InsightII (Accelrys Inc.) and PyMOL (Schrödinger LLC).

Identification of aaRS domains was performed by visual assessment and refinement of the initial InterPro [32] definition. Assignment of secondary structure elements to Cterm domains was performed by DSSP [33].

The sequence alignment between yeast and human mt tRNAs aminoacylated by aaRS listed in Tables 1 and 2, or by their homologues of known 3D structure, were downloaded from the mitotRNAdb and tRNAdb databases (http://trnadb.bioinf.uni-leipzig.de/) [34]. Nucleotides in contact with the cognate aaRS in 3D structures were calculated using in house-built software (PDM et al., unpublished data). Two atoms were considered to be in contact if their distance was lower than or equal to 4.0 Å. The definition of identity nucleotides is from Giegé et al. [35].

The multiple sequence alignment between human mt IleRS, ValRS and LeuRS has been using the program Multialin obtained (http://multalin.toulouse.inra.fr/multalin/multalin.html) [36].

3. Results

3.1. Mt aaRS exhibit cross suppressing activities towards several mt tRNA mutants

We exploited our collection of yeast mutants, bearing different substitutions located in various mt tRNA genes, to investigate the suppressing and cross-suppressing ability of: i) the mt aaRS other than mt LeuRS, IleRS and ValRS; ii) Sequences from the Cterminal regions of mt IleRS and ValRS; and iii) β30_31 and β32_33 isolated regions derived from human mt LeuRS Cterm [7]. Fig. 1 shows the localization of the mutations studied in this work in the context of a generic tRNA cloverleaf structure [37]. The mutant strains were named by letters indicating the mt tRNA gene and the base substitution. Mutations were obtained either by random mutagenesis or biolistic transformation procedure. They reside in different regions of the mt tRNA molecule and in mt tRNAs aminoacylated by aaRS belonging to different Classes. To avoid effects due to different nuclear backgrounds [14,38,39], we compared growth phenotype and suppression activities using isogenic cells obtained by cytoduction crosses with MCC123 rho° cells. The characterization of the mutants has been previously reported (for references see Tables 1 and 2). The growth of mutants at 28 and 37 °C on a strictly respiratory medium (YP containing 3% of glycerol) was compared to that of the WT (Tables 1 and 2, column 3). We have previously shown that the defects in mt protein synthesis of the mutants can be ascribed to faulty tRNA aminoacylation [40], defective tRNA processing [20,21] or subsequent steps at the ribosomal level and that, very often, defects are accompanied by 3D structure alterations, which are indicated by altered electrophoretic migration of the mutated tRNA (Tables 1 and 2, column 2).

The results of representative suppression experiments are shown in Fig. 2. The defective glycerol growth of LeuC25T mutant with respect to the WT was suppressed by overexpression of both the cognate mt LeuRS and the β30_31 peptide derived from it, but not by Class II enzymes mt HisRS or PheRS (Fig. 2, panel A). The respiratory defect of the HisG51A mutant was suppressed by both cognate mt HisRS and non-cognate mt PheRS, both of which belong to Class II (intra-Class II suppression), but not by Class I mt LeuRS (Fig. 2, panel B). Conversely, the defects associated with mutant PheC2T were not suppressed by the cognate mt PheRS or non-cognate aaRS belonging to either the same (mt HisRS) or different (mt LeuRS) Class. However, it is important to note that the β30_31 sequence derived from mt LeuRS Cterm was able to suppress the defects of both HisG51A and PheC2T mutants with higher efficiency than any of the whole enzymes tested.

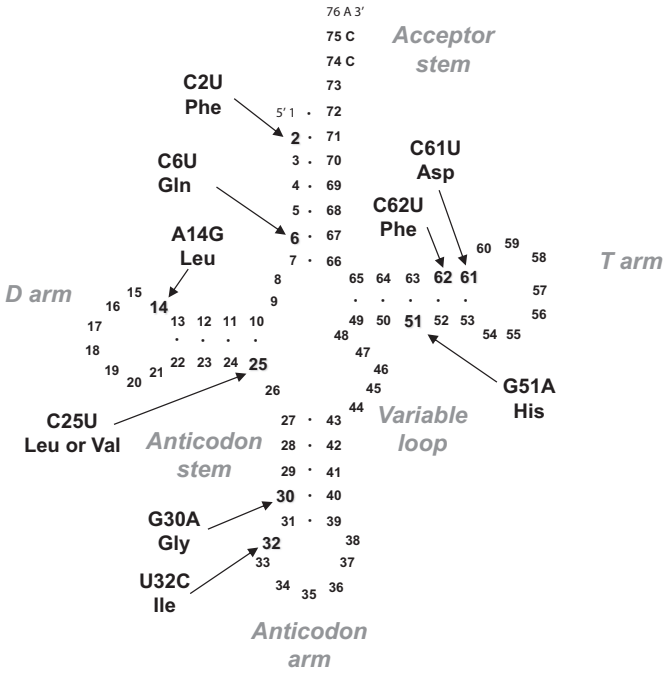

Fig. 1. Location of the mutations studied in this work in the context of a generic tRNA cloverleaf structure. Mutant strains are named by letters indicating the mt tRNA gene and base substitution. The mutated positions, indicated by arrows, are labelled according to the standard numbering referring to yeast cytoplasmic $tRNA^{Phe}$.

