Supporting Information

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SI Methods

Plasmids and Sequences. Methionyl-tRNA formyltransferase (MTF), methionyl-tRNA synthetase (MetRS), and the prokaryotic RFs (RF1 and RF3) were PCR-amplified from genomic DNA of the *E. coli* strain K-12 MG1655. The ORFs were amplified by the following primer pairs (all primer sequences are listed in Table S2): MTF, primers 1 and 2; MetRS, 3 and 4; RF1, 5 and 6; and RF3, 7 and 8. MTF, RF1, and RF3 were cloned in the BamHI/EcoRI sites of the pET-30a(+) vector. MetRS was introduced into the pET-30a(+) plasmid cut with EcoRI and NotI. The resulting plasmids were designated as pMTF, pMetRS, pRF1, and pRF3.

RF2 Thr246Ala (RF2 throughout this work; plasmid designated as pRF2) was expressed together with the HemK methyltransferase specific for Gln252 of the GGQ motif. The presence of the methylation of Gln252 was verified was MS (Fig. S8*A*).

The plasmid encoding RF1 Δ302–304 (RF1* throughout this work) was a gift of Andrei Korostelev, University of Massachusetts Medical School, Worcester, MA. A plasmid coding for RF2 E167K (RF2* throughout this work) was generated by using the Q5 site-directed mutagenesis kit (NEB) with pRF2 as a template. Mutagenesis was performed by using primers 9 and 10. All plasmids were Sanger-sequenced to ensure sequence integrity.

The eGFP cassette of the lentiviral destination vector pHR-DEST-SFFV-eGFP was amplified with primers 11 and 12. Primer 12 carries two consecutive 2′O-methylated nucleosides at the 5′ end. The PCR product served as a template for T7 in vitro transcription. Thereby, the 2′O-methylation prevented the addition of nontemplate nucleotides at the 3′ end of the transcript (1). The transcript was then ligated to the poly(A)-tailed ErmCL oligonucleotides yielding the Flag-eGFP-ErmCL-poly(A) mRNA for assaying stop codon recognition in HEK293T cells.

The firefly luciferase (FL) ORF was amplified from the PCD-FL1P plasmid (catalog no. 12567; addgene) by using primers 13 and 14 and was subcloned into the NdeI/*Bgl*II site of pT7CFE1-NHis (Thermo Scientific) vector. An IRES-His-FL fragment was amplified by using primers 15 and 16. The transcript was then ligated to poly(A)-tailed ErmCL oligonucleotides generating the IRES-His-FL-ErmCL-poly(A) reporter mRNA that was translated in the 1-Step human coupled IVT kit (Thermo Scientific).

Protein Purification. MTF, MetRS, RF1, RF1*, RF2, RF2*, and RF3 were overexpressed in *E. coli* BL21(DE3) and purified via the His-tag by using Ni-NTA agarose (Qiagen) as described (2). The purified proteins were dialyzed by using Spectra/Por membranes (molecular weight cutoff 3,500) against storage buffer (20 mM Hepes/KOH, pH 7.6, 6 mM $MgAc_2$, 150 mM NH_4Cl, 4 mM β-Mercaptoethanol, and 10% glycerol) for 5× 1 h at 4 °C. Proteins were quantified by using the Bradford assay and stored at −80 °C after flash-freezing in liquid nitrogen.

tRNA Charging and RF-Mediated Peptidyl-tRNA Hydrolysis. $tRNA^{fMet}$ was purchased from tRNA Probes. f[^{3}H]Met-$tRNA^{fMet}$ (20,000 cpm/pmol) was prepared with the aid of MTF and MetRS and purified by a reversed-phase C4 HPLC column as described (3, 4).

The peptide release assay was performed basically as described (5). The buffer that was used was 20 mM Hepes/KOH (pH 7.6), 150 mM NH_4Ac, 6 mM $MgAc_2$, 2 mM spermidine, 0.05 mM spermine, and 4 mM β-Mercaptoethanol. In short, tight-coupled 70S ribosomes [1.4 μM f.c. (final concentration)] were programmed with a synthetic mRNA (50 μM f.c.; 5′-AAGGAGGUAAAA**AUG**UAAGCU-3′; AUG in bold and the UAA stop codon underlined; the stop codon was modified or exchanged to UAG/UGA as indicated) and incubated with f[^{3}H]Met-$tRNA^{fMet}$ (0.1 μM f.c.) for 15 min at 25 °C, thereby forming the release complex. The final 70S concentration was adjusted to 0.2 μM for the peptidyl-tRNA hydrolysis reaction, which was initiated by adding *E. coli* RF at saturating levels (0.42 μM f.c.). The hydrolysis reaction was incubated for 10 min at 25 °C and was afterward quenched by the addition of 5 vol of 0.1 M HCl and quantified by using a Beckman LS6500 liquid scintillation counter.

Time course experiments were performed under saturating RF1 concentrations (420 nM) and at RF1 concentrations equimolar to f[^{3}H]Met-$tRNA^{fMet}$ (14 nM). The reactions were quenched at the indicated time points. Rate constants were obtained by fitting the time course data to single exponential equations using GraphPad Prism (Version 7).

RF K_D Titrations. Oligonucleotides with a 3′-amino-modifier C3 linker (sequence as for the peptide release assay; Dharmacon) were pyrene-labeled as reported (6).

