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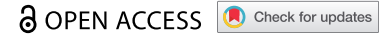Taylor & Francis Group

REVIEW

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The uniqueness of AlaRS and its human disease connections

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ABSTRACT

Among the 20 cytoplasmic aminoacyl-tRNA synthetases (aaRSs), alanyl-tRNA synthetase (AlaRS) has unique features. AlaRS is the only aaRS that exclusively recognizes a single G3:U70 wobble base pair in the acceptor stem of tRNA, which serves as the identity element for both the synthetic and the proofreading activities of the synthetase. The recognition is relaxed during evolution and eukaryotic AlaRS can mis-aminoacylate noncognate tRNAs with a G4:U69 base pair seemingly as a deliberate gain of function for unknown reasons. Unlike other class II aaRSs, dimerization of AlaRS is not necessarily required for aminoacylation possibly due to functional compensations from the C-terminal domain (C-Ala). In contrast to other 19 cytoplasmic aaRSs that append additional domains or motifs to acquire new functions during evolution, the functional expansion of AlaRS is likely achieved through transformations of the existing C-Ala. Given both essential canonical and diverse non-canonical roles of AlaRS, dysfunction of AlaRS leads to neurodegenerative disorders in human and various pathological phenotypes in mouse models. In this review, the uniqueness of AlaRS in both physiological and pathological events is systematically discussed, with a particular focus on its novel functions gained in evolution.

Introduction

Aminoacyl-transfer RNA (tRNA) synthetases (aaRSs) are a family of ubiquitously expressed enzymes functioning in translation of the genetic code. They catalyse the reactions to charge amino acids onto their corresponding cognate tRNAs in two steps [1]. In the first step, each aaRS binds to its specific amino acid (aa) which is activated by ATP to form a tightly enzyme-bound aminoacyl-adenylate intermediate (aa-AMP), while releasing pyrophosphate (PP$_i$). In the second step, the aa moiety of the adenylate is then transferred to the conserved 3′-end of the cognate tRNA to yield the aminoacyl-tRNA [2]. The charged tRNA further delivers the aa to the ribosome for protein synthesis.

Recent studies revealed that, in addition to aminoacylating tRNA, human aaRSs are also involved in post-translational addition of amino acids to lysine (Lys) residues in proteins [3]. Notably, the functions of these aminoacylated substrate proteins cover broad aspects of cell physiology, indicating that this expanded landscape of substrates endows aaRSs with more diverse and complex non-canonical roles, a topic requiring substantial future work [4].

Based on the structural feature of the catalytic domain, the 20 aaRSs each for one proteinogenic aa are divided into two classes with ten members in each class [5,6]. Alanyl-tRNA synthetase (AlaRS) belongs to the class II

tRNA synthetase and specifically attaches alanine (Ala) to the 3′-end of its cognate tRNA (tRNA^{Ala}) [5]. Throughout evolution, AlaRS is made up of four domains including the N-terminal catalytic domain, the tRNA binding domain, the editing domain, and the C-terminal domain (C-Ala), among which, the first two domains are collectively termed as the aminoacylation domain [7,8] (Fig 1 A,B). In contrast to most aaRSs, AlaRS recognizes its substrate tRNA in an anticodon-independent manner. While seryl-tRNA synthetase (SerRS) and leucyl-tRNA synthetase (LeuRS) are also featured with anticodon-independent tRNA recognition, which is facilitated by a long variable loop and a unique D-loop in the cognate tRNA, respectively [9,10], tRNA^{Ala} is recognized by AlaRS through a single wobble base pair in the acceptor stem. AlaRS features multiple editing mechanisms to correct the mistake when a wrong aa is charged onto tRNA^{Ala}. Although the presence of the C-Ala domain in AlaRS is evolutionarily conserved, its role in facilitating tRNA charging for supporting protein synthesis has been lost in the human enzyme. In this review, we will discuss the biological functions of AlaRS in physiological and pathological events, with a particular focus on novel roles of AlaRS that have been discovered in the past few years. Here AlaRS is exclusively referred to the cytosolic form, encoded by the AARS1 gene, with the mitochondrial AlaRS (mtAlaRS) omitted in our review unless specified otherwise.
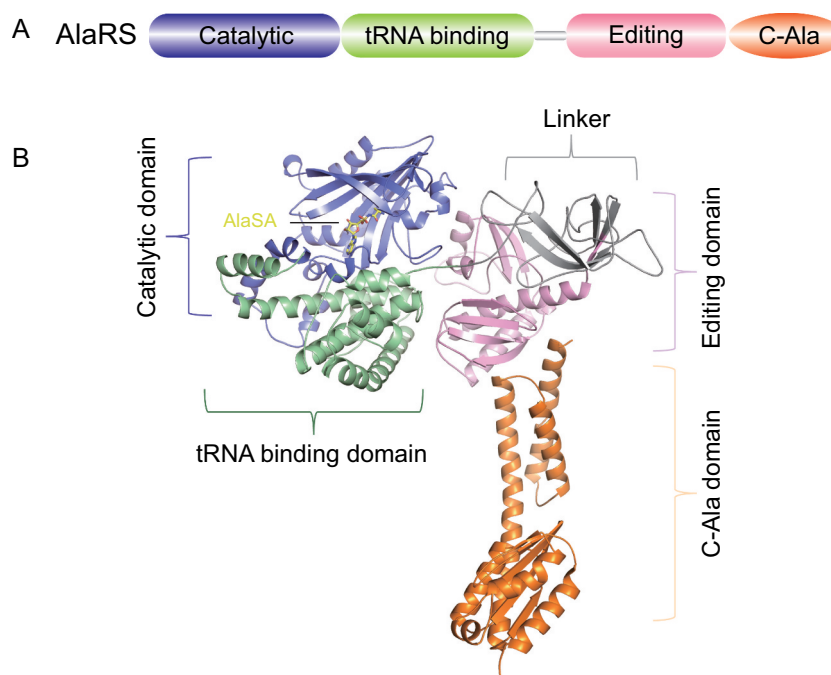
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Figure 1. Structure of AlaRS in human.
(**A**) Functional domains of AlaRS including catalytic domain (in blue), tRNA binding domain (in green), editing domain (in pink) and C-Ala domain (in orange). (**B**) The structure model of AlaRS was generated by the protein structure homology-modelling server SWISS-MODEL. Human AlaRS catalytic domain (PDB entry 5KNN) and C-Ala domain (PDB entry 5T5S) were further docked into the model and adjusted manually.