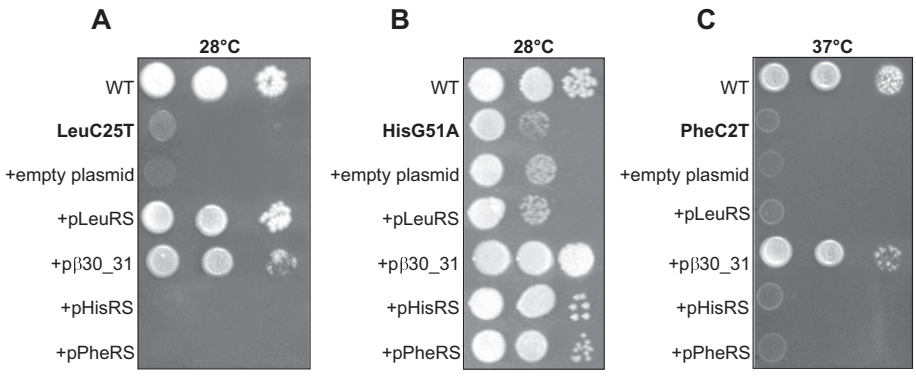

Fig. 2. Suppression activity of plasmids overexpressing mt aaRS sequences. Glycerol growth of serial dilutions of WT (MCC123) and isogenic mt tRNA mutants LeuC25T (A), HisG51A (B) and PheC2T (C) transformed with either empty multi-copy plasmid or plasmid containing the yeast gene coding for human mt leucyl-tRNA synthetase (pLeuRS), the Cterm β30_31 sequence (pβ30_31) coding for a short peptide derived from the Ctermimal domain of human mt leucyl-tRNA synthetase, the pHisRS and pPheRS, containing the yeast genes coding for mt histidyl- and phenilalanyl-tRNA synthetases, respectively. Pictures have been taken after 5 days of growth at the indicated temperature.

Suppression of the phenotype of mt tRNA mutants aminoacylated by Class I mt aaRS with multi-copy plasmids overexpressing different mt aaRS genes are reported in Table 1. The defects associated with mutations in mt $tRNA^{Leu(UUR)}$, $tRNA^{Ile}$ and $tRNA^{Val}$ are efficiently cross-suppressed by both yeast and human, cognate and non-cognate mt aaRS belonging to the same Class and subclass (Ia), but not by the tested mt aaRS from Class II or from different Class I subclasses Ib and Ic. The only exception is the suppression by mt TyrRS (Class Ic), of the phenotype of the ValC25T mutant, which is aminoacylated by Class I, subclass a, mt ValRS (Class Ia).

A different scenario is present in mutant GlnC6T. The defective phenotype of this mutant is only suppressed by mt LeuRS, at both temperatures in the case of the yeast gene (*NAM2*) and only at the permissive temperature in the case of the human gene (*LARS2*) and not by any other tested overexpressed gene. Therefore, the cross-suppression ability of Class Ia mt aaRS is not shared by the other Class I mt aaRS here analysed.

Table 2 reports the suppression, by Class I and II mt aaRS, of the defects associated with mutations in mt tRNAs aminoacylated by Class II mt aaRS. In this case, contrary to what has been reported in Table 1, the defective phenotype of only two mutants (AspC61T and HisG51A) is suppressed by the cognate aaRS and is cross-suppressed by other Class II and Class I aaRS.

In summary, comparison of the data reported in Tables 1 and 2 highlights that the rescuing effect is most extended for mt aaRS belonging to Class I, and in particular subclass a. A special case is the HisG51A mutant whose defect is suppressed by some Class II mt aaRS.

3.2. The cross-suppression activity of LeuRS carboxy-terminal domain and sequences thereof is not shared by the aligned sequences of mt ValRS and IleRS

We have previously demonstrated that the Cterm of human mt LeuRS (67aa) is able to suppress defective phenotypes of both yeast and human cells bearing mutations in mt tRNAs aminoacylated by Class Ia mt aaRS [7,9,10]. Here we extend the investigation of the suppressing ability of the Cterm by assessing its effect on the phenotype of mutant tRNAs aminoacylated by mt aaRS from Class Ib and Class II. Additionally, we examine the suppressing ability of the Cterminal regions of mt ValRS and IleRS towards the same mutants examined for mt LeuRS Cterm.

