Newly acquired N-terminal extension targets threonyl-tRNA synthetase-like protein into the multiple tRNA synthetase complex

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Received April 08, 2019; Revised June 19, 2019; Editorial Decision June 24, 2019; Accepted June 26, 2019

ABSTRACT

A typical feature of eukaryotic aminoacyl-tRNA synthetases (aaRSs) is the evolutionary gain of domains at either the N- or C-terminus, which frequently mediating protein–protein interaction. TARSL2 (mouse Tarsl2), encoding a threonyl-tRNA synthetase-like protein (ThrRS-L), is a recently identified aaRS-duplicated gene in higher eukaryotes, with canonical functions in vitro, which exhibits a different N-terminal extension (N-extension) from TARS (encoding ThrRS). We found the first half of the N-extension of human ThrRS-L (hThrRS-L) is homologous to that of human arginyl-tRNA synthetase. Using the N-extension as a probe in a yeast two-hybrid screening, AIMP1/p43 was identified as an interactor with hThrRS-L. We showed that ThrRS-L is a novel component of the mammalian multiple tRNA synthetase complex (MSC), and is reliant on two leucine zippers in the N-extension for MSC-incorporation in humans, and mouse cell lines and muscle tissue. The N-extension was sufficient to target a foreign protein into the MSC. The results from a Tarsl2-deleted cell line showed that it does not mediate MSC integrity. The effect of phosphorylation at various sites of hThrRS-L on its MSC-targeting is also explored. In summary, we revealed that ThrRS-L is a bona fide component of the MSC, which is mediated by a newly evolved N-extension domain.

INTRODUCTION

The genetic code is a universal algorithm for linking nucleotide triplets in mRNAs to the amino acids in proteins via mRNA translation (protein synthesis). Aminoacyl-tRNA synthetases (aaRSs) comprise a ubiquitously expressed family of enzymes that catalyze the esterification reaction to ligate transfer RNAs (tRNAs) with their cognate amino acids to generate aminoacyl-tRNAs for protein biosynthesis at the ribosome (1,2). Usually, tRNA charging (aminoacylation) occurs in two-step reactions involving amino acid activation, with the generation of intermediate aminoacyl-AMP, and transfer of the aminoacyl moiety of the intermediate to A76 of tRNA (1,3). Some aaRSs also catalyze a proofreading and editing reaction to remove mis-activated amino acids and mischarged tRNAs caused by an inability to correctly discriminate cognate amino acids from noncognate ones, which ensures a high level of accuracy in protein biosynthesis (4,5). The activities of aminoacylation and editing are canonical functions of aaRSs that maintain the speed and fidelity of genetic code transduction.

AaRSs have been present in the last common ancestor of the tree of life. Modern aaRSs are split into two distinct classes based on the architectures of the two distinct active sites, which are considered to be the scaffold of ancient aaRSs (6,7). All present-day aaRSs contain catalytic and anticodon binding domains to perform aminoacylation, together with editing domains in some aaRSs to carry out the editing activity. However, during evolution of aaRSs from prokaryotes to archaea and eukaryotes, certain aaRSs obtain new appended domains, usually at the N- or C-terminus (8), with unique structural characteristics that are not a part of the catalytic core, but frequently mediate
protein–protein interactions in various functions unrelated to canonical aminoacylation and editing, such as translation and transcription regulation, angiogenesis, inflammation and tumorigenesis (9–13).

Another well-characterized feature of aaRSs, especially in eukaryotes, is the presence of the high order multiprotein complex. For instance, in *Saccharomyces cerevisiae*, glutamyl-tRNA synthetase (GluRS) and methionyl-tRNA synthetase (MetRS) stably interact with a nonsynthetase accessory protein, Arc1p (14). An aaRS-containing complex is also observed in archaea (15). In human cytoplasm, nine aaRSs [including arginyl-, aspartyl-, glutaminyl-, glutamyl-prolyl-, isoleucyl-, leucyl-, methionyl- and lysyl-tRNA synthetases (ArgRS, AspRS, GlhRS, GluProRS, IleRS, LeuRS, MetRS, LysRS, respectively] with three auxiliary scaffold protein co-factors [aminoacyl-tRNA synthetase complex-interacting multifunctional protein (AIMP)1/p43, AIMP2/p38 and AIMP3/p18] (hereinafter referred to as p43, p38 and p18) form a multiple tRNA synthetase complex (MSC) (16). It has been proposed that the newly acquired domains are related with the occurrence of the MSC (8). The interaction pattern between the extra domains in human cytoplasmic aaRSs has been studied or confirmed by cross-linking (17), yeast two-hybrid (18), co-immunoprecipitation (19) assays and co-crystal structure determination with different components (20). The detailed mechanism of the formation of the MSC in higher eukaryotes is unclear; however, some models, such as facilitating tRNA channeling between various translation apparatus or functioning as a reservoir for regulatory proteins that, upon inducible release, acquire new auxiliary functions, or facilitating the tRNA transport from nucleus to cytoplasm, have been proposed (21,22). Indeed, several components of the human MSC, such as LysRS, GluProRS, LeuRS, GlnRS and MetRS, can be either released from the complex or have non-canonical functions in other cellular compartments under specific stresses or stimulations (9,10,23–25).

It is believed that there are 37 aaRS genes in human cells, encoding full sets of aaRSs for both cytoplasmic and mitochondrial protein synthesis (26). However, an aaRS-duplicated gene, *TARS* was identified in 2013 for the first time in our laboratory, which encodes a threonyl-tRNA synthetase (ThrRS)-like protein (ThrRS-L) (27). ThrRS-L shares high similarity with canonical ThrRS in the N1, N2, aminoacylation and C-terminal tRNA binding domains; however, it has evolved a quite different N-terminal extension (N-extension) from that of ThrRS. Later, human ThrRS-L (hThrRS-L) was found to be a component of the MSC by affinity purification-mass spectrometry in human cells, despite the fact that quantitative determination showed an obviously low abundance of ThrRS-L in human cell lines (28,29). In mouse, *Tars* encodes mouse ThrRS-L (mThrRS-L), which has 86% identity with hThrRS-L. Recently, we further revealed that the mRNA of mouse *Tars* is present at significantly lower levels than that of *Tars* in various mouse tissues and mouse cell lines with most enriched proteins in the muscle and heart (30). Interestingly, mThrRS-L is able to locate to the nucleus and, in vitro, can catalyze aminoacylation with similar efficiency despite having decreased editing activity and a distinct cross-species tRNA recognition capability compared with those of mouse ThrRS (mThrRS) (30). However, whether mThrRS-L is responsible for canonical tRNA aminoacylation to generate Thr-tRNA<sub>Thr</sub> for protein biosynthesis in vivo is unclear. Furthermore, its presence only in higher eukaryotes implies it has a non-canonical function. Harbouring a unique N-extension, whether it is a bona fide component of the MSC, and what is the mechanism of ThrRS-L-incorporation into MSC, are also unknown.

