D-amino acid-mediated translation arrest is modulated by the identity of the incoming aminoacyl-tRNA

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11 Abstract

12 A complete understanding of the determinants that restrict D-amino acid incorporation by
13 the ribosome, which is of interest to both basic biologists as well as the protein engineering
14 community, remains elusive. Previously, we demonstrated that D-amino acids are successfully
15 incorporated into the C-terminus of the nascent polypeptide chain. Ribosomes carrying the
16 resulting peptidyl-D-aminoacyl-tRNA (peptidyl-D-aa-tRNA) donor substrate, however, partition
17 into subpopulations that either undergo translation arrest through inactivation of the ribosomal
18 peptidyl-transferase center (PTC) or remain translationally competent. The proportion of each
19 subpopulation is determined by the identity of the D-amino acid sidechain. Here, we demonstrate
20 that the identity of the aminoacyl-tRNA (aa-tRNA) acceptor substrate that is delivered to
21 ribosomes carrying a peptidyl-D-aa-tRNA donor further modulates this partitioning. Our discovery
22 demonstrates that it is the pairing of the peptidyl-D-aa-tRNA donor and the aa-tRNA acceptor that
23 determines the activity of the PTC. Moreover, we provide evidence that both the amino acid and
24 tRNA components of the aa-tRNA donor contribute synergistically to the extent of arrest. The
25 results of this work deepen our understanding of the mechanism of D-amino acid-mediated
26 translation arrest and how cells avoid this precarious obstacle, reveal similarities to other
27 translation arrest mechanisms involving the PTC, and provide a new route for improving the yields
28 of engineered proteins containing D-amino acids.

29 Peptide bond formation is catalyzed by the ribosomal peptidyl-transferase center (PTC).
30 This reaction involves transfer of the nascent polypeptide chain from the peptidyl-tRNA donor in
31 the ribosomal peptidyl-tRNA binding (P) site to the aminoacyl-tRNA (aa-tRNA) acceptor in the
32 ribosomal aa-tRNA binding (A) site (Fig. 1A). Although the PTC has evolved to catalyze peptide
33 bond formation with a remarkable diversity of proteinogenic amino acids, these substrates are
34 exclusively L enantiomers or achiral. Nonetheless, D-amino acids can be found in the cell at high
35 concentrations[1]. Moreover, it is well-established that at least some aa-tRNA synthetase enzymes
36 are capable of aminoacylating D-amino acids onto tRNAs[2,3], suggesting the possibility that the
37 translation machinery (TM) may have to contend with D-aa-tRNAs *in vivo*. Consistent with this
38 possibility, a D-aa-tRNA deacylase enzyme has been identified that is nearly universally
39 conserved and serves to remove D-amino acids from the tRNAs onto which they have been
40 misacylated[4]. Although these observations suggest that D-aa-tRNAs might present a challenge to
41 the TM, how D-aa-tRNAs that are successfully delivered to the translating ribosome impact protein
42 synthesis remains poorly understood, with reported D-amino acid incorporation efficiencies
43 ranging anywhere from zero to 40% or higher for incorporation of a single D-amino acid[3,5–7].
44 Nonetheless, continued efforts to develop a complete mechanistic understanding of how D-aa-
45 tRNAs impede translation and/or how the TM might be engineered to facilitate the incorporation
46 of D-aa-tRNAs are important for elucidating the limits of basic biology and enabling new directions
47 in protein engineering[5,8].

48 Using a highly purified, *Escherichia coli*-based, *in vitro* translation system devoid of D-aa-
49 tRNA deacylase and a series of biochemical assays reporting on the major steps of the translation
50 elongation cycle (Fig. 1A), we recently published a mechanistic study of D-amino acid
51 incorporation by the TM[9]. The results of this study demonstrated that D-aa-tRNA acceptors in
52 complex with the GTP bound-form of elongation factor (EF)-Tu (*i.e.*, EF-Tu(GTP)D-aa-tRNA
53 complexes) can be delivered to the A site and participate in peptide bond formation with yields
54 mirroring those obtained from delivery of the corresponding L-aa-tRNA acceptors. Nonetheless,