Equilibrium K_D titrations were performed as described (7–9). In short, tight-coupled ribosomes (0.25 μM f.c.) were heat-activated for 10 min at 42 °C and subsequently cooled to 37 °C over 10 min in the same buffer as used in the peptide release assay. The pyrene-labeled mRNA was added (0.33 μM f.c.) and incubated for 10 min at 37 °C. Subsequently, the reaction was supplemented with $tRNA^{fMet}$ (0.5 μM f.c.) and kept for 30 min at 37 °C. The equilibrium K_D titrations were measured in a 160-μL fluorescence cuvette with a final concentration of the ribosome·mRNA·$tRNA^{fMet}$ complex of 5 nM. No difference in the K_D values was observed if the complex concentration was increased to 20 nM. The complex was incubated with the indicated amounts of the RFs for 30 min at room temperature (RT). Fluorescence intensities were measured by using a Jasco FP-8500 fluorometer. The fluorescence emission scans were conducted with an excitation and emission band pass of 1 nm. Pyrene was excited at 343 nm, and the emission intensity at 376 nm was measured. In parallel, the fluorescence emission of the RFs in the buffer was measured and subtracted from the data to compensate light scattering at high RF concentrations. Data were transformed and fit to the equilibrium K_D equation $Y = m \times \{(K + R + X) - [(K + R + X)^2 - 4 \times R \times X]/(2 \times R)$ with GraphPad Prism (Version 7) (Y is the observed fluorescence intensity, X is the concentration of the RF, m is the maximum fluorescence signal, K is K_D, and R is the complex concentration).

Synthesis of Ze- and Py-Modified Oligonucleotides. Purine, dap, inosine, and ribose-abasic–modified oligonucleotides were purchased from Dharmacon. Ze and Py-modified oligonucleotides were synthesized in-house. For the syntheses of these oligonucleotides, we used standard 2′-O-tert-butyldimethylsilyl ether (TBDMS)–protected building blocks (Sigma Aldrich) combined with commercially available Ze–CE and pyridin-2-one–CE phosphoramidites (Glen Research). The syntheses were carried out according to published procedures (10).

Removal of the RNA protecting groups and the cleavage from the solid support was accomplished in two different ways: (*i*) For sequences modified with a pyridin-2-one-CE phosphoramidite, the standard deprotection strategy, using aqueous methylamine (40%, 650 μL) and aqueous ammonium hydroxide (28%, 650 μL) for 3 h at 37 °C, was used. (*ii*) In case of Ze-modified RNA, the sequences were deprotected by using extremely mild conditions using a mixture of aqueous ammonia (28%, 900 μL) and ethanol (300 μL) with shaking for 40 h at RT. After removal of the alkaline deprotection solutions by evaporation, the residue

was dissolved in dry dimethyl sulfoxide (300 μL) and TEA.3 HF (200 μL) was added. After 30 h at 25 °C, the deprotection TBDMS deprotection reaction was quenched by the addition of 2 mL of Tris quenching buffer (Glen Research). The crude product was desalted on a HiPrep 26/10 desalting column (GE Healthcare). The RNA containing fractions (identified by UV absorption) were combined, and in a final step, the RNA was treated with a 100 mM triethylammonium bicarbonate solution before final desalting was accomplished by using a C18 Seppak cartridge (Waters).

The quality of the obtained crude RNA was checked by anion exchange chromatography (DionexDNAPac PA-100 column, 80 °C) and MS (Finnigan LCQ Advantage MAX ion trap connected to an Amersham Ettan micro-LC instrument; Fig. S8 *B*–*G*).

T7 in Vitro Transcription and Poly(A)-Tailing. The capped transcript encoding the 5′ part of the Flag-eGFP-ErmCL-poly(A) mRNA was generated with the HiScribe T7 ARCA mRNA kit (NEB). The 5′ fragment of the IRES-His-FL-ErmCL-poly(A) reporter mRNA was synthesized by using the HiScribe T7 High Yield RNA synthesis kit (NEB) as described by the manufacturer. The oligonucleotides used for assaying peptide release in cell culture were poly(A)-tailed using the A-Plus Poly(A) Polymerase tailing kit (CELLSCRIPT). The tailing reaction was incubated for 50 min at 37 °C to obtain at least 150-nt-long poly(A)-tails.

Splinted mRNA Ligation. Two RNA oligonucleotides were ligated to generate the prokaryotic reporter ErmCL mRNA. The oligonucleotide encoding the 5′ part (5′-GGGAGUUUUAUAAGGAGGAAAAAAU**AUG**GGCAUGUUUAGUAUUUUUGUAAUCAGCACAGUUC-3′; AUG in bold) and the 3′-part encoding oligonucleotide 5′-P-AUUAUUAACCAAACAAAAAAUAA-3′ (the two stop codons are underlined, of which the first stop codon was modified or exchanged to UAG/UGA as indicated) were bridged by the DNA oligonucleotide splinter 17. The ligation reaction using T4 DNA ligase (Thermo Scientific) was performed as described (11, 12). Ligated full-length mRNA was purified via 8% polyacrylamide-urea gels.

For the synthesis of the eukaryotic Flag-eGFP-ErmCL-poly(A) reporter mRNA, the capped 5′ transcript was ligated to the poly(A)-tailed ErmCL oligonucleotide 5′-P-AUUAUUAACCAAACAAAAAA$(A)_n$-3′ [modified stop codon underlined, poly(A) tail italicized] with the help of splinter 18. The enzymatic ligation was performed by T4 RNA ligase 2 (NEB) as described (11, 12). Ligated full-length mRNAs were purified by using a magnetic mRNA isolation kit (NEB), and mRNA purity and integrity was checked with a 2100 Bioanalyzer (Agilent).

