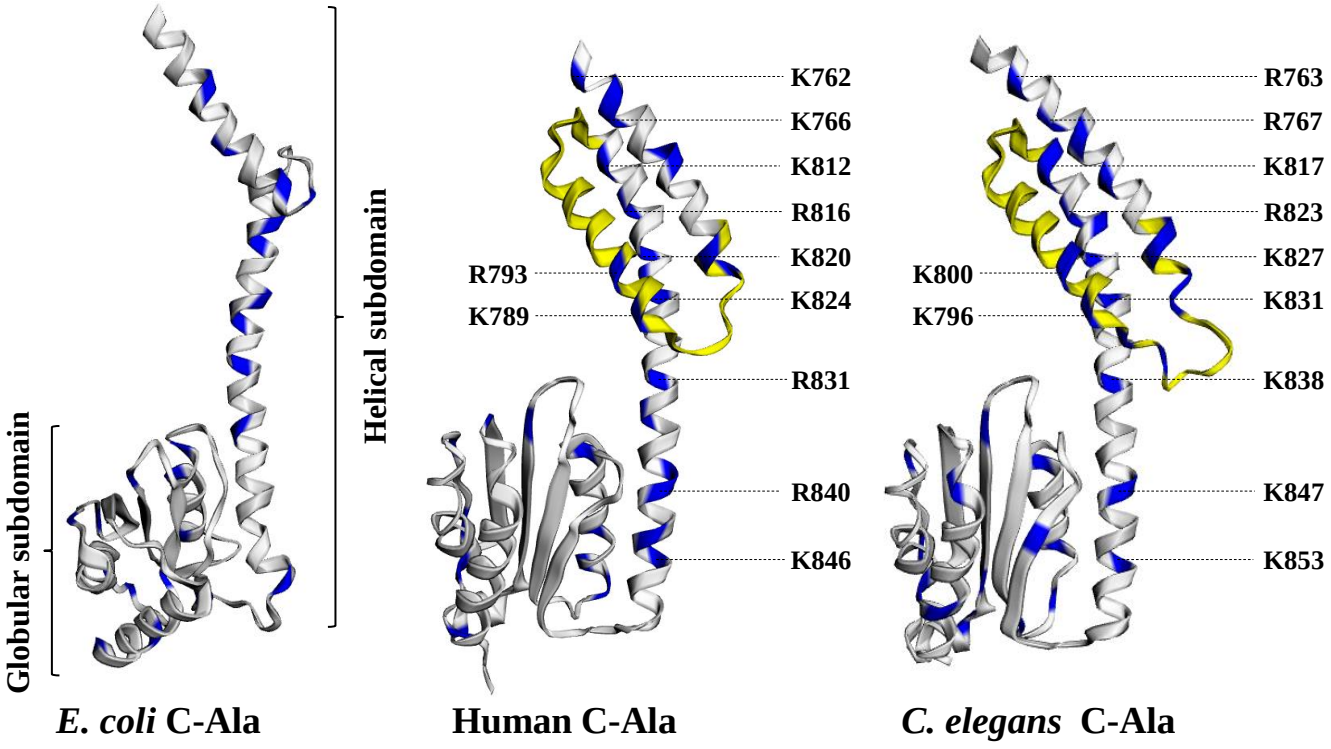


Figure S1. Comparison of C-Ala monomers. *E. coli* C-Ala consists of an N-terminal helical subdomain and a C-terminal globular subdomain. While human C-Ala (PDB ID: 5T76) retains the same overall structure, its helical subdomain contains an insertion sequence (highlighted in yellow) that is missing in a prokaryotic C-Ala. *C. elegans* C-Ala is predicted to form a structure more closely resembling that of human C-Ala. Positively charged amino acid residues in C-Ala are highlighted in blue. Conserved positively charged amino acid residues between the helical subdomains of human and *C. elegans* C-Ala domains are labeled. *E. coli* C-Ala naturally forms a dimer, while human C-Ala forms a dimer only under oxidizing conditions.

