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ORIGINAL ARTICLE

Evolutionary gain of highly divergent tRNA specificities by two isoforms of human histidyl-tRNA synthetase

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Abstract The discriminator base N73 is a key identity element of $tRNA^{His}$. In eukaryotes, N73 is an "A" in cytoplasmic $tRNA^{His}$ and a "C" in mitochondrial $tRNA^{His}$. We present evidence herein that yeast histidyl-tRNA synthetase (HisRS) recognizes both A73 and C73, but somewhat prefers A73 even within the context of mitochondrial $tRNA^{His}$. In contrast, humans possess two distinct yet closely related HisRS homologues, with one encoding the cytoplasmic form (with an extra N-terminal WHEP domain) and the other encoding its mitochondrial counterpart (with an extra N-terminal mitochondrial targeting signal). Despite these two isoforms sharing high sequence similarities (81% identity), they strongly preferred different discriminator bases (A73 or C73). Moreover, only the mitochondrial form recognized the anticodon as a strong identity element. Most intriguingly, swapping the discriminator base between the cytoplasmic and mitochondrial $tRNA^{His}$ isoacceptors conveniently switched their enzyme preferences. Similarly, swapping seven residues in the active site between the two isoforms readily switched their N73 preferences. This study suggests that the human HisRS genes, while descending from a common ancestor with dual function for both types of $tRNA^{His}$, have acquired highly specialized tRNA recognition properties through evolution.

Keywords Aminoacyl-tRNA synthetase · Evolution · Phylogenetic analysis · Protein synthesis · Subfunctionalization · tRNA

Abbreviations

AaRS	Aminoacyl-tRNA synthetase
cDNA	Complementary DNA
DAPI	4′,6-Diamidino-2-phenylindole
5-FOA	5-Fluoroorotic acid
GFP	Green fluorescence protein
HisRS	Histidyl-tRNA synthetase
MTS	Mitochondrial targeting signal
ORF	Open-reading frame
PCR	Polymerase chain reaction
PGK	Phosphoglycerate kinase
Thg1	$tRNA^{His}$ guanylyltransferase
WT	Wild-type
YPG	Yeast extract peptone glycerol.

Introduction

Aminoacyl-tRNA synthetases (aaRSs) are a group of essential enzymes that carry out the first step of protein synthesis—attaching amino acids to cognate tRNAs. The resultant aminoacyl-tRNAs are then delivered to ribosomes to decipher mRNA codons through base pairing with the anticodon of aminoacyl-tRNA [1]. As eukaryotic protein synthesis takes place in both the cytoplasm and organelles (such as mitochondria and chloroplasts), at least two sets of aaRSs are required for proper growth—one functioning in the cytoplasm and the other in mitochondria [2, 3]. The cytoplasmic and mitochondrial forms of a given aaRS are normally specified by two distinct nuclear genes. However, in some cases, both forms of an aaRS are specified by the

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same gene through alternative use of two initiator codons, examples of which include genes encoding yeast alanyl-, glycyl-, histidyl-, and valyl-tRNA synthetases [4–7]. As a result, the cytoplasmic and mitochondrial forms of an aaRS, for example yeast histidyl-tRNA synthetase (HisRS), possess essentially the same polypeptide sequence, except for a cleavable N-terminal mitochondrial targeting signal (MTS) [8].

Nearly all $tRNA^{His}$ species are distinguished by an extra nucleotide, G-1, at the 5′ end of the acceptor stem, which is positioned across from the discriminator base, N73. This feature is exclusive to $tRNA^{His}$, since position -1 is generally empty in mature tRNAs [9]. Two distinct pathways are known to ensure that all mature $tRNA^{His}$ species bear G-1. In almost all eubacteria, eukaryotic organelles, and some archaea, G-1 is encoded by $tRNA^{His}$ genes opposite C73 and is retained during processing by ribonuclease P [10]. However, in eukaryotes and other archaea, G-1 is post-transcriptionally added by $tRNA^{His}$ guanylyltransferase (Thg1) [11]. Thg1 adds G-1 opposite A73 in eukaryotes [12] and opposite C73 in archaeal species [13]. Thus, in eubacteria, archaea, and eukaryotic organelles, G-1:C73 forms an extra base pair, but in eukaryotic cytoplasm, the extra base pair is a G-1:A73 mismatch. To date, the only known $tRNA^{His}$ species that lack G-1 are found in the mitochondria of *Caenorhabditis elegans* [14], *Acanthamoeba castellanii* [15], and some α-proteobacteria [16].

The existence of two distinct mechanisms for preserving G-1 in $tRNA^{His}$ suggests that G-1 plays essential roles in the cell. One such role is in aminoacylation of $tRNA^{His}$. G-1, or its 5′-monophosphate, is a key identity element in histidylation of $tRNAs^{His}$ in vitro [17]. Other recognition elements include the discriminator base (C73 or A73), the second and third base pairs of the acceptor stem, and the anticodon. G-1 serves to present the 5′-monophosphate in the active site of HisRS [18]. Thus, deletion of G-1 from $tRNA^{His}$ leads to a defective tRNA unsuitable for histidylation. Similarly, deletion of the *THG1* gene from yeast results in the simultaneous loss of G-1 from $tRNA^{His}$ and the accretion of nonacylated $tRNA^{His}$ in cells [19]. Thg1 also possesses distinct 3′–5′ polymerase activity in vitro, which may play a role in the editing or repair of tRNA [20].

Saccharomyces cerevisiae HTS1 (*ScHTS1*) is a dual-functional gene that functions in both the cytoplasm and mitochondria. Yeast HisRS can efficiently charge both nucleus- and mitochondrion-encoded $tRNA^{His}$ isoacceptors ($SctRNA_n^{His}$ and $SctRNA_m^{His}$) [21]. In contrast, humans possess two distinct yet closely related HisRS genes, the expressions of which are driven by a bidirectional promoter [22]. The question arose as to whether these two human HisRS isoforms possess divergent tRNA specificities. Our study showed that despite these two isoforms sharing up to 81% identity, they have evolved highly divergent tRNA specificities. Moreover, the tRNA specificities of these two forms can be conveniently switched by exchanging a small peptide between their active sites. This study unveils an interesting scenario for the functional specialization of human HisRS genes.

Results

Distinguishing features of HisRS and $tRNA^{His}$

The 5′ extra nucleotide G-1 and discriminator base C73 of the acceptor stem are the primary identity elements of *Escherichia coli* $tRNA^{His}$ [17]. Figure 1A highlights the strong conservation of G-1:C73 in $tRNAs^{His}$ of bacteria and eukaryotic organelles and G-1:A73 in $tRNAs^{His}$ of eukaryotic cytoplasm. However, exceptions to this identity rule exist. The mitochondrial $tRNA^{His}$ isoacceptor of *C. elegans* lacks G-1, and contains U73 instead of C73 [14].

Previous studies suggested that the motif 2 loop of *E. coli* HisRS is part of a tRNA-binding pocket and is responsible for recognizing the G-1:C73 base pair [23]. Residues Q118 and R123 were, respectively, shown to interact with C73 and G-1. R123 was strictly conserved in all HisRSs analyzed, irrespective of whether they were bacterial or eukaryotic proteins (Fig. 1b). In contrast, Q118 was conserved only in bacterial HisRSs. Eukaryotic HisRSs possessed divergent residues at this position. For example, ScHisRS and *Drosophila melanogaster* HisRS (DmHisRS), both of which are dual functional, possessed "AMT" at this position; human cytoplasmic and mitochondrial HisRSs ($HsHisRS_c$ and $HsHisRS_m$), respectively, possessed "AMT" and "TIV" at this position; and *Arabidopsis thaliana* cytoplasmic and mitochondrial HisRSs ($AtHisRS_c$ and $AtHisRS_m$), respectively, possessed "S" and "MT" at this position. A schematic representation of $HsHisRS_c$ and $HsHisRS_m$ is shown in Fig. 1c.