Functional domains of AlaRS and their uniqueness

1. Aminoacylation domain

tRNA recognition is entirely based on a G3:U70 base pair throughout evolution

During aminoacylation, each of the 20 aaRSs pairs the cognate aa with a specific trinucleotide known as the 'anticodon' harboured in a tRNA [11,12]. Remarkably, AlaRS makes no contact with the anticodon; instead, its aminoacylation domain recognizes a single G3:U70 wobble base pair (where G and U represent the bases of guanine and uracil, respectively) in the acceptor stem of the cognate tRNA[Ala] [7]; this single base pair is close to the site of aa attachment, but 76 Å away from the anticodon [13]. A synthetic seven-base-pair hairpin microhelix mimicking the acceptor stem of tRNA[Ala] can be aminoacylated efficiently by *Escherichia coli* (*E. coli*) AlaRS in a G3:U70-dependent manner [14]; even a G3:U70-containing minimalist tetraloop with as few as four base pairs is a substrate for *E. coli* AlaRS [15] (Fig. 2A). More strikingly, introduction of the G3:U70 pair into non-cognate tRNAs, such as tRNA[Cys] or tRNA[Phe], enables each to be charged by AlaRS with Ala, both *in vitro* and in cells [16,17] (Fig. 2A). These findings established the G3:U70 base pair as the single, dominant identity determinant for tRNA aminoacylation by AlaRS.

Unlike in Watson-Crick pairs, the G3 and U70 bases slide towards the minor- and major-groove side, respectively, to form a wobble base pair, without using the 2-amino of G3 and the 4-carbonyl of U70 [18,19]. The non-hydrogen-bonded nature of the 2-amino group of G3 in the wobble pair is important for tRNA selection and aminoacylation by AlaRS [20]. Notably, the G3:U70 wobble pair as the identity determinant is universally conserved during evolution [21]; however, it is recognized by AlaRS through

distinct mechanisms across different species. In the archaeal *Archaeoglobus fulgidus* (*A. fulgidus*), AlaRS interacts with G3:U70 through residues asparagine 359 (Asn359) and aspartic acid 450 (Asp450) from the major and the minor grooves, respectively. Although these two G3:U70-recognizing residues are conserved throughout evolution (corresponding to Asn303 and Asp400 in *E. coli* and Asn 317 and Asp416 in human AlaRS) [19], a positive selection mechanism from the minor groove side is used in *E. coli* but not human AlaRS [13]. Moreover, the recognition from the major groove side is achieved by a negative selection mechanism against non-GU base pairs for both *E. coli* and human AlaRS [13]. Regardless, the G3:U70-dependent recognition of tRNA[Ala] is well conserved from bacteria to humans, and the distinct ways of recognition adopted by AlaRSs in different domains of life suggest a persistent and robust preference to link the G3:U70 wobble pair with Ala in the process of evolution.

Dimerization of human AlaRS is dispensable for aminoacylation

Most class II aaRSs require a dimer form (or tetramer [dimer of dimer]) for catalytic function [22], whereas AlaRS is an exception. Human AlaRS predominantly exists as monomers under reducing conditions that resemble the environment inside the cell. Oxidation stabilizes the human AlaRS dimer without affecting catalytic activity, indicating dimerization is not necessary for aminoacylation [23]. In contrast, dimerization is required for archaeal and bacterial AlaRS to charge tRNA. However, unlike the case for other class II aaRSs (Fig. 2B), dimerization of AlaRS is not mediated through the catalytic domain but the C-Ala domain instead (Fig. 2C). Prokaryotic C-Ala functions in enhancing tRNA binding (as a dimer) by providing contacts with the elbow
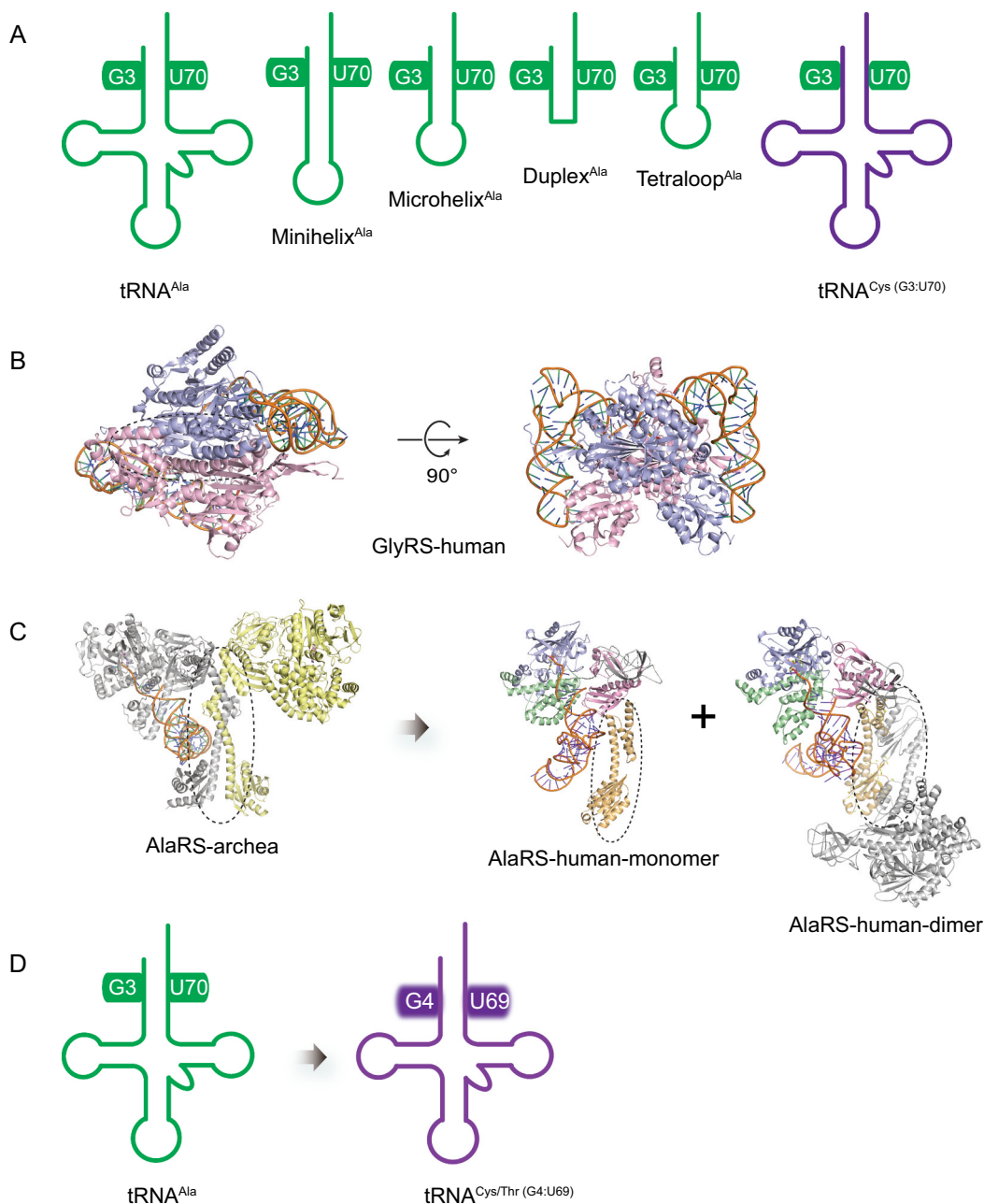
Figure 2. Uniqueness of the aminoacylation domain in AlaRS.

