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Review Article

Beyond protein synthesis: non-translational functions of threonyl-tRNA synthetases

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Aminoacyl-tRNA synthetases (AARSs) play an indispensable role in the translation of mRNAs into proteins. It has become amply clear that AARSs also have non-canonical or non-translational, yet essential, functions in a myriad of cellular and developmental processes. In this mini-review we discuss the current understanding of the roles of threonyl-tRNA synthetase (TARS) beyond protein synthesis and the underlying mechanisms. The two proteins in eukaryotes — cytoplasmic TARS1 and mitochondrial TARS2 — exert their non-canonical functions in the regulation of gene expression, cell signaling, angiogenesis, inflammatory responses, and tumorigenesis. The TARS proteins utilize a range of biochemical mechanisms, including assembly of a translation initiation complex, unexpected protein–protein interactions that lead to activation or inhibition of intracellular signaling pathways, and cytokine-like signaling through cell surface receptors in inflammation and angiogenesis. It is likely that new functions and novel mechanisms will continue to emerge for these multi-talented proteins.

Aminoacyl-tRNA synthetases

Aminoacyl-tRNA synthetases (AARSs) are a family of essential enzymes that catalyze aminoacylation — ligation of amino acids to their cognate tRNAs, thus creating substrates for protein synthesis [1,2]. There are two sets of these enzymes in eukaryotes — cytoplasmic and mitochondrial, encoded by distinct genes except for two of them (see below). There are various nomenclatures for this family of proteins. In this review we will follow the Human Genome Organisation gene nomenclature and use single-letter amino acid codes for the gene and protein names in higher organisms (e.g. TARS1 for cytoplasmic threonyl-tRNA synthetase and TARS2 for the mitochondrial counterpart). However, for the bacterial and yeast proteins we will follow the convention in the field and use three-letter amino acid codes (e.g. ThrRS for bacterial threonyl tRNA-synthetase). The human genome contains 37 genes encoding AARS proteins, with 18 in the cytoplasm, 17 in the mitochondria, and 2 in both compartments (KARS and GARS).

AARSs attach each amino acid to the 3′ end of its cognate tRNA, forming an ester linkage with the terminal adenosine [1,2]. In addition to accurate recognition of the tRNA, precise non-covalent binding of the cognate amino acid by the AARS is essential to prevent errors in protein synthesis [2]. However, AARSs can undergo misacylation under certain stress conditions like oxidative stress [3]. To circumvent the effects of misacylation, most AARSs possess proofreading and editing capabilities, which can occur either before the amino acid transfer step, involving hydrolysis of aminoacyl adenylate, or post-transfer by deacylation of mischarged aa-tRNA [4–6].

Despite the limited primary sequence similarities within the family, AARSs can be categorized into Class I and Class II based on distinct sequence motifs at the active sites [7]. Class I AARSs are primarily monomeric proteins with the conserved sequence motifs ‘HIGH’ and ‘KMSKS’, and a Rossmann dinucleotide binding domain. Class II AARSs are functional dimers or tetramers with the

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conserved Motifs 1, 2, 3 and an antiparallel β-fold in the active site. Class I enzymes transfer amino acids onto the 2′-OH group of the terminal adenosine of tRNA, whereas Class II enzymes mostly transfer amino acids to the 3′-OH.

AARSs have non-translational functions

While decades of studies have led to deep understanding of the house-keeping function and mechanisms of AARSs, more recent years have seen a surge in discoveries of non-canonical or non-translational functions for many cytoplasmic (as well as mitochondrial) AARSs in a broad range of cellular regulations [8–11]. For instance, AARSs are found to regulate gene expression at all levels, including transcription, splicing, and translation (non-canonically), via diverse mechanisms. Through dysregulation in their translational and non-translational functions, AARSs are known to be associated with many human diseases including but not limited to neuropathies, chronic inflammatory diseases, and cancer [10,12–16].