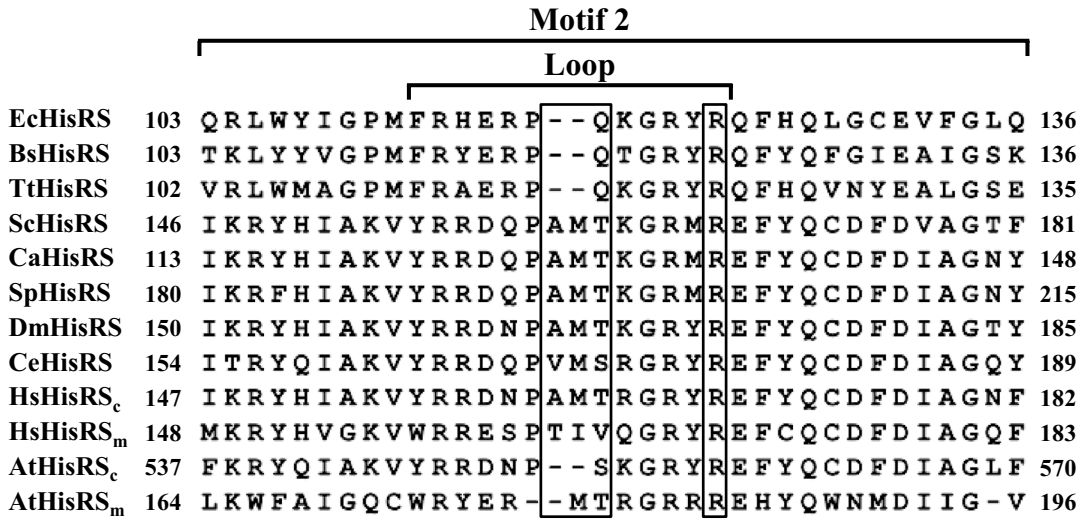
Yeast HisRS recognizes both A73 and C73 with a slight preference for A73

As yeast HisRS can efficiently charge both $tRNA_n^{His}$ and $tRNA_m^{His}$ [8], it is likely that the motif 2 loop of this enzyme is not as significant in N73 discrimination as the corresponding sequence of EcHisRS. To provide experimental evidence of this, "AMT" in the motif 2 loop of ScHisRS was mutated to "Q" (as in EcHisRS), and the resultant mutant's rescue activities were examined. To test the cytoplasmic rescue activity of the mutant, the test plasmid was transformed into a *HTS1* knockout strain that harbored a maintenance plasmid (with a *URA3* marker and the WT *HTS1* gene). The transformants evicted the maintenance plasmid in the presence of 5-FOA and thus could

A

Origin of $tRNA^{His}$	Nucleotides at -1:73 of $tRNA^{His}$		
Bacteria			
Escherichia coli		**G-1:C73**	
Bacillus subtilis		**G-1:C73**	
Thermus thermophilus		**G-1:C73**	
Eukaryote	**Cytoplasm**	**Mitochondrion**	**chloroplast**
Saccharomyces cerevisiae	**G-1:A73**	**G-1:C73**	
Drosophila melanogaster	**G-1:A73**	**G-1:C73**	
Caenorhabditis elegans	**G-1:A73**	**U73**	
Homo sapiens	**G-1:A73**	**G-1:C73**	
Arabidopsis thaliana	**G-1:A73**	**G-1:C73**	**G-1:C73**

B



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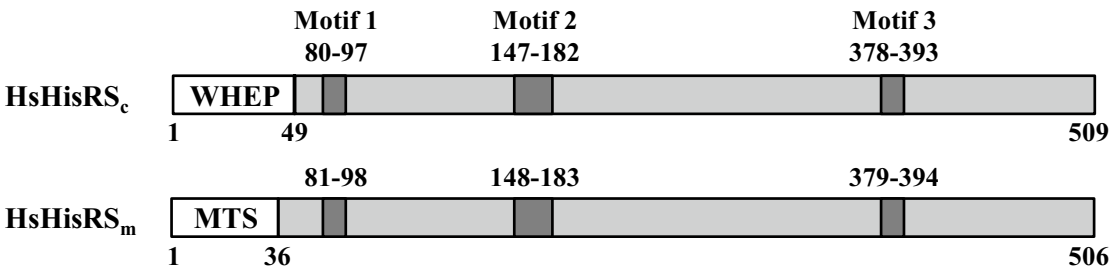


Fig. 1 Distinguishing features of histidyl-tRNA synthetase (HisRS) and tRNAHis. **a** Primary identity elements of $tRNA^{His}$—G-1:N73. **b** Alignment of the motif 2 sequences of HisRSs. Amino acid residues that are presumably important for recognition of G-1:N73 are boxed. Ec, *Escherichia coli*; Bs, *Bacillus subtilis*; Tt, *Thermus thermophilus*; Sc, *Saccharomyces cerevisiae*; Ca, *Candida albicans*; Sp, *Schizosaccharomyces pombe*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; Hs, *Homo sapiens*; At, *Arabidopsis thaliana*. **c** Schematic representation of the human cytoplasmic and mitochondrial HisRS isoforms

not grow on 5-FOA plates unless the test plasmid encoded a functional cytoplasmic HisRS. To test the mitochondrial rescue activity of the mutant, the test plasmid was co-transformed with a second maintenance plasmid (with a *HIS3* marker and an initiator mutant of *HTS1* that expressed only $ScHisRS_c$) into the yeast knockout strain. The co-transformants evicted the first maintenance plasmid with a *URA3* marker upon 5-FOA selection and thus could not further

grow on YPG plates (which contained glycerol as the sole carbon source) unless the test plasmid encoded a functional mitochondrial HisRS. As shown in Fig. 2a, this mutant efficiently supported the growth of the knockout strain on both 5-FOA and YPG (*number 2*), suggesting that this mutation does not severely impair its ability to charge $tRNA_n^{His}$ or $tRNA_m^{His}$.

To determine the aminoacylation activities of the WT and mutant ScHisRSs, ScHisRS proteins were purified to homogeneity through Ni–NTA affinity chromatography. Aminoacylation reactions were carried out at ambient temperature using 5 nM of the enzyme and 5 μM of in vitro-transcribed yeast $tRNA^{His}$ to measure the reaction rates. As shown in Fig. 2b, ScHisRS efficiently charged the WT $tRNA_n^{His}$ (with A73) (with a reaction rate of ~2.7 pmol of His incorporation per min) and slightly preferred A73 over C73 (~2-fold) within the context of this tRNA, a scenario consistent with previous observations [21]. Despite the fact that the WT $tRNA_m^{His}$ itself contains C73, ScHisRS preferred A73 over C73 (~3-fold) within the context of this tRNA (Fig. 2d). Mutation of AMT to Q had little effect on the enzyme's activity or preference for N73 (Fig. 2c, e). However, it was interesting to note that ScHisRS poorly charged $tRNA_m^{His}$ with U73 or G73 (Fig. 2d). Thus, ScHisRS slightly preferred A73 over C73 (two- to three-fold), irrespective of whether it was within the context of

