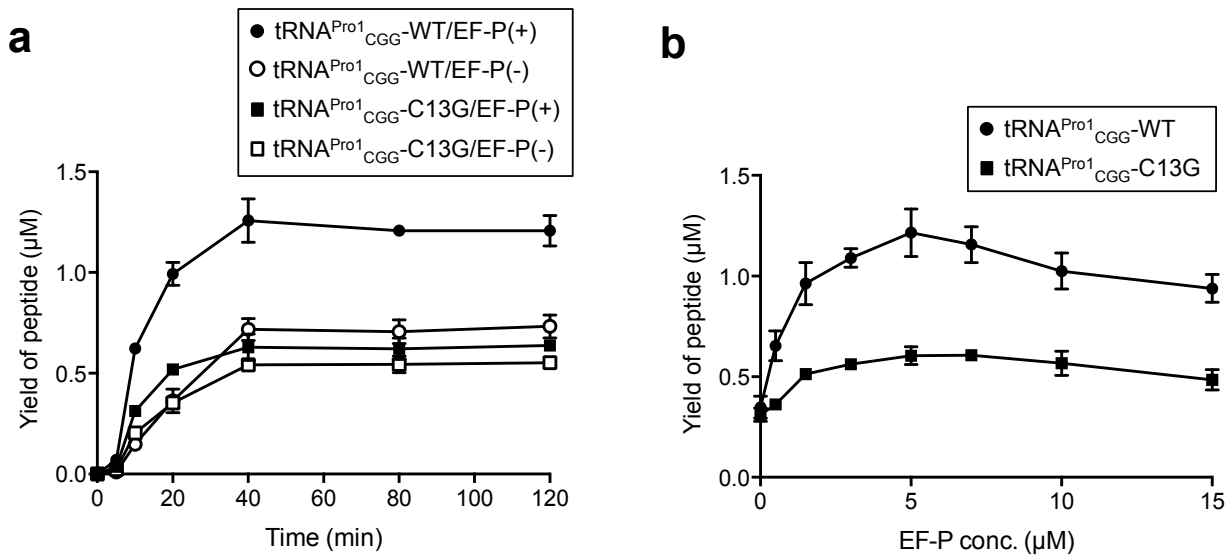
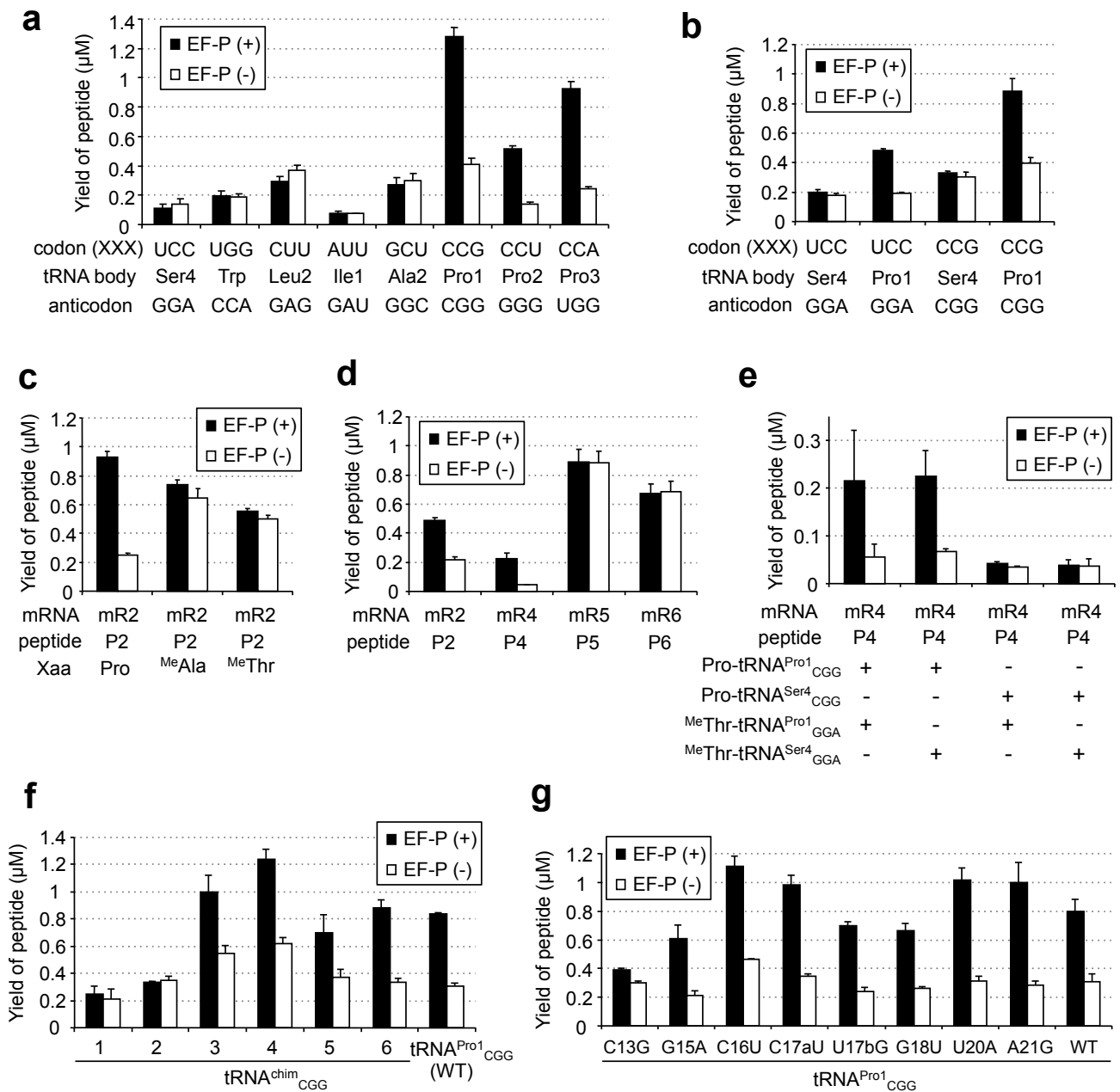


