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Translational fidelity maintenance preventing Ser mis-incorporation at Thr codon in protein from eukaryote

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ABSTRACT

Aminoacyl-tRNA synthetase (aaRS) catalyzes the first step of protein synthesis, producing aminoacyl-tRNAs as building blocks. Eukaryotic aaRS differs from its prokaryotic counterpart in terminal extension or insertion. Moreover, the editing function of aaRSs is an indispensable checkpoint excluding non-cognate amino acids at a given codon and ensuring overall translational fidelity. We found higher eukaryotes encode two cytoplasmic threonyl-tRNA synthetases (ThrRSs) with difference in N-terminus. The longer isoform is more closely related to the ThrRSs of higher eukaryotes than to those of lower eukaryotes. A yeast strain was generated to include deletion of the *thrS* gene encoding ThrRS. Combining *in vitro* biochemical and *in vivo* genetic data, ThrRSs from eukaryotic cytoplasm were systematically analyzed, and role of the eukaryotic cytoplasmic ThrRS-specific N-terminal extension was elucidated. Furthermore, the mechanisms of aminoacylation and editing activity mediated by *Saccharomyces cerevisiae* ThrRS (*Sc*ThrRS) were clarified. Interestingly, yeast cells were tolerant of variation at the editing active sites of *Sc*ThrRS without significant Thr-to-Ser conversion in the proteome even under significant environmental stress, implying checkpoints downstream of aminoacylation to provide a further quality control mechanism for the yeast translation system. This study has provided the first comprehensive elucidation of the translational fidelity control mechanism of eukaryotic ThrRS.

INTRODUCTION

Translation on the ribosome is the process by which the genetic code information in the messenger RNA (mRNA) determines the sequential order of the amino acids in the newly synthesized protein. Aminoacylation catalyzed by aminoacyl-tRNA synthetases (aaRSs) involves covalent linkage of amino acids with their cognate transfer RNAs (tRNAs) and represents the initiating step in the complex process of translation (1,2). Amino acids are first activated by aaRSs in an adenosine triphosphate (ATP)-dependent process leading to the synthesis of the aa-AMP intermediate with the release of pyrophosphate. The amino acid moiety of the intermediate is subsequently transferred to the cognate tRNA. Aminoacylation requires a high level of accuracy and provides a critical checkpoint for translational quality control (3). Mis-translation arising from disruption of translational fidelity has profound consequences in *Escherichia coli* (4,5) and mammalian cells (6). Even a slight decrease in aminoacylation accuracy can cause an intracellular accumulation of misfolded proteins and up-regulation of cytoplasmic protein chaperones in neurons, leading to severe mammalian neurodegeneration (7).

Multiple factors including structural differences, recognition elements and anti-determinants facilitate the selection of cognate tRNAs (8). However, accurate selection of cognate amino acids is much more challenging for the corresponding aaRS due to the lack of structural divergence between the 20 amino acids. Therefore, some aaRSs are prone to mis-activation of non-cognate amino acids. The editing function of some aaRSs has evolved to ensure removal of incorrect aa-AMPs (pre-transfer editing) and/or mis-charged tRNAs (post-transfer editing) (9). The pre-transfer editing may be further divided into tRNA-independent pre-transfer editing, in which the non-cognate aa-AMP is hydrolyzed into the amino acid and AMP molecule without the presence of cognate tRNA, as well as tRNA-dependent pre-transfer editing by which process, aa-AMP hydrolysis is triggered by the addition of tRNA (10–12).

Threonyl-tRNA synthetase (ThrRS) is a class IIa aaRS (13) and divided into two types (bacterial and archaeal)

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based on the primary structure. ThrRSs from both eukaryotes (such as yeast or human) and bacteria (such as *E. coli*) belong to the bacterial type of ThrRS. *E. coli* ThrRS (*Ec*ThrRS) has N1 (with unknown function), N2 (for post-transfer editing), class II-specific aminoacylation (for amino acid activation and tRNA charging) and C-terminal domains (for tRNA-anticodon binding) (14; Supplementary Figure S1). Despite being a bacterial type ThrRS, yeast mitochondrial ThrRS (ymThrRS) lacks the whole N-terminal region, including the N-extension and the N1- and N2-editing domain (15). The most striking feature of eukaryotic ThrRSs discriminating them from their bacterial counterparts is the presence of an extension appended to the N-terminus (here referred to as the N-extension) without details of the origin, structure and function information.

Threonine (Thr) is highly similar to valine (Val), serine (Ser) and cysteine (Cys). Furthermore, alanine (Ala) and glycine (Gly) have small side chains. These non-cognate amino acids may be accommodated and mis-activated by ThrRS. Notably, high-quality X-ray structures have clearly elucidated the amino acid selection and editing mechanism of *Ec*ThrRS (14,16–18). The mis-charged $tRNA^{Thr}$ is hydrolyzed at the N2 domain by post-transfer editing. Two residues, His^{73} and His^{77} in the HXXXH motif, play a pivotal role in editing. The accuracy of archaeal ThrRSs seems more divergent. In general, archaeal ThrRSs recruit a unique N-terminal D-aminoacyl-tRNA deacylase-like domain to remove D-amino acids and L-Ser (19,20). However, several crenarchaeal species contain individual genes encoding two ThrRSs with split function of aminoacylation (ThrRS-cat) and editing (ThrRS-ed). They work in concert to synthesize both Thr- and Ser-$tRNA^{Thr}$ by ThrRS-cat, and the latter is subsequently *trans*-edited by ThrRS-ed (21). However, the mechanism of amino acid selection and editing by eukaryotic cytoplasmic ThrRSs remains to be elucidated.

MATERIALS AND METHODS

Materials

L-threonine, L-serine, dithiothreitol, NTP, 5′-GMP, tetrasodium pyrophosphate, inorganic pyrophosphate, ATP, Tris–HCl, $MgCl_2$, NaCl and activated charcoal were purchased from Sigma (USA). [^{3}H]Thr was obtained from Biotrend chemicals (Destin, USA), [^{14}C]Ser and [α-^{32}P]ATP were obtained from Perkin Elmer Inc. *Pfu* DNA polymerase, the DNA fragment rapid purification kit and a plasmid extraction kit were purchased from Biotech Company (China). KOD-plus mutagenesis kit was obtained from TOYOBO (Japan). T4 ligase and restriction endonucleases were obtained from MBI Fermentas. Phusion high-fidelity DNA polymerase was purchased from New England Biolabs (USA). Ni^{2+}-NTA Superflow was purchased from Qiagen, Inc. (Germany). Polyethyleneimine cellulose plates were purchased from Merck (Germany). Pyrophosphatase was obtained from Roche Applied Science (China). Pyrobest DNA polymerase and the dNTP mixture were obtained from Takara (Japan). Oligonucleotide primers were synthesized by Invitrogen (China). *E. coli* BL21 (DE3) cells were purchased from Stratagene (USA).

Cloning and mutagenesis

The *Sc*ThrRS gene was amplified from the *S. cerevisiae* genome and cloned into pET22b with a C-terminal His_6-tag and into the yeast expression vector p425TEF with the *LEU2* gene (22). Expression of the recombinant protein was induced at 25°C for 8 h with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. Human genes encoding hcThrRS and hcThrRS-L were amplified from cDNA, obtained by reverse-transcription polymerase chain reaction (PCR) from total RNA obtained from human 293 T cells and cloned into p425TEF. Gene mutagenesis was performed according to the protocol provided with the KOD-plus mutagenesis kit.

tRNA gene transcription and enzyme preparation

In vitro *Sc*$tRNA^{Thr}$ (AGU) (*Sc*$tRNA^{Thr}$) transcripts were obtained using the T7 RNA polymerase run-off procedure as described previously (5). Proteins were purified using a previously reported method (23).

In vitro assays

ATP-PPi exchange measurement was carried out at 30°C in a reaction mixture containing 60 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mg/ml BSA, 2.5 mM ATP, 2 mM tetrasodium [^{32}P]pyrophosphate, 1 mM Thr or 400 mM non-cognate Ser, Val, Cys, Gly or Ala and 200 nM *Sc*ThrRS or its variant. Samples of the reaction mixture were removed at specific time points, added to 200 µl of quenching solution containing 2% activated charcoal, 3.5% $HClO_4$ and 50 mM tetrasodium pyrophosphate and mixed by vortexing for 20 s. The solution was filtered through a Whatman GF/C filter, followed by washing with 20 ml of 10 mM tetrasodium pyrophosphate solution and 10 ml of 100% ethanol. The filters were dried, and [^{32}P]ATP was counted using a scintillation counter (Beckman Coulter).

