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An unusual tRNA^{Thr} derived from tRNA^{His} reassigns in yeast mitochondria the CUN codons to threonine

Dan Su[1], Allyson Lieberman[1], B. Franz Lang[2], Miljan Simonović[3], Dieter Söll[1,4,*] and Jiqiang Ling[1,*]

[1]Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520-8114, USA, [2]Département de Biochimie, Robert Cedergren Centre, Université de Montréal, Montréal, Québec, Canada, [3]Department of Biochemistry and Molecular Genetics, University of Illinois, 900 S. Ashland Avenue, MBRB 1170, Chicago, IL 60607 and [4]Department of Chemistry, Yale University, New Haven, CT 06520-8114, USA

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ABSTRACT

The standard genetic code is used by most living organisms, yet deviations have been observed in many genomes, suggesting that the genetic code has been evolving. In certain yeast mitochondria, CUN codons are reassigned from leucine to threonine, which requires an unusual tRNA^{Thr} with an enlarged 8-nt anticodon loop (tRNA$_1^{Thr}$). To trace its evolutionary origin we performed a comprehensive phylogenetic analysis which revealed that tRNA$_1^{Thr}$ evolved from yeast mitochondrial tRNA^{His}. To understand this tRNA identity change, we performed mutational and biochemical experiments. We show that *Saccharomyces cerevisiae* mitochondrial threonyl-tRNA synthetase (MST1) could attach threonine to both tRNA$_1^{Thr}$ and the regular tRNA$_2^{Thr}$, but not to the wild-type tRNA^{His}. A loss of the first nucleotide (G_{-1}) in tRNA^{His} converts it to a substrate for MST1 with a K_m value (0.7 μM) comparable to that of tRNA$_1^{Thr}$ (0.3 μM), and addition of G_{-1} to tRNA$_1^{Thr}$ allows efficient histidylation by histidyl-tRNA synthetase. We also show that MST1 from *Candida albicans*, a yeast in which CUN codons remain assigned to leucine, could not threonylate tRNA$_1^{Thr}$, suggesting that MST1 has coevolved with tRNA$_1^{Thr}$. Our work provides the first clear example of a recent recoding event caused by alloacceptor tRNA gene recruitment.

INTRODUCTION

Transfer RNAs (tRNAs) are adaptor molecules that pair each amino acid with corresponding codons on the mRNA (1). Aminoacyl-tRNA synthetases (aaRSs) attach amino acids to the 3′-terminus of tRNAs (2), and the resulting aminoacyl-tRNAs (aa-tRNAs) are delivered by elongation factors to the ribosome, where codon–anticodon recognition defines the genetic code of life (3). The genetic code was once thought to be universal and frozen (4), but later studies revealed multiple codon reassignment events in nuclear and mitochondrial genomes (5–7). These reassignments include stop-to-sense, sense-to-sense and sense-to-stop codon changes; they have been found in nuclear genomes from all three domains of life. In some bacterial, archaeal and eukaryotic species the UGA codon is reassigned to allow insertion of selenocysteine (Sec), the 21st natural amino acid, in the presence of tRNA^{Sec} with a UCA anticodon (6,8). In a number of methanogenic archaea a UAG stop codon encodes the 22nd amino acid, pyrrolysine (Pyl), implemented by a tRNA^{Pyl} which recognizes UAG (9,10). A major reassignment in mitochondria is recoding UGA to tryptophan (Trp) implemented by a tRNA^{Trp} with a mutated anticodon (7). Furthermore, the mitochondria of several yeast species reassigned the AUA codon from isoleucine to methionine (Met) by the abnormal recognition of AUA by tRNA^{Met} (7).

One last codon reassignment in the well-studied organism *Saccharomyces cerevisiae* is still not understood. In certain budding yeasts (including *Saccharomyces*, *Nakaseomyces* and *Vanderwaltozyma*) the four CUN (N denotes U, C, A or G) codons in the mitochondria are reassigned from leucine (Leu) to threonine (Thr) (11,12). This results from the loss of tRNA$_{UAG}^{Leu}$ (with a UAG anticodon) that would translate CUN codons, and from the presence of an abnormal tRNA$_1^{Thr}$ with an enlarged 8-nt anticodon loop and a UAG anticodon (Figure 1) (11,13). This codon sense change was confirmed by protein sequencing of the *S. cerevisiae* mitochondrial ATPase (14). Mass spectrometry studies have also validated that at least three CUU and two CUA codons in *S. cerevisiae* mitochondrial-encoded proteins are recoded as Thr (15).

*To whom correspondence should be addressed. Tel: +1 203 432 6205; Fax: +1 203 432 6202; Email: jiqiang.ling@yale.edu
Correspondence may also be addressed to Dieter Söll. Tel: +1 203 432 6200; Fax: +1 203 432 6202; Email: dieter.soll@yale.edu