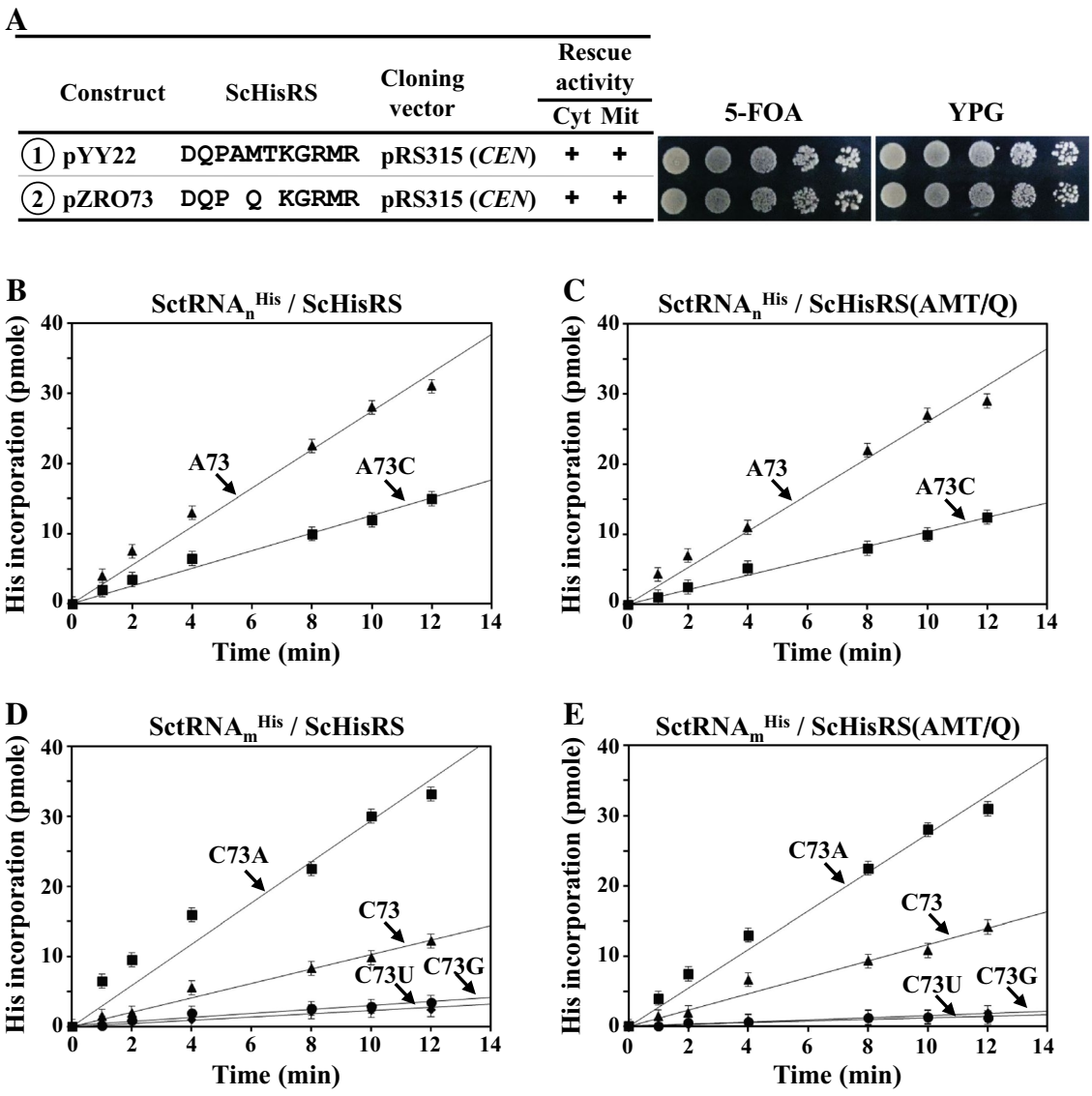


Fig. 2 tRNA preferences of yeast histidyl-tRNA synthetase (HisRS). **a** Summary of the constructs and their rescue activities. **b** Aminoacylation of $SctRNA_n^{His}$ (5 μM) by ScHisRS (5 nM). **c** Aminoacylation of $SctRNA_n^{His}$ (5 μM) by ScHisRS(AMT/Q) (5 nM). **d** Aminoacylation of $SctRNA_m^{His}$ (5 μM) by ScHisRS (5 nM). **e** Aminoacylation of $SctRNA_m^{His}$ (5 μM) by ScHisRS(AMT/Q) (5 nM)

$tRNA_n^{His}$ or $tRNA_m^{His}$. These results also confirmed our hypothesis that the motif 2 loop of ScHisRS is less significant in N73 recognition than the corresponding structure in bacterial enzymes.

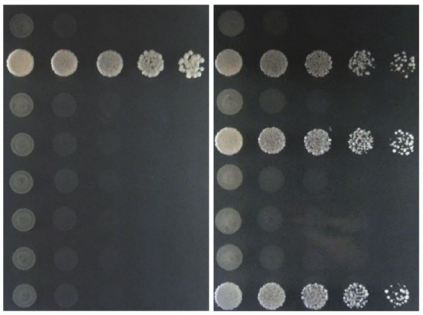
Bacterial HisRS recognizes only C73

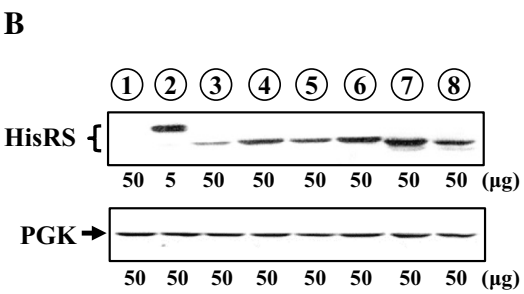
To explore whether a bacterial HisRS can functionally substitute for yeast HisRS in vivo, genes encoding *E. coli* HisRS (EcHisRS), *Bacillus subtilis* HisRS (BsHisRS), and their derivatives were cloned, and their rescue activities were tested. As shown in Fig. 3a, EcHisRS failed to rescue the growth defects of the yeast knockout strain on 5-FOA and YPG (*number 3*), but fusion of a heterologous MTS to the bacterial enzyme enabled it to rescue the mitochondrial defect of the null allele (*number 4*). Similarly, BsHisRS could be converted to a functional yeast mitochondrial enzyme by fusion of a heterologous MTS (*number 8*). The ability of the MTS used to import a fusion protein into mitochondria is well documented [24]. Thus, bacterial HisRSs can charge $SctRNA_m^{His}$ (with C73), but not $SctRNA_n^{His}$ (with A73). Mutating Q118 of EcHisRS to AMT (as in ScHisRS) yielded a defective enzyme that failed to rescue either defect of the yeast knockout strain (*numbers 5* and *6*), suggesting that Q118 is critical for recognition of C73-containing $tRNA^{His}$ by this enzyme.

A Western blot analysis showed that all constructs used were appropriately expressed in yeast, albeit at different levels (Fig. 3b). While BsHisRS had an expression level higher than that of EcHisRS, neither could rescue the yeast knockout strain on 5-FOA (*numbers 3* and *7*). Time course aminoacylation assays showed that EcHisRS efficiently charged $SctRNA_m^{His}$ (with a reaction rate of ~0.6 pmol of His incorporation per min), but not $SctRNA_n^{His}$ (Fig. 3c), which might account for the positive rescue activities of MTS-EcHisRS and MTS-BsHisRS on YPG (*numbers 4* and *8*).

A

	Construct	HisRS	Cloning vector	Rescue activity	
				Cyt	Mit
①	pADH	None	pADH (2μ)	-	-
②	pYRU47	ScHisRS	pADH (2μ)	+	+
③	pYRU29	EcHisRS	pADH (2μ)	-	-
④	pYRU30	MTS-EcHisRS	pADH (2μ)	-	+
⑤	pZRO64	EcHisRS(Q118/AMT)	pADH (2μ)	-	-
⑥	pZRO55	MTS-EcHisRS(Q118/AMT)	pADH (2μ)	-	-
⑦	pYRU27	BsHisRS	pADH (2μ)	-	-
⑧	pYRU28	MTS-BsHisRS	pADH (2μ)	-	+





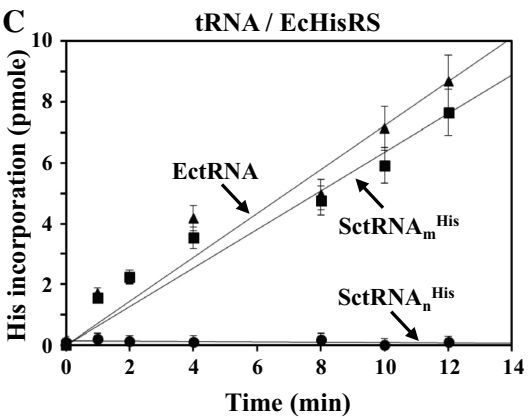


Fig. 3 tRNA preferences of various bacterial histidyl-tRNA synthetases (HisRSs). **a** Summary of the constructs and their cross-species rescue activities. **b** Western blotting. *Upper panel* HisRS, *lower panel* phosphoglycerate kinase (PGK). Amounts of cellular protein extracts loaded into each well are indicated at the *bottom* of the blots. *Numbers 1–8* (*circled*) represent constructs shown in (**a**). **c** Aminoacylation of $SctRNA_n^{His}$ (5 μM), $SctRNA_m^{His}$ (5 μM), and unfractionated EctRNA (100 μM) by EcHisRS (1 nM)

Human cytoplasmic and mitochondrial HisRSs recognize divergent discriminator bases (A73 or C73)

We next investigated whether HisRSs of other eukaryotes can functionally substitute for ScHisRS in vivo. As shown in Fig. 4a, both DmHisRS and CeHisRS were dual functional; each of these two genes effectively rescued the cytoplasmic and mitochondrial defects of the yeast knockout strain (*numbers 2–4*), suggesting that they can efficiently charge both A73- and C73-containing SctRNA^{His} isoacceptors. We should mention that pZRO10 possessed only the ORF of DmHisRS that specifies the cytoplasmic form (*number 2*). Its mitochondrial isoform appeared to be initiated from an upstream in-frame non-ATG codon, ATT(-27), of the gene. Hence, fusion of a heterologous MTS to the ORF of DmHisRS enabled the enzyme to rescue the mitochondrial defect (*number 3*).

Humans possess two distinct HisRS genes: *HARS* and *HARS2*. *HARS* encodes the cytoplasmic form (HsHisRS$_c$) with a characteristic N-terminal WHEP domain (N-terminal 1–49 amino acid residues), while *HARS2* encodes the mitochondrial isoform (HsHisRS$_m$) with a characteristic N-terminal MTS (N-terminal 1–36 amino acid residues) (Fig. 1c). These two forms shared up to 81% identity and respectively rescued the cytoplasmic and mitochondrial defects of the yeast knockout strain (*numbers 5* and *7*). However, targeting these two isoforms to opposite compartments by fusing a heterologous MTS to HsHisRS$_c$ or removing the endogenous MTS from HsHisRS$_m$ prevented them from functioning (*numbers 6* and *8*). Thus, HsHisRS$_c$ and HsHisRS$_m$, respectively, prefer A73- and C73-containing yeast tRNA^{His} isoacceptors.

A Western blot analysis showed that except for DmHisRS, all other constructs used were appropriately expressed in yeast. The expression of DmHisRS was almost negligible under the conditions used (Fig. 4b). It was therefore surprising to find that DmHisRS provided sufficient aminoacylation activity for the knockout strain (Fig. 4a).

