

1 Sequential action of a tRNA base editor in conversion of cytidine
2 to pseudouridine
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19 **Summary**

20 Post-transcriptional RNA editing modulates gene expression in a condition-dependent fashion. We
21 recently discovered C-to- Ψ editing in *Vibrio cholerae* tRNA. Here, we characterize the biogenesis,
22 regulation, and functions of this previously undescribed RNA editing process. We show that an enzyme,
23 TrcP, mediates the editing of C-to-U followed by the conversion of U to Ψ , consecutively. AlphaFold-2
24 predicts that TrcP consists of two globular domains, including a novel cytidine deaminase and a
25 pseudouridylase, along with a long helical domain. The latter domain tethers tRNA substrates during
26 both the C-to-U editing and pseudouridylation, likely enabling a substrate channeling mechanism for
27 efficient catalysis all the way to the terminal product. C-to- Ψ editing both requires and suppresses other
28 modifications, creating an interdependent network of modifications in the tRNA anticodon loop that
29 facilitates coupling of tRNA modification states to iron availability. Our findings provide mechanistic
30 insights into an RNA editing process that likely promotes environmental adaptation.

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33 **Introduction**

34 RNA nucleosides are considerably more diverse than the canonical A, U, G and C nucleosides that
35 correspond to their cognate DNA nucleosides, thereby expanding genetic information and modulating
36 gene expression (Bjork and Hagervall, 2014; Machnicka et al., 2013; Wiener and Schwartz, 2020;
37 Zaccara et al., 2019). Some modifications of RNA nucleosides result from processes, such as methylation
38 and acetylation, that add compounds to the base or ribose component of the nucleoside. Additional
39 modifications stem from processes, such as deamination or isomerization that alter the structure of the
40 base. RNA editing, the post-transcriptional conversion of one base to another, accounts for some of the
41 later set of modifications. To date, RNA editing of adenosine into inosine (A-to-I editing) and cytidine
42 into uridine (C-to-U editing) has been described (Eisenberg and Levanon, 2018; Ichinose and Sugita,
43 2016). These base conversions are mediated by adenosine deaminases and cytidine deaminases,
44 respectively. In contrast to genomic mutations, RNA editing is regulated, facilitating tissue- and
45 condition-specific control of gene expression (Eisenberg and Levanon, 2018). Base conversion
46 diversifies the transcriptome by modulating pre-mRNA splicing patterns as well as the translato-
47 me because edited bases can code for different amino acids (Eisenberg and Levanon, 2018). Furthermore,
48 A-to-I editing alters mRNA secondary structure, preventing innate immune responses triggered by
49 mRNAs' rigid double-stranded structures (Liddicoat et al., 2015). RNA editing is essential for viability
50 and development in many organisms (Chung et al., 2018; Wang et al., 2000; Wolf et al., 2002).

51 RNA editing occurs not only in mRNAs but also in non-coding RNAs, such as tRNAs. In bacteria and
52 eukaryotes, A-to-I editing occurs at the wobble position in a subset of tRNAs, thereby expanding their
53 decoding capacity (Crick, 1966; Juhling et al., 2009; Murphy and Ramakrishnan, 2004). In kinetoplastids
54 and marsupials, RNA editing in the anticodon sequence generates multiple species of tRNAs from a
55 single tRNA gene, enabling subcellular compartment specific translation (Borner et al., 1996; Janke and
56 Paabo, 1993). In addition, tRNA editing is not restricted to the anticodon loop in diverse organisms
57 (Dixit et al., 2019; Randau et al., 2009) and editing of other regions in tRNA structure are likely
58 associated with structural stability (Dixit et al., 2019). However, with some exceptions, the functions
59 and biosynthesis pathways of editing in tRNAs are largely unclear.

60 A-to-I and C-to-U RNA editing are mediated by zinc-dependent deaminases, from the ADAR
61 (adenosine deaminase acting on RNA) (Bass, 2002), ADAT (adenosine deaminase acting on tRNAs)
62 (Dixit et al., 2019; Gerber et al., 1998; Gerber and Keller, 1999; Maas et al., 1999), AID/APOBEC (Lerner
63 et al., 2018), and CDA (Cytidine deaminase) (Smith et al., 1994) families of proteins. These families are
64 distinguishable by their characteristic deaminase domains; the ADAR protein family contains the
65 Adenosine deaminase/Editase domain (A_deamin), whereas the other families possess the Cytidine and
66 deoxycytidylate deaminase domain (CMP_dCMP_dom) (Finn et al., 2017). These domains have a
67 consensus sequence that includes zinc-coordinating histidine and/or cysteine residues and a glutamate
68 residue that functions as a proton donor for facilitating the deamination reaction (Figure. S1). All
69 known RNA editing enzymes contain either the A_deamin or the CMP_dCMP_dom domains.

70 Recently, in studies characterizing the tRNA modification landscape in the cholera pathogen, *Vibrio*
71 *cholerae*, we discovered C-to- Ψ editing, a novel RNA editing process in which cytidine is converted into
72 pseudouridine (Ψ) (Kimura et al., 2020). C-to- Ψ editing was observed at position 32 in the anticodon
73 loop of *V. cholerae* tRNA-Tyr (Figure. 1A). A *V. cholerae* deletion mutant lacking *vca0104* (dubbed *trcP*),
74 which encodes a predicted pseudouridine synthase domain that likely mediates isomerization of U-to- Ψ ,
75 was found to be deficient for the entire editing process. However, TrcP lacks the consensus motif shared
76 in deaminases and the mechanisms underlying C-to- Ψ editing and its functional consequences were
77 unclear.

78 Here we characterize the mechanisms of C-to- Ψ editing. We show that TrcP mediates the stepwise
79 editing of C-to-U followed by the conversion of U to Ψ . Genetic and biochemical studies revealed that
80 TrcP relies on its two catalytic domains, including a novel cytidine deaminase domain and a
81 pseudouridine synthase domain, for C-to- Ψ editing. AlphaFold 2-based structural modeling uncovered a
82 third unique long helical domain within TrcP that likely binds and orients the substrate tRNA during
83 both reactions, suggesting TrcP mediated C-to- Ψ editing relies on a substrate channeling mechanism.
84 Additional studies of the control of C-to- Ψ editing uncovered a modification circuit in the tRNA
85 anticodon loop whereby C-to- Ψ editing at position 32 requires methylthiolation of A at position 37 as
86 ms²io⁶A and suppresses the conversion of G to Q at wobble position 34. Functionally, this modification
87 circuit facilitates coupling of tRNA modification states to environmental iron availability and impacts
88 decoding, likely for promoting environmental adaptation.

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96 **Results**

97 **TrcP mediates C-to- Ψ conversion**

98 Genetic analyses established that *vca0104* (*trcP*), is required for the unprecedented conversion of
99 C-to- Ψ in the *V. cholerae* tRNA-Tyr (Kimura et al., 2020). To address whether TrcP is sufficient to
100 catalyze C-to- Ψ editing, an *in vitro* editing assay was carried out using purified recombinant TrcP
101 protein and an unedited tRNA-Tyr isolated from a $\Delta trcP$ strain (Figure S2). In this assay, editing
102 frequency was measured by Sanger sequencing of cDNA derived from the tRNA-products of the reaction.
103 Note that in Sanger sequencing, both U and Ψ yield a T. Without addition of TrcP, the unedited
104 tRNA-Tyr was read as a C, whereas addition of increasing amounts of TrcP to the reaction increased the
105 frequency of T and decreased the frequency of C at position 32 of RNA-Tyr (Figure. 1B). These data
106 indicate that TrcP catalyzes C-to-U or C-to- Ψ conversion.

107 Cyanoethyl labeling coupled with MALDI-TOF mass spectrometry (MALDI-TOF-MS) was used to
108 distinguish between U and Ψ (Kimura et al., 2020; Suzuki and Suzuki, 2014). After incubation with
109 recombinant TrcP a fragment of tRNA-Tyr (position 10–46) was purified and treated with acrylonitrile,
110 which specifically attaches a cyanoethyl group on Ψ . MALDI-TOF-MS analyses of RNA fragments
111 generated by RNase A digestion revealed that the unmodified fragment (AGACp; m/z 1325.1) was
112 increasingly converted to the cyanoethylated fragment (AGA Ψ p + CE; m/z 1379.1) as the TrcP
113 concentration increased (Figure. 1C). Together these observations establish that TrcP catalyzes C-to- Ψ
114 base editing.

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116 **Phylogenetic distribution of TrcP**

117 Analysis of the TrcP primary amino acid sequence with the NCBI Conserved Domain Search tool
118 revealed it contains two recognizable domains. Its C-terminus (CTD, amino acids 351–570) encodes a
119 RluA family pseudouridine synthase (PUS) domain (E-value 5.43 e-74), which includes several highly
120 conserved residues implicated in catalysis (such as R507), that mediates U to Ψ conversion (Hoang et al.,
121 2006) (Figure 1D). Its N-terminus (NTD, amino acids 1–350) includes a region that has modest
122 similarity to the Smc super family (amino acids 132–258; E-value 7.32 e-6). BLAST searches were
123 carried out to probe the phylogenic distribution of TrcP. When the complete amino acid of TrcP was
124 used as the query, the search only yielded proteins that contain a PUS domain without sequences
125 corresponding to TrcP's NTD. However, when TrcP's NTD was the search query, TrcP homologs in other
126 Vibrionaceae, and genera related to vibrios, including *Shewanella* and *Pseudoalteromonas*, became
127 apparent; in addition, certain species in phyla that are phylogenetically distant from *V. cholerae*, such as
128 *Bacteroides*, *Nostoc*, *Synechococcus*, and *Myxococcus* also encode TrcP homologs (Figure. 1D and Figure.
129 S3), suggesting that though relatively uncommon, potential TrcP-like C-to- Ψ base editors are broadly
130 distributed among bacteria. Notably, a multiple sequence alignment of TrcP homologs showed that they
131 all contain sequences similar to TrcP's NTD and CTD (Figure. 1D). The co-occurrence of both the NTD
132 and PUS domains in TrcP homologs suggests that these two domains function together to mediate
133 C-to- Ψ conversion.

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135 **TrcP's N-terminal and C-terminal domains mediate C-to-U editing and pseudouridylation,**
136 **respectively**

137 Since TrcP's C-terminal PUS domain likely converts U to Ψ , we hypothesized that C-to- Ψ
138 conversion by TrcP is accomplished by sequential C-to-U and U-to- Ψ conversion mediated by its NTD
139 and CTD respectively (Figure. 2A). To test this idea, we purified recombinant TrcP-NTD, TrcP-CTD, and
140 a TrcP mutant in which a conserved catalytic arginine residue in the PUS domain was mutated to
141 alanine (R507A)(Figure. 2B and Figure. S2). The catalytic activity of these proteins was evaluated by
142 first incubating them in vitro with the unedited substrate tRNA-Tyr isolated from a $\Delta trcP$ strain, and
143 then analyzing the products by MALDI-TOF MS coupled with cyanoethylation as described above. The
144 TrcP-NTD fully converted the cytidine at position 32 into uridine but not to pseudouridine (Figure. 2C),
145 indicating that the TrcP-NTD is a cytidine deaminase sufficient for C-to-U base editing but not for
146 U-to- Ψ conversion. The TrcP-CTD did not catalyze C-to-U conversion, supporting the idea that the NTD
147 is necessary for C-to-U conversion (Figure. 2C). Like the TrcP-NTD, the R507A PUS domain mutant,
148 catalyzed C-to-U editing but failed to convert uridine to pseudouridine (Figure. 2C), confirming that the
149 intact NTD is sufficient for base editing and suggesting that the predicted catalytic site in the TrcP-CTD
150 is necessary for pseudouridylation. Together, these results support the idea that TrcP catalyzes C-to- Ψ
151 conversion in a stepwise reaction mechanism, where C is first converted to U, and then U is converted to
152 Ψ .