In addition to $tRNA_1^{Thr}$, yeast mitochondria also express a normal $tRNA_2^{Thr}$ with a UGU anticodon that reads the ACN threonine codon box (Figure 1). It was shown previously that while mitochondrial extracts from a wild-type (WT) *S. cerevisiae* strain could attach Thr to both $tRNA_1^{Thr}$ and $tRNA_2^{Thr}$, extracts from an *MST1* mutant strain could only threonylate $tRNA_2^{Thr}$ but not $tRNA_1^{Thr}$ (16). Thus it was suggested that MST1 serves as a mitochondrial threonyl-tRNA synthetase (ThrRS) specific for aminoacylation of $tRNA_1^{Thr}$, while a different ThrRS aminoacylates $tRNA_2^{Thr}$ in the mitochondria. Three decades after this discovery it is still a mystery how the unusual $tRNA_1^{Thr}$ emerged in the mitochondrial genome. Previous hypotheses suggest that $tRNA_1^{Thr}$ might have evolved from $tRNA_2^{Thr}$, or alternatively from the missing $tRNA_{UAG}^{Leu}$ (11,17,18). However, both hypotheses lack convincing experimental evidence. To our surprise, biochemical and phylogenetic analyses demonstrate that $tRNA_1^{Thr}$ directly evolved from mitochondrial $tRNA^{His}$. *Saccharomyces cerevisiae* mitochondrial $tRNA^{His}$ shares high (72%) sequence identity with $tRNA_1^{Thr}$, and a single-nucleotide change converts $tRNA^{His}$ to a substrate for MST1. Our work thus resolves the long-standing question regarding the origin of $tRNA_1^{Thr}$ and its coding response.

MATERIALS AND METHODS

Cloning, mutagenesis and general methods

Saccharomyces cerevisiae MST1, *S. cerevisiae HTS1*, *Candida albicans MST1* and *Schizosaccharomyces pombe MST1* genes were cloned into pET28a expression vector (Novagen) with an N-terminal six-His tag. Expression of recombinant proteins was induced at 37°C for 4 h with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) in *Escherichia coli* strain BL21-codon plus in Luria–Bertani (LB) media. His-tagged proteins were purified according to standard procedures. Mitochondrial tRNA genes were cloned into pUC18 vector (GenScript), and mutations were introduced using QuikChange Site-Directed Mutagenesis Kit (Stratagene).

In vitro assays

In vitro tRNA transcripts were obtained using the T7 RNA polymerase runoff procedure as described (19). Aminoacylation experiments were performed as described (20) in the presence of 100 mM Na-HEPES pH 7.2, 30 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 25 μM [^{3}H] Thr (100 μCi/ml), 25 μM [^{3}H] His (150 μCi/ml) or 50 μM [^{3}H] Leu (100 μCi/ml), 0.2–9 μM tRNA transcripts and 3–300 nM aaRSs.

Phylogenetic analyses

For phylogenetic analysis of mitochondrial tRNA sequences, tRNAs were predicted in mitochondrial genomes of interest with Erpin/RNAweasel (21,22) and a tRNA profile specific for fungal mitochondria that may be used *via* our website (http://megasun.bch.umontreal.ca/RNAweasel). Sequence columns representing the sequence positions in the variable loop between the anticodon- and T-loop were removed as the nucleotides in this region are extremely diverse in sequence and length, and phylogenetic analysis was conducted using either a Bayesian [PhyloBayes; (23)] or maximum likelihood approach [RaxML with GTR model; (24)]. For inference of a species tree, an application developed in-house (Mams) was used for automated mitochondrial protein alignment, removal of ambiguous regions in the alignment, and concatenation. Briefly, derived Cob, Cox1-3, Atp6, 9 and Nad1-6 protein sequences are pre-aligned with Muscle (25), alignments are iteratively refined with HMMalign (S. Eddy, http://hmmer.janelia.org) using *E*-values obtained with respective HMM models as an optimization criterion. Sequence positions that are not aligned with a posterior probability value of 1 are removed from the resulting alignment. This dataset with 22 taxa and

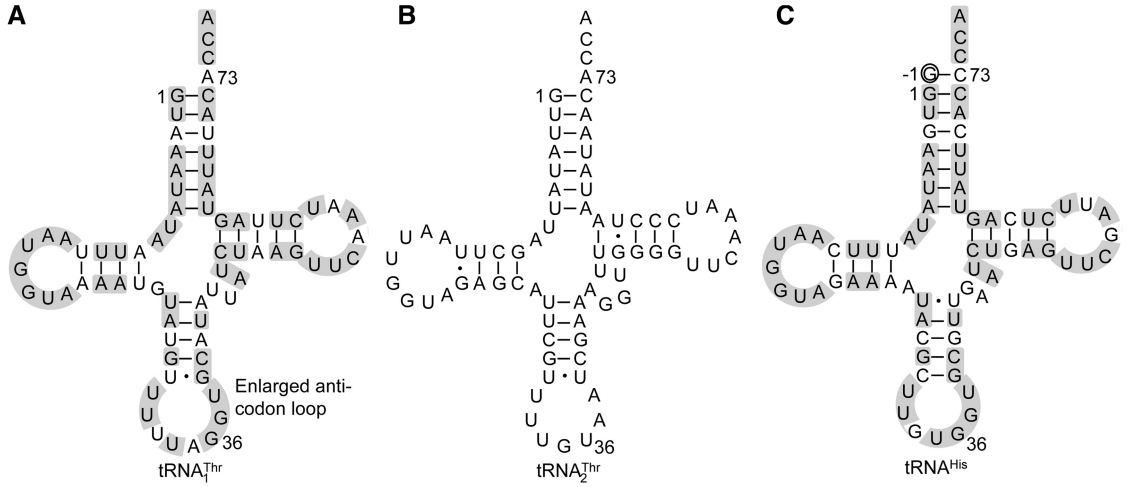

Figure 1. Nucleotide sequences of *S. cerevisiae* mitochondrial tRNAs. (**A**) $tRNA_1^{Thr}$ with an 8-nt anticodon loop and a UAG anticodon. (**B**) $tRNA_2^{Thr}$ with a canonical UGU anticodon. (**C**) $tRNA^{His}$ with a G_{-1} (circled, a major anti-determinant for ThrRS). The primary sequences of $tRNA_1^{Thr}$ and $tRNA^{His}$ are 72% identical (shaded).