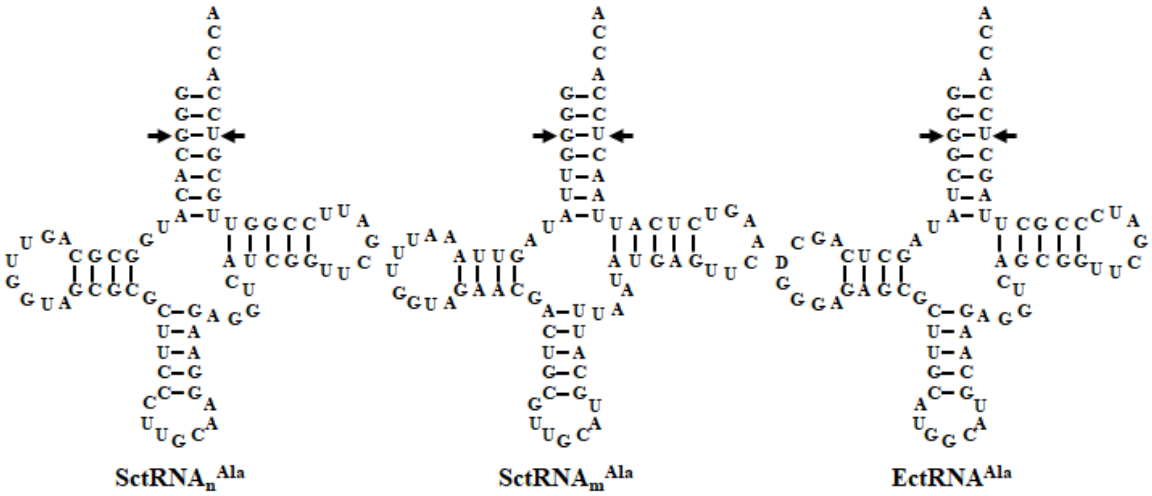


Figure S2. The cloverleaf structures of yeast and *E. coli* tRNAs^{Ala}. SctRNA$_n^{Ala}$, *S. cerevisiae* nuclear-encoded cytoplasmic tRNA^{Ala}; SctRNA$_m^{Ala}$, *S. cerevisiae* mitochondrial-encoded mitochondrial tRNA^{Ala}; EctRNA^{Ala}, *E. coli* tRNA^{Ala}. The universal identity elements G3:U70 are marked with an arrow.

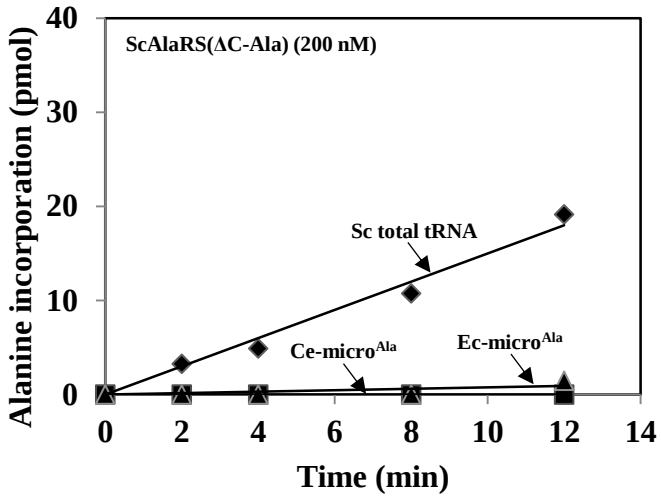


Figure S3. Aminoacylation of tRNA^{Ala} by ScAlaRS(ΔC-Ala). Aminoacylation assay was carried out using yeast total tRNA, Ec-micro^{Ala}, and Ce-micro^{Ala} as the substrates.

