Structural basis for selective stalling of human ribosome nascent chain complexes by a drug-like molecule

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The drug-like molecule PF-06446846 (PF846) binds the human ribosome and selectively blocks the translation of a small number of proteins by an unknown mechanism. In structures of PF846-stalled human ribosome nascent chain complexes, PF846 binds in the ribosome exit tunnel in a eukaryotic-specific pocket formed by 28S ribosomal RNA, and alters the path of the nascent polypeptide chain. PF846 arrests the translating ribosome in the rotated state of translocation, in which the peptidyl-transfer RNA 3’-CCA end is improperly docked in the peptidyl transferase center. Selections of messenger RNAs from mRNA libraries using translation extracts reveal that PF846 can stall translation elongation, arrest termination or even enhance translation, depending on nascent chain sequence context. These results illuminate how a small molecule selectively targets translation by the human ribosome, and provides a foundation for developing small molecules that modulate the production of proteins of therapeutic interest.

Most compounds that target the ribosome affect a large number of mRNAs through general inhibition of translation initiation or elongation1–3. Furthermore, these compounds almost exclusively act as broad-spectrum inhibitors, displaying little sequence specificity4. Many studies have revealed how these compounds bind the translating ribosome and inhibit its function5–9. Recently, we described the small molecule PF846 that selectively blocks the translation of individual mRNAs by the human ribosome5. However, a mechanism to account for the ability of PF846 and related compounds10,11 to selectively inhibit human protein synthesis remains unknown.

PF846 blocks production of proprotein convertase subtilisin/kexin type 9 (PCSK9)—an important target for regulating plasma low-density lipoprotein cholesterol levels—by interfering with the elongation phase of translation10,13. PF846 selectively stalls the ribosome on very few translated protein nascent chains, generally early in their formation and with no clear sequence pattern10. To gain insight into the mode of action of PF846 in targeting the human ribosome and specific nascent chains, and to identify principles for future drug development, we used single-particle cryo-EM to determine structures of PF846 stalled ribosome nascent chain (RNC) complexes. We also employed randomized mRNA libraries to identify sequence preferences for PF846-mediated translation stalling.

Since the stalling sequences in the few proteins affected by PF846 mostly occur near the N terminus and would therefore reside in the ribosome exit tunnel11, we first identified conditions for affinity purification of stable PF846-stalled RNCs from in vitro translation reactions in human cell extracts. The calcium-dependent cell–cell adhesion glycoprotein Cadherin-1 (CDH1)14 is stalled near its C terminus by PF846, enabling the extension of the peptide N terminus beyond the confines of the ribosome exit tunnel11. Appending an N-terminal affinity tag followed by the CDH1-V domain and the nascent chain sequences targeted by PF846 did not disrupt the ability of PF846 to stall translation and allowed formation and purification of stable PF846-stalled RNCs (Fig. 1a–c and Supplementary Fig. 1). All the following assays with PF846-stalled RNCs used an N-terminal 3X-FLAG tag unless specifically noted.

Results

Overall structures of PF846 stalled RNCs. To investigate how PF846 stalls specific nascent chain sequences in the human ribosome, we used cryo-EM to determine structures of PF846-stalled CDH1, PCSK9 and USO1 (USO1 vesicle transport factor) RNC complexes (CDH1-RNC, PCSK9-RNC and USO1-RNC, respectively) (Supplementary Figs. 2–4 and Tables 1–4). In both the CDH1-RNC and PCSK9-RNC samples, particle sorting of the purified RNCs revealed a major population of ribosomes in the rotated state that precedes mRNA and tRNA translocation14, containing predominantly hybrid A/P-site peptidyl-tRNA (that is, nascent chain-tRNA, NC-tRNA), but with some rotated-state RNCs containing A/A-site NC-tRNA. The USO1-RNC also adopts the rotated state, although the positioning of the A-site tRNA in the A/P or A/A site was less well defined. All three RNCs also included tRNA in the hybrid P/E site and PF846 bound in the ribosome exit tunnel (Fig. 1d,e and Supplementary Fig. 5). The following analyses describe the CDH1-RNCs due to the higher-quality of the CDH1-RNC cryo-EM maps, although we observe similar behavior for the PCSK9-RNC and USO1-RNC samples, as shown in the Supplementary Figures.

The CDH1-RNC complexes with hybrid A/P-site NC-tRNA or A/A-site NC-tRNA yielded cryo-EM maps with an average resolution of 3.0 and 3.9 Å, respectively (Fig. 1d,e and Supplementary Fig. 2). The hybrid A/P and P/E NC-tRNAs each represent a mixture of
tRNAs, as inferred from the EM map density for nucleotide bases in the codon-anticodon base pairs (Supplementary Fig. 6), consistent with the clustering of stalling sites spanning multiple mRNA codons observed by ribosome profiling. In the CDH1-RNC and PCSK9-RNC samples, we also observed a small population of PF846-stalled RNCs in the non-rotated state bearing a P/P-site NC-tRNA that exists after peptide bond formation (Supplementary Figs. 2, 3 and 5). This population appeared in higher abundance when the PCSK9-RNC sample was purified using a short isolation time (Supplementary Fig. 8). These results indicate that the non-rotated RNC is a transient state during PF846 mediated translation stalling. Collectively, our findings indicate that PF846 sequentially stalls RNCs in the rotated state at adjacent codons, whereas the non-rotated state with P/P-site NC-tRNA either participates more readily in translation elongation or can be resolved by ribosome quality control pathways; that is, those that involve PELO and HBS1L.

Eukaryotic-specific small molecule-binding pocket. In the stalled RNCs, PF846 occupies a binding pocket in a newly identified groove of the ribosomal exit tunnel formed by universally conserved 28S rRNA nucleotides (Fig. 2) and a stretch of the stalled nascent chain, best seen in the rotated-state RNCs with A/P NC-tRNAs. PF846 was fit as a rigid body to the density by evaluating its X-ray structures and low energy conformations based on quantum mechanical assessment of accessible torsion angles (Supplementary Fig. 7a–f and Supplementary Table 1), followed by adjustments of the chloropyridine ring (Fig. 2i). To explore the structural basis for PF846 action on eukaryotic but not bacterial ribosomes, we aligned the small molecule-binding pocket with the corresponding region in the Escherichia coli ribosome (Fig. 2i and Supplementary Fig. 7g–k). Phylogenetic analysis revealed key changes to the nucleotide pattern in 23S rRNA in the large ribosomal subunit of bacteria (Fig. 2i). Whereas nucleotides C1576, G1577 and G1579 in the human ribosome are highly complementary to the conformation of PF846 (Fig. 2e–h), nucleotides N1-methyl-G745 and ψ746 in bacterial 23S rRNA would clash with the compound (Supplementary Fig. 7i). Furthermore, whereas U4517 in the human ribosome stacks on the chloropyridine ring in PF846 (Fig. 2i), the corresponding nucleotide in bacterial 23S rRNA (U2609) would sterically occlude PF846 binding (Supplementary Fig. 7k). Taken together, these distinctions in nucleotide identity and positions help explain PF846’s specificity for eukaryotic ribosomes.

