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

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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 termed *pe*ptidyl-*t*RNA *e*nrichment using *o*rganic extraction and *s*ilica adsorption (PETEOS), and then identify their polypeptide moieties by mass spectrometry. As a proof-of-concept experiment using *Escherichia coli*, we identified ~800 proteins derived from the pep-tRNAs, which were markedly biased towards the N-termini in the proteins, reflecting that PETEOS captured the intermediate pep-tRNA population during translation. Furthermore, we observed the changes in the pep-tRNA set in response to heat shock or antibiotic treatments. In summary, PETEOS will complement conventional methods for profiling nascent chains such as ribosome profiling.

Significance Statement

In the central dogma of biology, RNA and protein 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 to enrich the pep-tRNAs by organic solvent extraction and silica column separation. The method, termed PETEOS, not only provides a unique approach to examine the nascent state of proteins but also may be effective in capturing snapshots of translation status in the cell.

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). For example, the translation product of the gene *secM* in *Escherichia coli* induces translation arrest (8, 9). The translation arrest by SecM is directly involved in the feedback regulation of its downstream gene, *secA* (8, 9). In addition to the ad hoc identification of translational pausing, 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 (10). Since it is evident that not only pausing but also many other biological events are regulated at the translational level, 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 (11). One is ribosome profiling, a deep sequencing technique for ribosome-protected mRNA fragments generated by RNase digestion (12). 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 (11–16). 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.* 17). 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 (18), QuaNCAT (19)) and puromycin or its analogs (*e.g.,* PUNCH-P (20, 21), OPP-ID (22), pSNAP (23)). 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 (24–26). In addition, all of these methods that rely on labeling usually require an hour-long timescale or more to accumulate the labeled products (27). 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 (28), 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 (29). 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 has, in principle, several advantages such as rapid capturing of translation status and non-labeling feature.

Results

Polypeptidyl-tRNA enrichment and identification using a proteomics approach.

As far as we know, there is no established method to purify whole pep-tRNAs from cells.

115 Our strategy to enrich pep-tRNAs takes advantage of their two distinctive properties as RNA
116 and polypeptide. The method, which we call PETEOS, is composed of four steps (Fig. 1A).
117 Step 1: Enrichment of pep-tRNAs and proteins from cells. Harvested cells are directly mixed
118 into a phenol-containing reagent (*e.g.* TriPURE, TRIzol) to separate most RNAs including
119 ribosomal RNAs (aqueous phase) from pep-tRNAs and proteins (organic phase). Note that
120 there is no need for labeling times in this method. After the chloroform addition, the pep-
121 tRNAs and proteins in the organic phase and interphase are precipitated by trichloroacetic
122 acid (TCA)/acetone, under conditions where the ester bonds between polypeptide and tRNA
123 in the pep-tRNAs are retained. Step 2: Isolation of the pep-tRNAs. The RNA is isolated by
124 using silica to adsorb the nucleic acid portions of pep-tRNAs, under highly concentrated
125 chaotropic salt conditions (30, 31). Step 3: Cleavage of peptide moieties from peptidyl-tRNAs.
126 The ester bond between peptide and tRNA is cleaved by high pH and heat treatment (29).
127 Step 4: Identification of the peptides after trypsin treatment, by LC-MS/MS-based shotgun
128 proteomics.
129

130 **Validation of the phenol extraction (Step 1).**

131 The validity of the PETEOS method using *E. coli* lysates was evaluated at each step. An
132 LC-MS/MS analysis of the TCA/acetone precipitate in Step 1 (organic phase) revealed that
133 ~79% of the identified proteins in the precipitate overlapped with proteins identified by a
134 conventional method (32) to extract proteins from cells (Fig. S1A), confirming that the
135 protein fraction in the organic phase seems to lack significant bias. Even if there was no bias
136 in the total protein fraction, we need to know how peptidyl-tRNAs are partitioned in organic
137 solvent extraction in Step 1 since the chemical properties of pep-tRNAs with various
138 polypeptide moieties are diverse. For example, when the peptide moiety is hydrophilic or
139 relatively short, the hydrophilic property of the RNA would be dominant, but when the
140 polypeptide portion is more hydrophobic or longer, the more protein-like property would be
141 manifested.

142 Then, we investigated this issue in a model system. First, we focused on the length of the
143 peptide moieties of pep-tRNAs. To prepare the pep-tRNA with various peptide lengths, we
144 translated nonstop mRNAs in a reconstituted *in vitro* translation system (33). The 3' end of *E.*
145 *coli* enolase (Eno), DHFR, *secM*, or superfolder GFP mRNAs were truncated at 30, 60, 120,
146 240, or 480 nucleotides (10, 20, 40, 80, or 160 amino acids) from the start codon, respectively
147 (Fig. 1B). The reaction mixture was then phenol-extracted. The pep-tRNA in each fraction
148 was analyzed by SDS-PAGE under neutral pH conditions (neutral-PAGE), where the ester
149 bonds between peptide and tRNA in the pep-tRNAs remain intact, as described previously

150 (34) (Fig. S1B). As shown in Fig. 1B, all the pep-tRNAs we tested were fractionated into the
151 organic phase in a length-dependent manner. Furthermore, the hydrophobicity of the
152 polypeptide moiety, though not as much as its length, was shown to have some influence on
153 the phenol-based fractionation (Fig. S1C). These results provided us with some tendencies in
154 the distribution of pep-tRNA in the phenol extraction, where pep-tRNAs with relatively short
155 peptide moieties do not distribute well in the organic phase.
156

157 **Validation of the later steps (Step 2-4) of the PETEOS method.**

158 Next, we analyzed the silica-based concentration of the pep-tRNA from the cell extract
159 (Step 2). Accordingly, we overexpressed a well-known pausing peptide, SecM. Then we
160 analyzed the eluate of silica-isolation by SDS-PAGE and following silver staining. SecM pep-
161 tRNA was successfully isolated from the cellular extract (Fig. 1C). Furthermore, the MS/MS
162 analysis identified the SecM-derived peptides including the $_{150}$FSTPVWISQAQGIRAG$_{165}$
163 fragment from this purified fraction (Fig. 1D). The translation of the gene *secM* is arrested at
164 the 165th glycine, where tRNA^{Gly} is covalently attached to the elongating end of the nascent
165 peptide (SecM$_{1-165}$-tRNA^{Gly}). This suggests that the PETEOS method has a potential to
166 identify paused peptides.