The IRES-His-FL-ErmCL-poly(A) reporter mRNA was generated by ligation of the IRES-His-FL fragment with the poly(A)-tailed ErmCL oligonucleotide bridged by splinter 19. The ligation was performed with T4 RNA ligase 2 (NEB).

Prokaryotic IVT. IVT using the PURExpress ΔRF123 system (NEB) was performed as described (11). In a typical reaction, mRNAs (0.8 μM f.c.) were translated in the presence of 10 μCi [^{35}S]Met (Hartmann Analytic) supplemented with the indicated RFs (0.25 μM f.c.) as recommended by the manufacturer (2 μM f.c. 70S ribosome). The IVT reactions were incubated for 1 h at 37 °C and were then resolved on Novex 16% Tris–Tricine gels (Thermo Scientific) and exposed to phosphorimager screens, which were scanned by using a STORM 840 scanner.

E. coli bulk tRNA (Sigma) was deacetylated by incubation in 50 mM Tris·HCl (pH 9.0) for 3 h at 37 °C, followed by ethanol precipitation. Precipitated bulk tRNA was incubated for 5 min at 65 °C and was then slowly cooled down to RT to refold the tRNAs. For the tRNA competition experiments, IVT reactions were performed with 0.125 μM RF2 and 320 μM bulk tRNA.

MS Analysis of IVT Products. ErmCL peptides were purified by using Vivaspin 2 (5 kDa; Hydrosart) columns as described (11). Peptides were analyzed by using a Dionex UltiMate 3000 nano-HPLC system coupled via nanospray ionization source to a Thermo Scientific Q Exactive HF mass spectrometer. Peptides were separated on a fritless fused-silica column (75 μm inner diameter × 280 μm outer diameter × 10 cm length) packed with 3 m reversed-phase C18 material (Reprosil). Solvents for HPLC were 0.1% formic acid (solvent A) and 0.1% formic acid in 85% acetonitrile (solvent B). The gradient started at 4% B. The concentration of solvent B was increased linearly from 4 to 35% during 53 min and from 35 to 100% during 5 min. A flow rate of 250 nL/min was applied.

The Q Exactive HF mass spectrometer was operating in data-dependent mode to switch between MS and tandem MS (MS/MS) acquisition. Full-scan MS spectra were acquired with a resolution of $R = 60{,}000$. Up to 20 of the most intense ions detected in the full-scan MS were sequentially isolated and fragmented by using higher-energy collision dissociation applying a normalized collision energy of 28.0. Fragments were scanned with a resolution of $R = 30{,}000$.

Database search was performed by using ProteomeDiscoverer (Version 1.4; Thermo Scientific) with search engine Sequest HT. MS/MS spectra were searched against an *E. coli* database (Uniprot, strain K12, last modified April 2015, 4,305 entries) to which 21 different ErmCL peptides sequences were added. The following settings were applied: The b and y ions were used for spectrum matching and scoring; precursor mass tolerance was set to 10 ppm; fragment mass tolerance was 0.05 Da; false discovery rate was set to 0.01 (1%); and N-terminal protein formylation was used as variable modification.

Eukaryotic in Vitro Translation. IRES-His-FL-ErmCL mRNAs (0.25 μM f.c.) were translated by using the 1-Step Human Coupled IVT kit (Thermo Scientific) as described by the manufacturer. The reaction was supplemented with 10 μCi [^{35}S]Met (Hartmann Analytic).

Cell Culture, Transfection, and Western Blotting. HEK293T cells were cultivated in DMEM with 4.5 g/L D-glucose and L-glutamine (Gibco), 100 U/mL penicillin/streptomycin (Gibco), and 10% heat-inactivated FBS (Gibco). A total of 40% confluent HEK293T cells were transfected with 10 pmol of the Flag-eGFP-ErmCL-poly(A) mRNA by using metafectene (Biontex) as described by the manufacturer.

Twenty-four hours after transfection, cells were lysed in lysis buffer (20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, pH 8.0, 1% Triton X-100, and Roche complete protease inhibitors), and protein was quantified via a Bradford assay. Thirty micrograms of total protein was separated on a Novex 16% Tris–Tricine gel (Thermo Scientific). The resolved proteins were transferred to 0.45-μm PVDF membranes (Amersham) by electroblotting by using a Novex XCell II blot module (30 min, 100 mA, 30 V; Thermo Scientific). The membrane was blocked with 5% BSA (Roth) in TBS-T buffer (10 mM Tris·HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) for 1 h at RT. The blot was probed with an anti-Flag M2 antibody (Sigma) at 1:3,000 dilution overnight at 4 °C. As a secondary antibody, a goat anti-mouse HRP-conjugated antibody (Dako) was used in a 1:3,000 dilution. The blot was developed by using the Pierce ECL Western blotting substrate (Thermo Scientific).