To gain further insights into the tRNA specificities of human HisRS isoforms, aminoacylation assays were carried out with purified HsHisRS and in vitro-transcribed HstRNA^{His} to measure the reaction rates. As shown in Fig. 4c, HsHisRS$_c$ efficiently charged the WT HstRNA$_n^{His}$ (with A73) (with a reaction rate of ~1.2 pmol of His incorporation per min), and mutation of A73 to C in this tRNA reduced its aminoacylation rate by 3.5-fold. Conversely, HsHisRS$_c$ poorly charged the WT HstRNA$_m^{His}$ (with C73) (with a reaction rate of ~0.15 pmol of His incorporation per min), but mutation of C73 to A in this tRNA enhanced its aminoacylation rate by fivefold (Fig. 4d). Hence, HsHisRS$_c$ preferred A73 over C73, irrespective of whether it was within the context of HstRNA$_n^{His}$ or HstRNA$_m^{His}$. On the contrary, HsHisRS$_m$ efficiently charged the WT HstRNA$_m^{His}$ (with C73) (with a reaction rate of ~1.1 pmol of His incorporation per min), and mutation of C73 to A in this tRNA reduced its aminoacylation rate by tenfold (Fig. 4e). Conversely, HsHisRS$_m$ poorly charged the WT HstRNA$_n^{His}$ (with A73) (with a reaction rate of ~0.1 pmol of His incorporation per min), but mutation of A73 to C in this tRNA enhanced its aminoacylation rate by sixfold (Fig. 4f). Hence, HsHisRS$_m$ preferred C73 over A73, irrespective of whether it was within the context of HstRNA$_n^{His}$ or HstRNA$_m^{His}$.

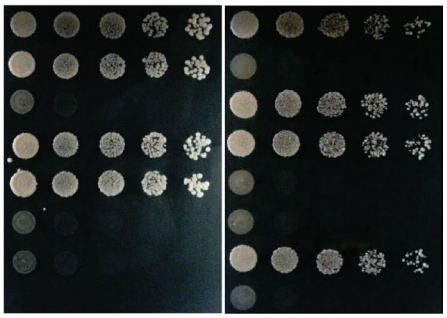
Kinetic assays provided a more-comprehensive picture of the effects of the identity elements. As shown in Table 1, mutation of A73 to C in HstRNA$_n^{His}$ reduced its aminoacylation efficiency (k_{cat}/K_M) by 13-fold, while mutation of C73 to A in HstRNA$_m^{His}$ reduced its aminoacylation efficiency (k_{cat}/K_M) by up to 54-fold. This may be why WT HstRNA$_m^{His}$ and WT HstRNA$_n^{His}$ are poor substrates for HsHisRS$_c$ and HsHisRS$_m$, respectively. In addition, deletion of G-1 from HstRNA$_n^{His}$ or HstRNA$_m^{His}$ led to a tRNA variant unsuitable for aminoacylation. As the first nucleotide of the anticodon (G34) is the most important nucleotide among the three on aminoacylation of yeast tRNA^{His} [21], we next tested its effect on aminoacylation of human tRNA^{His}. While mutation of G34 to U in HstRNA$_n^{His}$ reduced its aminoacylation efficiency (k_{cat}/K_M) by sixfold, a comparable mutation in HstRNA$_m^{His}$ led to a tRNA variant unsuitable for aminoacylation by HsHisRS$_m$. Thus, the anticodon is a much stronger identity element in HstRNA$_m^{His}$ than in HstRNA$_n^{His}$. Note that as the time course assays were done mostly at saturating histidine and tRNA concentrations (Fig. 4c–f), the fold changes observed in these assays are mainly due to differences in the k_{cat} values.

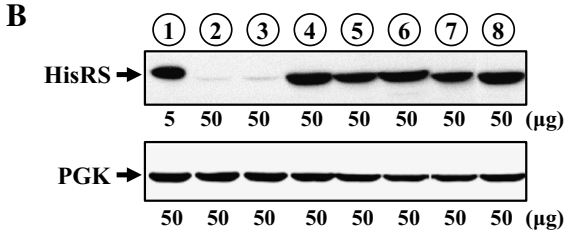
Switching tRNA preferences of human HisRS isoforms

A conspicuous difference between the motif 2 loop sequences of HsHisRS$_c$ and HsHisRS$_m$ was DNPAMTR (N-terminal residues 159–165) and ESPTIVQ (N-terminal residues 160–166) (Fig. 1b). We next swapped these seven residues between the two isoforms to test whether their tRNA specificities could be readily switched. As shown in Fig. 5a, b, replacing DNPAMTR of HsHisRS$_c$ with ESPTIVQ swiftly switched its tRNA preference. The resultant mutants rescued the mitochondrial but not the cytoplasmic defect of the yeast knockout strain (*numbers 3* and *4*). However, despite our expectations, replacing ESPTIVQ of HsHisRS$_m$ with DNPAMTR failed to yield a functional yeast cytoplasmic enzyme. The resultant mutants rescued neither defect of the knockout strain (*numbers 7* and *8*). MTS-HsHisRS$_c$ and HsHisRS$_m$ (ΔMTS), respectively denote adding a heterologous MTS to HsHisRS$_c$ and removing the endogenous MTS from HsHisRS$_m$. A

A

	Construct	HisRS	Cloning vector	Rescue activity	
				Cyt	Mit
①	pYRU47	ScHisRS	pADH (2μ)	+	+
②	pZRO10	DmHisRS	pADH (2μ)	+	-
③	pZRO11	MTS-DmHisRS	pADH (2μ)	-	+
④	pZRO39	CeHisRS	pADH (2μ)	+	+
⑤	pZRO8	$HsHisRS_c$	pADH (2μ)	+	-
⑥	pZRO9	MTS-$HsHisRS_c$	pADH (2μ)	-	-
⑦	pZRO7	$HsHisRS_m$	pADH (2μ)	-	+
⑧	pZRO74	$HsHisRS_m$(ΔMTS)	pADH (2μ)	-	-





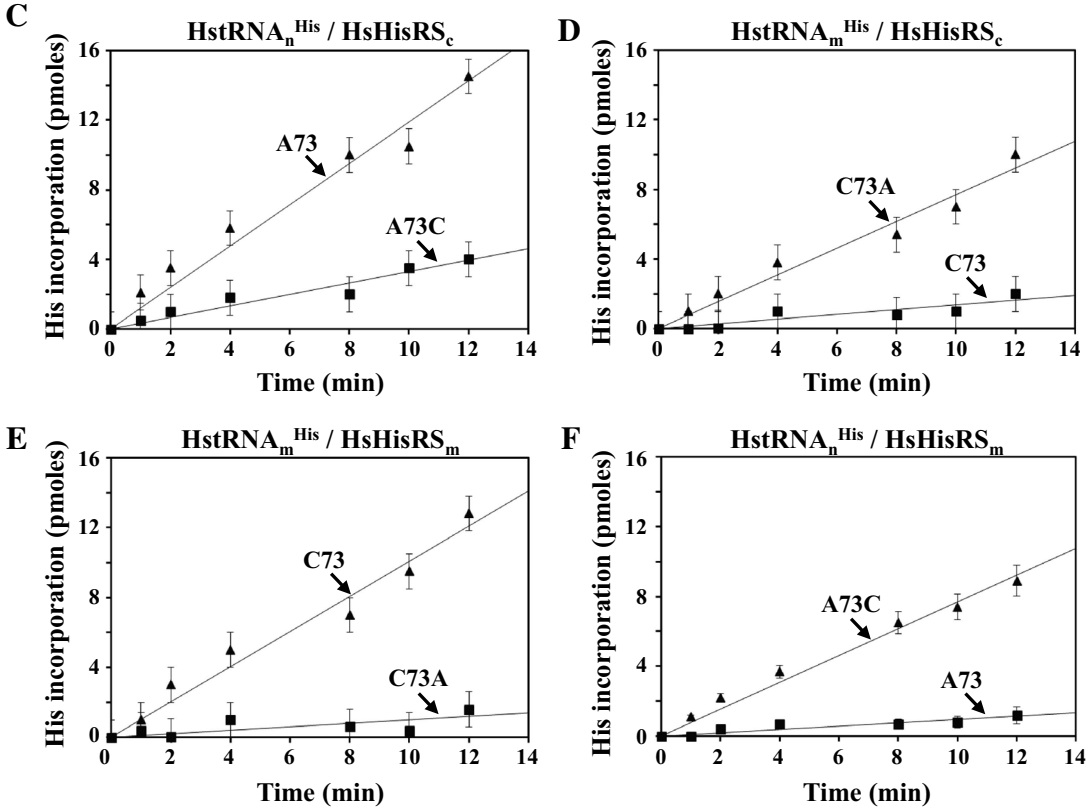


Fig. 4 tRNA preferences of various eukaryotic histidyl-tRNA synthetases (HisRSs). **a** Summary of the constructs and their cross-species rescue activities. **b** Western blotting. *Upper panel* HisRS, *lower panel* phosphoglycerate kinase (PGK). Amounts of cellular protein extracts loaded into each well are indicated at the *bottom* of the blots. *Numbers 1–8* (*circled*) represent constructs shown in (**a**). **c** Aminoacylation of $HstRNA_n^{His}$ (5 μM) by $HsHisRS_c$. **d** Aminoacylation of $HstRNA_m^{His}$ (5 μM) by $HsHisRS_c$. **e** Aminoacylation of $HstRNA_m^{His}$ (5 μM) by $HsHisRS_m$. **f** Aminoacylation of $HstRNA_n^{His}$ (5 μM) by $HsHisRS_m$. The final concentration of HsHisRS used in each reaction was 10 nM