The Cterm of both human and yeast mt LeuRS and the β30_31 and β32_33 sequences effectively suppress the defective phenotype of most mutants (Table 3). In particular, all of them equally and effectively suppress the phenotype due to mutations in all mt tRNAs aminoacylated by Class Ia aaRS. The only exception is mutant IleT32C whose phenotype is not suppressed by the Cterm from human LeuRS but is suppressed by the short peptides derived from it. All yeast and human mt LeuRS-derived fragments also suppress, albeit only at the permissive temperature, the phenotype due to mutation in mt $tRNA^{Asp}$, aminoacylated by Class IIb mt AspRS. Interestingly, the short (15 and 16 amino acid long, respectively) β30_31 and β32_33 sequences suppress the phenotype of several mutants, aminoacylated by Class IIa, much more effectively than the whole Cterm domain of both yeast and human mt LeuRS, from which they are derived. Strikingly, the ability of HisG51A and PheC2T mutants overexpressing β30_31 and β32_33 sequences to grow on respiratory substrate is even higher than that of the WT.

To evaluate whether the suppressing activity of human mt ValRS and IleRS resided in the Cterminal regions, as demonstrated for mt LeuRS, the IARS2 and VARS2 sequences corresponding to residues 642–706 (65 amino acids) of mt IleRS and 768–834 (67 amino acids) of ValRS were investigated (see Section 2). Fig. 3 shows the multiple alignment of the Cterminal regions of human mt IleRS, ValRS and LeuRS. The mt LeuRS Cterm domain region endowed with cross-suppression activity (see Table 3), is underlined. The mt IleRS and ValRS sequences aligned to this region have been used for suppression experiments. As shown in Table 3, only the ValC25T mutant is suppressed by the Cterminal sequences of both human mt IleRS and ValRS. These results indicate that the wide cross-rescuing activity of human mt LeuRS Cterm is not shared by the selected Cterminal sequences of mt IleRS and ValRS, and that the determinants of the suppressing and cross-suppressing activity possessed by the whole enzymes (see Tables 1 and 2) possibly reside, at least partially, in different regions of the molecules.

The phenotypes of mutants GlyG30A and PheC62T are not suppressed by any of the investigated Cterminal sequences, as they were not suppressed by any of the entire aaRS tested (Tables 1 and 2).

3.3. The β30_31 peptide derived from mt LeuRS Cterm interacts with human mt tRNAs

To investigate whether the 15 amino acid long β30_31 peptide is able to interact with human mt tRNAs we performed in vitro band shift experiments. WT mt $tRNA^{Leu(UUR)}$, $tRNA^{Ile}$ and $tRNA^{Gly}$ were cloned in the pGEM EASY vector and mutations m.3256C>T, equivalent to LeuC25T, and m.4277 T>C in $tRNA^{Ile}$ previously studied by Perli et al. [8], were introduced by site directed mutagenesis. In all cases, the in vitro transcription product was a unique band. Fig. 4 shows the electrophoretic migration of WT mt $tRNA^{Leu(UUR)}$ (panel A) and mt $tRNA^{Ile}$ reaction synthesis products (panel B). [α-^{32}P]UTP tRNA labelling was performed and 4mR of the reaction was used for the band shift experiments.

Fig. 4 (panels C and D) shows the electrophoretic mobility of labelled human mt tRNAs in the presence or absence of peptides. The results

Table 3
Suppression capability of Carboxy-terminal domain of mt LeuRS, and corresponding "Cterm" region of mt ValRS and mt IleRS.

	tRNA mutants	Glycerol growth 28 °C 37 °C	Glycerol growth of transformant strains					
			Cterm (*NAM2*)	Cterm (*LARS2*)	β30_31 (*LARS2*)	β32_33 (*LARS2*)	"Cterm" (*VARS2*)	"Cterm" (*IARS2*)
tRNA aminoacylated by Class I aaRS	**GLNC6T**	+/− −	+ −	+ −	+ −	+ −	+/− −	+/− −
	VALC25T	+/− +/−	+ [7] +	+ +	+ [7] +	+ [7] +	+ +	+ +
	LEUA14G	− −	+ +	+ +	+ +	+ +	− −	− −
	LEUC25T	− −	+ [7] +	+ +	+ +	+ +	− −	− −
	ILET32C	− −	+ [7] +	− −	+ +	+ +	− −	− −
tRNA aminoacylated by Class II aaRS	**ASPC61T**	− −	+ −	+ −	+ −	+ −	− −	− −
	GLYG30A	− −	− −	− −	− −	− −	− −	− −
	HISG51A	+/− −	+ +	+ +	++ ++	++ ++	+ +/−	+/− −
	PHEC2T	+ −	+ −	+ −	++ +	++ +	+ −	+ −
	PHEC62T	− −	− −	− −	− −	− −	− −	− −

"Cterm" is the Carboxy-terminal domain of the human mt ValRS and IleRS aligned with the suppressing Cterm domain of human mt LeuRS.
+ indicates growth similar to wild type; − indicates absence of growth; +/− indicates partial growth.

show that the mutated $tRNA^{Leu(UUR)}$ molecule has a different mobility compared to the WT (likely due to a conformational variation of the molecule) and that both WT and mutant mt $tRNA^{Leu(UUR)}$ and $tRNA^{Ile}$ undergo a band shift in the presence of the β30_31 peptide. The specificity of this interaction is demonstrated by the absence of a band shift in the presence of the 15 amino acid long peptide [Lys3]-Bombesin (Fig. 4, panel C).

