





Synthetic post-translational modifications of elongation factor P using the ligase EpmA

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Canonically, tRNA synthetases charge tRNA. However, the lysyl-tRNA synthetase paralog EpmA catalyzes the attachment of (*R*)- β -lysine to the ϵ -amino group of lysine 34 of the translation elongation factor P (EF-P) in *Escherichia coli*. This modification is essential for EF-P-mediated translational rescue of ribosomes stalled at consecutive prolines. In this study, we determined the kinetics of EpmA and its variant EpmA_A298G to catalyze the post-translational modification of K34 in EF-P with eight noncanonical substrates. In addition, acetylated EF-P was generated using an amber suppression system. The impact of these synthetically modified EF-P variants on *in vitro* translation of a polyproline-containing NanoLuc luciferase reporter was analyzed. Our results show that natural (*R*)- β -lysylation was more effective in rescuing stalled ribosomes than any other synthetic modification tested. Thus, our work not only provides new biochemical insights into the function of EF-P, but also opens a new route to post-translationally modify proteins using EpmA.

Introduction

In all domains of life, aminoacyl-tRNA synthetases (aaRSs) play an essential role in protein synthesis, as they catalyze the esterification of amino acids to the 3'-terminal hydroxyl group of tRNA [1]. Efficient and specific charging of tRNA is a prerequisite for the faithful translation of the genetic code. The reaction takes place in two steps. First, the amino acid is activated with ATP to form an aminoacyl-AMP. Then, the activated amino acid is transferred to the 3'-OH end of its cognate tRNA.

Despite this conserved reaction scheme, aaRSs can be divided into two subclasses based on their catalytic domains [2]. Class I aaRSs utilize an enzymatically

active Rossmann fold module that is typical for many nucleotide-binding proteins [3]. Members of this group are predominantly selective for hydrophobic amino acids. In contrast, the catalytic core of class II aaRS features an unusual α - β fold and is related to the biotin ligase BirA [4,5]. In this case, an invariant arginine is important for charging of tRNA [6] with predominantly small and/or polar amino acids.

Among the various amino acid substrates utilized by aaRSs, lysine stands out because members of both classes can recognize it [7–10]. Class II lysyl-tRNA synthetases (LysRSs) are typical for eukaryotes, Crenarchaeota, and the vast majority of bacteria, while

Abbreviations

aa-AMP, aminoacyl-adenylate; aaRS, aminoacyl-tRNA synthetase; ABC, ammonium bicarbonate; AC, aminocaproic acid; AcKRS, acetyl-lysine-tRNA synthetase; ACN, acetonitrile; DAP, diaminopimelic acid; DNase, deoxyribonuclease; EF-P, translation elongation factor P; FA, formic acid; IEF, isoelectric focusing; LysRS, lysyl-tRNA synthetase; MU, Miller Units; NLuc, NanoLuc luciferase; OB, oligonucleotide binding; PTM, post-translational modification; TEA, triethanolamine; TFA, trifluoroacetic acid.

class I LysRSs are restricted to Euryarchaeota, Spirochetes, and *Rickettsia* [2]. In addition to their well-studied canonical function, LysRSs have been found to perform other, noncanonical functions [11]. In eukaryotes, these include, but are not limited to, essential roles in HIV replication, cytokine-like signaling, and transport of proteins to the cell membrane [11]. Strikingly, even in bacteria, the role of LysRSs is not necessarily restricted to tRNA loading. For example, *Escherichia coli* encodes two class II LysRS variants, LysS and LysU. The first is constitutively expressed, while the second is induced by heat stress [12] and carries out moonlighting functions, namely the synthesis of the dinucleotide alarmone diadenosine tetraphosphate [13], and capping of mRNA [14].

A third bacterial LysRS homolog termed EpmA (also known as YjeA, PoxA, or GenX) deviates in structure and function from the archetypal class II LysRS. Whereas the entire catalytic domain is present, members of the EpmA subfamily lack the OB-fold (oligonucleotide binding) anticodon binding domain [15–17]. Accordingly, EpmA is unable to bind and charge tRNA. Instead, it modifies a single protein – the translation elongation factor P (EF-P). EF-P alleviates ribosome pausing at polyproline (XPPX) motifs by assisting in facilitating peptide-bond formation [18–22]. This translation factor is a molecular tRNA mimic [23], consisting of three β -barrel domains arranged in an L-shape structure. A positively charged residue (in *E. coli* lysine 34), which protrudes toward the peptidyltransferase center of the ribosome, is crucial for EF-P function [24]. Notably, this residue is the structural equivalent of the CCA end of the tRNA and target of the ligation reaction mediated by EpmA. Like the tRNA charging reaction of aaRSs, the first step in the post-translational modification (PTM) of EF-P is an ATP-dependent condensation to form enzyme-bound aminoacyl-adenylate (aa-AMP) (Fig. 1) [25]. However, the cognate donor substrate of EpmA is not (*S*)- α -lysine (L-lysine), but the nonproteinogenic amino acid (*R*)- β -lysine [26]. The second step involves the transfer of the (*R*)- β -lysyl moiety to the ϵ -amino group of lysine 34 (K34) of the protein acceptor and the simultaneous release of AMP from the catalytic pocket. We note here that other bacterial species modify EF-P in different ways, for example, 5-aminopentanylation is used by *Bacillus subtilis* [27,28] and α -rhamnosylation is found in pseudomonads [17,29]. Like β -lysylation in *E. coli*, these PTMs improve EF-P functionality and reveal the high degree of chemical flexibility. It is therefore plausible that EF-P might also be amenable to activation by other noncanonical modifications.

It is known that recognition of donor substrates by tRNA synthetases in general, and more specifically by EpmA, is error prone [25,30,31]. Initially, the following compounds were reported to serve as substrates for EpmA: (*S*)- α -lysine, 5-hydroxy-(*S*)- α -lysine, diamino pimelic acid (DAP), and thialysine ((*S*)-(2-aminoethyl)-L-cysteine) [25]. At the time of that study, EpmA was not known to be a protein ligase, and its (mis)charging capability was analyzed in the absence of EF-P. In addition, substrate selection was based on the assumption that the α -amino acid L-lysine is the enzyme's cognate donor substrate. In a more recent report, the noncanonical substrate spectrum was further extended by methyl-(*S*)- α -lysine and ethyl-(*S*)- α -lysine but also (*S*)- β -lysine [26]. In this present study, we reinvestigated substrate recognition using 10 lysine analogs and discovered four novel compounds that could be adenylated by EpmA and transferred to the protein acceptor EF-P. Thus, we were able to generate completely novel PTM of EF-P, which have allowed us to gain further insights into the function of this elongation factor.

Results

EpmA and its variant EpmA_A298G aminoacylate various noncanonical substrates

Our study builds on the investigations by Ambrogelly *et al.* and Roy *et al.*, which focused on substrate promiscuity of EpmA [25,26]. We set out to learn more about the role of the modification in the EF-P-mediated rescue of polyproline-stalled ribosomes by exploring the range of substrates to modify this translation factor. To do so, we chose the 10 lysine derivatives depicted in Fig. 2A [32]. The spectrum of substances tested encompasses not only known noncanonical donors, such as (*S*)- α -lysine, 5-hydroxy-(*S*)- α -lysine, and (*S*)- β -lysine, but also novel compounds [25,26]. In addition to the enantiomeric form of (*S*)- α -lysine [(*R*)- α -lysine], we included (*S*)- α - and (*R*)- β -ornithine, whose main chains are one carbon shorter than those of their lysine counterparts. By testing the enantiopure substrates of 3-aminohexanoic acid, ((*R*)-3-AC and (*S*)-3-AC), as well as 6-aminohexanoic acid (6-AC), we also assessed if lack of the β - or ϵ -amino group hinders EF-P ligation or alters its functionality.

The substrate specificity of EpmA can be redirected from (*R*)- β -lysine to (*S*)- α -lysine by a single amino acid replacement [26], namely substitution of the alanine present at position 298 in the wild-type enzyme by a glycine residue (Fig. 1). This relieves the steric