153 The capacity of the TrcP-CTD to catalyze U-to- Ψ conversion on tRNA-Tyr U32 appears to depend
154 on its linkage to TrcP's NTD. WT TrcP catalyzed pseudouridylation of tRNA-Tyr U32 generated by
155 TrcP-NTD (Figure. 2C, NTD \square TrcP), but TrcP-CTD did not (Figure. 2C, NTD \square CTD). Furthermore, U-to- Ψ
156 conversion was not observed when the TrcP-CTD and the TrcP-NTD were added to a reaction mixture
157 as separate proteins (Figure. 2C, NTD+CTD). We reasoned that TrcP's CTD must be coupled to its NTD
158 for pseudouridylation because the NTD facilitates substrate tRNA binding and/or stabilizes the proper
159 folding of the CTD.

160

161 **TrcP is predicted to consist of two globular enzymatic domains and one long helical domain**

162 ColabFold(Mirdita et al., 2021), a version of the structure prediction algorithm AlphaFold-2(Jumper
163 et al., 2021) was used to gain further insights into the mechanism of C-to- Ψ editing by TrcP. The
164 program yielded five nearly identical high confidence structural models (LDDT generally > 80) (Figure.
165 S4). These models all suggest that TrcP is composed of two globular domains along with a long helical
166 (LHL) domain consisting of three long helices (Figure. 3A). The TrcP-CTD forms a globular domain,
167 strongly suggesting that this domain corresponds to the PUS domain. The TrcP-NTD includes the other
168 globular domain with the insertion of the long helical domain.

169 Additional structural homology searches using Dali(Holm, 2020) strongly suggest that the globular
170 domain in the TrcP-NTD corresponds to a cytidine deaminase (CDA) domain. When the full-length
171 TrcP-NTD, containing the globular domain and the LHL domain, was used as a query to identify

172 structurally similar proteins, most of the hits were proteins that contain long helices, such as cohesin
173 and membrane proteins (Supplementary Data 1). However, when the query was the sequences
174 corresponding to the TrcP-NTD globular domain without the LHL domain (Figure 3A and Figure 1D), the
175 search identified several proteins annotated as deaminases (Supplementary Data 2). These hits
176 included deaminases targeting cytidine such as blastcidine-S deaminase (PDB 3oj6) and cytidine
177 deaminase (PDB 4eg2). The structure of Blastcidine-S deaminase (BSD) is highly similar to the
178 predicted structure of TrcP-NTD globular domain (Figure 1D, Figure. 3B and Figure. S5). Together these
179 findings are consistent with the idea that the TrcP-NTD globular domain encodes a cytidine deaminase.
180 Notably, the deaminase proteins identified in the structural search were not identified in the BLAST
181 search with the TrcP-NTD sequence, raising the possibility that the TrcP-CDA domain evolved
182 independently of other deaminase domains, including known C-to-U RNA editors such as CDAT8 in
183 archaea (Randau et al., 2009) and ADAT2/ADAT3 in Trypanosoma (Rubio et al., 2017).

184

185 **Reaction mechanism of C-to-U conversion by the TrcP CDA domain**

186 Deaminases that target adenine and cytosine rely on a zinc ion as a co-factor to activate a substrate
187 water molecule (Polson et al., 1991). In these enzymes, the zinc ion is usually coordinated by three
188 cysteine residues or one histidine and two cysteine residues (Betts et al., 1994) (Figure. S1). In the
189 structural model of the TrcP CDA domain there are three conserved cysteine residues, C282, C324, and
190 C328 (Figure 1D) that form a cluster with spatial coordinates (Figure. 3B) very similar to those of the
191 Zn-coordinating cysteines in blastcidine-S deaminase (Figure. 3B and Figure. S5), suggesting that the
192 TrcP-CDA also utilizes zinc ion as a co-factor. To assess this possibility, we measured the zinc
193 concentration in TrcP. Without denaturation, only trace amount of free zinc was detected in TrcP
194 solution (0.91 μM in 49 μM TrcP), whereas with acid denaturing, free zinc ion was detected in TrcP
195 solution (27.9 μM in 49 μM TrcP) (Figure. 3C). An *in vivo* complementation assay was carried out to
196 assess the requirement for these conserved cysteines for C-to-U editing. A ΔtrcP mutant strain was
197 transformed with vectors encoding cysteine mutants and the editing frequency was measured by
198 Sanger sequencing. No editing activity was observed for any of the three cysteine substitution mutants
199 even though all three mutant proteins were expressed at levels similar to the WT protein (Figure. 3D
200 and Figure. S6). Together these observations suggest that zinc is coordinated by a cysteine cluster in the
201 TrcP CDA catalytic site and the absence of any of these residues impairs TrcP's capacity for C-to-U
202 editing.

203 Known nucleoside base deaminases rely on a glutamate in the catalytic pocket to facilitate the
204 deamination reaction (Wilson et al., 1991). Like BSD, the TrcP CDA contains a conserved glutamate
205 within its catalytic pocket (Figure 1D, 3B and Figure. S5). This E303 residue was found to be critical for
206 C-to-U editing in the *in vivo* complementation assay (Figure. 3D and Figure. S6). Catalytic carboxyl
207 groups of E59 and E303 are located within the active sites of BSD and TrcP respectively (Figure 3B),
208 suggesting that the TrcP-CDA also relies on glutamate to facilitate the cytidine deamination reaction.
209 Collectively, our findings suggest that even though the TrcP CDA domain and BSD lack primary amino

210 acid sequence similarity, their respective catalytic sites both include a zinc ion coordinated by a cluster
211 of three cysteine residues and a glutamate residue. All four of these residues are also present in the
212 homologues identified in the TrcP-NTD BLAST search (Figure 1D, Figure. S1), suggesting that these
213 proteins also correspond to unrecognized cytidine deaminases.

214 During C-to-U editing, an enamine group in cytosine is converted to a carbonyl group (Figure 3E).
215 This process requires an external source of oxygen and characterized nucleoside base deaminases use
216 water as the oxygen source (Polson et al., 1991; Wilson et al., 1991). An *in vitro* editing assay with water
217 containing ^{18}O , which is 2 Da heavier than naturally abundant ^{16}O , was used to test whether TrcP also
218 uses water as an oxygen source (Figure. 3E). When the reaction mixture included 50% ^{18}O -labeled
219 water, we observed the heavier Ψ ($\Psi+2$) (Figure. 3F), indicating that labeled oxygen was incorporated
220 into Ψ . Detection of this labeled product required the addition of TrcP protein and ^{18}O to the reaction
221 (Figure. 3F). Thus, TrcP uses water as an oxygen source in the deamination process in C-to- Ψ editing.
222

223 **The TrcP long helical domain facilitates tRNA-Tyr binding**

224 TrcP's two globular domains appear to catalyze C-to- Ψ editing. We speculated that the enzyme's
225 long helical (LHL) domain facilitates substrate tRNA binding. To predict potential RNA binding regions
226 in TrcP, we calculated the surface potential in the model structures, since positively charged regions can
227 bind to the negatively charged RNA mainchain. Three positively charged TrcP regions were identified:
228 two regions in the pockets of the globular domains and one in a patch near the tip of the long helical
229 domain (Figure. 4A). To assess the possibility that the positively charged patch in the LHL domain is
230 involved in substrate tRNA binding, a mutant TrcP (KRmutant) in which six arginine and lysine residues
231 within the positively charged patch (R168, R171, K172, R175, K201, and K208) were substituted for
232 alanine was created. The KRmutant did not have C-to-U editing activity in the *in vivo* complementation
233 assay (Figure. 4B and Figure. S6), indicating that this positively charged patch in the LHL domain is
234 required for TrcP editing.

235 The binding capacity of TrcP to substrate tRNAs isolated from the $\Delta trcP$ strain was assessed with a
236 gel mobility shift assay. As the concentration of WT TrcP protein was increased, a shifted band that
237 presumably represents a complex of TrcP protein and tRNA-Tyr was observed; in contrast, a shifted
238 fragment was not detected when the same concentration of the TrcP-KRmutant was used (Figure. 4C).
239 Furthermore, TrcP appears to specifically bind tRNA-Tyr, since a shifted band was not observed when
240 tRNA-Asp was incubated with the WT TrcP protein (Figure. 4C).

241 The TrcP positively charged patch in its LHL domain is predicted to be relatively distant from the
242 catalytic centers of both the CDA and PUS domains, suggesting that this patch binds to the upper body of
243 the tRNA-Tyr molecule. To assess which parts of the tRNA-Tyr structure are required for TrcP editing
244 activity, we conducted *in vitro* editing assays with mutant tRNAs that lacked the CCA end (ΔCCA),
245 acceptor arm (ΔCArm), T-arm (ΔTarm), D-arm (ΔDarm), or variable loop (ΔVloop) (Figure. S7). The
246 tRNA substrates used in these reactions were generated by *in vitro* transcription and therefore lacked
247 modifications. Partial C-to-U editing was observed with full-length tRNA-Tyr and the mutant lacking the

248 CCA end, whereas the other four mutant tRNA-Tyr substrates were not edited (Figure. 4D). These
249 observations suggest that the properly folded structure of tRNA-Tyr is necessary for editing by TrcP.

250 Next, we asked whether the positively charged patch in the LHL domain facilitates TrcP's
251 pseudouridylation activity. For these experiments, substrate tRNA-Tyr isolated from the $\Delta trcP$ strain
252 was initially incubated with TrcP-NTD and ^{18}O -labeled water, yielding tRNA-Tyr with ^{18}O -labeled
253 uridine at position 32. The ^{18}O -labeled tRNAs were then reacted with WT or KRmutant TrcP and the
254 amount of labeled uridine was tracked (Figure. 4E). WT TrcP converted a larger fraction of the labeled
255 uridine to pseudouridine than the KRmutant (Figure. 4F), indicating that KRmutant has less
256 pseudouridylation activity than WT TrcP. This observation strongly suggests that the positively charged
257 patch in the LHL domain promotes TrcP pseudouridylation activity and at least partly accounts for the
258 lower activity of the CTD vs full-length TrcP in isomerization of U to Ψ (Figure 2C and Figure. 4F).