The same experiment was performed using WT mt $tRNA^{Gly}$. In this case, in agreement with the lack of suppression of the GlyG30A mutant phenotype following overexpression of the β30_31 sequence, we did not observe changes in tRNA electrophoretic mobility in the presence of the β30_31 peptide (Fig. 4, panel D).

3.4. Structure analysis shows that Cterminal domain regions of several aaRS have different lengths, topologies and tRNA binding properties

To investigate the mode of interaction of aaRS listed in Tables 1 and 2 with cognate tRNA molecules, we analysed the 3D structures of aaRS–tRNA complexes available from the PDB (see Supplementary Table S1). Analysis of the domain architecture in these aaRS showed that: i) not all aaRS comprise a Cterminal region after the anticodon binding (or, in the case of AspRS, catalytic) domain (Fig. 5, panel A); and ii) Cterminal regions belonging to different aaRS have different 3D structures, as highlighted by their different lengths and/or topologies (Fig. 5, panel B). Additionally, although all Cterminal regions present in known structures make contact with tRNA molecules, the specific tRNA nucleotides and Cterminal amino acids involved in the interaction vary for different aaRS. Fig. 6 shows the tRNA nucleotides interacting with different domains of cognate aaRS molecules. The aaRS catalytic and anticodon binding domains are generally involved in contacts with the tRNA acceptor and anticodon arms, respectively. Conversely, both the specific position and identity of nucleotides interacting with the Cterminal region show very little conservation between aaRS belonging not only to different subclasses, but also to the same subclass. Further, the Cterminal domain of LeuRS from species belonging to the bacterial and archaeal domains of life, have quite different lengths and topologies and interact with different regions of the cognate tRNA molecules. While LeuRS–$tRNA^{Leu}$ is the only complex for which 3D structures from different domains of life are available, the higher sequence similarity of human mt and cytoplasmic isoforms with bacterial and archaeal LeuRS (data not shown), indicates that these differences have been conserved in LeuRS of higher eukaryotes localized in different compartments.

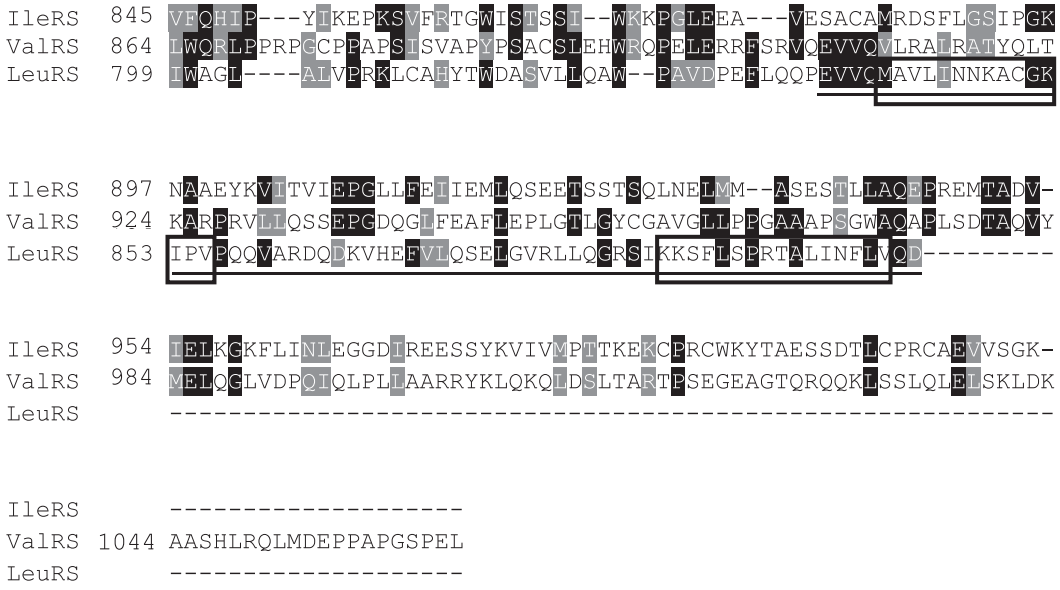

Fig. 3. Multiple sequence alignment of the Cterminal region of human mt IleRS (*IARS2* gene), ValRS (*VARS2* gene) and LeuRS (*LARS2* gene). Identical amino acids are highlighted in black; the amino acids with similar features are highlighted in grey. The 67 Cterm sequence of mt LeuRS and the sequence of 65 amino acids from mt IleRS (aa 642–706) and of 67 amino acids from mt ValRS (aa 768–834) aligned with the suppressing Cterm domain of mt LeuRS are underlined. Boxes indicate sequences β30_31 and β32_33 of human mt LeuRS.

