

# Gain of C-Ala enables AlaRS to target the L-shaped tRNA<sup>Ala</sup>

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## ABSTRACT

Unlike many other aminoacyl-tRNA synthetases, alanyl-tRNA synthetase (AlaRS) retains a conserved prototype structure throughout biology. While *Caenorhabditis elegans* cytoplasmic AlaRS (CeAlaRS<sub>c</sub>) retains the prototype structure, its mitochondrial counterpart (CeAlaRS<sub>m</sub>) contains only a residual C-terminal domain (C-Ala). We demonstrated herein that the C-Ala domain from CeAlaRS<sub>c</sub> robustly binds both tRNA and DNA. It bound different tRNAs but preferred tRNA<sup>Ala</sup>. Deletion of this domain from CeAlaRS<sub>c</sub> sharply reduced its aminoacylation activity, while fusion of this domain to CeAlaRS<sub>m</sub> selectively and distinctly enhanced its aminoacylation activity toward the elbow-containing (or L-shaped) tRNA<sup>Ala</sup>. Phylogenetic analysis showed that CeAlaRS<sub>m</sub> once possessed the C-Ala domain but later lost most of it during evolution, perhaps in response to the deletion of the T-arm (part of the elbow) from its cognate tRNA. This study underscores the evolutionary gain of C-Ala for docking AlaRS to the L-shaped tRNA<sup>Ala</sup>.

## INTRODUCTION

### Eukaryotes contain two sets of aaRSs

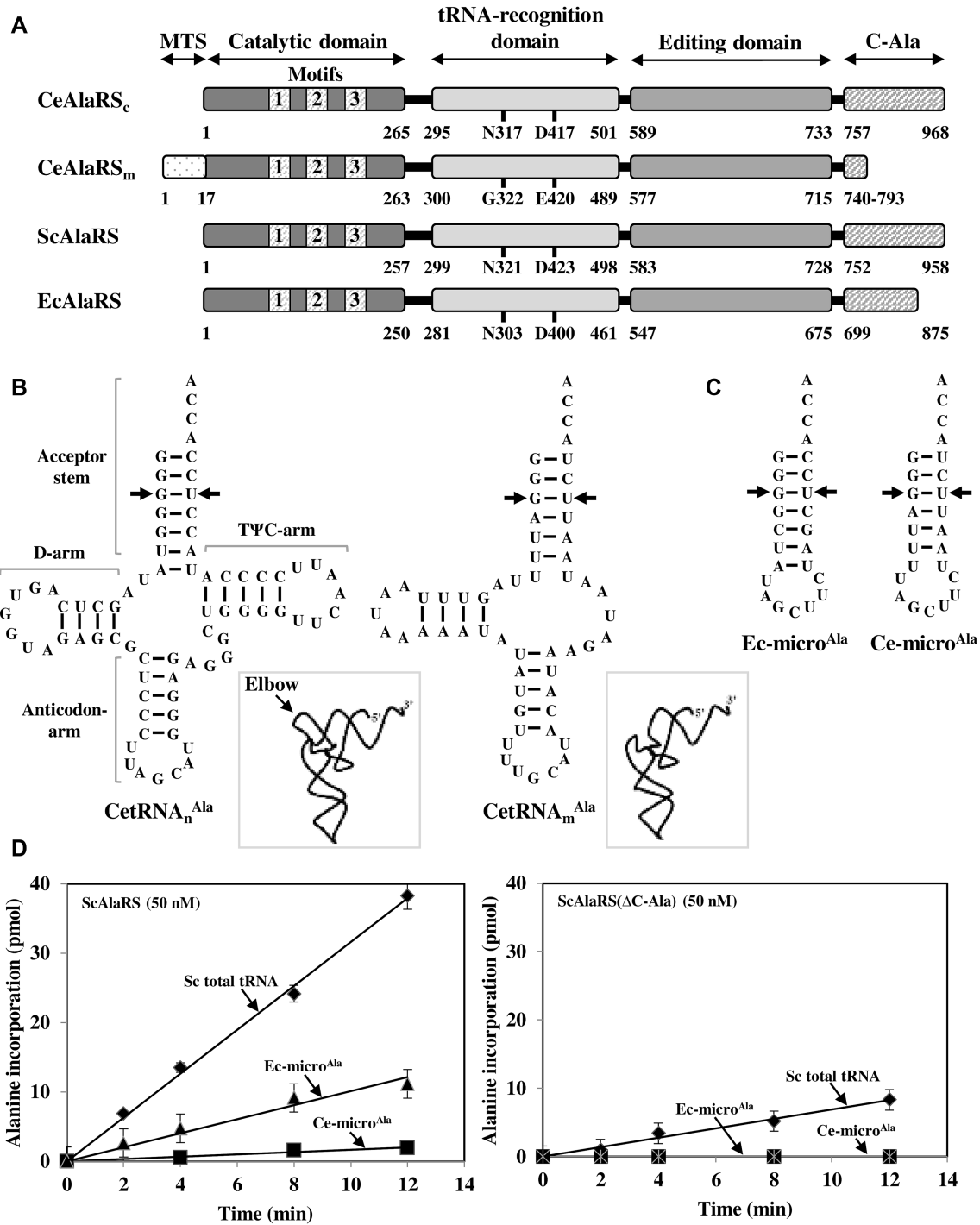
Aminoacylation of tRNA by aminoacyl-tRNA synthetase (aaRS) establishes the universal genetic code (1). The accuracy of tRNA aminoacylation is crucial for maintaining the fidelity of protein synthesis. AaRSs verify a tRNA's identity by recognizing certain identity elements present on the tRNA, which can be single nucleotides, base pairs or even post-transcriptional modifications (1–3). Identity elements often reside in the acceptor stem or the anticodon loop of

a tRNA, and they are normally conserved across a wide range of species. In eukaryotic cells, the nuclear genome normally encodes two distinct sets of aaRSs, one functioning in the cytoplasm and the other functioning in the mitochondria. For example, *Vanderwaltozyma polyspora*, *Homo sapiens* and *Caenorhabditis elegans* each possess two distinct nuclear alanyl-tRNA synthetase (AlaRS) genes, one encoding the cytoplasmic form and the other encoding its mitochondrial counterpart (4–6). Nevertheless, in some cases, both isoforms of a given aaRS are produced from a single gene through alternative transcription and translation, examples of which include the histidyl-tRNA synthetase genes of *C. elegans* and *Saccharomyces cerevisiae* (7,8).

### AlaRS retains an evolutionarily conserved modular structure

Most aaRSs expanded by recruiting novel noncatalytic appended domains during evolution. Conversely, almost all AlaRSs retained the same prototype structure across all three domains of life (Figure 1). The prototype structure consists of four functional domains that are responsible for catalysis, tRNA recognition, editing and oligomerization (9). Prokaryotic AlaRSs are normally  $\alpha_4$  tetramers, while those from eukaryotes are monomers (10). The catalytic and tRNA recognition domains are often referred to collectively as the aminoacylation domain. The editing domain is responsible for hydrolysis of the mischarged Ser- or Gly-tRNA<sup>Ala</sup> and is therefore critical for maintaining translational fidelity and preventing cytotoxicity. In mice, even a mild defect in editing activity leads to neural degeneration (11). The C-terminal domain (C-Ala) is highly diverse across species and plays an important role in both aminoacylation and editing (12). A prokaryotic C-Ala domain consists of an N-terminal helical subdomain, which mediates dimer formation, and a C-terminal globular subdomain, which binds tRNA (Supplementary Figure S1) (13,14). Recent studies further showed that C-Ala has been repurposed