259 To corroborate the hypothesis that the TrcP-NTD facilitates the pseudouridylation activity of the
260 CTD by promoting substrate binding, we created a chimeric enzyme. The TrcP-NTD was fused to RluA, a
261 *V. cholerae* pseudouridylase that does not ordinarily target tRNA-Tyr for pseudouridylation (Figure 4F).
262 However, the chimeric TrcP-NTD-RluA protein had pseudouridylation activity on tRNA-Tyr at position
263 32 (Figure. 4F). We speculate that tethering of the tRNA-Tyr substrate by the LHL domain facilitates
264 RluA's pseudouridylation activity.

265

266 **An iron-responsive modification network in *V. cholerae* tRNA-Tyr**

267 The synthesis of certain tRNA modifications depends on the presence or absence of other
268 modifications, likely due to substrate recognition by modification enzymes (Barraud and Tisne, 2019;
269 Han and Phizicky, 2018). *In vitro* transcribed (unmodified) tRNA-Tyr was used to test whether
270 modifications in tRNA-Tyr influence the efficiency of C-to- Ψ editing. TrcP C-to- Ψ editing of an
271 unmodified *in vitro* transcribed substrate was less efficient than on partially modified tRNAs extracted
272 from the $\Delta trcP$ strain; even with 50 pmol TrcP and 50 pmol of unmodified tRNA-Tyr, less than half of
273 position 32 cytidine was converted to pseudouridine, whereas all of the cytidine 32 was converted to
274 pseudouridine when the substrate tRNA-Tyr was derived from $\Delta trcP$ *V. cholerae* (compare Figure 5A
275 and Figure 1C). This observation suggests that other modifications within tRNA-Tyr facilitate TrcP
276 C-to- Ψ editing.

277 We suspected that modifications in the anticodon could modulate the efficiency of C-to- Ψ editing
278 because in eukaryotes modifications at position 32 often depend on other modifications in the
279 anticodon loop (Han and Phizicky, 2018). Besides the Ψ at position 32, there are two additional
280 modifications, at positions 34 (queuosine, Q) and 37 ($\text{ms}^2\text{i}^6\text{A}$), in the anticodon loop of tRNA-Tyr
281 (Figure. S8). To test whether these modifications influence C-to- Ψ editing, editing in total tRNAs
282 extracted from mutant strains lacking the enzymes that create these modifications was assessed. At
283 position 37, $\text{ms}^2\text{i}^6\text{A}$ is synthesized in three steps: 1) addition of an isopentenyl group to adenine by
284 MiaA, 2) methyl-thiolation of i^6A by MiaB, and 3) hydroxylation of $\text{ms}^2\text{i}^6\text{A}$ by MiaE (Figure. S8A). No
285 editing of tRNA-Tyr was observed in tRNA prepared from either $\Delta miaA$ or $\Delta miaB$ mutant strains but

286 editing of RNA derived from the $\Delta miaE$ strain was detected (Figure. 5B), suggesting modification of
287 adenine at position 37 by both an isopentenyl and a methyl-thio group are required for TrcP C-to- Ψ
288 editing at position 32 of tRNA-Tyr. Since the isopentenyl group is required for methyl-thiolation by
289 MiaB in *E. coli* (Pierrel et al., 2004), our findings strongly suggest that the methyl-thio group in ms^2io^6A
290 is critical for TrcP C-to- Ψ editing. *In vitro* editing assay showed that tRNA-Tyr with ms^2io^6A37 ($\Delta trcP$) is
291 a better substrate than tRNA-Tyr containing io^6A37 ($\Delta miaB$), consistent with the idea that methyl-thio
292 group facilitate editing reaction by TrcP (Figure. 5C). Note that in the presence of excess amount of TrcP
293 protein, tRNA-Tyr from the $\Delta miaB$ strain can be edited, suggesting that methyl-thio group is not
294 essential for editing. Conversely, frequency of editing was not altered by the absence of queuosine at
295 position 34 when tRNA from the *tgt* mutant strain was tested (Figure. 5B), indicating that the presence
296 of this modified Q34 does not influence TrcP editing.

297 The observation that TrcP C-to- Ψ editing is dependent on ms^2io^6A introduced the possibility that
298 editing is modulating by environmental conditions, particularly iron availability because generation of
299 ms^2io^6A in *E. coli* is controlled by the availability of this nutrient (Griffiths and Humphreys, 1978). To
300 test this hypothesis, we analyzed editing frequency in RNA derived from cells grown in iron replete or
301 iron depleted conditions, generated with the iron-chelator, 2',2'-dipyridyl (dip). As the concentration of
302 dip increased, a decrease in editing frequency was observed (Figure. 5D). In the presence of >175 μM
303 dip, editing was completely eliminated. Together these observations reveal that TrcP editing of C-to- Ψ
304 at position 32 is controlled by iron availability likely because efficient editing in the cell relies on
305 iron-controlled methyl-thio modification at position 37.

306 Since we observed that modification of position 37 to ms^2io^6A modulates the frequency of C-to- Ψ
307 editing, we wondered if editing at position 32 can affect the synthesis of other modifications. To
308 investigate this possibility, tRNA-Tyr from WT and $\Delta trcP$ strains were harvested from log and
309 stationary phase cultures and liquid chromatography mass spectrometry (LC-MS) was used to analyze
310 nucleoside modifications. In log phase samples, there were lower signals of Q and its precursor oQ in
311 the WT strain than those in the $\Delta trcP$ strain (Figure. 5E, S8B). The elevated signals of Q and oQ in the
312 $\Delta trcP$ strain were also observed in $\Delta miaB$ strain, where C-to- Ψ editing is also eliminated (Figure. 5E). In
313 stationary phase samples, the oQ signal level was similar in the two strains, whereas the Q signal
314 remained low in the WT (Figure. 5E). These data strongly suggest that biogenesis of Q at position 34 is
315 impaired by TrcP's C-to- Ψ editing (Figure. 5F).

316

317 C-to- Ψ editing promotes Tyr decoding

318 Finally, we assessed the effects of TrcP derived C-to- Ψ editing on the decoding capacity of
319 tRNA-Tyr to begin to investigate the function of editing. A reporter system in which the translation
320 efficiency of specific codons is evaluated with the efficiency of frameshifting was engineered (Sakai et al.,
321 2019; Urbonavicius et al., 2001) (see Methods, Figure 6A). The reporter system was introduced into the
322 WT, $\Delta trcP$, Δtgt , and $\Delta trcP/\Delta tgt$ strains to assess the decoding efficiency of two tyrosine codons in early
323 stationary phase. Absence of *trcP* did not affect the decoding ability of either codon, but absence of *tgt*

324 decreased UAU decoding but not UAC decoding (Figure. 6B), as reported in *Salmonella typhimurium*
325 (Urbonavicius et al., 2001). In the absence of both *tgt* and *trcP*, UAU decoding was even more reduced
326 than in the single *tgt* knockout strain (Figure. 6B), suggesting that C-to-Ψ editing facilitates UAU
327 decoding partially redundantly with Q.
328

329 Discussion

330 Here, we characterized the biogenesis of C-to- Ψ RNA editing, a new type of RNA editing. A single
331 enzyme, TrcP, was found to be sufficient to catalyze the conversion of C-to- Ψ . Examples of nucleosides
332 undergoing both editing and modification, such as A-to-I and C-to-U editing followed by methylation,
333 have been described (Dixit et al., 2019; Rubio et al., 2017), but those reactions are carried out by
334 independent editing and modifying enzymes. TrcP catalyzes both editing and modification in two
335 consecutive reactions (Figure. 7). First, TrcP's cytidine deaminase (CDA) domain in its N-terminus
336 catalyzes cytidine deamination to form uridine using water as a substrate. Then, TrcP's pseudouridine
337 synthase (PUS) domain isomerizes uridine into pseudouridine. AlphaFold-2-based structural modeling
338 was instrumental for uncovering TrcP's structure function relationships. This machine learning
339 algorithm predicted that TrcP contains three domains. Its two globular domains proved to correspond
340 to its novel CDA and pseudouridylase. The unexpected third long helical (LHL) domain proved critical
341 for TrcP substrate binding. This unique domain appears to enable the enzyme to adopt a substrate
342 channeling mechanism to carry out its consecutive deamination and pseudouridylation reactions.
343 Additional studies of the control of C-to- Ψ editing uncovered an interdependent network of
344 modification in the tRNA anticodon loop whereby C-to- Ψ editing at position 32 requires methyl-thio
345 modification of A at position 37 and suppresses the conversion of G to Q at position 34. Functionally,
346 this modification circuit facilitates coupling of tRNA modification states to environmental iron
347 availability and impacts decoding.

348 We propose that the positively charged patch in TrcP's LHL domain enables tRNA substrate
349 channeling. Since this patch facilitated both C-to-U editing and pseudouridylation, this interaction likely
350 keeps substrate tRNAs bound to the enzyme during both the deamination and isomerization reactions
351 (Figure. 7). It is tempting to speculate that continuous binding of the upper part of substrate tRNA by
352 the positively charged patch and flexible movement of the long helical domain facilitates the two
353 reactions by directing the movement of the anticodon loop from CDA to PUS domain without diffusion.
354 Metabolic enzymes adopt substrate channeling mechanisms for efficient consecutive reactions in which
355 substrate molecules are conveyed from one enzyme to another without diffusion. Biosynthesis of some
356 macromolecules, including tRNAs, depend on channeling by their associated protein factors. For
357 example, in the biogenesis of cysteinyl- or glutaminyl-tRNAs, SepRS and SepCysS or GatCAB and GluRS
358 form complexes catalyzing two-step aminoacylation, respectively, thereby sequestering misacylated
359 tRNAs from the active aminoacyl-tRNA pool (Ito and Yokoyama, 2010; Liu et al., 2014). In these cases,
360 the complexes stably bind the tRNA core during two consecutive reactions and channel 3' end of tRNAs
361 from one catalytic site to the other. This mechanism is analogous to the proposed mechanism of TrcP
362 channeling based on the tethering of tRNA substrates by LHL domain.

363 Our results suggest that the TrcP-CDA domain is a novel zinc-dependent RNA deaminase domain.
364 TrcP structurally resembles zinc-dependent deaminases (Figure. 3B, Figure. S5), although its primary
365 sequence is distinct from other deaminases (Figure. S1). The conserved cysteines in TrcP essential for
366 C-to-U editing are structurally well aligned with the cysteines in the Blastidine-S deaminase (BSD)

367 that coordinate a zinc ion (Figure. 3B, Figure. S5), strongly suggesting that TrcP also utilizes zinc ion for
368 its reaction. A notable difference between TrcP and other deaminases is in the putative catalytic
369 glutamate residue (E303 in TrcP and E59 in Blastocidin-S deaminase, Figure. 3B and Figure. S5).
370 Although the spatial coordinates of the cysteine cluster and the catalytic glutamate's carboxylate group
371 in the active sites of TrcP and BSD are similar, the orientation of the side chain and position of these
372 glutamate residues in TrcP and BSD (E303 and E59, respectively) differ (Figure S1). This difference
373 raises the possibility that these structurally similar deaminase catalytic pockets arose via distinct
374 evolutionary pathways.