55 EF-G(GTP)-catalyzed translocation of the resulting peptidyl-D-aa-tRNA from the A- to P sites
56 resulted in ribosomal elongation complexes (ECs) that partition into two subpopulations. One
57 subpopulation is unable to undergo peptidyl transfer with the next aa-tRNA acceptor and becomes
58 translationally arrested. The second subpopulation can undergo peptidyl transfer with the next
59 aa-tRNA acceptor and remains translationally competent. Remarkably, the fraction of ECs that
60 undergo translation arrest or remain translationally competent is dependent on the side chain of
61 the D-amino acid, suggesting that the observed arrest arises from a D-amino acid side chain-
62 dependent stabilization of an inactive conformation of the PTC. This conclusion was supported
63 by chemical probing experiments and molecular dynamics simulations indicating that the peptidyl-
64 D-aa-tRNA donor stabilizes conformations of 23S ribosomal RNA nucleotides A2058, A2059,
65 A2062, A2063, G2505, and U2506 that are different from those which the corresponding peptidyl-
66 L-aa-tRNA donor stabilizes. These nucleotides comprise part of the ribosomal nascent
67 polypeptide exit tunnel entrance (ETE), a region of the ribosome that evidence suggests is
68 conformationally and functionally coupled to the PTC[10].

69 A striking feature of the study described above is that several of the nucleotides we
70 identified are also implicated in macrolide[11]-, chloramphenicol[12]-, and nascent polypeptide-
71 mediated translation arrest mechanisms[13,14]. Perhaps most interestingly, a growing number of
72 studies are finding that the extent of arrest in these mechanisms is modulated by the identity of
73 the peptidyl-aa-tRNA donor and aa-tRNA acceptor pair in the P- and A sites of the arrested EC[15–
74 17]. Motivated by these studies, we hypothesized that such a 'pairing effect' may likewise modulate
75 the extent of D-amino acid-mediated translation arrest. To test this hypothesis, we have
76 investigated whether and how the identity of the aa-tRNA acceptor can modulate the fraction of
77 ECs carrying a peptidyl-D-aa-tRNA donor that undergo translation arrest or remain translationally
78 competent.

79 Using our previously described *in vitro* translation system[9], we began our investigation by
80 performing fMet-L-Lys-X and fMet-D-Lys-X tripeptide synthesis reactions in which the identity of

81 the third amino acid, denoted by the 'X', was varied by delivering EF-Tu(GTP)aa-tRNAs formed
82 using one of four different aa-tRNA acceptors (Supporting Materials and Methods and Figs. S1
83 and 1B). The choice of a peptidyl-D-Lys-tRNA^{Lys} donor in these experiments was driven by our
84 previous demonstration that the fraction of ECs that undergo translation arrest is closest to 50 %
85 when a peptidyl-D-Lys-tRNA^{Lys} donor is paired with a Phe-tRNA^{Phe} acceptor[9]. We therefore
86 reasoned that using the peptidyl-D-Lys-tRNA^{Lys} donor and Phe-tRNA^{Phe} acceptor pairing as a
87 reference would allow us to most easily detect both increases and decreases in the fraction of
88 ECs that undergo translation arrest. For the aa-tRNA acceptors, we chose aa-tRNAs in which the
89 amino acids differed by hydrophobicity (Phe-tRNA^{Phe}), electrostatics (Arg-tRNA^{Arg} and Glu-
90 tRNA^{Glu}), and beta-branching (Val-tRNA^{Val}). To validate our experimental system, we performed
91 an initial pair of tripeptide synthesis reactions using Phe-tRNA^{Phe} as the aa-tRNA acceptor (Figs.
92 S2 and 2). The fMet-L-Lys-Phe and fMet-D-Lys-Phe tripeptide synthesis reactions exhibited end-
93 point yields of 89 ± 1 % and 64 ± 1 %, respectively, in excellent agreement with our previous
94 observations of 80 ± 1 % and 58 ± 5 %, respectively[9].

95 Subsequently, we performed tripeptide synthesis reactions using Arg-tRNA^{Arg} as the aa-
96 tRNA acceptor (Figs. S2 and 2). In this case, the fMet-L-Lys-Arg and fMet-D-Lys-Arg tripeptide
97 synthesis reactions exhibited end-point yields of 83 ± 1 % and 5 ± 2 %, respectively. The latter
98 result stands in stark contrast to the end-point yield of 64 ± 1 % observed in the fMet-D-Lys-Phe
99 tripeptide synthesis reaction, revealing that the fraction of ECs that undergo D-amino acid-
100 mediated translation arrest depends on the identity of the aa-tRNA acceptor.