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**Figure 1.** *Caenorhabditis elegans* AlaRS and tRNA<sup>Ala</sup>. (A) Modular organization of CeAlaRS<sub>c</sub>, CeAlaRS<sub>m</sub>, ScAlaRS and EcAlaRS. Relative positions of the functional domains and motifs in CeAlaRSs are labeled. The amino acid residues that presumably make contacts with G3:U70 are labeled. (B) CetRNA<sub>n</sub><sup>Ala</sup> and CetRNA<sub>m</sub><sup>Ala</sup>. The identity elements G3:U70 are marked with an arrow for clarity. Additionally, the representative three-dimensional structures of CetRNA<sub>s</sub><sup>Ala</sup> (boxed) are shown, with an emphasis on the elbow. (C) Ec-micro<sup>Ala</sup> and Ce-micro<sup>Ala</sup>. (D) Aminoacylation of tRNA by ScAlaRS and ScAlaRS(ΔC-Ala).

from tRNA binding in prokaryotes to DNA binding in humans (14). Exceptions to this four-domain rule have been recovered. For example, *Leishmania major* AlaRS contains an extra insertion domain upstream of the editing domain (15), while *Nanoarchaeum equitans* AlaRS is split into two parts (16).

### G3:U70 are the universal identity elements of tRNA<sup>Ala</sup>

AlaRS identifies its cognate tRNA through a single GU base pair, G3:U70, in the acceptor stem without making contact with the anticodon. Three aaRSs, alanyl-, leucyl- and seryl-tRNA synthetases, have been shown to lack an anticodon-binding domain (17). G3:U70 is absent from all other tRNAs but is strictly conserved in tRNA<sup>Ala</sup> ranging from *Escherichia coli* to human cytoplasm (18–20). Transferring G3:U70 into a nonalanine tRNA also converts it into an alanine acceptor both *in vitro* and *in vivo* (21). In a sense, AlaRS exemplifies an ancient aaRS, which identifies its tRNA through recognition of an operational RNA code (a specific sequence or structure) embedded in the acceptor stem (22). A structural study on AlaRS of the archaea *Archaeoglobus fulgidus* in complex with tRNA<sup>Ala</sup> revealed that two amino acid residues—N359 and D450—in the tRNA recognition domain make specific hydrogen bonds with G3:U70, and thereby establish it as an alanine acceptor (13). These two amino acid residues are highly conserved in AlaRS, regardless of its evolutionary origin. Nevertheless, noncanonical AlaRSs that deviate from the conserved features have been found. For example, human mitochondrial AlaRS recognizes the variable loop in its cognate tRNA<sup>Ala</sup> (5), and *Drosophila melanogaster* mitochondrial AlaRS recognizes a shifted GU base pair, G2:U71, in its cognate tRNA<sup>Ala</sup> (23).

### *Caenorhabditis elegans* mitochondrial AlaRS lost most of its C-Ala

In contrast to canonical AlaRSs, *C. elegans* mitochondrial AlaRS (CeAlaRS<sub>m</sub>) lost almost all of its C-Ala (6), and its cognate tRNA (CetRNA<sub>m</sub><sup>Ala</sup>) lacks the T-arm, which together with the D-arm forms the elbow of the L-shaped tRNA. As all the mitochondrial tRNAs in this nematode are defective in either the D- or T-arm (24), they cannot fold into the typical L form. An earlier study reported that CeAlaRS<sub>m</sub> still retains G3:U70 specificity (6). However, little is known about the evolutionary significance of gaining or losing C-Ala and its true impact on enzyme activity. We showed herein that C-Ala of the nematode cytoplasmic AlaRS (CeAlaRS<sub>c</sub>) is both a tRNA- and a DNA-binding domain. Removal of C-Ala from CeAlaRS<sub>c</sub> sharply reduced its aminoacylation activity, while fusion of this domain to CeAlaRS<sub>m</sub> selectively and distinctly enhanced its activity toward the elbow-containing tRNA<sup>Ala</sup>. This study highlights the evolution and function of the C-Ala domain.

## MATERIALS AND METHODS

### Construction of plasmids

Cloning of the gene encoding CeAlaRS<sub>c</sub> or CeAlaRS<sub>m</sub> into pADH (a high-copy-number yeast vector with an *ADH* promoter) and pTEF1 (a high-copy-number yeast vector with

a *TEF1* promoter) followed a standard protocol. In brief, a set of gene-specific primers was designed to amplify the open reading frame of the gene via polymerase chain reaction (PCR) using *C. elegans* complementary DNA as the template. The forward primer with an NdeI site was annealed to the 5'-end sequence of the open reading frame, while the reverse primer with an XhoI site was annealed to the 3'-end sequence immediately upstream of the stop codon. Note that the native MTS (mitochondrial targeting signal) sequence of CeAlaRS<sub>m</sub> was not included in the construct. The PCR-amplified DNA fragment was treated with NdeI and XhoI before cloning into the aforementioned vectors. Fusion of a heterologous MTS to CeAlaRS<sub>m</sub> followed a strategy described earlier (25). To fuse C-Ala to the N- or C-terminus of CeAlaRS<sub>m</sub>, an NdeI–NdeI or XhoI–XhoI fragment (containing bp +2269 to +2904 of CeAlaRS<sub>c</sub>) was PCR amplified and cloned into the 5' NdeI or 3' XhoI site of the open reading frame encoding CeAlaRS<sub>m</sub>, yielding (C-Ala)-CeAlaRS<sub>m</sub> or CeAlaRS<sub>m</sub>-(C-Ala). To delete C-Ala from CeAlaRS<sub>c</sub>, an NdeI–XhoI fragment (containing bp +1 to +2268 of CeAlaRS<sub>c</sub>) was PCR amplified and cloned into appropriate vectors. Cloning of the genes encoding ScAlaRS and its C-Ala deletion mutant followed a similar strategy.

For protein purification, the target genes were individually cloned into pET21b (an *E. coli* expression vector), and the resultant plasmids were transformed into BL21-CodonPlus(DE3) for protein expression. His<sub>6</sub>-tagged proteins were purified to homogeneity through Ni-NTA column chromatography as previously described (25). Western blotting was carried out using an HRP-conjugated anti-His<sub>6</sub> tag antibody as the probe (26). Protein–RNA or protein–DNA binding affinities were determined by electrophoretic mobility shift assay as described previously (27). GFP microscopy followed a protocol described earlier (28).

### Rescue assay for cytoplasmic activity on 5-FOA

Construction of a yeast haploid *ALAI* knockout (KO) strain (*MATα*, *alal::kanMX4*, *his3Δ1 leu2Δ0 lyS3Δ0 ura3Δ0*) was reported earlier (29,30). Note that *ALAI* is a dual-functional gene that encodes cytoplasmic and mitochondrial forms of AlaRS through alternative initiation of translation from two in-frame initiator codons (30). To test whether a heterologous AlaRS gene can functionally substitute for the cytoplasmic activity of *ALAI*, a test plasmid carrying the target gene and a *LEU2* marker was transformed into the KO strain, and the resultant transformant was tested for its ability to grow on 5-fluoro-orotic acid (5-FOA, 1 mg/ml) plates. Because 5-FOA can be metabolized by yeast to a toxic compound in the presence of *URA3*, the transformant must evict the maintenance plasmid [with a wild-type (WT) *ALAI* gene and a *URA3* marker] on 5-FOA to survive. Thus, the transformant cannot grow on 5-FOA unless the test plasmid encodes a functional cytoplasmic AlaRS.