(**A**) G3:U70-dependent tRNA recognition by AlaRS. (**B**) Dimerization of GlyRS is mediated through the catalytic domain (dashed circle) (PDB entry 5E6M). (**C**) Dimerization of archaeal AlaRS is mediated through C-Ala domain (dashed circle), whereas human AlaRS has two folding state: one is monomer and the other is dimer mediated by C-Ala in antiparallel organization (dashed circle). The structure model of monomeric human AlaRS was aligned to archaeal AlaRS in complex with tRNA^Ala (PDB entry 3WQY) according to the aminoacylation domain, and the archaeal tRNA^Ala sequence was replaced by human tRNA^Ala. The structure model of dimeric human AlaRS was aligned to the crystal structure of dimeric human C-Ala followed by manual adjustment. (**D**) The mischarged tRNA (e.g., tRNA^Cys/Thr) has a G4:U69 base pair in the acceptor stem, shifted from the G3:U70 position in tRNA^Ala.

region of the L-shaped tRNA [24](Fig. 2C). This function is lost in humans, which explains why monomeric human AlaRS is sufficient for tRNA aminoacylation [23]. Interestingly, the human C-Ala domain can also dimerize, however through a different orientation (head-to-tail) than that in archaea (head-to-head) [23] (Fig. 2C). As dimerization of human C-Ala no longer facilitates charging, at least *in vitro*, the emergence of the new dimer interface suggests additional functions of AlaRS that are not yet identified [23]. Notably, AlaRS is also the only aaRS that has not acquired a new domain or motif during evolution. Therefore, it is

possible that the gained flexibility of C-Ala resulting from its loss of tRNA binding enables its functional expansion, that is otherwise supported by acquisition of new domains in other aaRSs.

Evolutionary gain of mischarging

As AlaRS recognizes its cognate tRNA through a single, dominant identity determinant (G3:U70), this particularly simplified mechanism also implies a likelihood for mischarging by AlaRS. By contrast, most aaRSs such as tyrosyl-tRNA synthetase (TyrRS) or glycyl-tRNA synthetase (GlyRS) require both

the acceptor stem and the anticodon for recognition. True as it is, human AlaRS, but not human TyrRS or GlyRS, shows substantial mischarging activity. Strikingly, all the mischarged tRNAs have a G4:U69 base pair in the acceptor stem, shifted from the G3:U70 position in tRNA^{Ala} [25] (Fig. 2D). Indeed, all the G4:U69-containing tRNAs such as tRNA^{Cys} and tRNA^{Thr} can be mischarged by AlaRS, and mutating the G4:U69 base pair in both tRNAs results in a completely loss of mischarging activity, suggesting that the G4:U69 base pair is responsible for AlaRS mischarging [25,26]. Of particular interest, the capacity of AlaRS to mischarge G4:U69-tRNAs is unique to eukaryotes and not found in bacteria, while some G4:U69-containing tRNAs are particularly abundant in higher eukaryotic organisms such as vertebrates and mammals [25,26]. This evolutionary gain-of-function acting on noncognate tRNAs reflects the relaxed specificity of human AlaRS in tRNA recognition, suggesting diversity and regulation of protein translation in higher eukaryotic organisms. The deliberate mischarging of G4:U69-tRNAs indeed can generate mistranslated proteins [25], raising the possibility that these mistranslated proteins might participate in cellular stress responses and adaptations, as previously suggested [27].

2. Editing domain

Pre-transfer and post-transfer editing

Two crucial steps of protein synthesis involve the selection of cognate aa and tRNA by an aaRS to provide the correctly charged tRNA, and the subsequent selection of a correct aminoacyl-tRNA in the codon-programmed ribosome [28]. In fact, the accuracy of protein synthesis largely relies on the precise aminoacylation reactions, and the frequency of errors involving noncognate tRNA aminoacylation is very low (10^{-4} or lower) [1,28]. Once errors occur, the proofreading activities of aaRSs prevent mistranslation by hydrolysing those mistakenly activated amino acids or mischarged tRNAs in the editing site/domain. Generally speaking, editing by aaRSs can be carried out in two manners: pre-transfer editing and post-transfer editing. Pre-transfer editing hydrolyzes the misactivated aa-AMP before the aminoacyl moiety is transferred

to the tRNA, whereas post-transfer editing targets the mischarged tRNA for hydrolysis to remove the incorrect aa [29] (Fig. 3). In this regard, the editing domain of an aaRS, including AlaRS, plays a fundamental role in the accurate processing of genetic information for living organisms.