167 A previous nascentome study showed that Tris-base in the buffer stimulates the ester
168 bond cleavage of pep-tRNAs (29). A nucleophilic attack of
169 tris(hydroxymethyl)aminomethane (Tris) on the carboxyl group of the C-terminal amino acid,
170 which is ester-bonded to tRNA in the pep-tRNA, produces a Tris-adduct during the pep-
171 tRNA hydrolysis (Fig. S1D). Therefore, we speculated that some peptides derived from pep-
172 tRNAs could become conjugated with Tris. A peptide search assuming the Tris-addition at
173 the C-terminus revealed ~10 Tris-conjugated peptides, including a SecM-derived paused
174 peptide (Fig. S1E). The conjugation of the Tris-base confirmed the handling of intact pep-
175 tRNAs until they are exposed to high pH and high temperature.
176

177 **Proof-of-concept analysis using *Escherichia coli* cell lysates.**

178 The silica-eluate isolated from the organic phase including the components of
179 exponentially growing *E. coli* cells (at Step 2) was separated by neutral-PAGE analysis. We
180 observed smeared silver-stained bands (Fig. 2A). Since an RNase treatment before the
181 adsorption to the silica column drastically reduced the smeared bands (Fig. 2A), they would
182 be derived from pep-tRNA species.

183 After the peptide moieties from the pep-tRNAs were obtained by the alkaline and heat
184 treatment (Step 3), the LC-MS/MS analysis (Step 4) identified ~5,000 peptides derived from

185 ~850 proteins (Fig. 2B, Fig. S2A, and Supplementary Dataset S1). The number of the
186 detected peptides and proteins was drastically decreased in the RNase-treated fraction (Fig.
187 2B, Fig. S2A, and Supplementary Dataset S1), indicating that more than 95% of the identified
188 proteins would be derived from pep-tRNAs. In addition, the signal intensities of the proteins
189 derived from the RNase-treated fraction were much lower than those without the RNase
190 treatment (Fig. 2C). Furthermore, the proteins detected in the RNase-treated fraction include
191 many high abundant proteins such as chaperones, translation factors, and several outer
192 membrane proteins, suggesting that these proteins were probably derived from non-specific
193 interaction with the silica column (Supplementary Dataset S1). From these results, we
194 concluded that the PETEOS method can enrich and identify the cellular pep-tRNAs.
195 We then investigated the overlap between the detected proteins by the PETEOS method
196 and a conventional proteome method. The result showed that ~80% of the identified proteins
197 by the PETEOS method were also identified by a conventional method (Fig. S2B).
198 Furthermore, we examined the reproducibility of this method by comparing the results
199 between two biological replicates. Among ~850 detected proteins in each replicate, ~80 %
200 were overlapped (Fig. 2D). At the peptide level, ~70 % of the peptides were overlapped (Fig.
201 S2C). Quantitative comparison by MS1 intensities revealed that the abundance ratios between
202 two replicates were within the 5-fold range (Fig. S2D). Although precise quantitative
203 assessment seems difficult compared to conventional quantitative proteomics for whole-cell
204 lysates (usually ~2-fold changes are detectable), the PETEOS method has the potential to
205 detect somewhat large proteomic changes.
206 We next investigated whether the MS-identified peptides derived from the pep-tRNAs in
207 *E. coli* are in fact molecular species during the process of translation. Since nascent peptides
208 elongate from the N- to C-terminus, we expected that the tryptic peptides derived from pep-
209 tRNAs would be enriched with the N-terminus as compared to those derived from pre-
210 existing total cellular proteins (Fig. 2E, upper panel). A meta-analysis demonstrated that the
211 tryptic peptides from the pep-tRNAs were highly biased toward the N-terminal regions (Fig.
212 2E, lower panel). In contrast, a meta-analysis of the total pre-existing proteins showed equally
213 distributed tryptic peptides (Fig. 2E, lower panel), as expected since the total proteins are
214 basically composed of the full-length polypeptides after translation. The analysis confirmed
215 that the MS-identified peptides derived from the pep-tRNAs in *E. coli* are indeed intermediate
216 species during the translation elongation.
217
218 **Nascentome change in response to heat stress.**
219 To test whether the PETEOS method could capture the rearrangement of the nascentome

220 upon environmental perturbations, we evaluated the nascentome change by heat treatment
221 with the PETEOS method. The cells were cultivated at 45 °C for 30 min and disrupted with
222 the phenol reagent. Then, pep-tRNAs were isolated, and the peptides moieties in the pep-
223 tRNAs were analyzed. The peptides detected from the samples again showed quite high
224 sensitivities to RNase treatment and positional biases toward the N-termini, confirming that
225 the PETEOS method is robust to heat treatment (Fig. S3A). Quantitative assessment of the
226 peptides obtained from the heat-treated and non-treated cells showed that several peptide
227 fragments were specifically increased or decreased by the heat treatment (Fig. 3A and
228 Supplementary Dataset S2). Among them, the peptides derived from heat shock proteins and
229 proteases (GroEL/ES, DnaK, ClpB, HtpG, Lon, and FtsH) were strongly biased toward the
230 enriched fraction (Fig. 3A and Supplementary Dataset S2). Importantly, the abundance ratios
231 (fold changes) of these Hsps and proteases obtained by the PETEOS method were much
232 larger than those by the conventional whole-cell proteome experiment (Fig. 3B and
233 Supplementary Dataset S3). These results suggest that acute protein expression under the heat
234 shock condition is strongly biased beyond the changes observed by the conventional
235 proteome analysis. In other words, we can investigate such a "snapshot" of the translation
236 status using the PETEOS method.
237
238 **Nascentome change in response to antibiotic treatments.**
239 As another example of the application of the PETEOS method, we examined the
240 rearrangement of the nascentome by the treatment of several antibiotics that inhibit translation
241 reactions on ribosomes. For this purpose, we employed three conditions: chloramphenicol,
242 kasugamycin+puromycin, and erythromycin treatment. Chloramphenicol inhibits the
243 peptidyl-transferase reaction of ribosomes, so that the change in the snapshot of the protein
244 synthesis is expected to be small, or the efficiency of pep-tRNA detection may be better (35).
245 Puromycin accepts the polypeptide part from pep-tRNA in a ribosome function-dependent
246 manner(36). Therefore, the pep-tRNAs that are detectable by PETEOS are anticipated to be
247 decreased upon the puromycin treatment. In addition, the property of puromycin may allow us
248 to assess the puromycin-resistant pep-tRNAs that impair ribosomal function to pause the
249 translation elongation. For this purpose, we added the kasugamycin in parallel to highlight the
250 effect of puromycin by inhibiting the following translation initiation. Erythromycin, a kind of
251 macrolide antibiotic, binds to the exit tunnel of the ribosome to inhibit translation elongation.
252 Previous studies unveiled that translation inhibition by the macrolide has some specificity for
253 the nascent peptide sequence within the tunnel (37, 38).
254 The cells were treated with each antibiotic or cocktail of antibiotics for 10 min and

255 disrupted with the phenol reagent. The identified peptide sets, which had properties as
256 nascentome (Fig. S3B), showed antibiotic-dependent characteristics (Fig. 3C, D). As expected,
257 chloramphenicol treatment caused a small increase in the number of detected peptides (Fig.
258 3C) and the abundance ratio of a subset of proteins (Fig. 3D). On the other hand, the
259 kasugamycin/puromycin treatment caused a strong decrease in the number of the detected
260 peptides (Fig. 3C) and fold changes of the many proteins (Fig. 3D). However, we observed
261 several pep-tRNAs such as a fragment of SecM with unchanged abundances even after the
262 treatment, suggesting that ribosomes accommodating these pep-tRNAs poorly react with
263 puromycin.