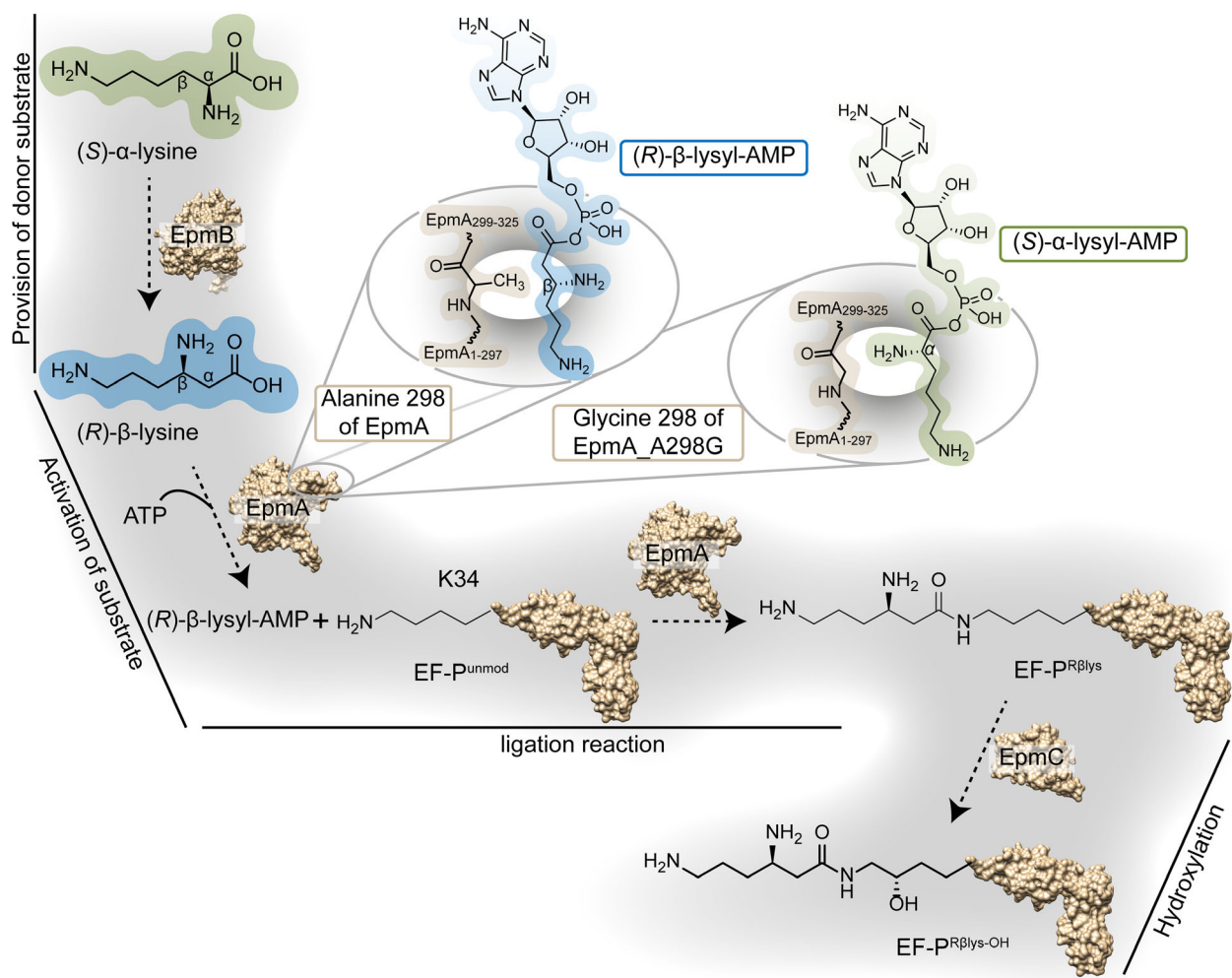
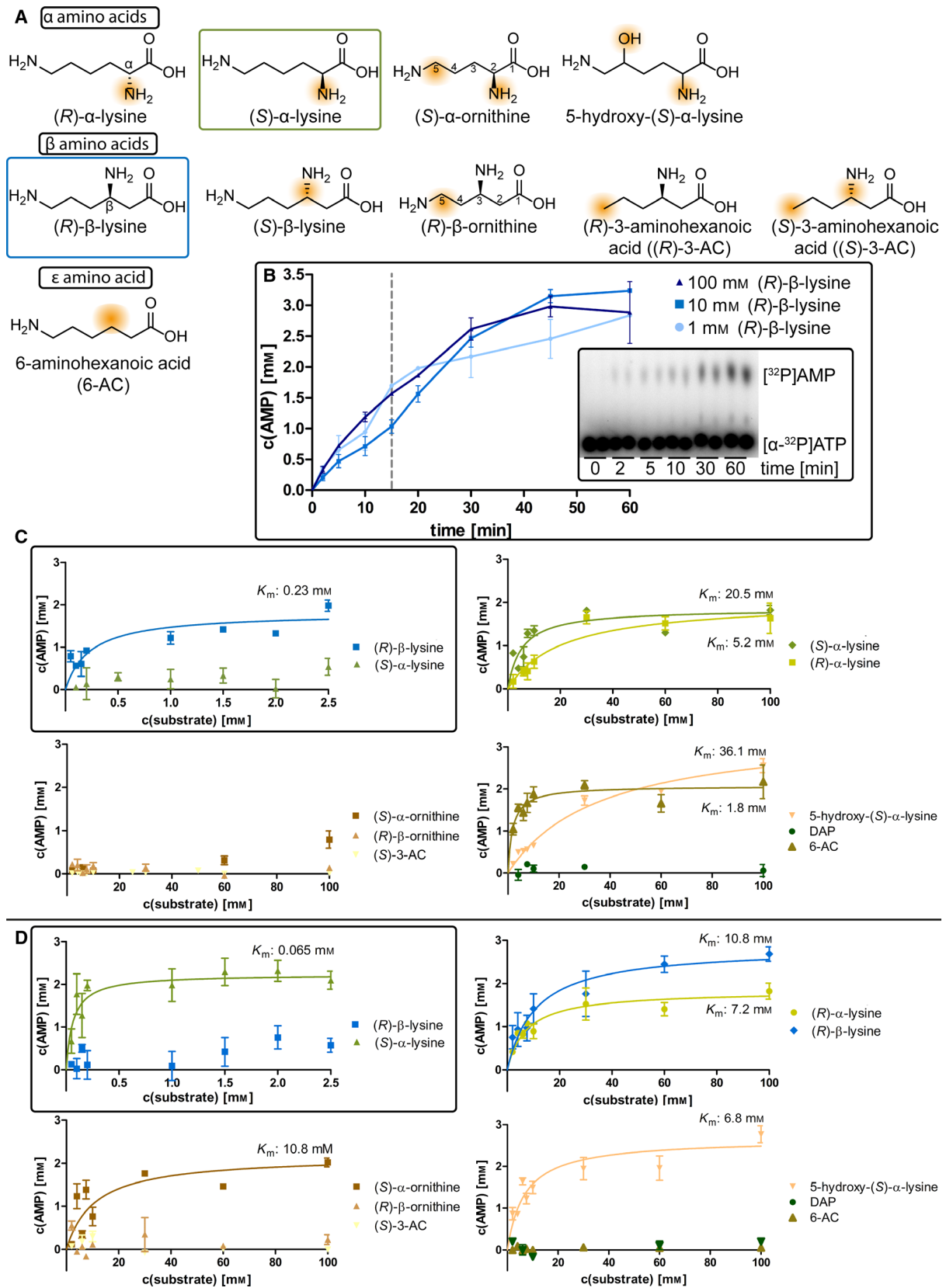


Fig. 1. Post-translational modification of EF-P by EpmABC with a focus on the catalytic pockets of EpmA and its variant EpmA_A298G. (S)-α-lysine (green) is first converted into (R)-β-lysine (blue) by the aminomutase EpmB [44]. This donor substrate is then activated by ATP hydrolysis, forming (R)-β-lysyl-AMP. This step, as well as the attachment of (R)-β-lysine to K34 of EF-P, is catalyzed by the ligase EpmA to yield modified EF-P (EF-P^{Rβlys}). In the catalytic pocket of EpmA, residue 298 (highlighted) predetermines the structure of the entering substrate. This explains why replacement of the wild-type alanine at position 298 by glycine (A298G) results in the use of (S)-α-lysine in preference to (R)-β-lysine as the substrate for activation. EF-P^{Rβlys} is further hydroxylated at K34 by EpmC, but this is of minor importance for the protein's function [22,46].

hindrance between the methyl group of A298 and the α-amino group of amino acids. Hence, the alanine-to-glycine replacement creates space for (S)-α-lysine, as demonstrated by the lower K_m of the EpmA variant

A298G (~ 300 μM), which is more than one order of magnitude less than that of the wild-type enzyme (> 5 mM) [26]. This significant difference motivated us to include the EpmA_A298G in our analysis.

Fig. 2. [³²P]AMP formation assay monitors *in vitro* activation of (R)-β-lysine and related compounds by EpmA. (A) Overview of the lysine derivatives tested in this study. The natural EpmA substrate (R)-β-lysine is framed in blue, while (S)-α-lysine, the high-affinity substrate for EpmA_A298G, is framed in green. Constitutional and spatial alterations of the cognate EpmA substrate are highlighted. (B) [³²P]AMP formation assay. A representative autoradiograph and time course of ATP hydrolysis in the presence of 1, 10 or 100 mM (R)-β-lysine and 1 μM EpmA is shown. Such plots were used to determine the linear segment of the reaction. Consequently, K_m values were determined at 15-min reaction time (dashed line). The error bars indicate the standard error of the technical duplicates. (C, D) Binding affinities (K_m values) of (C) EpmA and (D) EpmA_A298G for various lysine analogs. Low K_m values were determined using the 0–2.5 mM substrate range (framed). Higher K_m values were determined using the 0–100 mM substrate range. Error bars indicate SEM. Curves were fitted according to the Michaelis–Menten equation (GRAPHPAD PRISM) to determine K_m values.



Promiscuity was initially tested by utilizing the pre-transfer editing capability of aaRSs, which allows the enzyme to cleave (mis)activated aminoacyl-adenylates, thus releasing AMP [33,34]. Notably, this happens even in the absence of the acceptor substrate, enabling us to study EpmA activity without adding EF-P [35,36]. Kinetic parameters were determined using thin-layer chromatography in combination with radio-labeled [α - 32 P]ATP, which allows for monitoring and quantification of AMP release via autoradiography. First, the AMP production was monitored over time.

Figure 2B depicts the results of a representative time course experiment. Irrespective of the concentration of the donor substrate, accumulation followed a linear course over the first 20 min. Consequently, the reaction kinetics for both EpmA and EpmA_A298G were determined at the 15-min time point. Then, the initial velocities for several substrate concentrations were measured (based on the 15-min time points), and the kinetic parameters K_m and k_{cat} were determined. The resulting curves were fitted according to the Michaelis–Menten equation (Fig. 2C,D, Table 1 [37]). In agreement with earlier studies [26] of EpmA, (*R*)- β -lysine and (*S*)- α -lysine were activated by EpmA as well as EpmA_A298G, with the latter exhibiting the expected switch in affinity. Note that our K_m values match those previously reported, with one exception – the K_m determined for (*R*)- β -lysine here was almost two orders of magnitude higher for EpmA_A298G than reported in Ref. [26]. In line with previous work was activation of 5-hydroxy-(*S*)- α -lysine [25].

Of the newly tested substrates (Fig. 2A), (*R*)-3-AC and 6-AC, as well as (*R*)- α -lysine and (*S*)- α -ornithine, were adenylated. Whereas K_m values could be ascertained for the latter three (Table 1), this was not possible for (*R*)-3-AC. Here, we observed substrate inhibition effects that led to a reduction of AMP formation at concentrations above ~ 10 mM (Fig. S1). As

a consequence, saturation was not reached, which prohibited exact curve fitting and the determination of kinetic parameters.

Besides the known differences in specificity between wild-type EpmA and EpmA_A298G, we also observed clear preferences in the charging efficiency for other tested substrates. In this regard, EpmA_A298G was superior in adenylating (*R*)- α -lysine and 5-hydroxy-(*S*)- α -lysine. In addition, only this substitution variant could charge (*S*)- α -ornithine, whereas only EpmA activated 6-AC.