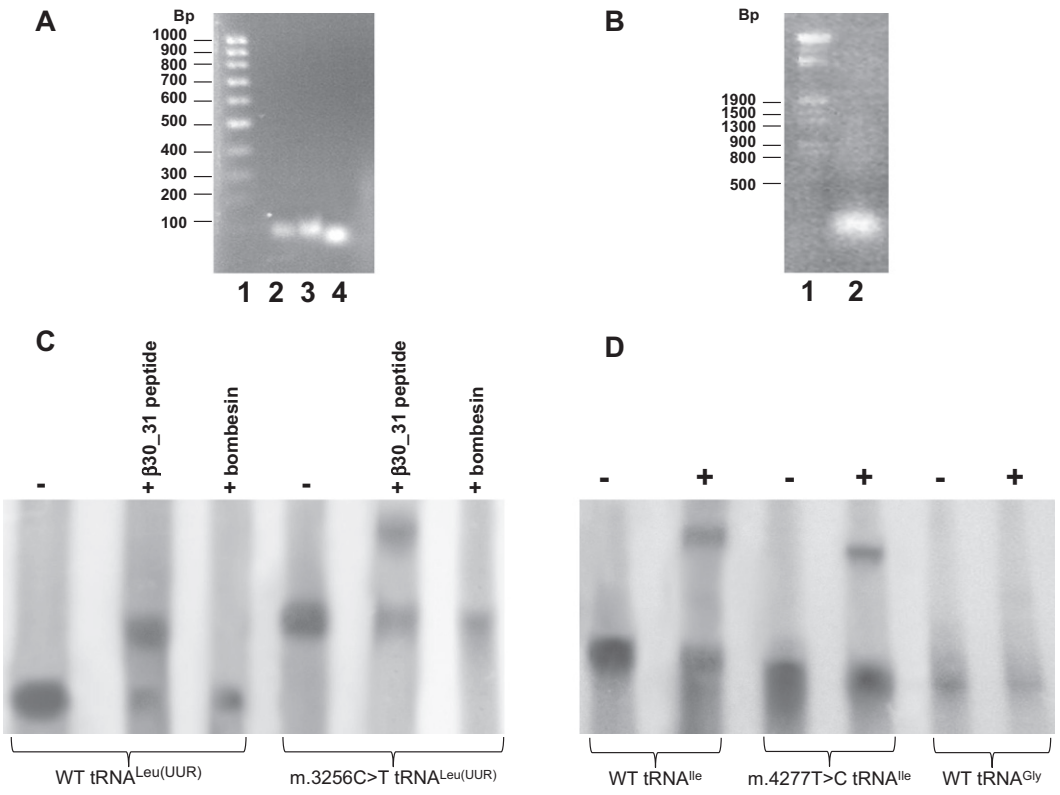

Fig. 4. Products of in vitro transcription reaction and Band shift experiments. (A) Human mt $tRNA^{Leu(UUR)}$ and *E. coli* commercial $tRNA^{Val}$, as reference, were loaded on 1% TBE agarose gel and visualized by EtBr staining. Lanes: 1. PCR 100 bp LADDER; 2. Mt $tRNA^{Leu(UUR)}$ (1/40 of reaction mixture); 3. Mt $tRNA^{Leu(UUR)}$ (1/20 of reaction mixture); 4. Commercial *E. coli* $tRNA^{Val}$ (3 μg). (B) Human mt $tRNA^{Ile}$ was loaded on 1% TBE agarose gel and visualized by Ethidium Bromide staining. Lanes: 1. EcoRI/HindIII digested λ DNA; 2. $tRNA^{Ile}$ (1/40 of reaction mixture). (C) Band shift experiments with in vitro [α^{32}P]UTP labelled human WT and mutated m.3256C > T mt $tRNA^{Leu(UUR)}$ loaded without (−) or after incubation with 7 mM β30_31 peptide and 7 mM [Lys3]-Bombesin (+), as control. (D) Band shift experiments with WT, mutated m.4277 T > C mt $tRNA^{Ile}$ and WT mt $tRNA^{Gly}$ loaded without (−) or after incubation with 7 mM β30_31 peptide (+).

3.5. The Cterm from mt LeuRS effectively targets mt EF-Tu to mitochondria

We previously observed that human mt LeuRS Cterm domain can be imported into the mitochondria [9,10] and that both human and yeast peptides have a positive/negative residue ratio similar to the N-terminal MTS of the whole enzymes [41,42]. To further investigate the mt import we constructed a chimeric protein in which the endogenous MTS of the mt protein synthesis elongation factor EF-Tu (*TUF1* gene) has been deleted and substituted by the Cterm of mt LeuRS.

