An antimicrobial peptide that inhibits translation by trapping release factors on the ribosome

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Many antibiotics stop bacterial growth by inhibiting different steps of protein synthesis. However, no specific inhibitors of translation termination are known. Proline-rich antimicrobial peptides, a component of the antibacterial defense system of multicellular organisms, interfere with bacterial growth by inhibiting translation. Here we show that Api137, a derivative of the insect-produced antimicrobial peptide apidaecin, arrests terminating ribosomes using a unique mechanism of action. Api137 binds to the Escherichia coli ribosome and traps release factor (RF) RF1 or RF2 subsequent to the release of the nascent polypeptide chain. A high-resolution cryo-EM structure of the ribosome complexed with RF1 and Api137 reveals the molecular interactions that lead to RF trapping. Api137-mediated depletion of the cellular pool of free release factors causes the majority of ribosomes to stall at stop codons before polypeptide release, thereby resulting in a global shutdown of translation termination.

RESULTS
Api137 arrests translation at the stop codon of mRNAs

To identify the stage of translation inhibited by Api137, we used in vitro toeprinting analysis, which determines the location of stalled ribosomes on mRNA13. In contrast to Onc112, which arrests translation at the start codon12,13 (Fig. 1a), Api137 arrested translation when the stop codon entered the A site of the ribosome (Fig. 1b). Similar stalling at the stop codon was obtained with other tested mRNAs when translation was carried out in the presence of Api137 or the unmodified natural apidaecin 1a (Supplementary Fig. 1). These results show that Api137, unlike other ribosome-targeting PrAMPs or any other known antibiotic, has the unique ability to specifically arrest the terminating ribosome.

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Mutations in RF1, RF2 and the ribosome confer resistance to Api137

In order to identify the components of the translation apparatus that are involved in the mechanism of Api137 action, we carried out an unbiased selection of spontaneous Api137-resistant mutants in two E. coli strains. We isolated three types of mutants. The resistance in the first type of mutant was caused by nonsense mutations in the sbmA gene (Supplementary Fig. 2a) encoding the transporter responsible for importing PrAMPs into the cell.

Resistant mutants of the second type carried mutations in the prfA or prfB genes encoding RF1 and RF2, respectively. RF1 and RF2 recognize the stop codon of the mRNA and facilitate hydrolysis of the peptidyl-tRNA ester bond, releasing the completed protein (reviewed in ref. 1). Mutants isolated using E. coli strain SQ110 carried a mutation in the prfA gene, which resulted in the replacement of Asp241 of the encoded RF1 with a glycine residue (Supplementary Fig. 2). The Api137-resistant mutant isolated with the E. coli strain BL21 had mutations in the prfB gene, resulting in substitutions R262C or Q280L in RF2 (Supplementary Fig. 2). The difference in the results obtained using these two strains probably reflects the fact that SQ110, as a derivative of the K12 strain, carries an alteration in the prfB gene that results in the replacement of Ala246 of RF2 with a threonine20 (Supplementary Fig. 2d). This mutation affects the properties of RF2 (ref. 21) and could conceivably alter the interactions of the K12-type RF2 with Api137. The RF1 and RF2 mutations found in Api137-resistant strains are located in proximity to the catalytically important GGQ motif (Supplementary Fig. 2b,c), suggesting that Api137 interferes with the function of RF1 and RF2.

The third type of Api137-resistant mutants had a mutation in the gene rplP encoding ribosomal protein uL16 (Supplementary Fig. 2). Subsequent testing of other ribosomal-protein mutants showed that mutations in the proteins uL22 and uL4, which are located in the nascent peptide exit tunnel, also increased resistance to Api137 (Supplementary Fig. 2). In agreement with this observation, mutations of nearby 23S rRNA nucleotides A2059 and A2503 rendered cells resistant to Api137 (Supplementary Fig. 2). Consistently, Api137 did not induce pronounced arrest of the A2059C or A2503G mutant ribosomes at the stop codons in vitro (Fig. 1c). Taken together, these results indicate that Api137 interferes with translation termination by influencing functional interactions between RF1 or RF2 and the ribosome.