Almost all the eukaryotic AARSs contain 'new domains' (distinct for each AARS) appended to the catalytic core, the acquisition of which coincided with the emergence of increasing complexity of eukaryotic organisms [9]. Some of these newly appended domains are found in multiple AARSs, including leucine zipper (LZ), glutathione *S*-transferase, WHEP (named after its presence in WARS1, HARS1, and EPRS1), whereas other domains are unique to individual AARSs (e.g. UNE-L for LARS1, UNE-S for SARS1, etc.). Once acquired, the new domains became conserved in higher organisms, suggesting that AARSs may perform essential, non-translational functions through these domains. Indeed, there are numerous cases of non-translational functions involving the new domains of AARSs [8,9]. For example, the WHEP domain of WARS1 is involved in the activation of p53 in the nucleus, whereas the multiple WHEP domains in EPRS1 play a critical role in γinterferon activated inhibition of translation (reviewed in [10,17]). Along with the emergence of the new domains in AARSs, higher eukaryotes evolved to have a multi-synthetase complex (MSC). The mammalian MSC contains eight cytoplasmic synthetase proteins — DARS1, EPRS1 (glutamyl- and prolyl-tRNA synthetase), KARS1, IARS1, LARS1, MARS1, QARS1, and RARS1, and three scaffold proteins — AARS-interacting multifunctional protein 1, 2 and 3 (AIMP1, AIMP2, and AIMP3). The MSC is believed to facilitate the translational function of the AARSs, as well as serving as a depot for AARSs with non-canonical functions [18–22].

In many cases the catalytic core of AARSs can also confer non-catalytic functions. For instance, the amino acid binding sites of several AARSs are utilized for non-translational functions, such as leucine-sensing by LARS1 in the activation of mammalian target of rapamycin complex 1 (mTORC1) signaling, glutamine regulation of apoptosis signal-regulating kinase 1 signaling through QARS1, and binding to VE-cadherin by an extracellular N-terminal fragment of WARS1 to exert anti-angiogenesis effects (reviewed in [23]). The readers are referred to excellent reviews on non-translational functions of AARSs cited above [8–11]. In this mini-review we focus on TARS. Recent developments and future prospects in uncovering the non-translational functions of TARS are discussed.

TARS

TARS is a class II dimeric enzyme, further categorized as a subclass IIa protein [7,24]. The first structural analyses of TARS were conducted on *Escherichia coli* ThrRS. The dimeric core of *E. coli* ThrRS is formed by the catalytic and C-terminal anticodon binding domains, while the N-terminal editing domain is situated on the opposite side of the core [25,26]. One copy of tRNA interacts with both monomers, engaging the catalytic domain, C-terminus, and editing domain. The very N-terminus of *E. coli* ThrRS is the TGS domain (named after its presence in TARS, GTPase, and SpoT) [27], which is conserved among prokaryotes and eukaryotes. In the course of evolution eukaryotic TARS gained an N-terminal extension, the UNE-T domain. Despite bacterial origin of the mitochondrion, the mammalian mitochondrial TARS2 exhibits greater sequence similarities to cytoplasmic TARS1 than to bacterial ThrRS [28,29]. Like its cytoplasmic counterpart, the mammalian mitochondrial TARS2 has an editing activity that is critical for translation fidelity and important for mitochondrial function and cell proliferation [30]. Vertebrates express an additional TARS-like protein named TARSL2, which exhibits marked sequence similarities to TARS1 except for a larger N-terminal domain [31] (Figure 1).

Like other AARSs, TARS can perform various non-canonical or non-translational functions involved in gene expression, cell signaling, angiogenesis, inflammation, and tumorigenesis as discussed below. The functional diversification of TARS is evident across both higher- and lower-order organisms.