Aminoacylation was performed at 30°C in a reaction mixture containing 60 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mg/ml BSA, 2.5 mM ATP, 20 µM [^{3}H]Thr, 10 µM *Sc*$tRNA^{Thr}$ and 200 nM *Sc*ThrRS or its variant. The mis-aminoacylation experiment was performed at 30°C in the presence of 60 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mg/ml BSA, 2.5 mM ATP, 320 µM [^{14}C]Ser, 10 µM *Sc*$tRNA^{Thr}$ and 2 µM *Sc*ThrRS or its variant. Post-transfer editing of pre-formed [^{14}C]Ser-$tRNA^{Thr}$ or [^{14}C]Ser-$tRNA^{Thr}$ variants (prepared with H151A/H155A) was conducted at 30°C in a reaction mixture containing 60 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mg/ml BSA, 2 µM [^{14}C]Ser-$tRNA^{Thr}$ and 200 nM *Sc*ThrRS or its variant. Samples of the reaction mixture were removed at specific time points, quenched on Whatman filter pads and equilibrated with 5% trichloroacetic acid (TCA). The pads were washed three times for 15 min each with cold 5% TCA and then three times for 10 min each with 100% ethanol. The pads were then dried under a heat

lamp. The radioactive content of the precipitates was quantified using a scintillation counter (Beckman Coulter).

The AMP formation assay [thin-layer chromatography (TLC)] was carried out at 30°C in a reaction mixture containing 60 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mg/ml BSA, 10 U/ml PPiase (Roche), 40 mM Ser (or 4 mM Thr), 3 mM [α-^{32}P]ATP and 2 μM *Sc*ThrRS or its variant in the presence and absence of *Sc*tRNA^{Thr}. Samples (1.5 μl) were quenched in 6 μl of 200 mM NaAc (pH 5.0). The quenched aliquots (1.5 μl of each sample) were spotted on polyethyleneimine cellulose plates pre-washed with water. Separation of Ser-[α-^{32}P]AMP, [α-^{32}P]AMP and [α-^{32}P]ATP was performed in 0.1 M NH_4Ac and 5% acetic acid. The plates were visualized by phosphorimaging, and the data were analyzed using Multi Gauge Version 3.0 software (FUJIFILM). Quantification of [α-^{32}P]AMP was achieved by densitometry in comparison with [α-^{32}P]ATP samples of known concentrations.

The rate of non-enzymatic hydrolysis of adenylate was measured in pulse-chase experiments wherein a large excess of unlabeled ATP was added to the reaction mixtures following the initiation of Ser-AMP synthesis (24). The excess of unlabeled ATP induces the release of the non-cognate aminoacyl-adenylate from the active site into solution, where its spontaneous hydrolysis is monitored. A solution of 2 μM *Sc*ThrRS was first incubated with 60 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mg/ml BSA, 10 U/ml PPiase, 40 mM Ser, 100 μM ATP and 0.25 [α-^{32}P]ATP for 10 min at 30°C to prepare Ser-[^{32}P]AMP. Subsequently, unlabeled ATP (50 mM, equal to a 500-fold molar excess of initial unlabeled ATP) was added, and the hydrolysis activity was quenched at various time points (1–10 min) by mixing 1.5 μl of the reaction mixture with 6 μl of 200 mM sodium acetate (pH 5.0) and 0.1% sodium dodecyl sulphate (SDS). Separation of Ser-[α-^{32}P]AMP by TLC was then performed, and reactions were quantified as described previously.

ScΔthrS complementation assay

For complementation assays, all genes of interest were cloned into the yeast expression vector, p425TEF. The constructs were introduced into *ScΔthrS* using the lithium acetate method. Transformants were selected on SD/Ura$^-$/Leu$^-$/G418 plates, and a single clone was cultured in liquid SD/Leu$^-$/G418 medium. The culture was then diluted to a concentration equivalent to 1 OD_{600}, and a 4-fold dilution of the yeast was plated onto the SD/Leu$^-$/G418 in the presence of 5-FOA to induce the loss of the rescue plasmid (pRS426-*ScthrS*). Complementation was observed by comparing the growth rates of *ScΔthrS* expressing *Sc*ThrRS or its variants.

Phylogenetic analysis of ThrRSs

For phylogenetic analysis of ThrRS and ThrRS-L from bacteria and eukaryotes, the protein sequences of various ThrRSs and ThrRSs-L homologs were obtained by Protein Blast (pBlast) analysis using the NCBI database. Sequences were aligned using the Clustal X2 program (25). Ambiguous regions were removed from the alignment manually. The phylogenetic tree was constructed using the minimal evolution algorithm of the MEGA 4 program (26). Bootstrap analysis was performed with 1000 replicates.

RESULTS

Analysis of eukaryotic cytoplasmic ThrRSs

Sequence alignment revealed the presence of an N-extension in all eukaryotic ThrRSs that is absent in the bacterial counterparts. pBlast analysis of human cytoplasmic ThrRS (hcThrRS, accession no. P26639) unexpectedly revealed the existence of two human cytoplasmic ThrRSs (hcThrRS and hcThrRS-L, accession No. A2RTX5). The hcThrRS and hcThrRS-L proteins are composed of 723 and 802 amino acid residues, respectively. The latter is only encoded in higher eukaryotes and is differentiated from hcThrRS by the evolution of a further N-terminus (Met^1-Cys^{74}) upstream the eukaryotic ThrRS-specific N-extension (Met^1-Ile^{83} for hcThrRS and Leu^{75}-Ile^{162} for hcThrRS-L) (Supplementary Figure S1A). However, these proteins share a high level of sequence similarity and identity in other regions. pBlast analysis revealed that the hcThrRS-L-specific N-terminus (Met^1-Cys^{74}) is homologous to the N-terminal extension of the human arginyl-tRNA synthetase (ArgRS), which is responsible for interacting with human LeuRS (Supplementary Figure S1B; 27). The reason for the existence of hcThrRS-L and the role of the N-extension of eukaryotic ThrRSs in aminoacylation is unclear. Phylogenetic analysis revealed that the hcThrRS-L is more closely related to ThrRS in higher eukaryotes than ThrRS in lower eukaryotes, suggesting that it may be derived from gene duplication in the predecessors of higher eukaryotes (Supplementary Figure S2).

Construction of the yeast *thrS* knockout strain

The *thrS* gene was first amplified by PCR using a yeast genome template and digested with *Sac*I and *Xho*I. The fragment was then ligated into the pRS426 plasmid (pre-digested with *Sac*I and *Xho*I) to generate pRS426-*ScthrS* (pRS426: *thrS*$^+$, *Ura*$^+$). The diploid BY4743 strain (BY4743-*thrS*$^{+/-}$), which contains a chromosomal copy of *thrS* replaced by the G418 resistance gene, was transformed with the rescue plasmid (pRS426-*ScthrS*). The transformants were cultured on minimal media with uracil dropout (SD/Ura$^-$). *Ura*$^+$ colonies were selected and cultured on sporulation plates, and the tetrads were dissected on YPD (1% yeast extract, 2% peptone and 2% glucose) plates. Spore growth of the *thrS* knockout strain is supported by SD/Ura$^-$ supplemented with 250 μg/ml G418 (Figure 1A).