ApI137 inhibits turnover of RF1 and RF2

To understand the mode of inhibition of translation termination by Api137, we used a fully reconstituted in vitro translation system. We prepared a model termination complex corresponding to the state of the ribosome before hydrolysis of peptidyl-tRNA (prehydrolysis complex, PreHC)4,22 (Fig. 2a). Mixing the PreHC with RF1 or RF2 results in the hydrolysis of the ester bond linking fMet to the P-site tRNA, emulating the polypeptide-release reaction. At a high concentration of RF1 or RF2, when recycling of the factors was not required for the reaction to progress to completion, rapid and complete hydrolysis of peptidyl-tRNA was observed even in the presence of high Api137 concentrations (Fig. 2b), suggesting that Api137 does not inhibit peptidyl-tRNA hydrolysis. In contrast, at limiting concentrations of RF1 or RF2, when multiple rounds of binding and dissociation of the factors from PreHC were needed to achieve termination on all PreHGs, the reaction was dramatically inhibited in the presence of as little as 1 µM Api137 (Fig. 2c). This result suggested that Api137 either competes with the RFs for binding to the PreHC or traps the RFs in the posthydrolysis (PostHC) complex, abolishing recycling of the factor.

To distinguish between these scenarios, we directly examined the effect of Api137 on RF1 binding or dissociation using a fluorescent derivative of fMet-tRNAfMet (PreHCFlu) and a quencher-dye-labeled

Figure 1 ApI137 stalls ribosomes at the termination step of translation. (a) Amino acid sequences of PrAMPs Api137 and Onc112. gu, N,N′-tetramethylguanidino; OH, hydroxyl; r = D-arginine. (b,c) In vitro toeprinting analysis comparing the Onc112- or Api137-mediated (labeled Onc and Api, respectively) translation arrest on model mRNA templates derived from the yrbA (b) or ermCL (c) genes. Positions of the toeprint bands (indicated by arrowheads on the gene sequence) are 16–17 nt downstream from the first nucleotide of the P-site codon. The P- and A-site codons of the stalled ribosomes are indicated by brackets. Toeprints in c were produced by wild-type ribosomes (WT) or by ribosomes with mutations in specific RNA nucleotides (Supplementary Fig. 2a). Gray arrowheads indicate toeprint bands in b and c generated by Onc112-arrested ribosomes at the initiation codon; white arrowheads indicate bands from ribosomes arrested by Api137 at termination. The similar intensities of the PrAMP-independent toeprint bands marked with a black arrowhead in c shows that WT and mutant ribosomes translate with comparable efficiencies. Sequencing reactions are marked. The gels are representatives of six (b) and two (c) independent biological replicates.
Figure 2 Apis137 allows peptide hydrolysis but inhibits turnover of RF1 and RF2. (a) Schematics of the peptidyl-tRNA hydrolysis experiments. PreHC carrying [3H]Met-tRNA^Met is reacted with RF1 (shown) or RF2, and the release of f[3H]Met is measured. (b) Time courses of peptide hydrolysis in PreHC in the presence of excess RF1 without (black) or with the indicated concentrations of Apis137 (colored traces). (c) Time courses of peptide hydrolysis in PreHC by RF1 (black) and RF2 (red) under turnover conditions in the absence (open circles) or presence (closed circles) of 1 µM Apis137. RF3-GTP was present in all reactions. Control experiments (blue) lacked RF1 and RF2 in the absence (open circles) or presence (closed circles) of Apis137. 100% corresponds to ten cycles of RF binding, catalysis and dissociation. Error bars represent the range of two independent replicates. (d) Schematics of the RF1-binding experiments. PreHC carries fluorescein-labeled fMet-tRNA^Met (PreHCFlu) and RF1 carries fluorescence quencher dye (RF1 Qsy). (e) Time courses of binding of RF1Qsy to PreHCFlu in the absence (red) or presence (blue) of Apis137. Gray trace, no RF1. The fluorescence traces represent the average of five to seven technical replicates. a.u., arbitrary units. (f) Time course of RF1 dissociation. RF1Qsy was incubated with PreHCFlu to generate PostHCFlu and then mixed with a ten-fold excess of unlabeled RF1 and RF3-GTP in the absence (gray) or in the presence (black) of Apis137. The traces represent the average of up to seven technical replicates. Details in Online Methods.