	Construct	AlaRS	Cloning vector	Rescue Cyt
①	pTT17	ScAlaRS	pTEF1	+++
②	pTT106	ScAlaRS(ΔC-Ala)	pTEF1	-
③	pTEF1	None	pTEF1	-

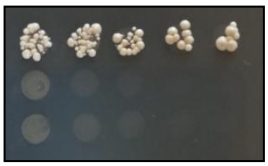


Figure S4. Complementation assay for ScAlaRS(ΔC-Ala). The rescue activity of ScAlaRS(ΔC-Ala) was determined by transforming the test plasmid into a yeast *ALA1* KO strain and plating the resultant transformants on 5-FOA. The symbols "+" and "-" denote positive and negative complementation, respectively.

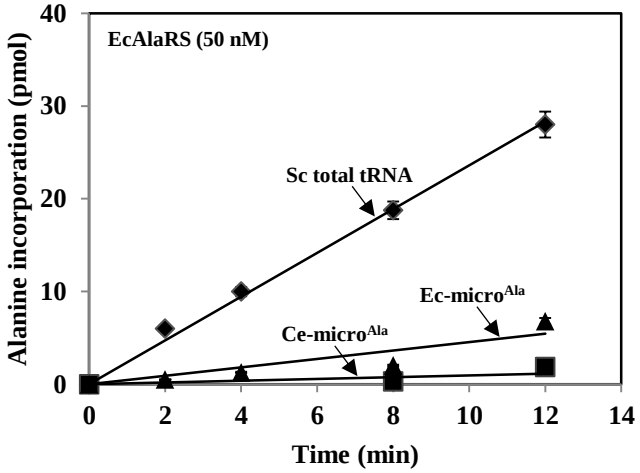


Figure S5. Aminoacylation of tRNA^{Ala} by *E. coli* AlaRS. Aminoacylation assay was carried out using yeast total tRNA, Ec-micro^{Ala}, and Ce-micro^{Ala} as the substrates.

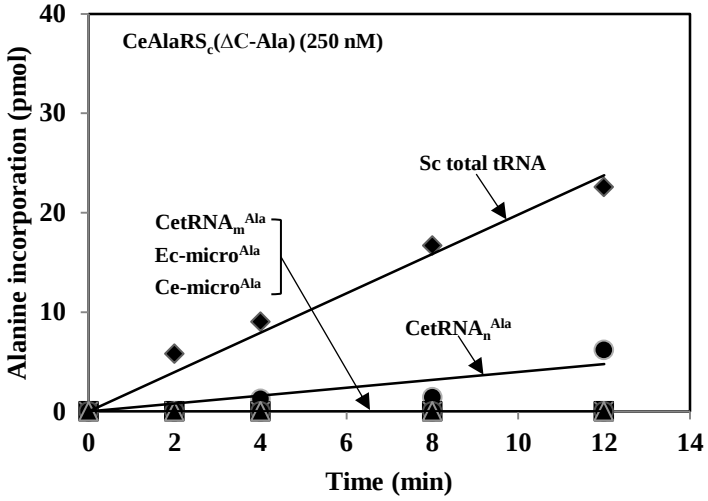


Figure S6. Aminoacylation of tRNA^{Ala} by CeAlaRS$_c$(ΔC-Ala). Aminoacylation assay was carried out using yeast total tRNA, CetRNA$_n^{Ala}$, CetRNA$_m^{Ala}$, Ec-micro^{Ala}, and Ce-micro^{Ala} as the substrates.

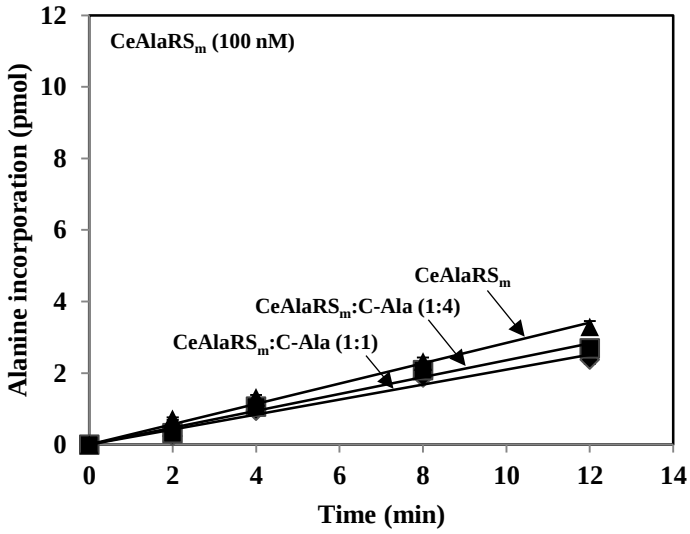
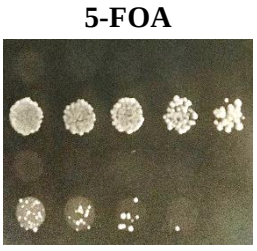


