A method to enrich polypeptidyl-tRNAs to capture snapshots of translation in the cell

Ayako Yamakawa[1], Tatsuya Niwa[1,2,*], Yuhei Chadani[2,*] and Hideki Taguchi[1,2,*]

[1] School of Life Science and Technology, Tokyo Institute of Technology, S2-19, Nagatsuta 4259, Midori-ku, Yokohama, 226-8503, Japan.

[2] Cell Biology Center, Institute of Innovative Research, Tokyo Institute of Technology, S2-19, Nagatsuta 4259, Midori-ku, Yokohama, 226-8503, Japan.

*Correspondence to: tniwa@bio.titech.ac.jp, chadani.y.aa@m.titech.ac.jp, taguchi@bio.titech.ac.jp

Abstract

Life depends on proteins, which all exist in nascent states when the growing polypeptide chain is covalently attached to a tRNA within the ribosome. Although the nascent chains; *i.e.,* polypeptidyl-tRNAs (pep-tRNAs), are considered as merely transient intermediates during protein synthesis, recent advances have revealed that they are directly involved in a variety of cell functions, such as gene expression control. An increasing appreciation for fine-tuning at translational levels demands a general method to handle the pep-tRNAs on a large scale. Here, we developed a method to enrich the pep-tRNAs by organic solvent extraction and silica column separation, and then identify their polypeptide moieties by mass spectrometry. As a proof-of-concept experiment using *Escherichia coli*, we identified ~500 peptides derived from the pep-tRNAs, which were markedly biased towards the N-terminal regions, reflecting that the method captured the intermediate pep-tRNA population during translation. Furthermore, the method identified a subset of pep-tRNAs that were resistant to puromycin treatment, suggesting that these pep-tRNAs accumulated due to nascent-chain dependent translational pausing. Although the current specificity and comprehensiveness of the pep-tRNA enrichment have room for improvement, this new method will complement conventional methods for profiling nascent chains.

Significance Statement

In the central dogma of biology, RNA, an informational molecule, and protein, a functional molecule, are usually regarded as two completely independent molecular species. However, they are combined into a single species called peptidyl-tRNA (pep-tRNA) during the translation process in the ribosome. Despite the importance of pep-tRNAs as precursors of all proteins in the cell, a general method to analyze pep-tRNAs on a large scale was lacking. Taking advantage of the properties of pep-tRNAs as RNA and protein, we developed a method termed *pe*ptidyl-*t*RNA *e*nrichment using *o*rganic extraction and *s*ilica adsorption (PETEOS). PETEOS not only provides a unique approach to examine the nascent state of proteins but also may be effective in exploring peptides that cause translational pauses.

Keywords: translation, nascent polypeptide chain, polypeptidyl-tRNA, proteomics, ribosome, mass spectrometry

Introduction

Every protein in the cell is generated through translation. The formation of the proteome involves the regulation of various steps in gene expression. In addition to the well-known importance of gene regulation at transcriptional levels, gene expression control at translational levels has been increasingly appreciated (1, 2).

The translational control is diverse, flexible, and rapid, depending on cellular contexts or stimuli such as a variety of stresses. In addition to the control at the translation initiation step, which is a major rate-determining step in translation, even the translation elongation could be regulated (3, 4). One of the fine-tuning events is elongation rate control, as exemplified by elongation pausing, which can affect the folding, function, and quality control of proteins (3–7). Besides the pausing induced by mRNA secondary structures or tRNA shortages (4, 5, 7, 8), both of which could be essentially predictable, nascent polypeptides within the ribosome could pause the elongation (9–12). For example, the translation product of the gene *secM* in *Escherichia coli* induces translation arrest (13, 14). The ribosomal arrest peptide of SecM, still tethered to its tRNA as a polypeptidyl-tRNA (pep-tRNA), interacts with the inner wall of the ribosomal exit tunnel (3, 14, 15). The translation arrest by SecM is directly involved in the feedback regulation of its downstream gene, *secA* (13, 14). Other genes identified in bacteria and eukaryotes also cause translation arrest (16–19). In addition to the ad hoc identification, biochemical analyses of over 1,000 *E. coli* genes revealed that a majority of them indeed undergo single or multiple pauses in translation, indicating the widespread occurrence of translational pausing (20). Notably, more than half of the pauses are resistant to puromycin (20), suggesting that the ribosomal function has been impaired, as shown for the arrest by SecM (21). However, so far, a comprehensive picture of amino acid sequence motifs that induce pausing remains obscure. Collectively, there is an increasing demand for methods to globally investigate the translation process in the cell.

So far, two main methodologies based on completely different concepts are used to survey the translation process in cells (22). One is ribosome profiling, a deep sequencing technique for ribosome-protected mRNA fragments generated by RNase digestion (23). Ribosome profiling is a very powerful genome-wide analysis with nucleotide resolution, and has been established as a method to monitor the translation status in cells (22–27). However, this method, which relies on a huge amount of "ribosome footprint" sequencing data, is inherently indirect in detecting the nascent proteins or pep-tRNAs, and might cause some controversy about how to collect and interpret the data (*e.g.*, 28). In addition, the method is still time-consuming and costly.

The other methodology is based on proteomics with liquid chromatography-tandem mass

spectrometry (LC-MS/MS). Several "translatome" methods have been developed to distinguish nascent proteins from the pre-existing protein population. The proteomics-based approaches rely on the labeling of nascent polypeptide chains, using noncanonical amino acids such as azide homoalanine (*e.g.,* BONCAT (29), QuaNCAT (30)) and puromycin analogs (*e.g.,* PUNCH-P (31, 32), OPP-ID (33)). Since the vast majority of nascent proteins labeled by the noncanonical amino acids have already been released from the ribosome, these proteins are newly but already "synthesized" mature proteins, and not nascent proteins during translation. The puromycin-related methods rely on the principle that puromycin or its analogs terminate translation by entering the A-site of the ribosome, and are then covalently incorporated into the growing end of the nascent peptide moiety in pep-tRNAs. However, certain nascent peptides are resistant to puromycin (16, 18, 21). In addition, all of these methods that rely on labeling usually require an hour-long timescale or more to accumulate the labeled products (34). In summary, these analytical methods for the nascent proteome do not directly deal with pep-tRNAs and do not capture the moments of the translation elongation status in the cell.

In view of these points, a direct methodology to handle pep-tRNAs is required to complement the prevailing methods for the translatome analyses. Although the point is not always appreciated, it is obvious that all proteins are born via pep-tRNAs, which possess the properties of both RNA and protein. In other words, pep-tRNA is an essential translation intermediate that lies between RNA and protein in the central dogma of biology. Despite their importance in life, pep-tRNAs are low-abundant. Assuming that the approximate numbers of proteins and ribosomes in *E. coli* are ~3 million and ~70,000, respectively (35), the maximum amount of pep-tRNAs is at most only ~2% of total proteins, even if all ribosomes participate in translation.