RF1 (RF1Qsy) and following changes in fluorescence resonance energy transfer (Fig. 2d). Though Apis137 did not affect binding of RF1 (Fig. 2e), it entirely blocked RF1 dissociation (Fig. 2f), demonstrating that Apis137 prevents turnover of RF1 and RF2 by trapping them on the ribosome. When similar experiments were carried out with the Apis137-resistant mutant of RF1 (Supplementary Fig. 2a), Apis137 was unable to abolish RF1 dissociation (Supplementary Fig. 3a), indicating that the mutation allowed RF1 to escape Apis137-mediated trapping in the PostHC complex. Similarly, the RF2 A246T mutation endemic in the K12 E. coli strain and located in the vicinity of the selected Apis137-resistance mutations (Supplementary Fig. 2d) showed considerably increased tolerance of Apis137 inhibition compared to the unaltered RF2 (Supplementary Fig. 3b). Collectively, these results showed that Apis137 traps RF1 and RF2 on the ribosome after the release of the nascent protein, abolishes RF turnover and prevents disassembly of the termination complex and recycling of the ribosome for new rounds of translation.

Interactions of Apis137 with the ribosome and RF1 illuminate molecular mechanisms of RF trapping

To obtain insights into the molecular mechanism of RF trapping, we determined a cryo-EM structure of Apis137 bound to a terminating ribosome (Fig. 3). The ribosome–nascent chain complex bearing a UAG stop codon in the A site was prepared by translating in vitro4 the ermCL ORF in the presence of Apis137 and then purified and subjected to cryo-EM analysis. In silico sorting of the cryo-EM data revealed a major subpopulation of ribosomes bearing a tRNA in the P site and RF1 bound in the A site (Supplementary Fig. 4 and Table 1). A final cryo-EM reconstruction with an average resolution of 3.4 Å enabled the generation of a molecular model for the entire complex (Fig. 3a). In the Apis137-stalled complex, the conformation of RF1 is similar to that observed previously in the PostHC during canonical termination23,24 (Supplementary Fig. 5a–c). Consistent with our kinetics data, the P-site tRNA is deacylated, showing that RF1 has catalyzed hydrolysis of the polypeptide chain in the presence of Apis137. A distinct electron density observed within the ribosomal exit tunnel could be unambiguously assigned to residues 5–18 of Apis137 bound in an extended conformation (Fig. 3b and Supplementary Fig. 4g,h). The orientation of Apis137 within the tunnel matches that of a nascent peptide.
peptide (Supplementary Fig. 5d) but is opposite from that observed for other investigated PrAMPs (Supplementary Fig. 5e). The C-terminal Arg17 and Leu18, which are critical for the activity of Api137 (ref. 16), are positioned close to the PTC (Supplementary Fig. 5a). However, in contrast to other PrAMPs that encroach upon the PTC A site, Api137 is positioned entirely within the exit tunnel, allowing it to bind when the A site is occupied by RF1 or RF2 (Supplementary Fig. 5e).

Api137 makes multiple interactions with the exit tunnel, including stacking and van der Waals interactions with the 23S rRNA nucleotides (Fig. 3d,e) and a potential hydrogen bond with the ribosomal protein uL4 (Fig. 3f), clarifying how rRNA and ribosomal protein mutations could confer resistance (Supplementary Figs. 2 and 6).

The interactions of the central and N-terminal segments of Api137 with the tunnel elements help to place the functionally critical C-terminal amino acids of Api137 in the vicinity of the GGQ motif of RF1 in the PTC (Supplementary Figs. 2a–c). The side chain of the penultimate residue Arg17 of Api137 is fixed in place by hydrogen bonding with the 2′-hydroxyl of the G2505 ribose and the O2 of the C2452 base (Supplementary Fig. 2b). This network of hydrogen bonds with the nucleotides of the 23S rRNA positions Arg17 for interaction with RF1. The Gln235 side chain carboxyl of RF1 is within hydrogen bond distance from the terminal nitrogen of the Arg17 guanidinium group (Supplementary Fig. 2b). The contact between the Arg17 side chain and RF1 is likely to be critical, because mutations of the penultimate residue of Api137 decrease the affinity of the PrAMP for the ribosome and reduce its inhibitory activity (ref. 16). Additionally, the backbone carbonyl of Arg17 of Api137 is also within hydrogen bond distance of the Gln235 side chain amine of RF1 (Supplementary Fig. 2b). Interaction between Api137 and RF1 not only helps to trap the RF on the ribosome but also stabilizes binding of Api137 itself. RNA probing experiments showed that in the absence of RF1, Api137 only minimally shielded A2058, A2059 and A2062 from modification, whereas the PrAMP readily protected these nucleotides when RF1 was present (Supplementary Fig. 2d). The C-terminal hydroxyl of Api137 is within hydrogen bond distance of the ribose hydroxyls of A76 of the deacetylated P-site tRNA (Supplementary Fig. 2c). These interactions could further contribute to RF1 or RF2 trapping by preventing the ribosome from undergoing the RF3-stimulated transition into the rotated state required for RF1 or RF2 dissociation (refs. 2, 25).