101 Given the striking effect that we observed when the aa-tRNA acceptor carried a positively
102 charged amino acid, we used the next tripeptide synthesis reactions to test the performance of
103 an aa-tRNA acceptor carrying a negatively charged amino acid, Glu-tRNA^{Glu} (Figs. S2 and 2).
104 Here, we observed end-point yields of 92 ± 1 % and 19 ± 2 % for the synthesis of the fMet-L-Lys-
105 Glu and fMet-D-Lys-Glu tripeptides, respectively. The data we have obtained here using aa-tRNA

106 acceptors carrying charged amino acids are notably similar to our previous findings in which
107 delivery of a Lys-tRNA^{Lys} acceptor to an EC carrying either fMet-D-Phe-tRNA^{Phe} or fMet-D-Val-
108 tRNA^{Val} donors results in 18 ± 3 % and 11 ± 1 % of the ECs undergoing translation arrest,
109 respectively[9]. Collectively, our results strongly suggest that charged aa-tRNA acceptors
110 exacerbate D-amino acid mediated translation arrest.

111 We next performed tripeptide synthesis reactions in which Val-tRNA^{Val} served as the
112 incoming aa-tRNA (Figs. S2 and 2). Surprisingly, the fMet-L-Lys-Val and fMet-D-Lys-Val tripeptide
113 synthesis reactions exhibited end-point yields of 88 ± 6 % and 97 ± 5 %, respectively. This result
114 again highlights the ability of the aa-tRNA acceptor to modulate the extent of D-amino acid-
115 mediated translation arrest. Perhaps most interestingly, these findings demonstrate that, by
116 judiciously choosing the identity of the aa-tRNA acceptor, it is possible to completely bypass D-
117 amino acid-mediated translation arrest and incorporate a D-amino acid into a ribosome-
118 synthesized protein or polypeptide as effectively as the corresponding L-amino acid can be
119 incorporated.

120 The ability of the aa-tRNA acceptor to modulate the extent of D-amino acid-mediated
121 translation arrest can originate from either its amino acid- or tRNA component. In order to dissect
122 the contributions of these components, we misacylated phenylalanine onto tRNA^{Glu} to generate
123 Phe-tRNA^{Glu} (Supporting Materials and Methods and Fig. S1)[9,18] and used it as the aa-tRNA
124 acceptor in the next tripeptide synthesis reactions (Figs. S2 and 3). If the phenylalanine
125 component is the major contributor, then we would expect an fMet-D-Lys-Phe tripeptide end-point
126 yield that is similar or identical to that obtained upon delivery of Phe-tRNA^{Phe} (*i.e.*, 64 ± 1 %). In
127 contrast, if the tRNA^{Glu} component is the major contributor, then we would expect an fMet-D-Lys-
128 Phe tripeptide end-point yield that is similar or identical to that obtained upon delivery of Glu-
129 tRNA^{Glu} (*i.e.*, 19 ± 2 %). Contrary to either expectation, fMet-L-Lys-Phe and fMet-D-Lys-Phe
130 tripeptide synthesis reactions using Phe-tRNA^{Glu} as the aa-tRNA acceptor exhibited end-point

131 yields of 95 ± 3 % and 95 ± 2 %, respectively. These results demonstrate that both the amino acid
132 and tRNA[19] components of the aa-tRNA acceptor contribute to modulating the extent of D-amino
133 acid-mediated translation arrest and do so synergistically.

134 In this communication, we demonstrate that the identity of the incoming aa-tRNA acceptor
135 can modulate the fraction of ECs carrying a peptidyl-D-aa-tRNA donor that undergo D-amino acid-
136 mediated translation arrest. In addition, we show that the amino acid- and tRNA components of
137 the incoming aa-tRNA contribute synergistically to modulating the extent of translation arrest.
138 Extending our previously proposed model for D-amino acid-mediated translation arrest[9], our data
139 strongly suggests that translocation of the peptidyl-D-aa-tRNA into the P site alters the
140 conformational dynamics of the PTC. Specifically, the peptidyl-D-aa-tRNA likely limits the
141 conformational space that the PTC can sample and, to an extent that depends on the identity of
142 the D-amino acid side chain, predisposes the PTC to stably adopt an inactive conformation. The
143 incoming aa-tRNA acceptor can then further modulate the dynamics of the PTC, biasing the PTC
144 towards stably adopting an inactive conformation that induces translation arrest or an active
145 conformation that enables continued translation. The ability of a particular aa-tRNA acceptor to
146 bias the PTC towards stably adopting an inactive conformation establishes the fraction of ECs
147 that will undergo translation arrest. Thus, the extent of D-amino acid-mediated translation arrest
148 is not determined solely by the side chain of the D-amino acid, as we originally proposed, but
149 rather by the identity of a particular peptidyl-D-aa-tRNA donor and aa-tRNA acceptor pair (Fig. 4).