264 The treatment by erythromycin resulted in both an increase and decrease in subsets of
265 proteins. We then evaluated the relationship between the abundance changes and the sequence
266 motif of erythromycin ("[K/R]-X-[K/R]") reported previously (37, 38). The result showed the
267 decreasing tendency of the abundances as the number of erythromycin-dependent pausing
268 motifs increased (Fig. S3C). This bias could be caused by ribosome rescue reaction at the
269 erythromycin-paused ribosome (39).

270 Finally, since the PETEOS method relies on the shotgun proteomics approach with
271 tryptic digestion and electrospray ionization mass spectrometry, a large bias exists in the
272 detectable peptides. Hence, it is usually difficult to discuss the precise position of translational
273 pausing. However, we detected several peptides whose C-termini were not lysine or arginine,
274 implying that these peptides are the C-terminal region of the pep-tRNAs since they were not
275 considered to be products of the tryptic digestion (Supplementary Table S1). In addition,
276 some of these peptides were also detected as C-terminal Tris-added peptides, confirming that
277 they were in the C-terminal region of the pep-tRNAs. Although the number of these peptides
278 was not large, these peptides would be potential candidates for transient translational pausing
279 under each condition.

280

281 **Discussion**

282 We developed a method for the direct isolation and analysis of pep-tRNAs in cells. Pep-
283 tRNAs are "in-between" molecules with both RNA and protein properties. The method
284 developed here exploited the "in-between" property to enrich pep-tRNAs from cellular RNAs
285 (mRNAs, tRNA, and ribosomal RNAs) and proteins that have already completed translation.
286 The organic solvent extraction and silica column purification with high concentrations of
287 guanidium salt were effective for isolating pep-tRNAs. Considering several results, including
288 the RNase treatment, the meta-analysis showing N-terminally-biased peptides derived from
289 pep-tRNAs, and the Tris-conjugated C-terminal ends, we conclude that the method, termed

290 PETEOS, indeed enriches and identifies cellular pep-tRNAs.

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

299 The PETEOS method has several limitations. First, the inherently low abundance of pep-
300 tRNAs in the cell makes it difficult to analyze them using LC-MS/MS. Second, the acidic
301 phenol/chloroform extraction, which concentrates proteins and pep-tRNAs while removing
302 RNAs, is not sufficient. Some of the pep-tRNAs move into the aqueous phase, as partly
303 revealed by our systematic separation analysis using model genes (Fig. 1B). Such incomplete
304 separation would lead to unidentified peptidyl-tRNAs, causing some biases in the population
305 and a lack of quantitative assessment. Third, since the peptide moieties derived from pep-
306 tRNAs are mainly identified by tryptic fragments, PETEOS does not have a codon-level
307 resolution, precluding a direct comparison of the method with ribosome profiling. Finally,
308 identifying pep-tRNA-derived peptides, including the C-terminal end of the growing
309 polypeptide, is inherently difficult. The possible reasons for this are as follows. (i) Unless the
310 translation is paused, the C-terminus of pep-tRNA moves continuously, making it difficult to
311 accumulate detectable amounts of the same peptides for MS/MS. (ii) The peptide fragments
312 closer to the N-terminus are, in principle, more abundant (Fig. 2E). These drawbacks are
313 inherent but could be overcome by improving the enrichment efficiency of C-terminal
314 peptides, which are generally considered problematic even in conventional LC-MS/MS-based
315 proteomics.

316 Despite its incompleteness, PETEOS has potential in that it is complementary to
317 established translatome methods and enables analyses that cannot be achieved by other
318 translatome methods. In addition, although *E. coli* lysates were used as a proof-of-concept
319 experiment, the pep-tRNA analysis by PETEOS could be applicable to any organism.

320

321

322 **Materials and Methods**

323 ***E. coli* strains and plasmids.**

324 The *E. coli* BW25113 {genotype: *Δ(araD-araB)567*, *ΔlacZ4787*(::*rrnB*-3), λ^-, *rph-1*,

325 *Δ(rhaD-rhaB)568*, *hsdR514*} was used as the standard strain. Phage P1-mediated transduction
326 was used to introduce the Δ*tolC* mutation from Keio collection JW5503 (40). The plasmids
327 used in this study are the pCA24N vector and pCA24N-*secM* (from the ASKA library) (41).
328

329 **Bacterial growth.**

330 *E. coli* cells cultured in LB medium overnight were inoculated in fresh LB medium, and
331 grown at 37 °C with shaking at 180 rpm, unless otherwise noted. The cells were incubated
332 with the antibiotics for 10 min at 37 °C if indicated; final 100 μg/ml of chloramphenicol, 10
333 μg/mL of puromycin and 50 μg/mL of kasugamycin, or 100 μg/ml of erythromycin,
334 respectively. In case of heat stress experiment, *E. coli* cells were inoculated in LB at 30 °C
335 with shaking at 180 rpm until OD_{660} reached ~0.4. The culture was then moved into water
336 bath set at 45 °C and incubated for another 30 min with shaking.

337 *E. coli* cells harboring pCA24N or its derivatives were grown overnight in LB medium
338 containing 20 μg/ml of chloramphenicol, and were then inoculated into fresh LB medium
339 containing chloramphenicol. They were grown at 37 °C with shaking at 180 rpm until the
340 OD_{660} reached ~0.5. The expression of SecM was then induced by adding 100 μM isopropyl
341 β-D-1-thiogalactopyranoside (IPTG) for 30 min.
342

343 **SDS-PAGE under neutral pH conditions and silver staining.**

344 Samples were diluted in 2× SDS sample buffer (final 62.5 mM Tris-HCl, pH 6.8, 2% SDS,
345 10% glycerol, 5% β-mercaptoethanol, a trace of bromophenol blue), as described by
346 Laemmli. SDS sample buffer was treated with RNA Secure (Ambion) according to the
347 manufacturer's specifications. The samples were separated by the 11% WIDE Range SDS-
348 PAGE system (Nacalai Tesque), as described previously (10, 34).