With this plasmid we transformed the strain deleted of the *TUF1* gene unable to grow on glycerol containing media (mt EF-Tu in fact is essential for mt protein synthesis). Fig. 7 shows that the rescue obtained with the chimera construct (pECterm-ΔMTSTUF1) is equivalent to that obtained with the plasmid containing the WT *TUF1* gene (pETUF1)

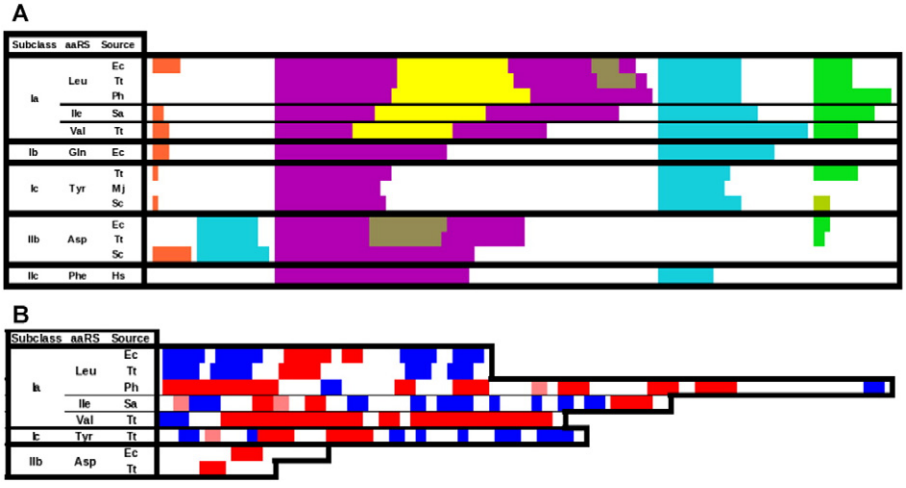

Fig. 5. Structure analysis of Cterminal regions in aaRS structures listed in Supplementary Table S1. (A) AaRS domain architecture. Domains are indicated by rectangles and colour-coded as follows: orange, N-terminal region; magenta, catalytic domain; yellow, editing domain; cyan, anticodon binding domain; green, Cterminal region; yellow–green, Cterminal region present in the sequence of *S. cerevisiae* mt TyrRS, but not visible in the structure; brown, other domain types. Rectangle width is proportional to domain length. (B) Secondary structure content of Cterminal regions indicated in panel A. Secondary structure elements are indicated by rectangles and colour-coded as follows: blue, β-strands; red, α-helices; salmon, 3–10 helices. Rectangle width is proportional to the length of secondary structure elements.