EpmA/EpmA_A298G-mediated EF-P ligation with noncanonical substrates generates novel post-translational protein modifications

Having successfully shown substrate promiscuity in the condensation reaction with AMP, we asked whether ligation of the activated lysine derivatives to K34 of EF-P is possible. We set up an *in vitro* assay to post-translationally modify EF-P with (*S*)- α -ornithine, (*R*)/(*S*)- α -lysine, 5-hydroxy-(*S*)- α -lysine, (*R*)/(*S*)- β -lysine, (*R*)-3-AC, and 6-AC. Successful ligation was visualized by immunodetection of isoelectrically focused EF-P [38]. If a PTM affects the net charge of the acceptor protein, its isoelectric point is altered. Indeed, EF-P was successfully modified *in vitro* (Fig. 3A). Electrophoretic separation from unmodified EF-P in a pH gradient was seen for all tested substrates, except for 3-AC and 6-AC, which are not expected to change the pI value of the acceptor. Whereas (*S*)- β -lysine-modified EF-P ran exactly at the same height as the variant bearing (*R*)- β -lysine, those EF-Ps bearing (*S*)/(*R*)- α -lysine ran slightly lower in the gradient. This is due to the fact that α -amino acids in general show a lower pK_a value than β -amino acids (e.g., $pK_a[\alpha\text{-alanine}] = 9.87$ [39], $pK_a[\beta\text{-alanine}] = 10.24$ [40]). The pI value for EF-P modified with 5-hydroxy-(*S*)- α -lysine

Table 1. K_m and k_{cat} values of EpmA and EpmA_A298G for different lysine derivatives. K_m values (\pm standard error) are given in mM concentrations and k_{cat} values (\pm standard error) in turnovers per second. Low K_m substrates are framed. (Accurate) K_m and k_{cat} determination was not possible for (*R*)- β -ornithine and (*S*)-3-AC due to lack of detectable AMP formation, for (*R*)-3-AC due to substrate inhibition and for (*S*)- β -lysine due to uncertain stock solution concentration. n. r.: no reaction detected in the investigated substrate concentration range.

	(<i>R</i>)- α -lysine	(<i>S</i>)- α -lysine	(<i>S</i>)- α -ornithine	5-hydroxy-(<i>S</i>)- α -lysine	(<i>R</i>)- β -lysine	6-AC
K_m (mM)						
EpmA	20.5 (\pm 9.3)	5.2 (\pm 1.3)	n. r.	36.1 (\pm 6.4)	0.23 (\pm 0.07)	1.8 (\pm 0.8)
EpmA_A298G	7.2 (\pm 2.0)	0.065 (\pm 0.032)	10.8 (\pm 4.1)	6.8 (\pm 1.7)	10.8 (\pm 4.0)	n. r.
k_{cat} (s^{-1})						
EpmA	2039 (\pm 308)	1851 (\pm 116)	n. r.	3416 (\pm 242)	1812 (\pm 128)	2069 (\pm 163)
EpmA_A298G	1830 (\pm 147)	2234 (\pm 192)	2167 (\pm 269)	2651 (\pm 187)	2834 (\pm 314)	n. r.

Colours correspond to Fig. 2: Low K_m substrates are framed in blue (EpmA) or green (EpmA_A298G).

differs only slightly from that of unmodified EF-P. Here, the pI-elevating effects of the α - and ϵ -amino groups are counteracted by the reducing effect of the hydroxyl group.

For all substrates tested, the modification status of EF-P was also confirmed by top-down mass spectrometric analyses (Fig. 3, Fig. S2). These tests also verified the attachments of 3-AC and 6-AC to EF-P K34, which could not be detected by isoelectric focusing (IEF).

These data demonstrate that EpmA and its variant EpmA_A298G are capable of transferring activated noncanonical donor substrates onto a protein acceptor, and thus enable the *in vitro* generation of novel PTMs.

In parallel, we asked if EpmA_A298G is able to modify EF-P in an *E. coli* *epmB* background *in vivo* (Fig. 1). Lack of the lysine aminomutase EpmB prevents the formation of (*R*)- β -lysine [41], thus precluding activation of EF-P by this substrate [42–44]. We

overexpressed either *epmA* or *epmA*_A298G from an arabinose-inducible promoter in this strain in order to force modification of EF-P with noncanonical substrates. We then purified the protein and subjected it to IEF and immunodetection. Strikingly, overexpression of both EpmA and EpmA_A298G resulted in synthetically modified EF-P (Fig. 4A). The migration height was the same as that of the EF-P isolated from wild-type cells, indicating the attachment of a compound with a similar effect on the protein's pI to that of (*R*)- β -lysine. Notably, only in the case of EpmA_A298G was the EF-P population shifted fully toward the modified state, whereas with wild-type EpmA 36% of EF-P remained unmodified. We therefore hypothesized that (*S*)- α -lysine was the attached substrate. This is a plausible assumption, insofar as the intracellular concentration of (*S*)- α -lysine can reach up to 0.4 mM [45], which is saturating for enzyme EpmA_A298G, but below the K_m of EpmA (Table 1). Indeed, subsequent analysis by mass spectrometry

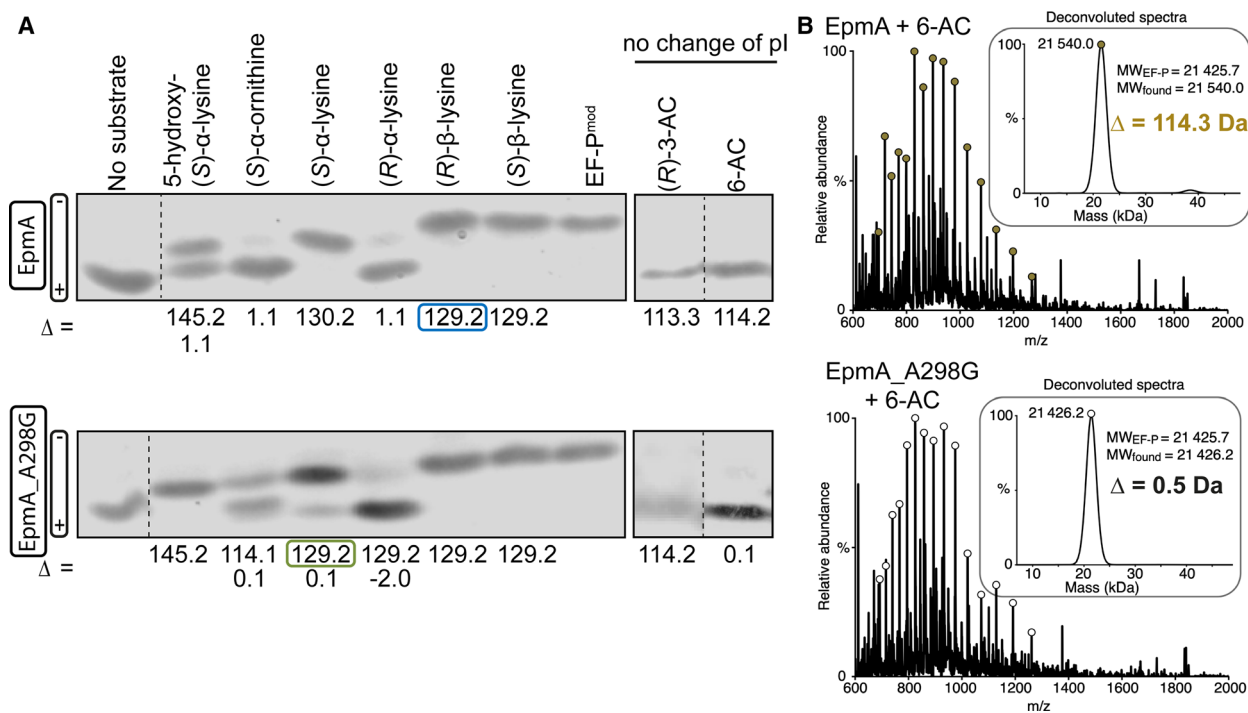


Fig. 3. *In vitro* modification of EF-P by EpmA and EpmA_A298G with different lysine derivatives. (A) In the *in vitro* reaction, purified unmodified EF-P and purified enzyme were incubated with 10 mM substrate overnight. Unmodified and modified EF-P were separated in an IEF gel and visualized by western blotting (with anti-EF-P_{*E. coli*} antibody). Experiments were performed in duplicates, whereby the running behaviors of the tested samples were identical. Dashed lines indicate splices. Mass spectrometry was performed with the same samples to determine the mass shift of EF-P caused by the corresponding modification. Here, the mass of unmodified EF-P was subtracted from the measured mass and is presented underneath each blot. *In vivo* modified EF-P^{R β lys} served as positive control. Low K_m substrates are framed in blue (EpmA) or green (EpmA_A298G). (B) Representative mass spectra of EF-Ps, which were modified with 6-AC *in vitro* by EpmA (top) but not by EpmA_A298G (bottom). The intact EF-Ps resulting from the *in vitro* modification reactions were measured using native mass spectrometry. The MS spectra obtained were deconvoluted using UNDEC software to reveal the difference in selectivity between EpmA and EpmA_A298G with high accuracy.