375 The AlphaFold-2 assisted identification of a new deaminase family suggests that additional new
376 deaminase families, which cannot be identified by *in silico* searches based solely on primary protein
377 sequences without biochemical or genetic data await discovery. Such editing enzymes may account for
378 editing reactions in plants, kinetoplastids, and marsupials, where editing enzymes remain unidentified
379 (Dixit et al., 2019). Coupling structural prediction and structural homology search has great potential
380 for identification of new deaminases and other enzyme families.

381 C-to-Ψ editing is part of an interdependent network of modifications within the tRNA-Tyr
382 anticodon loop. C-to-Ψ editing is promoted by 2-methylthio modification (ms²) in ms²io⁶A at position
383 37 and partially suppresses queuosine (Q) formation at position 34 (Figure. 5F). Interdependencies in
384 tRNA modifications between individual modifications have been reported, especially in the anticodon
385 loop, but networks of more than two modifications are unusual. One example is in the thermophilic
386 bacterium *Thermus thermophilus*, where Ψ⁵⁵ modification facilitates both m¹A⁵⁸ and m⁵s²U⁵⁴
387 modifications (Ishida et al., 2011). These latter two modifications are critical for tRNA thermostability,
388 suggesting that Ψ⁵⁵ optimizes tRNA stability depending on the temperature by controlling the
389 frequency of the two modifications. Similarly, the modification network found in *V. cholerae* tRNA-Tyr
390 can function as a circuit that couples modification states to environmental conditions. We found that the
391 amount of C-to-Ψ editing varies with environmental iron concentrations, presumably because iron is
392 required for generation of iron-sulfur cluster in MiaB (Hernandez et al., 2007) (Figure. 5F). Such
393 changes in modification states can facilitate adaptation to iron-depleted conditions, including within
394 host niches. Environmental control of post-transcriptional editing can provide a fitness advantage that
395 does not depend on genomic mutations in tRNA genes.

396

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404

405 **Methods**

406 **Strains and culture conditions**

407 The strains used in this study are listed in [Supplementary Data 3](#). *V. cholerae* C6706, a clinical
408 isolate, was used as the wild-type strain (Millet et al., 2014). All *V. cholerae* strains were grown in LB
409 containing 1% NaCl at 37 °C. *E. coli* SM10 (lambda pir) harboring derivatives of pCVD442 was cultured
410 in LB plus carbenicillin (Cb). Antibiotics were used at the following concentrations: 200 µg/mL
411 streptomycin, 50 µg/mL Cb, 50 µg/ml kanamycin (Km). A transposon insertion strain (*tgt::Tn*) is
412 derived from the strain library (Cameron et al., 2008).

413

414 **Strain and plasmid construction**

415 All mutations in C6706 were created using homologous recombination and a derivative of the
416 suicide vector pCVD442. Targeting vectors for gene deletions contained ~1000 bp of DNA flanking each
417 side of the target gene cloned into pCVD442's *Sma*I site using isothermal assembly (Kimura and Waldor,
418 2019).

419 For complementation experiments, *trcP* or fragments of the *trcP* ORF were cloned into pHL100's
420 *Sma*I site with the 23 nt upstream sequence and the Flag tag sequence on the C-terminal using
421 NEBuilder HiFi DNA Assembly Master Mix (NEB); subsequently, plasmids encoding *trcP* mutants were
422 generated with the PrimeSTAR Mutagenesis Basal Kit (TAKARA-bio).

423 TrcP protein expressing vector pET28-TrcP was generated by integrating *trcP* ORF and a linearized
424 pET28 using the NEBuilder HiFi DNA Assembly Master Mix (NEB). The mutant derivatives of this vector
425 were generated with the PrimeSTAR Mutagenesis Basal Kit (TAKARA-bio).

426 Reporter protein-expressing plasmids for measuring Tyr decoding ability were generated by
427 integrating frameshift sequences testing Tyr codon decoding and linearized from pMMB207 encoding
428 mCherry and bright GFP using NEBuilder HiFi DNA Assembly Master Mix (NEB).

429 Plasmids used in this study are listed in [Supplementary Data 4](#).

430

431 **RNA extraction**

432 Total RNA was extracted with TRIzol (Life Technologies) according to the manufacturer's instructions.

433

434 **Isolation of individual tRNA species**

435 One liter or 500 ml cultures of log-phase ($OD_{600} = 0.3-0.4$) and stationary phase (24h) *V. cholerae* cells
436 were harvested, and total RNA was extracted¹⁰. Briefly, cells were resuspended in 5 mL buffer (50 mM
437 NaOAc, pH 5.2, 10 mM Mg(OAc)₂), mixed with 5 mL water-saturated phenol, and agitated vigorously for
438 1 h in a 50 ml Erlenmeyer flask with a stir bar. The aqueous phase was separated by centrifugation,
439 washed with chloroform, and recovered by isopropanol precipitation. RNA was run through a manually
440 packed DEAE column (GE healthcare) to remove contaminants and the rRNA fraction. Typically for a 1 L
441 culture, total RNA resolved in 10 ml of the equilibration buffer (100 mM Hepes-KOH pH 7.4) and was
442 loaded on 2 ml of DEAE beads equilibrated with 20 ml of the equilibration buffer. After sequential

443 washes with 10 ml of the equilibration buffer and 10 ml of wash buffer (100 mM Hepes-KOH pH 7.4 and
444 300 mM NaCl), the RNA fraction was eluted with 10 ml of the elution buffer (100 mM Hepes-KOH pH 7.4
445 and 1 M NaCl) and recovered by isopropanol precipitation. Individual tRNA species were purified using
446 biotinylated DNA probes anchored to a high-capacity streptavidin agarose resin (GE Healthcare).
447 Typically for this purification, 2 mg of RNA treated with DEAE beads is mixed with 200 μ l of beads
448 which are bound to 4 nmol of probes in 30 mM Hepes-KOH, pH 7.0, 1.2 M NaCl, 15 mM EDTA, and 1 mM
449 DTT at 68 °C for 30 min with shaking. Beads were washed three times with 15 mM Hepes-KOH, pH 7.0,
450 0.6 M NaCl, 7.5 mM EDTA, and 1 mM DTT and seven times with 0.5 mM Hepes-KOH, pH 7.0, 20 mM NaCl,
451 0.25 mM EDTA, and 1 mM DTT. Purified tRNAs were extracted from beads with TRIzol. After Turbo
452 DNase (Thermo Fisher Scientific) treatment to remove residual DNA probes, tRNAs were purified on
453 10 % TBE-Urea gels. The probes used in this study are listed in [Supplementary Data 5](#).

454

455 **Purification of recombinant proteins**

456 The BL21(DE3) strain transformed with pET28b encoding WT and mutant TrcP proteins, RluA, or the
457 chimeric TrcP-RluA protein (RluA-NTD), was grown in 10 ml LB medium (Km50) overnight and
458 inoculated into 1 L LB medium (Km50) and grown at 37°C with shaking. When optical density (OD_{600})
459 reached 0.3, the flask was moved to 18 °C an incubator and shaken for 30 min. Protein expression was
460 induced by the addition of 1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) and the flask was
461 incubated with shaking at 18 °C for 24 h. Harvested cells were resuspended in 40 ml lysis buffer (50
462 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 10% glycerol, 300 mM NaCl, 0.2 U/mL DNase I, 1 mM PMSF,
463 complete proteinase inhibitor mixture; Roche) and homogenized with an EmulsiFlex for 20 min.
464 Cleared lysate (35 ml) supplemented with 700 μ l of 2 M imidazole (final concentration 40 mM) was
465 mixed with 1.5 ml Ni-NTA beads equilibrated with 10 ml lysis buffer and incubated at 4 °C for 2.5 h with
466 gentle rotation. Protein bound beads were loaded on an open column (Biorad) and washed twice with
467 10 ml wash buffer (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 10% glycerol, 300 mM NaCl, 40 mM
468 imidazole). Protein was eluted with Elution buffer 1 (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 10%
469 glycerol, 300 mM NaCl, 250 mM imidazole) and Elution buffer 2 (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$,
470 10% glycerol, 300 mM NaCl, 400 mM imidazole). The two elution fractions were mixed and dialyzed
471 overnight in Dialysis buffer 1 (20 mM Tris-HCl pH 8.0, 300 mM NaCl, 10% Glycerol, 1 mM DTT) and 8 h
472 in Dialysis buffer 2 (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% Glycerol, 1 mM DTT). Protein
473 concentration was measured by Qubit (Invitrogen).

474 For measurement of zinc concentration, highly purified, untagged TrcP was prepared using a previously
475 described SUMO2-based protein purification strategy(Zhou et al., 2018). Briefly, *V. cholerae* TrcP was
476 sub-cloned into a custom pET vector and expressed as a 6 \times His-tagged N-terminal human SUMO2 fusion
477 in *E. coli* BL21 RIL bacteria (Agilent). A 50 ml starter culture grown overnight at 37°C in MDG medium
478 (1.5% Bacto agar, 0.5% glucose, 25 mM Na_2HPO_4 , 25 mM KH_2PO_4 , 50 mM NH_4Cl , 5 mM Na_2SO_4 , 0.25%
479 aspartic acid, 2–50 μ M trace metals, 100 μ g mL^{-1} ampicillin, 34 μ g mL^{-1} chloramphenicol) was used to
480 seed growth of 2 \times 1 L induction cultures grown in M9ZB medium (47.8 mM Na_2HPO_4 , 22 mM KH_2PO_4 ,

481 18.7 mM NH₄Cl, 85.6 mM NaCl, 1% Cas-Amino acids, 0.5% glycerol, 2 mM MgSO₄, 2–50 μM trace metals,
482 100 μg mL⁻¹ ampicillin, 34 μg mL⁻¹ chloramphenicol). M9ZB cultures were grown at 37°C with shaking
483 at 230 rpm until an optical density at 600 nm (OD₆₀₀ nm) of ~2.5. Cultures were then transferred to an
484 ice bath for 20 min, supplemented with 0.5 mM IPTG and incubated at 16°C with shaking at 230 rpm for
485 overnight growth. Harvested cell pellet was lysed by sonication in lysis buffer (20 mM Hepes-KOH pH
486 7.5, 400 mM NaCl, 30 mM imidazole, 10% glycerol and 1 mM DTT) and recombinant TrcP was purified
487 from clarified lysates using Ni-NTA resin (Qiagen) and gravity chromatography. Ni-NTA resin was
488 washed with lysis buffer adjusted to 1 M NaCl concentration and eluted with lysis buffer adjusted to 300
489 mM imidazole concentration. Purified TrcP was then supplemented with ~250 μg of human SENP2
490 protease (D364–L589, M497A) and dialyzed overnight at 4°C in buffer (20 mM HEPES-KOH pH 7.5, 200
491 mM NaCl and 1 mM DTT). TrcP was next purified by ion-exchange chromatography by binding to a 5 ml
492 HiTrap Heparin HP column (Cytiva) and eluting across a 150–1000 mM NaCl gradient. Target protein
493 fractions were pooled and further purified by size-exclusion chromatography using a 16/600 S75
494 column (Cytiva) with a running buffer of 20 mM Hepes-KOH pH 7.5, 250 mM KCl and 1 mM TCEP-KOH.
495 Final protein was concentrated to ~6.3 mg/mL, flash-frozen in liquid nitrogen, and stored at –80°C
496