The results of the structural analysis not only corroborate the findings of biochemical and genetic experiments but also illustrate the possible molecular mechanism of trapping RF1 and RF2 on the terminating ribosome after the release of the nascent peptide.

Api137-mediated RF depletion inhibits nascent peptide release

The number of ribosomes in the bacterial (E. coli) cell exceeds the number of RF2 and RF1 molecules by ~25-fold and ~200-fold, respectively (refs. 2, 3). Api137-mediated trapping of RF1 or RF2 on a relatively small number of ribosomes should lead to a rapid depletion of the RFs. As a consequence, there would be no RF1 or RF2 available to facilitate the peptide release when the remaining translating ribosomes reach a stop codon. Therefore, although Api137 arrests the ribosome in a posthydrolysis state, in the cells treated with Api137, most of the ribosomes should stall at stop codons in a prehydrolysis state carrying an intact peptidyl-tRNA.

We first tested this hypothesis in a cell-free translation system using the TnaC-stalling peptide as a model. At high tryptophan concentrations (5 mM), the RF2-mediated release of TnaC peptide is impeded, leading to a well-documented accumulation of TnaC–tRNA (Supplementary Fig. 5a). By contrast, at low concentrations of tryptophan (0.3 mM), the TnaC peptide is rapidly released at the RF2-specific UGA stop codon. Strikingly, when Api137 was present, TnaC–tRNA also accumulated at low tryptophan concentrations. A similar result was obtained with the tnaC template carrying an RF1-specific UAG stop codon (Supplementary Fig. 5a). These results demonstrated that as a consequence of RF1 or RF2 depletion due to Api137-mediated trapping on a fraction of ribosomes, the majority of ribosomes are unable to release the TnaC peptide. Consistent with this conclusion, the Api137-induced accumulation of TnaC–tRNA was largely rescued by supplementing the reaction with a five-fold molar excess of RF1 over the ribosomes (Supplementary Fig. 5b).

When the translating ribosome reaches a stop codon, the conditional binding of a near-cognate aminoacyl-tRNA instead of the RFs may promote a stop codon readthrough event. The Api137-induced depletion of the pools of free RF1 and RF2 is expected to bias this competition in favor of aminoacyl-tRNA binding. Indeed, Api137 strain carrying a mutant lacZ allele with a premature UAG stop codon (Supplementary Fig. 5c). Notably, the efficiency of Api137-induced readthrough was considerably higher than that induced by the miscoding antibiotic streptomycin (Supplementary Fig. 5c). These results confirm that while Api137 traps RF1 and RF2 on the ribosome after the nascent protein release, the main downstream effect of Api137 action is the arrest of the ribosomes in the prehydrolysis state (Supplementary Fig. 5d,e).

DISCUSSION

Our biochemical, genetic and structural data reveal Api137 as the first known inhibitor that is specific for translation termination. Though several inhibitors can potentially interfere with polypeptide release, these antibiotics also target other steps of protein synthesis; in these cases, inhibition of termination is just a collateral effect of the antibiotic binding to the ribosomal centers critical for various ribosomal activities. In contrast, Api137 does not inhibit initiation or elongation of translation but specifically arrests the ribosome at the stop codons. Api137 achieves...
its inhibitory action in two related but functionally distinct ways. The primary effect of Api137 is to trap RF1 and RF2 on the ribosomes after the release of the nascent peptide (Fig. 5d). This leads to depletion of the free RF pool and, as a result, the majority of cellular ribosomes are arrested at the stop codons in the prehydrolysis state (Fig. 5e). The arrested ribosome may additionally block other ribosomes on the same ORF from completing translation. Thus, treatment of cells with Api137 results in the formation of two populations of ribosomes stalled at the stop codons: a small fraction is arrested in a posthydrolysis state, whereas the majority carries unhydrolyzed peptidyl-tRNA.