Table 1 Aminoacylation of $tRNA^{His}$ by wild-type and mutant $HsHisRS_c$ and $HsHisRS_m$

	$HsHisRS_c$				$HsHisRS_m$		
	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (μM^{-1} min^{-1})		K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (μM^{-1} min^{-1})
$tRNA_n^{His}$				$tRNA_m^{His}$			
WT	0.5 ± 0.2	17 ± 3	34	WT	0.6 ± 0.4	16 ± 3	27
A73C	2.8 ± 0.3	7 ± 1	2.6	C73A	11 ± 1	6 ± 1	0.5
G34U	2.4 ± 0.5	13 ± 2	5.4	G34U	ND	ND	ND
ΔG-1	ND	ND	ND	ΔG-1	ND	ND	ND
$tRNA_m^{His}$				$tRNA_n^{His}$			
WT	4.8 ± 0.6	5.4 ± 0.5	1.1	WT	9 ± 2	2.3 ± 0.4	0.3
	$HsHisRS_c^{Mut}$				$HsHisRS_m^{Mut}$		
	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (μM^{-1} min^{-1})		K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (μM^{-1} min^{-1})
$tRNA_n^{His}$	2.6 ± 0.3	2.4 ± 0.3	0.9	$tRNA_n^{His}$	2.2 ± 0.7	6.9 ± 0.6	3.1
$tRNA_m^{His}$	1.6 ± 0.5	11 ± 2	6.9	$tRNA_m^{His}$	6.0 ± 0.5	3.1 ± 0.2	0.5
					WHEP-$HsHisRS_m^{Mut}$		
					K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (μM^{-1} min^{-1})
				$tRNA_n^{His}$	1.4 ± 0.4	14 ± 3	10
				$tRNA_m^{His}$	5.8 ± 0.7	2.2 ± 0.1	0.4

ND not determined due to low activity

Western blot analysis showed that except for $HsHisRS_m$ mutants (*numbers 7* and *8*), all other constructs used were expressed in yeast at a significant level (Fig. 5c). This result implies that the negative growth phenotypes of $HsHisRS_m$ mutants might be caused by poor protein expression.

To provide further insights, the mutant enzymes $HsHisRS_c^{Mut}$ and $HsHisRS_m^{Mut}$ were purified from *E. coli* transformants through Ni–NTA affinity chromatography, and aminoacylation assays were carried out using in vitro-transcribed $HstRNA_n^{His}$ and $HstRNA_m^{His}$ as substrates to measure the reaction rates. As expected, replacing DNPAMTR of $HsHisRS_c$ with ESPTIVQ reduced its aminoacylation rate by 7.5-fold toward $HstRNA_n^{His}$, while enhancing its aminoacylation rate by sixfold toward $HstRNA_m^{His}$ (compare Fig. 6a, b). Similarly, replacing ESPTIVQ of $HsHisRS_m$ with DNPAMTR reduced its aminoacylation rate by fourfold toward $HstRNA_m^{His}$, while enhancing its aminoacylation rate by fourfold toward $HstRNA_n^{His}$ (compare Fig. 6c, d). Thus, the tRNA specificities of human HisRS isoforms can be switched by swapping the seven amino acid residues of the motif 2 loop. Results obtained from kinetic assays further supported this finding. As shown in Table 1, $HsHisRS_c^{Mut}$ preferred $tRNA_m^{His}$ over $tRNA_n^{His}$ (an eightfold difference in k_{cat}/K_M). In contrast, $HsHisRS_m^{Mut}$ preferred $tRNA_n^{His}$ over $tRNA_m^{His}$ (a sixfold difference in k_{cat}/K_M).

As $HsHisRS_c$ possesses an extra N-terminal WHEP domain, we wondered whether this domain affects the aminoacylation of $HstRNA_n^{His}$ by the WT or mutant $HsHisRS_m$. Pursuant to this objective, the WHEP domain was fused to the N terminus of the WT or mutant $HsHisRS_m$, yielding WHEP-$HsHisRS_m$ and WHEP-$HsHisRS_m^{Mut}$. Paradoxically, the WHEP domain had no discernible effect on the aminoacylation activity or tRNA preference of the WT $HsHisRS_m$ (Fig. 6e), but it selectively enhanced the aminoacylation rate (by 2.5-fold) of the mutant $HsHisRS_m$ toward $HstRNA_n^{His}$ (compare Fig. 6d, f). As a result, the fusion enzyme WHEP-$HsHisRS_m^{Mut}$ had tRNA specificity nearly identical to that of the WT $HsHisRS_c$ (compare Fig. 6a, f). Kinetic assays further showed that WHEP-$HsHisRS_m^{Mut}$ strongly preferred $HstRNA_n^{His}$ over $HstRNA_m^{His}$ (a 29-fold difference in k_{cat}/K_M) (see Table 1). It should be noted that one of the tRNA substrates used was not at saturating concentration in some of the time course assays, such as $tRNA_n^{His}$ in Fig. 6c and $tRNA_m^{His}$ in Fig. 6d, f.

A proposed evolutionary history of human HisRS isoforms

To provide insight into the evolutionary history of the two human HisRS isoforms (*HARS* and *HARS2*), we arbitrarily selected several representative species (whose genomes

A

	Construct	HsHisRS variant	Motif 2 loop	Cloning vector	Rescue activity	
					Cyt	Mit
①	pZRO8	$HsHisRS_c$	DNPAMTR	pADH (*2μ*)	+	-
②	pZRO9	MTS-$HsHisRS_c$	DNPAMTR	pADH (*2μ*)	-	-
③	pZRO187	$HsHisRS_c^{Mut}$	ESPTIVQ	pADH (*2μ*)	-	-
④	pZRO188	MTS-$HsHisRS_c^{Mut}$	ESPTIVQ	pADH (*2μ*)	-	+
⑤	pZRO7	$HsHisRS_m$	ESPTIVQ	pADH (*2μ*)	-	+
⑥	pZRO74	$HsHisRS_m$(ΔMTS)	ESPTIVQ	pADH (*2μ*)	-	-
⑦	pZRO126	$HsHisRS_m^{Mut}$	DNPAMTR	pADH (*2μ*)	-	-
⑧	pZRO127	$HsHisRS_m$(ΔMTS) ^{Mut}	DNPAMTR	pADH (*2μ*)	-	-

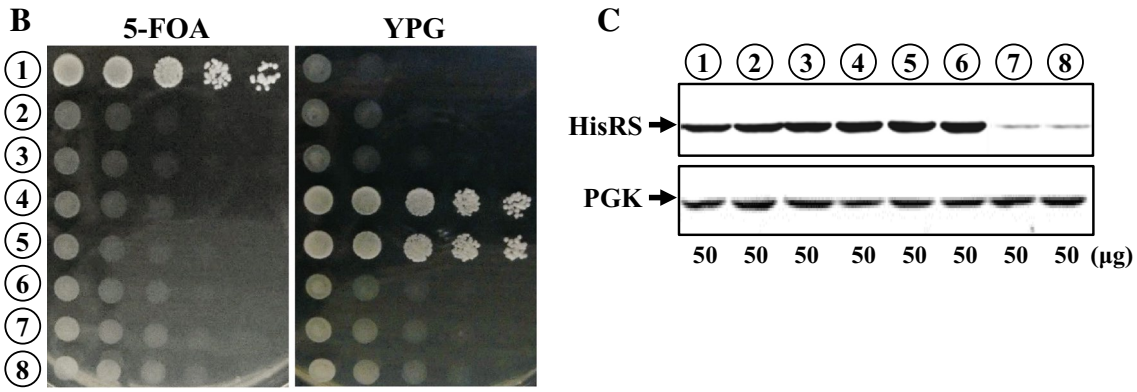


Fig. 5 tRNA preferences of human histidyl-tRNA synthetases (HisRSs). **a** Summary of the constructs and their cross-species rescue activities. **b** Complementation assays on 5-FOA and YPG. **c** Western blotting. *Upper panel* HisRS, *lower panel* phosphoglycerate kinase (PGK). Amounts of cellular protein extracts loaded into each well are indicated at the *bottom* of the blots. *Numbers 1–8* (*circled*) in **b, c** represent constructs shown in (**a**)