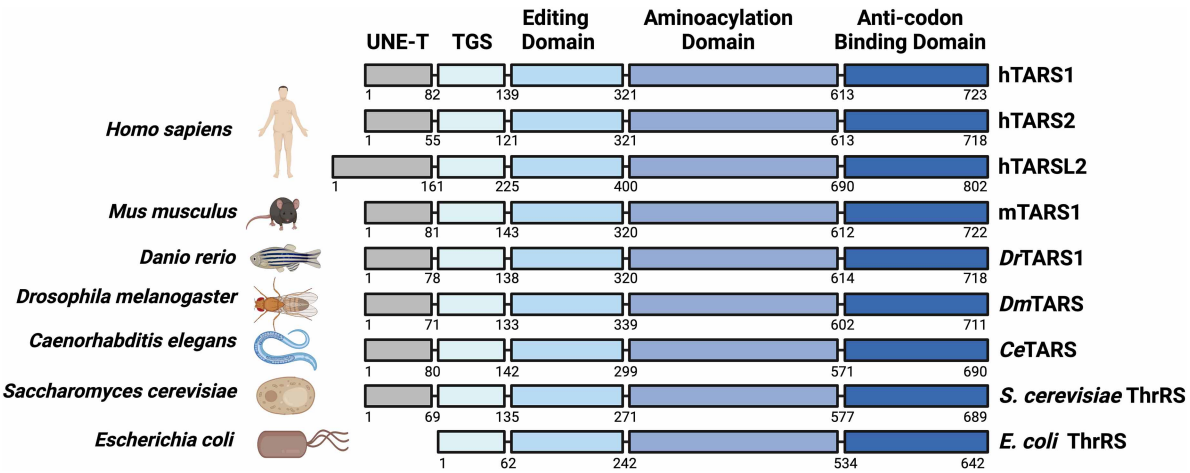

Figure 1. Schematic representation of the domain structures of TARS across species.

Amino acid residue numbers defining the domains are obtained from Uniprot (https://www.uniprot.org/) and/or determined by sequence alignment in Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo). For ease of alignment, not all domains are drawn to scale. Figure created with BioRender.com.

TARS in non-canonical regulation of translation

As one of the earliest non-canonical functions identified, negative regulation of the translation of its own mRNA by *E. coli* ThrRS exemplifies how an AARS can achieve autoregulation [32–36]. ThrRS binds to regions upstream of translational start site of its mRNA and subsequently inhibits translation by preventing binding of the 30S ribosome. The control element in the ThrRS leader mRNA, known as operator, consists of four structural domains: the ribosome binds domain I and 3′ part of the linker domain III; domains II and IV create two hairpins, recognized by ThrRS due to their structural similarity to the anticodon loop of $tRNA^{Thr}$. Replacing the anticodon-like sequence in the ThrRS mRNA with that of $tRNA^{Met}$ in the operator switches the regulation of translation from ThrRS to MetRS [37,38], suggesting that this autoregulatory mechanism may apply to other AARSs.

More recently, Jeong et al. [39] uncovered an unexpected function of human TARS1 in assembling a translation initiation machinery. Through affinity purification and mass spectrometry analysis, TARS1 was found to interact with 4EHP (eIF4E2), a homolog of the 5′cap-binding protein eIF4E. The UNE-T domain of TARS1 directly interacts with cap-bound 4EHP and, furthermore, TARS1 also interacts with eIF4A and the polyA-binding protein PABP, but not eIF4G [39]. Hence, TARS1 mimics eIF4G as a scaffold to assemble a cap-dependent translation initiation complex containing 4EHP, eIF4A, and PABP (Figure 2A). This TARS1-mediated translation initiation machinery most likely regulates a subset of mRNAs specifically recognized by 4EHP, and many of those mRNAs encode proteins that control biological processes specific for vertebrates, such as neuronal, skeletal, and vascular development. Vascular endothelial growth factor (VEGF) expression at the translational level was shown to be dependent on TARS1–4EHP interaction, and so was angiogenesis in human cells and zebrafish [39]. Indeed, the TARS1–4EHP interaction is only observed in vertebrates, consistent with sequence divergence found in the UNE-T domain as well as in 4EHP's TARS1-binding site in lower organisms [39].

Extracellular TARS1 and angiogenesis

The first clue of TARS involvement in angiogenesis came from the strong anti-angiogenic effect of borrelidin [40], a potent non-competitive TARS inhibitor isolated from *Streptomyces rochei* [41,42]. However, borrelidin interacts with the threonine-binding pocket in the TARS1 active site, and consequently elicits amino acid starvation response and apoptosis in endothelial cells [43]. It is therefore not possible to uncouple the anti-angiogenic effect of this inhibitor from its inhibition of TARS catalytic activity or its general toxicity. More recent studies using the borrelidin analog BC194, which has a drastically weakened affinity for the threonine