497 **Nucleoside analysis**

498 Purified and sometimes derivatized tRNA-Tyr (100 ng – 1 μg) was digested with 0.5 unit Nuclease P1
499 and 0.1 unit of phosphodiesterase I in 22 μl reactions containing 50 mM Tris-HCl pH 5.3, 10 mM ZnCl₂ at
500 37 °C for 1 h. Then, reaction mixtures were mixed with 2 μl 1M Tris-HCl pH 8.3 and 1 μl of 1 unit/μl Calf
501 Intestine phosphatase and incubated at 37 °C for 30 min. Enzymes were removed by filtration using 10
502 K ultrafiltration columns (VWR) and 5–10 μl of digests were injected into an Agilent 1290 uHPLC
503 system bearing a Synergi Fusion-RP column (100 × 2 mm, 2.5 μm, Phenomenex) at 35 °C with a flow
504 rate of 0.35 ml/min and a solvent system consisting of 5 mM NH₄OAc (Buffer A) and 100 % Acetonitrile
505 (Buffer B). The gradient of acetonitrile was as follows: 0 %; 0–1 min, 0–10 %; 1–10 min, 10–40 %;
506 10–14 min, 40–80 %; 14–15 min, 80–100 %; 15–15.1 min, 100 %; 15.1–18 min, 100–0 %; 18–20 min,
507 0 %; 20–26 min. The eluent was ionized by an ESI source and directly injected into an Agilent 6460 QQQ.
508 The voltages and source gas parameters were as follows: gas temperature; 250 °C, gas flow; 11 L/min,
509 nebulizer; 20 psi, sheath gas temperature; 300 °C, sheath gas flow; 12 L/min, capillary voltage; 1800 V,
510 and nozzle voltage; 2000 V. Dynamic multiple reaction monitoring (MRM) was carried out to detect
511 labeled and unlabeled known modifications. The retention time windows and *m/z* values of precursor
512 and product ions for dynamic MRM analyses are listed in [Supplementary Data 6](#).

513

514 **Direct sequencing**

515 cDNAs were synthesized from 10 ng purified tRNA-Tyr using Super Script III (Invitrogen) using the RT
516 primer. cDNA was amplified by PCR using primers: RT-PCR primers. PCR products were sequenced by
517 Sanger sequencing using the sequencing primer. The sequences of the primers are listed in
518 [Supplementary Data 5](#).

519

520 **Oligo protection**

521 50 pmol of tRNA-Tyr was mixed with 500 pmol of two DNA oligos complementary to C32 and U32
522 tRNA-Tyr ([Supplementary Data 5](#)) in 50 μ l aliquots containing 50 mM Hepes KOH pH 7.6, 150 mM KCl
523 and heated to 90 °C for 1 min and gradually cooled down to room temperature at 1 °C/min for
524 annealing, followed by RNase digestion with 50 ng RNase A and 50 unit RNase T₁ on ice for 15 min.
525 Protected DNA/RNA duplexes were purified on 10 % TBE-Urea gels and recovered by isopropanol
526 precipitation and dissolved in 5 μ l milliQ water. A 2 μ l aliquot was subjected to fragment analysis with
527 RNase A using a MALDI-TOF spectrometer as described above.

528

529 **Cyanoethylation**

530 Cyanoethylation was done as described¹⁹. 4 μ l of RNA (1–2 μ g) was mixed with 30 μ l CE solution (50%
531 ethanol, 1.1 M triethylamine pH 8.6) and 4 μ l of 15.2 M acrylonitrile, followed by incubation at 70 °C for
532 1 h. The reaction was stopped by addition of 162 μ l milliQ water and by placing the reaction tube on ice.
533 RNA was recovered by ethanol precipitation.

534

535 **Fragment analysis**

536 400–1000 ng partial fragments of tRNA-Tyr were digested in 3 μ l aliquot with 20 ng RNase A (QIAGEN)
537 in 10 mM NH₄OAc pH 7, or 20 unit RNase T₁ in 10 mM NH₄OAc pH 5.3 at 37 °C for 1 hr. On a MALDI steel
538 plate, 0.5 μ l of matrix (0.7 M 3-hydroxypicolinic acid (HPA) and 70 mM ammonium citrate in 50 %
539 milliQ water and 50 % acetonitrile) was mounted and dried, followed by mounting of 0.5 μ l RNase
540 digests and drying. The samples were analyzed with Bruker Ultraflex Xtreme MALDI-TOF mass
541 spectrometer.

542

543 ***In vitro* editing reaction**

544 Cytidine deamination activity of TrcP was measured by direct Sanger sequencing of cDNA derived
545 from tRNA-Tyr in Figure. 1B, Figure. 4D, and Figure. 5C. tRNA-Tyr (5 pmol) was incubated with 0–15
546 pmol TrcP recombinant protein in 20 μ l reaction mixtures (50 mM Tris-HCl pH 8.0, 5 mM MgCl₂, 30 mM
547 NaCl) for 1 hr at 37 °C. The reaction was stopped by an addition of 100 μ l phenol chloroform and
548 vigorous vortexing; subsequently, tRNAs were washed with chloroform and recovered by isopropanol
549 precipitation.

550 C-to-U editing and pseudouridylation activity was measured by MALDI-TOF-MS in Figure. 1C,
551 Figure. 2C, Figure. 5A. tRNA-Tyr isolated from cells or *in vitro* transcription reactions (50 pmol) was
552 incubated with 0, 5, or 50 pmol TrcP and TrcP mutant proteins in 200 μ l reaction mixture (50 mM
553 Tris-HCl pH 8.0, 5 mM MgCl₂, 30 mM NaCl) for 1 hr at 37 °C, and tRNAs were recovered as described
554 above. The partial fragment of tRNA-Tyr containing position 32 was purified by oligo protection, and
555 pseudouridines were labelled by cyanoethylation. C, U, and Ψ containing fragments were measured by
556 fragment analysis by MALDI-TOF-MS as described above.

557 Stable isotope labelled water ($H_2^{18}O$) was used for identifying the oxygen source of deamination
558 (Figure 3F) and tracking pseudouridylation by nucleoside analysis (Figure 4F). In Figure. 4F, 5 pmol
559 tRNA-Tyr isolated from the $\Delta trcP$ mutant was incubated for 1 hr at 37 °C with 10 pmol TrcP protein in a
560 20 μ l reaction in (50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$ 30 mM NaCl) containing 10 μ l ^{18}O -labelled water.
561 tRNAs were recovered as described above and signals of natural and heavier pseudouridines were
562 detected by nucleoside analysis as above. In Figure 5E, 200 pmol tRNA was incubated with 200 pmol
563 TrcP-NTD in a 40 μ l reaction in (50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 60 mM NaCl) containing 20 μ l
564 ^{18}O -labelled water. tRNAs were recovered and subject to reaction with WT and mutant TrcP proteins,
565 RluA, and chimeric RluA proteins. 5 pmol tRNA was incubated with 5 pmol or 500 fmol proteins in a 20
566 μ l reaction in (50 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 75 mM NaCl) for 1 hr at 37 °C. tRNAs were
567 recovered and subject to nucleoside analysis as described above and signals of U+2 normalized with
568 m^5U signals were tracked as the readout of uridine to pseudouridine conversion.

569

570 **Gel mobility shift assay**

571 The substrate tRNA binding capacities of TrcP and the KRmutant were evaluated using a gel mobility
572 shift assay. Isolated tRNA-Tyr from the $\Delta trcP$ strain and tRNA-Asp from the WT strain were labeled with
573 ^{32}P at their respective 5' ends. Briefly, calf intestinal alkaline phosphatase (CIP) (NEB) was used to
574 dephosphorylate tRNAs, which were purified with gel electrophoresis, and labeled with $[\gamma\text{-}^{32}P]ATP$ by
575 T4 PNK (NEB), followed by gel purification. WT or KRmut TrcP proteins (0–5 μ M) were incubated with
576 1 μ M cold tRNA-Tyr or tRNA-Asp and a trace amount of ^{32}P -labeled tRNAs ($< 0.1 \mu$ M) in a 5 μ l reaction
577 in 5 mM $MgCl_2$, 50 mM Tris-HCl pH 8.0, and 60 mM NaCl at 37 °C for 10 min, followed by mixing with
578 loading dye (80% glycerol and 0.05% bromophenol blue) and gel electrophoresis for 30 min at 180 V
579 and 4 °C on 4% polyacrylamide gel containing 50 mM Tris-Acetic acid pH 8.0 and 5 mM $Mg(OAc)_2$; the
580 running buffer was the same composition as the gel. Gels were dried with a gel dryer (Biorad) and
581 bands were detected using an FLA-5000 phosphoimager (Fuji Film).

582

583 **Translation reporter assay**

584 The WT, $\Delta trcP$, Δtgt , and $\Delta trcP/\Delta tgt$ strains were transformed with the frameshift and 0-frame
585 reporters. Cells were cultured overnight in 2 ml LB medium (Cm 5 μ g/ml) at 30 °C and then diluted to
586 OD_{600} 0.01 in 5 ml LB medium (Cm 1 μ g/ml) with 100 μ M IPTG and cultured at 37 °C and harvested one
587 hour after the OD_{600} reached 0.5 (~ 4 hr and $\sim 0.8 OD_{600}$, early stationary phase). The cells were spun
588 down and resuspended in 100 μ l PBS and fixed by incubation for 20 min at room temperature in 33 μ l
589 16% paraformaldehyde. The fixed cells were then spun down and resuspended in 1 ml PBS and left at
590 room temperature overnight. The cell suspension was diluted 100-fold in 1 ml PBS and analyzed with
591 FACS (Sony). The signals of GFP and mCherry were measured in 100,000 particles and analyzed with a
592 custom R script. To decrease background, particles with signal intensity of less than 500 in the of GFP or
593 mCherry channels were removed from the analysis. Average values of the ratio of GFP to mCherry
594 signals derived from 0 frame and +1 frame constructs were calculated as R_t and R_s , representing

595 efficiency of in-frame decoding and +1 frameshift, respectively. Then, $\log_2(R_t/R_s)$ values were
596 calculated to estimate the Tyr decoding efficiency.

597

598 **Measuring zinc ion concentration**

599 The zinc concentration of TrcP in solution was measured with a Zinc Assay Kit (Abcam) according to the
600 manufacturer's instruction. Twenty-five μl of TrcP solution (97.6 μM) was mixed with 25 μl 14%
601 trichloroacetic acid or water, followed by centrifugation and recovery of the supernatant. A 50 μl serial
602 dilution series of a Zn standard solution (0–100 μM) and TrcP samples were mixed with a 200 μl
603 reaction mix and incubated for 10 min at room temperature. Light absorption at A_{560} was measured.
604 Zinc concentrations of TrcP solutions were calculated with a standard curve generated with the
605 standard Zn solutions.