In the present study, based on the unique N-extension of hThrRS-L, we first analyzed its primary sequence and then used it as a bait protein to perform a yeast two-hybrid screening. We identified p43 is one of the proteins that interact with hThrRS-L, and further provided extensive data showing that ThrRS-L is a bona fide member of the MSC in humans and mice. We also revealed detailed mechanism of hThrRS-L targeting into the MSC. Furthermore, based on a *Tars*-deleted cell line, we showed that absence of mThrRS-L has no effect on MSC integrity.

**MATERIALS AND METHODS**

**Materials**

Dithiothreitol (DTT), Tris-base, phenyl methylsulfonyl fluoride (PMSF) and NaCl, were purchased from Sangon (Shanghai, China). KOD-plus mutagenesis kits were obtained from TOYOBO (Osaka, Japan). All restriction endonucleases, T4 DNA ligase, T4 Polynucleotide Kinase, Dynabeads protein G and Lipofectamine 2000 transfection reagent were obtained from Thermo Scientific (Waltham, MA, USA). All materials for the yeast two-hybrid screening and assay, including the Matchmaker Gold Yeast Two-Hybrid System, media, reagents, and a normalized Mate & Plate human cDNA library, were purchased from Clontech (Shiga, Japan). *Escherichia coli* Rosetta (DE3) cells were purchased from Stratagene (Santa Clara, CA, USA). Polyvinylidene fluoride (PVDF) membranes were obtained from Millipore (Darmstadt, Germany). DNA sequencing was performed by Biosune (Shanghai, China).

**Antibodies**

Anti-mThrRS-L, anti-FLAG, anti-His<sub>6</sub>-tag, anti-α-tubulin and anti-glycyl-tRNA synthetase (GlyRS) were detailed in a previous report (30). A glutathione-S-transferase (GST) fusion protein containing the N-terminal region of hThrRS-L (Met<sup>1</sup>–Leu<sup>50</sup>) or that of human ThrRS (hThrRS) (Met<sup>1</sup>–Glu<sup>52</sup>) was used as an antigen to generate anti-hThrRS-L or anti-hThrRS antibodies. Anti-Myc (M4439), anti-β-actin (A1978), anti-enhanced green fluorescent protein (EGFP) (G1544), HRP (horse radish peroxidase)-labeled anti-mouse and anti-rabbit secondary antibodies were obtained from Sigma-Aldrich (St. Louis, MO, USA). Details for the anti-p43, anti-LysRS, anti-ArgRS, anti-GluProRS, anti-LeuRS and anti-GlnRS were provided in a previous report (31).

**Cloning**

Genes encoding hThrRS and hThrRS-L were amplified from cDNA, obtained by reverse-transcription PCR from total RNA extracted from Human Embryonic Kidney 293T...
(HEK293T) cells. DNA encoding a C-terminal FLAG- or His$_6$-tagged hThrRS-L was cloned into pCMV-3Tag-3A. DNA encoding an N-terminal HA- or Myc-tagged hThrRS was inserted into pcDNA3.1. The DNA fragment (named as TLN161) encoding the N-terminal appended domain of hThrRS-L (Met$^1$–Ile$^{161}$, TLN161) was inserted into the gap between the NdeI and BamHI sites of pGBK7 to produce the recombinant plasmid pGBK7-TLN161. Co-expression of the two genes encoding hThrRS-L and any of the three (p43, LysRS or ArgRS, respectively) was performed by inserting the two genes into the gaps between the two cloning sites of pRSFDuet1; and the recombinant plasmid was then transformed into E. coli. Gene mutagenesis or fragment deletion was performed according to the protocol provided with the KOD-plus mutagenesis kit.

**Yeast two-hybrid assay**

All the yeast two-hybrid screening or assays were performed according to the user manual with Matchmaker Gold Yeast Two-Hybrid System. Briefly, pGBK7-TLN161 was transformed into the Y2HGold yeast strain to express a Gal4 DNA binding domain fused TLN161, which was then tested for autoactivation and toxicity. Y2HGold cells expressing TLN161 were mated with Y187 yeast strains containing a normalized Mate & Plate human cDNA library and plated on Quadruple Dropout (QDO) (SD/Ade$^−$/His$^−$/Leu$^−$/Trp$^−$) medium supplemented Aureobasidin A and X-α-Gal (QDO/X/A). The blue colonies that grew on the QDO/X/A plate were selected and their plasmids were extracted. The DNA fragment in the pGADT7 vector was sequenced. To confirm the interaction between TLN161 with other targeted proteins, the gene encoding targeted protein was cloned into the pGADT7. Subsequently, the construct was transformed into the Y2HGold yeast strain together with pGBK7-TLN161. The transformant was plated on QDO/X/A and its growth was observed.

**Cell culture and transfection**

HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, high glucose) supplemented with 10% fetal bovine serum (FBS) in a 37°C incubator with 5% CO$_2$. Transfection was performed using the Lipofectamine 2000 transfection according to the manufacturer’s protocol. Twenty-four hours after transfection, the cells were washed with 5 ml of ice-cold phosphate-buffered saline (PBS) twice, and lysed with 1 ml of ice-cold lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100] supplemented with a protease inhibitor cocktail. The supernatant was collected using centrifugation at 12,000 $\times$ g for 30 min. All procedures were performed on ice.