These "in-between" properties of both RNA and protein and the low abundance make pep-tRNAs challenging to handle on the "pep-tRNA-ome" scale. Ito *et al.* developed an ingenious method to collectively detect cellular pep-tRNAs, the "nascentome," using a combination of pulse-labeling and two-dimensional electrophoresis (36). The "nascentome" analysis can detect the whole pep-tRNAs but cannot identify which protein is part of each pep-tRNA. Therefore, we developed a non-labeling method to enrich and identify pep-tRNAs in cells. Briefly, pep-tRNAs in cells are enriched by organic solvent extraction, followed by isolation on a silica column. The polypeptide moieties in the enriched pep-tRNAs are cleaved under high pH and high temperature conditions. The resultant polypeptides are digested by proteases such as trypsin and then identified using conventional LC-MS/MS-based shotgun proteomics. This method, termed *pe*ptidyl-*t*RNA *e*nrichment using *o*rganic extraction and *s*ilica adsorption (PETEOS), is not only complementary to prevailing translation measurement methods, but also

103 has, in principle, several advantages such as rapid capturing of translation status, non-labeling
104 feature, and compatibility with puromycin treatment to detect elongation pausing candidates.
105
106 **Results**
107 **Polypeptidyl-tRNA enrichment and identification using a proteomics approach.**
108 The chemical properties of pep-tRNAs with various polypeptide moieties are diverse. For
109 example, when the peptide moiety is hydrophilic or relatively short, the hydrophilic property
110 of the RNA is dominant, but when the polypeptide portion is more hydrophobic or longer, the
111 more protein-like property is manifested. As far as we know, there is no established method to
112 purify whole pep-tRNAs from cells. Our strategy to enrich pep-tRNAs takes advantage of their
113 two distinctive properties as RNA and polypeptide. The method, which we call PETEOS, is
114 composed of four steps (Fig. 1). Step 1: Enrichment of pep-tRNAs and proteins from cells.
115 Harvested cells are directly mixed into a phenol-containing reagent (TriPURE) to separate most
116 RNAs (aqueous phase) from pep-tRNAs and proteins (organic phase). Note that there is no
117 need for labeling times in this method. After the chloroform addition, the pep-tRNAs and
118 proteins in the organic phase are precipitated by trichloroacetic acid (TCA)/acetone, under
119 conditions where the ester bonds between polypeptide and tRNA in the pep-tRNAs are retained.
120 Step 2: Isolation of the pep-tRNAs. The RNA is isolated by using silica to adsorb the nucleic
121 acid portions of pep-tRNAs, under highly concentrated chaotropic salt conditions (37, 38). Step
122 3: Cleavage of peptide moieties from peptidyl-tRNAs. The ester bond between peptide and
123 tRNA is cleaved by high pH and heat treatment. Step 4: Identification of the peptides after
124 trypsin treatment, by LC-MS/MS-based shotgun proteomics.
125
126 **Proof-of-concept analysis using *Escherichia coli* cell lysates.**
127 The validity of the PETEOS method was evaluated at each step. An LC-MS/MS analysis
128 of the TCA/acetone precipitate in Step 1 revealed that ~79% of the identified proteins in the
129 precipitate overlapped with proteins identified by a conventional method (39) to extract proteins
130 from cells (Fig. S1), confirming that the protein fraction in the organic phase seems to lack
131 significant bias. Next, when we analyzed the eluate at Step 2 by SDS-PAGE under neutral pH
132 conditions (neutral-PAGE), where the ester bonds between peptide and tRNA in the pep-tRNAs
133 remain intact, smeared silver-stained bands were observed (Fig. 2A). Since an RNase treatment
134 of the mixture at Step 1 before the adsorption to the silica column drastically reduced the
135 smeared bands (Fig. 2A), they are derived from pep-tRNA species.
136 After the peptide moieties from the pep-tRNAs were obtained by the alkaline and heat
137 treatment (Step 3), the LC-MS/MS analysis (Step 4) identified more than 500 proteins

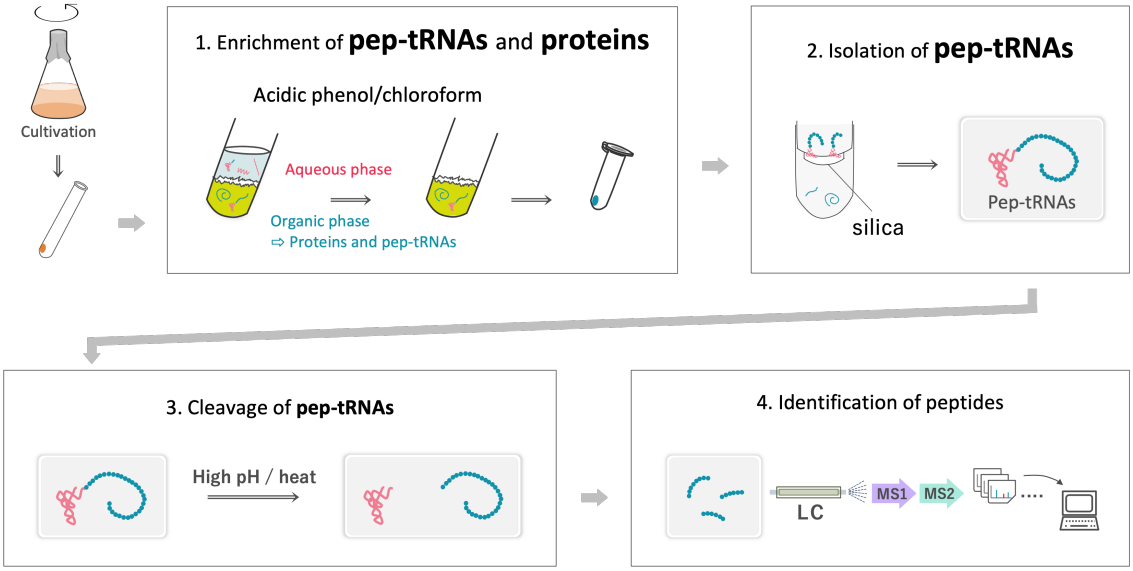

Figure 1. PETEOS workflow to enrich and identify polypeptidyl-tRNAs (pep-tRNAs) in cells
Step 1: Enrichment of pep-tRNAs and proteins. Log phase *E. coli* cells ($OD_{660} \approx 0.5$) cultured at 37 °C are extracted by phenol/chloroform to separate the hydrophilic RNA molecules into the aqueous phase. Relatively hydrophobic proteins and pep-tRNAs in the organic phase are precipitated by TCA/acetone. **Step 2**: Isolation of the pep-tRNAs by silica column chromatography in the presence of concentrated chaotropic salts. **Step 3**: Hydrolysis of the ester bond between peptide and tRNA in pep-tRNA under high pH and high temperature conditions. **Step 4**: Identification of the peptide moieties of the pep-tRNAs by conventional shotgun proteomics after trypsin digestion.

(Fig. 2B). The number of identified proteins was reduced to ~50 proteins in the RNase-treated fraction after Step 1 (Fig. 2B), indicating that ~90% of the identified proteins would be derived from pep-tRNAs. In addition, the intensity distribution of the MS/MS-identified peptides was significantly higher in the proteins obtained without the RNase treatment (Fig. 2C). Collectively, the PETEOS method can enrich and identify the cellular pep-tRNAs.