Analysis of stalled nascent chains within the exit tunnel. The triazolopyridine ring system of PF846 (Fig. 2c,d) faces the ribosome tunnel and is the only part of the molecule with direct interaction with the stalled nascent chain (Fig. 3a and Supplementary Fig. 8). The triazolopyridine moiety has incomplete density (Supplementary Fig. 7a–f) that may be caused by the flexibility of this region during nascent chain elongation. The density for the nascent chains in the RNCs with A/P NC-tRNA was mostly well defined in the ribosome exit tunnel with a local resolution of ~4-5 Å, although they probably consist of different sequences superimposed on each other corresponding to the cluster of stalled states spanning multiple mRNA codons (Supplementary Figs. 6 and 8). All three of the nascent chains (CDH1, PCSK9, USO1) have similar geometry and adopt a predominantly extended conformation in the exit tunnel (Supplementary Fig. 8). The nascent chain spans roughly nine residues between the C terminus of the nascent chains covalently linked to the 3′-CCA end of A/P-site tRNA in the peptidyl transferase center (PTC) and the small molecule-binding site (Fig. 3a and Supplementary Fig. 8). These residues engage in multiple

![Fig. 1 | Structural analysis of PF846 stalled RNCs. a. Schematic representation of the DNA constructs used to prepare PF846 stalled RNCs. b. Protein sequences from CDH1, PCSK9 and USO1 tested for stalling. The arrow indicates the main stalling site predicted from ribosome profiling data. c. Luciferase reporter assays using the chimeric PCSK9 nascent chain in the absence (white bar) or presence (gray bar) of PF846 (data represent mean ± s.d., n=3 independent experiments). d. Cryo-EM structure of the stalled CDH1-RNC in the rotated state showing A/P IRNA (tRNA bound to the 40S A site and 60S A site, orange) and P/E tRNA (tRNA bound to the 40S P site and 60S E site, magenta). CDH1 nascent chain (purple) and PF846 (dark green) with small and large subunit colored in cyan and gray, respectively. e. Structure of the stalled CDH1-RNC in the rotated state with A/A IRNA (tRNA bound to the 40S A site and 60S A site, orange) and P/E tRNA (magenta). Source data for c are available in Supplementary Data Set 3. NC, nascent chain.]
interactions with nucleotides in 28S rRNA of the 60S subunit, including U4493, U4414 and A3879 (Fig. 3a and Supplementary Fig. 8d,e). Past the compound-binding pocket (that is, in the N-terminal direction), the nascent chain contacts ribosomal proteins uL4 and uL22, along with A1582 and C2773 in 28S rRNA (Fig. 3a and Supplementary Fig. 8d,e). These contacts distal from the PTC are likely important for the stalling induced by PF846, based on N-terminal deletion analysis of the PCSK9 sequence10.

Most reported RNC structures have ‘kinks’ in the nascent chain residing in the ribosome exit tunnel proximal to the PTC 19. The CDH1, PCSK9 and USO1 nascent chains also have a kink in their structures between the PTC active site and PF846-binding pocket (Fig. 3a and Supplementary Fig. 8), but these adopt a different geometry when compared to stalled human cytomegalovirus (hCMV) and signal recognition particle (SRP) nascent chains20,21 (Fig. 3b,c). In the PF846-stalled RNC structures, the kinks assume more acute angles and occur closer to the PTC, to make enough space for the bound small molecule (Fig. 3a and Supplementary Fig. 8), whereas the hCMV and SRP nascent chain positions would occlude PF846 binding (Fig. 3b,c).

Destabilized interaction between the A/P tRNA and the P-loop in PF846 stalled RNCs.

### Table 1 | Cryo-EM data collection, refinement and validation statistics

|              | CDH1-RNC_ AP tRNA (EMD-0600, PDB 6OLE) | CDH1-RNC_ AA tRNA (EMD-0599, PDB 6OLF) | CDH1-RNC_ PP tRNA (EMD-0601, PDB 6OLG) |
|--------------|---------------------------------------|---------------------------------------|---------------------------------------|
| **Data collection and processing** |                                       |                                       |                                       |
| Magnification | 43,478                                | 43,478                                | 43,478                                |
| Voltage (kV)  | 300                                   | 300                                   | 300                                   |
| Electron exposure (e⁻/Å²) | 50                                   | 50                                   | 50                                   |
| Defocus range (μm) | 1.0–2.0 | 1.0–2.0 | 1.0–2.0 |
| Pixel size (Å) | 1.15 | 1.15 | 1.15 |
| Symmetry imposed | C1 | C1 | C1 |
| Initial particle images (no.) | 213,412 | 213,412 | 213,412 |
| Final particle images (no.) | 73,110 | 10,350 | 16,594 |
| Map resolution (Å) | 3.0 | 3.9 | 3.4 |
| FSC threshold | 0.143 | 0.143 | 0.143 |
| Map resolution range (Å) | 2.5–7.5 | 3.1–9 | 2.8–8 |
| **Refinement** |                                       |                                       |                                       |
| Initial model used (PDB code) | 40S subunit 6ek0, 60S subunit 5aj0, tRNA 3j7r | 40S subunit 6ek0, 60S subunit 5aj0, tRNA 3j7r | 40S subunit 6ek0, 60S subunit 5aj0, tRNA 3j7r |
| Model resolution (Å) | 3.0 | 3.9 | 3.4 |
| FSC threshold | 0.5 | 0.5 | 0.5 |
| Model resolution range (Å) | 2.5–7.5 | 3.1–9 | 2.8–8 |
| Map sharpening B factor (Å²) | −71 | −73 | −62 |
| R.m.s. deviations | Bond lengths (Å) | 0.007 | 0.019 | 0.009 |
| Bond angles (°) | 1.014 | 1.188 | 1.193 |
| **Validation** |                                       |                                       |                                       |
| MolProbity score | 1.81 | 2.02 | 2.04 |
| Clashscore | 4.89 | 7.02 | 6.89 |
| Poor rotamers (%) | 1.00 | 1.05 | 1.34 |
| Ramanchandran plot | Favored (%) | 88.90 | 87.03 | 89.28 |
| Allowed (%) | 9.76 | 12.63 | 10.08 |
| Disallowed (%) | 0.36 | 0.35 | 0.64 |