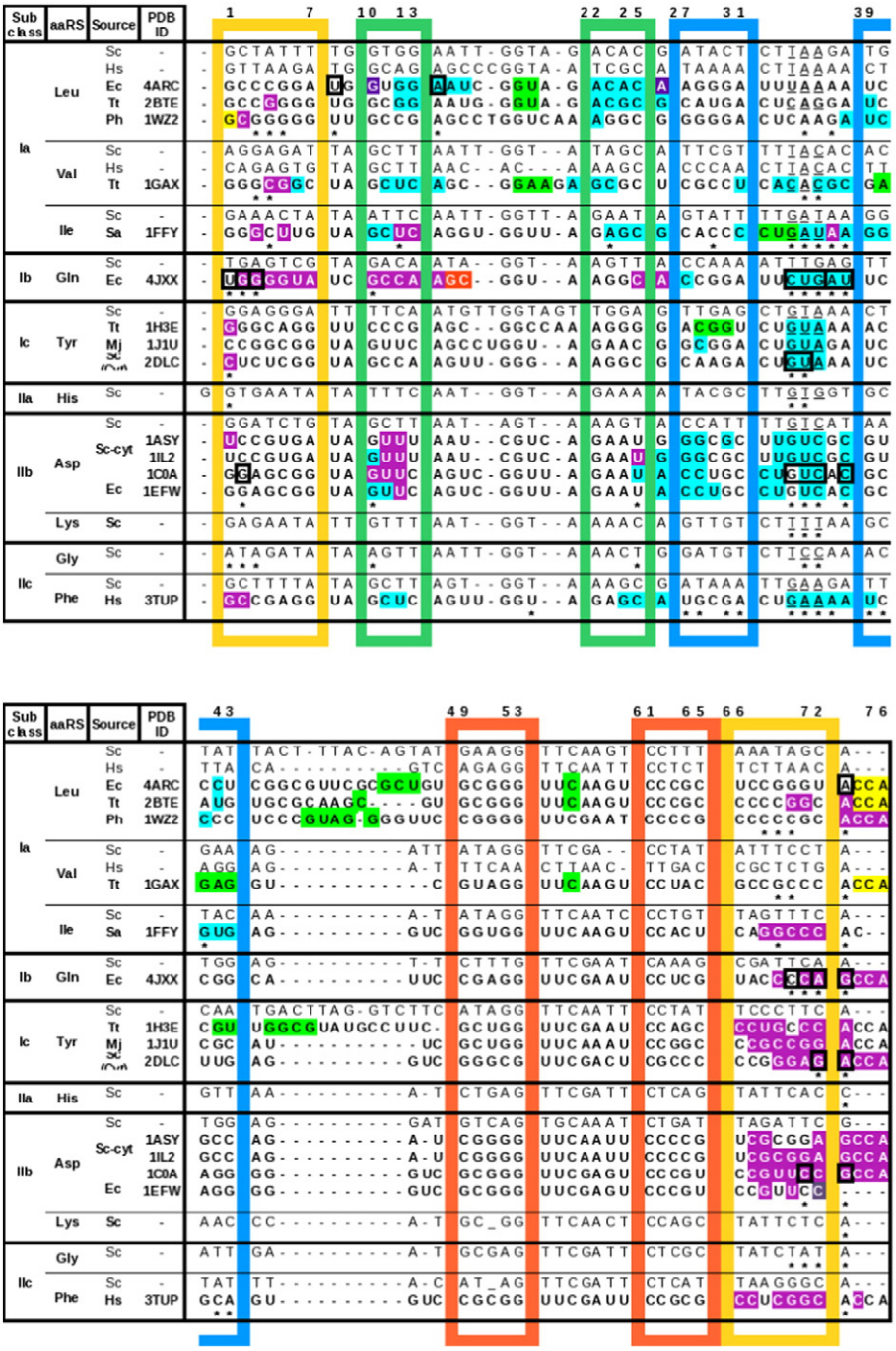

Fig. 6. Sequence alignment of tRNA molecules aminoacylated by aaRS studied in this work, and of homologous aaRS of known 3D structure (bold). Sub: aaRS subclass. Source: species abbreviations are as in Supplementary Table 1. PDB ID: identifier of the 3D structure available from the PDB. TRNA stem regions are indicated by coloured boxes: orange, acceptor stem; green, D-stem; blue, anticodon stem; red, T-stem. Numbers indicate the starting and ending nt of each stem region. TRNA nts in contact with different aaRS regions in experimentally determined 3D structures are indicated with differently coloured background: N-terminal region, orange; catalytic domain, magenta; editing domain, yellow; other inserted domains, violet; anticodon binding domain, cyan; and Cterminal region, green. Identity nts (see text) identified for tRNAs whose sequences are reported in the alignment are indicated by black borders. The positions corresponding to identity nts identified for other tRNAs, whose sequences are not present in the alignment, are indicated by asterisks.

indicating that the mt LeuRS Cterm has besides a suppression capability also a MTS function.

4. Discussion

Mutations in mt tRNA genes cause the majority of mt diseases. Since no cure for these diseases is currently available, the exploitation of the suppression effect exerted by mt aaRS or their fragments for therapeutic applications could be of highest importance.

For this reason we have investigated the extension and the characteristics of the suppression effects exerted by several mt aaRS towards the respiratory defects due to mutations in cognate and non-cognate mt tRNAs.

The results reported in this work represent an advancement towards the elucidation of the mechanism by which the effects of mutations in mt tRNAs are rescued by overexpression of cognate or non-cognate mt aaRS.

The results reported in Tables 1 and 2 show that, with some exceptions (e.g. TyrRS), cross-suppression takes place in Class Ia mt aaRS,

while it is limited among Class II mt aaRS with important exceptions for mt $tRNA^{Asp}$ and $tRNA^{His}$ mutants.

In principle, the suppressing ability of mt aaRS might be connected with an increase of aminoacylation activity. However, as previously mentioned we demonstrated that, at least in the case of mt LeuRS, the rescuing of respiratory defects takes place even if catalytic activity is absent [14]. This led us to suggest that the rescuing effect is mediated by a chaperone-like activity, by which the altered structure of mutated tRNA is corrected by the contact with the aaRS. Indeed, the Cterm of human mt LeuRS, which does not possess catalytic activity, has been shown to be endowed of the same suppression activity of the whole enzyme [7].

Here we demonstrate by a chimeric construct (Fig. 7) that the yeast mt LeuRS Cterm contains the specific sequence for mt targeting.

Additionally, short sequences derived from the human mt LeuRS Cterm domain (β30_31 and β32_33), when overexpressed, can suppress not only the respiratory defects of the mutants aminoacylated by Class I mt aaRS, but also of some additional mutants aminoacylated by Class II mt aaRS (AspC61T, HisG51A and PheC2T) which were not rescued by the whole enzyme (Tables 2 and 3). In some cases (i.e., HisG51A and PheC62T mutants), the suppression is even more effective than that obtained with the entire mt aaRS). This might be explained by a less specific tRNA binding ability of the β30_31 peptide compared with the entire enzyme. We can suggest that the short peptide might interact with several regions of the mutated tRNA restoring a structure required to exert its function thereby facilitating its rapid utilization in the mt protein synthesis process. In the case of HisG51A, the growth higher than WT may be related to the presence of high levels of mt histidylated tRNA previously reported for this mutant [40].