349 Silver staining of the gels was performed using a PlusOne Silver Staining Kit, Protein (GE
350 Healthcare), according to the manufacturer's specifications in protocol "E," except that
351 glutardialdehyde (25% w/v) was omitted from the *sensitizing solution* in the kit.
352

353 **Phenol extraction and TCA/acetone precipitation to fractionate polypeptides**
354 **and polypeptidyl-tRNAs from nucleic acids.**

355 *E. coli* cells grown in 200 mL of LB medium until the mid-log phase were mixed with an
356 approximately equal volume of crushed ice, and collected by centrifugation at 4 °C for 10
357 min. After supernatant removal, the cell pellet was suspended in 20 mL of TriPURE isolation
358 reagent (Roche) and incubated for 5 min at room temperature. The lysate was then mixed with
359 4 ml of chloroform, vortexed for 20 sec, incubated for 5 min at room temperature, and

360 centrifuged at 12,000 ×g for 5 min at 4 °C. The colorless to pale yellow transparent upper
361 layer (aqueous phase, 10-12 mL) was removed, leaving an organic phenol phase and a solid
362 middle layer. After the addition of an equal volume (12-15 mL from a 200 mL culture) of ice-
363 cooled 20% TCA/acetone containing 5 mM dithiothreitol (DTT), the mixture was vortexed
364 for 20 sec and incubated for at least 3 hrs at -20 °C. The sample was centrifuged at 15,600 ×g
365 for 20 min at 4 °C, and the supernatant was carefully discarded. The precipitate was vortexed
366 with 10 mL of acetone and centrifuged at 15,600 ×g for 5 min at 4 °C, and then the
367 supernatant was discarded by aspiration. This washing procedure was repeated twice.
368

369 ***In vitro* translation**

370 Coupled transcription-translation reaction was performed using PURE*frex 1.0*
371 (GeneFrontier) in the presence of 0.2 μM of anti-SsrA oligonucleotide
372 (TTAAGCTGCTAAAGCGTAGTTTTCGTCGTTTGCGACTA) and pre-charged Cy5-Met-
373 $tRNA^{fMet}$ at 37 °C for 40 min (34). DNA templates were prepared by PCR as summarized in
374 Table S2. The reaction was stopped by dilution into twenty volumes of TriPURE isolation
375 reagent. Following phenol-extraction and the precipitation of pep-tRNAs in the aqueous
376 phase were performed according to the manufacturer's instructions. The pep-tRNAs in the
377 organic phase were precipitated by TCA/acetone precipitation as described above. The
378 precipitates were resolved in 1× SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 2 % SDS,
379 10% glycerol, 50 mM DTT, treated with RNasecure) and separated by the 11% WIDE Range
380 SDS-PAGE. Then Cy5-labeled pep-tRNAs were detected by using Amersham Typhoon
381 scanner RGB. The signal intensities were quantified by MultiGauge (FUJIFILM) and the ratio
382 of pep-tRNA in the organic phase was calculated as below.
383

384 Pep-tRNA (organic phase) / [Pep-tRNA (organic phase) + Pep-tRNA (aqueous phase)]
385

386 **Peptidyl-tRNA purification using a silica column.**

387 The precipitate obtained from the organic phenol phase was resolved in a 1 mL volume of
388 phase-transfer surfactants (PTS) buffer {100 mM Tris-HCl, pH 6.8, 24 mM sodium
389 deoxycholate, 24 mM sodium N-lauroyl sarcosinate (32)} by vortex mixing.

390 As the next step, the peptidyl-tRNAs in the PTS buffer were isolated by using a High
391 Pure miRNA Isolation Kit (Roche). After adding 2080 μL of *binding buffer*, the lysate was
392 vortexed and mixed at 37 °C for 30 min with shaking. Then 660 μL of *binding enhancer* was
393 added and the lysate was centrifuged at 12,000 × g for 3 min at 4 °C to remove the
394 precipitates. The supernatant was loaded into 4 columns, and the following steps were

395 performed according to the manufacturer's instructions. The silica-bound RNA was eluted
396 with 100 μL of PTS buffer, and the collected eluate was divided into two equal portions, and
397 one was treated with 100 μg/mL of RNase A (Promega) at 37 °C for 1 hr. This purification
398 was repeated twice.
399
400 **LC-MS/MS sample preparation for the PETEOS experiment.**
401 The purified RNA sample in the previous section (100 μL) was mixed with 10 μL of 2 M
402 Tris base (pH= ~11) and incubated at 80 °C for 20 min to hydrolyze the ester bond of the
403 peptidyl-tRNA. After the addition of 90 μL deionized water, the sample was reduced by the
404 addition of 10 mM DTT at room temperature for 30 min, and then alkylated with 50 mM
405 iodoacetamide in the dark at room temperature for 20 min. The mixture was diluted 5-fold
406 with 50 mM ammonium bicarbonate. For the limited digestion of denatured polypeptides into
407 peptide fragments, 0.5 μg of Lys-C / Trypsin mix (Promega) was added, and the solution was
408 incubated at room temperature for at least 3 hrs. Subsequently, 0.5 μg of Lys-C / Trypsin mix
409 was further added and incubated at 37 °C overnight, whereas for the SecM analysis, 0.5 μg of
410 Lys-C was used. After the digestion, an equal volume of ethyl acetate and 0.5% trifluoroacetic
411 acid (final concentration) was added. The mixture was shaken vigorously for 2 min and
412 centrifuged at 15,700 ×g for 2 min. The upper ethyl acetate layer was removed, and the
413 solvent was removed by a centrifugal evaporator. The residual pellet was dissolved in 0.1%
414 TFA and 2% acetonitrile and desalted, as follows. The solution was applied to an SDB-XC
415 StageTip made by an Empore disk (3M, U.S.A.), equilibrated with 0.1% TFA and 2%
416 acetonitrile, washed with 0.1% TFA and 2% acetonitrile, and eluted with 0.1% TFA and 80 %
417 acetonitrile. The solvent was removed by a centrifugal evaporator, and the residue was
418 dissolved in 0.1% TFA and 2% acetonitrile. This solution was centrifuged at 20,000 ×g for 5
419 min, and the supernatant was collected and subjected to the LC-MS/MS measurement.
420
421 **LC-MS/MS measurement for the PETEOS experiment.**
422 The LC-MS/MS measurements were conducted with a Q-Exactive tandem mass
423 spectrometer and an Easy-nLC 1000 nano HPLC system (Thermo Fisher Scientific). The trap
424 column used for the nano HPLC was a 2 cm × 75 μm capillary column packed with 3 μm
425 C18-silica particles (Thermo Fisher Scientific), and the separation column was a 12.5 cm × 75
426 μm capillary column packed with 3 μm C18-silica particles (Nikkyo Technos, Japan). The
427 flow rate for the nano HPLC was 300 nL/min. The separation was conducted using a 10–40%
428 linear acetonitrile gradient over 70 min, in the presence of 0.1% formic acid. The nanoLC-
429 MS/MS data were acquired in the data-dependent acquisition (DDA) mode controlled by the