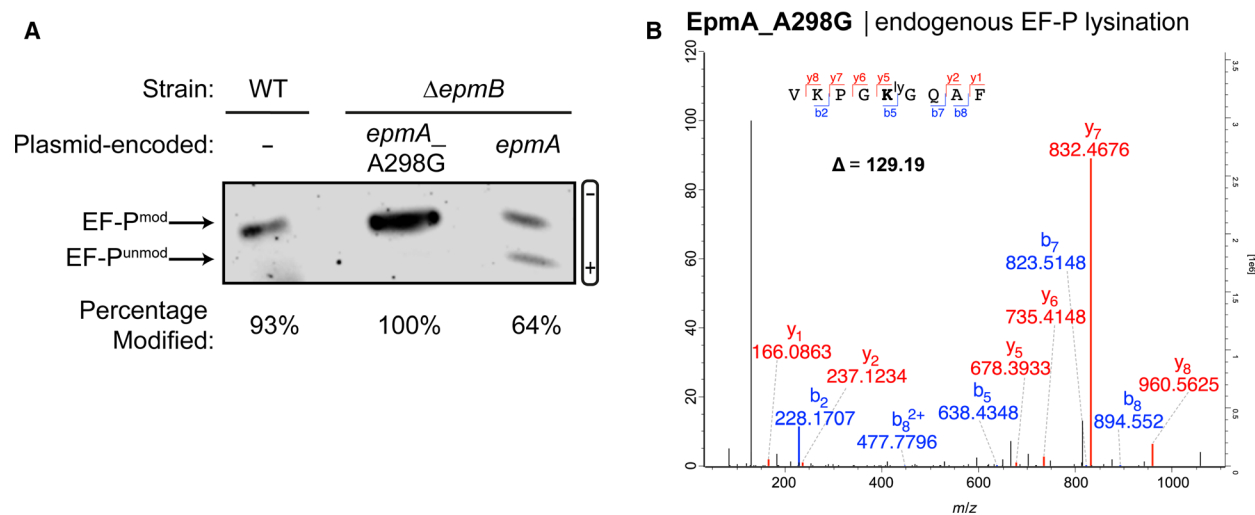


Fig. 4. Overproduction of EpmA_A298G allows for modification of EF-P in an *epmB* deletion mutant. (A) IEF gel of purified native EF-P from wild-type cells and the *Escherichia coli* BW25113 $\Delta epmB$ mutant after overexpression of *epmA* and *epmA*_A298G, respectively. Modified EF-P was separated from unmodified EF-P on the basis of their pI values and detected by western blotting using anti-EF-P_{*E. coli*} antibody and quantified by gray density analysis. Shown is the result of a single experiment. (B) Endogenous EF-P was isolated from the EpmA_A298G-overproducing strain and subjected to enzymatic digestion and MS analysis. The fragmentation spectrum of the EF-P peptide containing the critical K34 reveals that this residue is modified with lysine. The MS spectra were searched for all other tested modifications without positive result, confirming that lysylation is indeed the endogenously occurring modification of the EF-P in *E. coli* $\Delta epmB$.

revealed a shift of 129.2 Da at K34 (Fig. 4B), as expected for lysylation.

Notably, natural *E. coli* EF-P is not only lysylated but also hydroxylated at the C4 atom of K34 by an enzyme termed EpmC (Fig. 1) [22,46,47]. Accordingly, we also looked for a modification corresponding to 146 Da, but were unable to find it. This agrees with the previous finding that EpmC recognizes EF-P exclusively in its lysylated form [47]. Hence, the lack of hydroxylation of (*S*)- α -lysylated EF-P implies that EpmC has a strong preference for the β -form of the amino acid. As the acceptor substrate spectrum of the hydroxylase remains elusive, our data provide an unexpected hint as to its specificity.

Noncanonically modified EF-P variants yield new mechanistic insights into ribosome rescue with (*R*)- β -lysine

We were particularly curious to know whether the novel PTMs can support EF-P-mediated rescue of polyproline-stalled ribosomes. Therefore, an *in vitro* transcription/translation system reconstituted from purified components (PURExpress; New England Biolabs, Ipswich, MA, USA) was set up [18]. As readout for translation efficiency, we used NanoLuc luciferase (NLuc; Promega, Fitchburg, MA, USA) [48]. NLuc is a small protein (19.1 kDa) that converts furimazine

into furimamide and thereby emits light. The enzyme itself lacks polyproline stretches. Accordingly, we extended the *nluc* open reading frame by introducing a short sequence coding for RPPP (PPP-NLuc) at the 5' end, which causes a translational arrest that can be alleviated by modified EF-P (Fig. 5A) [18]. The relative efficiency of ribosome rescue can be assayed based on the level of light emitted by NLuc over time (Fig. S3) [19]. In line with earlier studies, *in vitro* translation with the polyproline-containing luciferase variant was boosted by a factor of about five when (*R*)- β -lysylated EF-P was present. Having shown that the system works robustly, we assessed the functionality of EF-Ps bearing the synthetic modifications (Fig. 5B). Moreover, we decided to test the acetylated EF-P variant, because the equivalent lysine ϵ -amino group in *B. subtilis* EF-P is acetylated *in vivo* when the modification system is missing [49]. Acetylated EF-P was generated by using the amber suppression system [50]. To this end, we introduced the amber stop codon into *efp* at position 34 (Am34). Supplementation of the medium with 5 mM acetyl-lysine allowed the production of EF-P^{AcK} in an *E. coli* strain that contained the tRNA synthetase/tRNA pair AcKRS/pyIT^{CUA} and thereby allowed suppression of Am34 (Fig. S4) [50,51].

As all noncanonically modified EF-Ps produced significantly less light than the (*R*)- β -lysylated protein, we conclude that both amino groups in their native

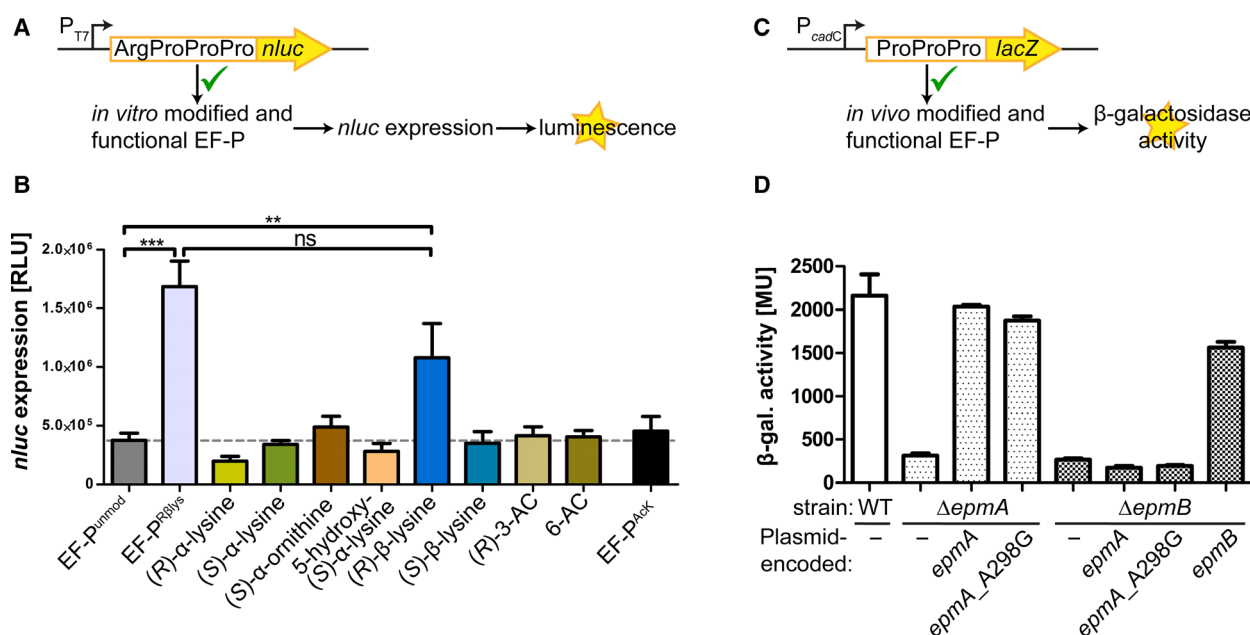


Fig. 5. Translation efficiencies of EF-Ps modified *in vitro* and *in vivo*. (A) Schematic depiction of the cell-free transcription/translation experiment designed to produce Nluc with RPPP-motif. Since it requires functional EF-P for robust translation of the luciferase, the functionality of the *in vitro* modified EF-Ps could be assayed by measuring luminescence. (B) *In vitro* modified EF-Ps (verified by IEF or MS analysis) were subjected to *in vitro* transcription/translation. Translation efficiencies of unmodified EF-P (dashed gray line), *in vivo* modified EF-P (EF-P^{Rβlys}), and EF-Ps modified at K34 with the indicated lysine derivatives were determined after a reaction time of 30 min in at least three independent assays. EF-P^{AcK} was obtained by using the amber suppression system [50]. Error bars indicate s. e. m. Results of the two-tailed *t*-test are indicated as follows ***P* value < 0.01, ****P* value < 0.001, ns: not significant. (C) Schematic depiction of the reporter assay [18]. Translation of the hybrid CadC'-PPP-LacZ requires a functional EF-P and correlates with high β-galactosidase activity. (D) The β-galactosidase activities of CadC₁₋₃₀-PPP-LacZ were determined in the *Escherichia coli* mutants Δ*epmA* and Δ*epmB* bearing empty vectors (-) or complemented with the indicated genes *in trans*. The experiment was performed at least in triplicates. Error bars indicate SEM.

configuration are crucial for EF-P-mediated ribosome rescue. Notably however, bioluminescence output with the (R)-α-lysylated and the 5-hydroxy-(S)-α-lysylated EF-P was even lower than that seen with the unmodified protein, which might indicate an inhibitory effect.

Finally, we tested the functionality of (S)-α-lysylated EF-P *in vivo*. For this purpose, we used an already established test system based on a PPP-LacZ hybrid (Fig. 5C). Accordingly, β-galactosidase activity is high in wild-type cells but low in strains lacking a functional EF-P (*E. coli* Δ*epmA* and Δ*epmB*). As expected, complementation of the mutants with the corresponding genes *in trans* restored the wild-type phenotype (Fig. 5D). By contrast, the overproduction of neither EpmA nor EpmA_A298G in *E. coli* Δ*epmB* led to an increase of β-galactosidase activity, indicating nonfunctional EF-P. Thus, the attached (S)-α-lysine moiety cannot substitute (R)-β-lysine in order to functionalize EF-P. This result is consistent with our *in vitro* data (Fig. 5B), where (S)-α-lysylated EF-P did not alleviate ribosome stalling of the PPP-NLuc construct.