### Table 2 | Cryo-EM data collection, refinement and validation statistics

|              | PCSK9-RNC_ AP tRNA (EMD-0596, PDB 6OM0) | PCSK9-RNC_ AA tRNA (EMD-0597, PDB 6OM7) | PCSK9-RNC_ PP tRNA (EMD-0598, PDB 6OLZ) |
|--------------|---------------------------------------|---------------------------------------|---------------------------------------|
| **Data collection and processing** |                                       |                                       |                                       |
| Magnification | 43,478                                | 43,478                                | 43,478                                |
| Voltage (kV)  | 300                                   | 300                                   | 300                                   |
| Electron exposure (e⁻/Å²) | 50                                   | 50                                   | 50                                   |
| Defocus range (μm) | 1.0–2.5 | 1.0–2.5 | 1.0–2.5 |
| Pixel size (Å) | 1.15 | 1.15 | 1.15 |
| Symmetry imposed | C1 | C1 | C1 |
| Initial particle images (no.) | 148,817 | 148,817 | 148,817 |
| Final particle images (no.) | 43,666 | 9,564 | 7,214 |
| Map resolution (Å) | 3.1 | 3.7 | 3.9 |
| FSC threshold | 0.143 | 0.143 | 0.143 |
| Map resolution range (Å) | 2.5–7.5 | 3–9 | 3–9 |
| **Refinement** |                                       |                                       |                                       |
| Initial model used (PDB code) | 40S subunit 6ek0, 60S subunit 5aj0, tRNA 3j7r | 40S subunit 6ek0, 60S subunit 5aj0, tRNA 3j7r | 40S subunit 6ek0, 60S subunit 5aj0, tRNA 3j7r |
| Model resolution (Å) | 3.1 | 3.7 | 3.9 |
| FSC threshold | 0.5 | 0.5 | 0.5 |
| Model resolution range (Å) | 2.5–7.5 | 3–9 | 3–9 |
| Map sharpening B factor (Å²) | −75 | −77 | −80 |
| R.m.s. deviations | Bond lengths (Å) | 0.007 | 0.020 | 0.009 |
| Bond angles (°) | 1.036 | 1.190 | 1.222 |
| **Validation** |                                       |                                       |                                       |
| Clashscore | 4.86 | 6.36 | 7.51 |
| Poor rotamers (%) | 1.25 | 1.03 | 0.83 |
| Ramanchandran plot | Favored (%) | 90.02 | 87.57 | 88.18 |
| Allowed (%) | 9.54 | 12.11 | 11.36 |
| Disallowed (%) | 0.45 | 0.32 | 0.45 |
predominantly in the rotated state that precedes mRNA and tRNA translocation to the next codon, presumably impeding the action of eEF2, the GTPase that promotes the translocation reaction.\(^\text{16,17}\) In bacterial translation, which is better understood\(^\text{18-20}\), translocation proceeds through a series of ribosome rotated states, in which the peptidyl-tRNA body and 3′-CCA end move independently from one another, transiting from an A/A site with 3′-CCA end base pairing with the large subunit tRNA A-loop, to an A/P site in the rotated state in which the 3′-CCA end base pairing with the large subunit tRNA A-loop, to an A/A site in the PTC than may normally occur\(^\text{20,21}\). This alteration would then prevent the peptidyl-tRNA from binding stably in the hybrid A/P site, which requires the tRNA 3′-CCA end to properly base pair with 28S rRNA nucleotides in the P-loop.

**Sequence determinants of PF846-induced stalling.** The few nascent chain sequences stalled by PF846 as previously identified by ribosome profiling do not reveal a simple amino acid ‘motif’ responsible for stalling\(^\text{10}\). We therefore used in vitro translation reactions to identify the sequence determinants in the nascent chain required for PF846-induced stalling of RNCs. To assay the sequence-dependent ability of PF846 to induce stalling of RNCs; that is, ribosomes in the middle of the luciferase open reading frame, we first used a poly-asparagine scan, analogous to ribosome display\(^\text{26}\). We ran- domized four nascent chain amino acids at a time near the PTC and near the PF846 binding site, by introducing (NNK)\(_4\) sequences (N, any nucleotide; K, G or U) into mRNAs encoding the CDH1 stalling sequence (Fig. 1a,b and Supplementary Fig. 10a). We used these mRNA libraries in in vitro translation reactions, in the presence or absence of PF846, and then used the 3X-FLAG tag at the N terminus of the nascent chain to pull down stalled RNCs, which would retain the mRNA and tRNAs\(^\text{23,24}\). In the PF846-stalled complexes, we observe conformational changes required for complete translocation of the bacterial analog of eukaryotic eEF2) can catalyze the remaining translocation reaction\(^\text{22}\). In bacterial translation, which is better understood\(^\text{23,24}\), translocation proceeds through a series of ribosome rotated states, in which the peptidyl-tRNA body and 3′-CCA end move independently from one another, transiting from an A/A site with 3′-CCA end base pairing with the large subunit tRNA A-loop, to an A/P site in the rotated state in which the 3′-CCA end base pairing with the large subunit tRNA A-loop, to an A/A site in the PTC than may normally occur\(^\text{20,21}\). This alteration would then prevent the peptidyl-tRNA from binding stably in the hybrid A/P site, which requires the tRNA 3′-CCA end to properly base pair with 28S rRNA nucleotides in the P-loop.

**Table 3 | Cryo-EM data collection**

| PCSK9s-RNC_AP tRNA (EMD-0526) | PCSK9s-RNC_PP tRNA (EMD-0534) |
|---------------------------------|---------------------------------|
| Data collection and processing  |                                  |
| Magnification (kV)              | 40,983                          |
| Voltage (kV)                    | 300                             |
| Electron exposure (e-/Å\(^2\))  | 50                              |
| Defocus range (μm)              | 1.0-2.5                         |
| Pixel size (Å)                  | 1.22                            |
| Symmetry imposed                | C1                              |
| Final particle images (no.)     | 8,631                           |
| Map resolution (Å)              | 4.68 Å                          |
| FSC threshold                   | 0.143                           |

**Table 4 | Cryo-EM data collection, refinement and validation statistics**

| USO1-RNC_AP tRNA (EMD-0526, PDB 6O1L) |
|----------------------------------------|--------------------------------------|
| Data collection and processing         |                                      |
| Magnification (kV)                     | 43,478                               |
| Voltage (kV)                           | 300                                  |
| Electron exposure (e-/Å\(^2\))        | 55                                   |
| Defocus range (μm)                     | 1.5-2.5                              |
| Pixel size (Å)                         | 1.15                                 |
| Symmetry imposed                       | C1                                   |
| Initial particle images (no.)          | 79,322                               |
| Final particle images (no.)            | 38,314                               |
| Map resolution (Å)                     | 3.5                                  |
| FSC threshold                          | 0.143                                |
| Map resolution range (Å)               | 2.8-8                                |

**Refinement**

| Initial model used (PDB code)         |                                   |
|---------------------------------------|-----------------------------------|
| 40S subunit                           | 6ek0                              |
| 60S subunit                           | 5aj0                              |
| tRNA                                   | 3j7t                              |
| Model resolution (Å)                  | 3.1                               |
| FSC threshold                         | 0.5                               |
| Model resolution range (Å)            | 2.5-7.5                           |
| Map sharpening B factor (Å\(^3\))     | -91                               |
| R.m.s. deviations                     |                                    |
| Bond lengths (Å)                      | 0.009                             |
| Bond angles (°)                       | 1.17                              |