In E. coli, specific mutations within the AlaRS editing domain result in increased production of mischarged glycine-tRNA^{Ala} (Gly-tRNA^{Ala}) or serine-tRNA^{Ala} (Ser-tRNA^{Ala}). High concentrations of either Gly or Ser lead to cell death in editing-deficient strains, indicating that AlaRS-editing is specific to both Gly and Ser [30]. Loss of AlaRS proofreading in E. coli also induces a global stress response, leading to gross dysregulation of the proteome homoeostasis [30,31]. For example, the single point mutation (C666A) of E. coli AlaRS affected editing in almost all aspects including ATP hydrolysis, mischarging, diacylation and in vivo toxicity, suggesting the vital importance of C666 in maintaining proofreading [30]. In mice, point mutations in the editing domain of AlaRS lead to severe neurodegenerative and/or cardiomyopathic phenotypes [32,33]. These findings not only highlight the essential roles of the editing activity of AlaRS for maintaining proteome homoeostasis but also suggest potential pathological mechanisms underlying human diseases.

G3:U70-dependent recognition by the editing domain

The post-transfer editing function of AlaRS is also dependent on the G3:U70 recognition. In E. coli, installing the G3:U70 base pair into tRNA^{Thr} triggers recognition and subsequent hydrolysis by the AlaRS editing domain. In fact, diverse chimeric non-Ala-tRNAs grafted with the acceptor stem of tRNA^{Ala} can be deacylated by the AlaRS editing domain [2]. Therefore, the E. coli AlaRS contains two motifs for specific tRNA^{Ala} recognition, with one well-studied motif embedded in the aminoacylation domain as described above, and the second one within the editing domain between residues 680 and 699, which is unrelated to that for aminoacylation [2]. Notably, each of these two motifs can recognize tRNA^{Ala} in the absence of the other [2]. In addition to the two independent tRNA^{Ala} recognition elements within AlaRS, a third mechanism to recognize mischarged tRNA^{Ala} is provided by
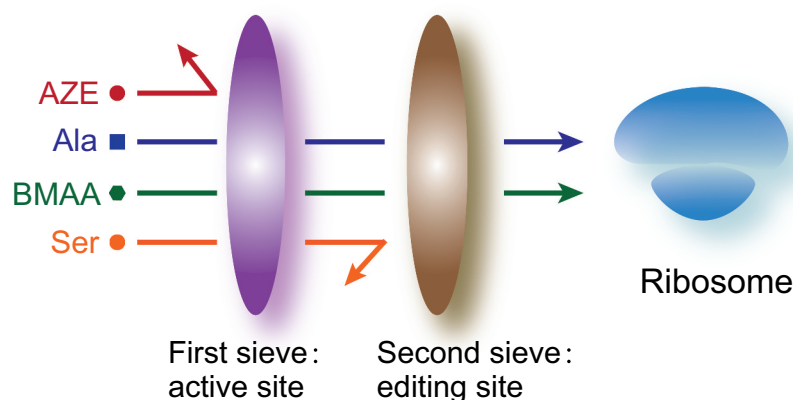

Figure 3. Editing for different amino acids by AlaRS.
The editing activities of AlaRS prevent mistranslation in two manners: pre-transfer editing (the first sieve, purple shield) and post-transfer editing (the second sieve, brown shield). Pre-transfer editing hydrolyzes the misactivated aa (e.g., AZE) before transferred to the tRNA, whereas post-transfer editing hydrolyzes the mischarged tRNA to clear the incorrect aa (e.g., Ser). In contrast, BMAA can slip through the proofreading sieves by AlaRS.

AlaXp proteins (AlaXps), which are genome-encoded free-stranding homologs of the editing domain of AlaRS widely distributed through all three kingdoms of life [34]. Taken together, three distinct proofreading activities are found to ensure the accuracy of tRNA^{Ala} aminoacylation to prevent mistranslation.

It is important to point out that the mischarging of Ala onto G4:U69-containing non-Ala tRNAs cannot be hydrolysed within the editing domain of AlaRS [25,26], because the editing domain would not fit with the cognate aa stably or in a productive manner [35], suggesting that the editing function of AlaRS can only handle noncognate aa, but not a noncognate tRNA. However, the mischarge tRNA can be edited *in trans* by the aaRS cognate to the mischarged tRNA species [25,26].

Editing for non-proteinogenic amino acids

In addition to the 20 standard proteinogenic amino acids, aaRSs have also evolved proofreading mechanisms to limit the incorporation of non-proteinogenic (np) amino acids into cellular proteins. For instance, Azetidine-2-carboxylic acid (AZE), a np aa found in plants [36], can be misactivated by both human AlaRS and prolyl-tRNA synthetase (ProRS). However, because AlaRS, but not ProRS, has editing function, AZE is exclusively misincorporated into proline (Pro) positions of proteins, leading to cell death, as demonstrated in zebrafish embryos [37]. Consistently, the toxicity can only be rescued by administration of Pro rather than Ala [37]. This work highlights the capacity of AlaRS to use its pre-transfer editing function to clear away misactivated AZE (Fig. 3). On the contrary, misactivation of another np aa β-*N*-methylamino-L-alanine (BMAA) escapes from being edited by AlaRS [38], because BMAA can inhibit the deacylation activity of AlaRS [38,39], revealing an exceptional case of how a np aa can evade proofreading by an aaRS (Fig. 3).