Figure S7. Aminoacylation of tRNA^{Ala} by CeAlaRS$_m$ in the presence and absence of C-Ala. Aminoacylation assay was carried out using yeast total tRNA as the substrate to explore whether C-Ala can act via a trans-acting mechanism to promote aminoacylation of tRNA^{Ala} by CeAlaRS$_m$. Different ratios of CeAlaRS$_m$ and C-Ala were added into the reactions.

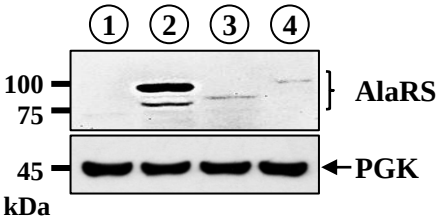
A

	Construct	AlaRS	Cloning vector	Rescue Cyt
①	pTEF1	None	pTEF1	-
②	pTT17	ScAlaRS	pTEF1	+++
③	pTT81	$CeAlaRS_m$	pTEF1	-
④	pND78	(C-Ala)-$CeAlaRS_m$	pTEF1	+



ΔScALA1

B



C

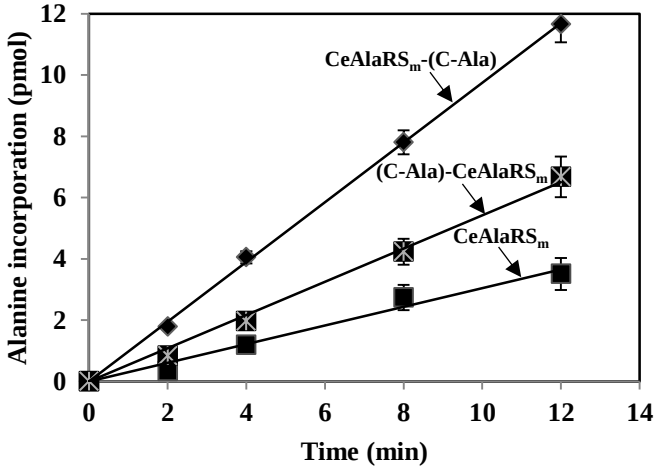


Figure S8. Fusion of C-Ala enhances the aminoacylation activity of $CeAlaRS_m$ towards the L-shaped $tRNA^{Ala}$. (A) Complementation assay. C-Ala was fused to the N-terminus of $CeAlaRS_m$, yielding (C-Ala)-$CeAlaRS_m$, and the rescue activity of the resultant fusion enzyme was determined by transforming the test plasmid into a yeast *ALA1* KO strain and plating the resultant transformants on 5-FOA. The symbols "+" and "-" denote positive and negative complementation, respectively. (B) Western blotting. The protein expression levels of these constructs were determined by Western blotting using an anti-His_6-tagged antibody as the probe. Constructs used in (A-B) are numbered for clarity. (C) Aminoacylation assay. Aminoacylation of yeast total tRNA (100 μM) by $CeAlaRS_m$ or its fusions (100 nM) was carried out under conditions described in the Materials and Methods.