Next, we validated the potential of the PETEOS method to identify peptides paused at a specific position within the ribosome. A particular length of peptides in a pep-tRNA mixture would accumulate if the C-terminal end of the pausing peptide is tethered to a tRNA at the pausing site. Accordingly, we overexpressed a well-known pausing peptide, SecM. The translation of the gene *secM* is arrested at the 165th glycine, where $tRNA^{Gly}$ is covalently attached to the elongating end of the nascent peptide ($SecM_{1\text{-}165}$-$tRNA^{Gly}$). As expected, the MS/MS analysis identified the SecM-derived pausing peptide ($_{150}$FSTPVWISQAQGIRAG$_{165}$) from the SecM-overexpressing cells (Fig. 2D), indicating that the method could identify paused peptides at an amino acid sequence resolution. We also overexpressed the known pausing peptide, TnaC ($_{1}$MNILHICVTS KWFNIDNKIV DHRP$_{24}$-stop codon), which induces ribosome pausing at the C-terminus. The MS/MS analysis identified a TnaC peptide ($_{12}$WFNIDNK$_{18}$), which would be derived from the trypsinized product of the TnaC pausing

peptide. Note that the MS analysis also detected a TnaC-derived peptide in the aqueous phase at Step 1, indicating that the separation of the TnaC-tRNA is incomplete with the phenol/chloroform extraction. The insufficient separation is probably due to the hydrophilic nature of the TnaC-tRNA, since its polypeptide moiety is relatively short (24 amino acids). Indeed, a silver-stained gel revealed the aqueous layer-biased accumulation of pep-tRNAs in the TnaC-overexpressing cells (Fig. S2).

A previous nascentome study showed that Tris-base in the buffer stimulates the ester bond cleavage of pep-tRNAs (36). A nucleophilic attack of tris(hydroxymethyl)aminomethane (Tris) on the carboxyl group of the C-terminal amino acid, which is ester-bonded to tRNA in the pep-tRNA, produces a Tris-adduct during the pep-tRNA hydrolysis (Fig. S3). Therefore, we speculated that some peptides derived from pep-tRNAs could become conjugated with Tris. A peptide search assuming the Tris-addition at the C-terminus revealed ~10 Tris-conjugated peptides, including a SecM-derived paused peptide (Fig. 2D, Table S1). Since the conjugation of the Tris-base confirmed the handling of intact pep-tRNAs until they are exposed to high pH and high temperature, the production of these Tris-replaced fragments is one of the hallmarks of the PETEOS method.

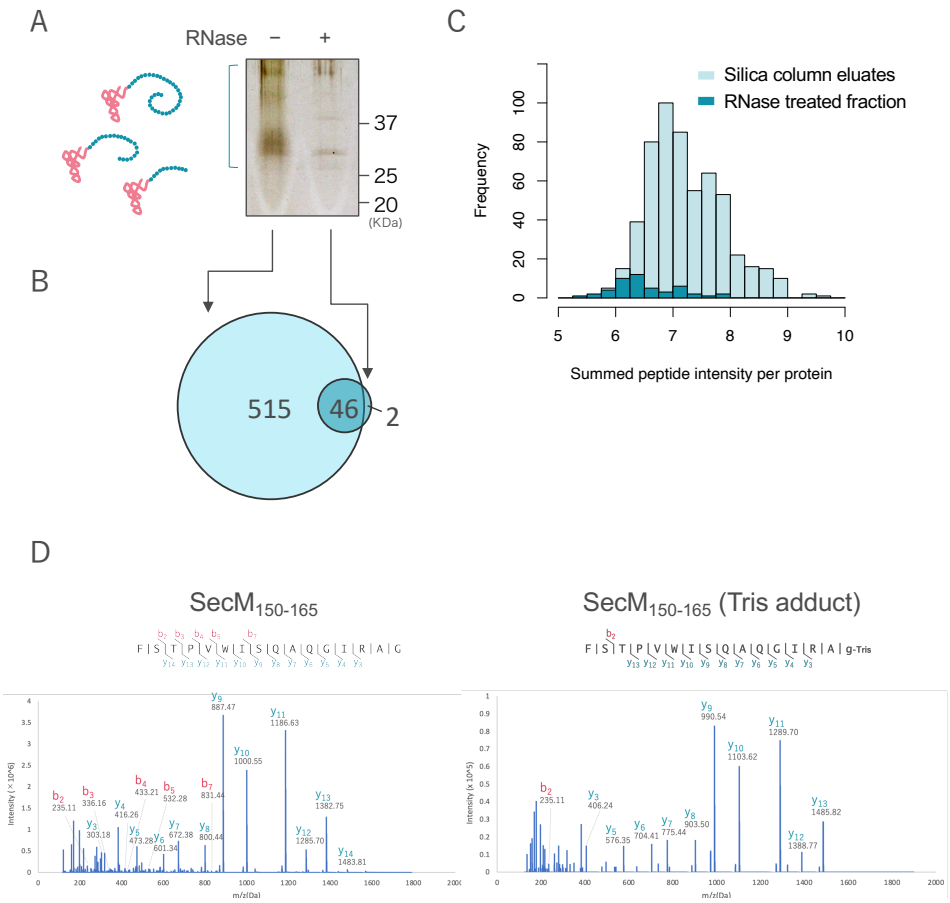

Figure 2. Enrichment and identification of pep-tRNAs in *E. coli*

A. The eluate from the silica column (Step 2) was analyzed by neutral pH SDS-PAGE, and detected by silver staining. The eluate treated with RNase after Step 1 was also analyzed. Pep-tRNA molecules are schematically indicated (tRNA moiety, orange; peptide moiety, blue). **B**. Number of proteins identified as pep-tRNA molecules in the method. The peptides detected more than once in the MS/MS analysis were counted. Among the 48 proteins identified in the RNase-treated fraction, 46 overlapped with the proteins derived from pep-tRNAs. **C**. Histogram of summed peptide intensity per protein ($\log_{10}$ scale) obtained by the MS1 peaks for proteins from the silica column eluate or the RNase-treated fraction. **D**. MS2 spectra of the C-terminal peptides of SecM from *E. coli* cells overexpressing SecM. (*Left*) $_{150}$FSTPVWISQAQGIRAG$_{165}$. (*Right*) The C-terminal peptide modified by Tris-base, $_{150}$FSTPVWISQAQGIRAG$_{165}$-Tris.

173

174 **The polypeptidyl-tRNA population reflects the translation intermediates.**

175 The MS-identified peptides derived from the pep-tRNAs in *E. coli* confirmed that the
176 peptides are intermediate species during the translation elongation. Since nascent peptides
177 elongate from the N- to C-terminus, we expected that the tryptic peptides derived from pep-
178 tRNAs would be enriched with the N-terminus as compared to those derived from pre-existing
179 total cellular proteins (Fig. 3A). A meta-analysis demonstrated that the tryptic peptides from
180 the pep-tRNAs were highly biased toward the N-terminal regions (Fig. 3B). In contrast, a meta-
181 analysis of the total proteins showed equally distributed tryptic peptides (Fig. 3B), as expected
182 since the total proteins are basically composed of the full-length polypeptides after translation.