**Gel filtration chromatography of cell lysates**

Cytosolic or mouse tissue extracts were applied to a Superose-6 column for high-performance liquid chromatography (HPLC) and eluted at a flow rate of 0.5 ml/min by using a buffer containing 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 1 mM phenyl methylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT). Fractions were collected for immunoblotting (western blotting).

**Western blotting**

The different proteins of interest in whole cell lysates were separated using 10% SDS-PAGE with pre-stained molecular protein standards; and then transferred to a PVDF membrane. The PVDF membrane was then cropped to blot different proteins in the same lane. After blocking with 5% (w/v) non-fat dried milk, the membranes with targeted proteins were incubated and detected with the corresponding primary antibodies overnight at 4°C. The membranes were then washed three times using PBS plus 0.05% Tween-20 (PBST) (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$ and 0.5% Tween-20) and incubated with HRP-conjugated secondary antibody at room temperature for 30 min. After washing three times with PBST, the membranes were treated with the chemiluminescent substrate, and imaging was performed using the Amersham imager 680 system (GE, CA, USA).

**Immunoprecipitation**

Whole cell lysates with different proteins of interest were incubated with specific primary antibody with agitation overnight, and then the mixture was incubated with Dynabeads protein G for 3 h. Recovered immune complexes were washed three times using PBST. All procedures are performed at 4°C. Proteins were eluted from the beads in 2× protein loading buffer (100 mM Tris–HCl, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM DTT), boiled for 10 min at 95°C. The retrieved proteins were subjected to SDS-PAGE and further analyzed by western blotting or visualized and identified by silver staining and mass spectrometry (MS), respectively.

**Obtaining a Tarsl2 deletion NIH/3T3 cell line**

The Tarsl2 deletion NIH/3T3 cell line was constructed using CRISPR/Cas9 mediated gene targeting technology (32). Briefly, a guide RNA (sgRNA) (5′-GGCGGAGCAGCGGCAGCCGGAGG-3′ with the PAM sequences underlined) was designed that targeted exon 1 of the transcript of Tarsl2 (ENSMUST00000032728.8). Sense and antisense oligonucleotides for the guide RNA were cloned into pX330-mCherry plasmid to produce pX330-mCherry-sgRNA. The NIH/3T3 cells were transfected with pX330-mCherry-sgRNA using lipofectamine 2000 according to the manufacturer’s instructions. Twelve hours after transfection, the NIH/3T3 cells expressing sgRNA with the red fluorescence protein were sorted using flow cytometry (FACS Aria SORP) and plated at 96-well cell culture plates. The genotypes of NIH/3T3 cell lines were analyzed by DNA sequencing of PCR products amplified from targeted sites.
RESULTS

The N-terminal extension of hThrRS-L is homologous to that of the long isoform of human cytoplasmic ArgRS

Sequence analysis among bacterial ThrRSs, canonical eukaryotic cytoplasmic ThrRSs and mammalian ThrRS-Ls showed that both eukaryotic cytoplasmic ThrRSs and ThrRS-Ls have acquired newly evolved N-terminal extensions (N-extensions) when compared with bacterial ThrRSs (except truncated yeast mitochondrial or Mycoplasma ThrRSs) (33, 34) (Figure 1A). For the human enzymes, the N-extensions of hThrRS (Uniprot: P26639) and hThrRS-L (Uniprot: A2RTX5) are peptides with 82 or 161 amino acid residues. The two extensions share conserved residues in the last sections but with 64% identity in the full-length enzymes. Besides, the sequence identity between hThrRS-L and human mitochondrial ThrRS precursor is only 46%. A similar observation was made for the N-extensions of mThrRS (Uniprot: Q9D0R2) and mThrRS-L (Uniprot: Q5BLY2), with 81 and 149 residues, respectively. However, the N-extensions between various ThrRS-Ls or ThrRSs are highly conserved (Figure 1B). These observations suggested that the N-extensions of ThrRSs and ThrRS-Ls are likely to be of different origins.

We searched for any potential homologous peptide of N-extensions of ThrRS-Ls using BLASTP analysis. The N-terminal region (Met1–Glu78) of hThrRS-L was significantly homologous to the N-extension of the long isoform of human cytoplasmic arginyl-tRNA synthetase (ArgRS), demonstrating that the formation of an hThrRS-L/hThrRS complex could be readily precipitated by hThrRS-L-His6, demonstrating the formation of an hThrRS-L/hThrRS complex (Figure 2C). In fact, we also revealed that overexpressed hThrRS-L forms a complex with native hThrRS in vivo and that native mThrRS-L and mThrRS formed a complex in muscle tissue (see text below). Consistently, our recent data showed that native hThrRS-L and hThrRS could be pulled down by overexpressed mThrRS-L (30). Taken together, these data clearly showed that ThrRS-L has the potential to form a homodimer and a complex with ThrRS. Considering the dimerization characteristics of class II tRNA synthetase, we hypothesized that the complex of ThrRS-L and ThrRS is a heterodimer. We further showed that the N-extensions of ThrRS-L is not involved in interaction with hThrRS (see details below), consistent with dimerization of ThrRS relying on the aminoacylation domain.

The N-terminal extension of hThrRS-L is not involved in interaction with hThrRS

The results of a co-precipitation (Co-IP) assay showed that hThrRS-L could be readily precipitated by hThrRS-L-His6, demonstrating the formation of an hThrRS-L/hThrRS complex (Figure 2C). In fact, we also revealed that overexpressed hThrRS-L forms a complex with native hThrRS in vivo and that native mThrRS-L and mThrRS formed a complex in muscle tissue (see text below). Consistently, our recent data showed that native hThrRS-L and hThrRS could be pulled down by overexpressed mThrRS-L (30). Taken together, these data clearly showed that ThrRS-L has the potential to form a homodimer and a complex with ThrRS. Considering the dimerization characteristics of class II tRNA synthetase, we hypothesized that the complex of ThrRS-L and ThrRS is a heterodimer. We further showed that the N-extension of ThrRS-L is not involved in interaction with hThrRS (see details below), consistent with dimerization of ThrRS relying on the aminoacylation domain.

p43 was identified to interact with ThrRS-L based on a yeast two-hybrid screening