Although Api137 belongs to the broad group of ribosome-targeting PrAMPs, its mode of binding is fundamentally different from those of the previously studied derivatives of oncocin, bactenecin, pyrrhocorin and metalnikowin10–14. Whereas the binding sites of all PrAMPs overlap, the orientation of Api137 is opposite to that observed for other PrAMPs. Furthermore, the N termini of other PrAMPs enroach upon the A site of the PTC, completely blocking it and hindering binding of any A-site substrates10–13, whereas Api137 binds entirely within the exit tunnel. Therefore, the binding of RF1 or RF2 to the A site is incompatible with the placement of oncocin and similar PrAMPs, whereas Api137 actually requires RF1 or RF2 for efficient binding.

Due to the spatial constraints of the tunnel, direct binding of Api137 promoted by its interactions with RF1 or RF2 is likely to occur only after the peptidyl-tRNA ester bond has been hydrolyzed and the newly synthesized protein has vacated the ribosome. Therefore, apidaecins have a rather narrow time window to exert their inhibitory action: namely, after the departure of the newly made protein but before RF1

**Figure 4** Inhibitory action of Api137 is mediated by its interactions with RF1 and P-site tRNA. (a) Position of Api137 (salmon) relative to RF1 (orange) and P-site tRNA (green). The boxed regions are enlarged in b and c. (b) Interactions of Api137 with RF1. Arg17 of Api137 is coordinated by bonding with 23S rRNA nucleotides C2452, G2505 and U2506 (gray) to form direct contacts with Gin236 of the GQG motif of RF1 (orange). (c) The C-terminal hydroxyl of Leu18 of Api137 interacts with the ribose of A76 of deacylated P-site tRNA (green). (d) Dimethylsulfate (DMS) probing of Api137 interaction with PostHC 23S rRNA in the absence or presence of RF1. The gel is representative of two independent experiments.

**Figure 5** Api137 induces accumulation of peptidyl-tRNA and stop codon readthrough. (a) Gel electrophoresis analysis of the [35S]-labeled products of the *in vitro* translation of the *tnaC* gene with its original UGA stop codon (lanes 1–6) or with the UAG stop codon (lanes 7–9), in the absence or presence of Api137. Lane 1, control reaction without mRNA. Lane 2 (M, marker) shows RNase-sensitive TnaC–tRNA accumulated at high concentration of tryptophan (Trp). Lanes 5 and 8 show Api137-induced accumulation of TnaC–tRNA at low concentration of tryptophan. The bands corresponding to TnaC–tRNA and the released TnaC peptide are indicated with filled and open arrowheads, respectively. The gel is representative of five independent biological replicates. (b) Excess of RF1 rescues Api137-induced accumulation of peptidyl-tRNA. Cell-free translation with a low tryptophan concentration was carried out in standard conditions (lanes 1 and 3) or with five-fold molar excess of RF1 over the ribosomes (lane 2). The gel is representative of two independent biological replicates. (c) Expression of the chromosomal mutant *lacZ* with a premature stop codon, mediated by stop codon readthrough stimulated by the miscoding antibiotic streptomycin (Str) or by Api137. The central circles indicate where droplets of Str or Api137 were placed on a lawn of *E. coli* cells grown on an LB-agar plate supplemented with ampicillin, IPTG and X-Gal. This plate represents one of three independent experiments. (d) A control plate. (e) The dual mode of Api137 action. (d) Api137 binds to the ribosome after RF1 or RF2 catalyzes the release of the complete protein and traps RF1 or RF2, thereby preventing their turnover. (e) Trapping of RF1 or RF2 depletes their available pool, causing the stalling of most of the ribosomes at the stop codons, unable to release the nascent proteins.
or RF2 dissociation. Within this window, Api137 has to traverse the entire length of the exit tunnel to reach its binding site close to the PTC where it can establish interactions with the RF. Thus, Api137-dependent trapping of RF1 and RF2 is probably a fairly rare event in the context of the global cellular translation. However, the resulting complex is long lived (Fig. 2d), and the majority of RF1 and RF2 molecules will eventually be sequestered.

Recycling of RF1 and RF2 in the cell is facilitated by RF3, but RF3 does not prevent trapping of RF1 or RF2 by Api137 in vitro (Fig. 2f). Nevertheless, minimal inhibitory concentration (MIC) testing shows that cells lacking RF3 are eight times more sensitive to Api137 than those expressing RF3 (Supplementary Table 1). This suggests that RF3 can partly mitigate the Api137 effect, probably by speeding up RF1 or RF2 dissociation before Api137 binding or by stimulating the dissociation of the already trapped factors.