**Validation**

| MolProbity score                      | 1.98                              |
| Clashscore                             | 5.36                              |
| Poor rotamers (%)                      | 1.48                              |
| Ramachandran plot                     |                                    |
| Favor (%)                              | 89.71                             |
| Allowed (%)                            | 9.95                              |
| Disallowed (%)                         | 0.35                              |
Fig. 2 | Interactions of PF846 with the human ribosome. a, b, Phylogenetic analysis of the 28S rRNA binding pocket in eukaryotes (a) and bacteria (b). Nucleotides that interact with PF846 are labeled with an asterisk (*). Nucleotides with capital letters (AUCG), are over 98% conserved, nucleotides with small letters (aucg) are 90–98% conserved. Nucleotides denoted by filled or open circles represent a conservation of 80–90%, or less than 80%, respectively. c, d, PF846 chemical and three-dimensional structure, respectively. e, The 28S rRNA residues with direct interactions with the piperidine ring of PF846. Surfaces represent the van der Waals radii of the C, N and O atoms. f–h, Interactions of PF846 and nucleotides from 28S rRNA. i, Interaction of the chloropyridine ring with U4517, with van der Waals surfaces as in e.

Fig. 3 | Nascent chains in the exit tunnel and their effects on the A/P tRNA 3′-CCA end. a, Molecular model of CDH1 nascent chain (magenta) following the 3′-CCA end of A/P tRNA (green, at the top) in the ribosome exit tunnel with 28S rRNA (gray), uL22 (orange), uL4 (light green) and PF846 (dark green) highlighted. b, c, Superposition of hCMV20 (blue) and SRP21 (cyan) stalled nascent chains within the exit tunnel, showing the predicted steric clashes with PF846 (dark green), using van der Waals surfaces. d, Poor positioning of A/P NC-tRNA 3′-CCA end near the ribosomal P-loop. High-resolution structure of the 3′-CCA end of peptidyl-tRNA (PDB: 1vy4)37 is docked as a rigid body, using nucleotides from 28S rRNA in the PTC as reference. e, Nucleotide C75 in A/P NC-tRNA modeled as flipped to nearby density without pairing with the P-loop (starting model from PDB: 1vy4). The asterisks in d and e indicate the positioning of nucleotide C75 in A/P NC-tRNA.
The mRNAs isolated in this manner were then converted into a DNA library and analyzed by deep sequencing. In the mRNA library with randomized sequences near the PTC, we observed strong enrichment for PF846-sensitive stalling sequences very different from the original CDH1 sequence (Fig. 5a and Supplementary Table 2). In particular, nascent chain residues −2 and −4 from the predicted stall site are enriched in histidine rather than leucine (Fig. 5a, inset). Other polar and charged amino acids are also enriched in the PF846-sensitive nascent chains. We validated two of the newly identified stalling sequences using in vitro luciferase assays. We found PF846 stalled HYHS and RSCK sequences with a similar half-maximum inhibitory concentration (IC50) compared to LLLL in the original CDH1 sequence (Fig. 5b and Supplementary Fig. 10c), with the HYHS sequence being more potently stalled at high PF846 concentrations (Fig. 5b). Both sequences were also enriched in stalled RNCs, as determined by western blotting (Fig. 5d and Supplementary Fig. 10d). We tested the HYHS motif in the context of the PCSK9 stalling sequence, and found it does not induce PF846-dependent stalling as seen in the CDH1 sequence background (Fig. 5f). The effects of the HYHS sequence in fact track the poly-asparagine scan of the PCSK9 sequence (Supplementary Fig. 9b), in terms of PF846-dependent stalling. Thus, the HYHS motif on its own is not sufficient to induce PF846-dependent stalling, but requires additional sequences in the nascent chain.

We also tested two sequences that were depleted from the mRNA library in a PF846-dependent manner (Fig. 5a). In the context of the CDH1 nascent chain, sequences SFRD and ATHF increase overall translation in a PF846-dependent manner (Fig. 5e), with ATHF increasing translation roughly four-fold. The results with these sequences indicate that the original sequences identified as stalled by PF846 (ref. 10) may be inherently poorly translated. We observed no enrichment of PF846-stalling dependent sequences in the mRNA library encoding randomized nascent chains in the ribosome exit tunnel near the PF846 binding site (PF library) (Supplementary Fig. 10e). This is in contrast to the Asn-scanning results, in which mutation of nascent chain sequences near the PF846 binding site disrupted stalling. This is likely due to the fact that Asn-scanning introduced negative determinants of PF846-dependent stalling, whereas enrichment from the mRNA libraries revealed positive determinants of stalling.

Finally, we also identified many sequences with a stop codon at the final position enriched in a PF846-dependent manner in the PTC-proximal mRNA library (Fig. 5c), indicating that PF846 blocked proper translation termination of these nascent chains. These sequences, roughly 6–7% of the total termination sequences identified, have an entirely different amino acid consensus compared to those predicted to stall translation elongation (Fig. 5a,c, insets). Since PF846 and related compounds had not been observed previously to block translation termination, we tested whether
PF846 affects termination at the identified sequences. Consistent with the enrichment observed from the PTC mRNA library, PF846 prevented nascent chain release from ribosomes when sequences NPN, DPC, NVI and CVT—but not GCV—preceded the stop codon (Fig. 5d and Supplementary Fig. 10d). We did not observe an enrichment of eRF1 in the pelleted RNCs (Supplementary Fig. 10d), indicating that aberrant termination induced by PF846 may prevent eRF1 binding.

**Model for PF846 induced translation stalling.** The present structures of PF846-stalled CDH1-, PCSK9- and USO1-RNCs provide the first molecular detail for the selective stalling of nascent chains in human ribosomes by a small molecule. PF846 binds a newly identified small-molecule-binding pocket in the exit tunnel of the human ribosome and traps select RNCs predominantly in the rotated state (Fig. 6). The ability of PF846 to stall highly specific nascent chain sequences seems to require a distributed set of