606

607 **Complementation assay**

608 A *V. cholerae* $\Delta trcP$ strain was transformed with plasmids expressing TrcP or mutant derivatives. The
609 resultant strains were cultured in LB medium (Km 50 $\mu\text{g}/\text{ml}$) overnight at 30 °C. Overnight cultures
610 were diluted to OD_{600} 0.01 in 5 ml LB (Km 50 $\mu\text{g}/\text{ml}$, IPTG 10 μM) and cultured at 37 °C until OD_{600}
611 reached 0.3. Culture (1 ml) was transferred to two tubes, and cells were harvested by centrifugation.
612 One tube was used to assess RNA editing, and the other was used for measuring TrcP and mutant
613 protein expression levels. RNA editing frequency was measured by Sanger sequencing as described
614 above. TrcP and mutant protein expression levels were measured by western blotting. For the latter
615 assays, cell pellets were resuspended in 200 μl lysis buffer (50 mM Tris-HCl pH 8.0, 10% glycerol, and
616 300 mM NaCl) and homogenized by sonication, and 10 μl of lysate mixed with loading solution and
617 boiled and loaded on NuPAGE 4–12% Bis-Tris Gel and electrophoresis was carried out for 40 min at
618 200 V. An iBlot2 (Invitrogen), was used to transfer proteins nitrocellulose membranes, followed by
619 blocking with TBST buffer (20 mM Tris-HCl pH 7.6, NaCl 150 mM, 0.1% Tween-20) supplemented with
620 5% milk, primary (Sigma anti-Flag M2 antibody) and secondary antibody (Invitrogen anti-mouse
621 antibody) binding, signals were detected with Supersignal West Pico Plus Chemiluminescent substrate
622 (Thermo Fisher) using a Chemidoc (Biorad).

623

624 **Structural modeling**

625 The amino acid sequences of full-length TrcP, its CDA domain (1–114 aa and 250–339 aa), and KR
626 mutant (R168A, R171A, K172A, R175A, K201A, and K208A) were input to ColabFold2 program (Mirdita
627 et al., 2021). The first model among five nearly identical models was used for further analyses. A pdb
628 file was generated and used for structural homology searches using the Dali Server (Holm, 2020). The
629 resultant structures were overlaid on the structural model of the CDA domain (Figure. 3B and Figure.
630 S5). Electro potential maps were generated by APBS Electrostatics in PyMOL with a default setting.

631

632 **Phylogenetic analysis of TrcP**

633 TrcP-NTD (1–339 aa) homologs were searched using BLAST with default algorithm parameters except
634 for Max target sequences (increased to 20000), yielding 4862 hits, including 4567 sequences derived
635 from 959 species with E-value less than 1 e-20 ([Supplementary Data 7](#)). To depict the distribution of
636 TrcP-NTD homologs, a phylogenetic tree was generated by phyloT with manually picked 757 organisms,
637 including 38 organisms having TrcP homologs with E-value less than 1 e-20, and displayed by
638 iTOL(Letunic and Bork, 2021) ([Supplementary Data 8 and Figure. S3](#)). Sequence alignment was
639 performed with Clustal X2(Larkin et al., 2007) and displayed with Bioedit(Hall, 1999).
640

641 **Figure Legends**

642 **Figure 1. TrcP is sufficient to catalyze C-to- Ψ editing reaction.**

643 **A.** Secondary structure of *V. cholerae* tRNA-Tyr. The C at position 32 is edited to Ψ .

644 **B.** Sanger sequencing of amplified cDNA derived from tRNAs incubated with recombinant TrcP protein.
645 TrcP concentrations are shown on the right. In the traces, red corresponds to T and blue corresponds to
646 C.

647 **C.** MALDI-TOF analysis of an oligo-protected portion (positions 10–46) of tRNA-Tyr after incubation
648 with recombinant TrcP protein. The tRNA fragment was treated with acrylonitrile to introduce a
649 cyanoethyl-group (53 Da) on to Ψ . RNase A digests of a cyanoethylated RNA fragment were subjected to
650 MS. The m/z values and assigned fragments are shown.

651 **D.** Multiple sequence alignment of TrcP homologs and *V. cholerae* RluA, a paralog of the pseudouridine
652 synthetase domain of TrcP. Similar amino acids that are observed in more than 80% of sequences are
653 shaded with colors. Amino acid residues that are mutated in this study are indicated. TrcP NTD and CTD,
654 and domains assigned by structural modeling, i.e., the cytidine deaminase (CDA) domain (1–114 aa and
655 250–339 aa), the long helical (LHL) domain (115–249 aa), and the pseudouridine synthase (PUS)
656 domain (351–570 aa) are shown.

657

658 **Figure 2. TrcP includes a C-to-U RNA editor and a pseudouridylase.**

659 **A.** Schematic of the C-to- Ψ editing reaction by TrcP.

660 **B.** TrcP domain structure. The residues that are mutated in this study are indicated.

661 **C.** MALDI-TOF analysis of an oligo-protected portion (positions 10–46) of tRNA-Tyr after incubation
662 with recombinant TrcP and mutant derivatives. The tRNA fragment was treated with acrylonitrile to
663 introduce a cyanoethyl-group (53 Da) to Ψ . RNase A digests of a cyanoethylated RNA fragment were
664 subjected to MS. The m/z values and assigned fragments are shown.

665

666 **Figure 3. Reaction mechanisms of TrcP catalyzed C-to-U editing.**

667 **A.** A structural model of TrcP protein generate by ColabFold(Mirdita et al., 2021). TrcP is composed of
668 two globular domains, cytidine deaminase (CDA) domain (light blue) and pseudouridine synthetase
669 (PUS) domain (orange) and a long helical (LHL) domain (green).

670 **B.** Alignment of the predicted catalytic site in the TrcP CDA domain (light blue) with the Blasticidin-S
671 deaminase (BSD) (yellow). The residues of BSD and its coordinating zinc ion are shown in the yellow
672 background, whereas the residues of TrcP are shown in the light blue background. Oxygen and sulfur
673 residues are also colored in red and gold, respectively.

674 **C.** Colorimetric measurement of zinc concentration of TrcP solution.

675 **D.** *In vivo* complementation analysis of TrcP mutants. Sanger sequencing results of tRNA-Tyr cDNA
676 derived from RNA isolated from *trcP* knockout strains expressing TrcP (WT) or the indicated mutant
677 derivatives.

678 **E.** A schematic of the C-to- Ψ editing reaction by TrcP. TrcP's NTD uses water as a source of oxygen for
679 C-to-U editing. In U and Ψ , the expected positions of ^{18}O -labeled oxygen from water are shown in red.

680 **F.** Nucleoside analysis of tRNAs incubated with or without TrcP and stable isotope-labeled water
681 (^{18}O -water). The reaction conditions and the detected nucleosides are shown above and right,
682 respectively.

683

684 **Figure 4. The long helical domain facilitates substrate tRNA binding.**

685 **A.** Predicted surface electrostatic potential of the structural model of TrcP. The color key is shown
686 below (unit $K_{\text{b}}T/e_c$).

687 **B.** *In vivo* complementation analysis of the KR mutant. Sanger sequencing results of tRNA-Tyr cDNA
688 derived from RNA isolated from *trcP* knockout strains expressing TrcP (WT) or mutant derivatives.

689 **C.** Gel mobility shift assay with TrcP (WT and KR mutant) and tRNAs (tRNA-Tyr and tRNA-Asp). Upper
690 and lower arrowheads represent wells and free tRNA signals, respectively. The black line indicates the
691 shifted band presumably representing the soluble tRNA-TrcP complex. Protein concentrations were 0,
692 0.1, 1, 2, and 5 μM , and the tRNA concentration was 1 μM .

693 **D.** Sanger sequencing of cDNA derived from tRNA-Tyr (WT) and the indicated mutants incubated with
694 recombinant TrcP protein. The secondary structures of mutant tRNAs are shown in [Figure S7](#).

695 **E.** A schematic of the experiment in F. TrcP-NTD was used for generating ^{18}O -labelled U32 in tRNA-Tyr.
696 Purified tRNA-Tyr was then incubated with indicated enzymes.

697 **F.** Nucleoside analysis of tRNA-Tyr after the reaction with the indicated proteins. Conversion of U into Ψ
698 was assessed by the signals of ^{18}O -labelled U (U+2). The signals were normalized by m^5U . The bar
699 represents the average values of two independent reaction results represented by the points.

700

701 **Figure 5. An iron-responsive modification network in *V. cholerae* tRNA-Tyr.**

702 **A.** MALDI-TOF analysis of the oligo-protected portion (positions 10–46) of *in vitro* transcribed
703 tRNA-Tyr. tRNA was reacted with 0 pmol (top), 5 pmol (middle), and 50 pmol (bottom) recombinant
704 TrcP. Oligo-protected portions were incubated with acrylonitrile, which specifically cyanoethylates (CE)
705 pseudouridine, increasing its mass by 53 Da. *m/z* values and assigned fragment sequences are shown.

706 **B.** Sanger sequence of tRNA-Tyr cDNA from RNA isolated from WT (Top), ΔmiaA (second top), ΔmiaB
707 (middle), ΔmiaE (second bottom), and *tgt::Tn* (bottom) strains.

708 **C.** Sanger sequence of cDNA of tRNA-Tyr isolated from a ΔtrcP strain (left) or a ΔmiaB strain (right).
709 tRNAs were incubated with 0 pmol, 1 pmol, and 10 pmol TrcP.

710 **D.** Sanger sequence of tRNA-Tyr cDNA from RNA isolated from the WT strain cultured with or without
711 dipyriddy (dip) at the indicated concentrations.

712 **E.** Nucleoside analysis of tRNA-Tyr from WT, ΔtrcP and ΔmiaB strains. The left panel shows log phase
713 results, and the right panel shows stationary phase results. The detected nucleosides are shown on the
714 right. The signal intensity is normalized with m^5U signals in each sample.

715 **F.** Schematic of proposed interdependency network of modifications in the tRNA-Tyr anticodon loop. In
716 WT under iron replete conditions, ms^2io^6A37 facilitates C-to- Ψ editing at position 32, which in turn
717 suppresses Q formation at position 34 (Left). In contrast, the absence of *miaB* or low iron conditions
718 eliminate methyl-thio modification at position 37, suppressing C-to- Ψ editing and thereby relieving
719 inhibition of Q34 biogenesis (Right).

720

721 **Figure 6. C-to- Ψ editing facilitates Tyr decoding.**

722 **A.** Schematic of reporter construct for measuring decoding ability of tRNA-Tyr.

723 **B.** Reporter assays evaluating decoding of Tyr codons. $\log_2(Rt/Rs)$ value (see methods) is indicated.

724 The left and right panels show decoding efficiency of UAU and UAC codons, respectively. The strains
725 analyzed are shown below.

726

727 **Figure 7. Model of TrcP mediated C-to- Ψ editing.**

728 The anticodon loop of tRNA-Tyr locates in the catalytic pocket of the CDA (light blue) domain and
729 undergoes C-to-U conversion. Then the anticodon moves to the PUS domain (orange) and undergoes
730 U-to- Ψ conversion. During both reactions, the positively charged patch (dark blue) in the LHL domain
731 (green) supports the reaction by binding to upper part of the tRNA. The LHL domain appears to enable
732 the enzyme to adopt a substrate channeling mechanism to carry out its consecutive deamination and
733 pseudouridylation reactions.