183 In addition, a substantial population of pep-tRNAs (228 out of 515) was not identified in
184 the cells treated with puromycin (Fig. 3C), indicating that the puromycin released a subset of
185 the pep-tRNAs from the ribosome by replacing the tRNA moiety with puromycin.

186 Finally, the amount of Tris-adducted peptides was significantly increased in the pep-tRNA-
187 derived peptides, as compared to that in the total proteins (Fig. S4), confirming that the tRNAs
188 had been tethered to the C-terminus of the peptide moieties before the heat and high pH
189 treatments. Taken together, PETEOS allowed us to capture a global snapshot of nascent
190 polypeptide chains during translation.

191

192 **Puromycin-resistant pep-tRNAs accumulate in the cells.**

193 We next applied the PETEOS method to explore novel pausing peptide candidates, since
194 in principle, the method can enrich potential SecM-like pausing peptides after the puromycin
195 treatment, which is impossible by puromycin-labeling methods such as PUNCH-P.

196 Using an *E. coli* strain lacking the AcrAB-TolC multidrug efflux pump, to prevent the
197 efflux of puromycin (40), we identified 400 proteins derived from the pep-tRNAs after the
198 puromycin treatment (Fig. 3C), whereas the number of identified proteins was significantly

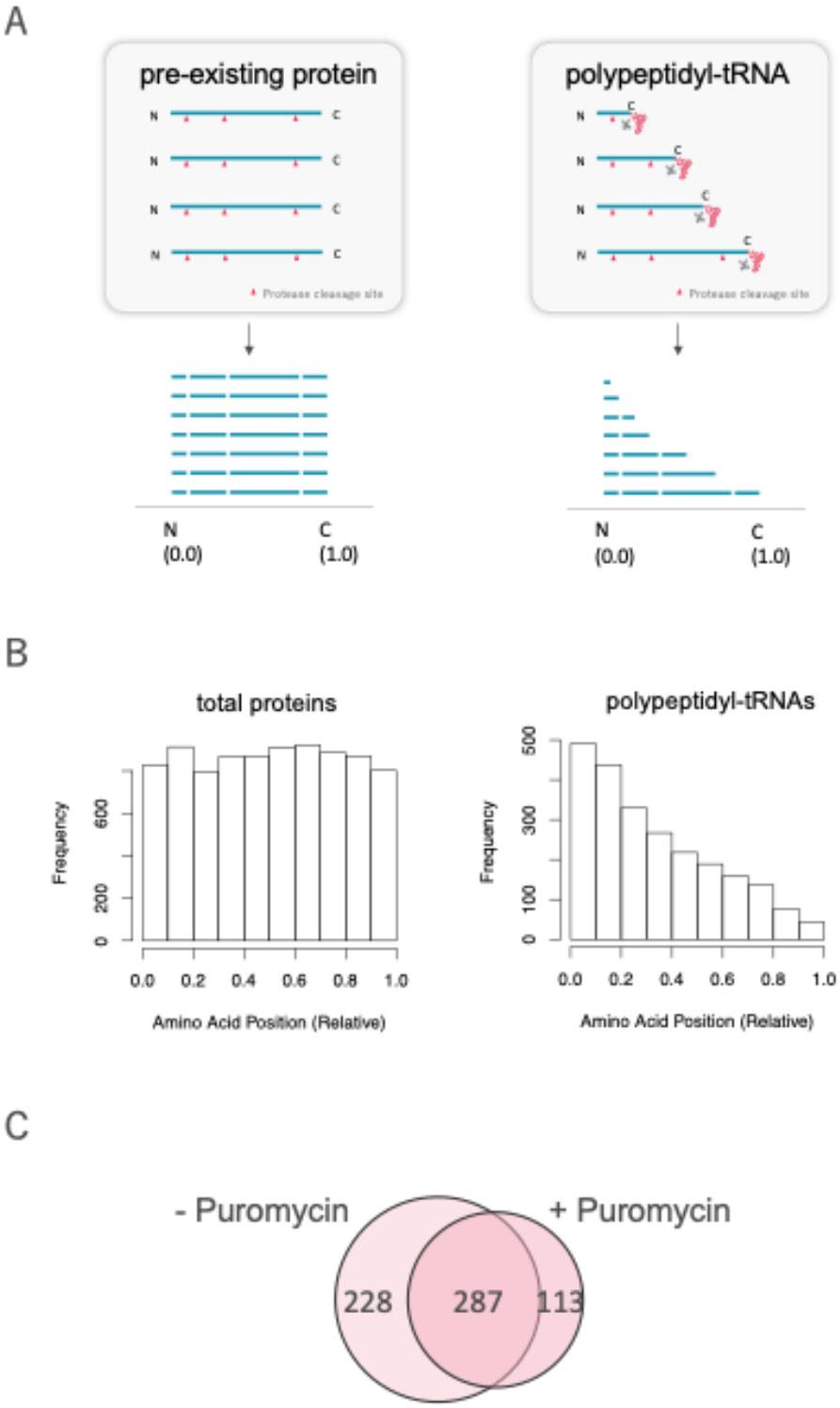

Figure 3. Indication that the peptides derived from pep-tRNAs are intermediates during translation elongation.

A. Illustration of pep-tRNAs and pre-existing proteins digested by trypsin. Trypsin digestion of pep-tRNAs, which elongate from the N- to C-terminus, should accumulate more peptides containing the N-terminal portion. In contrast, for pre-existing proteins, which are basically full-length polypeptides, the amounts of trypsin-digested peptides should be equal for all peptides. Red arrowheads indicate the trypsin cleavage sites. The relative positions of the N-terminus and C-terminus are defined as 0 and 1, respectively. **B**. Meta-analysis of peptides identified by LC-MS/MS for pep-tRNAs and total proteins. **C**. Venn diagram representing the identified pep-tRNAs obtained from cells, with or without puromycin treatment.

199

200 reduced to ~18 when RNase was added to the mixture after the phenol/chloroform extraction
201 (Step 1) (Fig. S5). About 70% of the 400 puromycin-resistant pep-tRNAs (287 out of 400)
202 overlapped with those derived from the puromycin-untreated sample (Fig. 3C), implying that
203 the puromycin-resistant pausing events might be prevalent.

204 The list of detected peptides included two known pausing peptides, SecM
205 ($_{150}$FSTPVWISQAQGIR$_{163}$) and TnaC ($_{19}$IVDHRP$_{24}$), only under the puromycin treatment
206 even though the *E. coli* strain used in this experiment did not overexpress these ORFs (Table
207 S2). In addition, a portion of the TnaC-derived pausing peptide was detected as a Tris-adduct
208 ($_{19}$IVDHRP$_{24}$-Tris), a hallmark of the peptidyl-tRNA. Taken together, the puromycin treatment
209 could be effective in identifying puromycin-resistant pausing peptides.

210 Indeed, several peptides that accumulated during translation could be candidates for
211 previously unknown pausing peptides (Table S2). One is the *raiA*-derived peptide,
212 $_{44}$EPQGFVADATINTPNGVL$_{61}$, along with its Tris-adduct ($_{44}$EPQGFVADATINTPNGVL$_{61}$-
213 Tris) (Fig. 4, Table S3). In fact, the C-terminal 61st leucine is consistent with a peak position in
214 the ribosome profiling data (Fig. 4), suggesting that the RaiA peptide could be derived from
215 translation pausing.