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**Fig. 5 | Selections of PF846-dependent stalling sequences from randomized mRNA libraries.** a, MA plot (data with M (log ratio) and A (mean average) scales) of sequences enriched for PF846-induced translation elongation stalling, compared to translation reactions in the absence of PF846. Sequences were enriched from the mRNA library of CDH1-derived nascent chains with four amino acid positions randomized near the predicted stall site in the PTC. log_{2}-fold enrichment of sequences is plotted against total read count, for experiments carried out in duplicate. Green, sequences enriched with an adjusted \( P < 0.01 \). Inset, consensus sequence logo \(^{28}\) for enriched sequences. b, In vitro translation assays with luciferase reporters containing the wild-type (WT) CDH1 stalling sequence or with the HYHS motif near the PTC, as a function of PF846 concentration (data represent mean ± s.d., \( n = 3 \) independent experiments). c, MA plot of sequences enriched for PF846-induced inhibition of translation termination, compared to translation reactions in the absence of PF846. Sequences were enriched from the mRNA library of CDH1-derived nascent chains with four amino acid positions randomized near the predicted stall site in the PTC, with only sequences containing stop codons shown. log_{2}-fold enrichment of sequences is plotted against total read count, for experiments carried out in duplicate. Green, sequences enriched with an adjusted \( P < 0.01 \). Inset, consensus sequence logo for enriched sequences\(^{28}\). d, Western blot of affinity-purified stalled CDH1-derived nascent chains. Reactions in the absence or presence of 50 µM PF846 are shown. Western blot of ribosomal protein RPS19 for each sample is also shown, as a loading control. Original gels are shown in Supplementary Data Set 1. e, In vitro translation assays of the WT CDH1 stalling sequence, or with the ATHF or SFRD motif near the PTC, in the context of the CDH1 sequence (data represent mean ± s.d., \( n = 3 \) independent experiments). f, In vitro translation assays with the PCSK9 nascent chain sequence containing the HYHS motif in different positions in the absence (white bar) or presence (gray bar) of PF846. The stalled PCSK9 sequence is shown in full at the top, and the positions of the HYHS motif are indicated. Luciferase, luciferase-only control sequence. (Data represent mean ± s.d., \( n = 3 \) independent experiments.) The statistical significance values in a and c were tested by DESeq2 using the Wald test (two-sided) and adjusted for multiple comparisons by the Benjamini–Hochberg method\(^{27}\). Source data for b, e and f are available in Supplementary Data Set 3.
interactions between the nascent chain and the exit tunnel wall (Figs. 3a and Sf and Supplementary Figs. 8d,e and Fig. 9). PF846-dependent stalling may also be favored on nascent chain sequences that are not translated efficiently, as suggested by the fact that certain amino acid changes in stalled sequences can actually enhance rather than repress translation in a PF846-dependent manner (Fig. 5a,c,e). Furthermore, we find that the wild-type and RCSK-containing CDH1 nascent chains are enriched in pelleted ribosomes, even in the absence of PF846 (Fig. 5d and Supplementary Fig. 10d), further supporting the notion that these sequences may inherently pause in the ribosome. Finally, the fact that PF846-induced stalling occurs across multiple adjacent codons (Supplementary Fig. 6) also supports a model where these compounds target sequences that are not translated efficiently.

Discussion

The specific sequence determinants required for PF846-induced stalling of translation elongation extend roughly one-third of the way through the exit tunnel, beginning at the PTC and extending past the binding pocket for PF846 (Supplementary Fig. 8). Sequences close to the PTC exert a more dominant effect on stalling potency, as revealed from the mRNA libraries (Fig. 5 and Supplementary Fig. 10). However, sequences close to the PTC are not sufficient for stalling, as revealed in the Asn scans (Supplementary Fig. 9) and in experiments with the HYS sequence embedded in the PCSK9 context (Fig. 5f). In the case of endogenous PCSK9, the nascent chain likely needs to span the length of the exit tunnel, as N-terminal truncations of PCSK9 reporters prevented PF846-dependent stalling (16). The distributed nature of PF846-induced stalling is reminiscent of those observed in naturally occurring stalling sequences in bacteria (19,27,28) and in eukaryotes (29). By contrast, the more general stalling induced by antibiotics exit tunnel may only require localized rearrangements between the drug binding site and the PTC, as observed for macrolide antibiotics (16,11).

Redirection of the nascent chain by PF846 seems to prevent stable base pairing of the NC-tRNA 3′-CCA end with the ribosomal P-loop, likely making these RNCs poor substrates for the GTPase eEF2 that is responsible for mRNA and tRNA translocation. Consistent with this model for stalling, mutations in the 3′-CCA end of A/P tRNA or P-loop of the bacterial ribosome that disrupt base pairing slow or prevent translocation (32). Furthermore, the SecM stalling peptide in bacteria is also capable of trapping the ribosome in the rotated state with hybrid A/P NC-tRNA, in which the 3′-CCA end of the tRNA is not properly base paired with the P-loop (27). Proper 3′-CCA end positioning has also been shown to be important for the peptidyl transferase reaction. Peptide bond formation is disrupted by certain amino acid sequences such as proline stretches that prevent pairing of the 3′-CCA end with the P-loop in rRNA. These sequences require dedicated translation elongation factors, EF-P in bacteria and eIF5A in eukaryotes, to rescue stalled ribosome by stabilizing the CCA end of the P-site tRNA in the conformation that favors peptide bond formation (31). Although PF846 does not stall elongating ribosomes on nascent chains with proline sequences enriched near the PTC (Fig. 5a, inset), PF846 blocks termination when proline-rich sequences occur immediately adjacent to a stop codon positioned at the C terminus of the CDH1 stalling sequence (Fig. 5c, inset). The broader sequence determinants for PF846-induced inhibition of termination remain to be elucidated, but may be distributed over a longer stretch of the nascent chain, as seen for the hCMV stalling peptide, which ends in the sequence IPP but requires specific sequences in the nascent chain that are span at least one-third the length of the exit tunnel (26,24). The mechanism of PF846-induced inhibition of translation termination differs from that of the hCMV peptide, which traps termination factor eRF1 on the ribosome in an inactive conformation (26,32). By contrast, the PF846-induced block of termination seems to have no observable effect on eRF1 binding (Supplementary Fig. 10d). This may be due to the PF846-stalled termination complex occupying the rotated state, thereby preventing eRF1 from accessing the tRNA A site on the small subunit, and/or preventing the 3′-CCA end of P-site tRNA from properly docking in the PTC (27).

Stalling of RNCs in the rotated state may serve as a critical determinant of PF846 selectivity by evading cellular quality control pathways that recognize aberrantly stalled ribosomes and initiate recycling (36). For example, Pelota (PELO, Dom34 in yeast) and its cofactors are involved in ribosome-associated quality control pathways that recognize stalled ribosome with an empty A site, to which Pelota binds and promotes ribosome subunit dissociation (36). Consistent with the hypothesis that quality control pathways resolve PF846-stalled RNCs, ribosome profiling experiments did not reveal a buildup of stalled RNCs in cells treated with PF846 (ref. 10). Furthermore, PELO and HBS1L are genetically linked to resolution of PF846-stalled RNCs, ribosome subunit dissociation (36).
Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41594-019-0236-8.

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Author contributions
K.F.M., S.L., R.G.D. and J.H.D.C. came up with the concept. W.L., F.R.W., K.F.M., S.L., R.G.D. and J.H.D.C. conducted the investigation and formal analysis. W.L. and K.F.M. were employed by Pfizer, Inc. The authors have filed a patent application related to this work.