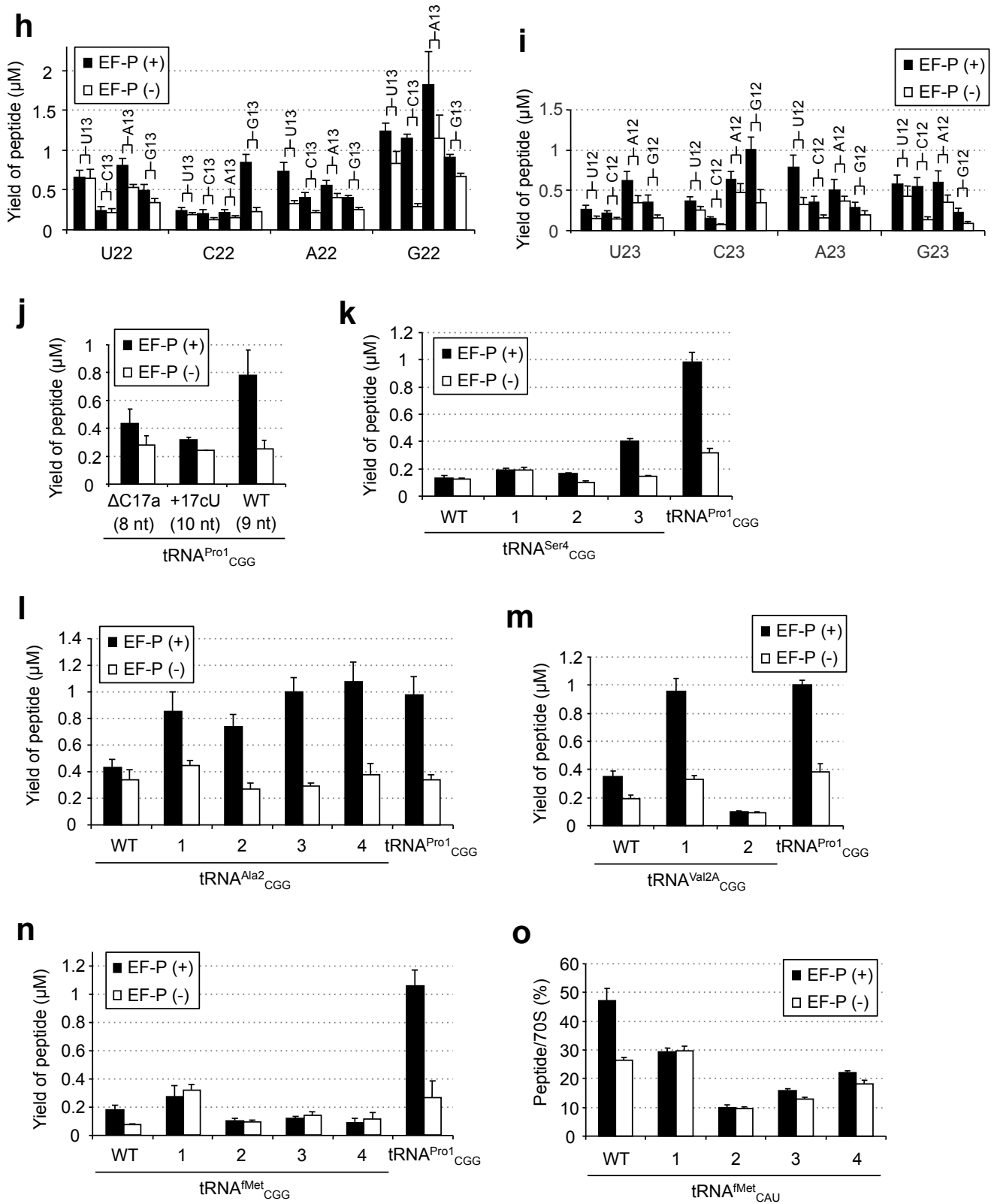
**Supplementary Figure 1 EF-P effect on the synthesis of Pro-containing sequences in the FIT system.** (a) Schematic depiction of peptide translation in the FIT system. For incorporation of Pro, *in vitro* transcribed *E. coli* tRNA<sup>Pro1</sup> was pre-charged with Pro by means of the flexizyme technology and added to the translation system. Other amino acids were charged on natural *E. coli* tRNAs *de novo* by the corresponding aminoacyl-tRNA synthetases (ARSs) present in the FIT system. [<sup>14</sup>C]Asp was used for autoradiographic detection of peptides. (b) Sequences of mRNAs (mR1–3) and the corresponding peptide sequences (P1–3). These mRNAs were transcribed from the corresponding DNA templates by T7 RNA polymerase present in the FIT system. (c) Tricine SDS-PAGE analysis of the peptides (P1–P3) synthesized in the FIT system. Translation was carried out for 20 min at 37°C in a 2.5-μL reaction mixture in the presence (3 μM) or absence of EF-P. (d) Yields of the full-length peptides (P1–3) synthesized by the FIT system. The [<sup>14</sup>C]Asp-labeled peptide in the tricine SDS-PAGE gel was quantified by autoradiography. Numbers above the bars indicate the relative translation yields of the peptides calculated by [EF-P(+)/EF-P(-)]. N.D.: “not detected”. Error bars, s.d. (n = 3).