Discussion

The generation of EF-Ps with synthetic PTMs provides new insights into the rescue of polyproline-mediated ribosome pausing. In 2017, Huter *et al.* [24] presented the cryo-EM structure of EF-P bound to the ribosome, demonstrating how (R)-β-lysine might stabilize the P-site tRNA (Fig. 6A). Specifically, hydrogen bonds can be formed between the ε-amino group of the β-lysyl moiety and the ribose 2' OH of tRNA^{Pro} C75, as well as the bridging oxygen of A76 [24]. Furthermore, the β-amino group makes contact with the conserved nucleotide A2439 of the 23S rRNA and would therefore be reminiscent to what was found for hypusine of eIF5A and A2808 [52,53]. Our biochemical analysis now complements these structure-based assumptions and demonstrates the importance of the (R)-β-lysyl moiety for the functionality of EF-P. Neither a lack of the β-amino group (in the case of 6-AC) nor loss of the ε-amino group (in the case of 3-AC) is tolerated (Fig. S5A,B). Similarly, shortening of the carbon chain as in (R)-β-ornithine weakens hydrogen bonding with

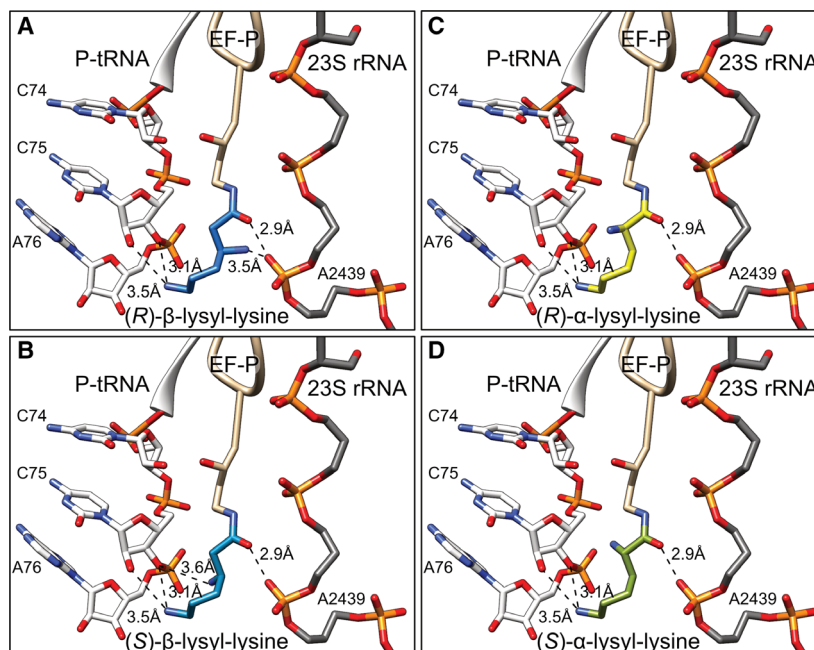


Fig. 6. Model of altered EF-P-tRNA and EF-P-ribosome (23S rRNA) interactions attributable to synthetic PTMs. Models were generated using UCSF Chimera [68]. (A) Interaction of (*R*)- β -lysyl-lysine with the CCA end of the P-site tRNA and 23S rRNA as determined by cryo-EM [24]. Putative interactions of (B) (*S*)- β -lysyl-lysine, (C) (*R*)- α -lysyl-lysine, and (D) (*S*)- α -lysyl-lysine with the CCA end of the P-site tRNA and 23S rRNA. For clarity, only the phosphate backbone of the 23S rRNA is shown.

the tRNA^{Pro} CCA end (Fig. S5C). In an α -lysylated EF-P (Fig. 6C,D), irrespective of its precise stereoconfiguration, the distance to A2808 increases to more than 5 Å and is therefore too large to permit the establishment of the stabilizing interaction. Even with (*S*)- β -lysine, this connection is lost (Fig. 6B). However, in that configuration the β -amino group could still make contact with an A76 phosphate oxygen, which in turn might have a mildly stimulatory effect on Pro-Pro peptide bond formation. This idea is corroborated by the partial compensation of a Δ *epmB* growth defect upon the external addition of (*S*)- β -lysine [23]. Taken together, the comprehensive biochemical dataset presented here explains the selective pressure in favor of (*R*)- β -lysine for the activation of EF-P.

Our new molecular insights into how (*R*)- β -lysylated EF-P mediates ribosome rescue are one notable aspect of our study. The other concerns EpmA substrate specificity and how it might be exploited as novel tool for protein engineering. The generation of polypeptides containing noncanonical amino acids is a central pillar of efforts to generate new functionalities. In this regard, synthetic biology predominantly relies on the very elegant system developed by Schultz and Yarus [54]. Their method is based on an orthologous tRNA/tRNA synthetase pair with which the amber stop signal is turned into a sense codon to enable the translational incorporation of noncanonical amino acids [55]. To date, engineering of the synthetase by directed evolution or the utilization of natural substrate

promiscuity allows researchers to site specifically incorporate more than 150 different substrates [56]. With this study, we demonstrate an alternative way to extend the spectrum of synthetic PTMs. Based on the promiscuity of EpmA, we have demonstrated site-directed ligation of (*S*)- α -ornithine, (*R*)/(*S*)- α -lysine, 5-hydroxy-(*S*)- α -lysine, (*R*)/(*S*)- β -lysine, 3-AC, and 6-AC to the ϵ -amino group of lysine K34 of EF-P. While this approach allows one to carry out alternative PTMs of EF-P, the specificity of both EpmA and EpmA_A298G for this protein acceptor currently limits the application of the lysyl-tRNA synthetase paralog as a more general molecular tool. Consequently, the next step will focus on engineering of EpmA to modify other proteins. Notably, only 25% of all bacteria rely on β -lysylation of EF-P, whereas other EF-Ps (e.g., in *Pseudomonas*) are activated by arginine rhamnosylation with EarP. The latter enzyme is phylogenetically unrelated to EpmA and thus favored strong co-evolution of the corresponding EF-P at the sequence level, while the structure has remained highly conserved [17]. Although the EF-Ps of *P. putida* and *E. coli* share only 26% sequence identity, we have recently shown that cross-modification and even activation by EarP is possible with only few amino acid replacements in EF-P [57]. Conversely, future studies will clarify whether EpmA can recognize the *P. putida* EF-P. If it were possible to narrow down acceptor substrate specificity to a short sequon, EpmA-mediated ligation could serve as a useful extension to our

molecular toolbox for protein engineering with non-canonical amino acids.

Materials and methods

General

Escherichia coli cells were routinely grown aerobically in lysogeny broth [LB; 1% (w/v) NaCl, 1% (w/v) tryptone, 0.5% (w/v) yeast extract, pH 7.0] at 37 °C. Antibiotics were used in the following concentrations: 100 µg·mL⁻¹ ampicillin sodium salt, 50 µg·mL⁻¹ kanamycin sulfate, 30 µg·mL⁻¹ chloramphenicol, 15 µg·mL⁻¹ tetracycline hydrochloride. Growth curves were recorded using the Spark 20M multimode microplate reader (Tecan, Männedorf, Switzerland) at 37 °C. Optical density at 600 nm was measured every 15 min.

ATP disodium salt, deoxyribonuclease I from bovine pancreas (DNase), (*S*)-3-aminohexanoic acid hydrochloride, (*R*)-3-aminohexanoic acid hydrochloride, (*S*)- α -ornithine hydrochloride, 6-aminohexanoic acid, and 2,6-diaminopimelic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). (*R*)- α -lysine hydrochloride and (*R,S*)-5-hydroxy-(*S*)- α -lysine were purchased from Fluka (Buchs, Switzerland). (*S*)- α -lysine hydrochloride was purchased from Roth (Karlsruhe, Germany). (3*R*)-3,5-diaminopentanoic acid dihydrochloride ((*R*)- β -ornithine) was provided by AKos GmbH (Steinen, Germany). (*R*)- β -lysine was synthesized by Carbolution Chemicals (Saarbrücken, Germany), and (*S*)- β -lysine was purchased from PepTech Corporation (Burlington, VT, USA). All lysine derivatives were dissolved in ddH₂O.

Cloning

The resistance cassette present in *E. coli* BW25113 Δ *epmB* Cm^R was removed by FLP/FLPe expression with the Quick & Easy *E. coli* Gene Deletion Kit (Gene Bridges, Heidelberg, Germany) in accordance with the manufacturer's instructions, yielding *E. coli* BW25113 Δ *epmB* Cm^S.

Synthetic ribosome binding sites were designed using RBS Calculator (<https://salislab.net/software/>, De Novo DNA). To create pBAD33_sRBS_His₆*epmA*, *epmA*_{Eco} was amplified from pBAD33_*epmA* under standard PCR conditions using P1/2 (Table S1) as primers and cloned into pBAD33. To introduce the amino acid substitution A298G, the resulting plasmid was used as template, and the primer pairs P3/4 and P5/6, respectively, were used to generate the upstream and downstream fragments. pBAD24-*efp*_{Eco} served as the template for introduction of the amber mutation TAG. The resulting plasmid pBAD24_sRBS_*efp*(*Eco*) K34TAG_His₆ was constructed by overlap extension PCR using P3/7 and P6/8. To construct pBAD33_His₁₀*epmB*, the corresponding fragment was amplified from

pBAD24_His₁₀*epmB* using P3/P6, digested at restriction sites NsiI and XbaI, and ligated with pBAD33.

Protein overproduction

Unmodified N terminally His₆-tagged EF-P was overproduced in *E. coli* BW25113 Δ *epmA* harboring the pBAD33_*efp*_His₆ plasmid. Furthermore, *E. coli* BW25113 was transformed with pBAD33_*efp*_His₆*epmAB* to produce (*R*)- β -lysylated EF-P. N terminally His₆-tagged EpmA and its corresponding variant were overproduced in *E. coli* LMG194 cells that harbored pBAD33_sRBS_His₆*epmA* or pBAD33_sRBS_His₆*epmA*_A298G. C terminally His₆-tagged EF-P with acetyl-lysine instead of lysine at position 34 was overexpressed from pBAD24_sRBS_*efp*(*Eco*) K34TAG_His₆ in *E. coli* LMG194 which contained the additional plasmid pACycDuet_AcKRST described by Volkwein *et al.* [50]. This allowed for amber suppression with the acetyl-lysine-tRNA synthetase (AcKRS). LB medium was supplemented with 5 mM *N*^ε-acetyl-L-lysine and 1 mM nicotinamide to prevent deacetylation by CobB [58].