To identify any potential proteins that interact with hThrRS-L, and to exclude those interacting with hThrRS, we used hThrRS-L-specific TLN161 as a probe to perform a yeast two-hybrid screen on a human cDNA library. After confirming that TLN161 did not act as a transcription factor (data not shown), we found 78 positive clones containing proteins that interacted with TLN161. Among these clones, some encoded the same proteins or domains and some do not harbor in-frame coding sequences in comparison with human Open Reading Frames (ORFs). At the end, 12 proteins were screened. Among these proteins, p43 was the only protein functionally associated with tRNA synthetase (2# and 4# in Figure 3A). All other 11 proteins are non-aaRS proteins. Based on our previous study showing that ThrRS-L is active in canonical functions as an aaRS (30), we initially focused on potential interaction between ThrRS-L and p43. To confirm the interaction between ThrRS-L and p43 in vitro, we overexpressed hThrRS-L-His6 in HEK293T cells and performed a Co-IP assay. The data showed that native p43 was indeed co-precipitated with the FLAG antibody (Figure 3B). We also expressed hThrRS-L-His6 and p43 in E. coli. Despite that hThrRS-L forms extensive inclusion bodies in E. coli; however, after purification using Nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography, western blotting analysis showed that p43 could be co-precipitated with hThrRS-L-His6; however human cytoplasmic LysRS could not. The above data clearly showed that hThrRS-L interacts with p43 in vivo (Figure 3C).

In spite of its main localization in the MSC, p43 is a multi-functional protein present in several cellular locations, including the nucleus, secretion out of the plasma membrane, and in mitochondria (37, 38). The C-terminal region of p43, homologous to the endothelial monocyte-activating polypeptide-II (EMAP-II), is responsible for tRNA binding, while its N-terminal portion [p43(1–78), Met1–Lys78] mediates protein–protein interactions (37). Indeed, based on the co-crystal structure (PDB: 4R3Z) (20),
Figure 1. Primary sequence alignment and comparison of the N-extension of ThrRS-Ls with that of ThrRSs and ArgRSs. (A) Schema showing the N-extension of ThrRS-L, ThrRS, and ArgRS. (B) Sequence comparison between N-extensions of ThrRS-L (ThrRS-L-N) and ThrRS (ThrRS-N). (C) Sequence comparison between N-extensions of ThrRS-L and ArgRS. The two helixes (Ha and Hb) as revealed by the structure of ArgRS-GlnRS-p43 (PDB 4R3Z) are shown at the bottom. hThrRS and hThrRS-L represent human ThrRS and ThrRS-L; similarly, mThrRS and mThrRS-L represent mouse ThrRS and ThrRS-L. The amino acid residues for mutagenesis in hThrRS-L-Ha/A or hThrRS-L-Hb/A were indicated by the inverted triangles. Br, Bos Taurus; Rn, Rattus norvegicus; Ss, Sus scrofa.

The N-extension of ArgRS, comprising two α-helices, and the H1 helix of the core domain of ArgRS sandwich p43 by interacting with its long helix in the N-terminus. p43 has two isoforms with different N-terminus lengths, comprising 312 and 336 amino acid residues, respectively (Uniprot No. Q12904-1 and Q12904-2, respectively). The clone we screened contained the ORF of the 336-aa isofrom; therefore, in this work we focused on the interaction of TLN161 in hThrRS-L with the longer p43. The homology of the N-extension between hThrRS-L and ArgRS (Figure 1C) prompted us to split p43 into two fragments, p43(1-78)(Met1–Lys78) and p43(79-336)(Leu79–Ly336), to map the region mediating its interaction with hThrRS-L. Expressing each fragment as a fusion protein with the GAL4 DNA activation domain, we found that p43(1-78) interacts with TLN161 (5# in Figure 3A); however p43(79–336) does not (6# in Figure 3A). The shorter p43 isoform only lacked the 25 N-terminal amino acid residues. The peptide consisting of Met26–Lys78 [p43(26–78)] still interacted with TLN161 (7# in Figure 3A). To determine whether the ArgRS-homologous region, the N-terminal part of TLN161 [TLN161(1–74)(Met1–Cys74)], or the C-terminal part of TLN161 [TLN161(75–161)(Leu75–Ile81)], was the binding element with p43, the two peptides were fused separately with the GAL4 DNA binding domain. The results showed that the N-terminal part of TLN161 is responsible for binding p43 (8# in Figure 3A), and the C-terminal part of TLN is not (9# in Figure 3A).

Taken together, these data showed that hThrRS-L and p43 interact in vivo and the N-terminuses of both proteins are responsible for the interaction.

**ThrRS-L is a bona fide component of multiple-synthetase complex (MSC)**

Based on the interaction of hThrRS-L with p43, and the potential association of hThrRS-L with the MSC, we further explored whether hThrRS-L is a component of the MSC. Gel filtration chromatography was performed to enrich the MSC-containing fraction from the whole cell lysate of mammalian cells and mouse muscle tissue, in which Tarsl2 (mThrRS-L gene) is abundantly expressed. ThrRS-L clearly eluted simultaneously with known MSC components from HEK293T cell extracts (LeuRS and ArgRS, Figure 4A), mouse NIH/3T3 cell extracts (p43, Figure 4B), and extracts of mouse muscle tissue (p43 and ArgRS, Figure 4C). These data implied that ThrRS-L was a potential member of the MSC. To confirm the direct interaction of
Figure 2. Overexpressed hThrRS-L forms homodimers or heterodimers with overexpressed hThrRS in vivo. (A) After co-expression of genes encoding hThrRS-L-His6 and hThrRS-L-FLAG in HEK293T cells, hThrRS-L-His6 was readily pulled down by hThrRS-L-FLAG in a Co-IP assay. (B) After co-expression of genes encoding HA-hThrRS and Myc-hThrRS in HEK293T cells, HA-hThrRS was readily pulled down by Myc-hThrRS in a Co-IP assay. (C) After co-expression of genes encoding Myc-hThrRS and hThrRS-L-FLAG in HEK293T cells, Myc-hThrRS was readily precipitated by hThrRS-L-FLAG in a Co-IP assay.