Competing interests
The authors declare competing interests. R.G.D. is employed by Pfizer, Inc. K.F.M. and S.L. were employed by Pfizer, Inc. The authors have filed a patent application related to this work.

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Methods

DNA constructs and in vitro transcription. The DNA fragments needed to assemble the constructs encoding the affinity tagged nascent chains (EMCV IRES, CDH1 sequence encoding amino acids 586–750 and PCKS9 sequence encoding amino acids 1–35). Five sets and one Supplementary Table were amplified from previous plasmids encoding full length PCKS9 and CDH1 (ref. 17). For the CDH1 nascent chain used for structural studies, we fused the EMCV IRES, affinity tags, CDH1 sequence encoding amino acids 586–750 and a NanoLuc luciferase reporter gene together by overlap extension PCR (Supplementary Table S3). For the PCKS9 nascent chain, CDH1 amino acids 716–750 were replaced with PCKS9 amino acids 1–35, leaving the CDH1 V-domain intact (Supplementary Table S3). The USO1 construct was made by replacing the sequence encoding CDH1 amino acids 709–750 with the USO1 nascent chain sequence spanning that found previously to cause PFK846-induced stalling (Supplementary Table S3) 17. The NanoLuc control without stalling sequence was obtained by fusing the EMCV IRES and the NanoLuc open reading frame using overlap PCR (Supplementary Table S3). All PCR products were purified via spin columns (Qiagen) before their use in in vitro transcription reactions.

In vitro transcription reactions were performed using PCR products generated with primers encoding a flanking T7 RNA polymerase promoter and a poly-A tail. Reactions were set up with 20 mM Tris-Cl pH 7.5, 75 mM MgCl2, 2 mM spermidine, 10 mM DTT, 1 U/ml T7 polymerase (Promega), 0.1 mg/ml T7 RNA polymerase and 40 ng/ml T7 RNA polymerase. After 3h incubation at 37 °C, 0.1 U/ml DTT and DTT of Promega) was added to the reactions, and incubated at 37° C for 30 min to remove the templates. The precipitated mRNA was collected by 20% ethanol and dissolved with RNase free water. The mRNA was further purified by Zymo RNA Clean and Concentrator (Zymo research) before use in in vitro transcription reactions.

In vitro translation reactions. The HeLa cell extract was made as described previously 17. Briefly, a frozen HeLa cell pellet was thawed and suspended with an equal volume of lysis buffer (20 mM HEPES pH 7.5, 10 mM potassium acetate, 1.8 mM magnesium acetate and 1 mM DTT). After incubation on ice an equal volume of lysis buffer (20 mM HEPES pH 7.5, 10 mM potassium acetate, 1.8 mM magnesium acetate and 1 mM DTT) was added. After 5 min. The supernatant after centrifugation was used for translation. As control. Translation reactions were incubated for 23 min at 30 °C, after which the translation reactions were used in in vitro transcription reactions.

Purification of stalled RNCs. In vitro translations of 1.5 ml with 0.2 mM PFK846 were incubated at 30°C for 23 min and then centrifuged at 11,400 r.p.m. for 5 min. The supernatant was incubated with 50 µl (packed volume) of anti-FLAG M2 agarose beads (Sigma) for 30 min at room temperature with gentle mixing. To avoid binding to HEPES and other non-specific ribosomes and other proteins, the beads were washed at room temperature 3 times with 500 µl RNC buffer (20 mM HEPES pH 7.4, 300 mM potassium acetate, 5 mM magnesium acetate, 1 mM DTT, 0.2 mM PFK846), then 3 times with 500 µl RNC buffer plus 0.1% TritonX-100, followed by 3 times with 300 µl RNC buffer plus 0.5% TritonX-100, and finally washed twice with 300 µl RNC buffer. The PFK846 stalled RNCs, bound to the FLAG beads by the N-terminal 3X-FLAG tag, were eluted twice at room temperature for 20 min each, with 30 µl 0.2 mg/ml 3X-FLAG peptide (Sigma) in RNC buffer. The eluted fractions were combined and loaded onto a 50% sucrose cushion prepared with cushion buffer (25 mM HEPES-KOH pH 7.5, 120 mM KOAc and 2.5 mM Mg(OAc)2), 1 M sucrose, 1 mM DTT, 0.2 mM PFK846 and centrifuged for 1 h in an MLA rotor (Beckman Coulter) with 100,000 r.p.m. (~603,000 g) at 4°C. The pellet was suspended in ice-cold RNC buffer and was immediately used for cryo-EM grid preparation. The concentration of purified RNC was determined using a NanoDrop Microvolume Spectrophotometer and calculated using a 1A260 unit corresponding to 20 pmol of ribosome.

RNaseA digestion and western blot. RNCs released from the anti-FLAG beads and pelleted through the sucrose cushion were resuspended in 20 µl to an A260 optical density of around 1 and then incubated with or without 100 ng/ml DNase free RNase A (ThermoFisher) and 50 mM EDTA at 37°C for 30 min followed by western blot of the FLAG-tagged peptides. For the western blots, Monoclonal ANTI-FLAG M2 Peroxidase (HRP) antibody (SIGMA, catalog number A5992) at 1:10,000 dilution was used.

Cryo-sample preparation and data collection. Approximately 3 µl of freshly made RNC at a concentration of 40–60 nM were incubated for 1 min on plasma-cleaned 300-mesh holey carbon grids (C-flat R2/2), on which a home-made continuous carbon film was pre-deposited. Grids were blotted for 4 s with 100% humidity at 4 °C. One drop of 1X stain-frozen in liquid ethane using an FEI Vitrobot. Automatic data collection was performed on a Titan Krios electron microscope (FEI) equipped with a K2 Summit direct detector and GIF Quantum filter (Gatan) at 300 kV (Tables S1–S4). The total exposure time was 9 s, with a total dose of 50 electrons Å–2 (frame dose 1.3 electrons Å−2).

Data processing. Frames were aligned using MotionCor2 with FReST 2 and dose weighting17. The final two frames were not used for image alignment to avoid excessive beam damage. We used Gautomatch (http://www.rnc-lmb.cam.ac.uk/zhanglei/1) for automatic particle picking. Power spectra, defocus values and astigmatism were determined with Gei software with per-particle CTF (contrast transfer function) estimation17, and classification into distinct classes. All the final reconstructions used dose-weighted images. For the CDH1–RNC data set, we divided the whole data set into three subsets and carried out particle sorting as shown in Supplementary Fig. 2a. After initial three-dimensional classification and refinement, an additional round of three-dimensional classification was performed with a local angular search that separated CDH1–RNCs in rotated states from a class with apo-805 ribosomes or ribosomes with weak density for tRNA (Supplementary Fig. 2a). A total number of 84,437 particles representing the CDH1–RNC in the rotated state were combined and subjected to three-dimensional refinement, which generated a map of 4 Å without the use of a mask. Signal subtraction was conducted to improve the signal density of the nascent peptide chain17. Starting with the aligned 84,437 particles, a region with a CDH1 nascent chain, PFK846 and A/P tRNA was extracted and classified by applying a soft mask generated in RELION. For the CDH1–RNCs with either hybrid tRNAs, the overall resolutions of the CDH1–RNCs with A/P NC-tRNA and A/A NC-tRNA were 3.9 and 3.9 Å, respectively, using the gold standard Fourier shell correlation (FSC = 0.145) criterion (Supplementary Fig. 2b and Table S1).