The identity of the knockout strain was confirmed by PCR-based analysis using a combination of four primers. The diploid BY4743-*thrS*$^{+/-}$ strain contains copies of both the *thrS* and G418 genes. Therefore, two PCR fragments (~5 kb and 4.4 kb, respectively) were amplified from the

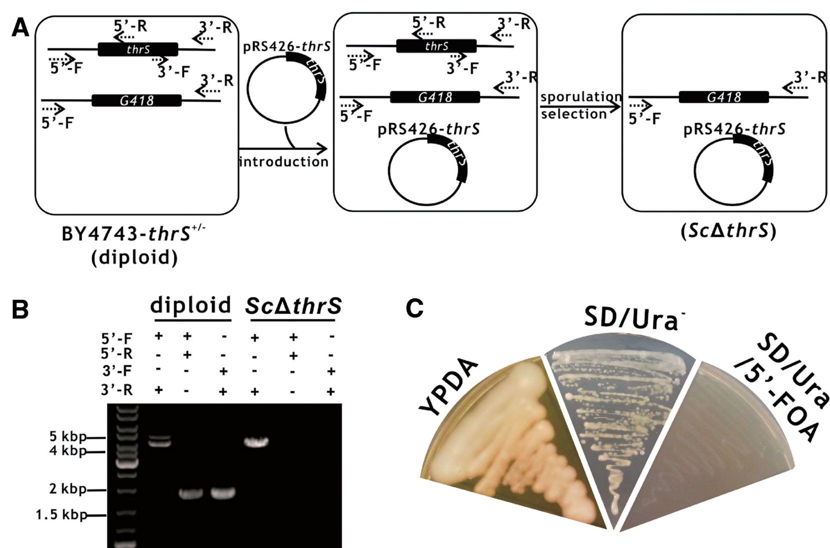

Figure 1. Construction of *ScΔthrS*. (**A**) Schematic representation of the construction process of *ScΔthrS*. Four primers were used to confirm the strain indicated. (**B**) PCR products using different combinations of primers and the extracted genome from the diploid strain or *ScΔthrS*. (**C**) Growth of *ScΔthrS* on YPDA, SD/Ura$^-$ and SD/Ura$^-$/5-FOA plates.

genome using the 5′-F (5′ ATTCATAGTCAAGCAGGT TG 3′) and 3′-R (5′ TCCGCGGGTGTAAGTCAAGC 3′). Furthermore, a single fragment (2.0 kb) was obtained using the 5′-F and 5′-R (5′ TATACTTCGAATAATGAA AC 3′) or 3′-F (5′ ATGTGCCACCATCCAATTAG 3′) and 3′-R primers, respectively. In contrast, a single fragment containing the G418 resistance gene (~4.4 kb) was amplified from the genome of the *thrS* knockout strain with the 5′-F and 3′-R primers, and no product was obtained with the combination of the 5′-F and 5′-R or 3′-F and 3′-R primers (Figure 1A and B). Furthermore, sensitivity to 5-fluoroorotic acid (5-FOA) was evaluated to confirm deletion of *thrS*. 5-FOA is converted to toxic 5-flurouracil in strains expressing the functional *URA3* gene encoding orotidine-5′-phosphate decarboxylase, which is involved in the synthesis of uracil. The knockout strain was unable to grow on the SD/Ura$^-$ plates containing 5-FOA (5-FOA sensitive, 5-FOA^s) although normal growth was observed on SD/Ura$^-$ plates and YPDA plates, thus confirming deletion of the chromosomal *thrS* copy (Figure 1C). The knockout strain, designated *ScΔthrS*, was used as the platform for *in vivo* complementation assays. The normal metabolism of *ScΔthrS* critically relies on the *thrS* in the rescue plasmid, the loss of which can be forced following the introduction of a gene encoding a functional ThrRS in the presence of 5-FOA. Using this approach, the function of the introduced gene was detected under *in vivo* physiological conditions.

The role of the eukaryote-specific N-extension of ThrRS

To clarify the role of the N-extension in hcThrRS and hcThrRS-L, the relevant genes were cloned and expressed using a bacterial expression system. However, the expressed proteins formed inclusion bodies despite extensive efforts, rendering *in vitro* studies of these human enzymes impossible. Therefore, *Sc*ThrRS was used to investigate the role of N-extension by *in vitro* methods. In comparison with *Ec*ThrRS, the N-extension of *Sc*ThrRS ranges from Met^1 to Val^{73} (Figure 2A; Supplementary Figure S1A). In the absence of available tertiary structure information, sequential deletion mutations targeting the N-extension were constructed to generate various mutated genes encoding *Sc*ThrRS-Δ40, *Sc*ThrRS-Δ65, *Sc*ThrRS-Δ73 and *Sc*ThrRS-Δ78 (Figure 2A). Unfortunately, only *Sc*ThrRS-Δ40 was successfully expressed and purified from *E. coli* transformants, whereas the other proteins formed inclusion bodies during induction, implying a stability-regulating role of the complete N-extension.

Biochemical investigation was performed using *Sc*ThrRS-Δ40, which activated Thr with equivalent activity to *Sc*ThrRS (Figure 2B) although a decrease in the aminoacylation activity was observed (Figure 2C). Detailed aminoacylation kinetics analysis showed that the mutation led to an increased K_m and a decreased k_{cat} during tRNA^{Thr} threonylation with a 76% loss in the catalytic efficiency (k_{cat}/K_m) (Table 1).

To further understand its role *in vivo*, the constructed *ScΔthrS* was used for transformation with the shuffling plasmids p425TEF-*ScthrS*, p425TEF-*ScthrS-Δ40*, p425TEF-*ScthrS-Δ65*, p425TEF-*ScthrS-Δ73* and p425TEF-*ScthrS-Δ78*. Only *Sc*ThrRS-Δ40 and *Sc*ThrRS-Δ65 supported normal growth of yeast cells on SD/Leu$^-$/G418/5-FOA plates with comparable efficiency to the *Sc*ThrRS. Yeast harboring the gene encoding *Sc*ThrRS-Δ73 or *Sc*ThrRS-Δ78 did not survive (Figure 2D). Furthermore, for investigation of the human enzyme *in vivo*, p425TEF-*hcThrRS*, p425TEF-*hcThrRS-Δ82*, p425TEF-*hcThrRS-L*, p425TEF-*hcThrRS-L-Δ74* and p425TEF- *hcThrRS-L-Δ161* were constructed (Supplementary Figure S1A) and introduced into *ScΔthrS*. It was observed that hcThrRS, hcThrRS-L and hcThrRS-L-Δ74 functionally compensated for the loss of *Sc*ThrRS but with an obvious decrease in efficiency. However, growth of yeast containing hcThrRS-L

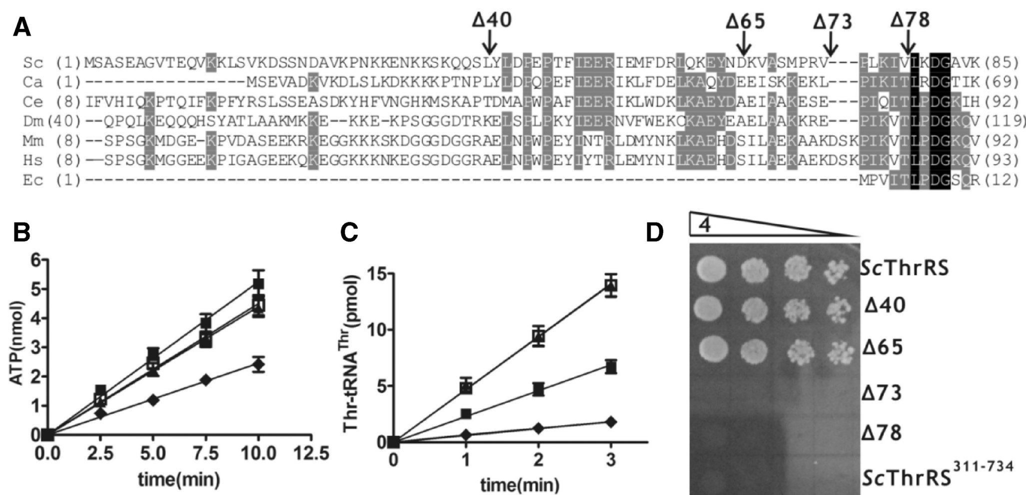

Figure 2. Role of the N-extension of *Sc*ThrRS. (**A**) Sequence alignment of the N-extensions of ThrRSs from eukaryotes and *Ec*ThrRS. Conserved and similar residues are highlighted in black and gray, respectively. Construction of deletion mutants is indicated by the arrows. (**B**) ATP-PPi exchange and (**C**) aminoacylation activities of *Sc*ThrRS (open squares), *Sc*ThrRS-Δ40 (closed squares), H151A/H155A (filled triangles) and *Sc*ThrRS$^{311-734}$ (filled diamonds) were assayed. (**D**) Complementation analysis of *Sc*ThrRS, *Sc*ThrRS-Δ40, *Sc*ThrRS-Δ65, *Sc*ThrRS-Δ73, *Sc*ThrRS-Δ78 and *Sc*ThrRS$^{311-734}$. A 4-fold dilution of yeast with initial 1 OD_{600} was performed. Sc, *Saccharomyces cerevisiae*; Ca, *Candida albicans*; Ce, *Caenorhabditis elegans*; Dm, *Drosophila melanogaster*; Mm, *Mus musculus*; Hs, *Homo sapiens*; Ec, *Escherichia coli*.