Figure 3. Identification of p43 as an interactor of hThrRS-L. (A) Left, Y2H Gold yeast cells expressing either native p43 (336-aa in length) alone (1# and 3#) or p43 plus TLN161 (2# and 4#) were grown on SD/Ade−/His−/Leu−/Trp−/Xα-Gal (SD/QDO/X) plates, identifying p43 as a binding protein of TLN161; middle and right, interaction-domain mapping of p43 (5#-7#) and TLN161 (8# and 9#) on an SD/QDO/X plate as constructs expressing various proteins or domains shown at the bottom. (B) Co-IP analysis confirming the interaction between native p43 and overexpressed hThrRS-L-FLAG in HEK293T cells. (C) NiNTA chromatography purification analysis confirming interaction between overexpressed p43 and overexpressed hThrRS-L-His6 in E. coli. As a control, LysRS and hThrRS-L-His6 were also overexpressed in E. coli and LysRS was not detected together with hThrRS-L-His6.

ThrRS-L with the components of the MSC, a Co-IP assay was performed with both native HEK293T cell lysates and anti-hThrRS-L antibodies; several MSC components, including GluProRS, ArgRS and p43, were co-precipitated (Figure 4D). To explore whether the native MSC could be enriched using anti-mThrRS-L antibodies (30) in mice, we performed a Co-IP assay with anti-mThrRS-L antibodies in an extract of mouse muscle tissue. After precipitation, the products were separated by SDS-PAGE and bands were cut out for MS identification. Mouse GluProRS, IleRS, LeuRS, LysRS and ArgRS were clearly detected as the strong bands (bands of lower intensity were not analyzed) (Figure 4E). Strikingly, native mThrRS was pulled down together with native mThrRS-L, suggesting the interaction between native ThrRS and native ThrRS-L in vivo, which was consistent with data after gene overexpression (Figure 2C). In addition, two novel non-aRS proteins were also detected (indicated by asterisks in Figure 4E).

Both gel filtration and Co-IP analysis clearly showed that mammalian ThrRS-L is a bona fide component of the MSC in human and mouse cell lines or mouse muscle tissue.

Two leucine-zipper motifs in TLN161 target hThrRS-L into the MSC

We overexpressed the gene encoding hThrRS-L-FLAG in HEK293T cells. Co-IP assays showed that several MSC components, such as LeuRS, GluRS, ArgRS, and p43, were readily pulled down using anti-FLAG antibodies (Figure 5A), which was consistent with data from gel filtration and Co-IP analyses using native cells (Figure 4A, B) or tissue (Figure 4C). Again, although it is not a member of the MSC, native hThrRS was also co-precipitated (Figure 5A), further confirming that ThrRS and ThrRS-L interact in vivo (Figures 2C, 4E).

The interaction of TLN161 with p43, one of the core components of the MSC, suggested that TLN161 is the most likely element that mediates MSC incorporation of ThrRS-L. To test this possibility, we overexpressed a deleted TLN161 mutant with a C-terminal FLAG-tag, hThrRS-L-Δ161-FLAG, in HEK293T cells. In HEK293T cells containing hThrRS-L-FLAG, LeuRS, GluRS, ArgRS or p43 could be co-precipitated (Figure 5A) using anti-FLAG antibodies; however in HEK293T cells containing hThrRS-L-Δ161-FLAG, these bands disappeared (Figure 5B). Notably, hThrRS was pulled down with hThrRS-L-Δ161-
FLAG (Figure 5B), indicating that the N-extension was not required for binding hThrRS, consistent with reports that the aminoacylation domain of ThrRS is the dimer interface (6,36). These data showed that the N-extension of hThrRS-L is necessary for its incorporation into the MSC but is not necessary for the potential interaction with hThrRS.

Recently, two leucine-zipper motifs in two α helices (Ha and Hb) in the N-terminus of ArgRS were shown to mediate protein–protein interactions in the MSC (20). Based on the two conserved leucine-zipper motifs between ArgRS and hThrRS-L (Figure 1C) and the region (Met1–Cys74) covering these leucine-zipper motifs being the p43-interaction element (Figure 3A), we mutated counterparts of the leucine-zipper motif in Ha (including Leu15, Ile22, Leu25, Val29 and Leu32) or in Hb (including Leu51, Leu54, Leu68, Leu71 and Leu75) in hThrRS-L to Ala residues, obtaining the mutants hThrRS-L-Ha/A and hThrRS-L-Hb/A, respectively (Figure 1C). hThrRS-L-FLAG, hThrRS-L-Ha/A-FLAG or -Hb/A-FLAG were overexpressed in HEK293T cells. Similar protein levels were observed in the wild-type and the two mutants of hThrRS-L, suggesting the two leucine zippers had little effect on the stability of hThrRS-L in the cells (Figure 5C). However, Co-IP with anti-p43 antibodies could only precipitate hThrRS-L-FLAG, but not hThrRS-L-Ha/A-FLAG or hThrRS-L-Hb/A-FLAG (Figure 5C), indicating the importance of both leucine-zippers for MSC incorporation. Consistently, components of the MSC (including p43, ArgRS, GluProRS and LeuRS) were only pulled down with anti-FLAG antibodies in cells expressing hThrRS-L-FLAG but not in those expressing the two mutants (Figure 5C). However, hThrRS was readily precipitated with both wild-type and the two mutants of hThrRS-L, further confirming that its N-extension was not responsible for the interaction between hThrRS-L and hThrRS in vivo, consistent with data from N-extension truncated hThrRS-L (Figure 5B). We further obtained the precipitated hThrRS-L-FLAG, hThrRS-L-Ha/A-FLAG or hThrRS-L-Hb/A-FLAG as described in a previous report (30); their aminoacylation activities showed that the mutations did not affect the tRNA charging activity of hThrRS-L in vitro (Supplementary Figure S1). These results showed that both leucine-zippers in the N-terminal region of TLN161 (Met1–Cys74) are required to target hThrRS-L into the MSC, but have little effect on the stability and activity of hThrRS-L.

hThrRS-L also interacts with ArgRS and the two leucine-zippers mediate interaction with ArgRS and p43