During exponential growth, 0.2% (w/v) L(+)-arabinose was added to induce gene expression from pBAD vectors, and 1 mM IPTG served to induce gene expression of the pACycDuet-based system. Cells were grown overnight at 18 °C and harvested by centrifugation on the next day. The resulting pellet was resuspended in HEPES buffer [50 mM HEPES, 100 mM NaCl, 50 mM KCl, 10 mM MgCl₂, 5% (w/v) glycerol, pH 7.0] and incubated with DNase for 30 min. Cells were then lysed using a continuous-flow cabinet from Constant Systems Ltd. (Daventry, UK) at 1.35 kbar. The resulting lysates were clarified by centrifugation at 4 °C at 234 998 *g* for 1.5 h. The His₆-tagged proteins were purified using Ni-NTA beads (Qiagen, Hilden, Germany) according to the manufacturer's instructions, using 20 mM imidazole for washing and 400 mM imidazole for elution. In the final step, the purified protein was dialyzed overnight against HEPES buffer to remove imidazole from the eluate.

Assay for the formation of [³²P]AMP

Hydrolysis of ATP by EpmA in the presence of various concentrations of lysine derivatives was monitored in HEPES buffer in a total volume of 10 µL.

For time course experiments, 1, 10, or 100 mM (*R*)- β -lysine, 5 mM DTT, and 3 mM [α -³²P]ATP (20 Ci·mol⁻¹; PerkinElmer, Waltham, MA, USA) were incubated at 37 °C (time point 0 min) and the reaction was started by the addition of purified EpmA (to 1 µM). Aliquots were taken at 2, 5, 10, 15, 20, 30, 45, and 60 min and quenched with four volumes of 200 mM sodium acetate (pH 5.0). Then, duplicates of 1 µL samples of the mixture were analyzed by thin-layer chromatography on PEI cellulose plates (Merck Millipore, Burlington, VT, USA, prewashed with

water), which were developed in 0.1 M ammonium acetate and 5% (w/v) acetic acid. [α - 32 P]ATP and [32 P]AMP were visualized and quantified by phosphor imaging. Gray densities of the [32 P]AMP spots were compared to the gray density of a known amount of [α - 32 P]ATP using IMAGEJ [59]. For K_m determination, the concentration of the lysine derivative was varied from 0 to 100 mM for low-affinity substrates and from 0 to 2.5 mM for high-affinity substrates. Reaction mixtures contained 5 mM DTT, 3 mM [α - 32 P]ATP (20 Ci·mol $^{-1}$; PerkinElmer), were incubated at 37 °C and initiated by adding either EpmA or EpmA_A298G (to 1 μ M). After 15 min, the reaction was quenched and processed as described above. K_m values were estimated using nonlinear fitting [least-squares (ordinary) fit] of the Michaelis–Menten equation, which was performed with GRAPHPAD PRISM (version 5.03 for Windows; GraphPad Software, La Jolla, CA, USA, www.graphpad.com). The k_{cat} values were determined with the same method.

***In vitro* EF-P modification**

For *in vitro* modification reaction, 25 μ M unmodified N terminally His $_6$ -tagged EF-P, 2 mM ATP, 5 μ M N terminally His $_6$ -tagged EpmA or EpmA_A298G, respectively, and 10 mM lysine derivative were mixed in a total volume of 10 μ L (HEPES buffer, pH 7.0) and incubated overnight at room temperature. On the next day, samples were either mixed with 2 \times IEF buffer (SERVA, Heidelberg, Germany) and stored at 4 °C prior to IEF analysis or stored at –20 °C for mass spectrometric analysis of intact proteins.

Isoelectric focusing and western blotting

For IEF analysis, 0.5 μ g of protein per lane was loaded onto a native vertical IEF gel with a pH gradient range of 4–7 (SERVAGel) containing approx. 3% (w/v) SERVAPLUT. Prior to loading, samples were mixed with 2 \times IEF sample buffer (SERVA) and wells were rinsed with SERVA IEF cathode buffer. Focusing was conducted at 4 °C for 1 h at 50 V and 1 h at 300 V, and finally, bands were sharpened for 40 min at 500 V.

For western blotting, proteins were transferred onto a nitrocellulose membrane (Whatman, Maidstone, UK) using the Bio-Rad Trans-Blot Turbo Transfer System and incubated with 0.1 μ g/mL anti-EF-P $_{Eco}$ (Eurogentec, Lüttich, Belgium) as the primary antibody. The latter was targeted by 0.2 μ g·mL $^{-1}$ anti-rabbit phosphatase-conjugated secondary antibody (Rockland, Gilbertsville, PA, USA), and the resulting complexes were visualized using development solution [50 mM sodium carbonate buffer, pH 9.5, 0.01% (w/v) *p*-nitroblue tetrazolium chloride, and 0.045% (w/v) 5-bromo-4-chloro-3-indolyl-phosphate]. Where necessary, gray densities were quantified using IMAGEJ [59].

Mass spectrometry of intact proteins

Samples were desalted and measured using a MassPREP On-Line Desalting Cartridge (Waters, Milford, CT, USA) on an UltiMate 3000 HPLC system (Dionex, Sunnyvale, CA, USA) coupled to a Finnigan LTQ-FT Ultra mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with electrospray ionization (spray voltage 4.0 kV, tube lens 110 V, capillary voltage 48 V, sheath gas 60 arb, auxiliary gas 10 arb, sweep gas 0.2 arb). XCALIBUR XTRACT Software (Thermo Fisher Scientific) and UNDEC were used for data analysis and deconvolution [60].

Sample preparation for MS-based proteomics to identify putative PTMs at the peptide level

Prior to enzymatic digestion, 1- μ g aliquots of purified protein were diluted with 200 μ L of ABC buffer (25 mM ammonium bicarbonate), reduced in the presence of 1 mM DTT (0.2 μ L of 1 M stock in ddH $_2$ O) for 45 min at 25 °C, alkylated in 5.5 mM iodoacetamide (IAA, 2 μ L of 550 mM stock in ddH $_2$ O) for 30 min at 25 °C, and quenched with 4 mM DTT (0.8 μ L of 1 M stock) for 30 min at 25 °C. Following supplementation with 2 μ L of 1 M CaCl $_2$, 1 μ L of chymotrypsin (0.5 μ g· μ L $^{-1}$) was added, and the mixture was incubated for 16 h at 25 °C. Samples were acidified to pH 2–3 with 1% (v/v) formic acid (FA) and desalted using SepPak C18 cartridges (50 mg; Waters) with a vacuum manifold. The cartridges were washed with acetonitrile (ACN, 2 \times 1 mL) and equilibrated with 0.5% (v/v) FA (3 \times 1 mL) prior to loading the samples. After washing with 0.5% (v/v) FA (3 \times 1 mL), peptides were eluted with 80% (v/v) ACN containing 0.5% FA (2 \times 0.25 mL) and freeze-dried using a SpeedVac centrifuge. Samples were prepared for MS analysis by dissolving them in 30 μ L of 1% (v/v) FA and passing the solution through 0.22 μ m PVDF filters (Merck Millipore).

MS measurement and analysis of putatively modified peptides

MS analysis was performed on a Q Exactive Plus instrument coupled to an UltiMate 3000 Nano-HPLC via an electrospray easy source (all obtained from Thermo Fisher Scientific). Samples were loaded onto a 2-cm PepMap RSLC C18 trap column (particles 3 μ m, 100 Å, inner diameter 75 μ m; Thermo Fisher Scientific) with 0.1% trifluoroacetic acid, and peptides were separated on a 50-cm PepMap RSLC C18 column (particles 2 μ m, 100 Å, inner diameter 75 μ m; Thermo Fisher Scientific) at a constant temperature of 50 °C. The gradient 5–32% ACN in 0.1% FA was run over a period of 152 min (7 min 5%, 105 min to 22%, 10 min to 32%, followed by 10 min to 90%, then a 10 min wash at 90%, and a 10 min equilibration at 5%) at a flow rate of 300 nL·min $^{-1}$. Survey scans (m/z 300–1500) were acquired in the orbitrap with a resolution of

70 000 at m/z 200, and the maximum injection time was set to 80 ms (target value 3e6). Data-dependent HCD fragmentation scans of the 12 most intense ions in the survey scans were acquired in the orbitrap at a resolution of 17 500, using a maximum injection time of 50 ms and minimum and maximum AGC targets of 5e3 and 5e4, respectively. The isolation window was set to 1.6 m/z . Unassigned and singly charged ions were excluded from the measurement, and the dynamic exclusion of peptides was enabled for 60 s. The lock mass ion 445.12002 from ambient air was used for real-time mass calibration of the Q Exactive Plus. Data were acquired using XCALIBUR software version 3.1sp3 (Thermo Fisher Scientific).

The raw MS files were analyzed using MAXQUANT software (version 1.5.3.8) (<https://www.maxquant.org>). MS/MS-based peptide identification was carried out using the Andromeda search engine with the fasta file containing *E. coli* EF-P. The following parameter settings were used: peptide and protein FDR, 1%; enzyme, chymotrypsin; minimal number of amino acids required for peptide identification, 7; variable modification, methionine oxidation; and fixed modification, carbamidomethylation. At least one unique peptide was required for the identification of the protein. All other parameters were used according to the default settings. To identify putatively unknown modifications, the search for dependent peptides was enabled. Potential contaminants and reverse hits were removed from the result lists. Searches for lysine, hydroxylysine, ornithine, 6-aminohexanic acid (6-AC), and diaminopimelic acid (DAP) modifications were carried out with MAXQUANT, defining these targets as variable modifications on lysine with the compositions C₆H₁₂N₂O (MW = 128.09), C₆H₁₂N₂O₂ (MW = 144.09), C₅H₁₀N₂O (MW = 115.14), C₆H₁₁NO (MW = 113.084), and C₇H₁₂N₂O₃ (MW = 172.08), respectively.