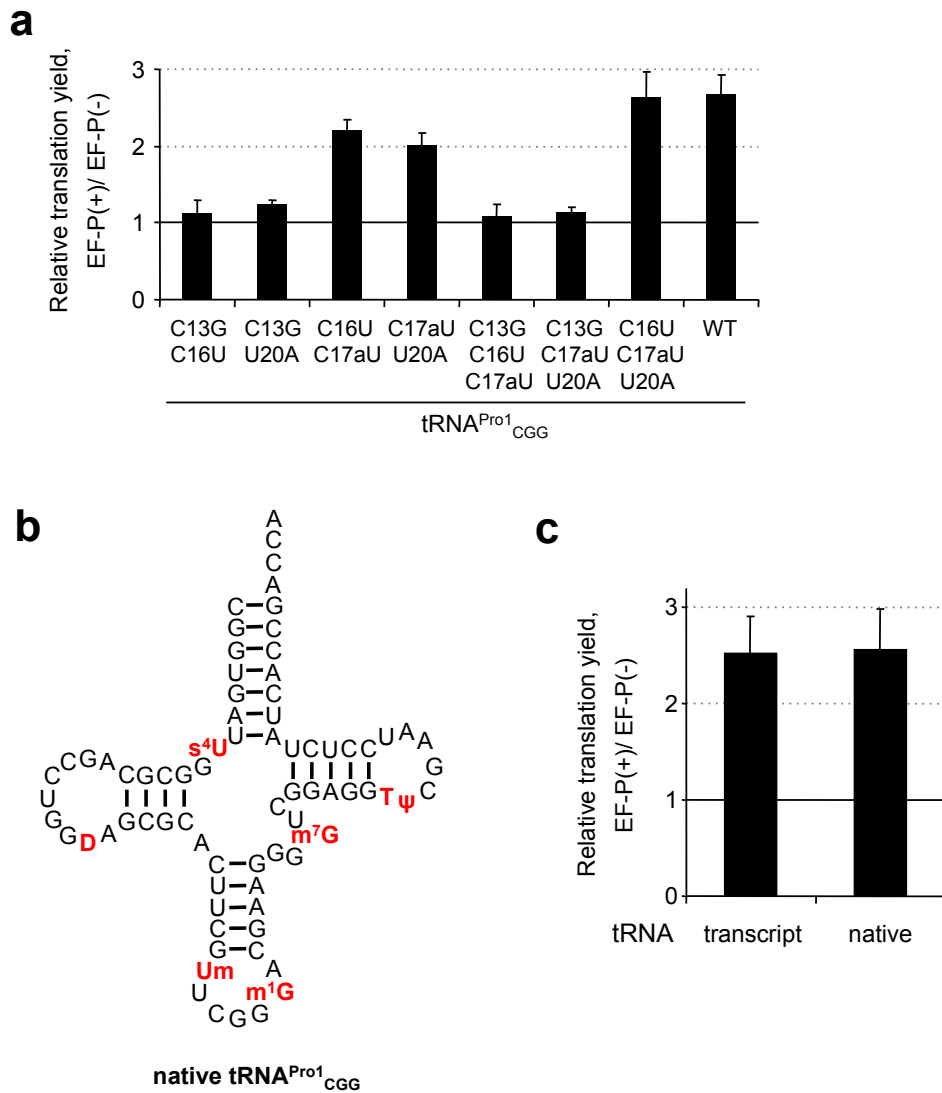
**Supplementary Figure 2 Time-course analysis and titration of EF-P concentration in translation.** (a) Time-course analysis in translation of P2-Pro<sub>2</sub> (Fig. 2a) using wildtype (WT) tRNA<sup>Pro1</sup><sub>CGG</sub> and C13G mutant in the presence (3 μM) and absence of EF-P. Error bars, s.d. (n = 3). (b) Titration of EF-P concentration in P2-Pro<sub>2</sub> (Fig. 2a) translation using the wildtype (WT) tRNA<sup>Pro1</sup><sub>CGG</sub> and C13G mutant for Pro incorporation. Translation time is 20 min. Error bars, s.d. (n = 3).