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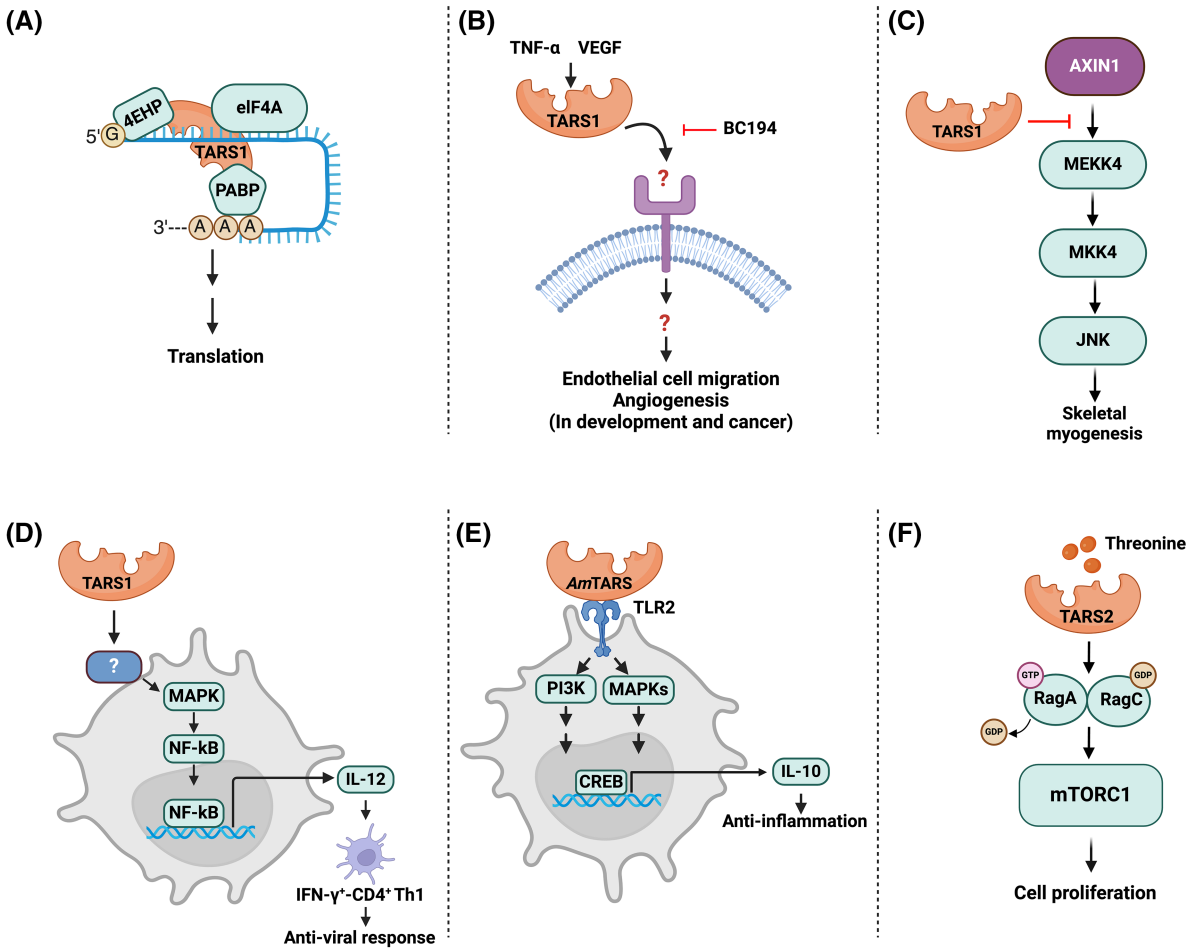

Figure 2. Non-canonical functions of TARS.

(**A**) TARS1 assembles a translation initiation complex involving 4EHP, eIF4A and PABP. (**B**) TNFα or VEGF induces secretion of TARS1, which through an unknown receptor regulates endothelial cell migration and angiogenesis (potentially relevant in both vascular development and cancer). (**C**) TARS1 negatively regulates skeletal myogenesis by inhibiting JNK signaling through interaction with Axin1. (**D**) Extracellular TARS1 induces dendritic cell maturation and activation through the activation of MAP kinases ERK and JNK followed by NF-κB activation. Activated dendritic cells produce IL-12, which promotes Th1 anti-viral response. (**E**) *Am*TARS binds TLR2 on macrophages and activates MAPK (ERK and p38) and PI3K signaling, resulting in activation of CREB and subsequent production of the anti-inflammatory IL-10. (**F**) TARS2 binds and activates the RagA/C dimer in response to threonine, and subsequently activates mTORC1 in the regulation of cell proliferation. Figure created with BioRender.com.

binding site while still exhibiting a full anti-angiogenic activity in human cells and zebrafish [44,45], have provided more definitive evidence for a non-canonical function of TARS1 in angiogenesis regulation.