have been completely sequenced) among the three domains and retrieved their HisRS sequences following a protocol described earlier [25]. The sequence alignment was performed using Expresso [26], and the phylogenetic tree was built using Maximum Likelihood with 1000 bootstrap replicates implanted in MEGA6 [27]. As shown in Fig. 6 in Appendix, all eukaryotic HisRSs examined were clustered within a monophyletic group. It is therefore likely that one of the two endosymbiotic HisRS genes was lost early in eukaryotic evolution, leaving a dual-functional homologue in the primordial eukaryotes. Interestingly, some eukaryotes (such as *S. cerevisiae, D. melanogaster, C. elegans*, and *Bombyx mori*) still possess a single dual-functional HisRS gene, while others (such as *H. sapiens, Xenopus laevis, Gallus gallus, A. thaliana*, and *Zea mays*) possess two or even three HisRS genes. The three analyzed mammals (human, chimp, and mouse) all possess two HisRS genes. The three mammalian cytoplasmic isoforms were clustered with bootstrap supporting value 99, while the three mammalian mitochondrial isoforms were clustered with supporting value 100. This result implies that the gene duplication event followed by subfunctionalization may correspond to the origin of mammals. A proposed evolutionary history of human HisRS isoforms is illustrated in Fig. 8 in Appendix. Such duplication and subfunctionalization events may have occurred multiple times independently among different eukaryotic lineages (e.g. the event in plants should be ancient and in *Gallus gallus* should be quite recent). A comprehensive analysis may shed light on their evolutionary history in the future.

Discussion

Human HisRS has attracted copious attention partly due to its close association with certain diseases. For example, mutations of $HsHisRS_c$ were linked to Usher syndrome type III [28], and mutations in $HsHisRS_m$ were shown to

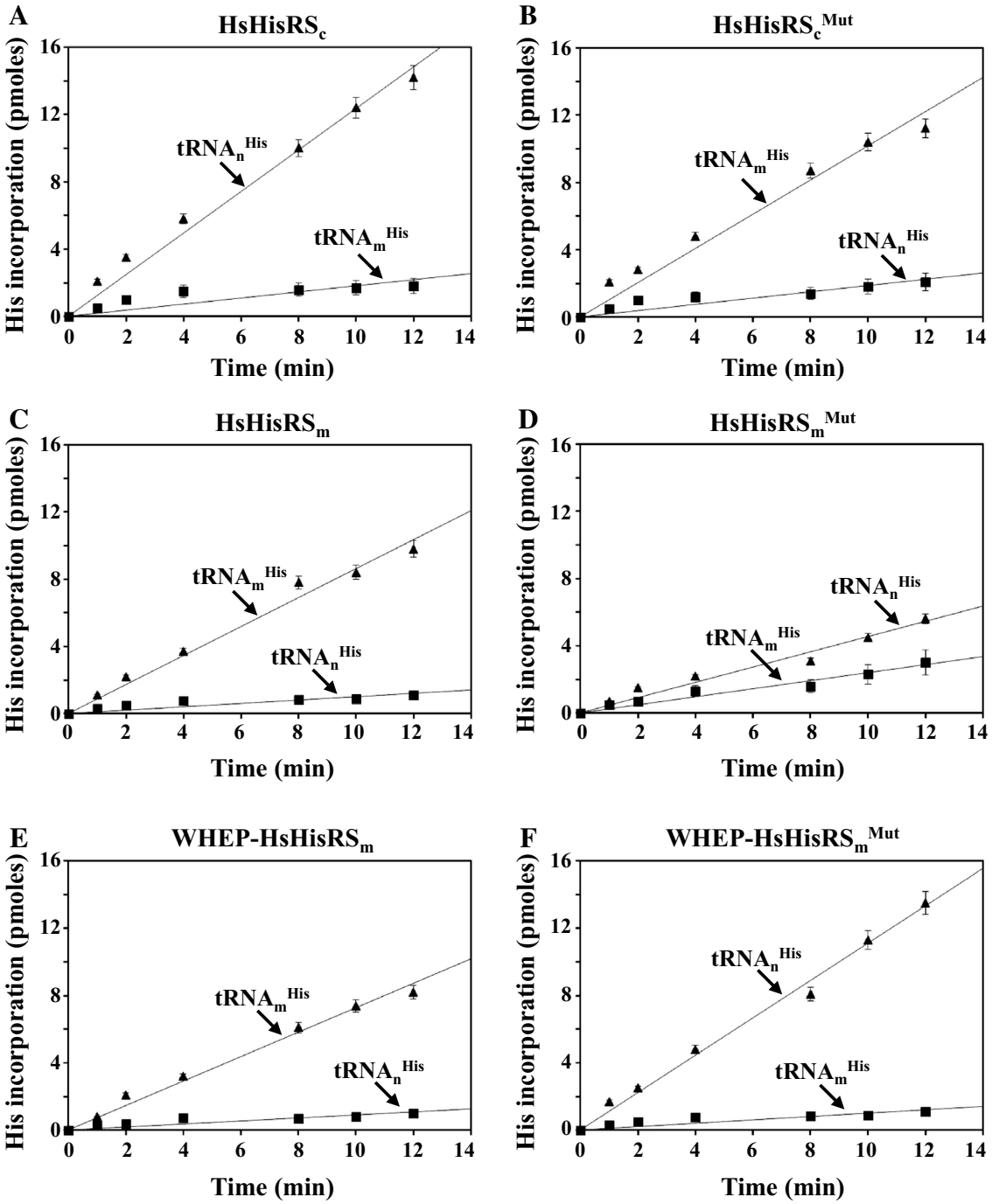


Fig. 6 tRNA preferences of the wild-type (WT) and mutant human histidyl-tRNA synthetases (HisRSs). Aminoacylation of HstRNA$_n^{His}$ (5 μM) and HstRNA$_m^{His}$ (5 μM) by **a** HsHisRS$_c$, **b** HsHisRS$_c^{Mut}$, **c** HsHisRS$_m$, **d** HsHisRS$_m^{Mut}$, **e** WHEP-HsHisRS$_m$, and **f** WHEP-HsHisRS$_m^{Mut}$. The final concentration of HsHisRS used in each reaction was 10 nM

cause ovarian dysgenesis and sensorineural hearing loss of Perrault syndrome [29]. Moreover, patients suffering from idiopathic inflammatory myositis often carry autoantibodies in their sera [30]. One of the most common autoantibodies is the anti-JO-1 antibody, which is directed against HisRS [31]. This antibody inhibits HisRS-catalyzed aminoacylation reactions and pulls down HisRS along with its cognate tRNA [32]. As it turns out, epitopes recognized by the autoantibody reside in the WHEP domain of human HisRS$_c$. Later on, the WHEP domain was shown to play as a secretion signal, directing the secretion of human HisRS$_c$ and two of its splice variants [30]. It is worth mentioning

that a point mutation (A5E) in the WHEP domain was identified from screening of the $HisRS_c$ gene in a large cohort of patients with peripheral neuropathies [28]. It remains to be determined whether this mutation alters the secretion, structure, or function of human $HisRS_c$.

The WHEP domain normally exists in metazoan TrpRS, HisRS, Glu-ProRS, GlyRS, and MetRS and plays a role in RNA or protein interactions [33]. We showed here that fusion of the WHEP domain of $HsHisRS_c$ to the WT $HsHisRS_m$ had little effect on its tRNA specificity or aminoacylation activity, but fusion of the same domain to a motif 2 loop-mutated $HsHisRS_m$–$HsHisRS_m^{Mut}$—— selectively enhanced its aminoacylation activity toward $tRNA_n^{His}$ (Fig. 6). It should be mentioned that swapping the divergent segment of the motif 2 loop between the two isoforms successfully switched their tRNA preferences, and fusion of the WHEP domain merely amplified this effect (Fig. 6; Table 1). As a result, the fusion enzyme WHEP-$HsHisRS_m^{Mut}$ bears tRNA specificity almost indistinguishable from that of the WT $HsHisRS_c$ (Fig. 6). To our knowledge, this is the first demonstration that the N73 preferences of HisRSs can be switched without losing activity. This finding also reinforces the hypothesis that the WHEP domain is not directly involved in N73 recognition. Instead, it may act through promoting an active conformation of HisRS.