**Supplementary Figure 3 Quantification of absolute translation yields of peptides.** (a-n) Absolute translation yields of the peptides obtained in the experiments of Fig. 2b (a), Fig. 2c (b), Fig. 3b (c), Fig. 3d (d), Fig. 3e (e), Fig. 4b (f), Fig. 4c (g), Fig. 4d (h), Fig. 4e (i), Fig. 4g (j), Fig. 5b (k), Fig. 5d (l), Fig. 5f (m) and Fig. 6b (n). Translation products of each experiment were separated by 15% tricine SDS-PAGE, and the corresponding peptide labeled with [<sup>14</sup>C]Asp were quantified by autoradiography. Black and white bars indicate results of translation with and without EF-P, respectively. Error bars, s.d. (n = 3). (o) Yields of f<sup>35</sup>S-Met-puromycin obtained in the experiment of Fig. 6d were analyzed by liquid scintillation counting. Black and white bars indicate results of reaction with and without EF-P, respectively. Error bars, s.d. (n = 3).



Supplementary Figure 3 (continued)



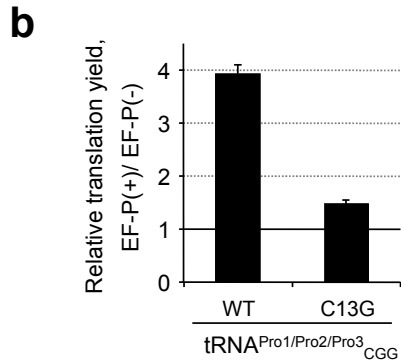
**Supplementary Figure 4 Effects of multiple point mutations and nucleotide modifications.** (a) Effect of double or triple point mutations introduced into tRNA<sup>Pro1</sup><sub>CGG</sub> on incorporation of Pro. Relative translation yields were analyzed using mR2-CCG<sub>2</sub> mRNA (Fig. 2a). Error bars, s.d. (n = 3). (b) Secondary structure of native tRNA<sup>Pro1</sup><sub>CGG</sub> with nucleotide modifications indicated by red. s<sup>4</sup>U: 4-thiouridine, D: dihydrouridine, Um: 2'-O-methyluridine, m<sup>1</sup>G: 1-methylguanosine, m<sup>7</sup>G: 7-methylguanosine, T: ribothymidine, ψ: pseudouridine (c) Incorporation of [<sup>14</sup>C]Pro at tandem CCG codons of mR2-CCG<sub>2</sub> mRNA using transcript and native tRNA<sup>Pro1/Pro2/Pro3</sup> mixture. Values represent relative translation yields calculated as [EF-P(+)/EF-P(-)]. Error bars, s.d. (n = 3).