216 We also found another candidate, IvbL-LLPTAPSAAVVVVR, which seems to be
217 consistent with the ribosome profiling data (Table S2, Fig. S6). The translation of consecutive
218 valine residues near the C-terminus of IvbL induces pausing, due to the depletion of the valyl-
219 tRNA (41). Since the pausing due to such tRNA depletion would be abolished by the puromycin
220 addition, the accumulation of puromycin-resistant IvbL-tRNA implies an alternative pausing
221 mechanism, such as an association of the nascent peptide with the exit tunnel. In addition to
222 these candidates, we also detected other pausing candidates for the translation products, such
223 as *rof* and *udp* (Table S2).

224

225 **Discussion**

226 We developed a method for the direct isolation and analysis of pep-tRNAs in cells. Pep-
227 tRNAs are "in-between" molecules with both RNA and protein properties. The method
228 developed here exploited the "in-between" property to enrich pep-tRNAs from cellular RNAs
229 (mRNAs, tRNA, and ribosomal RNAs) and proteins that have already completed translation.
230 The organic solvent extraction and silica column purification with high concentrations of
231 guanidium salt were very effective for isolating pep-tRNAs. Considering several results,
232 including the RNase treatment, the meta-analysis showing N-terminally-biased peptides
233 derived from pep-tRNAs, and the Tris-conjugated C-terminal ends, we conclude that the
234 method, termed PETEOS, indeed enriches and identifies cellular pep-tRNAs.

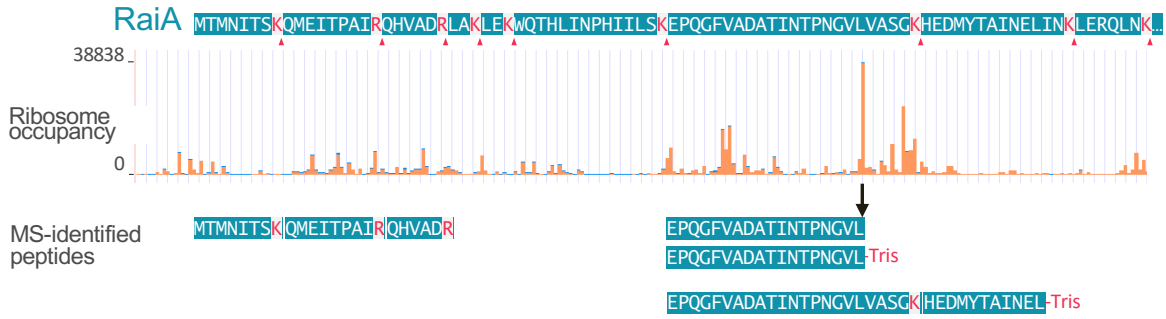

Figure 4. Example of an ORF that includes pep-tRNA-derived peptides from puromycin-treated cells.
Representation of the *raiA* ORF. Blue regions schematically indicate the MS/MS-identified peptides, including a peptide adducted with Tris base. The peptides at the bottom represent the frequencies of ribosome footprints around the *raiA* ORF, obtained from a ribosome profiling database (GWIPS-viz browser). The amino acid sequences of the peptides with C-termini that matched with a prominent peak in the ribosome footprints are shown. Arrowheads indicate the trypsin cleavage sites.

A unique feature of the PETEOS method is the direct handling and analysis of pep-tRNAs, providing a complementary approach to other established translatome analyses. Additionally, the PETEOS method has several technical advantages. The time required to halt the cellular processes before the pep-tRNA enrichment is very rapid, since an organic reagent is simply added to the cells. Such quick halting enables the method to capture snapshots of the translation status in the cell. In addition, the PETEOS procedure is relatively inexpensive and straightforward as compared to ribosome profiling.

We assumed that the pep-tRNAs enriched by the PETEOS method are the genuine intermediates during translation elongation. However, in addition to pep-tRNAs accommodated within the ribosome as translation intermediates, some pep-tRNAs might be the products that dropped off from the ribosome for some reasons. Although peptidyl-tRNA hydrolase cleaves the ester bond of pep-tRNAs released from the ribosome, some pep-tRNAs might remain. The current protocol cannot discriminate the "drop-off" pep-tRNAs from the pep-tRNAs-accommodating ribosomes (42, 43). The isolation of the translating ribosomes by sucrose density gradient centrifugation could enrich the pep-tRNAs committed to further elongation.

The PETEOS method has several limitations and points to improve. First, the inherently low abundance of pep-tRNAs in the cell makes it difficult to analyze them using LC-MS/MS. Second, the acidic phenol/chloroform extraction, which concentrates proteins and pep-tRNAs while removing RNAs, is not sufficient. Some of the pep-tRNAs move into the aqueous phase, probably due to their hydrophilic nature. For example, the distribution of TnaC-tRNA in the organic solvent extraction differs between our results (Fig. S2C) and those of an earlier study, where TnaC-tRNA was partitioned in the phenol phase (44). Although the protocols are

258 different, such as when the organic solvent is added, subtle differences in conditions and
259 methods may affect the distribution significantly, leading to the incomplete separation of pep-
260 tRNAs. Such incomplete separation would lead to unidentified peptidyl-tRNAs, causing some
261 biases in the population and a lack of quantitative assessment. Finally, identifying pep-tRNA-
262 derived peptides, including the C-terminal end of the growing polypeptide, is inherently
263 difficult. The possible reasons for this are as follows. (i) Unless the translation is paused, the
264 C-terminus of pep-tRNA moves continuously, making it difficult to accumulate detectable
265 amounts of the same peptides for MS/MS. (ii) The peptide fragments closer to the N-terminus
266 are, in principle, more abundant (Fig. 3). These drawbacks are inherent but could be overcome
267 by improving the enrichment efficiency of C-terminal peptides, which are generally considered
268 problematic even in conventional LC-MS/MS-based proteomics.
269 Despite its incompleteness, PETEOS has potential in that it is complementary to
270 established translatome methods and enables analyses that cannot be achieved by other
271 translatome methods. In addition, although *E. coli* lysates were used as a proof-of-concept
272 experiment, the pep-tRNA analysis by PETEOS could be applicable to any organism.
273
274
275 **Materials and Methods**
276 ***E. coli* strains and plasmids.**
277 The *E. coli* DH5α strain was used for cloning, and the *E. coli* BW25113 {genotype:
278 *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), λ^-, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*} was used as
279 the standard strain. Phage P1-mediated transduction was used to introduce the Δ*tolC* mutation
280 from Keio collection JW5503 (45). The plasmids used in this study are the pCA24N vector,
281 pCA24N-*secM* (from the ASKA library) (46) and pYA004(pCA24N-*tnaC*). To construct
282 pYA004, a DNA fragment amplified by PCR from a template *E. coli* using primers AY0003
283 and AY0004 and a DNA fragment amplified from pCA24N using primers PM0066 and PT0123
284 were assembled by Gibson assembly (47). The primers used in this study are as follows.
285 AY0003(AAAGAGGAGAAATTAACTATGAATATCTTACATATATGTGTG),
286 AY0004(TTAGCGGCCGCATAGGCCGATCCCTGTGAATATTACATC),
287 PM0066(GGCCTATGCGGCCGCTAAGGGTCGACCTGC),
288 PT0123(CATAGTTAATTTCTCCTCTTTAATGAATTC).
289
290 **Bacterial growth.**
291 *E. coli* cells cultured in LB medium overnight were inoculated in fresh LB medium, and
292 grown at 37°C with shaking at 180 rpm, unless otherwise noted. Puromycin was added to a