In-gel protein digestion

EF-P modified *in vitro* by EpmA_A298G with (*S*)- α -ornithine as substrate was subjected to IEF, and the gel was stained with Coomassie Blue. The bands were excised and washed successively (at 25 °C and 550 r.p.m.) with 100 μ L H₂O for 15 min, 200 μ L ACN : ABC buffer 1 : 1 (ABC buffer: 50 mM ammonium bicarbonate) for 15 min, 100 μ L ACN for 10 min, 100 μ L ABC buffer for 5 min, 100 μ L ACN for 15 min, and a further 100- μ L aliquot of ACN for 10 min, and dried for 15 min in a SpeedVac. The proteins were reduced by adding 100 μ L of 10 mM DTT in ABC buffer to each gel piece, and incubating for 50 min at 45 °C, alkylated, digested with chymotrypsin, and subjected to MS analysis essentially as described above [61].

In vitro transcription/translation assay

The PURExpress *In Vitro* Protein Synthesis Kit (New England Biolabs) was used according to the manufacturer's

instructions, but reactions were supplemented with unmodified EF-P, *in vivo* modified EF-P, EF-P^{R^{Blys}} (as positive control) or *in vitro* modified EF-P (described above), and a plasmid coding for the EF-P-dependent RPPP-*nluc*. Luminescence was measured over time.

For a 12.5- μ L reaction mixture, 5 μ L of PURExpress solution A and 3.75 μ L of solution B, 0.25 μ L of Murine RNase inhibitor (New England Biolabs), 5 μ M EF-P, and 1 ng pET16b_ProProPro-*nluc* are incubated under agitation (300 r.p.m.) at 37 °C. At various time points, a 1- μ L aliquot was quenched with 1 μ L of 50 mg-mL⁻¹ kanamycin and stored on ice. Afterward, 2 μ L of Nano-Glo Luciferase Assay Reagent (Promega) and 18 μ L ddH₂O were added to induce luminescence development, which was detected by the Infinite F500 microplate reader (Tecan). At least three independent replicates were analyzed, and the statistical significance of the result was determined by two-tailed *t*-test using GRAPHPAD PRISM.

β -galactosidase activity assay

Escherichia coli BW25113 and the deletion mutants BW25113 Δ *epmA* and BW25113 Δ *epmB* were transformed with the plasmid p3LC-TL30-3P-CCG [18]. Complementation of the deletion mutants as well as cross-complementation was performed using plasmids pBAD33_sRBS_His₆*epmA*, pBAD33_sRBS_His₆*epmA*_A298G, or pBAD33_His₁₀*epmB*, respectively. Cotransformants were grown in LB medium under inducing conditions overnight and harvested by centrifugation. Pellets were resuspended in sodium phosphate buffer, and β -galactosidase measurements were performed as previous described [62]. Values are given in Miller Units (MU), which were calculated according to Miller [63].

Native purification

Escherichia coli BW25113 cells co-transformed with the plasmids p3LC-TL30-3P-CC G and pBAD33, or *E. coli* BW25113 Δ *epmB* co-transformed with the plasmids p3LC-TL30-3P-CCG and pBAD33_sRBS_His₆*epmA* or pBAD33_sRBS_His₆*epmA*_A298G were grown to the exponential growth phase in 1 L of LB medium at 37 °C. Expression of *epmA* was induced by adding 0.2% (w/v) L (+)-arabinose, and cells were grown at 18 °C overnight. The cells were then harvested, and the pellet was resuspended in 20 mM triethanolamine (pH 7.5). After 30 min of incubation with DNase and phenylmethylsulfonyl fluoride, cells were disrupted in a continuous-flow cabinet from Constant Systems Ltd. at 1.35 kbar. The resulting lysates were clarified by centrifugation at 4 °C at 235 000 *g* for 1.5 h. Anion-exchange chromatography was performed on an ÄKTA pure chromatography system (GE Healthcare, Chicago, IL, USA) equipped with the fraction collector F9-C and the Mono Q 10/100 GL column. Proteins were

eluted using a salt gradient from 0 to 1 M KCl. The fraction with the highest EF-P concentration was determined by SDS/PAGE followed by western blotting and detection with anti-EF-P *E. coli* western blotting. Size-exclusion chromatography was performed in HEPES buffer on a Superdex 200 Increase 10/300 GL (GE Healthcare) column. Samples were assayed for their EF-P modification status by IEF on gels followed by western blotting, or by MS analysis.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

MP, JL, and KJ designed research. MP, PK, RK, and WV performed experiments. MP, PK, and SAS analyzed data. MP, RK, JL, and KJ wrote the paper.

References

- Schimmel P (1987) Aminoacyl tRNA synthetases: general scheme of structure-function relationships in the polypeptides and recognition of transfer RNAs. *Annu Rev Biochem* **56**, 125–158.
- Wolf YI, Aravind L, Grishin NV & Koonin EV (1999) Evolution of aminoacyl-tRNA synthetases – analysis of unique domain architectures and phylogenetic trees reveals a complex history of horizontal gene transfer events. *Genome Res* **9**, 689–710.
- Sugiura I, Nureki O, Ugaji-Yoshikawa Y, Kuwabara S, Shimada A, Tateno M, Lorber B, Giege R, Moras D, Yokoyama S *et al.* (2000) The 2.0 Å crystal structure of *Thermus thermophilus* methionyl-tRNA synthetase reveals two RNA-binding modules. *Structure* **8**, 197–208.
- Artymiuk PJ, Rice DW, Poirrette AR & Willet P (1994) A tale of two synthetases. *Nat Struct Biol* **1**, 758–760.
- Smith TF & Hartman H (2015) The evolution of class II aminoacyl-tRNA synthetases and the first code. *FEBS Lett* **589**, 3499–3507.
- Lu Y & Hill KA (1994) The invariant arginine in motif 2 of *Escherichia coli* alanyl-tRNA synthetase is important for catalysis but not for substrate binding. *J Biol Chem* **269**, 12137–12141.
- Siatecka M, Rozek M, Barciszewski J & Mirande M (1998) Modular evolution of the Glx-tRNA synthetase family – rooting of the evolutionary tree between the bacteria and archaea/eukarya branches. *Eur J Biochem* **256**, 80–87.
- Ibba M, Bono JL, Rosa PA & Soll D (1997) Archaeal-type lysyl-tRNA synthetase in the Lyme disease spirochete *Borrelia burgdorferi*. *Proc Natl Acad Sci USA* **94**, 14383–14388.
- Ibba M, Morgan S, Curnow AW, Pridmore DR, Vothknecht UC, Gardner W, Lin W, Woese CR & Soll D (1997) A euryarchaeal lysyl-tRNA synthetase: resemblance to class I synthetases. *Science* **278**, 1119–1122.
- Koonin EV & Aravind L (1998) Genomics: re-evaluation of translation machinery evolution. *Curr Biol* **8**, R266–R269.
- Motzik A, Nechushtan H, Foo SY & Razin E (2013) Non-canonical roles of lysyl-tRNA synthetase in health and disease. *Trends Mol Med* **19**, 726–731.
- Clark RL & Neidhardt FC (1990) Roles of the two lysyl-tRNA synthetases of *Escherichia coli*: analysis of nucleotide sequences and mutant behavior. *J Bacteriol* **172**, 3237–3243.
- Despotovic D, Brandis A, Savidor A, Levin Y, Fumagalli L & Tawfik DS (2017) Diadenosine tetraphosphate (Ap₄A) – an *E. coli* alarmone or a damage metabolite? *FEBS J* **284**, 2194–2215.
- Luciano DJ, Levenson-Palmer R & Belasco JG (2019) Stresses that raise Np₄A levels induce protective nucleoside tetraphosphate capping of bacterial RNA. *Mol Cell* **75**, 957–966.e8.
- Kong L, Fromant M, Blanquet S & Plateau P (1991) Evidence for a new *Escherichia coli* protein resembling a lysyl-tRNA synthetase. *Gene* **108**, 163–164.
- Bailly M & de Crécy-Lagard V (2010) Predicting the pathway involved in post-translational modification of elongation factor P in a subset of bacterial species. *Biol Direct* **5**, 3.
- Lassak J, Keilhauer EC, Furst M, Wuichet K, Godeke J, Starosta AL, Chen J-M, Sogaard-Andersen L, Rohr J, Wilson DN *et al.* (2015) Arginine-rhamnosylation as new strategy to activate translation elongation factor P. *Nat Chem Biol* **11**, 266–270.
- Ude S, Lassak J, Starosta AL, Kraxenberger T, Wilson DN & Jung K (2013) Translation elongation factor EF-P alleviates ribosome stalling at polyproline stretches. *Science* **339**, 82–85.
- Peil L, Starosta AL, Lassak J, Atkinson GC, Virumae K, Spitzer M, Tenson T, Jung K, Remme J & Wilson DN (2013) Distinct XPPX sequence motifs induce ribosome stalling, which is rescued by the translation elongation factor EF-P. *Proc Natl Acad Sci USA* **110**, 15265–15270.
- Starosta AL, Lassak J, Peil L, Atkinson GC, Virumae K, Tenson T, Remme J, Jung K & Wilson DN (2014) Translational stalling at polyproline stretches is modulated by the sequence context upstream of the stall site. *Nucleic Acids Res* **42**, 10711–10719.