3728 amino acid positions was analyzed using Bayesian inference by PhyloBayes that implements the CAT + GTR model, known to be among the least sensitive to LBA artifacts [(23,26,27), and references therein].

RESULTS

Phylogenetic analysis reveals that $tRNA_1^{Thr}$ originated from $tRNA^{His}$

The unsolved question of the yeast mitochondrial CUN codon reassignment is the evolutionary origin of $tRNA_1^{Thr}$ with an 8-nt anticodon loop and a UAG anticodon. To analyze the $tRNA_1^{Thr}$ recruitment, we performed a phylogenetic analysis of all mitochondrial tRNAs of *S. cerevisiae* and related yeast species using Bayesian inference (Figure 2). Not surprisingly, the 10 organisms with

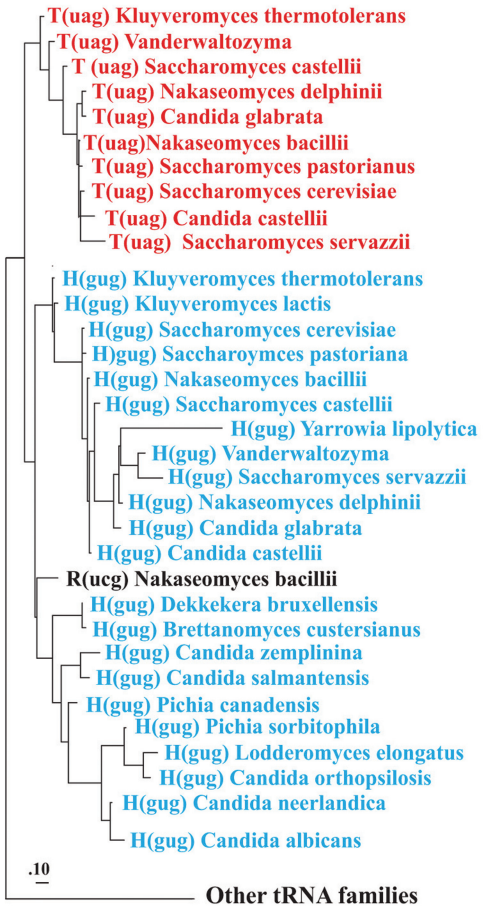

Figure 2. Phylogeny of yeast mitochondrial tRNAs. The phylogenetic analysis with PhyloBayes (default model parameters) contained all tRNA sequences from the species shown in Figure 3. Only the section of the tRNA phylogeny covering the $tRNA_1^{Thr}$ and $tRNA^{His}$ clusters are shown (marked red and blue, respectively), confirming monophyly of $tRNA_1^{Thr}$ and a sister group relationship to $tRNA^{His}$. The posterior probability support for the two tRNA groups is 1.0 (note that phylogenetic analysis with tRNA sequences depends on only few informative nucleotide positions, which does not allow to resolve the branching order within these groups). Removal of the anticodon sequence positions from the dataset did not change clustering into the two tRNA groups, nor did an analysis of the same datasets with maximum likelihood and the GTR model.

$tRNA_1^{Thr}$ are closely related; yet this $tRNA_1^{Thr}$ cluster is related to $tRNA^{His}$ (72% sequence identity between the two respective *S. cerevisiae* tRNAs; Figure 1). In contrast, $tRNA_1^{Thr}$ and $tRNA_2^{Thr}$ are definitively distant in the phylogeny (Figures 2, Supplementary Figure S1) and the respective *S. cerevisiae* tRNAs share only 52% sequence identity. As the phylogenetic signal in tRNA sequences is limited by the small number of informative sequence sites, and not a reliable marker in species phylogenies because of occasional identity shifts [e.g. (28)], we have built a yeast species tree based on mitochondrion-encoded proteins to permit mapping of evolutionary changes to this tree. The result of this phylogenetic analysis (Figure 3) is consistent with a single origin of the 10 yeast species that have a $tRNA_1^{Thr}$ homolog with an 8-nt anticodon loop, a clade emerging close to the divergence of *Kluyveromyces* species and subsequent to *Pichia canadensis*. Together, these results strongly suggest that $tRNA_1^{Thr}$ derived from mitochondrial $tRNA^{His}$ in an ancestral yeast species.

Saccharomyces cerevisiae MST1 threonylates both $tRNA_1^{Thr}$ and $tRNA_2^{Thr}$