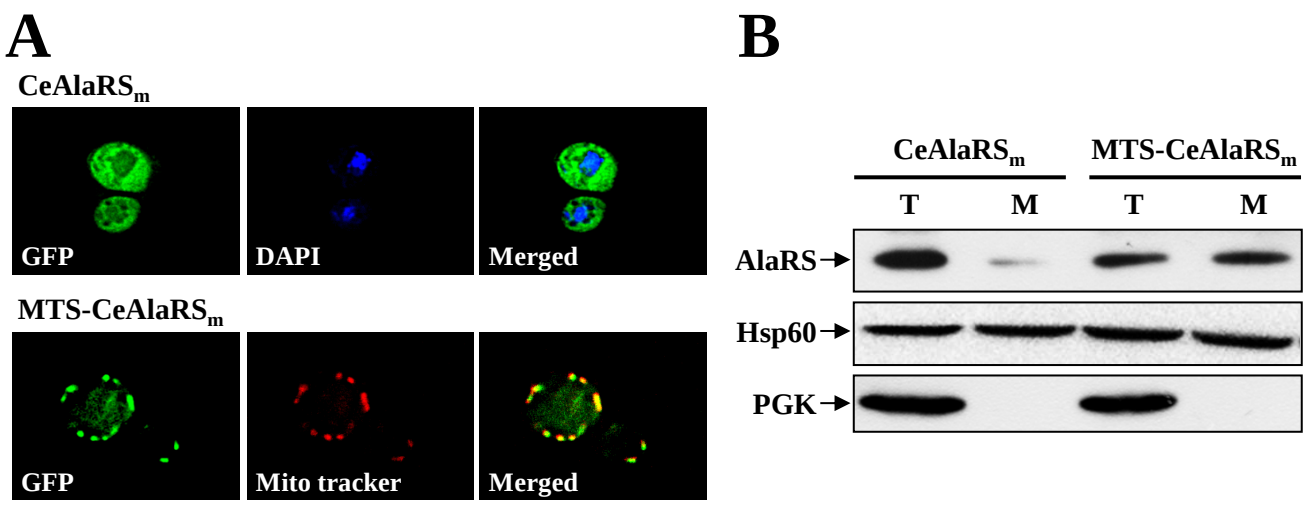


Figure S9. Cellular localization of CeAlaRS$_m$. (A) GFP microscopy. Subcellular localization of CeAlaRS$_m$ in yeast was determined via fluorescence microscopy. Constructs that express CeAlaRS$_m$-GFP and MTS-CeAlaRS$_m$-GFP were individually transformed into a yeast strain, INVSc1. The resultant transformants were then treated with a mitochondrion-specific dye (MitoTracker) or 4',6-diamidino-2-phenylindole (DAPI), and examined via fluorescence microscopy. MitoTracker and DAPI were respectively used to label mitochondria and nuclei. (B) Fractionation. Total (T) and mitochondrial (M) protein extracts were prepared from the yeast transformants possessing CeAlaRS$_m$ or MTS-CeAlaRS$_m$. The relative protein levels of CeAlaRS$_m$-His$_6$, Hsp60, and PGK were respectively determined by Western blotting using anti-His$_6$-tagged, anti-Hsp60, and anti-PGK antibodies as the probes.