- 21 Lassak J, Wilson DN & Jung K (2016) Stall no more at polyproline stretches with the translation elongation factors EF-P and IF-5A. *Mol Microbiol* **99**, 219–235.
- 22 Doerfel LK, Wohlgemuth I, Kothe C, Peske F, Urlaub H & Rodnina MV (2013) EF-P is essential for rapid synthesis of proteins containing consecutive proline residues. *Science* **339**, 85–88.
- 23 Gilreath MS, Roy H, Bullwinkle TJ, Katz A, Navarre WW & Ibba M (2011) β -Lysine discrimination by lysyl-tRNA synthetase. *FEBS Lett* **585**, 3284–3288.
- 24 Huter P, Arenz S, Bock LV, Graf M, Frister JO, Heuer A, Peil L, Starosta AL, Wohlgemuth I, Peske F *et al.* (2017) Structural basis for polyproline-mediated ribosome stalling and rescue by the translation elongation factor EF-P. *Mol Cell* **68**, 515–527.e6.
- 25 Ambrogelly A, O'Donoghue P, Söll D & Moses S (2010) A bacterial ortholog of class II lysyl-tRNA synthetase activates lysine. *FEBS Lett* **584**, 3055–3060.
- 26 Roy H, Zou SB, Bullwinkle TJ, Wolfe BS, Gilreath MS, Forsyth CJ, Navarre WW & Ibba M (2011) The tRNA synthetase paralog PoxA modifies elongation factor-P with (*R*)- β -lysine. *Nat Chem Biol* **7**, 667–669.
- 27 Rajkovic A, Hummels KR, Witzky A, Erickson S, Gafken PR, Whitelegge JP, Faull KF, Kearns DB & Ibba M (2016) Translation control of swarming proficiency in *Bacillus subtilis* by 5-amino-pentanoylated elongation factor P. *J Biol Chem* **291**, 10976–10985.
- 28 Hummels KR, Witzky A, Rajkovic A, Tollerson R, Jones LA, Ibba M & Kearns DB (2017) Carbonyl reduction by YmfI in *Bacillus subtilis* prevents accumulation of an inhibitory EF-P modification state. *Mol Microbiol* **106**, 236–251.
- 29 Rajkovic A, Erickson S, Witzky A, Branson OE, Seo J, Gafken PR, Frietas MA, Whitelegge JP, Faull KF, Navarre W *et al.* (2015) Cyclic rhamnosylated elongation factor P establishes antibiotic resistance in *Pseudomonas aeruginosa*. *MBio* **6**, e00823.
- 30 Schimmel P & Schmidt E (1995) Making connections: RNA-dependent amino acid recognition. *Trends Biochem Sci* **20**, 1–2.
- 31 Jakubowski H (1999) Misacylation of tRNA^{Lys} with noncognate amino acids by lysyl-tRNA synthetase. *Biochemistry* **38**, 8088–8093.
- 32 Starosta AL, Lassak J, Jung K & Wilson DN (2014) The bacterial translation stress response. *FEMS Microbiol Rev* **38**, 1172–1201.
- 33 Splan KE, Musier-Forsyth K, Boniecki MT & Martinis SA (2008) In vitro assays for the determination of aminoacyl-tRNA synthetase editing activity. *Methods* **44**, 119–128.
- 34 Baldwin AN & Berg P (1966) Transfer ribonucleic acid-induced hydrolysis of valyladenylate bound to isoleucyl ribonucleic acid synthetase. *J Biol Chem* **241**, 839–845.
- 35 Hati S, Ziervogel B, Sternjohn J, Wong F-C, Nagan MC, Rosen AE, Siliciano PG, Chihade JW & Musier-Forsyth K (2006) Pre-transfer editing by class II prolyl-tRNA synthetase: role of aminoacylation active site in "selective release" of noncognate amino acids. *J Biol Chem* **281**, 27862–27872.
- 36 Guth E, Farris M, Bovee M & Francklyn CS (2009) Asymmetric amino acid activation by class II histidyl-tRNA synthetase from *Escherichia coli*. *J Biol Chem* **284**, 20753–20762.
- 37 Michaelis L, Menten ML, Johnson KA & Goody RS (2011) The original Michaelis constant: translation of the 1913 Michaelis-Menten paper. *Biochemistry* **50**, 8264–8269.
- 38 An G, Glick BR, Friesen JD & Ganoza MC (1980) Identification and quantitation of elongation factor EF-P in *Escherichia coli* cell-free extracts. *Can J Biochem* **58**, 1312–1314.
- 39 Beyer H & Walter W (1991) Lehrbuch der Organischen Chemie, 22nd edn. S. Hirzel Verlag, Stuttgart.
- 40 Haynes WM (2017) CRC Handbook of Chemistry and Physics: A Ready-Reference Book of Chemical and Physical Data, 97th edn. CRC Press, Boca Raton, FL; London; New York, NY.
- 41 Behshad E, Ruzicka FJ, Mansoorabadi SO, Chen D, Reed GH & Frey PA (2006) Enantiomeric free radicals and enzymatic control of stereochemistry in a radical mechanism: the case of lysine 2,3-aminomutases. *Biochemistry* **45**, 12639–12646.
- 42 Navarre WW, Zou SB, Roy H, Xie JL, Savchenko A, Singer A, Edvokimova E, Prost LR, Kumar R, Ibba M *et al.* (2010) PoxA, yjeK, and elongation factor P coordinately modulate virulence and drug resistance in *Salmonella enterica*. *Mol Cell* **39**, 209–221.
- 43 Yanagisawa T, Sumida T, Ishii R, Takemoto C & Yokoyama S (2010) A paralog of lysyl-tRNA synthetase aminoacylates a conserved lysine residue in translation elongation factor P. *Nat Struct Mol Biol* **17**, 1136–1143.
- 44 Park J-H, Johansson HE, Aoki H, Huang BX, Kim H-Y, Ganoza MC & Park MH (2012) Post-translational modification by β -lysylation is required for activity of *Escherichia coli* elongation factor P (EF-P). *J Biol Chem* **287**, 2579–2590.
- 45 Bennett BD, Kimball EH, Gao M, Osterhout R, van Dien SJ & Rabinowitz JD (2009) Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nat Chem Biol* **5**, 593–599.
- 46 Peil L, Starosta AL, Virumae K, Atkinson GC, Tenson T, Remme J & Wilson DN (2012) Lys34 of translation elongation factor EF-P is hydroxylated by YfcM. *Nat Chem Biol* **8**, 695–697.
- 47 Starosta AL, Lassak J, Peil L, Atkinson GC, Woolstenhulme CJ, Virumae K, Buskirk A, Tenson T,

- Remme J, Jung K *et al.* (2014) A conserved proline triplet in Val-tRNA synthetase and the origin of elongation factor P. *Cell Rep* **9**, 476–483.
- 48 England CG, Ehlerding EB & Cai W (2016) NanoLuc: a small luciferase is brightening up the field of bioluminescence. *Bioconjug Chem* **27**, 1175–1187.
- 49 Witzky A, Hummels KR, Tollerson R, Rajkovic A, Jones LA, Kearns DB & Ibba M (2018) EF-P posttranslational modification has variable impact on polyproline translation in *Bacillus subtilis*. *mBio* **9**, e00306-18.
- 50 Volkwein W, Maier C, Krafczyk R, Jung K & Lassak J (2017) A versatile toolbox for the control of protein levels using N ϵ -acetyl-L-lysine dependent amber suppression. *ACS Synth Biol* **6**, 1892–1902.
- 51 Umehara T, Kim J, Lee S, Guo L-T, Soll D & Park H-S (2012) N-acetyl lysyl-tRNA synthetases evolved by a CcdB-based selection possess N-acetyl lysine specificity *in vitro* and *in vivo*. *FEBS Lett* **586**, 729–733.
- 52 Schmidt C, Becker T, Heuer A, Braunger K, Shanmuganathan V, Pech M, Berninghausen O, Wilson DN & Beckmann R (2016) Structure of the hypusylated eukaryotic translation factor eIF5A bound to the ribosome. *Nucleic Acids Res* **44**, 1944–1951.
- 53 Melnikov S, Mailliot J, Shin B-S, Rigger L, Yusupova G, Micura R, Dever TE & Yusupov M (2016) Crystal structure of hypusine-containing translation factor eIF5A bound to a rotated eukaryotic ribosome. *J Mol Biol* **428**, 3570–3576.
- 54 Schultz DW & Yarus M (1990) A simple and sensitive *in vivo* luciferase assay for tRNA-mediated nonsense suppression. *J Bacteriol* **172**, 595–602.
- 55 Brabham R & Fascione MA (2017) Pyrrolysine amber stop-codon suppression: development and applications. *ChemBioChem* **18**, 1973–1983.
- 56 Vargas-Rodriguez O, Sevostyanova A, Soll D & Crnkovic A (2018) Upgrading aminoacyl-tRNA synthetases for genetic code expansion. *Curr Opin Chem Biol* **46**, 115–122.
- 57 Volkwein W, Krafczyk R, Jagtap PKA, Parr M, Mankina E, Macošek J, Guo Z, Fürst MJLJ, Pfab M, Frishman D *et al.* (2019) Switching the post-translational modification of translation elongation factor EF-P. *Front Microbiol* **10**, 2100.
- 58 Gallego-Jara J, Écija Conesa A, de Diego Puente T, Lozano Terol G & Cánovas Díaz M (2017) Characterization of CobB kinetics and inhibition by nicotinamide. *PLoS ONE* **12**, e0189689.
- 59 Schneider CA, Rasband WS & Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* **9**, 671–675.
- 60 Marty MT, Baldwin AJ, Marklund EG, Hochberg GKA, Benesch JLP & Robinson CV (2015) Bayesian deconvolution of mass and ion mobility spectra: from binary interactions to polydisperse ensembles. *Anal Chem* **87**, 4370–4376.
- 61 Shevchenko A, Tomas H, Havlis J, Olsen JV & Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc* **1**, 2856–2860.
- 62 Tetsch L, Koller C, Haneburger I & Jung K (2008) The membrane-integrated transcriptional activator CadC of *Escherichia coli* senses lysine indirectly via the interaction with the lysine permease LysP. *Mol Microbiol* **67**, 570–583.
- 63 Miller JH (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, New York, NY.
- 64 Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL & Mori H (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2**, 2006.0008.
- 65 Platt R, Drescher C, Park SK & Phillips GJ (2000) Genetic system for reversible integration of DNA constructs and *lacZ* gene fusions into the *Escherichia coli* chromosome. *Plasmid* **43**, 12–23.
- 66 Guzman LM, Belin D, Carson MJ & Beckwith J (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. *J Bacteriol* **177**, 4121–4130.
- 67 Ude S (2013) The role of elongation factor EF-P in translation and in copy number control of the transcriptional regulator CadC in *Escherichia coli*. Dissertation, Munich.
- 68 Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC & Ferrin TE (2004) UCSF Chimera—a visualization system for exploratory research and analysis. *J. Comput. Chem* **25**, 1605–1612.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. (R)-3-AC exhibits a concentration-dependent substrate inhibition *in vitro*.

Fig. S2. Mass spectrometric analyses of *in vitro* modified EF-Ps.

Fig. S3. Transcription/translation efficiency in presence of successfully *in vitro* modified EF-P.

Fig. S4. Production of acetylated EF-P using amber suppression.

Fig. S5. Models of altered EF-P-tRNA and EF-P-ribosome (23S rRNA) interactions induced by synthetic post-translational modifications.

Table S1. Strains, plasmids and oligonucleotide sequences.