### Rescue assay for mitochondrial activity on YPG

To test whether a heterologous AlaRS gene can functionally substitute for the mitochondrial activity of *ALAI*, a test

plasmid (with the target gene and a *LEU2* marker) and a second maintenance plasmid (with an initiator mutant of *ALAI* that encodes only cytoplasmic AlaRS and a *HIS3* marker) were cotransformed into the yeast KO strain (30). The first maintenance plasmid (with a *URA3* marker) was evicted from the cotransformant on 5-FOA. Following 5-FOA selection, the cotransformant (with the second maintenance plasmid and test plasmid) was further tested on YPG plates. Because glycerol (a nonfermentable carbohydrate) is the sole carbon source in YPG, yeast cells must retain functional mitochondria to metabolize glycerol and survive in this medium. Thus, the cotransformant cannot grow on YPG unless the test plasmid encodes a functional mitochondrial AlaRS.

### In vitro transcription of tRNA

Preparation of the  $\text{CetRNA}_n^{\text{Ala}}$ ,  $\text{CetRNA}_m^{\text{Ala}}$ ,  $\text{Ce-micro}^{\text{Ala}}$  and  $\text{Ec-micro}^{\text{Ala}}$  transcripts followed a standard protocol. Briefly, a DNA duplex containing a T7 promoter followed by a sequence encoding the tRNA or microhelix was cloned into the *Sma*I site of pUC18. The transcription template was enriched by PCR amplification of the insert. *In vitro* transcription was performed at 37°C for 3 h with 0.3  $\mu\text{M}$  T7 RNA polymerase in 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 20 mM  $\text{MgCl}_2$ , 5 mM DTT, 1 mM spermidine and 2 mM of each NTP. The transcript was purified using a 15% denaturing urea-polyacrylamide gel. After ethanol precipitation and vacuum drying, the RNA pellet was dissolved in 1 $\times$  TE buffer [20 mM Tris-HCl (pH 8.0) and 1 mM EDTA], refolded by heating to 65°C and gradually cooled to room temperature in the absence of  $\text{MgCl}_2$ . *In vitro* transcription of  $\text{CetRNA}_n^{\text{Ala}}$  followed a similar protocol.

### Aminoacylation assay

Aminoacylation was performed at ambient temperature in a buffer containing 50 mM HEPES (pH 7.5), 50 mM KCl, 15 mM  $\text{MgCl}_2$ , 5 mM dithiothreitol, 10 mM ATP, 0.1 mg/ml bovine serum albumin, 100  $\mu\text{M}$  yeast total tRNA or 5  $\mu\text{M}$  tRNA (or microhelix), 20  $\mu\text{M}$  alanine (1.34  $\mu\text{M}$   $^3\text{H}$ -alanine; PerkinElmer, Waltham, MA, USA), and 50 or 100 nM AlaRS. Reactions were quenched at various time points by spotting 10  $\mu\text{l}$  aliquots of the reaction mixture onto Whatman filters (Maidstone, Kent, UK) that had been pre-soaked in 5% trichloroacetic acid and 2 mM alanine. The filters were washed three times for 15 min each in ice-cold 5% trichloroacetic acid before liquid scintillation counting. Active protein concentrations were determined by active site titration as previously described (31). Aminoacylation data were obtained from three independent experiments and averaged.

## RESULTS

### *Caenorhabditis elegans* contains a canonical and a noncanonical AlaRS

*Caenorhabditis elegans* possesses two distinct nuclear AlaRS genes, one encoding the cytoplasmic form ( $\text{CeAlaRS}_c$ ) and the other encoding its mitochondrial

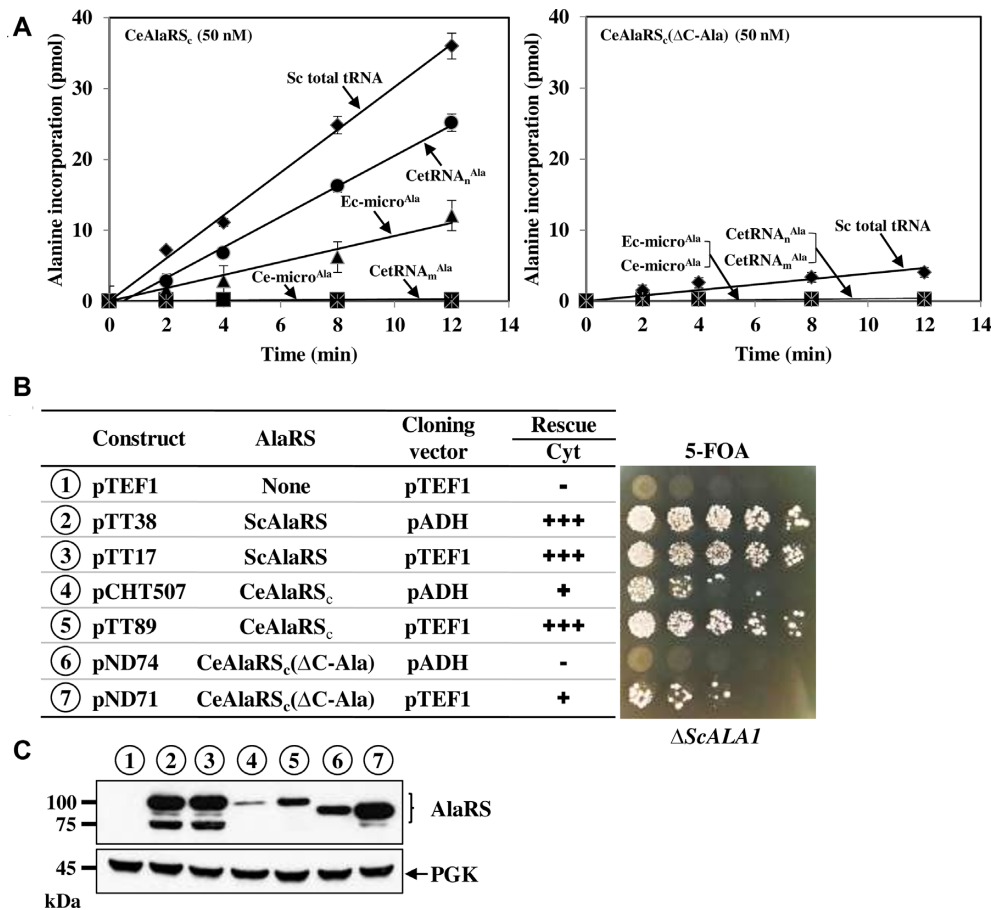
counterpart.  $\text{CeAlaRS}_m$  contains a residual C-Ala (with  $\sim 54$  amino acid residues) and an extra cleavable N-terminal MTS. Upon post-translational processing, the most distinct difference between the two isoforms is C-Ala (Figure 1A). The nematode nuclear-encoded cytoplasmic  $\text{tRNA}^{\text{Ala}}$  ( $\text{CetRNA}_n^{\text{Ala}}$ ) retained a cloverleaf structure and is predicted to fold into an L form, while its mitochondrial isoacceptor ( $\text{CetRNA}_m^{\text{Ala}}$ ) lacked the T-arm and is predicted to fold into an elbowless crescent-shaped structure (Figure 1B). To determine the tRNA preferences of  $\text{AlaRS}_c$  and its C-Ala deletion construct, three tRNA substrates, yeast total or unfractionated tRNA (that contains all 20 different tRNAs),  $\text{Ec-micro}^{\text{Ala}}$  (an acceptor-stem microhelix derived from  $\text{EctRNA}^{\text{Ala}}$ ) and  $\text{Ce-micro}^{\text{Ala}}$  (an acceptor-stem microhelix derived from  $\text{CetRNA}_m^{\text{Ala}}$ ) (Figure 1C), were used as the substrates for aminoacylation. It is worth mentioning that the G1:U72 base pair in the acceptor stem of  $\text{Ce-micro}^{\text{Ala}}$  has been shown to act as an anti-determinant for aminoacylation by  $\text{EcAlaRS}$  (6). The cloverleaf structures of  $\text{SctRNA}_n^{\text{Ala}}$ ,  $\text{SctRNA}_m^{\text{Ala}}$  and  $\text{EctRNA}^{\text{Ala}}$  are shown in Supplementary Figure S2.