In *E. coli*, both G-1 and C73 are essential identity elements for $tRNA^{His}$; mutation of either nucleotide leads to defective tRNA unsuitable for aminoacylation [34]. In contrast, the anticodon is not as important as G-1 or C73; mutation of the first nucleotide of the anticodon (G34U) reduces the aminoacylation efficiency (k_{cat}/K_M) by only sevenfold (mainly in K_M) [21]. In yeast, G-1 is also conserved and essential for aminoacylation (Table 1), but N73 is varied—an "A" in $tRNA_n^{His}$ and a "C" in $tRNA_m^{His}$. Yeast HisRS retains a relatively relaxed specificity for the discriminator base (Fig. 2). To maintain aminoacylation fidelity, the anticodon of $SctRNA^{His}$ becomes a much stronger identity element; mutation in the first nucleotide of the anticodon (G34U) reduces the aminoacylation efficiency (k_{cat}/K_M) by up to 75-fold (mainly in k_{cat}) [21]. While yeast HisRS prefers A73-containing $tRNA^{His}$ in the presence of G-1 (Fig. 2), this enzyme prefers C73-containing $tRNA^{His}$ in the absence of G-1 [35]. As for human $tRNA^{His}$ isoacceptors, G-1 is conserved and essential for aminoacylation (Table 1). Unexpectedly, human $HisRS_c$ and $HisRS_m$ possessed highly divergent tRNA specificities. The cytoplasmic form preferred A73 over C73, irrespective of whether it was within the context of $HstRNA_n^{His}$ or $HstRNA_m^{His}$. In contrast, the mitochondrial isoform preferred C73 over A73, irrespective of whether it was within the context of $HstRNA_n^{His}$ or $HstRNA_m^{His}$ (Table 1). Moreover, the anticodon remained a very strong identity element for recognizing $HstRNA_m^{His}$ but not $HstRNA_n^{His}$ (Table 1). All these newly acquired features make human HisRS paralogues an excellent paradigm for studies of gene subfunctionalization.

In contrast to all other bacteria, an α-proteobacterial clade comprising *Rhizobiales, Caulobacter crescentus*, and *Silicibacter pomeroyi* possessed A73 instead of C73. As it turns out, the HisRS gene in this clade was derived from a horizontal transfer event followed by in situ adaptation of the bacterial $tDNA^{His}$ [36]. Our study underlines the evolutionary histories of human HisRSs and their adaptation to cytoplasmic and mitochondrial $tRNA^{His}$ isoacceptors. It appears that human $HisRS_c$ and $HisRS_m$ relatively recently descended from duplication of a dual-functional predecessor (Fig. 8 in Appendix). In this regard, it is even more surprising to find that they recognize such distinct identity elements in $tRNAs^{His}$: G-1:A73 for $HsHisRS_c$ and G-1:C73 and the anticodon for $HsHisRS_m$. While a similar scenario of a gene-duplication event was reported in other eukaryotic aaRSs [37, 38], these events arose mainly because their cytoplasmic and mitochondrial tRNA isoacceptors retain the same identity elements, such as G3:U70 in $tRNA^{Ala}$ or A35/C36 in $tRNA^{Val}$. Thus, the scenario of human HisRS genes represents an interesting paradigm for the evolution of eukaryotic aaRS genes.

Materials and methods

Construction of plasmids

Cloning of genes encoding the WT or mutant ScHisRS into pRS315 (a low-copy-number yeast shuttle vector) followed a standard protocol. Briefly, a pair of gene-specific primers was used to amplify the gene via a polymerase chain reaction (PCR) using yeast genomic DNA as the template. The forward primer with a *Spe*I site was located 300 bp upstream of the first ATG codon of the open reading frame (ORF), while the reverse primer with an *Xho*I site was located immediately upstream of the stop codon. The PCR-amplified fragment was digested with *Spe*I and *Xho*I and then cloned into *Spe*I/*Xho*I sites of pRS315 for rescue assays. Cloning of genes encoding bacterial, yeast, and other eukaryotic HisRSs into pADH (a high-copy-number yeast shuttle vector with a constitutive *ADH* promoter and a short sequence encoding a C-terminal His_6 tag) or pET21b (an *E. coli* expression vector) followed a similar protocol, except that only the ORF was cloned. Target genes cloned in pADH were mainly used for heterologous rescue assays and for purification of yeast HisRS derivatives, while those cloned in pET21b were solely used for protein purification. Western blotting followed a previously described protocol

and used an HRP-conjugated anti-His_6 tag antibody as a probe [39].

Yeast HisRS proteins were purified from yeast transformants, and *E. coli* and human HisRS proteins were purified from *E. coli* transformants. His_6-tagged proteins were purified to homogeneity through Ni–NTA affinity chromatography as previously described [40]. Purity of the purified HisRS derivatives was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Mutagenesis was carried out using QuikChange II Site-Directed Mutagenesis Kit and following standard protocols provided by the manufacturer (Agilent, Santa Clara, CA, USA). To fuse a heterologous MTS to HisRS, the DNA sequence encoding amino acid residues 1–46 of the mitochondrial precursor form of yeast ValRS was amplified by a PCR as an *Xba*I–*Spe*I fragment and then inserted at the 5′ end of the ORF of HisRS.

Complementation assay for cytoplasmic activity

A yeast *HTS1* knockout heterozygous diploid strain (YPR033C) was purchased from Dharmacon (Lafayette, CO, USA). After sporulation and tetrad dissection, a haploid knockout strain (*MAT*α, *hts1::kanMX4, his3*Δ*1 leu2*Δ*0 lys2*Δ*0 ura3*Δ*0*) was obtained. This strain was maintained by a maintenance plasmid, which was constructed by cloning the WT *HTS1* gene into the *Spe*I/*Xho*I sites of pRS316 (with a *URA3* marker) [41]. We carried out a complementation assay for cytoplasmic HisRS activity by introducing a test plasmid carrying the target gene and a *LEU2* marker into the null allele and determining the transformants' ability to grow in the presence of 5-FOA (1 mg/ml) [42]. Starting with a cell density of 1.0 A_{600}, cell cultures were threefold serially diluted, and 10-µl aliquots of each dilution were spotted onto the designated plates containing 5-FOA. Plates were incubated at 30 °C for 3 days. The transformants evicted the maintenance plasmid (which carried the WT *HTS1* gene) with a *URA3* marker in the presence of 5-FOA and thus could not grow on the 5-FOA plates unless the test plasmid encoded a functional cytoplasmic HisRS.

Complementation assay for mitochondrial activity

The yeast *hts1*$^-$ strain was co-transformed with a test plasmid (which carried a *LEU2* marker) and a second maintenance plasmid (which carried a *HIS3* marker and an initiator mutant of *HTS1* that expressed only the cytoplasmic form of ScHisRS). In the presence of 5-FOA, the first maintenance plasmid (carrying a *URA3* marker) was evicted from the co-transformants, and the second maintenance plasmid was retained. Thus, all co-transformants could survive 5-FOA selection due to the presence of the cytoplasmic ScHisRS derived from the second maintenance plasmid. The co-transformants' mitochondrial phenotypes were further tested on yeast extract peptone glycerol (YPG) plates at 30 °C, with results documented on day 3 following plating. Because a yeast cell cannot survive on glycerol (a non-fermenting carbon source) without functional mitochondria, the co-transformants did not grow on the YPG plates unless the test plasmid encoded a functional mitochondrial HisRS.

In vitro transcription of $tRNA^{His}$

Preparation of the WT and mutant $tRNA^{His}$ transcripts followed a previously described protocol [34]. A synthetic DNA sequence containing both a T7 promoter and a gene encoding the WT or mutant $tRNA^{His}$ (GUG) was cloned into pUC18 using complementary primers with a *Sma*I site on both ends. The transcription template was enriched by PCR amplification of the cloned DNA fragments. The in vitro transcription reaction of $tRNA^{His}$ (with a 5′-GMP) was performed at 37 °C for 3 h with 0.3 µM T7 RNA polymerase in 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 20 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine, 2 mM of each NTP, and 20 mM GMP. The transcript was purified by a 15% denaturing urea-polyacrylamide gel. After ethanol precipitation, the tRNA pellet was dissolved in 1× TE buffer (20 mM Tris–HCl (pH 8.0) and 1 mM EDTA). tRNA was annealed by heating to 80 °C and gradually cooled to room temperature after the addition of 10 mM $MgCl_2$. ~80% of in vitro-transcribed $tRNA^{His}$ were active in aminoacylation. The annealed tRNA preparation was split into aliquots and stored at −80 °C.

Aminoacylation assay

Aminoacylation reactions were carried out at ambient temperature in a buffer containing 50 mM HEPES (pH 7.5), 50 mM KCl, 15 mM $MgCl_2$, 5 mM dithiothreitol, 10 mM ATP, 0.1 mg/ml bovine serum albumin, 5 µM in vitro-transcribed $tRNA^{His}$, and 26.25 µM histidine (6.25 µM ^{14}C-histidine; PerkinElmer, Waltham, MA, USA). Aminoacylation assay followed a previously described protocol [43]. The specific activity of ^{14}C-histidine used was 325 mCi/mmol. Final concentrations of the enzymes used in the reactions are indicated in the figure legends. Reactions were quenched by spotting 10-µl aliquots of the reaction mixture onto Whatman filters (Maidstone, Kent, UK) presoaked in 5% trichloroacetic acid (TCA) and 2 mM histidine. The filters were washed three times for 15 min each in ice-cold 5% TCA before liquid scintillation counting. Data were obtained from three independent experiments and averaged.