The MSC contains nine aaRSs and three auxiliary proteins. Elements responsible for MSC targeting in most aaRSs has been identified (18). To determine whether hThrRS-L interacts with other proteins in the MSC, TLN161 was used as a probe in yeast two-hybrid assays, which have successfully uncovered the interaction network in the MSC.
(8, 18), to identify its potential interaction with most components of the MSC. To this end, we constructed eight genes encoding various proteins and peptides, comprising p18, p38, the WHEP domain of human GluProRS (Leu753–Asn1023), the N-terminal half of human cytoplasmic LysRS (Met1–Asp222), the N-terminal leucine-zipper domain of ArgRS (Met1–Asn72), the N-terminal GST domain of human MetRS (Met1–Ile268), the C-terminal appended domain of human IleRS (Asp966–Phe1262), and the C-terminal appended domain of human LeuRS (Gly1066–His1176), and then ligated them into pGADT7 separately and expressed them as fusion proteins with the GAL4 DNA activation domain in Y2HGold yeast cells expressing TLN161. The data showed that TLN161 clearly interacted with N-terminal domain of ArgRS (2#, 3# and 13# in Figure 6A) besides having a weak interaction with the C-terminal appended domain of LeuRS (data not shown). In addition, we observed that the N-terminal ArgRS-homologous region of TLN161 [TLN161(1–74)] (4# in Figure 6A), but not the C-terminal part of TLN161 [TLN161(75–161)] (5# in Figure 6A) was involved in the interaction between ArgRS and hThrRS-L. We further confirmed the direct interaction in E. coli transformants containing the genes encoding hThrRS-L-His8 and ArgRS after co-expression and Ni-NTA purification. Again, LysRS was not co-purified after co-expression and purification (Figure 6B).

Based on the fact that peptide Met1–Cys74 is able to interact with both ArgRS and p43, and the two leucine-zippers being responsible for hThrRS-L MSC-targeting, we further explored role of the two leucine-zippers in these interactions. When TLN161 was introduced with Ha/A or Hb/A mutations, the resultant TLN161-Ha/A or TLN161-Hb/A lost their interactions with ArgRS, suggesting that both leucine-zippers are essential for the interaction between hThrRS-L and ArgRS (6# and 7# in Figure 6A). However, both mutants interacted with p43 (8# and 9# in Figure 6A), suggesting other elements in the peptide Met1–Cys74 or either leucine-zipper is sufficient for the interaction. To test the first possibility, three TLN161 mutants in the linker region between the Ha and Hb helices were constructed [TLN161-M1/A (8DEQL37 mutated to 8AAA37), TLN161-M2/A (8QAE36 to 8AAA36), and TLN161-M3/A (8LIREVAQ56 to 8AAA56)]. All the mutants interacted with p43 and ArgRS as well as TLN161 (data not shown). When Ha/A and Hb/A were simultaneously introduced into TLN161, the mutant TLN161-Ha/Hb/A could not interact with p43 (12# in Figure 6A) and ArgRS, as expected (14# in Figure 6A), suggesting that at least one leucine-zipper of TLN161 is necessary to interact with p43 and both leucine-zippers are crucial to interact with ArgRS.

TLN161 is sufficient to incorporate a foreign protein into the MSC

To determine whether TLN161 was sufficient to target a foreign protein into the MSC, we expressed genes encoding TLN161 fused with a C-terminal EGFP (TLN161-EGFP) or EGFP alone in HEK293T cells. The MSC was precipitated using anti-p43 antibodies, as observed by the presence of ArgRS, GluProRS and LeuRS, but the absence of ThrRS and α-tubulin in the precipitated products. The presence of TLN161-EGFP, but not EGFP, in the MSC was confirmed using anti-EGFP antibodies, showing that TLN161 was sufficient to incorporate a foreign protein into the MSC (Figure 7).

Deletion of ThrRS-L has no effect on MSC integrity in vivo in mouse NIH/3T3 cells

To explore any potential role of ThrRS-L in MSC integrity, especially for the assembly of p43 and ArgRS into the complex, we initially inactivated Tarsl2 in mouse NIH/3T3 cell lines using CRISPR/Cas9. A clone (designated CYB) was obtained with a nucleotide ‘A’ insertion at position 288 of the first exon of Tarsl2 gene (position 242 in mRNA),

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**Figure 5.** Two leucine-zippers in the N-extension mediate targeting of hThrRS-L into MSC. hThrRS-L-FLAG (A) or hThrRS-L-Δ161-FLAG (B) was overexpressed in HEK293T cells and Co-IP was performed using the anti-FLAG antibody. Various known MSC components and hThrRS were detected using their specific antibodies. β-Actin was used as a negative (non-component) control. (C) hThrRS-L-FLAG, hThrRS-L-Ha/A-FLAG, or hThrRS-L-Hb/A-FLAG were overexpressed in HEK293T cells and Co-IP was performed using either anti-p43 or anti-FLAG antibodies. Various known MSC components and hThrRS were detected using their specific antibodies.
Figure 6. Two leucine-zippers in the N-extension mediate hThrRS-L interaction with ArgRS. (A) Growth of Y2H Gold yeast cells expressing either N-extension of ArgRS (ArgRS-N) alone (1#) or ArgRS-N plus TLN161 (2#, 3# and 13#) on SD/QDO/X plates. Domain mapping (4# and 5#) and leucine-zipper identification (6# and 7#) in TLN161 with ArgRS-N or with p43 (8# and 9#) were also performed using Y2H gold yeast cells by expressing various proteins or domains. In contrast to wild-type TLN161 (2#, 3# and 13# with ArgRS-N; 10# and 11# with p43), after mutation of both helices, the resultant TLN161-Ha/Hb/A lost interaction with both p43 (12#) and ArgRS-N (14#). (B) Ni-NTA chromatography purification analysis confirming interaction between overexpressed ArgRS and overexpressed hThrRS-L-His$_6$ in E. coli. As a control, LysRS and hThrRS-L-His$_6$ were also overexpressed in E. coli and LysRS was not detected together with hThrRS-L-His$_6$.