1. Kao C, Zheng M, Rüdisser S (1999) A simple and efficient method to reduce nontemplated nucleotide addition at the 3 terminus of RNAs transcribed by T7 RNA polymerase. *RNA* 5:1268–1272.
2. Erlacher MD, Chirkova A, Voegele P, Polacek N (2011) Generation of chemically engineered ribosomes for atomic mutagenesis studies on protein biosynthesis. *Nat Protoc* 6:580–592.
3. Walker SE, Fredrick K (2008) Preparation and evaluation of acylated tRNAs. *Methods* 44:81–86.
4. Erlacher MD, et al. (2005) Chemical engineering of the peptidyl transferase center reveals an important role of the 2′-hydroxyl group of A2451. *Nucleic Acids Res* 33: 1618–1627.
5. Amort M, et al. (2007) An intact ribose moiety at A2602 of 23S rRNA is key to trigger peptidyl-tRNA hydrolysis during translation termination. *Nucleic Acids Res* 35:5130–5140.
6. Studer SM, Feinberg JS, Joseph S (2003) Rapid kinetic analysis of EF-G-dependent mRNA translocation in the ribosome. *J Mol Biol* 327:369–381.
7. Hetrick B, Lee K, Joseph S (2009) Kinetics of stop codon recognition by release factor 1. *Biochemistry* 48:11178–11184.
8. Field A, Hetrick B, Mathew M, Joseph S (2010) Histidine 197 in release factor 1 is essential for a site binding and peptide release. *Biochemistry* 49:9385–9390.
9. Trappl K, Mathew MA, Joseph S (2014) Thermodynamic and kinetic insights into stop codon recognition by release factor 1. *PLoS One* 9:e94058.
10. Wunderlich CH, et al. (2012) Synthesis of (6-(13)C)pyrimidine nucleotides as spin-labels for RNA dynamics. *J Am Chem Soc* 134:7558–7569.
11. Hoernes TP, et al. (2016) Nucleotide modifications within bacterial messenger RNAs regulate their translation and are able to rewire the genetic code. *Nucleic Acids Res* 44:852–862.
12. Hoernes TP, Erlacher MD (2017) Methylated mRNA nucleotides as regulators for ribosomal translation. *Methods Mol Biol* 1562:283–294.
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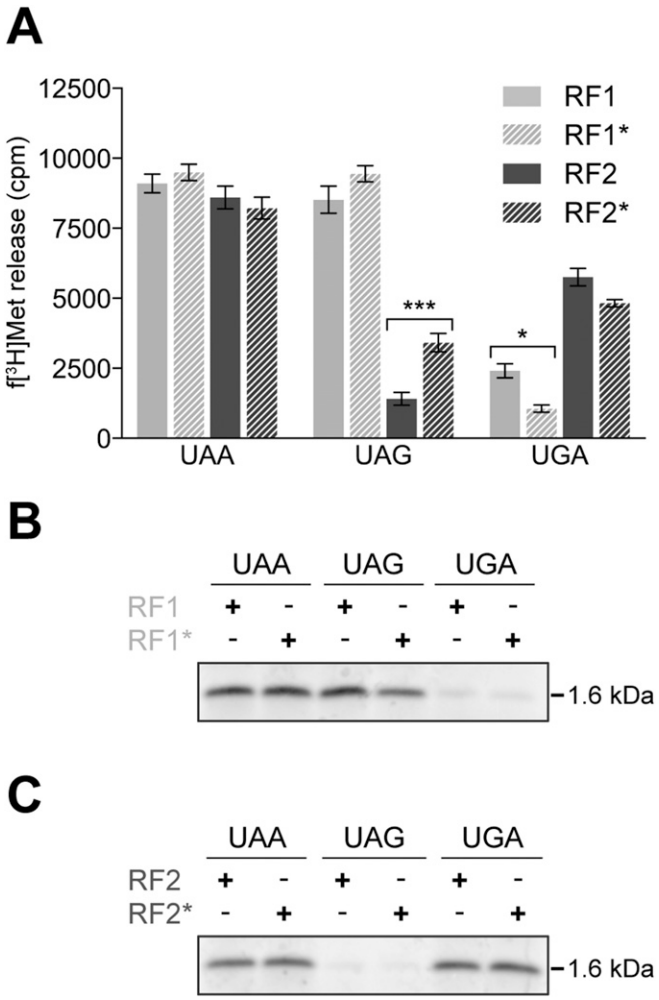


Fig. S1. Recognition of unmodified stop codons by RFs. (*A*) Peptide release assay with RF1/RF2 and the RF mutants, RF1* and RF2*, with UAA, UAG, or UGA stop codons in the ribosomal A site (error bars indicate SDs from the mean of at least three independent experiments). (*B* and *C*) Performance of the same RFs in the IVT-based assay by using ErmCL mRNAs encoding an UAA, UAG, or UGA stop codon. In *A*, significant differences between RF1, RF2 and RF1*, RF2*, respectively, were determined by using the two-tailed unpaired Student's *t* test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

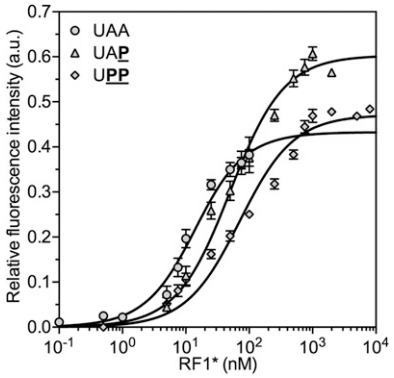


Fig. S2. K_D titration of RF1* binding to the ribosome. Increasing amounts of RF1* were added to ribosomes programmed with UAA (●), UAP (▲), or UPP (♦) stop codons in the A site. The relative changes in fluorescence activity were fit to the equilibrium K_D equation (black line; error bars show SDs from the mean in three independent experiments). The total amount of supplemented RF1* is indicated on the *x* axis.