Table 1. Aminoacylation kinetics of *Sc*ThrRS and its mutants for $tRNA^{Thr}$

Enzyme	k_{cat} (s^{-1})[a]	K_m (μM)[a]	k_{cat}/K_m ($mM^{-1}s^{-1}$)	Relative k_{cat}/K_m (%)
*Sc*ThrRS	0.049 ± 0.003	1.297 ± 0.23	37.779	100
ΔN40	0.028 ± 0.003	3.077 ± 0.49	9.100	24.09
Y291R	0.016 ± 0.002	0.602 ± 0.12	26.578	70.35
Y291E	0.003 ± 0.001	0.416 ± 0.09	7.212	19.09
R303A	0.013 ± 0.003	6.619 ± 0.72	1.964	5.20

[a]The results are the average of three independent repeats with standard deviations indicated.

and TARSL-Δ74 was inhibited after culture for 3–4 days, possibly due to instability of these proteins in yeast (Supplementary Figure S3). hcThrRSs lacking the entire N-extension (hcThrRS-Δ82 and hcThrRS-L-Δ161) did not support yeast cell growth, which was consistent with the effects of *Sc*ThrRS-Δ73 and *Sc*ThrRS-Δ78 (Figure 2D).

These data suggested that the N-extension plays an important role in the $tRNA^{Thr}$ charging activity by directly affecting the catalytic parameters and/or indirectly influencing the local conformation, thus contributing to the efficiency of translation and normal growth of yeast.

Amino acid activation and editing properties of *Sc*ThrRS

To investigate mis-activation of non-cognate amino acids by the eukaryotic ThrRSs, amino acid activation assays were performed with Val, Ser, Cys, Ala and Gly, which are isoteric with or smaller than Thr. The data showed that *Sc*ThrRS strongly mis-activated only Ser at a rate of 1:910 compared with the cognate Thr (Figure 3A and Table 2), implying the existence of editing activity for the removal of non-cognate Ser. To investigate the mechanism, AMP formation during editing of ThrRS was assayed using a TLC method, which has been extensively employed in the investigation of the editing mechanism of both class I (such as LeuRS, IleRS, ValRS and GlnRS) and class II aaRSs (such as ProRS) (11,24,28,29). In the presence of tRNA, the rate of AMP formation represents the global editing activity, including tRNA-independent and -dependent pre-transfer editing in addition to post-transfer editing. In the absence of tRNA, the rate of AMP formation is representative solely of tRNA-independent pre-transfer editing (5,10,11).

*Sc*ThrRS was incubated with Ser in the absence of $tRNA^{Thr}$ to detect the occurrence of tRNA-independent pre-transfer editing. The observed rate (k_{obs}) of AMP formation was $(1.32 \pm 0.19) \times 10^{-3}\,s^{-1}$ (Figure 3B and C and Table 3). The AMP is produced by *Sc*ThrRS-catalyzed hydrolysis of Ser-AMP or by the 'selective release' pathway, in which the Ser-AMP dissociated from the enzyme into solution undergoes non-enzymatic hydrolysis. Pulse-chase experiments were conducted to distinguish these possibilities and to measure the rate of non-catalytic hydrolysis. Radioactively labeled Ser-AMP was first prepared by incubating *Sc*ThrRS with Ser and [α-^{32}P]-ATP. The reaction was then quenched by the addition of a 500-fold molar excess of unlabeled ATP. Monitoring of the conversion of Ser-AMP to AMP yielded a k_{obs} value of $(0.068 \pm 0.005) \times 10^{-3}\,s^{-1}$ (Figure 3D), indicating a negligible rate of non-enzymatic hydrolysis after its release into solution. In the presence of

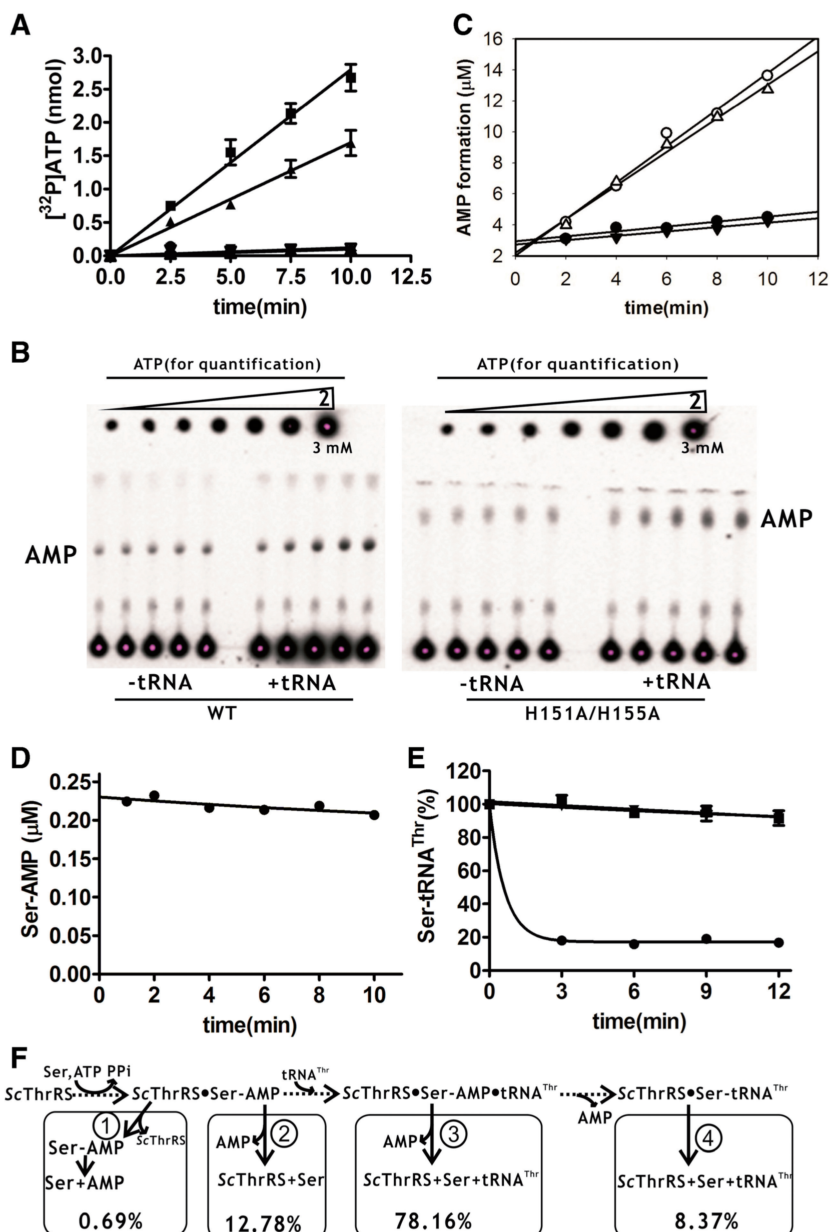

Figure 3. Amino acid activation and editing properties of *Sc*ThrRS. (**A**) Activation of Thr (closed squares), Ser (filled triangles), Ala (inverted filled triangles), Gly (filled diamonds), Cys (open triangles) and Val (inverted open triangles) by *Sc*ThrRS. (**B**) Generation of [^{32}P]AMP in the absence (-tRNA) or presence (+tRNA) of tRNA^{Thr} by *Sc*ThrRS (WT) and H151A/H155A, after incubations of 2, 4, 6, 8 and 10 min. A 2-fold dilution of [α-^{32}P]ATP (initial concentration, 3 mM) was included for quantification. (**C**) Quantification of AMP formation by WT with (open circles) or without (closed circles) tRNA or by H151A/H155A with (open triangles) or without (inverted filled triangles) tRNA. (**D**) Non-enzymatic hydrolysis of Ser-AMP in solution. (**E**) Post-transfer editing assay of *Sc*ThrRS (closed circles) and H151A/H155A (inverted filled triangles). Spontaneous hydrolysis (no enzyme addition) was performed as a control (closed squares). (**F**) Schematic representation of the editing module of *Sc*ThrRS with relative contributions to total editing indicated. Pathway 1 represents kinetic proofreading after selective release into solution of Ser-AMP from the active site; pathway 2 represents tRNA-independent pre-transfer editing; pathway 3 represents tRNA-dependent pre-transfer editing and pathway 4 represents post-transfer editing.

Table 2. Kinetic parameters of *Sc*ThrRS for threonine and serine in activation reaction

Amino acid	k_{cat} (s^{-1})[a]	K_m (mM)[a]	k_{cat}/K_m (mM^{-1}s^{-1})	Discrimination factor[b]
Thr	1.72 ± 0.21	0.21 ± 0.02	8.19	1
Ser	1.03 ± 0.13	112.83 ± 15.28	0.009	910

[a]The results are the average of three independent repeats with standard deviations indicated.
[b]Discrimination factor corresponds to the loss of catalytic efficiency relative to Thr.