293 final concentration of 10 μg/mL and the cells were incubated for 10 min at 37°C where indicated.
294 *E. coli* cells harboring pCA24N or its derivatives were grown overnight in LB medium
295 containing 20 μg/ml of chloramphenicol, and were then inoculated into fresh LB medium
296 containing chloramphenicol. They were grown at 37 °C with shaking at 180 rpm until the OD_{660}
297 reached ~0.5. The expression of SecM or TnaC was then induced by adding 100 μM isopropyl
298 β-D-1-thiogalactopyranoside (IPTG) for 30 min.
299
300 **SDS-PAGE under neutral pH conditions and silver staining.**
301 Samples were diluted in 2x SDS sample buffer (final 62.5 mM Tris-HCl, pH 6.8, 2% SDS,
302 10% glycerol, 5% β-mercaptoethanol, a trace of bromophenol blue), as described by Laemmli.
303 SDS sample buffer was treated with RNA Secure (Ambion) according to the manufacturer's
304 specifications. The samples were separated by the 11% WIDE Range SDS-PAGE system
305 (Nacalai Tesque), as described previously (20, 48).
306 Silver staining of the gels was performed using a PlusOne Silver Staining Kit, Protein (GE
307 Healthcare), according to the manufacturer's specifications in protocol "E," except that
308 glutardialdehyde (25% w/v) was omitted from the *sensitizing solution* in the kit.
309
310 **Phenol extraction and TCA/acetone precipitation to fractionate polypeptides and**
311 **polypeptidyl-tRNAs from nucleic acids.**
312 *E. coli* cells grown in 200 ml of LB medium until the mid-log phase were mixed with an
313 approximately 200 mL volume of crushed ice, and collected by centrifugation at 4 °C for 10
314 min. After supernatant removal, the cell pellet was suspended in 20 mL of TriPURE isolation
315 reagent (Roche) and incubated for 5 min at room temperature. The lysate was then mixed with
316 4 ml of chloroform, vortexed for 20 sec, incubated for 5 min at room temperature, and
317 centrifuged at 12,000 ×g for 5 min at 4 °C. The colorless to pale yellow transparent upper layer
318 (aqueous phase, 10-12 mL) was removed, leaving an organic phenol phase and a solid middle
319 layer. After the addition of an equal volume (12-15 mL from a 200 mL culture) of ice-cooled
320 20% TCA/acetone containing 5 mM dithiothreitol (DTT), the mixture was vortexed for 20 sec
321 and incubated for at least 3 hrs at -20 °C. The sample was centrifuged at 15,600 ×g for 5 min at
322 4 °C, and the supernatant was carefully discarded. The precipitate was vortexed with 10 mL of
323 acetone and centrifuged at 15,600 ×g for 5 min at 4 °C, and then the supernatant was discarded
324 by aspiration. This washing procedure was repeated twice.
325
326 **Peptidyl-tRNA purification using a silica column.**
327 The precipitate obtained from the organic phenol phase was resolved in a 1 ~ 3 ml volume

328 of phase-transfer surfactants (PTS) buffer {100 mM Tris-HCl, pH 6.8, 24 mM sodium
329 deoxycholate, 24 mM sodium N-lauroyl sarcosinate (39)} by vortexing at 37 °C for 15 min.
330 After centrifugation, the collected supernatant was divided into two equal portions, and one
331 was treated with 40 μg/ml of RNase A (Promega) at 37 °C for 1 hr.
332 As the next step, the peptidyl-tRNAs in the PTS buffer were isolated by using a High Pure
333 miRNA Isolation Kit (Roche) according to the manufacturer's instructions, except that 1/2
334 volume of the *binding enhancer* was used (48). About 750 μL of PTS lysate could be processed
335 by one silica column in the kit. The silica-bound RNA was eluted with 100 μl of PTS buffer,
336 and this purification was repeated twice.
337
338 **LC-MS/MS sample preparation.**
339 The purified RNA sample in the previous section (100 μl) was mixed with 10 μl of 2 M
340 Tris base (pH= ~11) and incubated at 80 °C for 20 min to hydrolyze the ester bond of the
341 peptidyl-tRNA. After the addition of 90 μl deionized water, the sample was reduced by the
342 addition of 10 mM DTT at room temperature for 30 min, and then alkylated with 50 mM
343 iodoacetamide in the dark at room temperature for 20 min. The mixture was diluted 5-fold with
344 50 mM ammonium bicarbonate. For the limited digestion of denatured polypeptides into
345 peptide fragments, 0.5 μg of Lys-C (Fujifilm Wako) was added, and the solution was incubated
346 at room temperature for at least 3 hrs. Subsequently, 0.5 μg of Trypsin Gold (Promega) was
347 added and incubated at 37 °C overnight, whereas for the SecM analysis, 0.5 μg of Lys-C was
348 used. After the digestion, an equal volume of ethyl acetate and 0.5 % trifluoroacetic acid (final
349 concentration) was added. The mixture was shaken vigorously for 2 min and centrifuged at
350 15,700 ×g for 2 min. The upper ethyl acetate layer was removed, and the solvent was removed
351 by a centrifugal evaporator. The residual pellet was dissolved in 0.1 % TFA and 2 % acetonitrile
352 and desalted, as follows. The solution was applied to an SDB-XC StageTip made by an Empore
353 disk (3M, U.S.A.), equilibrated with 0.1 % TFA and 2 % acetonitrile, washed with 0.1 % TFA
354 and 2 % acetonitrile, and eluted with 0.1 % TFA and 80 % acetonitrile. The solvent was removed
355 by a centrifugal evaporator, and the residue was dissolved in 0.1 % TFA and 2 % acetonitrile.
356 This solution was centrifuged at 20,000 ×g for 5 min, and the supernatant was collected and
357 subjected to the LC-MS/MS measurement.
358
359 **LC-MS/MS measurement.**
360 The LC-MS/MS measurements were conducted with a Q-Exactive tandem mass spectrometer
361 and an Easy-nLC 1000 nano HPLC system (Thermo Fischer Scientific). The trap column used
362 for the nano HPLC was a 2 cm × 75 μm capillary column packed with 3 μm C18-silica particles