To identify other CDH1–RNC classes, we combined the remaining classes after sorting the RNC with hybrid tRNAs (Supplementary Fig. 2a) for an additional three-dimensional refinement and classification with a local angular search, from which three major classes were observed: from left to right, 805 ribosome without tRNA, CDH1–RNC with P/P tRNA and the large (60S) subunit. Another round of signal subtraction for the CDH1–RNC with P/P tRNA was conducted based on the P/P tRNA density, resulting in roughly 61% of particles that were chosen for the final reconstruction of the non-rotated state RNC.

Two data sets for the PCKS9–RNC were collected on the same Titan Krios microscope within a week of each other. After overall refinement, an additional round of three-dimensional classification was performed with a local angular search, which separated RNCs in the rotated state (Supplementary Fig. 3). The overall resolutions for the USO1–RNC were collected in a single session and processed similarly to the PCKS9–RNC sample, except signal subtraction yielded only a single tRNA population, with A/P NC-tRNA (Supplementary Fig. 4 and Table S3).

For the data set of stalled PCKS9–RNC prepared using a short time interval, the selected particles after RELION two-dimensional classification were processed using CrysospARC24 for further three-dimensional classification and homogeneous refinement, which generated two maps representing different states of the ribosome at an average resolution of 4.6 and 4.8 Å, respectively (Fig. 3b and Table S2).

Model building and refinement. Initial rigid body fitting of the human 40S ribosomal subunit structure (PDBe: 6bux) and 60S subunit structure

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Modeling of codon–anticodon base pairs. Correlations between modeled nucleotides and observed cryo-EM density at 3.0 Å resolution are dominated by the phosphate-ribose backbone and the common position of the nucleobase in pyrimidines and five-membered ring in purines. Thus, it is difficult to distinguish purines from pyrimidines at this resolution using only real-space correlations. We therefore used helix h24 in human 18S rRNA (nucleotides 1,037–1,078) to assess the ability of real-space density differences to distinguish purines and pyrimidines in base pairs and guanosines from adenosines in base pairs, in ~3.0 Å resolution cryo-EM maps. We generated map fragments from the CDH1-RNC cryo-EM map with P/A NC-tRNA and made mutations in nucleotides using COOT, followed by real-space refinement of models in PHENIX (phenix.real_space_refine), with secondary structure restraints generated by phenoix.secondary_structure restrain and verified manually. We then generated real-space difference maps in Chimera, as follows. We set the minimum threshold of the observed cryo-EM map to zero (vop threshold minimum 0.), and used the model coordinates to generate a calculated map at 3.0 Å resolution (mollmap). Then, we generated (observed – calculated) difference maps by minimizing the root-mean-square of the differences (vop subtract minRMS true). From these maps, we found that negative difference density was diagnostic for positions that are incorrectly modeled as purine, due to negative density on the N2, C2, N1, C6 and O6/N6 positions of the modeled purine ring. We also observed positive difference density in these maps consistent with the position of a purine incorrectly modeled as a pyrimidine, due to the distribution in the density map calculated from model coordinates with ADP values grouped at the nucleotide level, currently the only setting available in phenix.real_space_refine.

DNA libraries with randomized codons in the CDH1 stalling sequence. DNA libraries encoding the open reading frame containing the CDH1 stalling sequence in Fig. 1b, with stretches of four random codons encoded by NNK sequences (N = any nucleotide, K = G or T) to introduce random mutations in different sites of the CDH1 nascent chain within the ribosome exit tunnel, were generated by PCR amplification. PCR products were purified using spin columns (Qiagen, catalog number 28506), then treated with DpnI nuclease at 37 °C for 1 h to remove template DNA. A double-stranded DNA fragment encoding a T7 RNA polymerase promoter was then ligated to the 5’ end of the treated DNA, along with a double-stranded DNA encoding a poly-A tail to the 3’ end, using T4 DNA ligase and T4 Polynucleotide Kinase at 16 °C overnight. The ligation reaction was followed by PCR amplification using primers flanking the T7 promoter and poly-A tail to generate the final DNA library. The oligonucleotides used to prepare the DNA libraries are presented in Supplementary Table 3.

Selection of RNC complexes stalled by PF846 using mRNAs encoding random libraries. The mRNAs encoding the NNK libraries were prepared by T7 RNA polymerase transcription of the above NNK DNA libraries, followed by mRNA purification as described above. The mRNA libraries were incubated in a 1.0 ml in vitro translation reaction (250 μM [35S]MTP, 0.25 μM T7 RNA polymerase, 100 μM Tris 7.5, 150 mM KCl, 2.5 mM MgCl₂, 2 mM DTT) and treated with 1 μl (5 units) RNase H and 1 μl of the targeting DNA oligonucleotide at 37 °C for 40 min. Total RNA was then extracted from the purified and RNase H treated RNAs using TRizol LS (ThermoFisher Scientific, catalog number 10296010). First-strand cDNAs were synthesized using a primer specific to a nucleotide sequence in the open reading frame of Fig. 1a located 3’ of the stalling sequence, using SuperScript II (Invitrogen, catalog number 18064014), according to the manufacturer’s protocol. The cdna was then used in a 70 μl PCR reaction with Q5 DNA polymerase with optimized cycles of 10 s at 98 °C, 30 s at 66 °C and 15 s at 72 °C, followed by a two-step purification with SPRIselect beads (Beckman Coulter, catalog number B23317), first by adding 42 μl beads (0.6x amount of the PCR reaction) and then applying the flowthrough to another 56 μl beads (0.6x amount of the PCR reaction). The resulting bound DNA was eluted in 15 μl molecular biology grade water. The DNA libraries were sequenced using an Illumina HiSeq 4000. The oligonucleotides used to prepare the sequencing libraries are presented in Supplementary Table 3.

Analysis of the Illumina sequencing libraries. UMIs were extracted from raw reads using umi_tools and sequences were aligned to an index of all 12-mer DNA sequences using segkit (ref. *) and bowtie2 (ref. *) (parameters—N=0,– 1-1mm-upfront, –norc–L 24). No mismatches were allowed in the 6 bp sequences flanking the library bases to ensure each read was a correctly generated library member. Alignments were sorted and indexed with samtools and deduplicated with umi tools using the ‘unique’ method. Deduplicated reads were tabulated by translated library sequence, replicate and condition (PF846 treated or untreated) in Python and PF846 sensitivity was analyzed using DESeq2 (ref. *). PF846-sensitive or -tolerant cDNAs were defined by a false discovery rate of 10%. The complete list of sequences recovered from sequencing the mRNA libraries is included in Supplementary Data Set 2 and sequencing data for those statistically enriched or depleted is included in Supplementary Table 2.