Kinetic parameters for the histidylation of tRNA were determined by measuring the initial rate of charging over the first 2 min [17]. Initial rates of aminoacylation were

determined at 25 °C with $tRNA^{His}$ concentrations ranging 0.16–20 μM and enzyme concentrations ranging 10–100 nM. Each initial rate at a given substrate concentration was determined in duplicate, and the slope of line was derived by linear regression. The parameters were derived from Lineweaver–Burk plots. Error values represent 2 × standard deviations. The data for each HisRS variant were obtained from a single protein prep. Determination of active protein concentrations by active site titration was as previously described [46]. The tRNA used for the assay was in vitro-transcribed WT or mutant $tRNA_n^{His}$ or $tRNA_m^{His}$. Data were obtained from three independent experiments and averaged.

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Appendix

See Figs. 7 and 8.

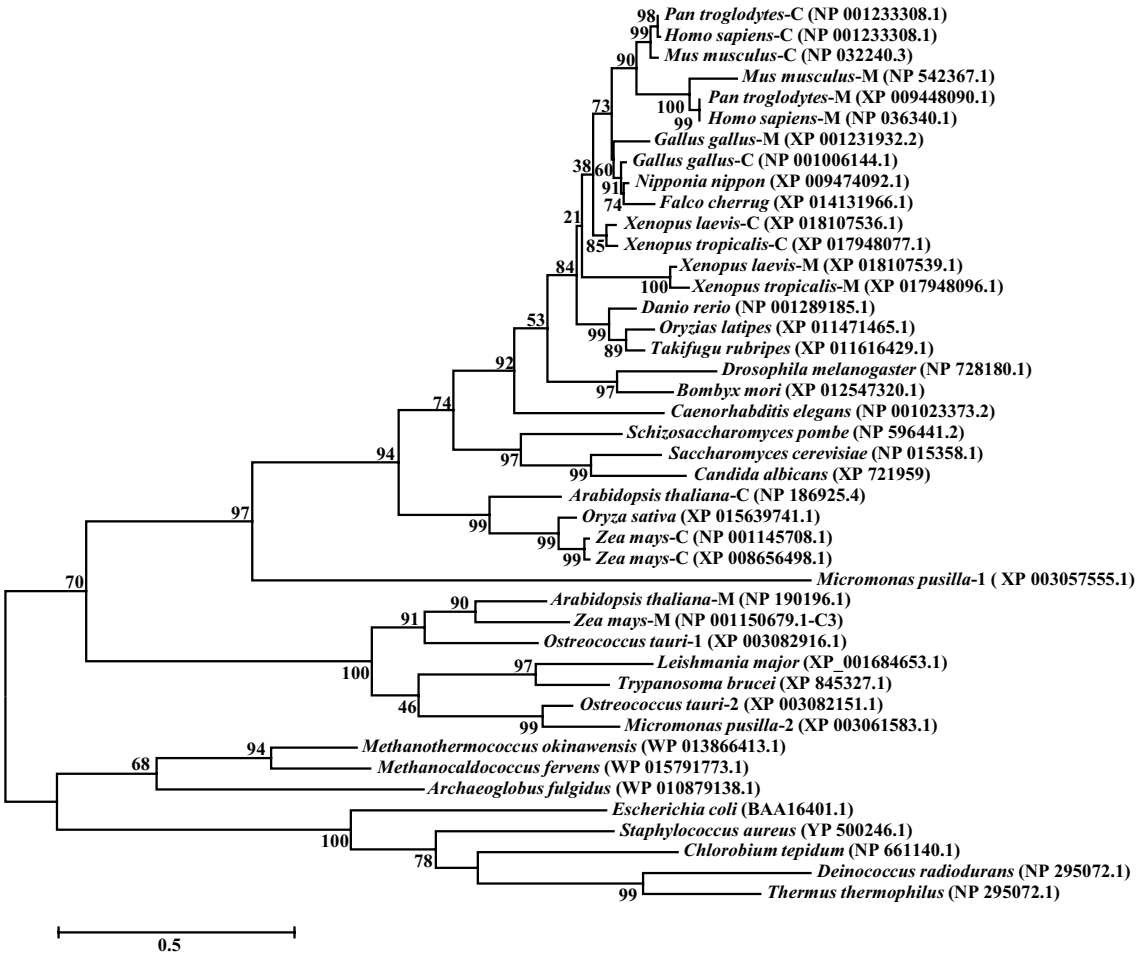

Fig. 7 Phylogenetic analysis of HisRS proteins. The evolutionary history was inferred using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al. [44]). The tree with the highest log likelihood (−9551.2796) is shown. Initial tree(s) for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using a JTT model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 43 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 205 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. [27]). Numbers at the nodes denote bootstrapping frequencies (shown as percent) calculated from 1000 trees. The accession number of the HisRS sequence is shown in the parentheses. The localizations of eukaryotic HisRSs were predicted by using the PSORTII software (Horton et al. [45]), and C and M, respectively, denote HisRSc and HisRSm. While *M. pusilla* and *O. tauri* also contain two HisRS homologues (denoted as 1 and 2), their localizations are uncertain

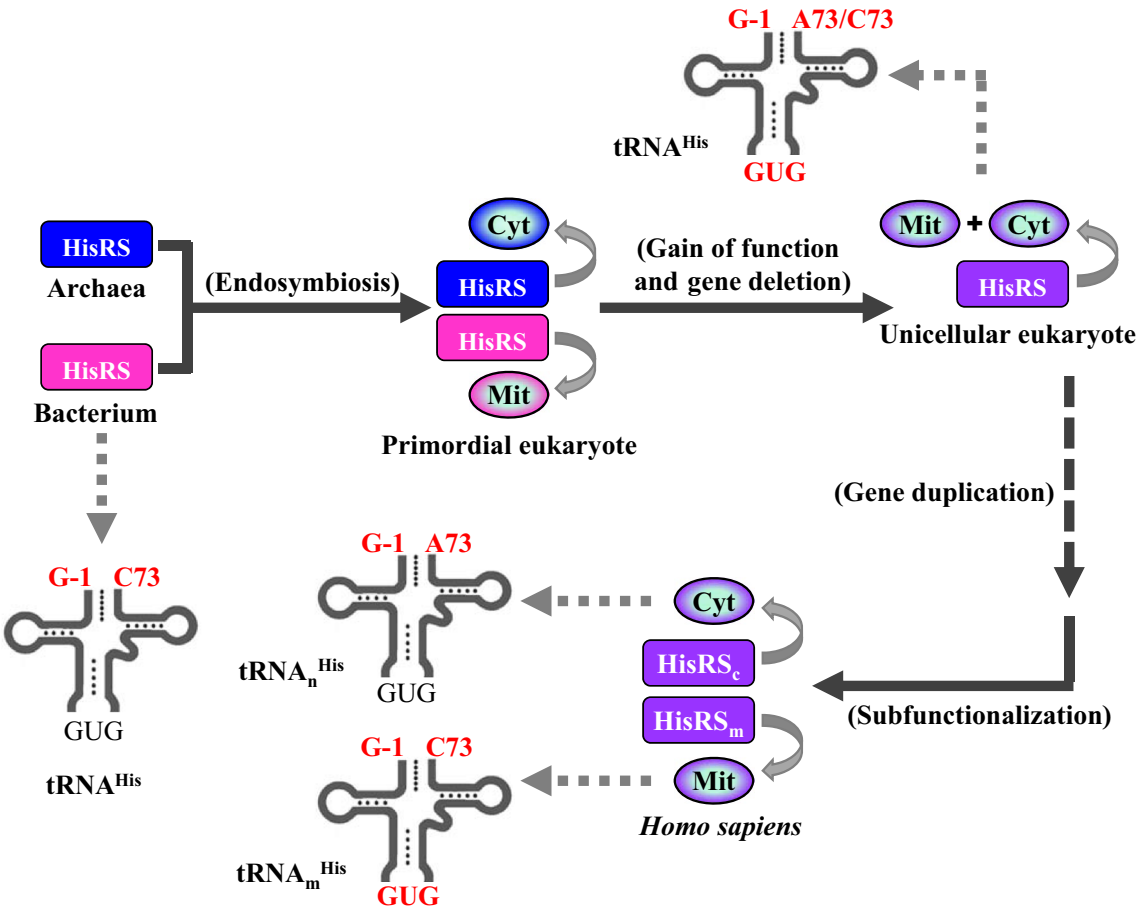

Fig. 8 A proposed evolutionary history of human HisRS homologues. Yeast contains a dual-functional HisRS gene, while humans contain two distinct yet closely related HisRS genes. The primary identity elements of tRNAHis are shown in red. *Cyt* cytoplasmic form, *Mit* mitochondrial form

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