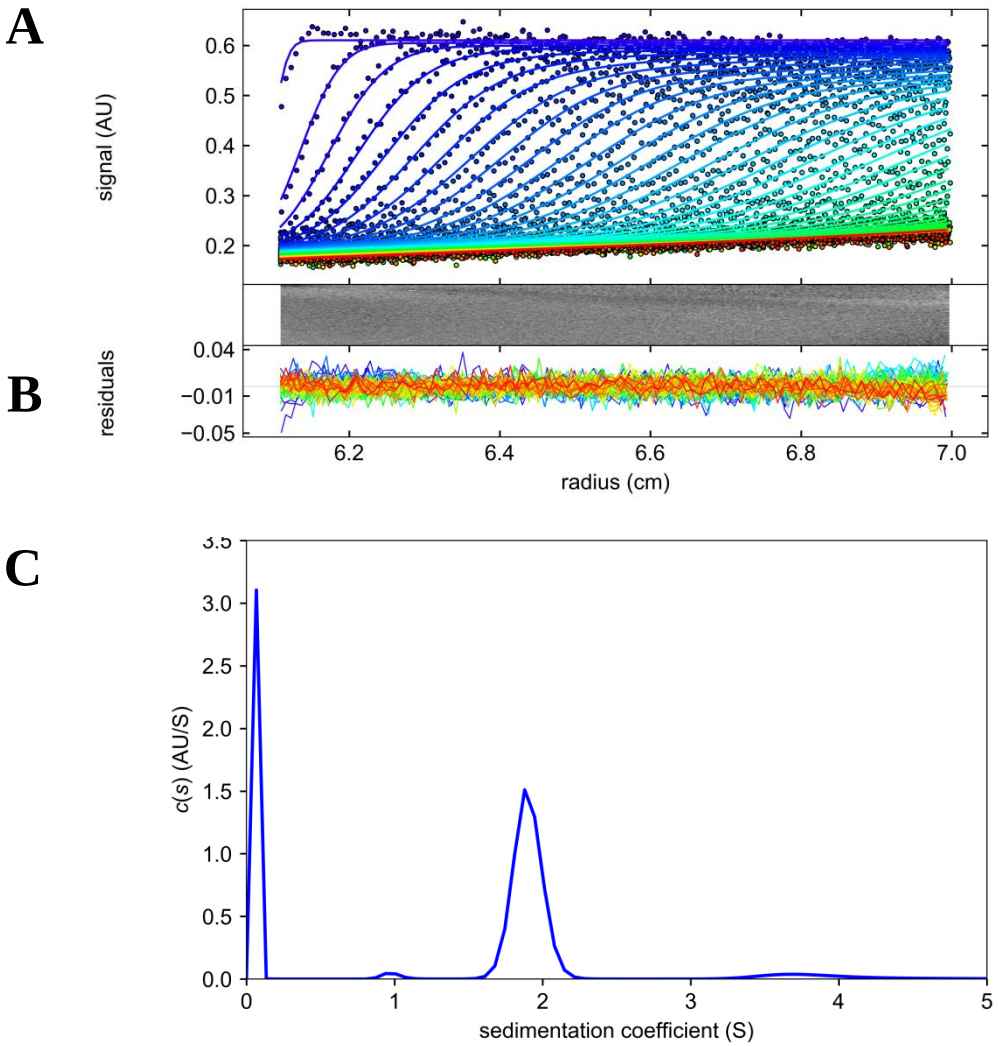


Figure S10. Sedimentation velocity-analytical ultracentrifugation experiment for Ce-C-Ala. (A) Typical raw sedimentation profiles of absorbance at 280 nm versus cell radius. The sedimentation scans were colored with the progressive rainbow colors according to the software default setting: from violet, for those scans with the best fit and lowest residual, to those scans with the worst adjustment and the highest residual value. (B) Residual plot supplied by SEDFIT software showing the fitting goodness. (C) Continuous sedimentation coefficient distribution, c(S) curve, obtained with a regularization procedure from data shown in panels (A) and (B) with a confidence level of 0.90 using SEDFIT software and frictional ratio (f/fo) values between 1.136116 and 1.96157. Position 2S corresponds to a protein with a molecular weight of ~24 kD.