150 Previously, we have demonstrated that translocation of a peptidyl-D-aa-tRNA into the P
151 site allosterically induces a conformational change of 23S rRNA nucleotides that comprise the
152 ETE. Intriguingly, several other translation arrest mechanisms involving these same ETE
153 nucleotides likewise demonstrate a pairing effect similar to that described here. For example, in
154 their studies of a nascent polypeptide-mediated translation arrest mechanism involving contacts
155 of the nascent polypeptide with these ETE nucleotides[20], Mankin and co-workers demonstrated
156 that the extent to which they could exacerbate or relieve the corresponding nascent polypeptide-

157 mediated translation arrest depended on the identity of the aa-tRNA acceptor[16]. Surprisingly, they
158 observed that Val-tRNA^{Val} and Phe-tRNA^{Phe} acceptors exhibited among the lowest arrest
159 efficiencies, while Arg-tRNA^{Arg} and Glu-tRNA^{Glu} acceptors exhibited among the highest, a trend
160 that is consistent with the trend that we have observed here. Mankin and co-workers have also
161 shown that binding of a macrolide antibiotic to these ETE nucleotides robustly induces translation
162 arrest primarily in the context of specific, problematic peptidyl-aa-tRNA donor and aa-tRNA
163 acceptor pairs[15]. Similar conformational and pairing effects were observed in the context of the
164 PTC-binding antibiotics chloramphenicol and linezolid[11,17]. Thus, our findings contribute to an
165 emerging understanding of a conformational and functional link between the ETE and the PTC,
166 in which translation arrest can be induced by particular peptidyl-aa-tRNA donor and aa-tRNA
167 acceptor pairs. In addition, the results we report here demonstrate the potentially devastating
168 effects that incorporation of a D-amino acid into the nascent polypeptide chain can have on
169 translation and highlight the important role of that D-aa-tRNA deacylase enzymes play in
170 protecting ECs under stress conditions in which D-amino acids may be misacylated onto tRNAs
171 and delivered to ECs[21]. Finally, our results reveal a straightforward approach for improving the
172 efficiency with which D-amino acids can be incorporated into proteins or polypeptides for protein
173 engineering applications: By simply optimizing the pairing of peptidyl-D-aa-tRNA donors and aa-
174 tRNA acceptors, the efficiency of incorporation can be improved dramatically without the need for
175 complex engineering of the TM.

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Author Contributions

R.C.F. and R.L.G. designed the research; R.C.F. performed the experiments and analyzed the data; R.C.F. and R.L.G. interpreted the results. R.C.F. and R.L.G. wrote the manuscript; all three authors approved the final manuscript.

Competing Financial Interests

The authors declare no competing financial interests.

243 **Figure Legends**

244 **Figure 1. The experimental framework used to examine the role of the incoming aa-tRNA**
245 **acceptor in D-amino acid-mediated translation arrest.** (A) The translation elongation cycle
246 begins with delivery of EF-Tu(GTP)aa-tRNA complexes to an EC carrying a peptidyl-tRNA donor
247 at the P site. Following selection of the correct, mRNA-encoded EF-Tu(GTP)aa-tRNA, GTP is
248 hydrolyzed, EF-Tu(GDP) dissociates from the aa-tRNA and the EC, and the aa-tRNA is
249 accommodated into the A site. Upon accommodation, peptide bond formation results in transfer
250 of the nascent polypeptide chain from the peptidyl-tRNA donor at the P site to the aa-tRNA
251 acceptor at the A site. Subsequently, EF-G(GTP) catalyzes translocation of the P- and A-site
252 tRNAs into the ribosomal tRNA exit (E) and P sites, with concomitant movement of the mRNA by
253 one codon. (B) Tripeptide synthesis reactions were performed by delivering a mixture of either
254 EF-Tu(GTP)L-Lys-tRNA^{Lys} (top row) or EF-Tu(GTP)D-Lys-tRNA^{Lys} (bottom row), EF-Tu(GTP)X-
255 tRNA^{X}, and EF-G(GTP) to ribosomal initiation complexes programmed with an mRNA encoding
256 an fMet-Lys-X tripeptide (Table S1) and carrying a P-site f-[^{35}S]-Met-tRNA^{fMet} (Supporting
257 Materials and Methods).
258