Multiple editing mechanisms gained in evolution

Over the course of evolution, AlaRS is not alone in minimizing errors in pairing the correct aa to tRNA^{Ala} for translational fidelity and cellular fitness. As aforementioned, AlaXps, the natural homologs of AlaRS editing domains, exhibit autonomous editing activity against mischarged Ser-tRNA^{Ala} and Gly-tRNA^{Ala} *in vitro* [40,41]. AlaXPs are also able to deacylate Ser-tRNA^{Ala} in cells and rescue Ser toxicity in the editing-defective AlaRS *E. coli* strain, indicating an essential role of AlaXP in compensation for editing deficiencies in AlaRS [34]. Another example is D-aminoacyl-tRNA deacylase (DTD), a key factor originally found to hydrolyse D-amino acids mistakenly attached to tRNAs. DTD also has proofreading activity towards the smaller and nonchral Gly misactivated by AlaRS [42]. In *E. coli*, cells lacking DTD in AlaRS editing-defective background display pronounced toxicity which can be alleviated by Ala supplementation [42]. Remarkably, DTD also specifically recognizes the G3:U70 wobble base pair of tRNA^{Ala}, and its activity to remove Gly mischarged on tRNA^{Ala} is conserved in bacteria and eukaryotes, suggesting an important role of DTD as a Gly deacylator to safeguard translational fidelity [42]. While DTD specifically acts on Gly-tRNA^{Ala}, the editing domain of AlaRS seems to correct primarily misactivated Ser, as a defect in the editing domain of AlaRS causes toxic effects only in the presence of high level of Ser but not Gly [32], although AlaRS can edit both Gly and Ser *in vitro*.

[30]. Recently, a vertebrate-specific co-factor ankyrin repeat domain 16 (ANKRD16) was identified as a direct interaction partner of AlaRS to help prevent the mischarging of Ser to tRNA^{Ala} in mice. This newly discovered proofreading mechanism in higher organisms will be further discussed in the review [43].

3. C-Ala domain

Crystal structures and functions of C-Ala in archaea and bacteria

The C-Ala domain was once termed 'dimerization domain' due to its role in mediating dimerization in *A. fulgidus* AlaRS [7]. In 2009, the crystal structures of the C-Ala domain in archaea and bacteria were successively unveiled [7,24]. In archaea, the C-Ala domain consists of a long helical subdomain and a globular subdomain. The helical subdomain mediates dimerization by forming a helix-loop-helix zipper, whereas the globular subdomain has a positively charged surface suitable for tRNA binding [7]. Indeed, further analysis revealed that the globular domain of *E. coli* C-Ala forms an ancient single-stranded nucleic acid binding motif, which facilitates cooperative binding of both aminoacylation and editing domains to the acceptor stem of tRNA^{Ala}, suggesting a C-Ala-dependent role in this process [24].

Functional transformation of C-Ala in human

During evolution, 19 out of 20 cytoplasmic aaRSs have appended new domains or motifs to accommodate higher organismal complexity, allowing new functions and interactions with a variety of cellular partners. AlaRS is the only exception with no additional domain found in human compared with simple organisms, such as bacteria and basal eukaryotes. It is believed that the existing C-Ala domain has been evolved to undergo a structural change to switch its function from facilitating tRNA binding to engendering new roles outside of aminoacylation in humans [44].

Among archaea, bacteria and eukaryotes, conservation analysis of AlaRS sequences demonstrated that the C-Ala diverges widely in the evolutionary progress to humans [23]. The low sequence conservation of C-Ala across species as well as the fact that human C-Ala is completely dispensable for aminoacylation raises the possibility that C-Ala may have developed distinct roles in higher organisms [23]. Indeed, comparison of the crystal structures between human C-Ala and *A. fulgidus* C-Ala revealed a completely different dimerization interface (Fig. 4A), which significantly affects the overall architecture of the full-length AlaRS in these two organisms (Fig. 2C). Notably, functional analyses indicated that human C-Ala, while losing the canonical tRNA binding activity, is able to enter the nucleus and to bind strongly to DNA [23]. The changes in C-Ala represent a unique evolutionary path for AlaRS through reshaping a pre-existing domain for novel functions instead of introducing a new domain or motif.

4. Alternative splice variants

Coinciding with the increasingly reported non-enzymatic functions of aaRSs [44,45], large numbers of splice variants that are
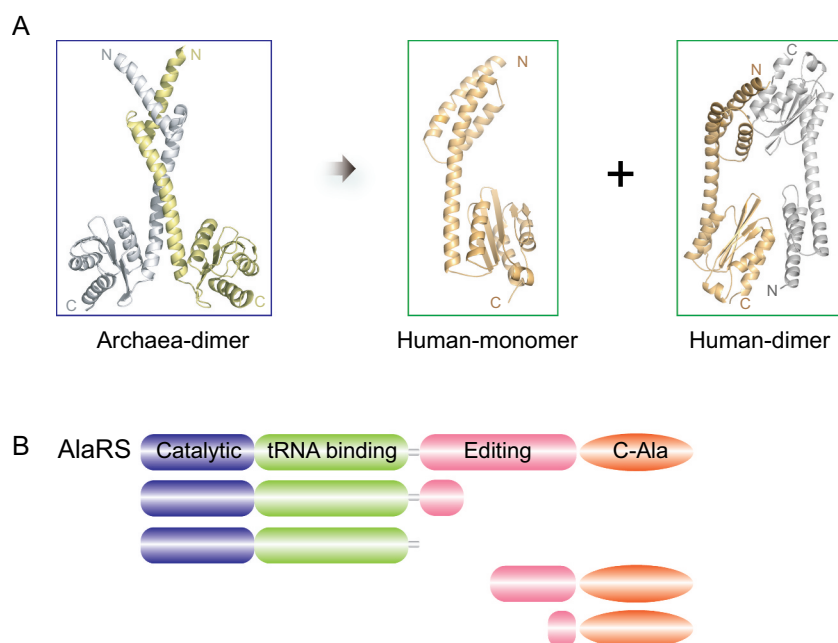
Figure 4. Uniqueness of the C-Ala domain in AlaRS.