The mitochondria of all yeast species containing $tRNA_1^{Thr}$ have lost the $tRNA_{UAG}^{Leu}$ gene, leaving $tRNA_1^{Thr}$ the only tRNA capable of reading CUN codons. Whereas *S. cerevisiae* mitochondrial LeuRS (*Sc*mtLeuRS) efficiently attaches Leu to $tRNA_{UAA}^{Leu}$ (29,30), it could not recognize $tRNA_1^{Thr}$ (Figure 4A), confirming that the *S. cerevisiae* mitochondrial CUN codons are assigned to Thr instead of Leu. It is known that the WT *S. cerevisiae* strain attaches Thr to $tRNA_1^{Thr}$, and an *MST1* deletion strain fails to threonylate $tRNA_1^{Thr}$, resulting in a respiration-deficient phenotype (16). This suggests that MST1 is a putative mitochondrial ThrRS. Compared with bacterial ThrRSs, yeast MST1 enzymes lack an N-terminal editing domain, but share homologous catalytic and tRNA anticodon binding domains. To test aminoacylation of $tRNA_1^{Thr}$ directly, the *S. cerevisiae MST1* gene was cloned into pET28a for protein overexpression in *E. coli*. N-terminal His-tagged MST1 was purified to homogeneity and tested in aminoacylation reactions in the presence of [^{3}H] Thr and *in vitro* transcribed *S. cerevisiae* tRNAs. Consistent with previous *in vivo* results (16), *Sc*MST1 was able to charge $tRNA_1^{Thr}$ with Thr *in vitro* (Figure 4B). MST1 could also threonylate $tRNA_2^{Thr}$, which was unexpected as it was previously thought that a second mitochondrial ThrRS was responsible for $tRNA_2^{Thr}$ aminoacylation. Steady-state kinetic experiments revealed that *Sc*MST1 recognized $tRNA_1^{Thr}$ and $tRNA_2^{Thr}$ with high affinity, with K_m values of 0.29 and 0.44 μM, respectively (Table 1), suggesting that tRNA modifications (17) are not critical for MST1 recognition. These data, together with previous *in vivo* results, establishes unequivocally that $tRNA_1^{Thr}$ is indeed threonylated by MST1. While we favor that MST1 threonylates $tRNA_2^{Thr}$ *in vivo*, we do not exclude the possibility that a second ThrRS activity is present in yeast mitochondria.

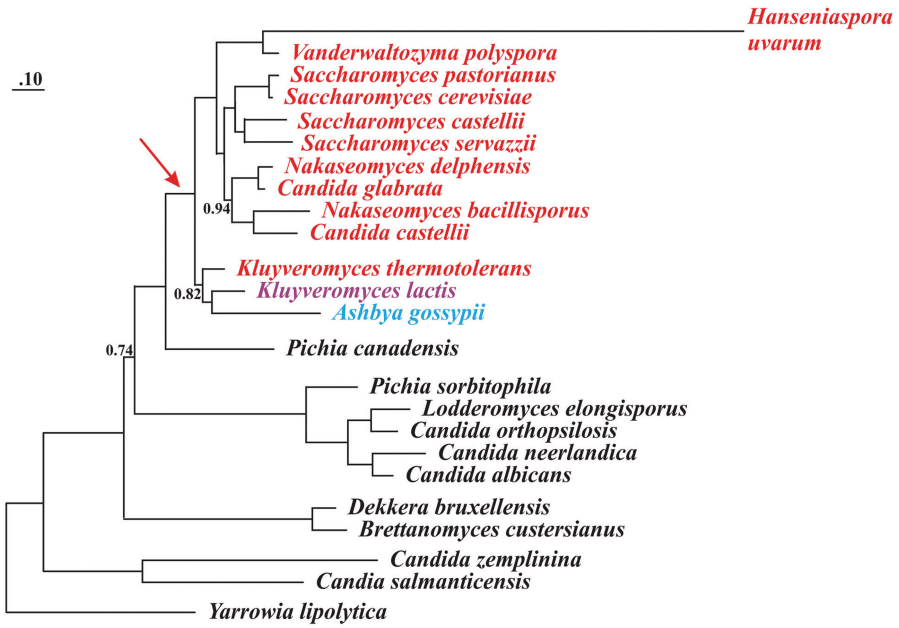

Figure 3. Phylogeny of yeast species based on concatenated mtDNA-encoded protein sequences. The phylogenetic analysis with PhyloBayes and the CAT model is based on 13 mtDNA encoded proteins. All divergence points are supported by posterior probability values of 1.0, except where indicated. The red arrow points to the concomitant loss of all seven *nad* genes and the start of mitochondrial codon reassignments, including AUA methionine, CUN threonine. Species shown in black possess mitochondrial $tRNA^{Leu}_{UAG}$ (but not $tRNA^{Thr}_{1}$), and in these organisms CUN codons are assigned to Leu. The yeast species marked red, such as *K. thermotolerans*, have lost mitochondrial $tRNA^{Leu}_{UAG}$ and obtained $tRNA^{Thr}_{1}$ that decodes CUN codons as Thr. *K. lactis* is marked magenta as it has no CUN codons and no corresponding tRNA with a UAG anticodon. *A. gossypii* (marked blue) contains a tRNA species with a UAG anticodon, yet its identity of this tRNA and the amino acid reading CUN codons in *A. gossypii* remain obscure (to be discussed by BFL in Organelle Genetics: Evolution of Organelle Genomes and Gene Expression, Springer 2011).