259 **Figure 2. The identity of the incoming aa-tRNA acceptor modulates the extent of D-amino**
260 **acid-mediated translation arrest.** (A) Tripeptide synthesis reaction products were collected at
261 reaction time points spanning 0–60 min in order to ensure that the reactions had gone to
262 completion and we were measuring true end-point yields. Products were separated using
263 electrophoretic thin layer chromatography (eTLC) and visualized by phosporimaging (Supporting
264 Materials and Methods). (B) End-point yields of tripeptide synthesis reactions. Reaction time
265 courses were performed in duplicate and in all cases were observed to go to completion by 30
266 min (Fig. S2). Consequently, the duplicate yield measurements collected at the 30 and 60 min
267 time points for each tripeptide synthesis reaction were clustered, averaged, and reported as the

268 mean reaction end-point. Error bars represent the standard deviation of the mean (Supporting
269 Materials and Methods).
270
271 **Figure 3. The amino acid- and tRNA components of the aa-tRNA acceptor contribute**
272 **synergistically to the extent of D-amino acid-mediated translation arrest.** (A) eTLC of the
273 fMet-L-Lys-Phe and fMet-D-Lys-Phe tripeptide synthesis reactions performed using Phe-tRNA^{Glu}
274 as the aa-tRNA acceptor. (B) End-point yields of tripeptide synthesis reactions. Reaction time
275 courses were performed in duplicate and in all cases were observed to go to completion by 30
276 min (Fig. S2). Consequently, the duplicate yield measurements collected at the 30 and 60 min
277 time points for each tripeptide synthesis reaction were clustered, averaged, and reported as the
278 mean reaction end-point. Error bars represent the standard deviation of the mean (Supporting
279 Materials and Methods).
280
281 **Figure 4. Extended mechanistic model for D-amino acid-mediated translation arrest.**
282 Translocation of a peptidyl-D-aa-tRNA donor into the P site predisposes the PTC to stably adopt
283 an inactive conformation. Pairing of a particular peptidyl-D-aa-tRNA donor with an aa-tRNA
284 acceptor that facilitates the ability of the PTC to stably adopt an inactive conformation results in a
285 large fraction of ECs undergoing translation arrest (red aa-tRNA acceptor and pathway), whereas
286 pairing with an aa-tRNA acceptor that interferes with the ability of the PTC to stably adopt an
287 inactive conformation results in a large fraction of ECs remaining translationally competent (green
288 aa-tRNA acceptor and pathway).

289 **Figures**

290 **Figure 1**

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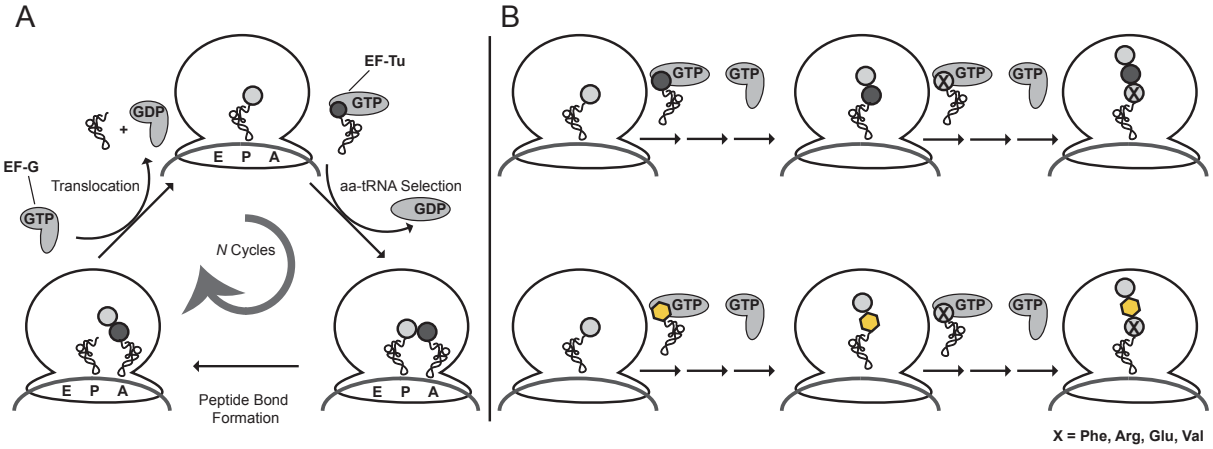