734

735 **Supplementary Information**

736 **Figure. S1 Sequence motifs in deaminases.**

737 **Figure. S2 Purified recombinant proteins**

738 **Figure. S3 Phylogenetic tree with the distribution of TrcP homologs**

739 **Figure. S4 Quality scores of structural predictions by ColabFold**

740 **Figure. S5 Comparison between the predicted structures of TrcP and Blasticidin-S deaminase**
741 **(BSD).**

742 **Figure. S6 Expression levels of mutant TrcP proteins in strains used for *in vivo* complementation**
743 **assays.**

744 **Figure. S7 Secondary structures of tRNA-Tyr mutants tested in Figure. 4D**

745 **Figure. S8 Biosynthesis of ms^2io^6A (A) and Q (B)**

746

747 **Supplementary Data 1 Structural homology search using full-length TrcP as a query**

748 **Supplementary Data 2 Structural homology search using the TrcP-CDA domain as a query**

749 **Supplementary Data 3 Strain list**

750 **Supplementary Data 4 Plasmid list**

751 **Supplementary Data 5 Primer list**

752 **Supplementary Data 6 Parameters of mass spectrometry for dynamic MRM analyses**

753 **Supplementary Data 7 A result of Blast search using the TrcP-NTD as a query**

754 **Supplementary Data 8 Phylogenetic distribution of TrcP-NTD homologs**

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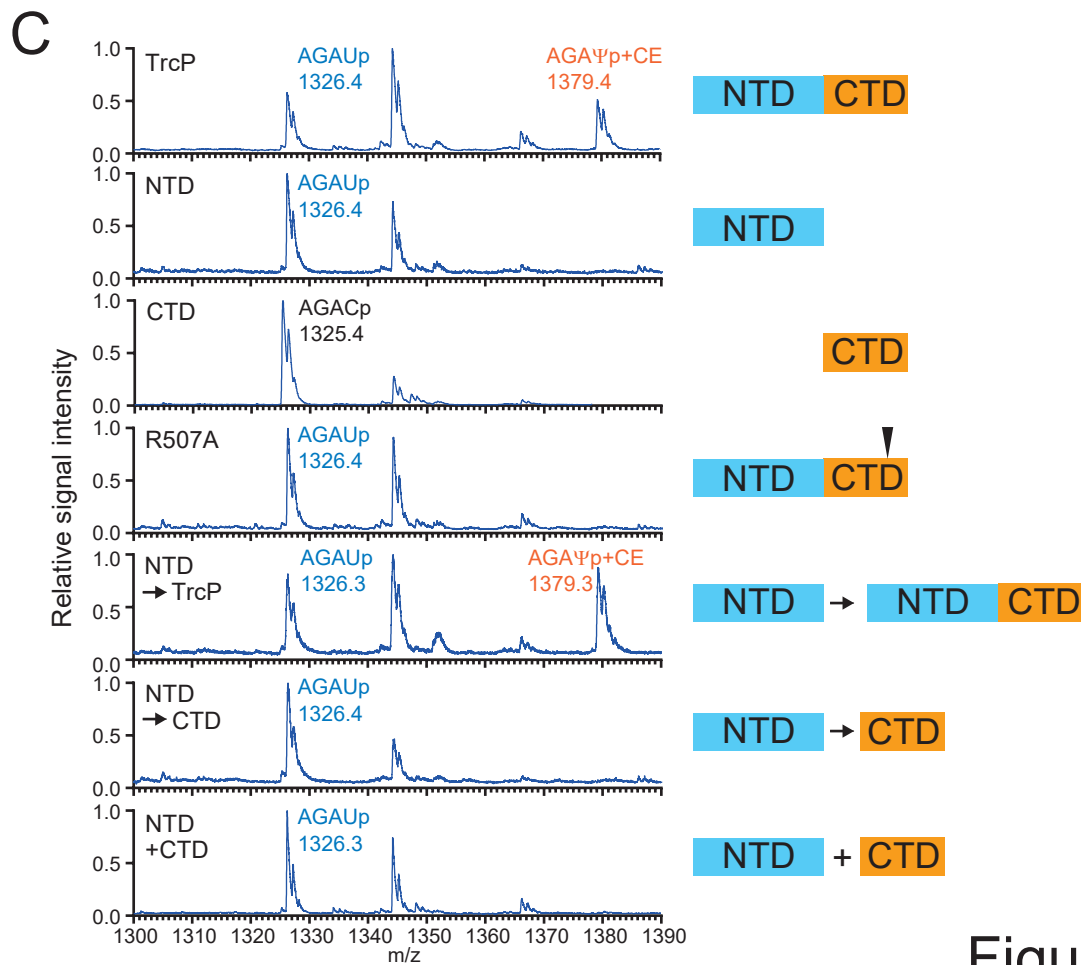
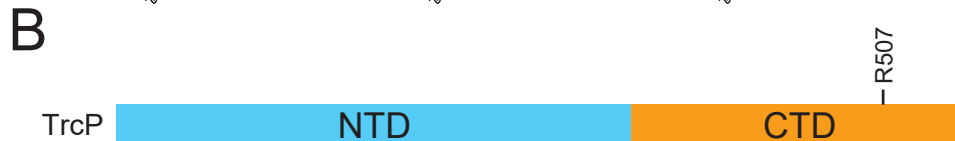
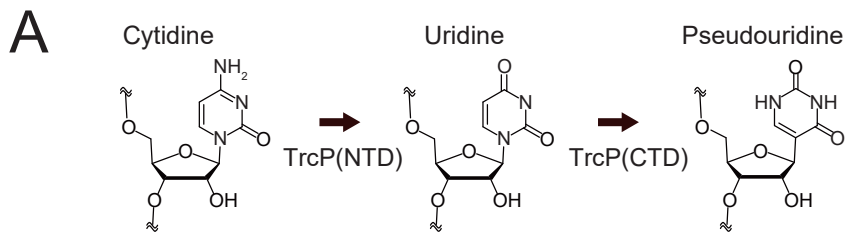