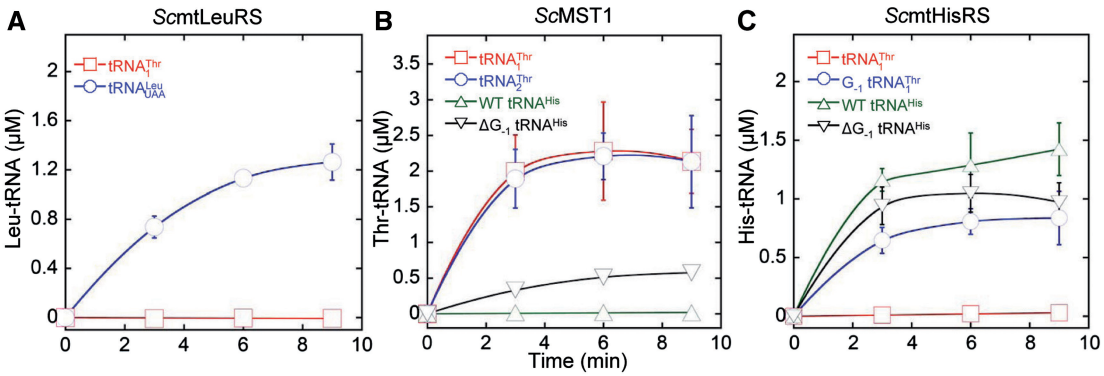

Figure 4. Aminoacylation by *S. cerevisiae* MST1 and HisRS. (**A**) Leucylation of $tRNA^{Thr}_{1}$ (3 μM) and $tRNA^{Leu}_{UAA}$ (3 μM) by *Sc*mtLeuRS (0.3 μM). (**B**) Threonylation of $tRNA^{Thr}_{1}$ (3 μM), $tRNA^{Thr}_{2}$ (3 μM) and $tRNA^{His}$ variants (3 μM) by *Sc*MST1 (0.3 μM). (**C**) Histidylation of $tRNA^{Thr}_{1}$ (3 μM) and $tRNA^{His}$ variants (3 μM) by *Sc*mtHisRS (0.3 μM).

Loss of G_{-1} converts *S. cerevisiae* $tRNA^{His}$ to a substrate for *S. cerevisiae* MST1

To provide experimental evidence for the evolution of $tRNA^{Thr}_{1}$, we examined recognition of $tRNA^{His}$ variants by MST1. Except in a few α-proteobacteria [(31), and references therein], all known $tRNA^{His}$ species contain a G at position −1, which is a critical identity element for histidyl-tRNA synthetase (HisRS) [(32,33) and references therein]. Sequence alignments of $tRNA^{Thr}_{1}$ and mitochondrial $tRNA^{His}$ genes revealed that G_{-1} addition in $tRNA^{His}$ comprises one major difference between the two tRNA species (Figure 5). *In vitro*, *Sc*MST1 failed to charge the WT *Sc*$tRNA^{His}$ transcript with Thr, but deleting G_{-1} stimulated threonylation of $tRNA^{His}$ by *Sc*MST1 (Figure 4B). Steady-state kinetic data showed that ΔG_{-1} $tRNA^{His}$ gained 4% threonylation activity of the WT $tRNA^{Thr}_{1}$ (Table 1). Compared with $tRNA^{Thr}_{1}$, ΔG_{-1} $tRNA^{His}$ displayed 10-fold lower k_{cat} and 3-fold higher K_m values for threonylation by *Sc*MST1. In addition to G_{-1}, other major differences between $tRNA^{His}$ and $tRNA^{Thr}_{1}$ include an A insertion in $tRNA^{Thr}_{1}$ at position 35, and the discriminator base at position 73 (Figure 5). Changing C73 to A did not improve the threonylation efficiency of ΔG_{-1} $tRNA^{His}$,

Table 1. Threonylation by *Sc*MST1

	Anticodon loop	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (μM min^{-1})	Relative k_{cat}/K_m
$tRNA_1^{Thr}$					
WT	UUUUAGGU	2.8 ± 0.4	0.29 ± 0.09	10.3 ± 2.3	100
G_{-1}	UUUUAGGU	0.094 ± 0.032	1.5 ± 0.6	0.065 ± 0.006	0.63
$tRNA_2^{Thr}$					
WT	UUUGUAA	2.3 ± 0.1	0.44 ± 0.04	5.4 ± 0.6	52
$tRNA^{His}$					
WT	UUGUGGU	ND	ND	ND	ND
InsA35	UUGUAGGU	ND	ND	ND	ND
InsA35/C73A	UUGUAGGU	ND	ND	ND	ND
ΔG_{-1}	UUGUGGU	0.27 ± 0.10	0.74 ± 0.41	0.39 ± 0.06	3.8
ΔG_{-1}/InsA35	UUGUAGGU	0.34 ± 0.07	0.20 ± 0.02	1.7 ± 0.5	17
ΔG_{-1}/C73A	UUGUGGU	0.23 ± 0.01	0.85 ± 0.32	0.29 ± 0.09	2.8
ΔG_{-1}/C73A/InsA35	UUGUAGGU	1.2 ± 0.4	0.57 ± 0.26	2.4 ± 1.0	23

ND, not determined due to low activity.