Because of its unique mechanism of action, Api137 and its analogs could serve as important tools for research and medicine. Api137 could have an application in synthetic biology in which interference with peptide release at engineered stop codons could stimulate the incorporation of noncanonical amino acids via stop codon suppression25. The use of Api137 for medicine could go far beyond its known antibacterial action. Many human genetic disorders are caused by nonsense mutations. Although enabling premature stop codon readthrough by using translation-error-inducing compounds is one of the promising strategies, the decrease in translational accuracy makes such drugs highly toxic26. The ability of Api137 to dramatically stimulate readthrough by interfering with the function of RFs provides new avenues for exploring this approach27, and our high-resolution structure of Api137 complexed with the bacterial ribosome can serve as a starting point for the rational design of specific inhibitors of eukaryotic translation termination.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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Supplementary Table 1

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ONLINE METHODS

Peptides and oligonucleotides. Api137 was synthesized by NovoPro Biosciences Inc. Onc112 was synthesized by GenScript. The ‘start-stop’ mRNA (Supplementary Table 2) was purchased from IBA GmbH. The 2XermCL_S100_UAG construct was synthesized by Eurofins. DNA oligonucleotides were synthesized by Integrated DNA Technologies.

Generation of templates for in vitro translation and toeprinting. The DNA templates for toeprinting (Supplementary Table 2) were generated by PCR using AccuPrime DNA Polymerase (Thermo Fisher Scientific) and primers listed in Supplementary Table 3. The synthetic template yrba-fs15 was prepared using three overlapping primers (T7-IR-AUG, URA-yRSA-615-RI1-F and pos-NT1-V) in a single PCR reaction. The ermCL template was created by PCR amplification of the gene from the plasmid PERMC7-M23 using primers T7 and ermCL-UAG. The complete sequences of the templates are shown in Supplementary Table 2.

Toeprinting reactions were carried out in 5 µl of PURExpress transcription–translation system (New England Biolabs) as previously described2,3. The reverse transcription on the ermCL template was carried out using the primer ermCL-TCP-term. The final concentrations of Api137 and Onc112 in the reactions were 50 µM; the PrfAmps were added as stock solutions in water.

Selection of Api137-resistant mutants. The first round of selection of Api137-resistant mutants was performed with the E. coli strain SQ110, derived from the K12 strain (Supplementary Table 4). An overnight culture grown in LB medium, was diluted 100-fold into fresh medium containing a subinhibitory concentration of Api137 (10 µM). After 24 h of growth at 37 °C, the culture was diluted 100-fold into 1 ml fresh LB medium containing 50 µM Api137. The culture was passaged one more time at 100 µM Api137 (eight-fold MIC). The dilutions of cell culture were plated on LB agar. After overnight incubation, the sbmA gene was PCR amplified from 20 individual colonies using primers Sbmα-seq-fwd and Sbmα-seq-rev and sequenced. All but one clone had mutations in the sbmA gene. The Api137-resistant clone with the WT sbmA sequence (clone SQ110 ApiR21 in Supplementary Table 4) was grown in liquid culture; genomic DNA was isolated and prepared for sequencing using a Nextera XT kit (Illumina). Sequencing was performed on an Illumina NextSeq500 instrument (paired-end, 2 × 150 base pairs). Sequencing was analyzed using a Nextera XT kit (Illumina) and sequenced. Five clones had mutations in the prfB gene: three had the C784T and two had the A839T.

Preparation of preHC for fast kinetics experiments. All experiments were performed in buffer A (50 mM Tris–HCl, pH 7.5, 70 mM MgCl2, 30 mM KCl, 7 mM MgCl2) at 37 °C unless stated otherwise. Ribosomes from the E. coli strain MRE600, E. coli initiation factors IF1, IF2 and IF3, [3H]Met–tRNAfMet and its fluorescent-labeled version [3H]Met–tRNAfMet(Flu) were prepared as described3,39. PreHC was assembled on the synthetic ‘start-stop’ mRNA (Supplementary Table 2) and purified through sucrose cushion as described38. The extent of [3H]Met–tRNAfMet binding was higher than 95% as determined by nitrocellulose filter binding. The pellets of PreHC were resuspended in buffer A, flash frozen in liquid nitrogen, and stored at −80 °C.

Single-cysteine mutants RFI S167C, RFI S167C D241G and the K12-type RFI2 A246T variant were generated by site-directed mutagenesis of the corresponding plasmids. C-terminally 6×His-tagged RFI2 and RFI2 were purified and in vitro methylated by PrfC according to the published protocol32. RFI3 was purified as described38.