Figure 2

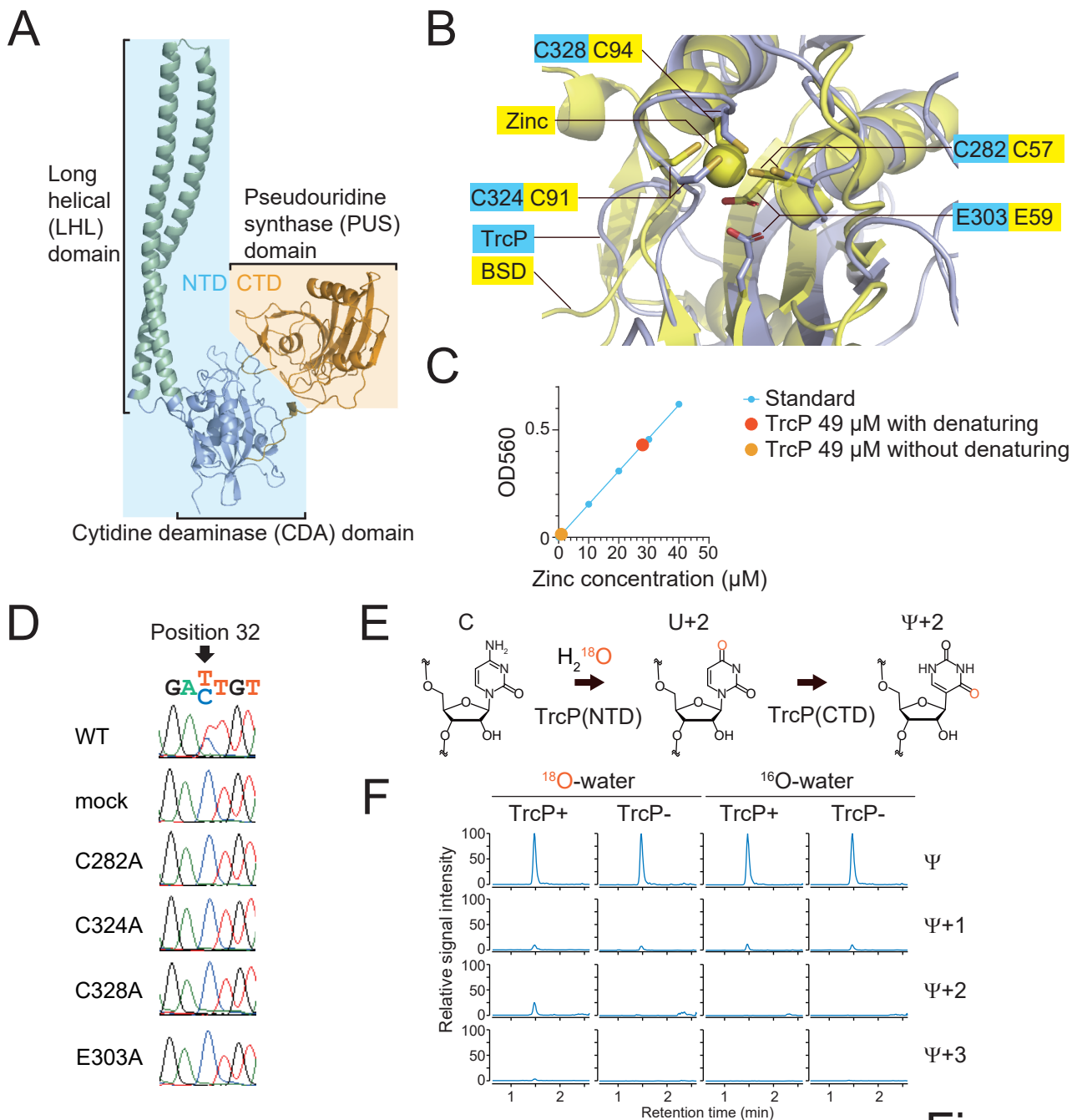


Figure 3

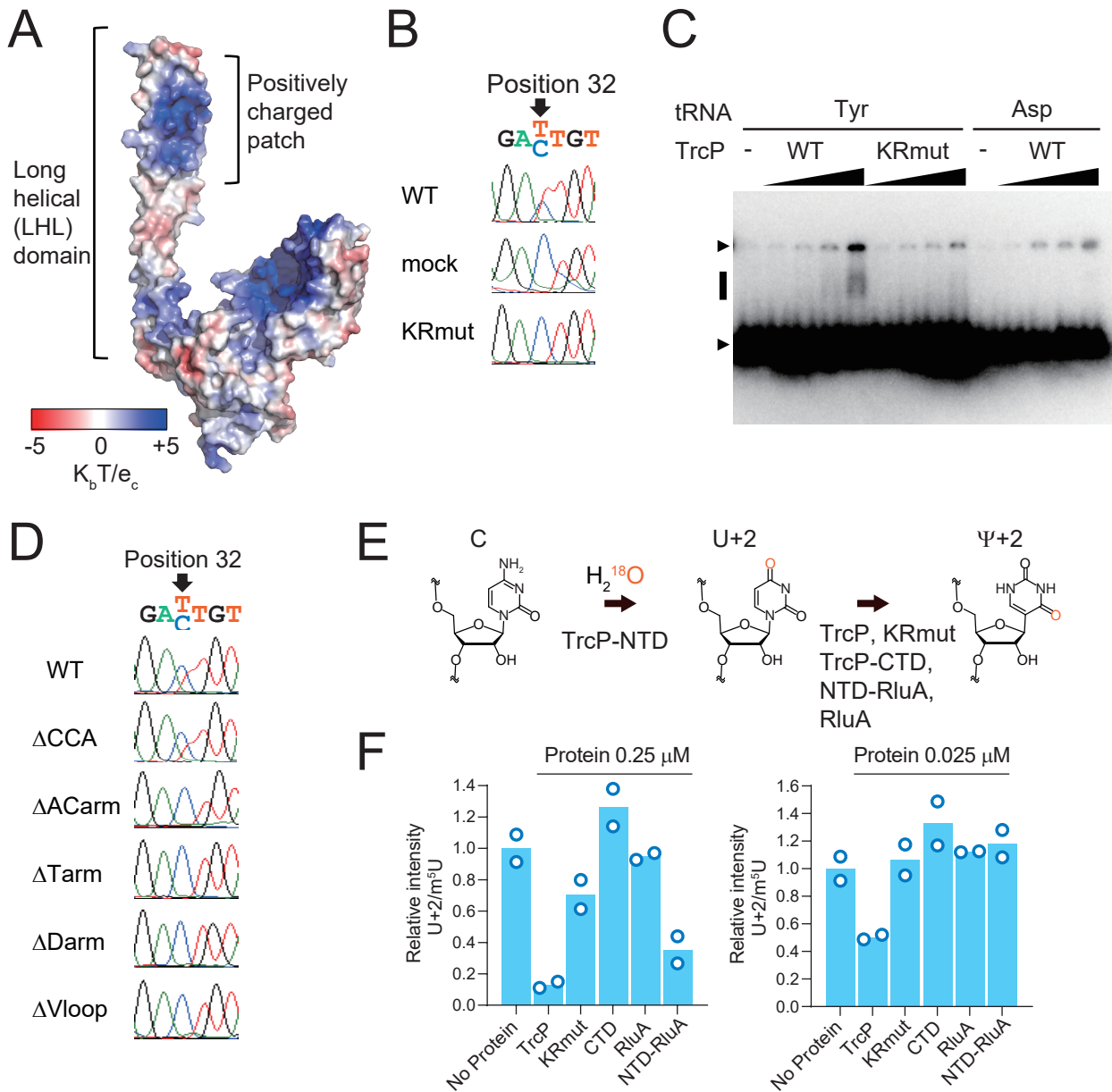


Figure 4

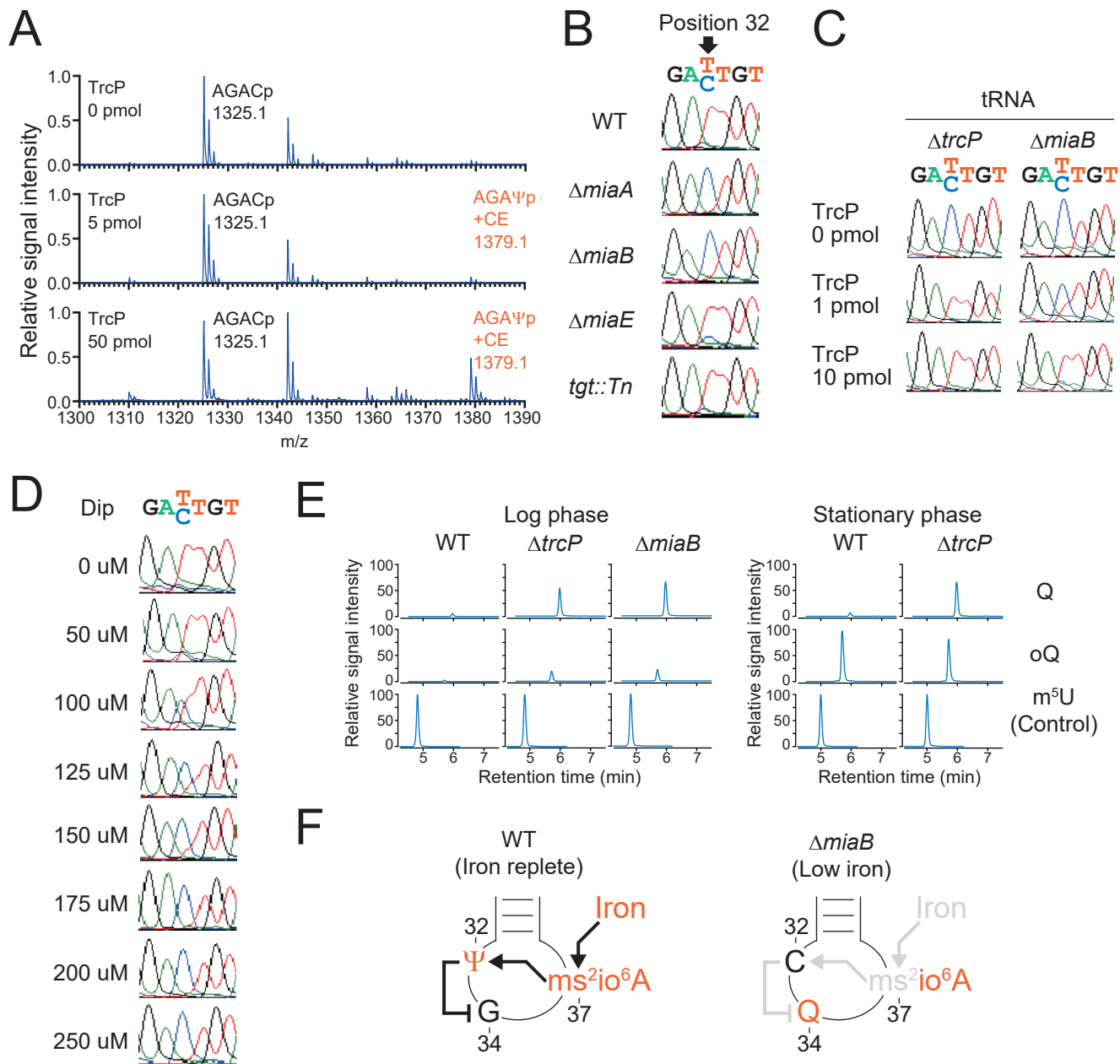


Figure 5

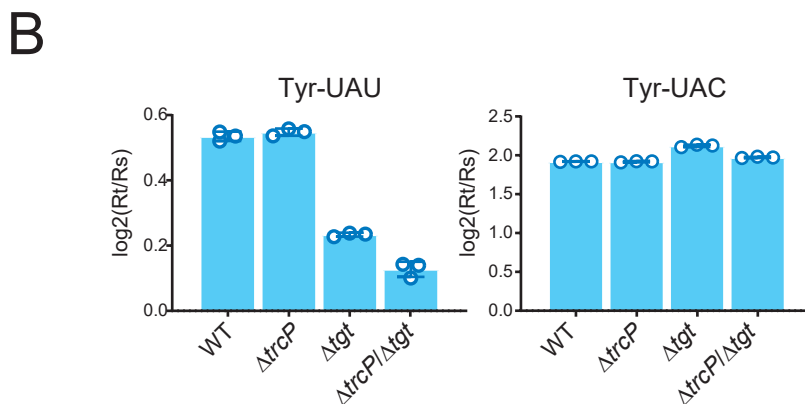
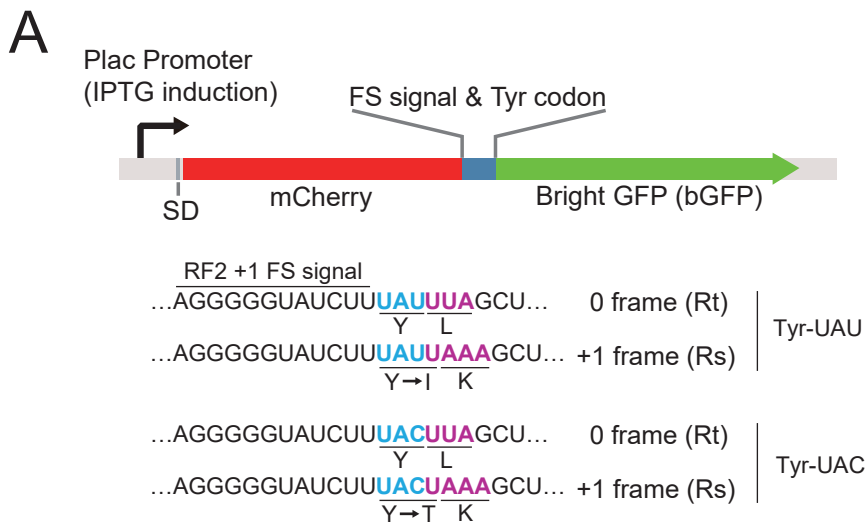


Figure 6

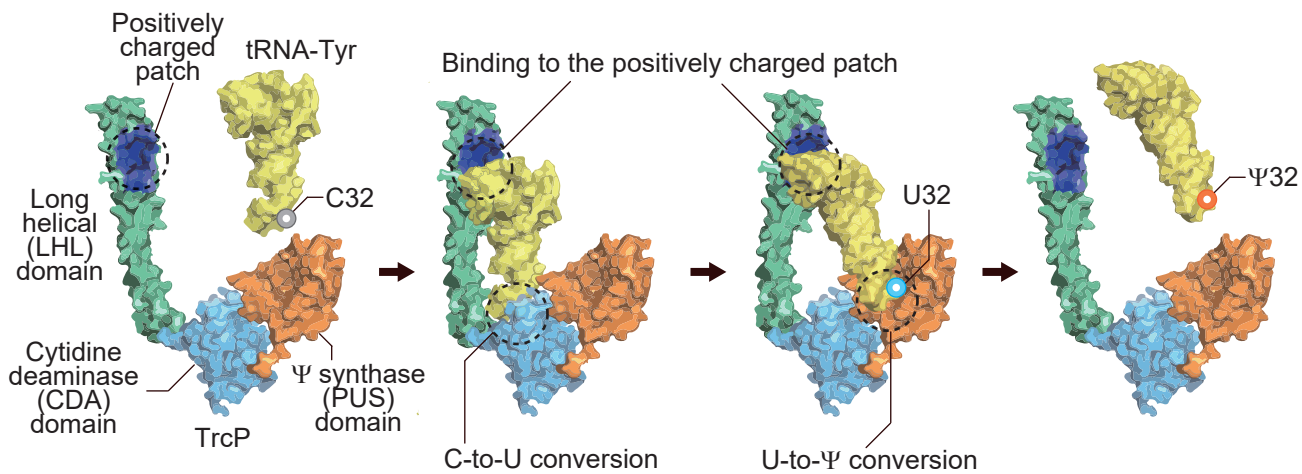


Figure 7