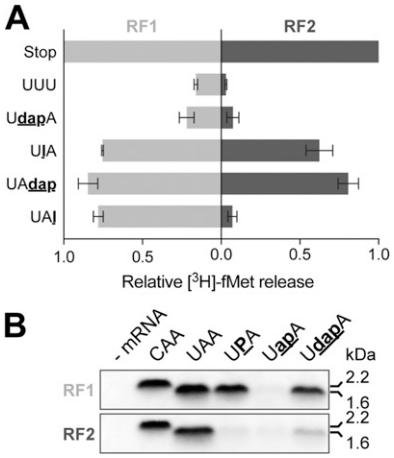


Fig. S3. Discrimination of RF1 and RF2 against UGA and UAG, respectively. (*A*) Peptide release assay using dap- and I-modified stop codons to reveal the chemical groups required for discrimination against noncognate stop codons. (*B*) Termination at 2-aminopurine (ap) and dap-modified stop codons using the PURExpress IVT system.

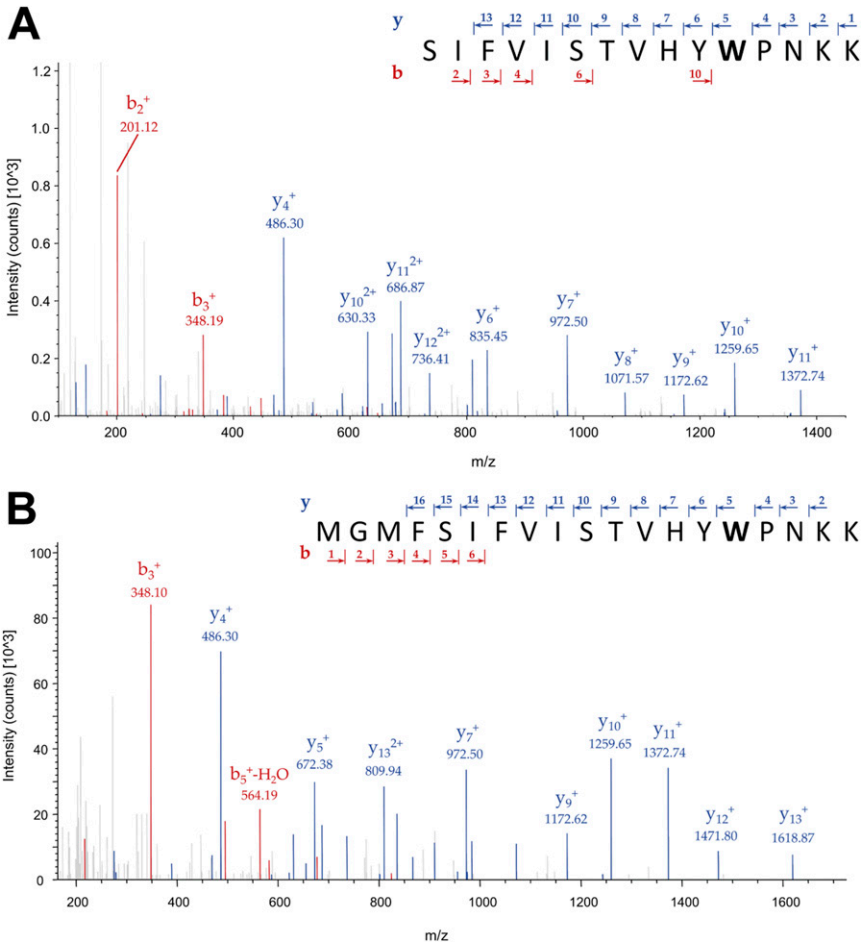
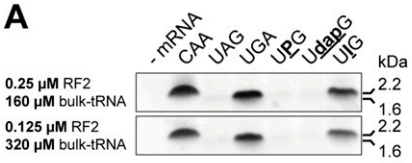


Fig. S4. MS/MS spectra of peptides originating from read-through events at modified stop codons. (*A*) Translation of an ErmCL mRNA encoding UIG at codon 15 resulted in the incorporation of Trp at this distinct position. (*B*) Translation of mRNAs harboring an UGP stop codon led to read-through and the incorporation of Trp in the presence of RF1. Annotated peptide fragments are shown in blue (y ions) and red (b ions), according to the general nomenclature of fragment ions.

A



B

mRNA sequence	Peptide	MH+ [Da]	#PSMs	XCorr
5'-[…] UAU **UAG** CCA AAC AAA AAA UAA-3'	SIFVISTVHY**Q**PNKK	1760.97086	3	5.00
	IFVISTVHY**Q**PNKK	1673.93900	2	4.29
	FVISTVHY**Q**PNKK	1560.85637	1	2.71
	SIFVISTVHY	1165.62639	6	2.67
	SIFVISTVHY_PNKK	1632.91166	2	4.36
	SIFVISTVHY**E**PNKK	1761.97075	1	2.86
5'-[…] UAU **UGA** CCA AAC AAA AAA UAA-3'	FVISTVHY**W**PNKK	1618.87798	1	3.00
	SIFVISTVHY	1165.62700	2	2.89
	IFVISTVHY	1078.59490	2	2.55
5'-[…] UAU **UPG** CCA AAC AAA AAA UAA-3'	-	-	-	-
5'-[…] UAU **UdapG** CCA AAC AAA AAA UAA-3'	SIFVISTVHY_PNKK	1632.91265	4	3.92
	VISTVHY_PNKK	1285.72978	3	2.45
	SIFVISTVHY**Q**PNKK	1760.97068	4	4.85
	ISTVHY**Q**PNKK	1413.78569	2	3.42
	SIFVISTVHY**S**PNKK	1719.94480	2	4.84
5'-[…] UAU **UIG** CCA AAC AAA AAA UAA-3'	SIFVISTVHY**W**PNKK	1818.99167	4	5.13
	IFVISTVHY**W**PNKK	1731.95981	4	5.02
	FVISTVHY**W**PNKK	1618.87541	3	4.46
	VISTVHY**W**PNKK	1471.80644	6	5.04
	ISTVHY**W**PNKK	1372.73845	7	5.02
	STVHY**W**PNKK	1259.65373	6	3.91
	TVHY**W**PNKK	1172.62200	6	3.17
	VHY**W**PNKK	1071.57366	4	2.62
	HY**W**PNKK	972.50566	3	2.61
	SIFVISTVHY**S**PNKK	1719.94352	1	5.04