Figure 7. The N-extension of hThrRS-L is sufficient to assemble EGFP into MSC. EGFP or TLN161-EGFP was expressed in HEK293T cells and Co-IP was performed using p43 antibodies. Various components of MSC, hThrRS or α-Tubulin (A) or EGFP and TLN161-EGFP (B) were detected in the input or precipitated sample.

leading to premature termination of translation of Tarsl2 mRNA at position 330 (Figure 8A and B). The absence of mThrRS-L was confirmed by western blotting analysis (Figure 8C). The Tarsl2-inactivated cell line could be obtained, which indicated that Tarsl2 is not an essential gene in the cells. In this work, we only focused on the role of mThrRS-L in MSC assembly.

Whole cell lysate of CYB was fractionated by gel filtration chromatography to study the MSC integrity. The data clearly showed that several members of the MSC, including mouse GluProRS, LeuRS, GlnRS, and especially ArgRS and p43 were all readily incorporated into the complex; however, mouse GlyRS eluted as a free aaRS away from the MSC, as expected (Figure 8D). These data showed that the MSC remained intact at the absence of mThrRS-L, suggesting that mThrRS-L is not a pre-requisite for ArgRS and p43 incorporation into the MSC.

Effect of potential phosphorylation at specific residues on MSC incorporation

Post-translation modification, in particular phosphorylation, of mammalian aaRSs is frequently involved in their MSC disassociation (9,10). So far, six residues in hThrRS-L, including Thr$^{163}$, Thr$^{449}$, Ser$^{453}$, Tyr$^{457}$, Ser$^{459}$ and Tyr$^{619}$, has been listed to be modified by phosphorylation in the PhosphoSitePlus database (www.phosphosite.org). To address whether potential phosphorylation at these sites regulates the release of hThrRS-L from the MSC, Asp was introduced at each site to mimic phosphorylated hThrRS-L. After expressing the genes encoding these single-point mutants with a C-terminal FLAG tag in HEK293T cells, the mutants were precipitated using anti-FLAG antibodies. Several components of the MSC (including GluProRS, ArgRS and p43) were clearly detected in the precipitated products of all mutants (Figure 9A), suggesting that po-
Figure 8. mThrRS-L does not affect MSC integrity \textit{in vivo} in NIH/3T3. (A) Using CRISPR/Cas9 mediated gene editing, a \textit{Tarsl2} deletion cell line (CYB) was obtained with an additional nucleotide ‘A’ insertion at position 242 of \textit{Tarsl2} mRNA (B). The result of DNA sequencing of CYB. (C) mThrRS-L was not detected by western blotting because of a premature termination in translation. (D) Whole cell lysate of CYB was fractionated by gel filtration chromatography and various components of MSC or a free tRNA synthetase (GlyRS) were detected.

Figure 9. Phosphorylation of various sites of hThrRS-L has little effect on MSC targeting. Gene expressing C-terminal FLAG tagged hThrRS-L, hThrRS-L-T163D, hThrRS-L-T449D, hThrRS-L-S453D, hThrRS-L-Y457D, hThrRS-L-S459D or hThrRS-L-Y619D was separately introduced into HEK293T (A) or HeLa (B) cells. Each protein was enriched by anti-FLAG antibodies and known components of MSC and hThrRS were detected using their specific antibodies. Empty vector was also introduced as a negative control.

**DISCUSSION**

AaRSs from higher eukaryotes frequently acquire new appended domains at either N- or C-terminus (8). For example, hThrRS evolves a new appended domain at N-terminus when compared with bacteria ThrRSs. These newly acquired domains frequently mediate protein–protein interaction in diverse pathways, including MSC formation (8). In a previous report, we have shown that ThrRS-L gene is likely duplicated and deviated from canonical cytoplasmic ThrRS gene (30). The N-extension of human or mouse ThrRS-L was absent from ThrRSs in bacteria and lower eukaryotes and was likely of different origin from that of human or mouse cytoplasmic ThrRSs. pBlast analysis showed that the N-extension of ThrRS-L was absent from any other proteins and only displayed similarity in the first part with N-terminal extension of cytoplasmic ArgRS, in contrast to that of canonical ThrRS. Therefore, we suggest that the N-extension is a newly acquired domain during evolution.

The MSC was identified more than two decades ago (16). It is generally thought that nine aaRSs reside in the MSC, in addition to three scaffold co-factors. Indeed, these nine aaRSs have been consistently revealed as enriched in the MSC using enzymatic determination, Co-IP, silver staining, and Coomassie Brilliant Blue staining. The presence of ThrRS-L in the MSC was only noticed recently using mass spectrometry after tag purification, which is more sensitive than the above methods (28,29). In addition, we have revealed that the mRNA level of mouse \textit{Tarsl2} is markedly lower than that of canonical mouse \textit{Tars} in mouse tissues (ranging from 1/3 to 1/100) and cell lines (approximately 1/120) (30). Consistently, the steady-state ThrRS-L level is much lower than that of canonical aaRSs, including ThrRS and ArgRS, in HEK293T cell lines (29). The significantly low abundance of ThrRS-L rel-
ative to other aaRSs might have delayed its identification as a component of the MSC. Notably, in a recent effort to map all human protein interaction networks, 15 proteins were identified as forming a complex with ThrRS-L, four of which (p43, ArgRS, LysRS and p38) are components of the MSC (39). We suggest that these four proteins were identified because of ThrRS-L’s incorporation into the MSC. This finding is also in agreement with our results showing that ThrRS-L is a *bona fide* component of the MSC. Other 11 proteins (CDC102B, ING2, SDCBP, BAF13, KIAA1683, FERMT1 and EIF4E2) are unrelated to tRNA synthetases, suggesting non-canonical functions of ThrRS-L (39).