Table 3. k_{obs} of *Sc*ThrRS and its variants in AMP formation with Ser

Enzyme	tRNA	k_{obs} ($\times 10^3$) (s^{-1})[a]	Relative k_{obs} (%)[b]
*Sc*ThrRS	+	9.80 ± 0.87	100
	–	1.32 ± 0.19	13.50
H151A/H155A	+	8.98 ± 0.91	91.63
	–	1.18 ± 0.20	12.04
Y291R	+	2.72 ± 0.45	27.76
	–	1.11 ± 0.16	11.33
Y291E	+	1.44 ± 0.29	14.70
	–	1.48 ± 0.22	15.10
R303A	+	3.05 ± 0.33	31.12
	–	1.27 ± 0.14	12.96

[a]The results are the average of three independent repeats with standard deviations indicated.
[b]The k_{obs} values are relative to that of *Sc*ThrRS (with $tRNA^{Thr}$).

$tRNA^{Thr}$, the AMP formation rate reflecting the total editing activity was $(9.80 \pm 0.87) \times 10^{-3} s^{-1}$ (Figure 3B and C and Table 3).

These data showed that *Sc*ThrRS possessed a detectable tRNA-independent pre-transfer editing activity. In contrast, *Sc*ThrRS displayed similar k_{obs} values of AMP formation either without ($[0.96 \pm 0.09] \times 10^{-3} s^{-1}$) or with ($[1.24 \pm 0.17] \times 10^{-3} s^{-1}$) $tRNA^{Thr}$ in the presence of cognate Thr, suggesting that *Sc*ThrRS editing is selective for Ser.

To confirm the existence of tRNA-dependent pre-transfer editing required isolation of the post-transfer editing activity from the total editing activity. Thus, it was necessary to identify a variant with an abolished post-transfer editing activity. It was speculated that the N2 domain is involved in the post-transfer editing based on knowledge of the *Ec*ThrRS editing mechanism (17,18). Sequence alignment allowed generation of a site-directed double mutation targeting the His^{151} and His^{155} of *Sc*ThrRS (counterparts of the editing-crucial His^{73} and His^{77} in *Ec*ThrRS) to obtain the H151A/H155A double mutant.

The H151A/H155A was found to display equivalent Thr activation and $tRNA^{Thr}$ charging activities compared with *Sc*ThrRS (Figure 2B and C). However, in contrast to *Sc*ThrRS, the mutant did not hydrolyze the pre-formed Ser-$tRNA^{Thr}$, indicating that the post-transfer editing was abolished by the double mutation (Figure 3E). The AMP formation rate of the post-transfer editing-deficient H151A/H155A was then determined in the presence of cognate $tRNA^{Thr}$ and Ser. The k_{obs} value was $(8.98 \pm 0.91) \times 10^{-3} s^{-1}$, thus the post-transfer editing contributed $0.82 \times 10^{-3} s^{-1}$ ($[9.80–8.98] \times 10^{-3} s^{-1}$) of the total editing activity (Figure 3B and C).

The following detailed editing mechanism for *Sc*ThrRS was postulated based on these data (Figure 3F): (i) negligible selective release accounts for only 0.69% (0.068/9.80 × 100%) of the total editing activity; (ii) *Sc*ThrRS possessed a detectable and obvious level of tRNA-independent pre-transfer editing, accounting for 12.78% ([1.32–0.068]/9.80 × 100%) of the total editing activity; (iii) apart from the tRNA-independent editing, tRNA also triggered an obvious and dominant level of tRNA-dependent pre-transfer editing, accounting for 78.16% ([8.98–1.32]/9.80 × 100%) of the total editing activity and (iv) post-transfer editing is a minor enzyme-catalyzed editing pathway, contributing only 8.37% (0.82/9.80 × 100%) of the total editing activity.

The anti-codon loop is the nucleic acid determinant for editing

The crucial recognition element of $tRNA^{Thr}$ by *Sc*ThrRS has been investigated *in vitro* (30). However, the tRNA elements for editing have not been identified. To investigate this, a series of tRNA mutants targeting the identified aminoacylation-crucial A73, G35 and U36 (A73U, A73C, A73G, G35A, G35U, G35C, U36A, U36C and U36G) were constructed (Figure 4A). Analysis of $tRNA^{Thr}$ aminoacylation showed charging with Thr was not detectable among all G35 and U36 mutants, whereas aminoacylation of three A73 mutants was detected to various extents (Figure 4B). These data are consistent with the reported catalytic efficiency (30).

Activation of Ser by *Sc*ThrRS in the presence of $tRNA^{Thr}$ or its variants A73U, A73C and A73G showed no or little decrease in the k_{obs} ($[10.78 \pm 1.32] \times 10^{-3} s^{-1}$, $(8.09 \pm 1.03) \times 10^{-3} s^{-1}$ and $(5.60 \pm 0.63) \times 10^{-3} s^{-1}$, respectively). However, the k_{obs} was markedly reduced for the variants altered in the anti-codon, particularly the three mutants of U36, which all displayed the basal editing activity ($[2.14 \pm 0.33] \times 10^{-3} s^{-1}$ for U36A, $(1.60 \pm 0.19) \times 10^{-3} s^{-1}$ for U36C and $(1.50 \pm 0.28) \times 10^{-3} s^{-1}$ for U36G) compared with that of the tRNA-independent pre-transfer editing alone ($1.32 \pm 0.19 \times 10^{-3} s^{-1}$) (Table 4).

The mis-charged $tRNA^{Thr}$s were prepared from the A73 mutants with Ser (mis-charged tRNAs from G35 and U36 could not be obtained) for analysis of post-transfer editing. These mis-charged $tRNA^{Thr}$s were readily edited by *Sc*ThrRS despite a small decrease in hydrolysis of Ser-$tRNA^{Thr}$-A73G (Figure 4C). Furthermore, mis-charged tRNAs were not detected in the mis-aminoacylation assays using the A73 mutants, in sharp contrast to that observed in the presence of $tRNA^{Thr}$ and the editing-defective H151A/H155A (Figure 4D).

These data clearly indicated that the anti-codon bases (G35 and U36) were both crucial for the aminoacylation and editing reactions. However, A73 significantly contributed only to the aminoacylation.

Tyr^{291} controls the tRNA-dependent pre-transfer editing

tRNA-dependent pre-transfer editing requires the correct interaction between the enzyme-tRNA amino acid acceptor end in class I aaRS, illustrated by the Y330D mutation in *Ec*LeuRS (5,11). The existence of a similar mechanism employed by class II ThrRS remains to be determined. Tyr^{205} of *Ec*ThrRS was found to be located for the specific binding of the G1 of the first base pair, thus allowing correct positioning of the helical tRNA CCA_{76} end within the aminoacylation active site of the *Ec*ThrRS-$tRNA^{Thr}$ structure (PDB code 1QF6)