430 Xcalibur 4.0 program (Thermo Fisher Scientific). The DDA settings were as follows: the
431 resolution was 70,000 for a full MS scan and 17,500 for an MS2 scan; the AGC target was
432 3.0E6 for a full MS scan and 5.0E5 for an MS2 scan; the maximum IT was 60 ms for both the
433 full MS and MS2 scans; the full MS scan range was 310-1,500 m/z; and the top 10 signals in
434 each full MS scan were selected for the MS2 scan. The DDA measurement was performed
435 three times for each sample, and two biological replicates were measured in each condition
436 except for the set of antibiotics experiment.
437

438 **Data analysis for the PETEOS experiment.**

439 The data analysis of the DDA measurements was performed with the Proteome Discoverer 2.4
440 program bundled with the SEQUEST HT search engine (Thermo Fisher Scientific). The
441 dataset of the amino acid sequences of *E. coli* genes was obtained from the UniProt database
442 (UP000000625, downloaded on Aug. 20th, 2019). The ratio calculation and the hypothesis test
443 were conducted by "Summed Abundance Based" and "Individual proteins" mode,
444 respectively. The detailed settings of Proteome Discoverer 2.4 were listed in Supplementary
445 Table S3. Only the peptides/proteins with a < 1 % false discovery rate (by the Percolator
446 algorithm) were selected. For the peptide analyses, the peptides whose number of peptide-
447 spectrum matches (PSMs) was less than three were excluded from the analyses. For the
448 analysis at the protein level, the proteins whose number of detected peptides was less than
449 two were excluded from the analyses. The processes and statistical analyses of the output data
450 of Proteome Discoverer 2.4 were conducted by the R software (version 4.1.3 with in-house
451 scripts).
452

453 **Sample preparation and LC-MS/MS measurement and analysis for conventional**
454 **proteome analysis.**

455 The sample preparation was performed according to reference (42). Briefly, the cells
456 were disrupted and containing proteins were precipitated by adding 10% TCA. The proteins
457 were re-dissolved in PTS buffer and alkylated with 55 mM iodoacetamide after reduction
458 with 10 mM DTT. Then, the proteins were digested by Trypsin/Lys-C Mix (Promega). After
459 the digestion, surfactants were removed by the ethyl acetate treatment at low pH, and the
460 resulting peptides were desalted with an SDB-XC StageTip.

461 The measurement for the comparison against the phenol-extracted proteins (Figure
462 S1A) was performed in DDA mode using a Q-Exactive tandem mass spectrometer and an
463 Easy-nLC 1000 nano HPLC system (Thermo Fisher Scientific). The detailed settings were as
464 described above. The data analysis was performed using Proteome Discoverer 2.4 with the

465 same settings as described above. For the heat shock experiment, the measurement was
466 performed in DIA/SWATH-acquisition mode using a TripleTOF 6600 tandem mass
467 spectrometer and an Eksigent nanoLC 415 nano HPLC system (SCIEX). The analysis of
468 DIA/SWATH data was performed using DIA-NN software (version 1.7.16) with default
469 settings (43). The library for DIA-SWATH analysis was obtained from the SWATHatlas
470 database (http://www.swathatlas.org/, accessed on 30 April 2021); the original data were
471 acquired by Midha *et al* (44). The following analysis was performed using in-house R scripts
472 (R.app for Mac, version 4.1.3). The detailed settings of the measurement and the analyses
473 were the same as described in the previous report (42).
474
475 **Data Availability Statement.**
476 Data in this manuscript have been uploaded to the Mendeley Dataset public repository
477 (https://doi.org/10.17632/xzycy8m6tb.1). The mass spectrometry data have been deposited in
478 the jPOST repository (45) (https://repository.jpostdb.org/), with reference number
479 JPST001572/PXD033726.
480
481
482 **Author contributions.**
483 YC, AY and AK performed experiments; TN and AY performed LC-MS/MS and analyzed
484 the MS data; YC, TN, and HT conceived the study, designed experiments, and analyzed the
485 results; YC, TN, and HT supervised the entire project; TN, AY, YC and HT wrote the
486 manuscript.
487
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494 Ohsumi Frontier Science Foundation to YC.
495
496 **Conflicts of Interest.**
497 The authors declare no competing interest.
498
499 **References.**

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608
609

610 **Figure Legends**

611 **Figure 1. PETEOS workflow to enrich and identify polypeptidyl-tRNAs (pep-**
612 **tRNAs) in cells**

613 **A. Step 1**: Enrichment of pep-tRNAs and proteins. Log phase *E. coli* cells (OD660 ~ 0.5)
614 cultured at 37 °C are extracted by phenol/chloroform to capture the hydrophilic RNA
615 molecules into the aqueous phase. Relatively hydrophobic proteins and pep-tRNAs in the
616 organic phase are precipitated by TCA/acetone. **Step 2**: Isolation of the pep-tRNAs by a silica
617 column in the presence of concentrated chaotropic salts. **Step 3**: Hydrolysis of the ester bond
618 between peptide and tRNA in pep-tRNA under high pH and high temperature. **Step 4**:
619 Identification of the peptide moieties of the pep-tRNAs by conventional shotgun proteomics
620 after trypsin digestion. **B**. Fractionation of pep-tRNAs by phenol-extraction. *Left*, schematic
621 of the truncated nonstop mRNAs to prepare the pep-tRNA with various lengths of the
622 polypeptide moiety. *Right*, the fractionation ratio of the pep-tRNA into the organic phase was
623 calculated from the gel image (representative in Fig. S1B) as described in materials and
624 methods. Pep-tRNAs carrying N-terminal 10, 20, 40, 80 or 160 aa of *E. coli* enolase (Eno),
625 DHFR, *secM*, or superfolder GFP mRNAs were examined. The mean ± SE values of three
626 independent experiments are shown.

627 **C**. Purification of SecM-tRNA by silica column. The lysate of *E. coli* cells overexpressing
628 *secM* was processed by silica column purification as described in materials and methods.
629 Cellular extract (lane 1) and the eluate with (lane 3) or without (lane 2) RNase treatment
630 during the isolation procedure were separated by neutral pH SDS-PAGE and developed by
631 silver staining.

632 **D.** MS2 spectra of the C-terminal peptides of SecM ($_{150}$FSTPVWISQAQGIRAG$_{165}$) from the
633 *E. coli* cells overexpressed SecM.