363 (Thermo Fisher Scientific), and the separation column was a 12.5 cm × 75 μm capillary column
364 packed with 3 μm C18-silica particles (Nikkyo Technos, Japan). The flow rate for the nano
365 HPLC was 300 nL/min. The separation was conducted using a 10－40% linear acetonitrile
366 gradient over 70 min, in the presence of 0.1% formic acid. The nanoLC-MS/MS data were
367 acquired in the data-dependent acquisition (DDA) mode controlled by the Xcalibur 4.0 program
368 (Thermo Fisher Scientific). The DDA settings were as follows: the resolution was 70,000 for a
369 full MS scan and 17,500 for an MS2 scan; the AGC target was 3.0E6 for a full MS scan and
370 5.0E5 for an MS2 scan; the maximum IT was 60 ms for both the full MS and MS2 scans; the
371 full MS scan range was 310－1,500 m/z; and the top 10 signals in each full MS scan were
372 selected for the MS2 scan. The DDA measurement was performed three times for each sample,
373 and two or three biological replicates were measured in each condition.
374
375 **Data analysis.**
376 The data analysis of the DDA measurements was performed with the Proteome Discoverer 2.4
377 program and the SEQUEST HT search engine (Thermo Fischer Scientific). The dataset of the
378 amino acid sequences of *E. coli* genes was obtained from the UniProt database (UP000000625,
379 downloaded on 181219). Only the peptides with a < 1 % false discovery rate (by the Percolator
380 algorithm) detected more than twice in the three measurements were selected. All processes
381 and statistical analyses were conducted by the R software (version 4.4.1 with in-house scripts).
382 The ribosome distributions on the mRNAs in the ribosome profiling were obtained using the
383 aggregation mode in the GWIPS-viz browser, a public web tool to visualize the ribosome
384 distribution (49, 50).
385
386 **Data Availability Statement.**
387 Data in this manuscript have been uploaded to the Mendeley Dataset public repository
388 (https://doi.org/10.17632/xzycy8m6tb.1).
389
390 **Author contributions.**
391 AY and YC performed experiments; AY and TN analyzed the LC-MS/MS data; AY, YC, TN,
392 and HT conceived the study, designed experiments, and analyzed the results; YC, TN, and HT
393 supervised the entire project; AY, YC, TN, and HT wrote the manuscript.
394
395 **Acknowledgments.**
396 We thank Eri Uemura for technical support; the Bio-support Center at Tokyo Tech for DNA
397 sequencing; and the Cell Biology Center Research Core Facility at Tokyo Tech for the Q-

398 Exactive mass spectrometry measurements. This work was supported by MEXT Grants-in-Aid
399 for Scientific Research (Grant Numbers 17K15073 to TN, 17K15062, 19K16038 to YC,
400 JP26116002, JP18H03984, JP20H05925 to HT) and a grant from the Ohsumi Frontier Science
401 Foundation to YC.
402

403 **Conflicts of Interest.**

404 The authors declare no competing interest.

405

406 **References.**

407 1. C. Vogel, E. M. Marcotte, Insights into the regulation of protein abundance from
408 proteomic and transcriptomic analyses. *Nature Reviews Genetics* **13**, 227–232 (2012).
409 2. S. Pechmann, F. Willmund, J. Frydman, The Ribosome as a Hub for Protein Quality
410 Control. *Molecular Cell* **49**, 411–421 (2013).
411 3. K. Ito, S. Chiba, Arrest peptides: Cis-acting modulators of translation. *Annual Review of*
412 *Biochemistry* **82**, 171–202 (2013).
413 4. K. C. Stein, J. Frydman, The stop-and-go traffic regulating protein biogenesis: How
414 translation kinetics controls proteostasis. *Journal of Biological Chemistry* **294**, 2076–
415 2084 (2019).
416 5. E. Samatova, J. Daberger, M. Liutkute, M. v Rodnina, Translational Control by Ribosome
417 Pausing in Bacteria: How a Non-uniform Pace of Translation Affects Protein Production
418 and Folding. *Frontiers in Microbiology* **11**, 619430 (2021).
419 6. D. N. Wilson, S. Arenz, R. Beckmann, Translation regulation via nascent polypeptide-
420 mediated ribosome stalling. *Current Opinion in Structural Biology* **37**, 123–133 (2016).
421 7. J. Choi, *et al.*, How Messenger RNA and Nascent Chain Sequences Regulate Translation
422 Elongation. *Annu Rev Biochem* **87**, 421–449 (2018).
423 8. J. L. Chaney, P. L. Clark, Roles for Synonymous Codon Usage in Protein Biogenesis.
424 *Annual Review of Biophysics* **44**, 143–166 (2015).
425 9. T. Sunohara, T. Abo, T. Inada, H. Aiba, The C-terminal amino acid sequence of nascent
426 peptide is a major determinant of SsrA tagging at all three stop codons. *RNA* **8**, 1416–
427 1427 (2002).
428 10. M. A. Sørensen, C. G. Kurland, S. Pedersen, Codon usage determines translation rate in
429 Escherichia coli. *Journal of Molecular Biology* **207**, 365–377 (1989).
430 11. C. S. Hayes, B. Bose, R. T. Sauer, Proline residues at the C terminus of nascent chains
431 induce SsrA tagging during translation termination. *Journal of Biological Chemistry* **277**,
432 33825–33832 (2002).

433 12. I. Rychlík, J. ČErná, S. Chládek, P. Pulkrábek, J. ŽEmlička, Substrate Specificity of
434 Ribosomal Peptidyl Transferase: The Effect of the Nature of the Amino Acid Side Chain
435 on the Acceptor Activity of 2'(3')-O-Aminoacyladenosines. *European Journal of*
436 *Biochemistry* **16**, 136–142 (1970).
437 13. H. Nakatogawa, K. Ito, Secretion monitor, secM, undergoes self-translation arrest in the
438 cytosol. *Molecular Cell* **7**, 185–192 (2001).
439 14. H. Nakatogawa, K. Ito, The ribosomal exit tunnel functions as a discriminating gate. *Cell*
440 **108**, 629–36 (2002).
441 15. J. Zhang, *et al.*, Mechanisms of ribosome stalling by SecM at multiple elongation steps.
442 *Elife* **4**, e09684 D (2015).
443 16. F. Gong, K. Ito, Y. Nakamura, C. Yanofsky, The mechanism of tryptophan induction of
444 tryptophanase operon expression: Tryptophan inhibits release factor-mediated
445 cleavage of TnaC-peptidyl-tRNAPro. *Proc Natl Acad Sci U S A* **98**, 8997–9001 (2001).
446 17. N. Vazquez-Laslop, C. Thum, A. S. Mankin, Molecular Mechanism of Drug-Dependent
447 Ribosome Stalling. *Molecular Cell* **30**, 190–202 (2008).
448 18. H. Onouchi, *et al.*, Nascent peptide-mediated translation elongation arrest coupled with
449 mRNA degradation in the CGS1 gene of Arabidopsis. *Genes and Development* **19**, 1799–
450 1810 (2005).
451 19. K. Yanagitani, Y. Kimata, H. Kadokura, K. Kohno, Translational pausing ensures
452 membrane targeting and cytoplasmic splicing of XBP1u mRNA. *Science* **331**, 586–589
453 (2011).
454 20. Y. Chadani, T. Niwa, S. Chiba, H. Taguchi, K. Ito, Integrated in vivo and in vitro nascent
455 chain profiling reveals widespread translational pausing. *Proc Natl Acad Sci U S A* **113**,
456 E829–E838 (2016).
457 21. H. Muto, H. Nakatogawa, K. Ito, Genetically Encoded but Nonpolypeptide Prolyl-tRNA
458 Functions in the A Site for SecM-Mediated Ribosomal Stall. *Molecular Cell* **22**, 545–552
459 (2006).
460 22. S. Iwasaki, N. T. Ingolia, The Growing Toolbox for Protein Synthesis Studies. *Trends*
461 *Biochem Sci* **42**, 612–624 (2017).
462 23. N. T. Ingolia, S. Ghaemmaghami, J. R. S. Newman, J. S. Weissman, Genome-wide analysis
463 in vivo of translation with nucleotide resolution using ribosome profiling. *Science (1979)*
464 **324**, 218–223 (2009).
465 24. N. T. Ingolia, G. A. Brar, S. Rouskin, A. M. McGeachy, J. S. Weissman, The ribosome
466 profiling strategy for monitoring translation in vivo by deep sequencing of ribosome-
467 protected mRNA fragments. *Nature Protocols* **7**, 1534–1550 (2012).