**a**

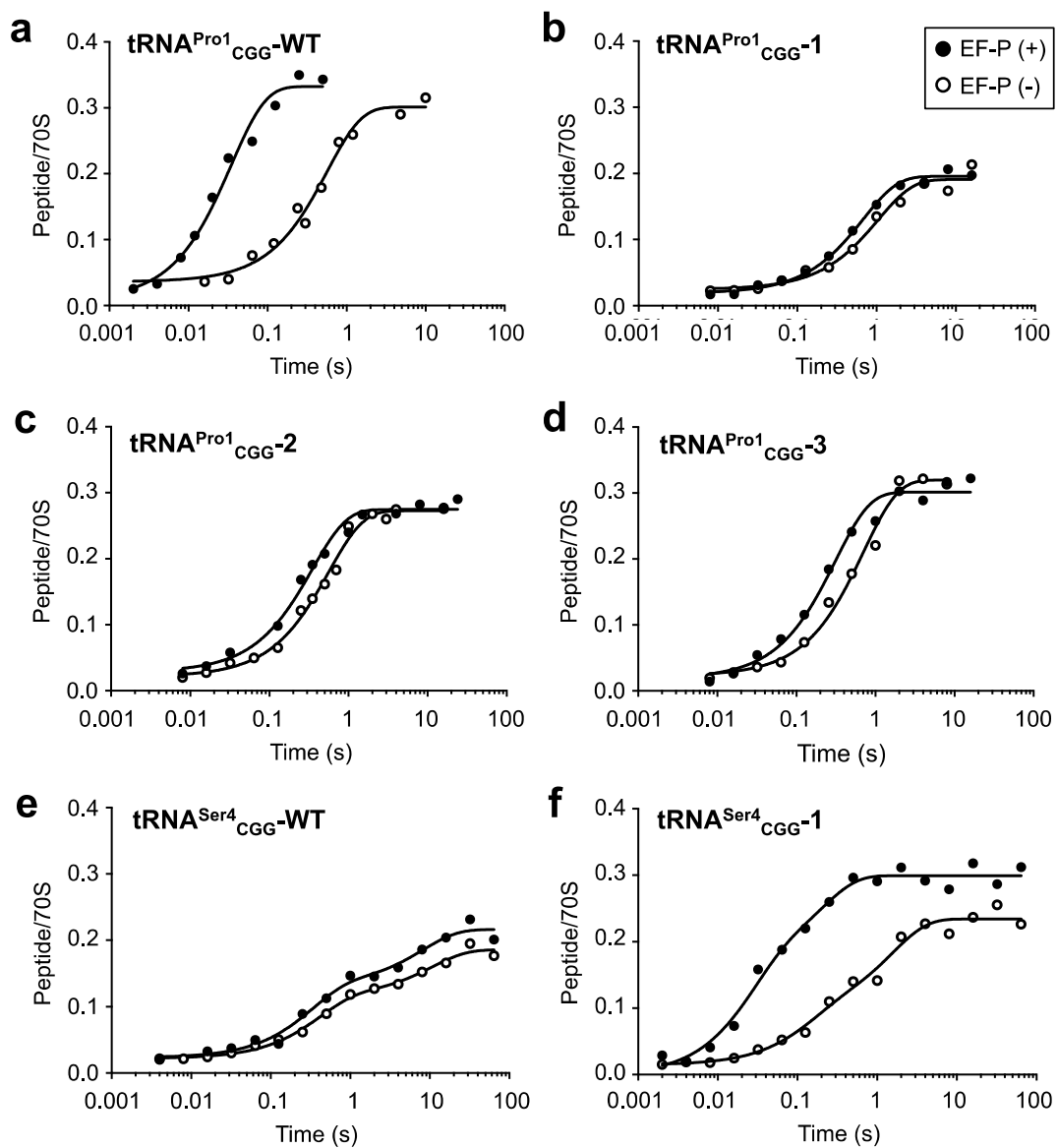
mRNA	AUG	AUA	CAA	<b>CCU</b>	AUU	UCC	GGC	<b>CCU</b>	<b>CCU</b>	<b>CCU</b>	GGG	CAA	<b>CCA</b>	<b>CCA</b>	GGU
FLK-FLAG	fM	I	Q	<b>P</b>	I	S	G	<b>P</b>	<b>P</b>	<b>P</b>	G	Q	<b>P</b>	<b>P</b>	G