634

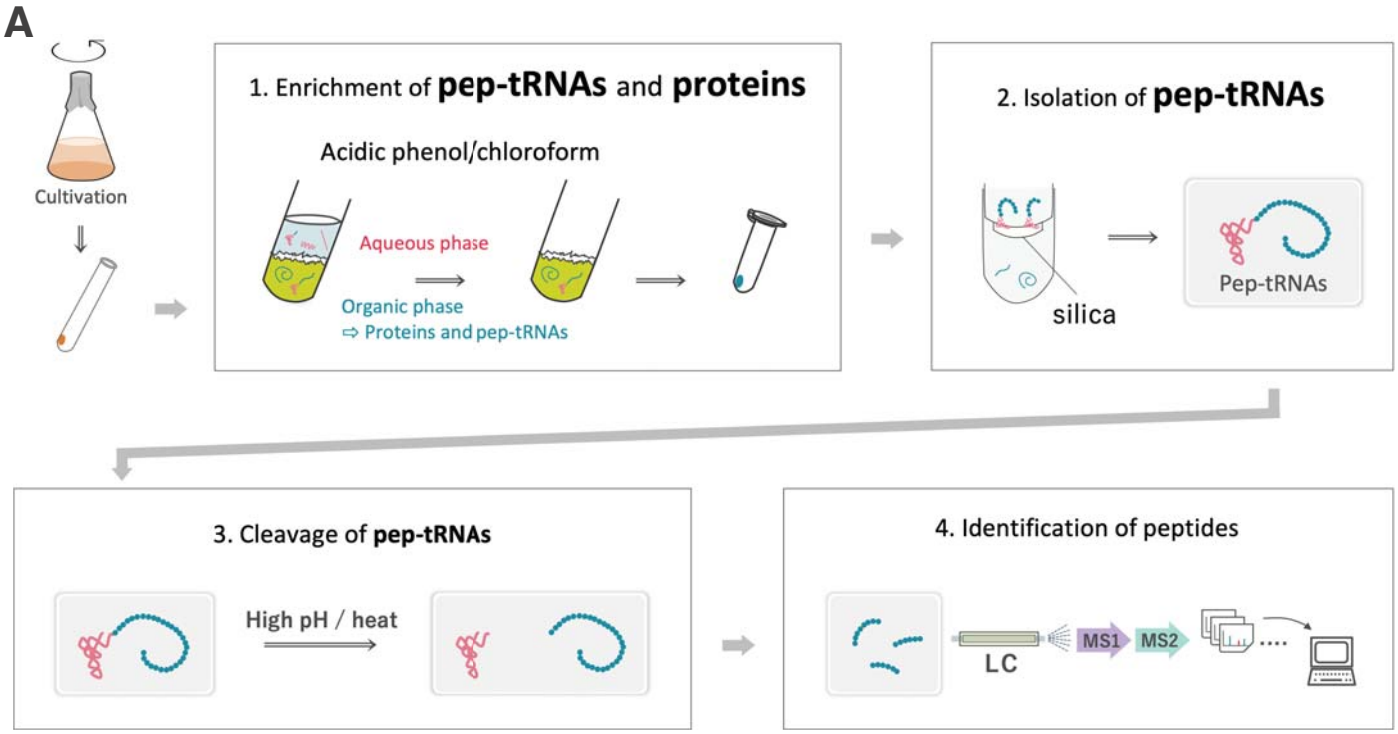
635 **Figure 2. Enrichment and identification of pep-tRNAs in *E. coli***

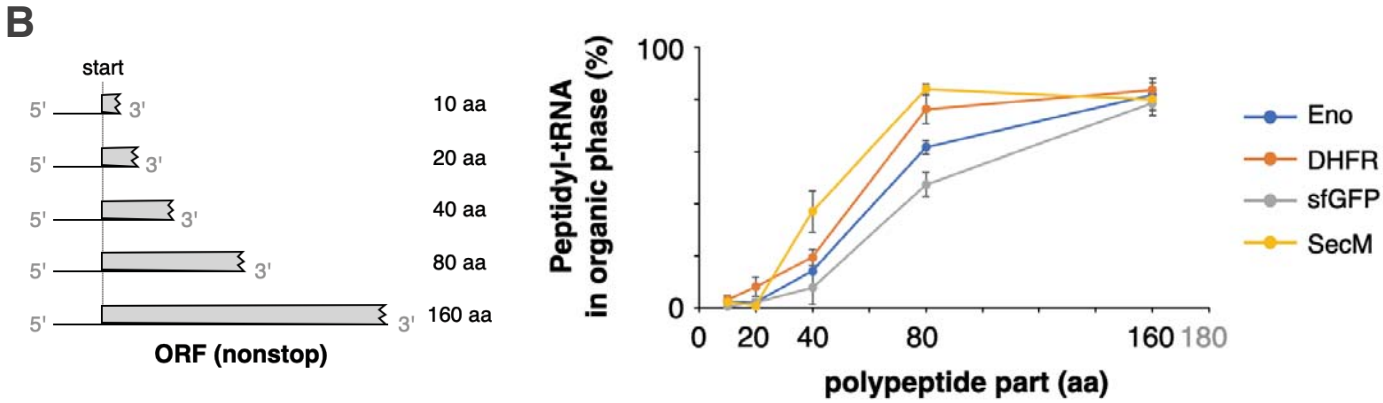
636 **A**. The eluate from the silica column (Step 2) was analyzed by SDS-PAGE under neutral pH,
637 and detected by silver staining. The eluate with an RNase treatment after Step 1 was also
638 analyzed. Pep-tRNA molecules are schematically indicated (tRNA moiety, orange; peptide
639 moiety, blue). **B**. Number of proteins identified as pep-tRNA molecules in the method. The
640 proteins with < 1% FDR and ≥ 2 number of detected peptides were counted. All 30 proteins
641 identified in the RNase-treated fraction were also detected from the no-treatment fraction. **C**.
642 Histogram of summed peptide intensity per protein (log10 scale) obtained by MS1 peaks for
643 proteins from the silica column eluate or the RNase-treated fraction. **D**. Overlap of the
644 detected proteins between two replicates. The proteins with < 1% FDR and ≥ 2 number of

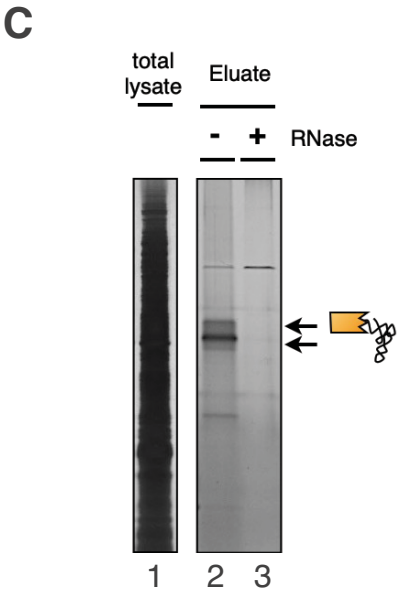
645 detected peptides were counted. **E** (Upper) Illustration of pep-tRNAs and pre-existing
646 proteins digested by trypsin. Trypsin digestion of pep-tRNAs, which elongate from the N- to
647 the C-terminus, should accumulate more peptides containing the N-terminal portion. In
648 contrast, for pre-existing proteins, which basically have full-length polypeptides, the amounts
649 of trypsin-digested peptides should be equal for all peptides. Arrowheads colored in red
650 indicate the sites for the trypsin cleavage. Relative positions of the N-terminus and C-
651 terminus are defined as 0 and 1, respectively. (Lower) Meta-analysis of the relative position of
652 the peptides identified by LC-MS/MS for pep-tRNAs and pre-existing proteins.
653