For comparison, we first checked the tRNA preferences of *S. cerevisiae* AlaRS ( $\text{ScAlaRS}$ ) and its C-Ala deletion construct. Figure 1D showed that  $\text{ScAlaRS}$  prefers  $\text{SctRNA}_n^{\text{Ala}}$  over  $\text{Ec-micro}^{\text{Ala}}$  (4-fold) and  $\text{Ec-micro}^{\text{Ala}}$  over  $\text{Ce-micro}^{\text{Ala}}$  (8-fold). Deletion of C-Ala drastically reduced its aminoacylation activity toward  $\text{SctRNA}_n^{\text{Ala}}$  (5-fold) and abolished its aminoacylation activity toward both microhelices (Figure 1D). No detectable aminoacylation activity toward the microhelices was observed even when the concentration of the deletion construct was increased 4-fold (Supplementary Figure S3). Complementation assay on 5-FOA using a yeast *ALAI* KO strain showed that deletion of C-Ala from  $\text{ScAlaRS}$  completely eliminates its rescue activity (Supplementary Figure S4). As expected, *E. coli* AlaRS exhibited a similar preference for these three tRNA substrates (Supplementary Figure S5). Conceivably, canonical AlaRSs prefer the L-shaped  $\text{tRNA}^{\text{Ala}}$ , and G1:U72 blocked the aminoacylation of  $\text{Ce-micro}^{\text{Ala}}$  by noncognate enzymes.

### Deletion of C-Ala impairs the aminoacylation activity of $\text{CeAlaRS}_c$

We next determined the aminoacylation activity of  $\text{CeAlaRS}_c$  under similar conditions, but with the inclusion of two more tRNAs,  $\text{CetRNA}_n^{\text{Ala}}$  and  $\text{CetRNA}_m^{\text{Ala}}$ . As shown in Figure 2A,  $\text{CeAlaRS}_c$  exhibited a tRNA preference similar to those of  $\text{ScAlaRS}$  and  $\text{EcAlaRS}$ . It strongly preferred  $\text{SctRNA}^{\text{Ala}}$  over  $\text{Ec-micro}^{\text{Ala}}$  ( $\sim 4$ -fold) and failed to charge  $\text{Ce-micro}^{\text{Ala}}$  to a detectable level. Like  $\text{SctRNA}_n^{\text{Ala}}$ ,  $\text{CetRNA}_n^{\text{Ala}}$ , but not  $\text{CetRNA}_m^{\text{Ala}}$ , was a preferable substrate for  $\text{CeAlaRS}$ . Deletion of C-Ala from this enzyme drastically impaired its aminoacylation activity toward all tRNA substrates used (Figure 2A). Notably, the deletion mutant failed to charge  $\text{CetRNA}_m^{\text{Ala}}$ ,  $\text{Ec-micro}^{\text{Ala}}$  and  $\text{Ce-micro}^{\text{Ala}}$  to a detectable level even when its concentration was increased 5-fold (Supplementary Figure S6). This result indicates that C-Ala plays an important role in the aminoacylation of  $\text{tRNA}^{\text{Ala}}$  by  $\text{CeAlaRS}_c$ .





**Figure 2.** C-Ala is important for the aminoacylation activity of CeAlaRS<sub>c</sub>. (A) Aminoacylation assay. The aminoacylation activities of CeAlaRS<sub>c</sub> and CeAlaRS<sub>c</sub>(ΔC-Ala) were determined using various tRNAs as substrates. (B) Complementation assay. The rescue activities of CeAlaRS<sub>c</sub> and its C-terminal deletion construct were determined by transforming the test plasmid into a yeast *ALAI* KO strain and plating the resultant transformants on 5-FOA. The symbols ‘+’ and ‘-’ denote positive and negative complementation, respectively. (C) Western blotting. The protein expression levels of these constructs were determined by western blotting using an anti-His<sub>6</sub>-tagged antibody as the probe. Constructs used in (B) and (C) are numbered for clarity.

We next examined whether CeAlaRS<sub>c</sub> and its C-terminal deletion mutant can rescue the growth defect of a yeast *ALAI* KO strain on 5-FOA (summarized in Figure 2B). The cDNA encoding CeAlaRS<sub>c</sub> or its derivative was cloned into two high-copy-number yeast shuttle vectors, pADH and pTEF1, with one having a strong *ADH* promoter and the other having a superstrong *TEF1* promoter (32). As expected, ScAlaRS cloned in either vector (as a positive control) robustly supported the growth of the KO strain on 5-FOA. In contrast, CeAlaRS<sub>c</sub> cloned in pADH only weakly rescued the KO strain. Its rescue activity was markedly increased when it was cloned into pTEF1. The C-terminal deletion construct showed a negative and a weak positive phenotype when cloned in pADH and pTEF1, respectively. Therefore, deletion of C-Ala from CeAlaRS<sub>c</sub> substantially reduced its ability to rescue *ALAI*-dependent growth defects.

To confirm the level of AlaRS expression in the KO strain, western blotting was carried out using an anti-His<sub>6</sub>-tagged antibody as the probe and phosphoglycerate kinase as the loading control (Figure 2C). As expected, CeAlaRS<sub>c</sub> cloned in pTEF1 had a protein expression level much higher than that of its pADH counterpart. The equivalent oc-

curred with its C-terminal deletion derivative. Unexpectedly, the C-terminal deletion construct had a protein expression level much higher than that of its WT counterpart when cloned in the same vector. However, contrary to our expectations, ScAlaRS cloned in these two vectors had a similar level of protein expression. It thus appears that the expression of this gene is under autogenous translational repression, a scenario reminiscent of yeast histidyl-tRNA synthetase (33). The strong double bands shown in ScAlaRS are likely to result from partial degradation of the overexpressed protein. It is noteworthy that although WT CeAlaRS<sub>c</sub> had a protein expression level lower than that of its deletion counterpart cloned in the same vector (Figure 2C), it showed a stronger rescue activity (Figure 2B), further underlining the involvement of C-Ala in rescuing *ALAI*-dependent growth defects through aminoacylation (Figure 2).