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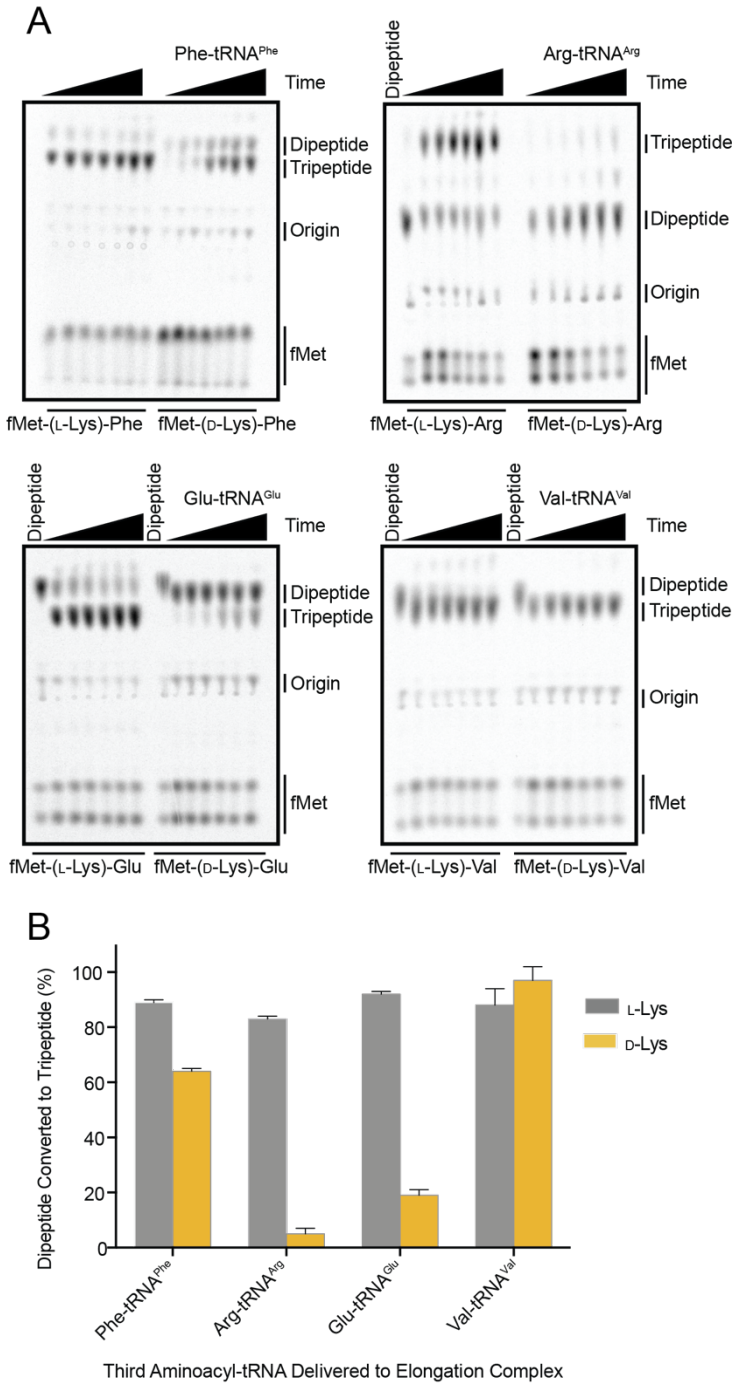