(**A**) The crystal structure of a dimeric form of archaeal C-Ala (PDB entry 2ZVF), with one molecule is shown in light yellow and the other is in grey. The crystal structure of monomeric human C-Ala (PDB entry 5T76), and dimeric human C-Ala with one molecule is shown in light orange and the other is in grey (PDB entry 5T5S). (**B**) Totally four alternative splice variants of AlaRS were identified with two as catalytic nulls (at the bottom).

deprived of catalytic activities were discovered in human aaRSs, including AlaRS [46]. In 2014, scientists made a comprehensive search for alternative splice variants of aaRSs based on gene-specific multiplex PCR of exon-exon junctions followed by deep sequencing [47]. A large number of splice variants were discovered for each aaRS. Interestingly, many of them have the catalytic domain removed or disrupted and thus are effectively catalytic nulls (CNs). Among them, four splice variants of AlaRS were identified with two as CNs, both containing the C-Ala domain (Fig. 4B). Immunoblotting validated the presence of one C-Ala-containing fragment in Jurkat T cells, suggesting that the CNs mRNAs can be translated into proteins [47]. Along with other aaRS splice variants, the recombinant AlaRS CNs were further tested in various cell-based assays and showed biological activities, including cytoprotection, inflammatory response and cell differentiation [47]. Thus, the existence of these AlaRS splice variants indicates broad, nonenzymatic functions of AlaRS acquired in evolution.

Disease connections

Given both the essential canonical and diverse non-canonical roles of AlaRS, it is not surprising that dysfunction of AlaRS leads to pathological phenotypes. To date, AlaRS has been predominantly linked to neurodegenerative disorders in human and mouse models (Fig. 5).

1. Dominant *AARS* mutations in Charcot-Marie-Tooth disease

Charcot-Marie-Tooth disease (CMT) is the most common heritable peripheral neuropathy with an estimated prevalence of 1 in 2,500 [48,49]. To date, more than 100 CMT-associated genes have

been identified [50]. Intriguingly, aaRS is the largest protein family implicated in CMT. AlaRS is one of the five aaRSs linked to CMT.

So far, ten mutations in *AARS* have been associated with CMT subtype 2 N (CMT2N [MIM613287]), an autosomal dominant axonal peripheral neuropathy characterized by muscle weakness, wasting and impaired sensation in the extremities [51]. Among them, five variants (*AARS*[N71Y], *AARS*[G102R], *AARS*[R326W] *AARS*[R329H], and *AARS*[R337K]) are located in the aminoacylation domain, two (*AARS*[S627L] and *AARS*[E688G]) in the editing domain and the remaining three (*AARS*[E778A], *AARS*[Q855R] and *AARS*[D893N]) in the C-Ala domain [51–58]. Interestingly, most of these mutations do not necessarily affect the essential aminoacylation activity of AlaRS. Based on yeast complementation assays, the recurrently identified *AARS*[R329H] mutation from multiple unrelated families, along with the *AARS*[N71Y], *AARS*[G102R], *AARS*[R326W] and *AARS*[S627L] variants, cause impaired enzyme function [53,56,57]. In contrast, the *AARS*[E778A] variant does not affect the growth of the yeast compared with the wide-type enzyme [53], and the *AARS*[R337K] variant even stimulates yeast cell growth with enhanced tRNA charging activity [57]. Despite the divergent enzyme activities of these *AARS* variants, similar levels of disease-relevant toxicities were observed in the zebrafish embryos after micro-injections of the human mutant mRNAs, indicating loss of enzymatic function is unlikely responsible for the development of the CMT disease [57]. Other mutations in *AARS* including *AARS*[D893N] from a Chinese family [54], *AARS*[E688G] from an Irish family [55] and *AARS*[Q855R] from a Korean family [58], were reported; however, no study of their impacts on gene function, enzyme activity, and their associated phenotype in animal models has been reported.

One should note that the activity of the *AARS* CMT variants was mostly evaluated in haploid yeast cells, which
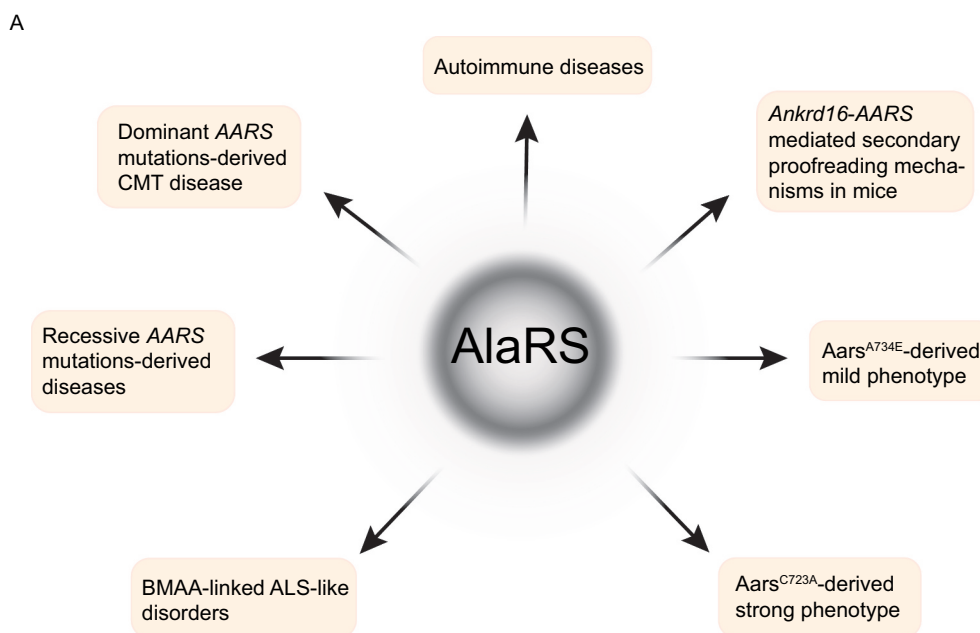
Figure 5. AlaRS and its disease connections.

Dysfunction of AlaRS leads to human diseases and pathological phenotypes in mouse models. Dominant and recessive *AARS* mutations result in CMT and other neurological diseases, respectively. Editing-defective AlaRS causes different levels of phenotypes in mouse models and BMAA-linked ALS-like disorders in human. Beyond neurodegenerative diseases, human AlaRS is also linked to human autoimmune diseases.

cannot fully represent the functional impact of these variants in mammalian cells, which are diploid. For example, the P134H mutation in histidyl-tRNA synthetase (HisRS), another CMT-linked aaRS, causes a complete loss of function in the yeast model [59]; however, the latest analyses using actual patient cells demonstrated no loss of enzymatic function. Therefore, the disease is most likely caused by a gain-of-function mechanism driven by a mutation-induced conformation opening [60]. This may apply to *AARS*-linked CMT, and more *in vitro* studies as well as *in vivo* animal models are necessary to provide essential information regarding the pathogenicity of above identified variations.