Peptide hydrolysis assay. [3H]Met–tRNAfMet hydrolysis was monitored under single-round conditions, by mixing [3H]PreHC (0.1 µM), preincubated with 0–100 µM Api137, with RFI1 (1 µM) in a quench-flow apparatus at 37 °C. Reactions were quenched with 10% trichloroacetic acid (TCA) solution in 50% ethanol. The extent of hydrolysis was assayed by means of liquid scintillation counting of the supernatants after centrifugation for 30 min at 16,000 × g at 4 °C. To measure peptide release under multturnover conditions, [3H]PreHC (0.1 µM) was preincubated with RFI3 (0.1 µM), GTP (1 mM), pyruvate kinase (0.1 mg/ml) and phosphoenol pyruvate (3 mM) for 15 min at 37 °C. The concentration of Api137, when present, was 1 µM. Time courses were started by addition of RFI1 or RFI2 (10 nM), and after quenching the reactions with a 10% TCA solution in 50% ethanol, the samples were processed as described above.

Preparation of quencher-labeled RFI1O2. Prior to labeling, RFI1 containing a single cysteine was incubated for 30 min at room temperature with a ten-fold molar excess of Tris(2-carboxyethyl)phosphine (TCEP, Sigma). The quencher dye QSY9 (Thermo Fisher) was dissolved in DMSO and added to the RFI1 solution at a ten-fold molar excess. The labeling reaction was incubated for 1 h at room temperature with vigorous shaking and stopped by the addition of 2 mM DTT. The excess dye was removed by gel filtration on a PD10 column (GE Healthcare), and protein purity was checked by means of SDS-PAGE. The extent of RFI1 labeling (as analyzed by absorbance) was greater than 80%.

Measuring kinetics of RFI1 binding and dissociation. Rapid kinetics measurements were performed on an SX-20MV stopped-flow apparatus (Applied Photophysics, Leatherhead, UK). Experiments were performed by rapidly mixing equal volumes (60 µl) of [3H]PreHC (18.5 µM) and RFI1 (0.15 µM) at 37 °C. The reaction mixture was preincubated with Api137, for 2 min at room temperature and RFI1O2 (0.15 µM) at 37 °C. Fluorescence was excited at 470 nm and fluorescence emission was monitored after passing a KV500 filter (Schott). Time courses were evaluated by fitting using exponential functions by GraphPad Prism software. Dissociation rates (kd) were determined by chase experiments. PreHC(O2) (0.05 µM) was preincubated with 0.15 µM RFI1O2 to generate PostHC(O2) in the absence or presence of 1 µM Api137. PreHC was then rapidly mixed with a ten-fold excess of unlabeled RFI1 and RFI3-GTP (1 mM); pyruvate kinase (0.1 mg/ml) and phosphoenol pyruvate (3 mM) were present in both syringes. The increase of fluorescence upon dissociation of RFI1O2 was monitored as described above.

Chemical probing of Api137 interaction with the ribosome. PostHC was prepared by incubating 70S ribosomes (9 µM) with tRNAfMet(18 µM) and start-stop mRNA (18 µM) at 37 °C for 30 min in buffer A containing 20 mM MgCl2. PostHC (0.2 µM) was incubated in 50 µl of reaction buffer B (250 mM K-Borate, 50 mM MgCl2, 300 mM NH4Cl) with RFI1 (1 µM) and/or Api137 (50 µM) at 37 °C for 10 min. Modification with dimethyl sulfate (Sigma–Aldrich) and quenching were carried out at 37 °C for 10 min as described39. mRNA was isolated using phenol extraction, and the distribution of modifications was analyzed by primer extension using primers L2667 and L2180.

Cell-free translation and analysis of peptide-tRNA accumulation. To prepare the templates for translation in the E. coli S30 Extract System for Linear Templates (Promega), the trnA gene was first amplified by PCR from genomic DNA of E. coli

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MG1655 using primer Ptec-tnarC-2 in combination with either tnaC-UGA-rev or tnaC-UAG-rev. These PCR fragments were cloned into the Smal site of pUC18, and the tnaC template was reamplified with primers Ptec-eC11 and rev-44.