Fig. S5. Read-through of modified stop codons in the presence of increased tRNA concentrations. (*A*) mRNAs with modified stop codons were translated in the presence of increased bulk tRNA concentrations (320 µM) and decreased RF2 concentrations (125 nM). No increase in read-through events was observed as deduced from SDS/PAGE. (*B*) The in vitro-translated peptides were analyzed by MS. Only trace amounts of peptides originating from read-through of the modified stop codons were detected. PSM, peptide spectrum match; XCorr, cross-correlation score.

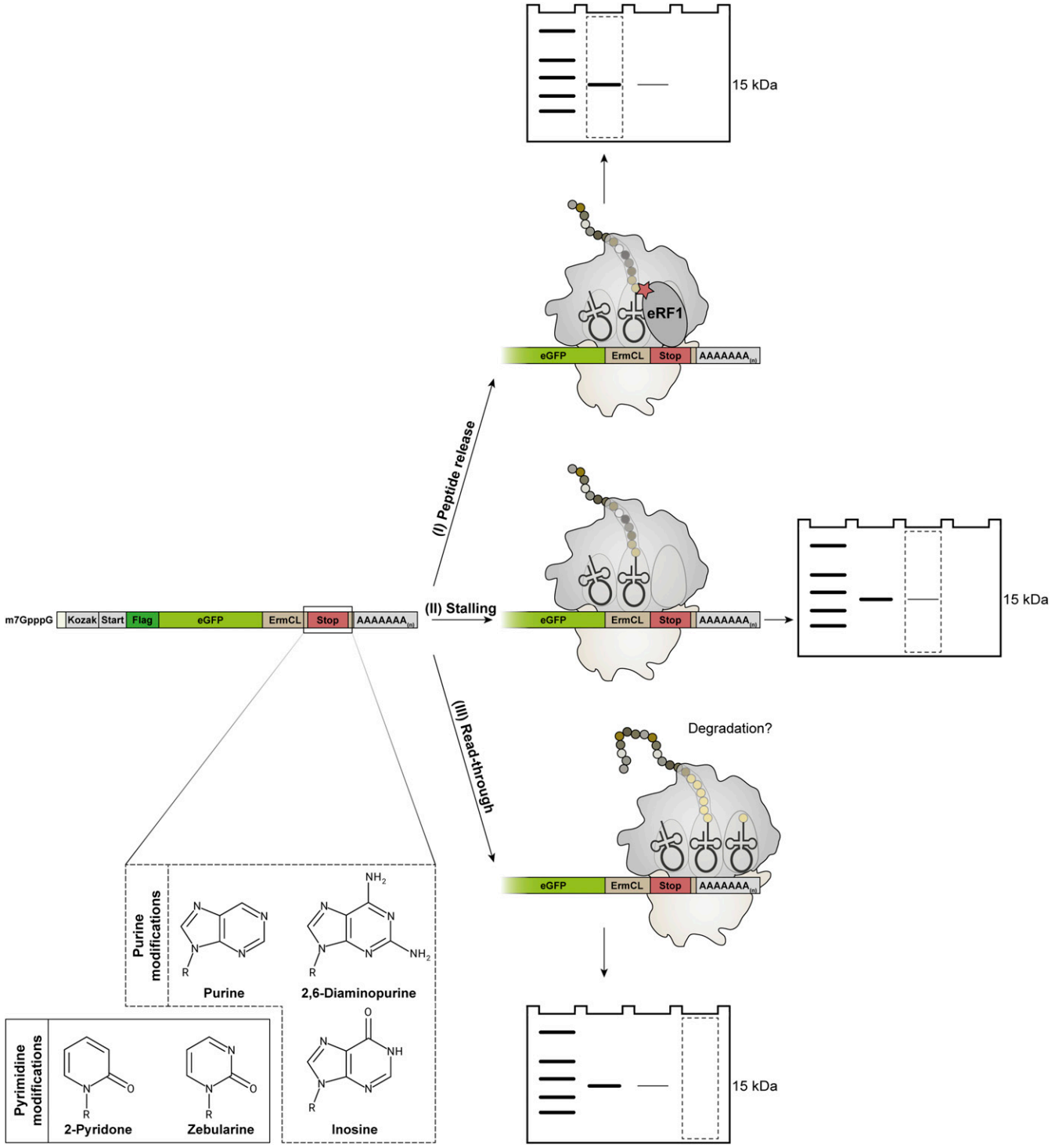


Fig. S6. Possible scenarios for recognition of modified stop codons by eRF1/tRNAs. The stop codon of a Flag-eGFP reporter mRNA was modified as depicted and transfected into HEK293T cells. Potential scenarios include (*i*) recognition of the modified stop codon by eRF1 and peptide release (*Top*); (*ii*) stalling at the modified stop codon, which is neither recognized by eRF1 nor tRNA ternary complexes (*Middle*); or (*iii*) recognition of the modified stop by tRNAs and subsequent read-through yielding a polylysine-tagged peptide inducing mRNA cleavage and "nonstop/no-go" decay (*Bottom*) (13).