Our data also clearly showed that the unique N-extension of ThrRS-L is responsible for its incorporation into the MSC. Two leucine-zipper motifs in the first part of the N-extension of ThrRS-L mediate its interaction with ArgRS and p43. It is notable that all leucine-zipper-containing aaRS proteins or co-factors (including ArgRS, p43 and p38) are components of the MSC and are associated in the complex, at least in part, through the leucine-zipper motifs. Therefore, our results provide a further example of leucine-zipper containing aaRS species as components of the MSC. The interaction occurs among ThrRS-L, ArgRS and p43. Indeed, after disrupting either leucine-zipper, ThrRS-L lost its ability to be incorporated into the MSC, suggesting that both leucine-zippers are essential elements for MSC localization. The N-termini of both ArgRS and p43 are long α-helices, protruding away from the main body of the proteins, as illustrated by the co-crystal structure of ArgRS-p43-GlnRS (20), making them ideal protein–protein interaction surfaces. As expected, the N-extension of ThrRS-L, especially the first half, which is similar to the N-extension of ArgRS, also likely extends out from the main body. We further modeled the structure of TLN161, which showed that the entire N-extension formed three tandem α-helices (Supplementary Figure S2). The two conserved leucine-zippers were imbedded in the first and the beginning of the second α-helix, potentially contributing to hydrophobic interactions with MSC components such as ArgRS and p43. The three termini of ArgRS, hThrRS-L, and p43 might form a highly ordered interaction network using leucine-zipper motifs in their respective N-terminal α-helices. It is expected that the shorter form of ArgRS cannot bind hThrRS-L because it lacks an N-terminal extension (35). Mutation at either leucine zipper had no effect on the protein level and enzymatic activities of ThrRS-L, suggesting that the two leucine zipper motifs have evolved for protein–protein interactions, but do not regulate the protein structure and enzymatic activities. Consistently, the activities of the shorter form of ArgRS were not decreased compared with those of the longer form of ArgRS, suggesting that the leucine zipper itself has no direct effect on enzymatic activity (40). However, the interaction between the N-terminal leucine zipper containing helices of ArgRS and p43 is important for the catalytic activity of the longer isoform (20).

Despite both ArgRS and ThrRS-L using conserved peptides to interact with p43, we believe there is little competition for the binding of p43 between ArgRS and ThrRS-L *in vitro*. This might be explained, at least in part, by the low level of ThrRS-L compared with that of ArgRS in the MSC fraction (29), making ThrRS-L a weaker competitor for ArgRS. In addition, one leucine-zipper motif can mediate an interaction with more than one partner in a single complex. For example, the leucine-zipper motif in p43 mediates its interaction with leucine zipper motifs in both ArgRS and p38 in the MSC. Furthermore, ThrRS-L and ArgRS can interact with each other using the leucine-zipper motifs in their respective N-termini. However, we have tested the leucine zipper motifs in ThrRS-L and showed that they cannot mediate its self-dimerization (data not shown). By contrast, the absence of ThrRS-L has no effect on the assembly of other components in the MSC, in line with p38 being a core scaffold of the complex (19).

Our results also explain why ThrRS, which is highly homologous to ThrRS-L over its whole sequence, is not a component of MSC: It lacks a distinct N-extension like that of ThrRS-L. However, it is striking that, despite forming a complex with ThrRS-L *in vivo*, ThrRS is not targeted to the MSC by ThrRS-L. It is possible that the interaction between ThrRS and ThrRS-L places the N-extension of ThrRS-L in an unfavorable conformation by an as-yet-unidentified mechanism (such as post-translational protein modification), stopping ThrRS-L from staying in the MSC. A similar mechanism has been observed in the assembly or release of other MSC components. For example, in monocytic cells, γ-interferon treatment induced phosphorylation at Ser886 and Ser999 of GluProRS by CDK5, dissociating it from the MSC to bind other protein partners and forming a GAIT complex to suppress the translation of various inflammatory mRNAs (9). Upon laminin signaling, LysRS is phosphorylated at Thr52 by p38MAPK, which dissociates it from the MSC to the cell membrane to interact with 67LR, thereby regulating cell migration (41). LysRS binds with MITF to regulate the immune response in mast cells after it is released from the MSC upon phosphorylation at Ser207 (42). In addition, our previous report showed that some fraction of ThrRS-L is localized in the nucleus (30). We suggest that under most conditions, ThrRS-L is incorporated in the MSC; but under some specific but unidentified conditions, ThrRS-L is modified and then translocated to the nucleus; the nucleus-localized ThrRS-L is likely not associated with the MSC.

Our recent data from native or overexpressed ThrRS-L showed that ThrRS-L could catalyze both aminoaacylation and editing functions *in vitro* (30). However, we hypothesized that ThrRS-L has not evolved for canonical tRNA aminoaacylation for protein synthesis because of its presence only in higher eukaryotes, the presence of a conserved *TARS* in the three domains of life, and the lower abundance of ThrRS-L *in vivo* (29,30). We also suggested that it is unlikely to solely catalyze editing activity to hydrolyze mischarged Ser-tRNA<sup>Thr</sup> *in vivo* under physiological conditions because, on the one hand, ThrRS itself retains the editing activity and on the other hand, it would not be evolutionarily economical to evolve a new gene just to perform another layer of editing. However, we cannot rule out the possibility that its residence in the MSC might be beneficial for recycling tRNA<sup>Thr</sup> to supplement ThrRS activity to some extent under specific conditions (such as under stress or when ThrRS is secreted outside of cells during angiogen-
esis) (43). In addition, its presence in the nucleus and its potential to bind both nucleic acid (via the tRNA binding domain) and proteins (via the leucine-zipper motifs) strongly implied that it has some non-canonical functions, at least in the nucleus, which are currently under investigation. The MSC might function as a reservoir to regulate its cellular localization, similar to LysRS and GluProRS, as one of its non-canonical functions (22).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank Dr Chao Peng, Ping Wu, Xiaoxu Tian and Yue Yin in the Mass Spectrometry System at the National Facility for Protein Science in Shanghai (NFPS), Zhangjiang Lab, China.

FUNDING

National Key Research and Development Program of China [2017YFA0504000]; Natural Science Foundation of China [31822015, 81940204, 31500644, 31570792]; Strategic Priority Research Program of the Chinese Academy of Sciences [XDB19010203]; Shanghai Rising-Star Program [16QA1404400]; Youth Innovation Promotion Association (Chinese Academy of Sciences) [Y119S41291]. Funding for open access charge: National Key Research and Development Program of China [2017YFA0504000]; Natural Science Foundation of China [2017YFA0504000]; Strategic Priority Research and Development Program of China [Y119S41291].

Conflict of interest statement

None declared.

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