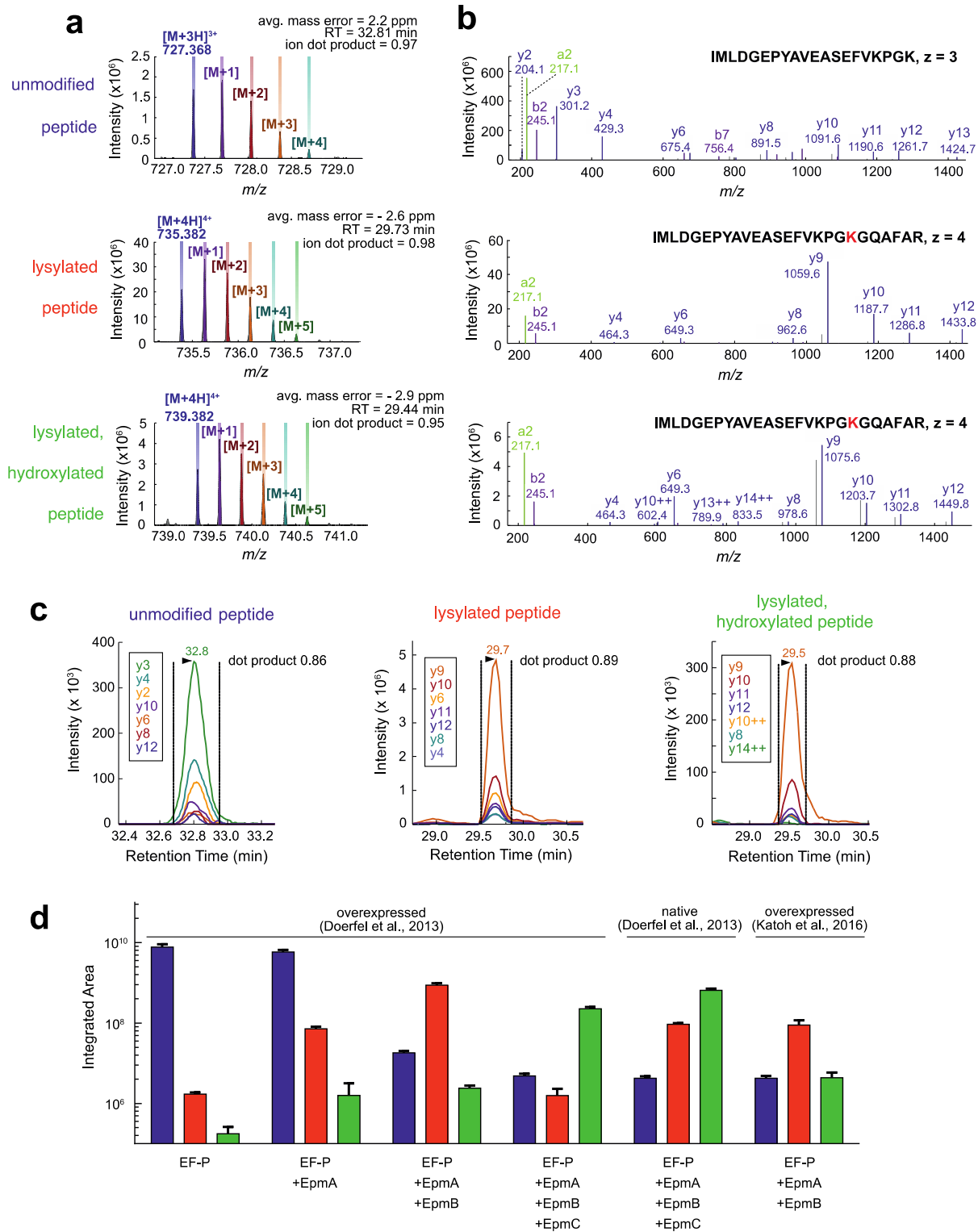
CAG	GGA	GAU	AAU	CUG	GAC	UAC	AAG	GAC	GAC	GAC	GAC	AAG	UAA
Q	G	D	N	L	D	Y	K	D	D	D	D	K	stop



**Supplementary Figure 5 Improvement of translation of FLK-FLAG by EF-P depending on D-arm structure of tRNA<sup>Pro</sup> used for Pro incorporation.** (a) Sequence of mRNA and FLK-FLAG peptide, 20 amino acids from the N terminus of FLK protein followed by FLAG sequence. (b) Improvement of translation yield of FLK-FLAG by EF-P. Pro was pre-charged on wildtype tRNA<sup>Pro1/Pro2/Pro3</sup> mixture or their D-arm mutants (C13G), and used for Pro incorporation in translation of FLK-FLAG. Translation product was separated by 15% tricine SDS-PAGE, and the desired peptide labeled with [<sup>14</sup>C]Asp was quantified by autoradiography. Values represent relative translation yields calculated as [EF-P(+)/EF-P(-)]. Error bars, s.d. (n = 3).



**Supplementary Figure 6 Time courses of peptide bond formation between fMet-Pro and Gly using D-arm variants of  $tRNA^{Pro1}$  and  $tRNA^{Ser4}$ .** The reaction was initiated by mixing posttranslocation complex containing [ $^{14}C$ ]fMet-Pro-tRNA and [ $^3H$ ]Gly-tRNA<sup>Gly3</sup>•EF-Tu•GTP ternary complex, and reaction products were analyzed by HPLC. Posttranslocation complexes were prepared with the 6 different Pro-tRNA variants shown in Fig. 6a,b.



**Supplementary Figure 7 Mass spectrometric analysis of EF-P modification.** (a) Mass spectra of the unmodified ( $z=3$ ;  $m/z=727.3696$  (monoisotopic ion)) and modified peptides ( $z=4$ ; lysylated  $m/z=735.3837$ ; lysylated/hydroxylated  $m/z=739.3825$  (monoisotopic ions)). Series of other isotopic ions are indicated as  $[M+1\sim5]$ . (b) Library MS/MS spectra of the unmodified ( $z=3$ ) and modified peptides ( $z=4$ ). The modified lysine residue is indicated in red in the peptide sequence. (c) Elution profile of parallel reaction monitoring transitions (PRM). Peptides were identified by co-elution of their PRM transitions and comparison to the reference library spectrum (dot product). Example PRM profiles show the modification status of EF-P used in this work. (d) Relative label-free quantification of the EF-P modification status by PRM. Bars represent the sum of 7 PRM transitions. Error bars indicate standard deviations of three independent technical replicates.