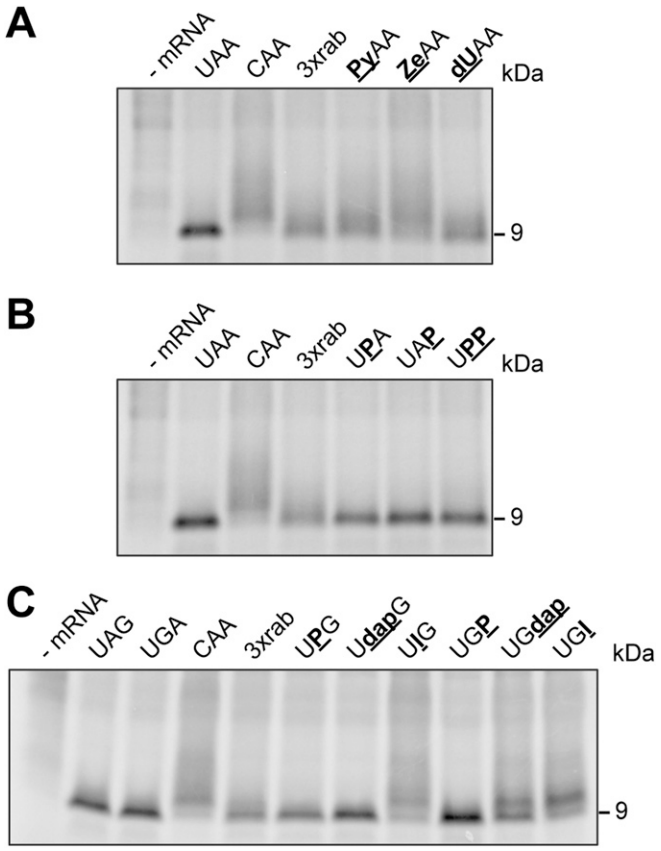


Fig. S7. Eukaryotic translational termination at modified stop codons investigated in vitro. IVT of modified IRES-His-FL-ErmCL-poly(A) reporter mRNAs allowed the discrimination of peptide release from stalling and read-through events. Modifications were introduced at the first (*A*), second, and third stop codon positions (*B* and *C*).