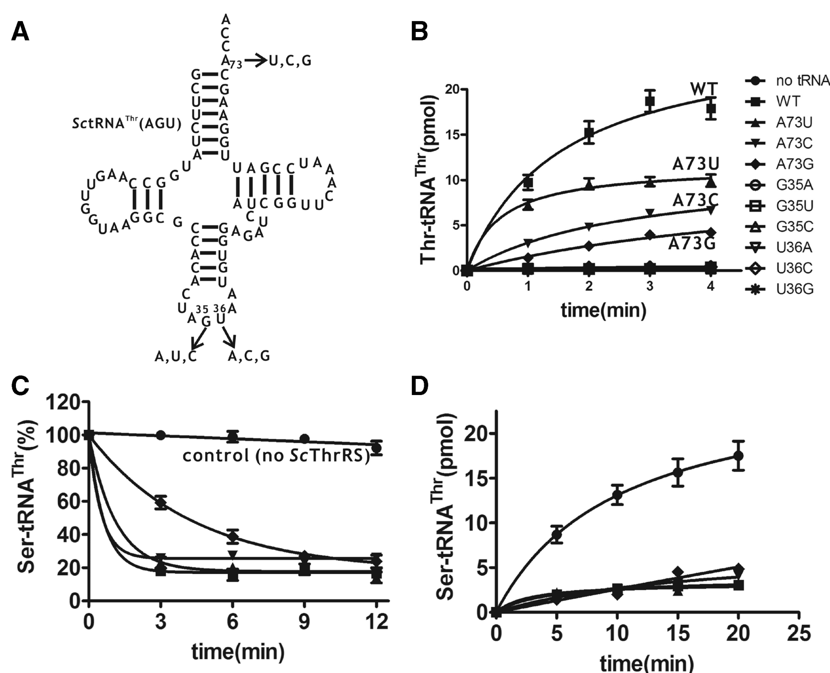

Figure 4. tRNA determinants for editing. (**A**) Cloverleaf structure of *Sc*tRNA^{Thr} (AGU) with mutation indicated. (**B**) Aminoacylation of WT *Sc*tRNA^{Thr} (closed squares), A73U (filled triangles), A73C (inverted filled triangles), A73G (filled diamonds), G35A (open circles), G35U (open squares), G35C (open triangles), U36A (inverted open triangles), U36C (open diamonds) and U36G (filled stars) by *Sc*ThrRS. (**C**) Post-transfer editing of various mis-charged tRNAs from WT *Sc*tRNA^{Thr} and A73 mutants by *Sc*ThrRS. (**D**) Mis-aminoacylation assays with WT *Sc*tRNA^{Thr} and A73 mutants by *Sc*ThrRS. Symbols in (C) and (D) are the same as in (B). Controls [aminoacylation without tRNA in (B), spontaneous hydrolysis of mis-charged tRNA^{Thr} in (C) and mis-aminoacylation of WT *Sc*tRNA^{Thr} by H151A/H155A in (**D**)] are indicated (closed circles).

(Figure 5A and B). This site is highly conserved among numerous ThrRSs. The corresponding site of *Sc*ThrRS (Tyr^{291}) was mutated to arginine (Y291R) or glutamic acid (Y291E), respectively, with the aim of destroying the enzyme-tRNA interaction.

Time-course analysis of aminoacylation showed that both mutants displayed an obvious decrease in tRNA-charging ability (Figure 5C). Kinetics analysis showed that Y291R and Y291E exhibited 70% and 19% catalytic efficiency, respectively, compared with *Sc*ThrRS, mainly resulting from the decrease in k_{cat} and slight increase in K_m (Table 1). These data showed that the correct tRNA G1-C72-ThrRS interaction was important for aminoacylation. Mis-charging assays of the two mutants revealed that mis-charged tRNAs were not generated in contrast to H151A/H155A (Figure 5D). Further analysis revealed equivalent or slightly decreased post-transfer editing (Figure 5E). Interestingly, TLC assays revealed a significant decrease in AMP formation by Y291R and Y291E in the presence of tRNA, produced almost exclusively by tRNA-independent pre-transfer editing ($[2.72 \pm 0.45] \times 10^{-3} s^{-1}$ and $(1.44 \pm 0.29) \times 10^{-3} s^{-1}$, respectively). However, the tRNA-independent pre-transfer editing activity of the mutants was not affected (Table 3). The retention of post-transfer editing activity and the absence of Ser-tRNA^{Thr} synthesis among the Y291 mutants suggest that shuffling of the tRNA amino acid acceptor end from the aminoacylation site to the editing active site was uninfluenced. Above all, these two Tyr^{291} mutants (especially Y291E) retained post-transfer editing activity but lost tRNA-dependent pre-transfer editing activity, demonstrating the correct ThrRS and tRNA acceptor stem interaction imposed by Tyr^{291} contributes to tRNA-dependent pre-transfer editing.

Arg^{303} directs the tRNA amino acid acceptor end from the aminoacylation site to the editing site

After charging, the 3′-end of tRNA^{Thr} translocates approximately 35 Å from the aminoacylation site to the editing site for amino acid scrutiny accompanied by the conformational change of the acceptor region from the helical to the hairpin structure (17). This also occurs in class I aaRSs although the conformational switch is from the hairpin to the helical states as proposed by Dock-Bregeon *et al.* (17). An element for the translocation by binding and directing the movable mis-charged end is necessary, and the translocation peptide has been identified in *Ec*LeuRS (31).

An absolutely conserved residue, Arg^{217} was identified in the *Ec*ThrRS-tRNA^{Thr} structure (PDB code 1QF6), which is located near the acceptor region and points toward the potential moving pathway, suggesting that this residue is the candidate for translocation. This residue is located very close to the phosphate backbone but does not directly contact the bases of tRNA in the aminoacylation conformation (Figure 6A).

The corresponding site (Arg^{303}) was mutated in *Sc*ThrRS to Ala to obtain the R303A mutant. Aminoacylation data showed that this mutation obviously

decreased the k_{cat} and increased the K_m, leading to the retention of only 5.20% activity, suggesting Arg^{303} is critical for aminoacylation (Figure 6B and Table 1). However, R303A synthesized a significant amount of Ser-tRNA^{Thr} in the mis-charging assay in contrast to the native enzyme (Figure 6C). Retention of the intact editing active site combined with the Ser-tRNA^{Thr} accumulation suggested that the mis-charged tRNA end bypasses the editing active site for hydrolysis after mis-aminoacylation. R303A was unable to clear pre-formed Ser-tRNA^{Thr}, also suggesting that the acceptor end was not directed to the editing active site (Figure 6D). In TLC assay, the k_{obs} of AMP formation catalyzed by R303A was reduced to $(3.05 \pm 0.33) \times 10^{-3} s^{-1}$ without affecting tRNA-independent pre-transfer editing (Table 3), showing that the translocation impairment was also detrimental to the tRNA-dependent pre-transfer editing activity. These data further confirmed the importance of the correct interaction between the enzyme and acceptor end as revealed by the Tyr^{291} mutants and showed that Arg^{303} contributes to aminoacylation and then directs the mis-charged 3′-end from the charging site to the editing site for mistake removal.

Table 4. k_{obs} of *Sc*ThrRS in AMP formation in the presence of Ser and tRNA^{Thr} or its variants

tRNA	k_{obs} ($\times 10^3$)(s^{-1})[a]	Relative k_{obs} (%)[b]
No tRNA^{Thr}	1.32 ± 0.19	13.50
tRNA^{Thr}	9.80 ± 0.87	100
A73U	10.78 ± 1.32	110.00
A73C	8.09 ± 1.03	82.55
A73G	5.60 ± 0.63	57.14
G35A	3.83 ± 0.54	39.08
G35U	4.75 ± 0.61	48.47
G35C	2.43 ± 0.24	24.80
U36A	2.14 ± 0.33	21.84
U36C	1.60 ± 0.19	16.33
U36G	1.50 ± 0.28	15.31

[a]The results are the average of three independent repeats with standard deviations indicated.
[b]The k_{obs} values are relative to that of tRNA^{Thr}.

Separation of the two active sites impairs tRNA-charging and -editing activities

ymThrRS is an atypical ThrRS containing only the aminoacylation and anti-codon-binding domains, suggesting that the N-terminal region of bacterial ThrRS is dispensable in the mitochondrial translational system (Supplementary Figure S1A) (15). To investigate whether its cytoplasmic counterpart encompassing Asp^{311}-Ala^{734} of *Sc*ThrRS (*Sc*ThrRS$^{311-734}$) is sufficient for cytosolic translation, *Sc*ThrRS was divided between Pro^{310} and Asp^{311} to obtain *Sc*ThrRS$^{1-310}$ and *Sc*ThrRS$^{311-734}$. The former contains the N-extension, N1 and N2 domains (Supplementary Figure S1A). Our results showed obvious decreases in both the amino acid activation and aminoacylation activities of *Sc*ThrRS$^{311-734}$ (Figure 2B and C). The residual activity failed to compensate for the loss of the native enzyme activity in *ScΔthrS* (Figure 2D).