2. Recessive AARS *mutations in neurological diseases*

In contrast to CMT2N, which is characterized by its dominant genetic traits, other severe neurological and developmental diseases are caused by recessive mutations in *AARS*. For example, homozygous ($AARS^{R751G}$) and compound heterozygous ($AARS^{R751G}$ and $AARS^{K81T}$) autosomal recessive mutations in *AARS* were identified in three individuals presenting primarily severe early-onset infantile epileptic encephalopathy with persistent myelination defect and peripheral neuropathy [61]. Both $AARS^{R751G}$ and $AARS^{K81T}$ mutations impair aminoacylation activity of AlaRS *in vitro* and reduce growth in yeast cells, suggesting a significant reduction in enzymatic function [61]. Although the $AARS^{R751G}$ variant is located in the editing domain, no detectable effect of this mutation was found on the editing activity. Later on, two more heterozygous autosomal recessive variants in *AARS* ($AARS^{Y690LS*}$ and $AARS^{G913D}$) were further identified in two siblings presenting progressive microcephaly, hypomyelination, and epileptic encephalopathy [62]. Again, *in vitro* assays indicated an

aminoacylation defect of these two mutants. Notably, the $AARS^{Y690LS*}$ variant disrupts the editing domain and would cause a loss of the editing activity for hydrolysing misacylated $tRNA^{Ala}$, whereas the $AARS^{G913D}$ mutation is located in the C-Ala domain and does not affect the deacylation activity compared to the wild-type enzyme. These findings, to a certain extent, broaden the genetic and clinical spectrum associated with *AARS* mutations in human. However, mechanistic studies in the context of AlaRS-linked human diseases are limited. Further investigations at the cellular and organismal levels are warranted to delineate the roles of AlaRS in the development and function of the neurological system, and the impact of its dominant and recessive mutations.

3. Phenotypes and mechanisms linked to mutations in mouse AARS

Mild editing-deficient phenotype in sticky mice

The sticky (*sti*) mutation is associated with rough and unkempt sticky appearance of fur in mice homozygous for the mutation, which also exhibit an overt ataxia and extensive loss of cerebellar Purkinje cells starting from six weeks of age. This mutation was identified as a missense mutation leading to A734E substitution in the editing domain of murine AlaRS ($Aars^{A734E}$, also termed $Aars^{sti}$). The mutation only causes a mild increase (2-fold) of mischarged Ser-$tRNA^{Ala}$, yet leads to substantial accumulation of misfolded proteins in neurons and subsequent degeneration of Purkinje cells [32]. Later studies from the same group of scientists revealed that the phenotypes found in the sticky mice were not only associated with the A734E mutation but also a partial loss of function of an epistatic modifier ANKRD16 (see below).

Secondary proofreading mechanisms in sticky mice

Interestingly, a strain-specific single nucleotide polymorphism (SNP) in *Ankrd16* was found in C57BL/6 J mice where the neurodegenerative phenotypes were observed with the *Aars*[sti/sti] mutation. The SNP generates an alternative splicing site that leads to a significantly reduced protein level of ANKRD16 in the cerebellum [43]. Further experiments revealed that ANKRD16 protein binds directly to the catalytic domain of AlaRS and specifically captures the misactivated Ser through its Lys side chains [43]. It is interesting to note that, other than serine, Ala can also be linked to the lysine side chains of ANKRD16; however, this reaction is negligible in the presence of tRNA[Ala], suggesting a selective preference of ANKRD16 for Ser. The interaction between AlaRS and ANKRD16 prevents misincorporation of Ser in peptides to ensure accurate protein translation [43]. This work identifies ANKRD16 as a new aa-accepting collaborator of AlaRS to facilitate the editing function of AlaRS in mammalian cells. It is tempting to speculate that the Ser-modified ANKRD16 would play additional roles beyond merely accepting a mischarged aa. Possibly, it would mediate a signalling pathway downstream of an AlaRS-mischarging event. The crosstalk between AlaRS and ANKRD16 introduces an additional layer in the understanding of AlaRS editing-defective pathologies and heralds new opportunities in therapeutic intervention for neurogenerative diseases.

Taken together, the elegant and comprehensive studies of the editing-deficient phenotypes in *sti* mice and the discovery of *Ankrd16* highlight the importance of AlaRS editing functions in higher organisms. Also, the requirement of *Ankrd16* for AlaRS fidelity reflects a high proteotoxic cost of AlaRS-mediated mistranslation events, which are unaffordable across species, especially in mammalian cells.

Strong editing-deficient phenotype in sticky mice

The same group of scientists also explored another mutation in the editing domain of murine AlaRS (*Aars*[C723A]), corresponding to C666A in bacterial AlaRS which is severely defective for editing [30]. In agreement, the *Aars*[C723A] mutation induced the production of mischarged Ser-tRNA[Ala] at a level at least 15-fold higher than that with the wild-type enzyme. Mouse embryos homozygous for the *Aars*[C723A] mutation died before midgestation [33]. To explore the phenotypes associated with different levels of editing deficiency, the authors generated compound heterozygotes *Aars*[stop/sti] and *Aars*[C723A/sti] mice. Strikingly, in addition to the loss of Purkinje cells, both *Aars*[stop/sti] and *Aars*[C723A/sti] mice show widespread ubiquitinated protein aggregates in cardiomyocytes and subsequent death of these cells, eventually leading to cardiomyopathy [33]. This study, together with their previous work [32], suggests protein aggregation as a common phenomenon induced by AlaRS editing deficiency in both neurons and cardiomyocytes, two postmitotic cell types in which protein aggregates cannot be diluted by cell division. Nonetheless, one cannot rule out the possibility that other mechanisms contribute to these neurodegenerative and cardiomyopathy phenotypes in mice.