Additionally, we did not obtain suppression activity overexpressing the Cterminal sequences of human mt IleRS and ValRS, aligned with the Cterm of mt LeuRS (Table 3 and Fig. 3).

We also demonstrate by band-shift experiments that the β30_31 peptide, derived from the Cterm domain of mt LeuRS, interacts directly with both WT and mutant human mt $tRNA^{Leu(UUR)}$ and mt $tRNA^{Ile}$ (see Fig. 4). These interactions might force the mutated tRNA to resume a structure that can restore normal mt protein synthesis. Conversely, we did not observe band shift for human mt $tRNA^{Gly}$ in the presence of the β30_31 peptide, consistent with the lack of suppression of the defective phenotype of GlyG30T mutant by the overexpressed β30_31 sequence. Taken together, our results suggest that direct binding between mutated tRNA and suppressive molecule is required for suppression. The chaperone-like suppressor activity is expected to be effective in the case of functional defects mediated at least in part, by structural defects. Conversely, in the case of substitutions that do not result in tRNA structural defects chaperone-like suppressor molecules may be less effective. Once the mutated tRNAs have recovered their functionality, all the cellular defects derived from loss of tRNA function are expected to be rescued.

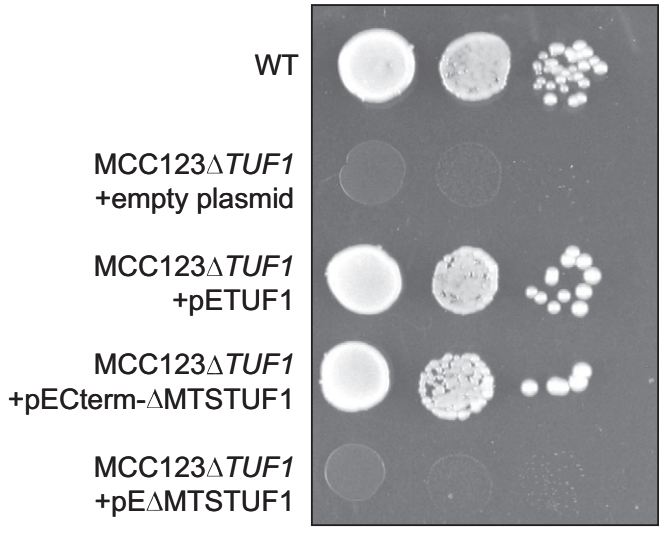

Fig. 7. The Cterm domain of yeast mt LeuRS can substitute the mt targeting sequence of mt EF-Tu. Glycerol growth capability of WT (MCC123), the same strain deleted of the *TUF1* gene (MCC123ΔTUF1) and of transformants bearing either the empty plasmid or one of the following plasmids: pETUF1, containing the *TUF1* WT gene; pECtermΔMTSTUF1 containing a chimeric construct where yeast mt LeuRS Cterm has substituted the endogenous mt targeting sequence of *TUF1*; pEΔMTSTUF1 containing a mutated version of *TUF1* without the mt targeting sequence. Picture was taken after 5 days of growth at 28 °C.

An important point to discuss concerns the existence of a relationship between the cross-suppression activity of mt aaRS and their splicing activity. Here we show that several mt aaRS that have not yet been reported to be involved in splicing are endowed with suppression capability. On the other side, mt LeuRS that has been first discovered and studied as a splicing factor for yeast mt introns (which have complex stem and loop RNA structures), is shown here to have an exceptionally wide range of suppressing activities. The splicing capability has been shown to be localized in short sequences of the Cterm and editing domains of mt LeuRS [43,44]. Both these domains and the splicing activity are conserved in the human orthologue [6] even if the human mtDNA does not contain introns. This character might suggest that aaRS sequences involved in splicing/suppression might rescue the phenotype due to several mutations in mt tRNA and possibly be used for the therapy of a range of diseases associated with mutations in mt tRNAs.

The pattern of aaRS–tRNA interactions observed in known structures indicates that, with the exception of a few regions, like the anticodon or the CCA tail, different aaRS recognize different regions of their cognate tRNA molecules.

However, inspection of the interactions established by Class Ia aaRS, which is the only one for which different aaRS structures are available, highlights that the position and, in a few cases, identity of contacting nucleotides is relatively conserved, in agreement with the extensive cross-suppression activity shown by aaRS belonging to this subclass.

In particular, the Cterm domain of LeuRS interacts with highly conserved G and C nucleotides in the D-loop and T-loop, respectively. Conversely, the lack of cross-suppressing ability by nucleotides contacted by the Cterminal domains of mt IleRS and ValRS may be explained, at least in part, by their low extent of conservation.

This extensive study adds new insights on the possibility to identify a single peptide endowed with suppressing activity and able to alleviate respiratory defects, due to mutations in most mt tRNAs. This might be the basis for the first specific cure for mt diseases due to mutations in mt tRNAs. For this purpose, sequences derived from the mt LeuRS Cterm should be preferentially investigated.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.09.011.

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