#### Fusion of C-Ala to CeAlaRS<sub>m</sub> selectively enhances its aminoacylation activity toward the L-shaped tRNA<sup>Ala</sup>

The elbow of the L-shaped tRNA is formed by interactions between the T- and D-arms. As CetRNA<sub>m</sub><sup>Ala</sup> lacks

the T-arm, it is expected to fold into an elbowless crescent-like shape (Figure 1B). This raises the question of whether its cognate enzyme CeAlaRS<sub>m</sub> can still efficiently recognize the L-shaped tRNA<sup>Ala</sup>. Figure 3A showed that CeAlaRS<sub>m</sub> can only charge SctRNA<sup>Ala</sup> poorly, with an efficiency ~15-fold lower than that of CeAlaRS<sub>c</sub> (compare Figures 2A and 3A). Unlike CeAlaRS<sub>c</sub>, which strongly preferred the L-shaped tRNA<sup>Ala</sup> (Figure 2A), CeAlaRS<sub>m</sub> had no such preference. This mitochondrial enzyme charged SctRNA<sub>n</sub><sup>Ala</sup>, Ec-micro<sup>Ala</sup> and Ce-micro<sup>Ala</sup> to a similar level. Strikingly, fusion of C-Ala of CeAlaRS<sub>c</sub> to CeAlaRS<sub>m</sub> enhanced its aminoacylation activity up to ~3-fold toward SctRNA<sup>Ala</sup> but had little effect on its activity toward the microhelices and CetRNA<sub>m</sub><sup>Ala</sup> (Figure 3A). This result suggests that, like a prokaryotic C-Ala (13,14), the nematode C-Ala domain is an elbow-specific tRNA-binding domain. To further explore whether C-Ala can act via a trans-acting mechanism to promote the aminoacylation activity of CeAlaRS<sub>m</sub>, C-Ala was purified and added in different ratios to the reactions catalyzed by CeAlaRS<sub>m</sub>. As shown in Supplementary Figure S7, C-Ala failed to act via a trans-acting mechanism to promote the aminoacylation activity of CeAlaRS<sub>m</sub>, emphasizing the role of C-Ala in targeting the fusion enzyme to L-shaped tRNA<sup>Ala</sup>.

We next examined whether CeAlaRS<sub>m</sub> and its fusions cloned in pTEF1 can be substituted for the cytoplasmic (on 5-FOA) and mitochondrial (on YPG) functions of yeast *ALAI* (summarized in Figure 3B). Note that *ALAI* is a dual-functional gene that encodes both the cytoplasmic and mitochondrial forms of ScAlaRS through alternative initiation of translation (29). As expected, ScAlaRS cloned in pTEF1 rescued the growth defects of the KO strain on both 5-FOA and YPG. Unexpectedly, CeAlaRS<sub>m</sub> (lacking its native MTS) effectively rescued the mitochondrial defect, but not the cytoplasmic defect, of the KO strain, regardless of whether it was fused with a heterologous MTS. This result suggests that CeAlaRS<sub>m</sub> can charge the yeast mitochondrial tRNA<sup>Ala</sup> and mitochondria require only a minimal AlaRS activity to maintain their normal function. In addition, it implies that the mature CeAlaRS<sub>m</sub> carries a cryptic MTS, which allows a portion of the enzyme to be imported into mitochondria to function there, a scenario often seen in aaRS rescue assays (7,30,34). Unfortunately, no transformants carrying the CeAlaRS<sub>m</sub>-(C-Ala) fusion were available for the rescue assays. As the editing activity of ScAlaRS is crucial for the survival of yeast (35), perhaps fusion of C-Ala to the C-terminus of CeAlaRS<sub>m</sub> disturbed the enzyme's editing activity, leading to mistranslation and cellular toxicity.

Interestingly, fusion of C-Ala to the N-terminus of the enzyme yielded a fusion enzyme, (C-Ala)-CeAlaRS<sub>m</sub>, that conferred a weak positive growth phenotype on the KO strain and slightly enhanced (1.6-fold) its activity on tRNA<sup>Ala</sup> *in vitro* (Supplementary Figure S8), lending further support to the observation that C-Ala enhances the aminoacylation activity of CeAlaRS<sub>m</sub> toward the L-shaped tRNA<sup>Ala</sup>. Given the long distance between the N-terminus of the aminoacylation domain and the elbow of the bound tRNA<sup>Ala</sup> (10), the fused C-Ala domain might act in cooperation with the aminoacylation and editing domains of CeAlaRS<sub>m</sub> to recruit tRNA<sup>Ala</sup>, instead of being di-

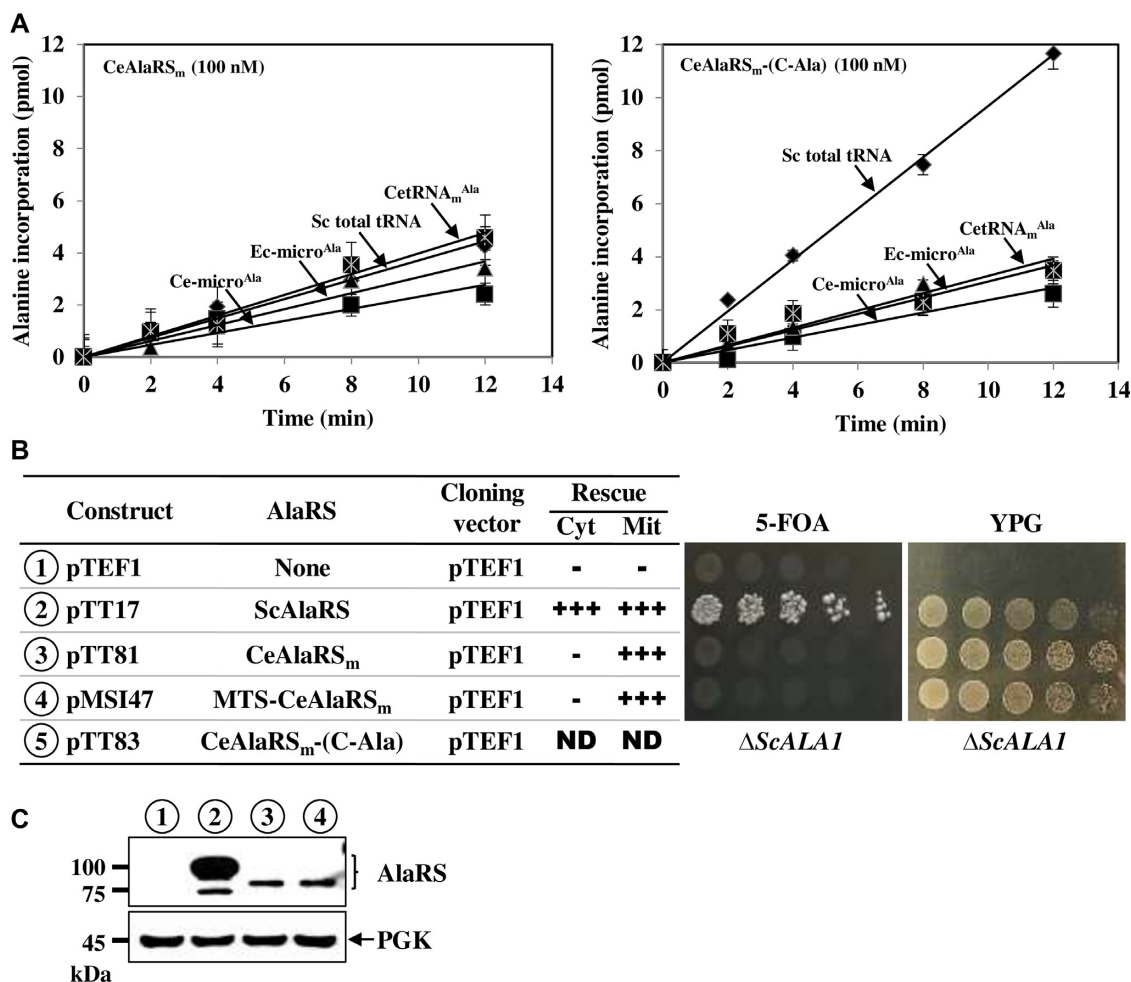
rectly involved in mediating the formation of a productive enzyme/tRNA complex.