	Sequence of D-arm	Loop (nt)	Stem (bp)	12,13/22,23
Ala1B	GCUCAGCUGGGAGAGC	8	4	UC/GA
Ala2	GCUCAGCUGGGAGAGC	8	4	UC/GA
Arg2	GCUCAGCUGGAUAGAGU	9	4	UC/GA
Arg3	GCUCAGCUGGAUAGAGC	9	4	UC/GA
Arg4	GCUCAGUUGGAUAGAGC	9	4	UC/GA
Arg5	GUUAAAUGGAUUAAC	8	4	UA/UA
Asn	GUUCAGUCGGUAGAAC	8	4	UC/GA
Asp	GUUCAGUCGGUUAGAAU	9	4	UC/GA
Cys	ACAAAGCGGUUAUGU	9	3	AA/AU
Gln1	GCCAAGCGGUAAAGGC	9	3	CA/AG
Gln2	GCCAAGCGGUAAAGGC	9	3	CA/AG
Glu	GUCUAGAGGCCAGGAC	9	4	CU/GG
Gly1	GUUCAAUUGGUAGAAC	7	4	UC/GA
Gly2	GUAUAAUGGCUAUUAC	10	3	AU/UU
Gly3	GCUCAGUUGGUAGAGC	8	4	UC/GA
His	GCUCAGUUGGUAGAGC	8	4	UC/GA
Ile1	GCUCAGGUGGUUAGAGC	9	4	UC/GA
Ile2	GCUCAGUGGUUAGAGC	8	4	UC/GA
Leu1	GCGGAAUUGGUAGACGC	11	3	GG/AC
Leu2	GUGGAAUUGGUAGACAC	11	3	GG/AC
Leu3	GCGAAAUUGGUAGACGC	11	3	GA/AC
Leu4	GUGGAAUCGGUAGACAC	11	3	GG/AC
Leu5	GCGAAAUCGGUAGACGC	11	3	GA/AC
Lys	GCUCAGUUGGUAGAGC	8	4	UC/GA
Met	GCUCAGUUGGUUAGAGC	9	4	UC/GA
fMet	GAGCAGCCUGGUAGCUC	9	4	GC/GC
Phe	GCUCAGUCGGUAGAGC	8	4	UC/GA
Pro1	GCGCAGCCUGGUAGCGC	9	4	GC/GC
Pro2	GCGCAGCCUGGUAGCGC	9	4	GC/GC
Pro3	GCGCAGCUUGGUAGCGC	9	4	GC/GC
Sec	UCGUCUCCGGUAGGCGG	4	7	GU/GC
Ser1	GCCGAGCGGUUGAAGGC	11	3	CG/AG
Ser2	CCGAGCGGCUGAACGG	11	3	GG/AC
Ser3	GCCGAGAGGCUGAAGGC	11	3	CG/AG
Ser4	UCCGAGUGGUUGAAGGA	11	3	CG/AG
Ser5	UCCGAGUGGCUGAAGGA	11	3	CG/AG
Thr1	GCUCAGUUGGUAGAGC	8	4	UC/GA
Thr2	GCUCAGUUGGUAGAGC	8	4	UC/GA
Thr3	GCUCAGUUGGUAGAGC	8	4	UC/GA
Thr4	GCUCAGUAGGUAGAGC	8	4	UC/GA
Trp	GUUCAAUUGGUAGAGC	8	4	UC/GA
Tyr1	CCCAGCGGCCAAAGGG	11	3	CG/AG
Tyr2	CCCAGCGGCCAAAGGG	11	3	CG/AG
Val1	GCUCAGCUGGGAGAGC	8	4	UC/GA
Val2A	GCUCAGUUGGUUAGAGC	9	4	UC/GA
Val2B	GCUCAGUUGGUUAGAGC	9	4	UC/GA

**Supplementary Table 1 Comparison of the D-arm sequences of *E. coli* tRNAs.** Watson-Crick base-pairing (A/U and G/C) and wobble base-pairing (G/U) nucleotides are shown in red and blue, respectively. G/U wobble base pairs are also considered as base pairs for counting the length of the D-loop and stem region.