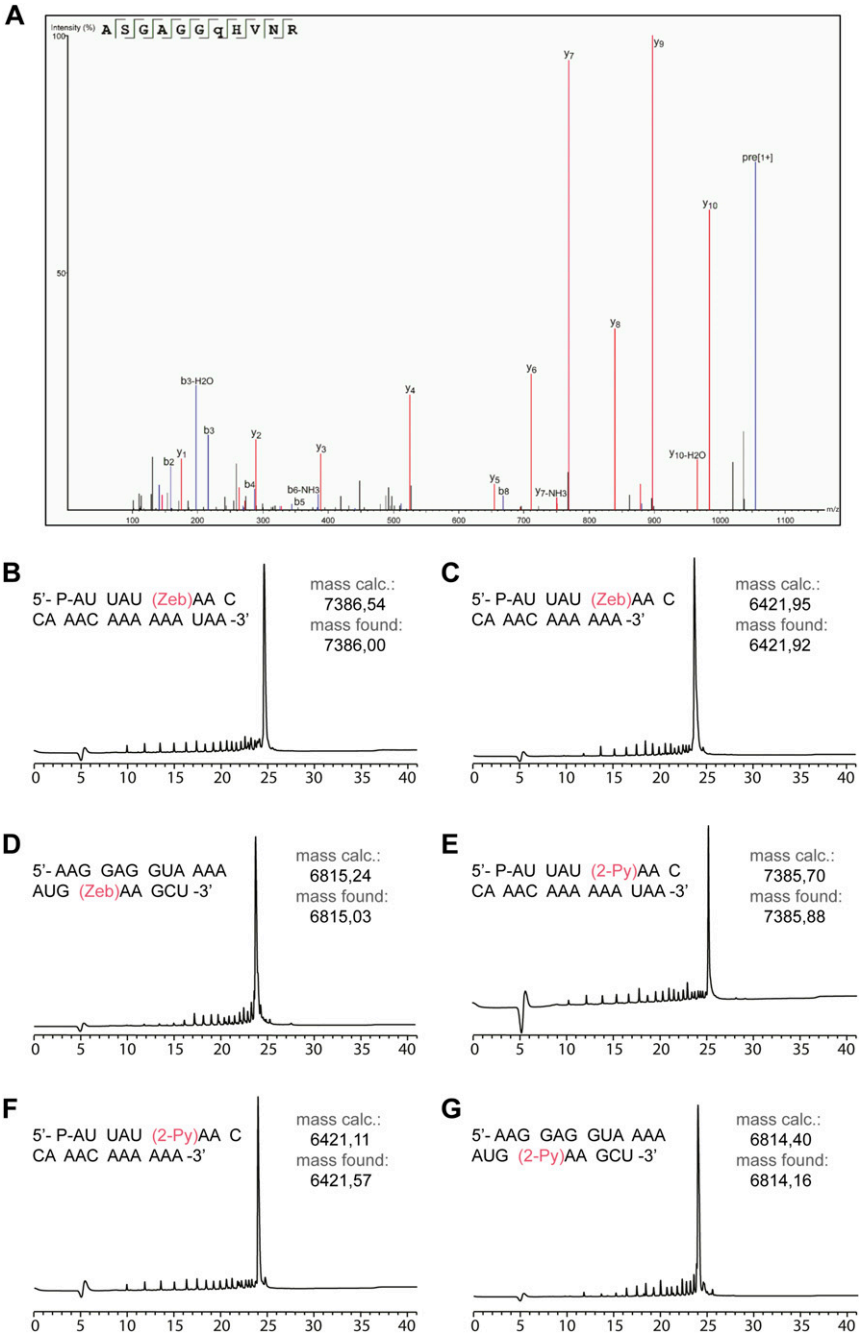


Fig. S8. Quality controls of RF2 and chemically synthesized RNA oligonucleotides. (*A*) Representative MS/MS spectrum of the RF2 Gln252 methylated peptides. Annotated peptide fragments are shown in red (y ions) and blue (b ions), according to the general nomenclature of fragment ions. (*B–G*) Various Ze-modified (*B–D*) and Py-modified (*E–G*) RNAs were validated by anion exchange chromatography and MS (expected and found masses are depicted). Calc., calculated.

Table S1. Kinetics and thermodynamics of bacterial RFs binding to the ribosome programmed with canonical or modified stop codons

A site codon	RF1	RF2	RF1 Δ302–304
UAA			
K_D, nM	<3.0	11.1 ± 2.1	11.4 ± 2.1
k_{cat}, s^{-1}	$(1.9 \pm 0.1) \times 10^{-2}$ (14 nM RF1) $(6.0 \pm 0.1) \times 10^{-2}$ (420 nM RF1)	Not measured	Not measured
UAG			
K_D, nM	Not measured	Not measured	Not measured
k_{cat}, s^{-1}	2.0×10^{-2} (14 nM RF1) $(6.1 \pm 0.1) \times 10^{-2}$ (420 nM RF1)	Not measured	Not measured
UAP			
K_D, nM	91.3 ± 6.6	50.2 ± 3.0	47.7 ± 4.1
k_{cat}, s^{-1}	1.6×10^{-2} (14 nM RF1) $(4.7 \pm 0.1) \times 10^{-2}$ (420 nM RF1)	Not measured	Not measured
UPG			
K_D, nM	Not measured	Not measured	Not measured
k_{cat}, s^{-1}	$(2.0 \pm 0.1) \times 10^{-2}$ (14 nM RF1) $(5.2 \pm 0.1) \times 10^{-2}$ (420 nM RF1)	Not measured	Not measured
UPP			
K_D, nM	158.9 ± 13.9	67.5 ± 7.6	68.9 ± 7.5
k_{cat}, s^{-1}	Not measured	Not measured	Not measured

Table S2. DNA oligonucleotide sequences used in this study

Oligo#	Sequence (5′ to 3′)	Description
1	CGCGGATCCGTGTCAGAATCACTACGTATT	BamHI-MTF fwd
2	CGGAATTCTCAGACCAGACGGTTGC	EcoRI-MTF rvs
3	CGGAATTCATGACTCAAGTCGCGAAGAAAA	EcoRI-MetRS fwd
4	ATAAGAATGCGGCCGCTTATTTCACCTGATGACCCG	NotI-MetRS rvs
5	CGCGGATCCATGAAGCCTTCTATCGTTGC	BamHI-RF1 fwd
6	CGGAATTCTTATTCCTGCTCGGACAAC	EcoRI-RF1 rvs
7	CGCGGATCCATGACGTTGTCTCCTTATTTG	BamHI-RF3 fwd
8	CGGAATTCTTAATGCTCGCGGGTCTG	EcoRI-RF3 rvs
9	TGAAATCATCAAAGAGTCGGAAGG	RF2 mutagenesis
10	GTTTTGAAACCACGCGATTC	RF2 mutagenesis
11	GCTCTAGATAATACGACTCACTATAGGGGGCCACCATGGACTACAAGGACGACGACGATAAGGTGAGCAAGGGCGAGG	5′ part of eGFP for ligation fwd
12	mCmGTCCTCCTTGAAGTCGATGCCCTTCAGCTC	5′ part of eGFP for ligation rvs
13	GGAATTCCATATGGAAGACGCCAAAAAC	NdeI-FL fwd
14	GAAGATCTTCCGGCTCGAGTCTAG	*Bgl*II-FL rvs
15	TAATACGACTCACTATAGGG	5′ part of FL for ligation fwd
16	mAmCATTTCGAAGTACTCAGCG	5′ part of FL for ligation rvs
17	TTTGTTTGGTTAATAATGAACTGTG	Splinter ErmCL
18	TTTTTTGTTTGGTTAATAATCGTCCTCCTTGAAGTCGATG	Splinter eGFP-ErmCL
19	TTATTTTTTGTTTGGTTAATAATACATTTCGAAGTACTCAGCGTAAG	Splinter FL-ErmCL

Fwd, forward; rvs, reverse.