Western blotting showed that CeAlaRS<sub>m</sub> or its fusions cloned in pTEF1 have a protein expression level much lower than that of ScAlaRS cloned in the same vector (Figure 3C). As CeAlaRS<sub>m</sub> can only poorly charge SctRNA<sup>Ala</sup> *in vitro* (Figure 3A), it was incredible to find that this enzyme can rescue the mitochondrial defect of the KO strain with such a low level of protein expression (Figure 3B and C). GFP microscopy and fractionation assay further showed that CeAlaRS<sub>m</sub> (without an MTS) is predominantly localized in the cytoplasm, while MTS-CeAlaRS<sub>m</sub> is predominantly localized in the mitochondria. However, we also noticed that a minor fraction of CeAlaRS<sub>m</sub> is colocalized in the mitochondria (Supplementary Figure S9). Conceivably, a minimal level of CeAlaRS<sub>m</sub> is sufficient to maintain the normal mitochondrial function.

### The nematode C-Ala is both a tRNA- and a DNA-binding domain

Although *C. elegans* C-Ala (Ce-C-Ala) has a protein sequence more closely related to human C-Ala than to prokaryotic C-Ala (14), our aminoacylation data suggested that this domain retains tRNA-binding activity (Figure 3). To provide direct evidence, Ce-C-Ala was cloned, purified and analyzed using an electrophoretic mobility shift assay. As a comparison, *E. coli* C-Ala (Ec-C-Ala) was also purified and analyzed. Six ligands were used in the assay, including CetRNA<sub>n</sub><sup>Ala</sup>, SctRNA<sub>n</sub><sup>Phe</sup>, SctRNA<sub>n</sub><sup>His</sup>, *Bacillus thuringiensis* tRNA<sup>Pro</sup> (BttRNA<sup>Pro</sup>), Ec-micro<sup>Ala</sup> and CetDNA<sup>Ala</sup> (which encodes CetRNA<sub>n</sub><sup>Ala</sup>). As shown in Figure 4A, Ce-C-Ala bound tRNA<sup>Ala</sup>, tRNA<sup>Phe</sup>, tRNA<sup>His</sup> and tRNA<sup>Pro</sup> (with  $K_d$  values of 0.8, 2.0, 3.4 and 4.0  $\mu$ M, respectively) but did not bind Ec-micro<sup>Ala</sup> to an appreciable level (with a  $K_d$  value of >16  $\mu$ M). This result clearly indicates that the Ce-C-Ala domain is a structure-specific tRNA-binding domain, with a distinct preference for tRNA<sup>Ala</sup>. Despite the high sequence divergence from Ce-C-Ala (~34% identity), Ec-C-Ala retained a very similar tRNA preference, with  $K_d$  values of 0.5, 2.6, 3.4, 5.5 and >16  $\mu$ M for tRNA<sup>Ala</sup>, tRNA<sup>Phe</sup>, tRNA<sup>His</sup>, tRNA<sup>Pro</sup> and Ec-micro<sup>Ala</sup>, respectively (Figure 4B). Unexpectedly, these two C-Ala domains possessed quite different DNA-binding properties. Ec-C-Ala poorly bound to DNA (with a  $K_d$  value of 5.0  $\mu$ M), while Ce-C-Ala bound to DNA robustly (with a  $K_d$  value of 0.5  $\mu$ M). Thus, the Ce-C-Ala domain is both a tRNA-binding domain and a DNA-binding domain (Figures 3 and 4). For comparison, we also purified human C-Ala under normal (or reducing) conditions as previously described (14). Unexpectedly, monomeric human C-Ala robustly bound DNA (with a  $K_d$  value of 0.5  $\mu$ M), but poorly bound tRNA<sup>Ala</sup> (with a  $K_d$  value of 4.5  $\mu$ M) (Figure 4C).

To determine the oligomeric status of Ce-C-Ala in solution, a sedimentation velocity analytical ultracentrifugation experiment was carried out. As shown in Supplementary Figure S10, Ce-C-Ala formed a monomer and no homodimer was detected. To test whether DNA binding is a property that is exclusive to the stand-alone C-Ala domain, the full-length CeAlaRS<sub>c</sub> was purified and tested. Supplemen-



**Figure 3.** C-Ala enhances the aminoacylation activity of CeAlaRS<sub>m</sub> toward the L-shaped tRNA<sup>Ala</sup>. (A) Aminoacylation assay. The aminoacylation activities of CeAlaRS<sub>m</sub> and CeAlaRS<sub>m</sub>-(C-Ala) were determined using various tRNAs as substrates. (B) Complementation assay. The rescue activities of CeAlaRS<sub>m</sub> and its C-Ala fusion construct were determined by transforming the test plasmid into a yeast *ALAI* KO strain and plating the resultant transformants on both 5-FOA and YPG. The symbols ‘+’ and ‘-’ denote positive and negative complementation, respectively. ND indicates not determined due to lack of transformants. (C) Western blotting. The protein expression levels of these constructs were determined by western blotting using an anti-His<sub>6</sub>-tagged antibody as the probe. Constructs used in (B) and (C) are numbered for clarity.

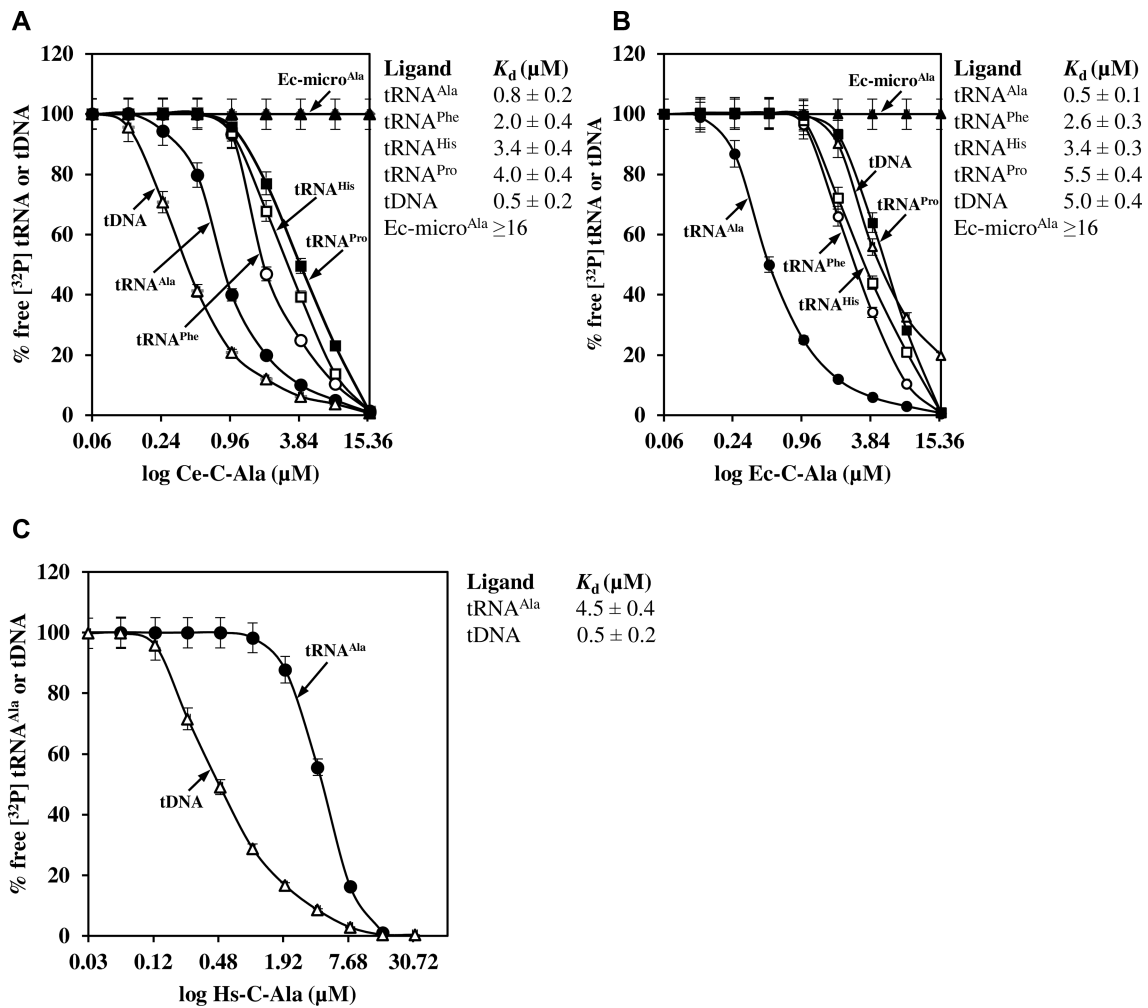
tary Figure S11 shows that CeAlaRS<sub>c</sub> bound DNA with a  $K_d$  value of 1.5  $\mu$ M (3-fold higher than that of Ce-C-Ala).