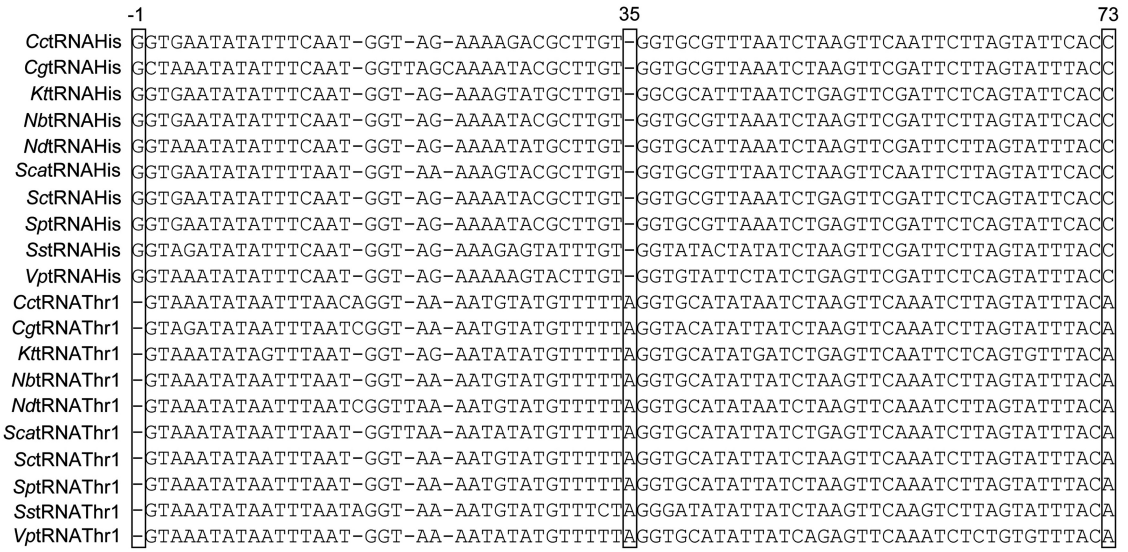

Figure 5. Sequence alignment of mitochondrial $tRNA_1^{Thr}$ and $tRNA^{His}$. Three major differences between $tRNA_1^{Thr}$ and $tRNA^{His}$ sequences are indicated by boxes. *Cc*, *Candida castellii*; *Cg*, *Candida glabrata*; *Kt*, *Kluyveromyces thermotolerans*; *Nb*, *Nakaseomyces bacillisporus*; *Nd*, *Nakaseomyces delphensis*; *Sca*, *Saccharomyces castellii*; *Sc*, *Saccharomyces cerevisiae*; *Sp*, *Saccharomyces pastorianus*; *Ss*, *Saccharomyces servazzii*; *Vp*, *Vanderwaltozyma polyspora*.

but inserting A35 in the anticodon loop of ΔG_{-1} $tRNA^{His}$ further increased its threonylation activity 5-fold (Table 1). In the presence of G_{-1}, A35 insertion did not allow threonylation of $tRNA^{His}$. These results suggest that G_{-1} is a major anti-determinant in $tRNA^{His}$ for MST1. In line with this notion, addition of G_{-1} to $tRNA_1^{Thr}$ reduced its threonylation activity 150-fold (Table 1).

G_{-1} addition allows $tRNA_1^{Thr}$ to be histidylated by *S. cerevisiae* HisRS

*Sc*MST1 efficiently aminoacylates mitochondrial $tRNA^{His}$ variants lacking G_{-1}. We next investigated the recognition of $tRNA^{His}$ and $tRNA_1^{Thr}$ variants by mitochondrial HisRS. In *S. cerevisiae*, a single nuclear gene *HTS1* containing two open reading frames encodes both the cytoplasmic and mitochondrial forms of HisRS (34). The mitochondrial HisRS (mtHisRS) harbors an extra mitochondria-targeting sequence, yet the predicted mature form of the mitochondrial enzyme is identical to the cytoplasmic HisRS. Previous studies revealed that *Sc*HisRS recognizes both the G_{-1} and the anticodon of the cytoplasmic $tRNA^{His}$ (35,36). We overexpressed mature *Sc*mtHisRS in *E. coli* and purified the enzyme to homogeneity. *In vitro* aminoacylation reactions showed that *Sc*mtHisRS efficiently charged His to the WT mitochondrial $tRNA^{His}$ (Figure 4C, Table 2), with a K_m value comparable to that of the cytoplasmic $tRNA^{His}$ (35). Surprisingly, neither deleting G_{-1} nor inserting A35 in the anticodon loop of mitochondrial $tRNA^{His}$ significantly affected the aminoacylation activity. However, when both changes were introduced into mitochondrial $tRNA^{His}$, the histidylation activity decreased by 80-fold. The discriminator base also appeared to be important for recognition by *Sc*mtHisRS. These results indicate that in the context of *S. cerevisiae* mitochondrial $tRNA^{His}$, G_{-1} is dispensable for HisRS recognition and the anticodon can

alternatively serve as the major identity element. *Sc*mtHisRS was unable to aminoacylate WT $tRNA_1^{Thr}$ (Figure 4C). Interestingly, addition of G_{-1} to $tRNA_1^{Thr}$ restored 7% histidylation activity of the WT $tRNA^{His}$ (Figure 4C, Table 2), further strengthening our argument that the mitochondrial $tRNA^{His}$ and $tRNA_1^{Thr}$ in yeast are closely related during evolution.

MST1 has coevolved with $tRNA_1^{Thr}$ to establish CUN codon reassignment in yeast mitochondria

The biochemical and phylogenetic evidence above suggests that $tRNA_1^{Thr}$ has evolved from mitochondrial $tRNA^{His}$, at a time point close to the divergence of *Kluyveromyces* species. To understand whether $tRNA_1^{Thr}$ could be recognized by MST1 enzymes from other fungal species, we overexpressed and purified MST1s from *C. albicans* and *S. pombe* [a non-hemiascomycete 'fission yeast' belonging to *Taphrinomycotina* (37)], and tested them for threonylation of $tRNA_1^{Thr}$. *Ca*MST1 and *Sp*MST1 share 49 and 43% sequence identity with *Sc*MST1, respectively, and both enzymes were able to aminoacylate *S. cerevisiae* mitochondrial $tRNA_2^{Thr}$ (Figure 6A). However, neither *Ca*MST1 nor *Sp*MST1 recognized $tRNA_1^{Thr}$ or the $tRNA^{His}$ variants tested above (Figure 6). These results strongly suggest that MST1 specifically evolved to recognize $tRNA_1^{Thr}$ in a group of yeasts comprising *S. cerevisiae*. Therefore, CUN codon reassignment was completed following the coevolution of MST1 and $tRNA_1^{Thr}$, which established specific protein–tRNA interactions.