654 **Figure 3. Investigation of the changes in the nascent peptides/proteins by heat-**
655 **shock and antibiotics.**

656 **A.** Quantitative assessment of the changes by the heat-shock (45 ºC, 30 min) at the peptide
657 level. Red dots indicate the peptides derived from the heat shock proteins and proteases
658 (GroEL/ES, DnaK, ClpB, HtpG, Lon, and FtsH). Red dotted lines represent 5 and 1/5 fold
659 change values, indicating around the 2σ point obtained by comparing two replicates (see Fig.
660 S2D). The horizontal axis indicates the value of the fold changes by the heat shock taken as
661 $\log_2$, and the vertical axis indicates *P*-values by t-test with three technical replicates in each
662 sample taken as $-\log_{10}$. The values of the fold changes and *P*-values were obtained by
663 Proteome Discoverer 2.4 (detailed settings were described in the Materials and Methods
664 section and Supplementary Table S3). The peptides with less than three number of peptide-
665 spectrum matches (PSMs) were omitted. **B.** Fold changes of the heat shock proteins evaluated
666 by the PETEOS method and a conventional proteome method. The fold change data by the
667 conventional method was acquired by the SWATH-MS acquisition method. **C.** Number of the
668 detected peptides from the antibiotics-treated samples. The number of the peptides whose C-
669 termini was not Lys (K) or Arg (R) were also shown. The peptides with less than three
670 number of PSMs were omitted. **D.** Quantitative assessment of the changes by treating the
671 antibiotics at the protein level. Red dotted lines represent 5 and 1/5 fold change values,
672 indicating around the 2-sigma point obtained by comparing two replicates (see Fig. S2D). The
673 horizontal axis indicates the value of the fold changes by the treatment taken as $\log_2$, and the
674 vertical axis indicates *P*-values by t-test with three technical replicates in each sample taken
675 as $-\log_{10}$. The values of the fold changes and *P*-values were obtained by Proteome Discoverer
676 2.4 (detailed settings were described in the Materials and Methods section and Supplementary
677 Table S3).
678


A
Cultivation
1. Enrichment of pep-tRNAs and proteins
Acidic phenol/chloroform
Aqueous phase
Organic phase
⇨ Proteins and pep-tRNAs
2. Isolation of pep-tRNAs
silica
Pep-tRNAs
3. Cleavage of pep-tRNAs
High pH / heat
4. Identification of peptides
LC
MS1
MS2


B
start
5'
3'
10 aa
20 aa
40 aa
80 aa
160 aa
ORF (nonstop)
Peptidyl-tRNA in organic phase (%)
100
0
0
20
40
80
160
180
polypeptide part (aa)
Eno
DHFR
sfGFP
SecM