<b>Prokaryote</b>	Sequence of D-arm	Loop (nt)	Stem (bp)	12,13/22,23
<i>Agrobacterium tumefaciens</i> (CGG)	GCGCAGUCUUGGUA <b>GCGC</b>	9	4	GC/GC
<i>Bacillus subtilis</i> (UGG)	GCU <b>C</b> AGCUUGGUA <b>GAGC</b>	9	4	UC/GA
<i>Escherichia coli</i> (CGG, Pro1)	GCGCAGCCUUGGUA <b>GCGC</b>	9	4	GC/GC
<i>Escherichia coli</i> (UGG, Pro3)	GCGCAGCUUGGUA <b>GCGC</b>	9	4	GC/GC
<i>Mycobacterium tuberculosis</i> (CGG)	GCGCAGCUUGGUA <b>GCGC</b>	9	4	GC/GC
<i>Mycoplasma pneumoniae</i> (UGG)	GCU <b>U</b> AGU <b>U</b> UGGUA <b>GAGC</b>	9	4	UU/GA
<i>Prochlorococcus marinus</i> (UGG)	GCGCAGCUUGGUA <b>GCGC</b>	9	4	GC/GC
<i>Pseudomonas aeruginosa</i> (CGG)	GCGCAGCUUGGUA <b>GCGC</b>	9	4	GC/GC
<i>Salmonella typhi</i> (CGG)	GCGCAGCCUUGGUA <b>GCGC</b>	9	4	GC/GC
<i>Streptococcus pneumoniae</i> (UGG)	<b>G</b> CU <b>C</b> AGCUUGGUA <b>GAGU</b>	9	4	UC/GA
<i>Thermus thermophilus</i> (CGG)	GCGCAGCCCGGUA <b>GCGC</b>	9	4	GC/GC
<b>Eukaryote</b>	Sequence of D-arm	Loop (nt)	Stem (bp)	12,13/22,23
<i>Arabidopsis thaliana</i> (CGG)	<b>G</b> UCUAGUGGUA <b>UGAU</b>	9	3	CU/UG
<i>Caenorhabditis elegans</i> (CGG)	<b>G</b> UCUAGAGGUA <b>UGAU</b>	9	3	CU/UG
<i>Drosophila melanogaster</i> (CGG)	<b>G</b> UCUAGAGGUA <b>UGAU</b>	9	3	CU/UG
<i>Homo sapiens</i> (UGG)	<b>G</b> UCUAGGGGUA <b>UGAU</b>	9	3	CU/UG
<i>Homo sapiens</i> (CGG)	<b>G</b> UCUAGGGGUA <b>UGAU</b>	9	3	CU/UG
<i>Mus musculus</i> (CGG)	<b>G</b> UCUAGGGGUA <b>UGAU</b>	9	3	CU/UG
<i>Plasmodium falciparum</i> (UGG)	<b>G</b> UCUAGUGGUA <b>UGAU</b>	9	3	CU/UG
<i>Saccharomyces cerevisiae</i> (UGG)	<b>G</b> UCUAGUGGUA <b>UGAU</b>	9	3	CU/UG
<i>Zea mays</i> (CGG)	<b>G</b> UCUAGUGGUA <b>UGAU</b>	9	3	CU/UG
<b>Archaea</b>	Sequence of D-arm	Loop (nt)	Stem (bp)	12,13/22,23
<i>Halobacterium salinarum</i> (CGG)	<b>G</b> GGUAGCUUGGUA <b>UCC</b>	6	5	GU/AU
<i>Haloferax volcanii</i> (CGG)	<b>G</b> GGUAGCUUGGUA <b>UCC</b>	6	5	GU/AU
<i>Methanococcus jannaschii</i> (UGG)	<b>G</b> GGUAGCCUGGUA <b>UCC</b>	6	6	GU/AU
<i>Pyrobaculum aerophilum</i> (CGG)	<b>G</b> UCUAGCCUGGUA <b>GGAU</b>	9	4	CU/GG
<i>Nitrosopumilus maritimus</i> (UGG)	<b>G</b> UCUAGCUUGGUA <b>UGAU</b>	11	3	CU/UG
<i>Pyrococcus abyssi</i> (CGG)	<b>G</b> GGUAGCUUGG <b>CCUAUCC</b>	6	6	GU/AU
<i>Sulfolobus islandicus</i> (CGG)	<b>G</b> UCUAGCCUGGACUA <b>GGAU</b>	11	4	CU/GG
<i>Thermoplasma volcanium</i> (CGG)	<b>G</b> GGUAGCCUGGUA <b>UCCU</b>	11	3	GU/UC
<i>Thermococcus gammatolerans</i> (CGG)	<b>G</b> GGUAGCUUGGUA <b>UCCU</b>	13	3	GU/UC

**Supplementary Table 2 Comparison of the D-arm sequences among different organisms.** Watson-Crick base-pairing (A/U and G/C) and wobble base-pairing (G/U) nucleotides are shown in red and blue, respectively. G/U wobble base pairs are also considered as base pairs for counting the length of the D-loop and stem.