BMAA-linked ALS-like neurodegenerative disorder

Although the cause behind CMT remains a mystery, scientists have recently answered the question that has been puzzling neurologists for more than 50 years. BMAA, a np aa isolated from cycad seeds in Guam in 1967, was once considered the cause of Guam symptom composed of amyotrophic lateral sclerosis (ALS) and Parkinson's/dementia comple (PDC) [63]. Decades later, BMAA is further linked to multiple neurodegenerative diseases including Alzheimer's disease (AD) [64]. However, the question of how such a np aa (i.e. BMAA) could bioaccumulate in the brains was still unaddressed.

In 2013, Dunlop and the colleagues revealed that BMAA is mistaken for Ser by a tRNA synthetase during protein synthesis, causing protein misfolding and aggregation [65]. Recently, another research group further pinpointed AlaRS as responsible for misacylating BMAA. Unlike AZE, BMAA can escape from the intrinsic AlaRS proofreading activity and is transferred to tRNA[Ala] to form BMAA-tRNA[Ala]. What's worse, BMAA acts as a competitive inhibitor to AlaRS in activation of the cognate Ala and also perturbs the deacylation activity of AlaRS on Ser-tRNA[Ala] [38]. This finding not only reveals the mechanisms by which BMAA can disrupt the integrity of protein synthesis but also addresses the pathogenesis regarding BMAA-linked neurodegenerative diseases that has been sealed for years to potentially open a new route for treating these lifelong diseases.

4. Autoimmune disease

Beyond neurodegenerative diseases, human AlaRS is also linked to autoimmune diseases through its autoantibodies.

Historically, HisRS is the first aaRS to be identified as an autoantigen (Jo-1 antigen) in myositis, an autoimmune disease characterized by weakness and wasting of muscles [66]. The autoantigenic HisRS, together with asparaginyl-tRNA synthetase (AsnRS) were later found to perpetuate the development of myositis by activating chemokine receptors on T lymphocytes and immature dendritic cells [67]. Meanwhile, more autoantibodies against aaRSs including anti-AlaRS (also known as anti-PL-12) were successively identified in patients with similar inflammatory symptoms, collectively termed autoimmune antisynthetase syndrome (ASS) [68,69].

Clinically, anti-AlaRS autoantibodies are frequently associated with myopathies [70–73], interstitial lung disease [74,75], and type 1 diabetes mellitus (DM) [76]; however, little is known about how the production of anti-AlaRS autoantibodies is triggered and the role of these autoantibodies in disease progression. Does AlaRS/anti-PL-12 play differential roles from other aaRSs/anti-aaRSs in the initiation and development of disease? Is there a connection between AlaRS-linked CMT and autoimmune myositis, as both diseases are manifested as muscle weakness and wasting? Being an autoantigen, is this unlikely that AlaRS or its fragment can be used in treating inflammatory diseases as suggested for HisRS [77]? To address these issues, further in-depth investigations of AlaRS in autoimmune diseases are necessary in order to provide insights into specific targeted therapies.

Final remarks and future directions

We have presented the uniqueness of AlaRS among other family members in the following aspects: 1) AlaRS is the only class II aaRS that does not require dimerization for aminoacylation. 2) AlaRS is the only aaRS exclusively recognizing a single identity determinant

(G3:U70 wobble base pair) in the acceptor stem of tRNA without other regions involved. 3) AlaRS can mischarge noncognate tRNAs with a G4:U69 base pair as a deliberate gain of function in eukaryotic organisms. 4) AlaRS is a single exception whose evolutionarily new functions are likely acquired by reshaping the ever-existing C-Ala domain rather than adding new domains or motifs.

With progressive in-depth research, more understanding on AlaRS, including both the cytosolic and mitochondrial forms encoded by different genes (*AARS1* and *AARS2*), are obtained. For example, mice bearing the milder editing mutation of A734E in cytosolic AlaRS (ctAlaRS) lead to neurodegenerative phenotype [32], whereas recapitulation of the same mutation into the mtAlaRS results in early embryonic lethality in mice [78], suggesting that the mitochondrial proteome is highly sensitive to mtAlaRS dysfunction, probably due to imperfect proofreading mechanisms in mitochondria and/or the lack of additional protective mechanisms such as that illustrated by ANKRD16 for ctAlaRS. Another example is from a recent study which identified multiple nucleotide elements in the acceptor stem for recognizing human mitochondrial tRNA^Ala, indicating a G3:U70-independent recognition mechanism in human mtAlaRS [79]. The dyad of Asn317 and Asp416 in human ctAlaRS that recognizes the G3:U70 pair is also lost during mtAlaRS evolution and replaced by Met343 and Gly444, respectively, in human mtAlaRS [80]. Although mtAlaRS is outside the scope of this review, the differences between the cytosolic and mitochondrial forms in mammalian cells, as exemplified above, emphasize the complex and distinct properties of AlaRS in two subcellular compartments, all of which need to be taken into account when we are trying to understand the pathophysiological roles of AlaRS.

The identification of AlaRS CNs splice variants with various biological activities provides additional evidence for diverse non-canonical functions of AlaRS during evolution. These newly-gained features of AlaRS offer new opportunities for therapeutic efforts to target a certain role involved in pathogenesis without affecting other physiologically important functions at the same time. Still unclear is how to find and develop new targets for drug discovery in human neurodegenerative diseases such as AlaRS-linked CMT. Advances in next-generation sequencing technology have promoted identification of more *AARS* mutations in CMT. However, drug development regarding these identified variants is still in its infancy, as the dysfunction of each variant in pathogenesis of CMT has not been fully uncovered. For most AlaRS variants, functional analyses are limited to yeast models which however are insufficient to interpret human diseases in the context of complex networks among genes, proteins, and microenvironmental factors. Generation of animal models such as mouse and fly models may allow for recapitulation of human disease relevant phenotypes, paving the way for better understanding diseases and offering therapeutic strategies.

Despite these unaddressed barriers, increasing research has strengthened our grasp of potential underpinnings of the initiation and development of AlaRS-associated human diseases. Future investigations into the unexplored territories of AlaRS could bring unforeseen surprises and define an unprecedented path to the next generation of well-tailored therapies.

Authors' contributions

X-L. Y and L.S. designed the review. All the authors wrote the paper and made the figures.

Disclosure statement

The authors report no conflicts of interest.

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