Several mammalian AARSs are secreted either as intact proteins, fragments, or splice variants, and they can act as extracellular signaling proteins [46]. Williams et al. found TARS1 to be secreted by human umbilical vein endothelial cells in response to stimulation by tumor necrosis factor-α (TNF-α) or VEGF [47]. Extracellular TARS1 is shown to stimulate angiogenesis both *in vitro* and *in vivo*, and TARS1 likely exerts this angiogenic effect by promoting endothelial cell migration (Figure 2B). A mutant of TARS1 (R442A) that no longer has any aminoacylation activity is fully effective in stimulating angiogenesis, further validating the non-translational nature of this function [47]. It remains to be determined in what form TARS1 is secreted and which receptor mediates the signaling by extracellular TARS1 in the regulation of angiogenesis.

Another connection between TARS1 and angiogenesis came from the characterization of a zebrafish mutant line (cq16) that displayed abnormal branching and patterning of brain vascular network during embryonic

development [48]. VEGF-A expression was increased in the mutant embryo, and pharmacological inhibition of VEGF receptor signaling suppressed the vascular phenotype. Interestingly, the cq16 gene was found to encode TARS1 with a missense mutation in the catalytic domain. However, since the biochemical effect of this mutation has not been characterized, it is not possible to speculate whether the wild-type TARS1 stimulates or inhibits angiogenesis, or whether TARS1 exerts its function canonically or non-canonically.

TARS1 inhibition of JNK signaling in myogenic differentiation

A few years ago, our group reported a non-translational role of LARS1 in regulating skeletal muscle differentiation and regeneration through the mTORC1 pathway [49]. Those findings prompted us to ask whether any other AARSs may also regulate skeletal myogenesis. An RNAi screen of cytoplasmic AARSs in the mouse myoblast C2C12 cells led to the identification of TARS1 as a potential regulator of myogenic differentiation. Through knockdown and overexpression experiments TARS1 is found to negatively control myoblast differentiation in culture and injury-induced muscle regeneration in mice [50]. This regulation by TARS1 is independent of global protein synthesis, and the catalytic activity of TARS1 is dispensable, indicating a non-translational function. To dissect the mechanism of this novel function, we surveyed major signaling pathways known to regulate myogenesis, and JNK signaling emerged to be specifically down-regulated by TARS1 in myoblasts. We further discovered that TARS1 physically interacted with Axin1, which disrupted Axin1 interaction with MEKK4, resulting in the inhibition of the Axin1–MEKK4–MKK4 pathway upstream of JNK [50] (Figure 2C). A positive role of JNK in regulating myogenic differentiation has been previously established [51,52].

Strikingly, the N-terminal UNE-T and TGS domains of TARS1 together are both necessary and sufficient for the inhibition of myogenic differentiation [50]. It is interesting to note that a naturally occurring splice variant of human TARS1 encodes an N-terminal fragment of the protein that encompasses UNE-T and TGS domains [53]. It is not known whether this splice variant is expressed in mouse myoblasts and, if so, whether its protein product is involved in myogenic regulation. Meanwhile, a potential link between this N-terminal protein product and JNK signaling in other cell types known to express the splice variant [53] will also be worthy of attention in future investigations.

TARS in immunity and inflammation

While pathogen AARSs often elicit immune responses and can serve as anti-infective targets, mammalian AARSs are involved in immune cell development as well as signaling in immune responses [54,55]. TARS1 is one of several AARSs found to be up-regulated in expression in response to viral infection [54]. In a recent report, Jung et al. [56] showed that extracellular TARS1 induced the maturation and activation of dendritic cells (DCs), likely through the activation of the MAP kinases ERK and JNK, and ultimately NF-κB activation. Treatment with extracellular TARS1 led to enhanced DC secretion of the cytokine IL-12, which in turn induced the polarization of $CD4^+$ T cells into Th1 cells ($IFN\gamma^+CD4^+$) [56] (Figure 2D). Notably, *in vivo* relevance of these findings was confirmed by the observation that infusion of TARS1-treated DCs induced a Th1 response and enhanced the antiviral effect of the DCs [56]. TARS1 presumably interacts with a yet-to-be-identified cell surface receptor to perform the function described here. These findings may have important implications in the association of TARS1 with autoimmune diseases such as polymyositis and dermatomyositis, in which TARS1 is the target of the autoantibody PL-7 [57].