Validation of PF846-dependent stalling sequences and inhibition of termination. To validate whether the sequences identified in the mRNA selections are bona fide targets of PF846-dependent stalling, we cloned reporter mRNAs with representative examples into the CDH1-chimeric mRNA construct as described above and shown in Fig. 1a. We replaced codons for amino acids L726–L729 in the CDH1 sequence with codons for the following sequences: HH in HYHS, R in RSCK for sequence stalled by PF846 during elongation, C in NNK, CVY in NNK, CYP6 in NNK, CV in NNK and NPV for sequences that lead to PF846-dependent inhibition of termination (*, stop codon). We also used GCV, which was not enriched in the experiment, as a negative control for PF846-dependent inhibition of translation termination. Codons used for each sequence are listed in Supplementary Table 3. As an additional negative control, we cloned nanoluciferase with an N-terminal 3X FLAG tag.

To test whether the sequences HYHS and RSCK, and the ones with stop codons, lead to PF846-dependent stalling in the context of the CDH1 sequence, we synthesized mRNAs as described above and subjected them to in vitro translation reactions, also as described above, with the following modifications. We carried out the in vitro translation reactions with ribosomes derived from the wild-type CDH1 sequences using different concentrations of PF846 to determine IC₅₀ values. To assess the amount of nascent chains that remain associated with ribosomes after the reactions, we also carried out in vitro translation reactions with or without 50 μM PF846, followed by pelleting ribosomes through a sucrose cushion and western blotting with an anti-FLAG antibody (Monoclonal ANTI-FLAG
Figures were generated with UCSF Chimera53, PyMOL (Schrödinger), ChemDraw (PerkinElmer) and WebLogo54.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The cryo-EM maps have been deposited with the Electron Microscopy Data Bank under the accession codes EMD-0599 (CDH1-RNC with A/A and P/E tRNA), EMD-0600 (CDH1-RNC with A/P and P/E tRNA), EMD-0601 (CDH1-RNC with P/P tRNA), EMD-0597 (PCSK9-RNC with A/A and P/E tRNA), EMD-0596 (PCSK9-RNC with A/P and P/E tRNA), EMD-0598 (PCSK9-RNC with P/P tRNA), EMD-20134 (PCSK9-RNC in rotated state with sample prepared with a short sample incubation time), EMD-20135 (PCSK9-RNC in non-rotated state with sample prepared with a short sample incubation time) and EMD-0526 (USO1-RNC with A/P and P/E tRNA). Atomic Coordinates have been deposited in the Protein Data Bank with accession codes 6OLF (CDH1-RNC with A/A and P/E tRNA), 6OLE (CDH1-RNC with A/P and P/E tRNA), 6OLG (CDH1-RNC with P/P tRNA), 6OM7 (PCSK9-RNC with A/A and P/E tRNA), 6OM0 (PCSK9-RNC with A/P and P/E tRNA), 6OL2 (PCSK9-RNC with P/P tRNA) and 6OLI (USO1-RNC with A/P and P/E tRNA). Sequencing data is available at the Sequence Read Archive (SRA) under accession codes 5PRNA533782. Analysis of all reads from the sequencing data for the PTC library and PF library is available in Supplementary Data Set 3. All other data that support the findings of this study are available from the corresponding author upon reasonable request. The uncropped images for the main text are available in Supplementary Data Set 1.

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Antibodies

Antibodies used:

- [A] FLAG antibody: Monoclonal ANTI-FLAG® M2 Peroxidase (HRP) antibody, SIGMA, A8592-2MG; (B) eRF1 antibody: Cell Signaling Technology, 13916; (C) RPS19 antibody: Bethyl Laboratories, A304-002A.

Validation:

- [A] FLAG antibody: M2, monoclonal; antibody from purified immunoglobulin; application: direct ELISA: 1:20,000. Also antibody titer from 1:100 to 1:1000 is recommended for immunocytochemistry and Western blot (https://www.sigmaaldrich.com/catalog/product/sigma/a8592?lang=en&region=US); Biological source mouse; citation: (1) Michael Waterfield et. al. (2014) Nature Immunology, 15. (2) Marcos Simões-Costa et. al. (2015) Developmental cell, 34. (3) Simon B Saucet et. al. (2015) Nature communications, 6. (4) Peter Dy et. al. (2008) Nucleic acids research, 36(9). (4) Mickael Leiek et. al. (2015) Nature communications, 6. (5) Yoonjin Lee et. al. (2014) Nature, 510. Ezra Burstein et. al. The EMBO journal, 23.

- (B) eRF1 antibody: Antibodies are purified by protein A and peptide affinity chromatography; Species: Human, Mouse, Rat, Monkey; Polyclonal; Validated by Western blot analysis of extracts from various cell lines using eRF1 antibody, which can be found in the manufacture’s website [https://media.celsignal.com/pdf/13916.pdf]; application: western blotting with 1:1000 dilution; citations are listed: (1) Zhouravleva, G. et al. (1995) EMBO J 14, 4065-72. (2) Song, H. et al. (2000) Cell 100, 311-21. (3) Cheng, Z. et al. (2009) Genes Dev 23, 1106-18. (4) Buvigyn, K.N. et al. (2010) RNA 16, 1902-14. (5) Frolova, L. et al. (2002) RNA 8, 129-36. (6) Feng, T. et al. (2014) Mol Cell 53, 645-54. (7) Frolova, L.Y. et al. (1999) RNA 5, 1014-20. (8) Merkulova, T.I. et al. (1999) FEBS Lett 443, 41-7.

- (C) RPS19 antibody: affinity purified; species: human, mouse; Polyclonal; application: IP and WB; validated by IP and WB which can be found in the manufacture’s website [https://www.bethyl.com/product/A304-002A/RPS19+Antibody]; citations are listed: (1) de la Parra, C., Ernlund, A., et al. (2018) Nature Communications 9:3068. (2) Fletcher, S. J., Pisareva, V. P., et al. (2018) RNA 7, 939-949. (3) Juszkiewicz, S. & Hegde, R. S. (2017) Molecular Cell 65, 743-750. (4) Lee, A. S., Kranzusch, P. J., et al. (2016) Nature 536 96-99. (5) Lee, A. S., Kranzusch, P. J., et al. (2015) Nature 522 111-114.
## Eukaryotic cell lines

Policy information about [cell lines](#)

| Cell line source(s)          | Hela          |
|------------------------------|---------------|
| Authentication               | Commonly used cell type, obtained from Pfizer |
| Mycoplasma contamination     | negative      |
| Commonly misidentified lines| negative      |

(See [CLAC register](#))