#### Lack of an intact C-Ala domain in CeAlaRS<sub>m</sub> resulted from secondary loss of this domain

To track the evolutionary origin of CeAlaRS<sub>m</sub> and its relationship with CeAlaRS<sub>c</sub>, phylogenetic analysis was carried out using AlaRSs retrieved from all three domains of life, with a particular emphasis on eukaryotes (36,37). As illustrated in Figure 5, the majority of eukaryotic AlaRSs are clustered into a monophyletic group that is closer to bacteria, and therefore they should be regarded as having a mitochondrial origin (4). Nevertheless, amitochondriate (eukaryotes without mitochondria) AlaRSs are clustered into two independent monophyletic groups. One group, including *V. culicis*, *N. ceranae* and *O. colligate*, is closer to the major eukaryotic group (mitochondrial origin), while the other, including *E. histolytica*, *G. lamblia* and *T. vaginalis*, is closer to the archaeal group instead (Figure 5). Thus, not all

eukaryotic AlaRSs are of the same evolutionary origin. It is also noteworthy that none of the contemporary eukaryotes retain both types of AlaRS (archaeal and mitochondrial types), suggesting that the functional substitution process between the two types of AlaRS occurred early in evolution.

As all the cytoplasmic and mitochondrial AlaRSs in the major eukaryotic group are of mitochondrial origin, the original eukaryote-type AlaRS must have been lost before speciation of these eukaryotes. Moreover, the AlaRS paralogs existing in each of these organisms, including those for CeAlaRS isoforms, were descended from duplication of a mitochondrion-type predecessor (Figure 5). Therefore, both CeAlaRS isoforms should retain the same organization with four domains. We determined that the selective absence of most of C-Ala from CeAlaRS<sub>m</sub> most likely resulted from secondary loss of this domain. That is, CeAlaRS<sub>m</sub> once possessed a full-length C-Ala, but lost it later during evolution, perhaps as a response to deletion of the T-arm from its cognate tRNA (or the other way around) (Supplementary Figure S12). To date, such a scenario has only been



**Figure 4.** C-Ala of CeAlaRS<sub>C</sub> robustly binds both tRNA and DNA. Protein–RNA or protein–DNA binding affinities were determined by an electrophoretic mobility shift assay with protein concentrations ranging from 16 to 0.0625 or 32 to 0.03  $\mu\text{M}$ . The <sup>32</sup>P-labeled CetRNA<sub>n</sub><sup>Ala</sup> (●), SctRNA<sub>n</sub><sup>Phe</sup> (○), SctRNA<sub>n</sub><sup>His</sup> (□), BttRNA<sub>n</sub><sup>Pro</sup> (■), Ec-micro<sup>Ala</sup> (▲) and CetDNA<sup>Ala</sup> (△) are shown. The tRNA- and DNA-binding affinities of (A) Ce-C-Ala, (B) Ec-C-Ala and (C) Hs-C-Ala. The equilibrium response at each concentration was fitted to a single-site binding model. Error bars are standard deviations from triplicates.

found in *C. elegans* and some of its close relatives, such as *Ascaris suum*. It is still unclear whether C-Ala or T $\psi$ C-arm was lost first during evolution.

## DISCUSSION

### Distinct features of the prokaryotic and eukaryotic C-Ala domains

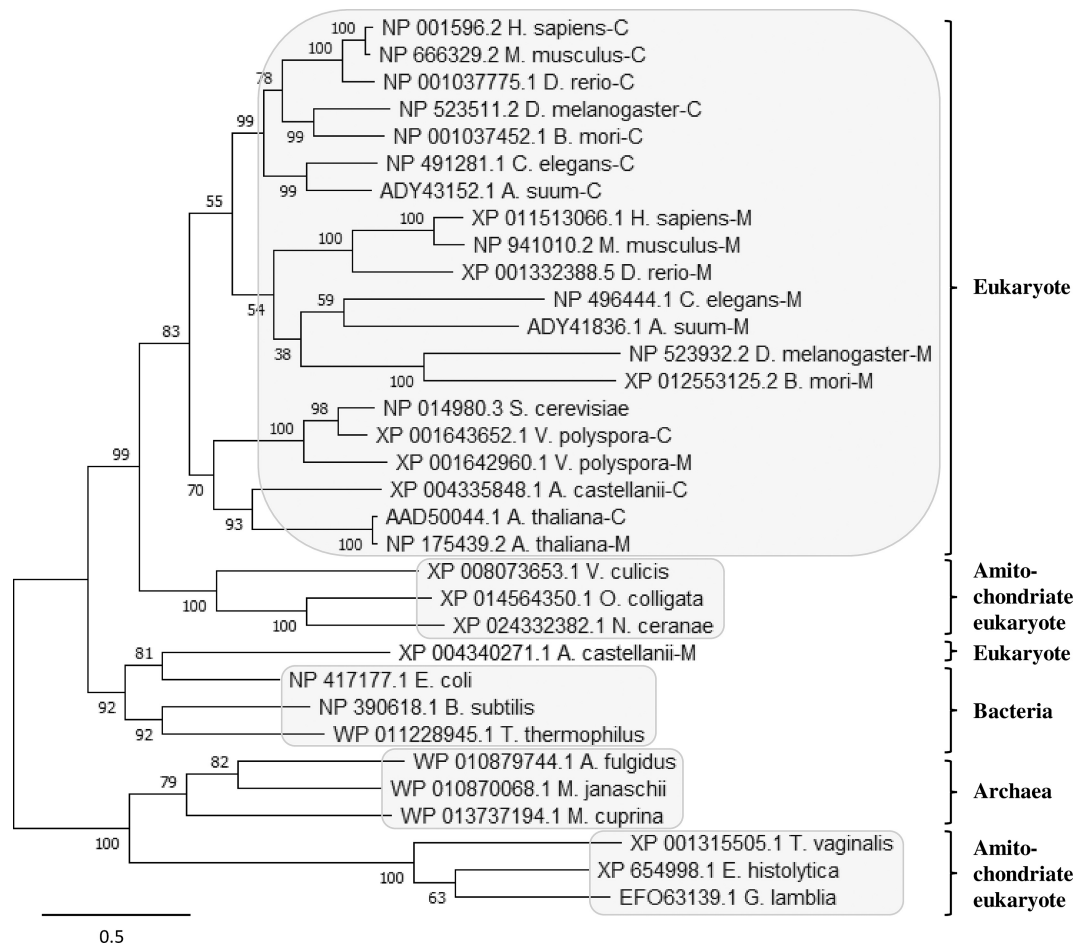
Despite AlaRS retaining a prototype structure throughout biology, C-Ala is highly diverged in protein sequences (12,14). Deletion of C-Ala from AlaRS of *E. coli* or *A. fulgidus* sharply reduced its aminoacylation activities (12,14,38). In contrast, deletion of C-Ala from human cytoplasmic AlaRS had little effect on its aminoacylation activity (14). It is believed that human C-Ala has little contact with the bound tRNA. In the case of CeAlaRS<sub>C</sub>, deletion of C-Ala from this enzyme severely impaired its aminoacylation activity (Figure 2), suggesting that similar to prokaryotic C-Ala, the nematode C-Ala plays an important role in tRNA aminoacylation. A structural study further unveiled