In contrast, *Sc*ThrRS$^{1-310}$ clearly hydrolyzed Ser-tRNA^{Thr}, indicating that the function of the isolated editing domain of ThrRS in post-transfer editing is independent of the aminoacylation and tRNA-binding

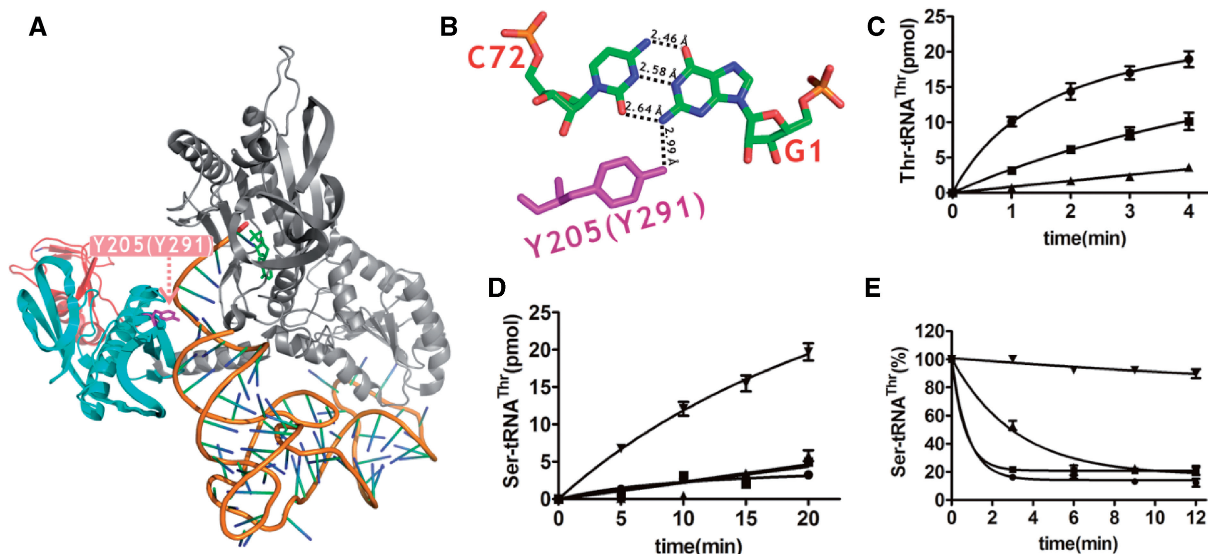

Figure 5. Tyr^{291} contributed to tRNA-dependent pre-transfer editing. (**A**) A crystal structure of *Ec*ThrRS-tRNA^{Thr} (PDB code 1QF6) showing the location of Tyr^{205} as indicated, corresponding to Tyr^{291} of *Sc*ThrRS. The AMP in the aminoacylation active site was shown in green. The N1, N2 and aminoacylation plus anti-codon-binding domains were shown in red, cyan, and light gray, respectively. (**B**) Schematic representation of the interaction between Tyr^{205} of *Ec*ThrRS and the G1-C72 base pair of tRNA with distances indicated. Aminoacylation of *Sc*tRNA^{Thr} (**C**), mis-aminoacylation of *Sc*tRNA^{Thr} (**D**) and post-transfer editing acitivity (**E**) of *Sc*ThrRS (closed circles), Y291R (closed squares) and Y291E (filled triangles). Controls [mis-aminoacylation of *Sc*tRNA^{Thr} by H151A/H155A in (**D**) and spontaneous hydrolysis of mis-charged *Sc*tRNA^{Thr} in (**E**)] are shown (inverted filled triangles).

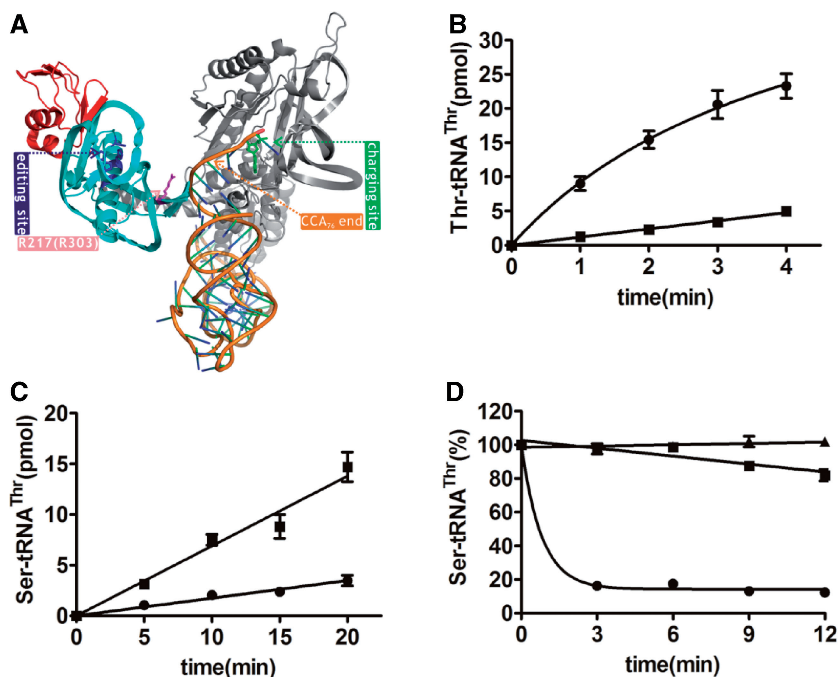

Figure 6. Arg[303] contributed to translocation of the tRNA 3′-end. (**A**) Crystal structures (PDB code 1QF6) showing the location of Arg[217] as indicated, corresponding to Arg[303] of *Sc*ThrRS. Aminoacylation (**B**), mis-aminoacylation (**C**) of *Sc*tRNA^{Thr} and post-transfer editing capacity (**D**) of *Sc*ThrRS (closed circles) and R303A (closed squares). Spontaneous hydrolysis of mis-charged *Sc*tRNA^{Thr} in (**D**) is shown as a control (filled triangles).

domains (Supplementary Figure S4). However, the editing efficiency was obviously decreased compared with that of native *Sc*ThrRS, suggesting the tRNA-binding domain and C-terminal domain contributed to the post-transfer editing activity.

Yeast tolerates mutation within the editing active site

To investigate the *in vivo* significance of ThrRS-mediated editing, *Sc*ThrRS and the editing-deficient H151A/H155A were expressed in *ScΔthrS*. Growth of the transformants was observed on SD/Leu^{-}/G418 plates containing increasing concentrations of Ser (0, 2, 5, 20, 50, 200 and 500 mM). Cell growth of the *ScΔthrS* strains expressing the native or editing-deficient enzymes was comparable. In the presence of 500 mM Ser, both strains displayed an obvious and equivalent decrease in the growth rate compared with that on media containing Ser at lower concentrations (Figure 7). Furthermore, there were no differences in the time-course growth curves of the two transformants (Supplementary Figure S5A). To exclude the possibility of reverse mutation of the two His codons to Ala codons under such a stress condition, DNA sequencing of the plasmids extracted from both transformants grown in liquid SD/Leu^{-}/G418 containing 500 mM Ser was performed, and no reverse mutations were detected (Supplementary Figure S5B). The native and mutant enzymes from both yeast transformants were purified by Ni-NTA affinity chromatography (Supplementary Figure S5C), and both enzymes were shown to possess similar aminoacylation activity (Supplementary Figure S5D) but only H151A/H155A clearly synthesized Ser-tRNA^{Thr} (Supplementary Figure S5E). These data collectively suggested that yeast is tolerant of the mutations in the crucial editing active sites, implying that Ser is not mis-inserted at Thr codons during protein biosynthesis. To test this implication, tandem mass spectrometry was performed to identify Ser mis-incorporation in the proteomes of the two transformants grown in SD/Leu^{-}/G418 containing 500 mM Ser. Approximately 855 or 860 types of proteins were analyzed from *ScΔthrS* expressing *Sc*ThrRS or H151A/H155A. Only six and nine points of Thr-to-Ser mis-incorporation were identified in the proteomes of *ScΔthrS* expressing *Sc*ThrRS and H151A/H155A, respectively. This comparable data suggested that no significant Ser mis-insertion occurred after editing active site mutation. Then, we introduced mutation on Tyr[291] (crucial for the tRNA-dependent pre-transfer) based on post-transfer editing-defective H151A/H155A to get H151A/H155A/Y291R and H151A/H155A/Y291E. The p425TEF constructs encoding H151A/H155A/Y291R and H151A/H155A/Y291E were introduced into *ScΔthrS*. After loss of rescue plasmid, the growth of the transformants expressing both triple mutants were comparable with that of yeasts expressing *Sc*ThrRS and H151A/H155A with increasing concentrations of Ser. Above all, it strongly suggests that discrimination against non-cognate Ser-tRNA^{Thr} by the elongation factor and/or the ribosome takes place downstream of the mis-charging step.