C
total lysate
Eluate
-
+
RNase
1
2
3

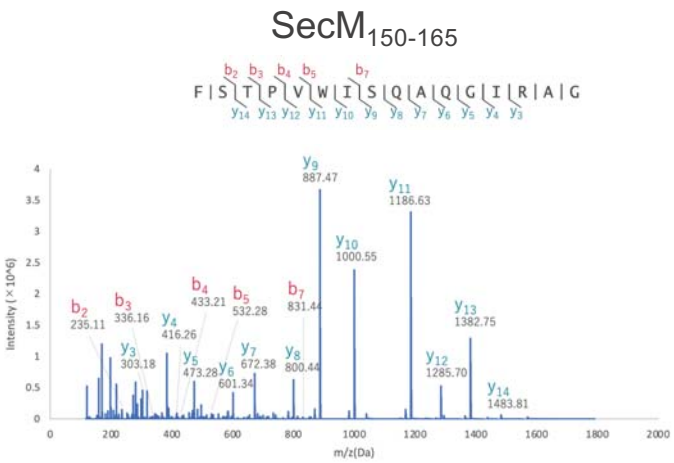
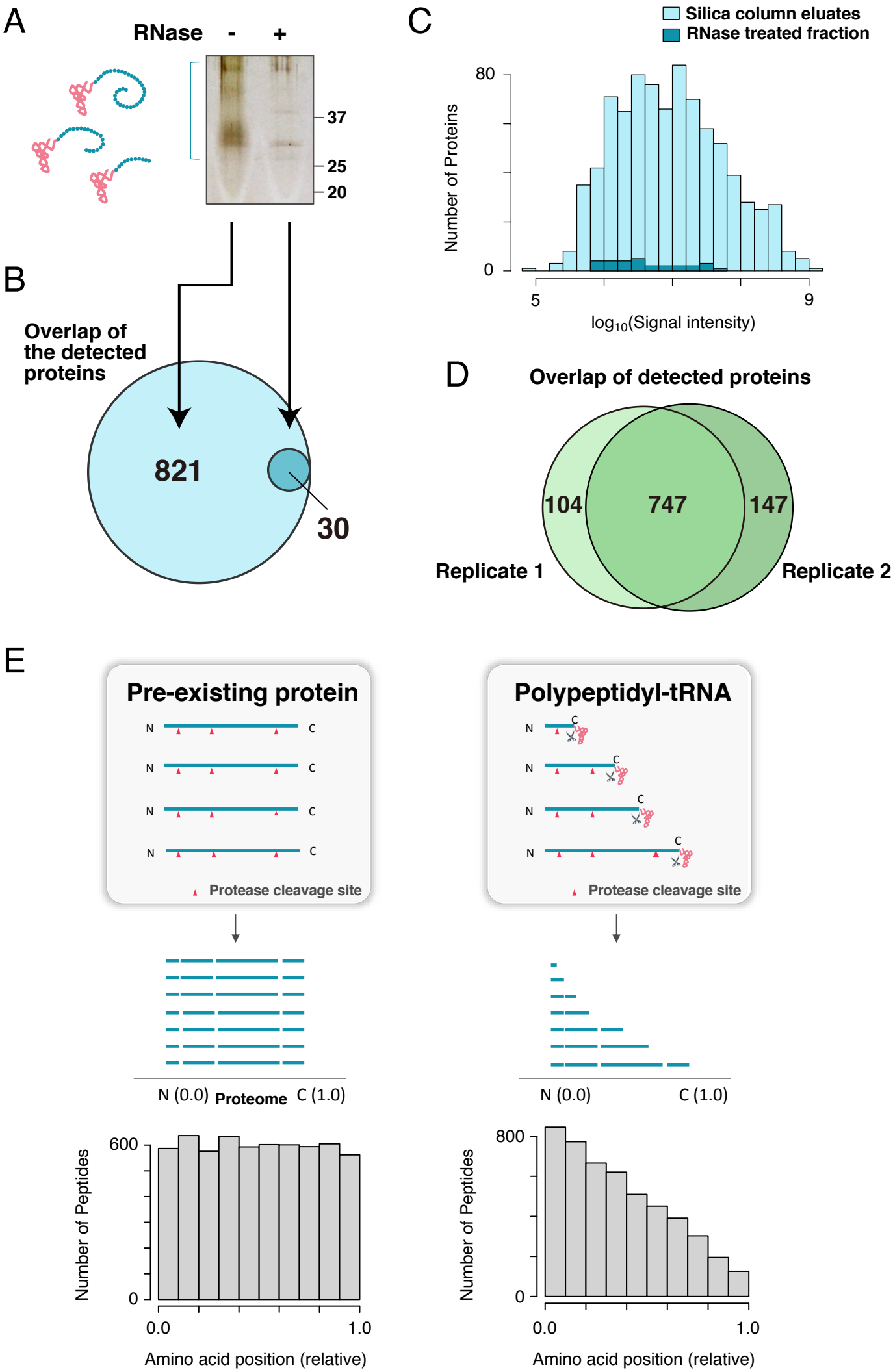
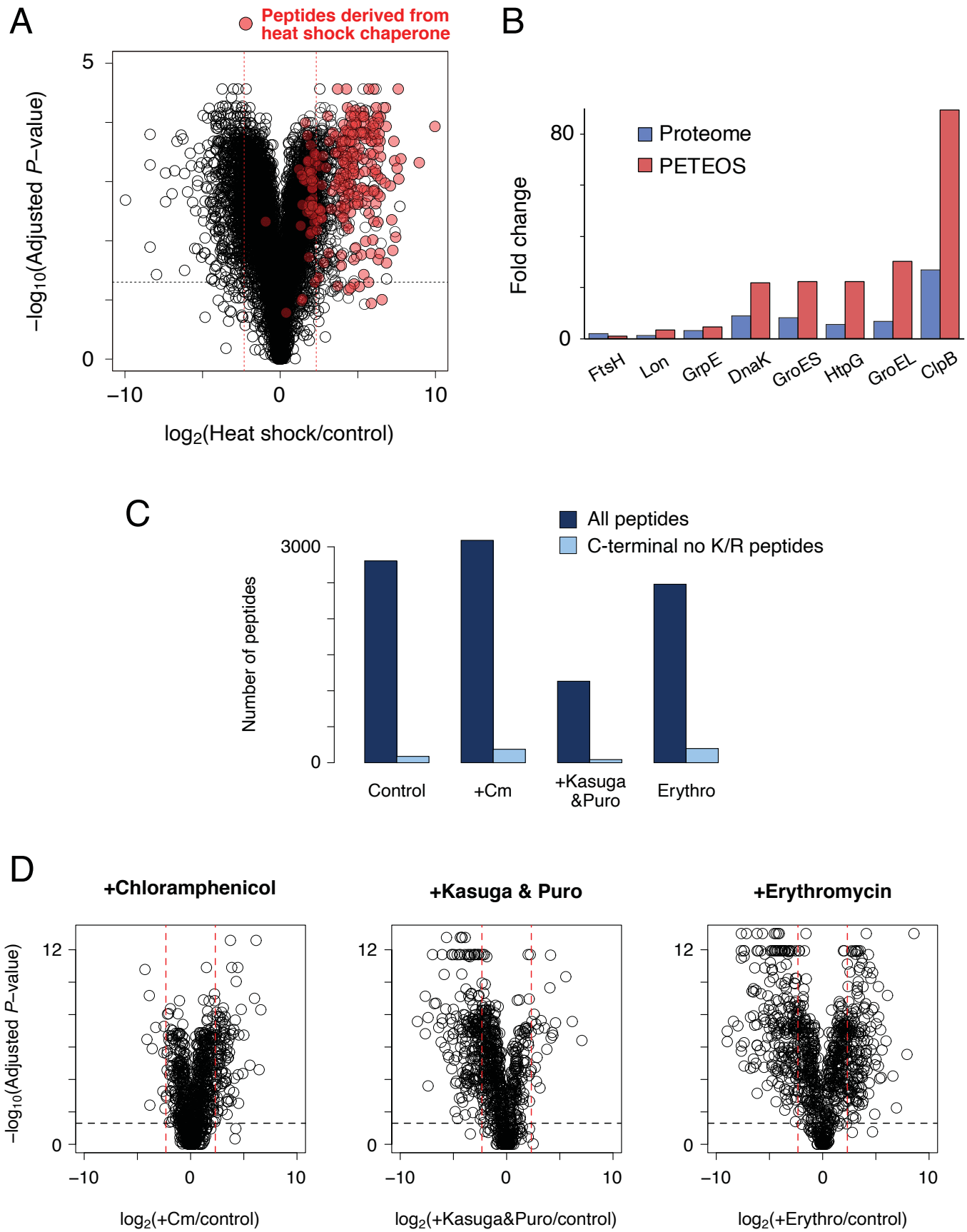

D
SecM150-165
F S T P V W I S Q A Q G I R A G
Intensity (×10^6)
m/z(Da)

Fig.1


A
RNase - +
37
25
20
B
Overlap of the detected proteins
821
30
C
Silica column eluates
RNase treated fraction
Number of Proteins
80
0
5
9
log10(Signal intensity)
D
Overlap of detected proteins
104
747
147
Replicate 1
Replicate 2
E
Pre-existing protein
N
C
Protease cleavage site
Polypeptidyl-tRNA
N (0.0)
Proteome
C (1.0)
Number of Peptides
600
0
0.0
1.0
Amino acid position (relative)
800


A
Peptides derived from heat shock chaperone
5
0
−10
0
10
−log10(Adjusted P−value)
log2(Heat shock/control)
B
Proteome
PETEOS
80
0
Fold change
FtsH
Lon
GrpE
DnaK
GroES
HtpG
GroEL
ClpB
C
All peptides
C-terminal no K/R peptides
3000
0
Number of peptides
Control
+Cm
+Kasuga &Puro
Erythro
D
+Chloramphenicol
+Kasuga & Puro
+Erythromycin
12
0
−10
0
10
−log10(Adjusted P−value)
log2(+Cm/control)
log2(+Kasuga&Puro/control)
log2(+Erythro/control)