DISCUSSION

Reassignment of CUN codons from Leu to Thr in yeast mitochondria

CUN codons in the mitochondria of *Saccharomyces*, *Nakaseomyces* and *Vanderwaltozyma* have been previously assigned to Thr (5,11,12). This recoding event is accompanied with the loss of $tRNA_{UAG}^{Leu}$ and the appearance of $tRNA_1^{Thr}$ with an unmodified UAG anticodon (17). Analysis of a mass spectrometry database (PeptideAtlas) confirms that CUU and CUA codons indeed encode Thr in *S. cerevisiae* mitochondria. CUG and CUC codons are rarely used in yeast mitochondria, and the nature of the amino acid translated by such codons has not been verified experimentally. Given that the unmodified U at the first anticodon position is able to recognize all 4 nt (6,38), and no tRNA bearing GAG or CAG anticodons have been shown to be encoded by or imported into yeast mitochondria (39), it is plausible that CUG and CUC are also decoded by $tRNA_1^{Thr}$ as Thr. $tRNA_1^{Thr}$ possesses an enlarged anticodon loop with 8 nt (UUUUAGGU), whereas a canonical tRNA anticodon loop consists

Table 2. Histidylation by *Sc*mtHisRS

	Anticodon loop	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (μM min^{-1})	Relative k_{cat}/K_m
$tRNA_1^{Thr}$					
WT	UUUUAGGU	ND	ND	ND	ND
G_{-1}	UUUUAGGU	1.8 ± 0.6	0.43 ± 0.20	4.3 ± 0.6	7.2
$tRNA^{His}$					
WT	UUGUGGU	25 ± 7	0.43 ± 0.18	60 ± 10	100
InsA35	UUGUAGGU	27 ± 8	0.60 ± 0.13	45 ± 4	74
InsA35/C73A	UUGUAGGU	38 ± 3	0.28 ± 0.01	130 ± 16	220
ΔG_{-1}	UUGUGGU	14 ± 3	0.40 ± 0.22	45 ± 25	75
ΔG_{-1}/InsA35	UUGUAGGU	1.8 ± 0.1	2.4 ± 0.8	0.78 ± 0.22	1.3
ΔG_{-1}/C73A	UUGUGGU	1.2 ± 0.2	0.54 ± 0.28	2.6 ± 1.0	4.3
ΔG_{-1}/C73A/InsA35	UUGUAGGU	0.75 ± 0.07	2.2 ± 0.5	0.35 ± 0.06	0.58

ND, not determined due to low activity.

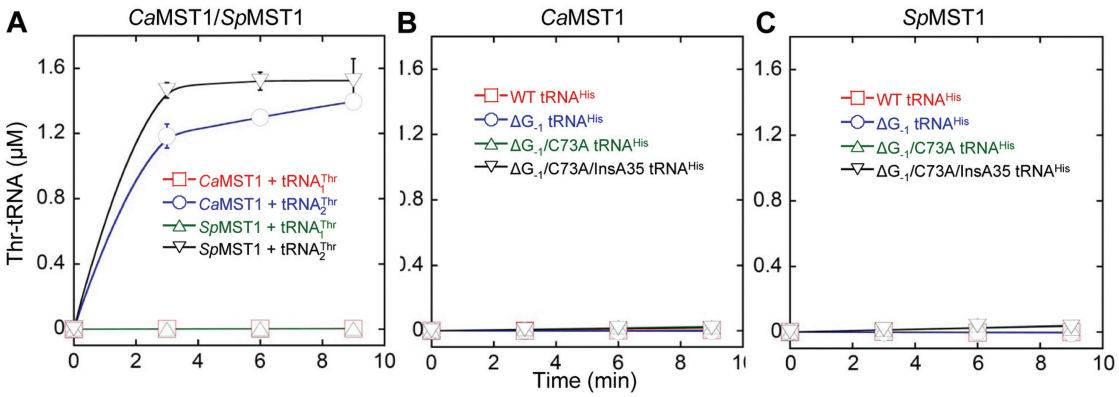

Figure 6. Threonylation of $tRNA_1^{Thr}$ and $tRNA^{His}$ variants (3 μM) by *C. albicans* and *S. pombe* MST1 (0.3 μM). (**A**) *Ca*MST1 and *Sp*MST1 threonylate $tRNA_2^{Thr}$ but not $tRNA_1^{Thr}$. (**B** and **C**) *Ca*MST1 and *Sp*MST1 are unable to threonylate $tRNA^{His}$ variants.

of 7 nt with the triplet anticodon centered at positions 34–36. In addition to $tRNA_1^{Thr}$, several natural tRNAs have been found to contain eight bases in the anticodon loop, yet such tRNAs decode quadruplet codons instead of triplet codons (40). A question that arises is whether $tRNA_1^{Thr}$ can also decode quadruplet codons. In yeast mitochondria, several protein-encoding genes, such as *cox1*, harbor in-frame CUAA sequences. Decoding of CUAA by a quadruplet anticodon could cause detrimental frameshift translation of these critical mitochondrial proteins. It is thus reasonable to think that $tRNA_1^{Thr}$ decodes all four CUN triplet codons but not quadruplet codons.