468 25. G. A. Brar, J. S. Weissman, Ribosome profiling reveals the what, when, where and how
469 of protein synthesis. *Nature Reviews Molecular Cell Biology* **16**, 651–664 (2015).
470 26. N. T. Ingolia, J. A. Hussmann, J. S. Weissman, Ribosome profiling: Global views of
471 translation. *Cold Spring Harbor Perspectives in Biology* **11**, a032698 (2019).
472 27. A. R. Buskirk, R. Green, Ribosome pausing, arrest and rescue in bacteria and eukaryotes.
473 *Philosophical Transactions of the Royal Society B: Biological Sciences* **372**, 20160183
474 (2017).
475 28. F. Mohammad, C. J. Woolstenhulme, R. Green, A. R. Buskirk, Clarifying the Translational
476 Pausing Landscape in Bacteria by Ribosome Profiling. *Cell Reports* **14**, 686–694 (2016).
477 29. D. C. Dieterich, A. J. Link, J. Graumann, D. A. Tirrell, E. M. Schuman, Selective
478 identification of newly synthesized proteins in mammalian cells using bioorthogonal
479 noncanonical amino acid tagging (BONCAT). *Proc Natl Acad Sci U S A* **103**, 9482–9487
480 (2006).
481 30. A. J. M. Howden, *et al.*, QuaNCAT: Quantitating proteome dynamics in primary cells.
482 *Nature Methods* **10**, 343–346 (2013).
483 31. R. Aviner, T. Geiger, O. Elroy-Stein, Genome-wide identification and quantification of
484 protein synthesis in cultured cells and whole tissues by puromycin-associated nascent
485 chain proteomics (PUNCH-P). *Nature Protocols* **9**, 751–760 (2014).
486 32. R. Aviner, T. Geiger, O. Elroy-Stein, Novel proteomic approach (PUNCH-P) reveals cell
487 cycle-specific fluctuations in mRNA translation. *Genes and Development* **27**, 1834–1844
488 (2013).
489 33. C. M. Forester, *et al.*, Revealing nascent proteomics in signaling pathways and cell
490 differentiation. *Proc Natl Acad Sci U S A* **115**, 2353–2358 (2018).
491 34. M. Dermit, M. Dodel, F. K. Mardakheh, Methods for monitoring and measurement of
492 protein translation in time and space. *Molecular BioSystems* **13**, 2477–2488 (2017).
493 35. R. Milo, P. Jorgensen, U. Moran, G. Weber, M. Springer, BioNumbers The database of
494 key numbers in molecular and cell biology. *Nucleic Acids Research* **38**, D750-3 (2010).
495 36. K. Ito, *et al.*, Nascentome analysis uncovers futile protein synthesis in Escherichia coli.
496 *PLoS ONE* **6**, e28413 (2011).
497 37. R. Boom, *et al.*, Rapid and simple method for purification of nucleic acids. *Journal of*
498 *Clinical Microbiology* **28**, 495–503 (1990).
499 38. B. Vogelstein, D. Gillespie, Preparative and analytical purification of DNA from agarose.
500 *Proc Natl Acad Sci U S A* **76**, 615–619 (1979).
501 39. T. Masuda, M. Tomita, Y. Ishihama, Phase transfer surfactant-aided trypsin digestion for
502 membrane proteome analysis. *Journal of Proteome Research* **7**, 731–740 (2008).

503 40. H. Okusu, D. Ma, H. Nikaido, AcrAB efflux pump plays a major role in the antibiotic
504 resistance phenotype of Escherichia coli multiple-antibiotic-resistance (Mar) mutants. *J*
505 *Bacteriol* **178**, 306–308 (1996).
506 41. P. Friden, T. Newman, M. Freundlich, Nucleotide sequence of the ilvB promoter-
507 regulatory region: A biosynthetic operon controlled by attenuation and cyclic AMP. *Proc*
508 *Natl Acad Sci U S A* **79**, 6156–6160 (1982).
509 42. S. Vivanco-Domínguez, *et al.*, Protein Synthesis Factors (RF1, RF2, RF3, RRF, and tmRNA)
510 and Peptidyl-tRNA Hydrolase Rescue Stalled Ribosomes at Sense Codons. *Journal of*
511 *Molecular Biology* **417**, 425–439 (2012).
512 43. V. Heurgué-Hamard, *et al.*, Ribosome release factor RF4 and termination factor RF3 are
513 involved in dissociation of peptidyl-tRNA from the ribosome. *The EMBO Journal* **17**,
514 808–816 (1998).
515 44. M. Gong, F. Gong, C. Yanofsky, Overexpression of tnaC of Escherichia coli inhibits growth
516 by depleting tRNA2Pro availability. *Journal of Bacteriology* **188**, 1892–1898 (2006).
517 45. T. Baba, *et al.*, Construction of Escherichia coli K-12 in-frame, single-gene knockout
518 mutants: The Keio collection. *Molecular Systems Biology* **2**, 0008 (2006).
519 46. M. Kitagawa, *et al.*, Complete set of ORF clones of *Escherichia coli* ASKA library (A
520 complete set of E. coli K-12 ORF archive): unique resources for biological research. *DNA*
521 *Research* **12**, 291–299 (2005).
522 47. D. G. Gibson, Enzymatic assembly of overlapping DNA fragments. *Methods Enzymol* **498**,
523 349–361 (2011).
524 48. Y. Chadani, *et al.*, Intrinsic Ribosome Destabilization Underlies Translation and Provides
525 an Organism with a Strategy of Environmental Sensing. *Molecular Cell* **68**, 528-539.e5
526 (2017).
527 49. A. M. Michel, S. J. Kiniry, P. B. F. O'Connor, J. P. Mullan, P. v. Baranov, GWIPS-viz: 2018
528 update. *Nucleic Acids Research* **46**, D823–D830 (2018).
529 50. A. M. Michel, *et al.*, GWIPS-viz: Development of a ribo-seq genome browser. *Nucleic*
530 *Acids Research* **42**, D859–D864 (2014).
531