The transcription–translation reactions were carried out in a total volume of 5 μl. The reactions contained 0.5 pmol of the tnaC DNA template, 2 μCi[35S]l-methionine (specific activity 1,175 Ci/mmol, MP Biomedicals). When needed, the reactions were supplemented with 50 μM of ApI37, 5 mM tryptophan or 3.7 μM of purified RF1. The reactions were incubated at 37 °C for 30 min and then, when needed, split into two aliquots, one of which was treated for 5 min at 37 °C with 0.5 μg RNase A (Sigma-Aldrich). The translation products were precipitated with four volumes of cold acetone and resolved in 16.5% Tris-Tricine gels that preserve the integrity of peptidyl-tRNA40. Gels were dried, exposed to the phosphoimager screen and scanned on a Typhoon scanner (GE).

**In vivo suppression of premature stop codon.** The E. coli strain with a premature stop codon in the lacZ gene was generated by subjecting the SQ171-ΔtolC strain (Supplementary Table 4) to chemical mutagenesis and selecting lacZ-deficient mutants. For this procedure, an overnight culture of SQ171-ΔtolC was diluted 1:200 into fresh LB medium supplemented with kanamycin (50 μg/ml), grown at 37 °C until reaching A600 of 0.1, then exposed to 0.1% of ethyl methane sulfonate for 1 h. Cells were washed twice with LB medium and plated at high density on LB agar supplemented with kanamycin (50 μg/ml), X-gal (40 μg/ml), and IPTG (0.2 mM). White colonies were selected and streaked on fresh kanamycin (50 μg/ml), X-gal (40 μg/ml), and IPTG (0.2 mM) LB-agar plates. The presence of mutations was detected by PCR amplification of the lacZ gene and sequencing.

The clone designated SQ171-tolC/3 (Supplementary Table 4) contained the C2035T mutation, which changed Gln679 of the encoded β-galactosidase to a UAG stop codon.

To test the stop codon suppressing activity of Api137, SQ171-ΔtolC/W3 cells were grown in LB medium supplemented with 50 μg/ml of kanamycin. Upon reaching A600 of 1.0, 0.5 ml were mixed with 3.5 ml of LB agar (0.6%) kept at 4 °C and 44,100 × g for 1 h. Cells were washed twice with LB medium and plated at high density on LB agar supplemented with kanamycin (50 μg/ml), X-gal (40 μg/ml), and IPTG (0.3 mM) LB-agar plates. The presence of mutations was detected by PCR amplification of the lacZ gene and sequencing.

For growth from overnight cultures, 0.5 ml were mixed with 3.5 ml of LB agar (0.6%) kept at 4 °C and 44,100 × g for 30 min. Purification of His-tagged RF1 was done with Protino Ni-NTA agarose beads (Macherey-Nagel). The final eluate was applied onto a Superdex HiLoad 70S-6000 column (GE Healthcare) to yield the final 70S ribosome as a reference structure. Subsequently, particles were subjected to 3D classification resulting in six classes with a maximum resolution extending to <3 Å (0.143 FSC) for class 1 (Supplementary Fig. 4a). 3D classification and initial alignment was performed using 3×-1-decimated data. The local resolution of the final maps was computed using ResMap47 (Supplementary Fig. 4e). The final maps were sharpened by dividing the maps by the modulation transfer function of the detector and by applying an automatically determined negative B factor to the maps using RELION48.

**Data availability.** The cryo-EM density map of the Api137-RF1-ribosome complex has been deposited in the Electron Microscopy Data Bank under accession code EMD 3730. The corresponding molecular model has been deposited in the Protein Data Bank under accession code PDB 5028. Source data for Figure 2b,c,e,f and Supplementary Figure 3 are available online. All other data are available from the corresponding author upon reasonable request.

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**Molecular modeling and map- docking procedures.** The molecular model of the 70S ribosome was based on E. coli-70S-EF-Tu structure49. RF1 was modeled based on the previously reported RF1 structure (PDB 513C)44. The Ile-tRNA model was generated based on the previously described P-site tRNA50. The models were initially adjusted and refined using Coot44. Api137 was modeled de novo into the map using Coot. The complete atomic model of the E. coli ribosome was refined using phenix.real_space_refine with secondary structure restraints calculated by PHENIX52. Cross-validation against overfitting (Supplementary Fig. 4d) was performed as described elsewhere53. The statistics of the refined model were obtained using MolProbity54 and are presented in Table 1.

**Figure preparation.** Figures showing electron densities and atomic models were generated using either UCSF Chimera55 or PyMol Molecular Graphics Systems (version 1.8, Schrödinger).

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