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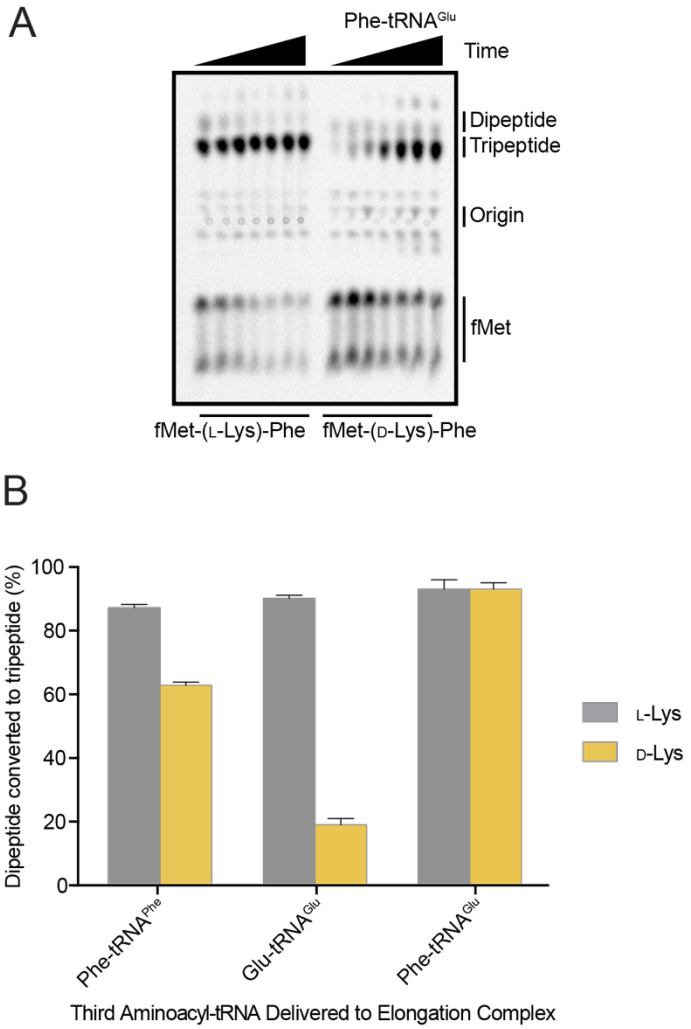
301 **Figure 3**



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306 **Figure 4**

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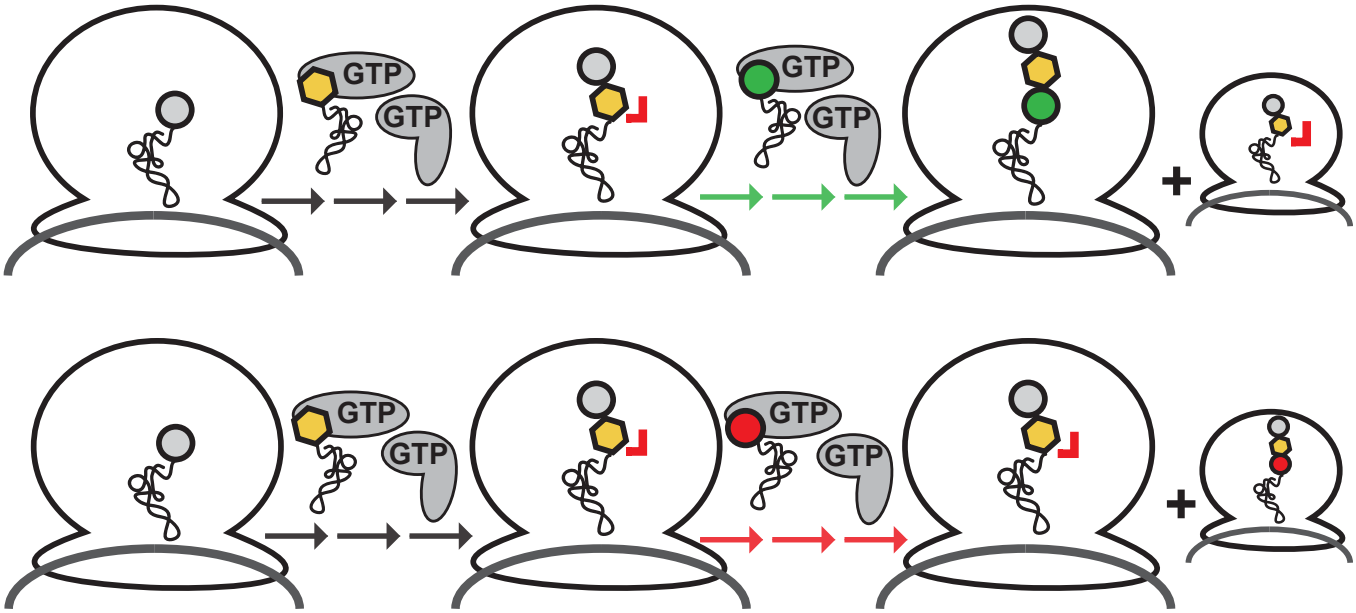

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