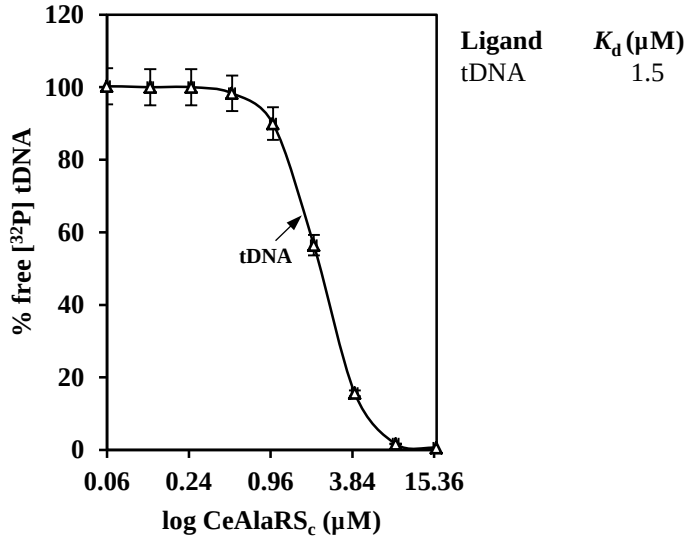


Figure S11. CeAlaRS$_c$ binds DNA. The protein-DNA binding affinity was determined by an electrophoretic mobility shift assay with protein concentrations ranging from 16 to 0.0625 μM. The ^{32}P-labeled CetDNA^{Ala} (Δ) is shown. The equilibrium response at each concentration was fitted to a single-site binding model. Error bars are standard deviations from triplicates.

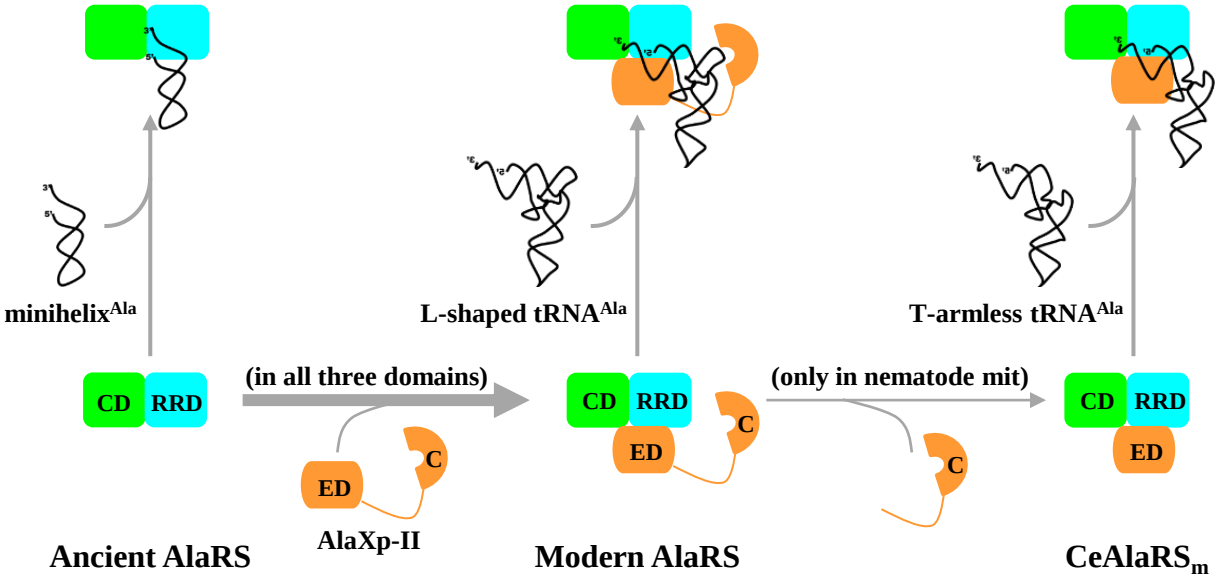


Figure S12. A proposed model for evolution of *C. elegans* mitochondrial AlaRS. An ancient AlaRS (without the editing and C-Ala domains) is thought to recognize a minihelix-like $tRNA^{Ala}$. As the minihelix-like $tRNA^{Ala}$ developed into the L-shaped $tRNA^{Ala}$, AlaRS recruited AlaXp-II (a free-standing editing factor) to edit the misacylated tRNA and to enhance tRNA binding. *C. elegans* mitochondrial AlaRS ($CeAlaRS_m$) lost C-Ala later in evolution, possibly as a response to the deletion of the T-arm (part of the elbow) from its cognate tRNA. CD, catalytic domain; RRD, tRNA-recognition domain; ED, editing domain; C, C-Ala domain.