Cross-species *in vivo* recognition toward yeast tRNA^{Thr}

ThrRSs are classified into two major groups, bacterial and archaeal types. The former one includes enzymes from bacteria (such as *E. coli*), mitochondria (such as human mitochondria) and eukaryotic cytoplasm (such as yeast

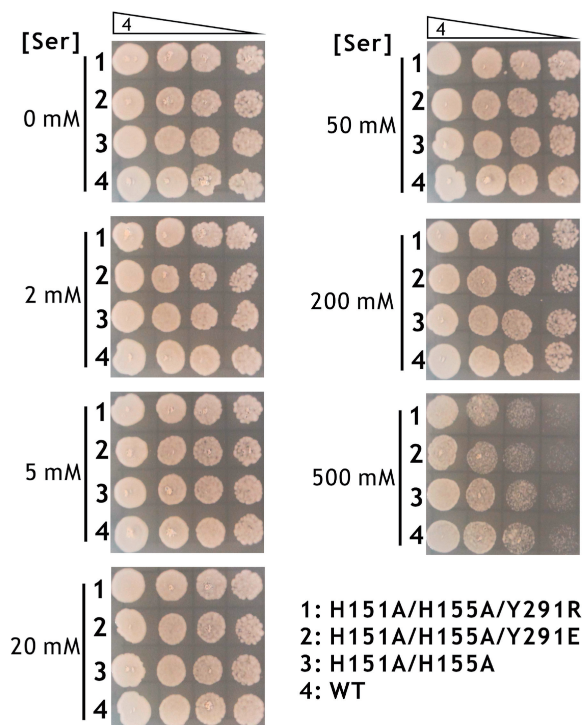

Figure 7. Yeast-tolerated editing active sites mutation. Growth of *ScΔthrS* expressing *Sc*ThrRS, H151A/H155A, H151A/H155A/Y291R and H151A/H155A/Y291E after loss of rescue plasmid-encoded *Sc*ThrRS was compared under different stress conditions in the presence of the indicated increasing concentrations of Ser. A 4-fold dilution of yeast with initial 1 OD_{600} was performed. Labels 1, 2, 3 and 4 indicate yeasts expressing H151A/H155A/Y291R, H151A/H155A/Y291E, WT *Sc*ThrRS and H151A/H155A, respectively.

and human). However, it is more divergent in ThrRSs from archaea, which is further composed of two situations. Majority archaea contains a single-polypeptide ThrRS (such as *Natrialba magadii* ThrRS), whereas some crenarchaeal species encodes a catalytically editing-domain-lacking protein for aminoacylation (such as *Sulfolobus islandicus* ThrRS, *Si*ThrRS-cat) besides a freestanding editing protein (21). To test whether the above ThrRSs could complement the *ScΔthrS*, we introduced these genes into *ScΔthrS,* and the complementation assay was performed (the mitochondrial targeting sequence of mtThrRS was removed). The data showed that only *Ec*ThrRS could support yeast growth despite lower efficiency when compared with *Sc*ThrRS. The remaining ThrRSs were all unable to replace *Sc*ThrRS for protein synthesis (Supplementary Figure S6).

DISCUSSION

As an essential component of protein translation apparatus, aaRS matches a specific tRNA and its cognate amino acid to provide aminoacyl-tRNA substrate for the ribosome. Although preserving the fundamental aminoacylation role, aaRSs from higher eukaryotes have developed other, non-canonical functions (32). Eukaryotes usually express only one aaRS in a single cell compartment for local protein biosynthesis. However, there is sporadic example of two tRNA synthetases in one compartment. For instance, human cell cytoplasm contains two ArgRSs, which are derived from two translational initiation sites by a single mRNA (33). The short ArgRS was proposed to participate in the protein modification at the N-terminus for the protein targeted for degradation by providing the substrate for argninyl-tRNA transferase (34). Interestingly and intriguingly, ThrRS-L is present and conserved only in higher eukaryotes, suggesting that it might function in some higher organism-specific pathways. Therefore, further functional clarification of hcThrRS-L is required. Furthermore, eukaryotic cytoplasmic ThrRSs have evolved an N-extension of previously unidentified origin and function. This study demonstrated that the N-extension is indispensable for the enzyme function. It can also be speculated that the N-extension regulates the overall protein conformation. Considering appended or inserted domain is often involved in cellular processed other than aminoacylation, it is likely that the N-extension of ThrRS or ThrRS-L may also participate in other non-canonical yet unidentified functions (35).

The editing of aaRS is critical for faithful translation of the genetic code by ensuring only the cognate amino acid is linked with a given tRNA molecule (1). As a small molecular with little recognizable difference element, the accurate selection of cognate amino acid seems to be a great challenge for some aaRSs. ThrRS is such an aaRS, which should discriminate cognate Thr against near cognate Ser to prevent mis-acylated Ser-$tRNA^{Thr}$ being used by the protein synthesis apparatus (16).

*Ec*ThrRS was previously thought to possess only post-transfer editing activity (18). Recently, *Ec*ThrRS was found to exhibit pre-transfer editing activity against Ser (36). Kinetic proofreading activity of *Sc*ThrRS was negligible compared with the total editing capacity. This phenomenon is also observed in class I LeuRS, IleRS, ValRS (24,28) and class II ProRS (29). Without tRNA, *Sc*ThrRS possessed measurable and comparable levels of tRNA-independent pre-transfer editing activity for both Thr and Ser. In sharp contrast, tRNA addition triggered robust tRNA-dependent pre-transfer editing of Ser but not Thr. This editing selectivity prevented hydrolysis of Thr-AMP. Strikingly, tRNA-dependent pre-transfer editing constituted a major contribution to the total editing of *Sc*ThrRS. By mutating the HXXXH motif, the post-transfer editing was effectively isolated from the tRNA-independent and tRNA-dependent pre-transfer editing. Furthermore, tRNA-dependent pre-transfer editing activity was successfully isolated from the tRNA-independent pre-transfer and post-transfer editing pathways by mutation of Tyr^{291} (especially Y291E). H151A/H155A produced a significant amount of Ser-$tRNA^{Thr}$ although the tRNA-independent and tRNA-dependent pre-transfer editing activities remained intact. However, the mutants Y291R and Y291E, which kept intact post-transfer editing activity, did not generate Ser-$tRNA^{Thr}$ despite totally or partially deficient tRNA-dependent pre-transfer editing activity. This evidence clearly suggested that post-transfer editing, despite the minor contribution to AMP formation,

constituted the most crucial and efficient aminoacylation quality control mechanism and that the tRNA-independent and tRNA-dependent pre-transfer editing pathways combined were unable to prevent formation of mis-charged tRNA.

This study demonstrated that Tyr^{291} mutants specifically influenced tRNA-dependent pre-transfer editing but not post-transfer editing. The data obtained using the Y291E and R303A mutants suggest that the tRNA acceptor end and enzyme interaction are required for tRNA-dependent pre-transfer editing. According to the *Ec*ThrRS-$tRNA^{Thr}$ structure (PDB code 1QF6, Figure 6A), we proposed Arg^{303} contributes to aminoacylation by binding the phosphate backbone of C72-A73 of $tRNA^{Thr}$, aiding in the formation of the specific interaction with the CCA_{76} tail during translocation. Similarly, in class Ia *Ec*LeuRS, the binding between the tRNA acceptor end with the conserved Tyr^{330} in the editing domain is also crucial for the tRNA-dependent pre-transfer editing (5,11). Thus, the triggering of tRNA-dependent pre-transfer editing by tRNA addition might require tRNA acceptor end and enzyme interaction in both aaRS classes.

Mutation of the editing active sites is detrimental to *E. coli* and mammalian cell metabolism (4–7). Considering the ThrRS system, editing deletion leads to obvious growth arrest even in the presence of trace amounts of Ser (0.5 mM) in *Sulfolobus solfataricus* (21). Strikingly, yeast cell growth was not obviously affected under normal or stress conditions after mutations in the editing active sites. Therefore, the significance of editing by an aaRS may be species specific. H151A/H155A retained intact tRNA-dependent pre-transfer editing activity, whereas post-transfer editing activity was completely abolished. However, the two triple mutants harbored mutations at the sites, which were essential for both tRNA-dependent pre-transfer editing and post-transfer editing. Therefore, it is very likely that the produced Ser-$tRNA^{Thr}$ is efficiently excluded from the protein biosynthesis machinery by the elongation factor and/or the ribosome. Despite the toleration of mutation at editing active site with the presence of Ser, however, the editing function by ThrRSs might be strictly kept because of the presence of various Thr non-cognate amino acids and/or metabolite.

In summary, our data investigated the aminoacylation and editing features of *Sc*ThrRS, clarified the editing mechanism by eukaryotic cytoplasmic ThrRS and provided the first comprehensive understanding of eukaryotic ThrRS-mediated translational quality control preventing Ser mis-incorporation at Thr codons in yeast.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–6.

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