Most recently, Kim et al. [58] have reported that the gut-associated bacteria *Akkermansia muciniphila* secrete TARS (*Am*TARS) to modulate immune homeostasis. They show that extracellular *Am*TARS induces the polarization of M2 macrophages and subsequent production of the anti-inflammatory cytokine IL-10, which ameliorates the inflammatory effects of colitis in mice [58]. The authors further identify amino acid regions unique to *Am*TARS that are responsible for interacting with the toll-like receptor TLR2. By engaging TLR2, *Am*TARS acts as a ligand for the receptor to activate intracellular signaling pathways including the MAP kinases ERK and p38, as well as PI3K–AKT, which converge on CREB activation in the nucleus [58] (Figure 2E). TLR2 represents the first cell surface receptor to be identified to receive signal from an extracellular TARS. Although mammalian TARS1 is unlikely to utilize the same receptor due to its lack of sequences homologous to those in *Am*TARS mediating TLR2 interaction, the work by Kim et al. should inspire others to pursue the elusive receptor for mammalian TARS1 in the regulation of angiogenesis or immune cell development.

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TARS2 regulation of mTORC1 signaling

mTORC1 is a master regulator of cell growth and proliferation by mediating and integrating environmental cues, including nutrient availability, growth factor signals, energy levels, and various types of stress [59–61]. mTORC1 has been known to transduce the signals of cellular amino acid sufficiency through a multitude of proteins as sensors specifically for leucine, glutamine, arginine, and methionine [62–69]. These amino acid sensors regulate the Rag small G proteins (RagA/B/C/D), which functions as a heterodimer to activate mTORC1 on the surface of the lysosome [70]. More recently, Kim et al. [71] discovered that the mitochondrial TARS2 physically associates with the protein complex responsible for amino acid-dependent mTORC1 activation at the lysosome. The authors went on to show that TARS2 is necessary for mTORC1 translocation to lysosomes and activation in response to threonine stimulation, and that TARS2 performs this role by binding to inactive RagC and promoting RagA GTP-loading (Figure 2F) [71]. Exactly how TARS2 modulates guanine nucleotide exchange of RagA is not known, but the collective evidence presented by Kim et al. supports a role of TARS2 in mediating the sensing of cellular threonine by mTORC1 in the regulation of cell proliferation. TARS2 represents another example of AARSs utilizing their specific amino acid binding to confer non-translational functions.

TARS in cancer

Genomic and transcriptomic analyses of cancer databases have revealed a strong cancer-associated profile for human AARSs as a family [72]. Individual AARSs have distinct profiles, some resembling tumor suppressors and others oncogenes [73]. Although the elevated expression levels of several AARSs in cancer could reflect a heightened demand for protein synthesis in cancer cells, non-translational functions of AARSs are implicated by the diverse genomic and transcriptomic profiles of AARSs in cancer. Indeed, several AARSs have been demonstrated to have direct or indirect roles in tumorigenesis independent of protein synthesis [72,74].

A potential link of TARS1 to cancer was speculated based on the epidemiological connection between myositis and cancer [75]. TARS1 is one of the most highly expressed AARSs in cancer when all cancer types are taken into consideration (our unpublished observation and [73]). More importantly, high expression levels of TARS1 significantly correlate with poor patient survival in several types of cancer including breast, lung, ovarian, liver, and pancreatic cancer (https://kmplot.com [76]). A canonical function of TARS1 has been suggested in pancreatic cancer, where the overexpression of Mucin1 (MUC1) and cell migration are dependent on high levels of TARS1 due to an unusually high number of threonine residues in the amino acid sequence of MUC1 [77]. It is not clear whether this mechanism occurs in other types of cancer.

Following the discovery of an angiogenic function of extracellular TARS1 [47], Wellman et al. [78] examined a potential role of TARS1 in the highly angiogenic ovarian cancer. Indeed, they have found that ovarian cancer cells secrete TARS1, and patient serum TARS1 levels correlate with TARS1 expression in tumor samples. Furthermore, overexpression of TARS1 strongly correlates with advanced stages of the disease as well as the angiogenic marker VEGF. However, paradoxically, in late-stage disease an inverse correlation is found between TARS1 expression and patient mortality [78]. Wellman et al. propose a complex role for TARS1 in ovarian cancer that involves the tumor microenvironment, angiogenesis, and immune cell response.