Evolutionary origin of $tRNA_1^{Thr}$

Our analyses reveal that $tRNA_1^{Thr}$ was recruited from mitochondrial $tRNA^{His}$. It has been long considered that tRNAs accepting the same amino acid (isoacceptors) evolved by gene duplication from the same common ancestor. However, studies in bacteria demonstrate that several tRNA genes may derive from different amino acid accepting groups (alloacceptors) (41). Naturally-occurring recruitment of alloacceptor tRNA genes was later reported in sponge mitochondria based on phylogenetic studies (28). Our work combines both biochemical and phylogenetic approaches to provide the first clear evidence that alloacceptor tRNA gene recruitment is directly responsible for a recent recoding event, suggesting that such a mechanism might have played an important role in the establishment of the genetic code during evolution.

Phylogenetic analysis suggests that $tRNA_1^{Thr}$ likely occurred after the split between *Candida* and *Saccharomyces*, subsequent to the divergence of *P. canadensis*. Interestingly, the *C. albicans* and *C. parapsilosis* mitochondrial genomes each contains two copies of the $tRNA^{His}$ gene but no $tRNA_1^{Thr}$, and the CUN codons in these mitochondria remain assigned to Leu. In *Kluyveromyces lactis*, the mitochondrial genome lacks both CUN codons and any tRNA reading such codons (7). Previous studies also suggest that current CUN codons decoded as Thr did not originate from ancestral Leu codons, but instead are derived from codons for other amino acids (18). Although we do not exclude the possibility that the original CUN codons could be ambiguously assigned to both Leu and Thr in certain intermediate yeast species, we favor that the emergence of $tRNA_1^{Thr}$ is a late event following the loss of original CUN codons, and such codons have reemerged in present-day yeast mitochondria. We propose that CUN codon reassignment initiated with duplication of the $tRNA^{His}$ gene, followed by the loss of CUN codons and $tRNA_{UAG}^{Leu}$ (Figure 7). Next, one copy of the $tRNA^{His}$ gene evolved to the present-day $tRNA_1^{Thr}$, and MST1 coevolved with $tRNA_1^{Thr}$ to form a cognate aaRS/tRNA pair. Finally, the CUN codons reappeared from various other codons and were reassigned to Thr. Our biochemical studies suggest that HisRS could still recognize the intermediate tRNA during the evolution from $tRNA^{His}$ to $tRNA_1^{Thr}$ (Table 2). However, the mitochondrial proteome remained largely unaffected as the CUN codons were not present in the mitochondrial genome during this period.

Several mechanisms have been proposed to explain the codon reassignment processes, including: (i) codon capture mechanism (42), hypothesizing that a specific tRNA and corresponding codons completely disappear from a genome before a novel tRNA evolves to read such codons; and (ii) ambiguous intermediate mechanism (43), postulating that a tRNA mutant is recognized by more than one aminoacyl-tRNA synthetases, and a codon may be assigned to multiple amino acids in an intermediate state. The latter mechanism is supported by the discovery that in several extant *Candida* species, the CUG codon is ambiguously decoded by both Leu and Ser due to the presence of a $tRNA_{CAG}$ charged by both leucyl- and seryl-tRNA synthetases (12,44). Our work suggests that CUN codons and $tRNA_{UAG}^{Leu}$ are lost in yeast mitochondria prior to the emergence of $tRNA_1^{Thr}$, which provides a paradigm for the codon capture mechanism and lends support to the evolving genetic code theory.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

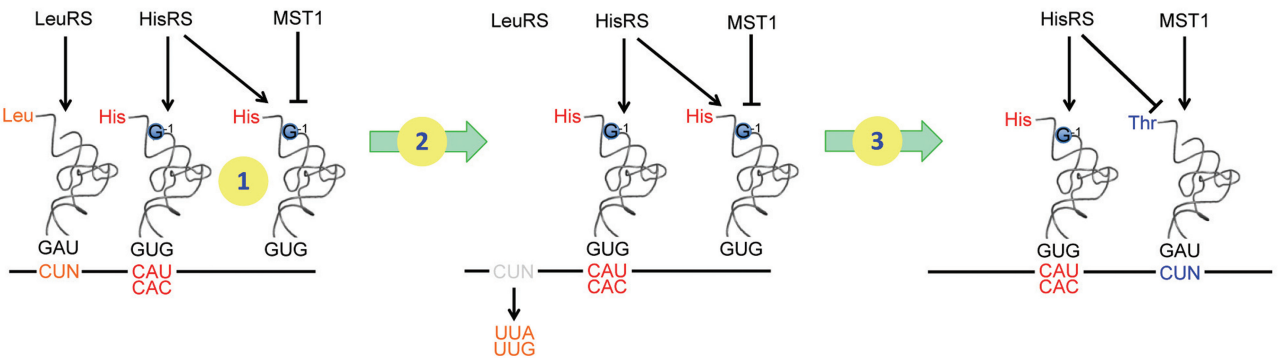

Figure 7. Proposed model for CUN codon reassignment in yeast mitochondria. (1) $tRNA^{His}$ duplicated in an ancestral yeast species while CUN codons remain assigned to Leu. (2) CUN codons were changed to UUA or UUG decoded by $tRNA_{UAA}^{Leu}$, and $tRNA_{UAG}^{Leu}$ was lost. (3) $tRNA^{His}$ evolved to $tRNA_1^{Thr}$, and CUN codons reemerged from various codons.

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