that C-Ala from prokaryotes folds into a homodimer with a parallel organization (10) and binds the elbow of the L-shaped tRNA<sup>Ala</sup> (12). In contrast, human C-Ala forms a homodimer with an antiparallel organization (under oxidizing conditions) and binds DNA (14). In spite of this, human C-Ala, when fused to the editing domain of *E. coli* AlaRS, enhances its editing activity (12), suggesting that human C-Ala retains at least partial tRNA-binding activity. This might be true for all eukaryotic C-Ala domains, as the nematode C-Ala also retains tRNA-binding activity (Figures 3 and 4). It should be noted that like other eukaryotic C-Ala domains (39–41), human C-Ala folds into a monomer under reducing conditions, and the cysteine residues required for disulfide bridge formation (under oxidizing conditions) exist only in human C-Ala (14).

### The nematode C-Ala is both a tRNA- and a DNA-binding domain

Previous studies showed that Ec-C-Ala binds the elbow of the L-shaped tRNA<sup>Ala</sup> (12) but does not bind DNA (14).





**Figure 5.** Phylogenetic tree of AlaRS. The evolutionary history was inferred by using the maximum likelihood method and JTT matrix-based model. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model. This analysis involved 33 AlaRS sequences. Evolutionary analyses were conducted in MEGA X.

In contrast, dimeric human C-Ala binds DNA (14). We demonstrated that Ce-C-Ala robustly binds both DNA and tRNA (Figure 4). This domain binds different tRNAs, albeit with a distinct preference for tRNA<sup>Ala</sup> (2.5–5.0-fold difference in the  $K_d$  values), but it does not bind an acceptor-stem microhelix to a discernible level (Figure 4). Consistent with this finding, fusion of Ce-C-Ala to CeAlaRS<sub>m</sub> selectively and distinctly enhances its aminoacylation activity toward the elbow-containing (or L-shaped) tRNA<sup>Ala</sup> (Figure 3). Thus, like Ec-C-Ala, the Ce-C-Ala domain is an elbow-specific tRNA-binding domain. We also demonstrated that Ec-C-Ala exhibits a tRNA preference very similar to that of Ce-C-Ala (Figure 4), despite the low overall similarity between them. Ec-C-Ala is missing ~34 amino acid residues in the helical subdomain (Supplementary Figure S1), with ~34% identity in the remaining amino acid residues (14). The broad tRNA specificity of C-Ala might be attributed to the fact that the elbow of the L-shaped tRNA, formed by the D- and T-loops, is clustered with conserved invariant bases (42). In addition to the C-Ala domain of AlaRS, the C domain of LeuRS (43), the N domain of human LysRS (44) and two freestanding proteins, Trbp111 (45) and Arc1p (46), have also been shown to bind the elbow of the L-shaped tRNA.

Interestingly, Ce-C-Ala also robustly binds DNA, with an affinity almost equivalent to that of tRNA<sup>Ala</sup> (Figure 4). To date, this is the only C-Ala domain known to possess high affinities (0.5–0.8  $\mu$ M) to both DNA and tRNA. In contrast, Ec-C-Ala binds to DNA poorly (with a 10-fold lower affinity), while monomeric human C-Ala binds to tRNA poorly (with a 6-fold lower affinity) (Figure 4). Consistent with our finding, an earlier study showed that Ec-C-Ala fails to bind DNA cellulose to a significant level (14). Sequence and structural analyses revealed that all the amino acid residues that have been predicted to be involved in DNA binding by human C-Ala (such as R793, K812, R816, K820, K824, R831 and K846) are conserved in the helical subdomain of Ce-C-Ala, but not Ec-C-Ala (Supplementary Figure S1). It therefore appears that the helical subdomains of eukaryotic C-Ala domains have evolved DNA-binding activity. Paradoxically, the full-length human AlaRS<sub>c</sub> failed to bind DNA (14), while the full-length CeAlaRS<sub>c</sub> bound DNA with a  $K_d$  value of 1.5  $\mu$ M (Supplementary Figure S11). An investigation is currently underway to find out whether Ce-C-Ala (or CeAlaRS<sub>c</sub>) plays a role in the nucleus. It is interesting to note in this regard that *E. coli* AlaRS binds to its own promoter in a site-specific manner to repress transcription (47).

### The amino acid residues responsible for G3:U70 recognition diverge in CeAlaRS<sub>m</sub>

G3:U70, the universal identity element of tRNA<sup>Ala</sup> found from *E. coli* to human cytoplasm, allows for the feasibility to perform cross-species and cross-compartmental rescue assays using a yeast *ALAI* KO strain (4,30). This wobble base pair is recognized by two evolutionarily conserved amino acid residues—N and D—in the tRNA recognition domain (13). As a result, mutation at either of the two amino acid residues in the *E. coli* enzyme eliminates its aminoacylation activity. Surprisingly, a comparable mutation in human cytoplasmic AlaRS has little effect on its aminoacylation activity (20). Even more surprising was the finding that mitochondrial tRNAs<sup>Ala</sup> in high eukaryotes, such as humans, mice, chickens, zebrafish and silkworms, lack the universal identity elements, as their AlaRSs lack the conserved N and D residues. Human mitochondrial AlaRS recognizes a short sequence—AGAU—in the variable loop, which is highly conserved in the abovementioned mitochondrial tRNAs<sup>Ala</sup> (5). While CeAlaRS<sub>m</sub> still recognizes the canonical identity element G3:U70 (6), the conserved N and D residues responsible for recognition of this wobble base pair are diverged in this enzyme (Figure 1). It remains elusive as to how CeAlaRS<sub>m</sub> recognizes this wobble base pair.

### AlaRS acquired (or lost) C-Ala to fit the new structure of its cognate tRNA

Except for the mitochondrial AlaRS in *C. elegans* (and its close relatives), all known AlaRSs possess an intact C-Ala domain (9). It is generally believed that the editing and C-Ala domains were acquired simultaneously, instead of successively, by an ancient AlaRS during evolution. An ancient freestanding editing factor, AlaXp-II, which possesses a sequence and function homologous to these two domains, was proposed to be fit for the purpose (12). Our phylogenetic analysis further indicated that the absence of most of the C-Ala domain from CeAlaRS<sub>m</sub> actually resulted from secondary loss of this domain (Figure 5). That is, CeAlaRS<sub>m</sub> used to possess a full-length C-Ala, but lost most of it later during evolution (Supplementary Figure S12). Upon deletion (or reduction) of C-Ala, this mitochondrial enzyme lost much of its capability to charge the elbow-containing tRNA<sup>Ala</sup> while being readapted to a T-armless tRNA<sup>Ala</sup> (or the other way around) (Supplementary Figure S12). Regardless of the detailed interpretation, our results highlight the functional diversity of a pre-existing appended domain. As prokaryotes evolved into eukaryotes, the C-Ala domain has been repurposed from tRNA binding to DNA binding, with Ce-C-Ala being an intermediate that robustly binds both ligands.

### SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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