Elevated expression of mitochondrial TARS2 is also found to correlate with patient mortality in non-small cell lung adenocarcinoma (LUAD). Tian et al. recently showed that knockdown of TARS2 in LUAD cells inhibited proliferation and increased mitochondrial reactive oxygen species-induced apoptosis, and that TARS2 knockdown also suppressed xenograft tumor growth *in vivo* [79]. However, mTORC1 signaling, a master regulator of cell growth/proliferation and reported to be activated by TARS2 [71], was not examined in the study. Future investigation will be necessary to probe the mechanism of TARS2 action in LUAD.

Function of TARSL2

A unique aspect of TARS is the duplication of the TARS1 gene in higher eukaryotes, resulting in TARSL2 [31]. Characterization of TARSL2 by En-Duo Wang and colleagues in recent years has shed significant light on this curious homolog. As expected from its high sequence similarity to TARS1, TARSL2 has aminoacylation and editing activities [80]. However, TARSL2 appears to be dispensable for protein synthesis, as deletion of the *Tarsl2* gene does not affect the aminoacylation of $tRNA^{Thr}$ [81]. While knockout of *Tars1* results in embryonic lethality in mice, *Tarsl2* knockout mice appear normal at birth [81]. Nevertheless, growth retardation becomes evident after 3 weeks of age in the *Tarsl2* knockout mice, with defects observed in bone development and skeletal muscle formation. Remarkably, the *Tarsl2* knockout mice are leaner than WT, with enhanced glucose and

lipid metabolism [81]. Future investigation will be necessary to characterize the mechanism of TARSL2 action that underlies those striking phenotypes.

Interestingly, biochemical evidence suggests that TARSL2 may be a component of the MSC [82–84]. The extended N-terminus, which contains two LZs, can interact with other components of the MSC to facilitate the incorporation of TARSL2 in the complex [83]. However, the functional relevance of TARSL2's presence in the MSC is not clear. Knockout of *Tarsl2* does not affect the integrity of MSC, ruling out a role of scaffolding for TARSL2 in the MSC [81]. Of note, TARS1 and TARSL2 can form a heterodimer when overexpressed in cells [83]. It is also noteworthy that the catalytic activity of TARSL2 has been maintained throughout evolution. All these biochemical features of TARSL2 should be kept in mind during future investigation of this fascinating protein.

Concluding remarks and future prospects

Many AARSs continue to surprise us with new non-canonical functions, and TARS is a striking example. The range of novel functions assigned to TARS1 and TARS2 discussed above may represent only a fraction of what these house-keeping proteins can do. While the regulation of some processes is unique to TARS (e.g. TARS2 assembly of the translational initiation machinery; threonine-dependent mTORC1 activation by TARS1), other aspects of biology can involve multiple AARSs although each with a distinct mechanism (e.g. TARS1 and LARS1 in skeletal myogenesis). It is remarkable that a family of proteins with well-conserved functional domains can device such wide-raging biochemical mechanisms to exert non-canonical regulation. The 'new domains' unique to each AARS certainly contribute to the diversity, but even the conserved catalytic domains can have specific non-canonical functions in individual AARSs. Cellular contexts, such as the presence of cofactors and/or distinct subcellular localization, can confer cell type-specific functions for TARS, as is likely the case for TARS1 regulation of JNK signaling exclusively in muscle cells. These context-specific regulatory mechanisms, as well as yet-to-be-discovered new functions, warrant significant future research efforts. The involvement of TARS in muscle regeneration, angiogenesis, immunity, and cancer promises tremendous potential in future therapeutic development.

Perspectives

- Like many other AARSs, TARS proteins play essential roles in a wide range of cellular and developmental processes, which are independent of their canonical function in protein synthesis.
- The two eukaryotic proteins, cytoplasmic TARS1 and mitochondrial TARS2, are found to non-canonically or non-translationally regulate gene expression, cell signaling, angiogenesis, inflammatory responses, and tumorigenesis, through diverse biochemical mechanisms.
- Dissecting mechanisms underlying the currently known TARS functions and identifying additional biological processes involving TARS will deepen our fundamental understanding of biological regulation and facilitate future therapeutic exploration against several human diseases including cancer.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

AARS, aminoacyl-tRNA synthetase; *Am*TARS, *Akkermansia muciniphila* secrete TARS; DC, dendritic cell; LUAD, lung adenocarcinoma; LZ, leucine zipper; MSC, multi-synthetase complex; mTORC1, mammalian target of rapamycin complex 1; MUC1, Mucin1; PABP, polyA-binding protein; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

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