Dynamic pathways of –1 translational frameshifting

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Spontaneous changes in the reading frame of translation are rare (frequency of $10^{-3}$ to $10^{-4}$ per codon), but can be induced by specific features in the messenger RNA (mRNA). In the presence of mRNA secondary structures, a heptanucleotide ‘slippery sequence’ usually defined by the motif X XXY YYZ, and (in some prokaryotic cases) mRNA secondary sequences that base pair with the 3’ end of the 16S ribosomal RNA (internal Shine–Dalgarno sequences), there is an increased probability that a specific programmed change of frame occurs, wherein the ribosome shifts one nucleotide backwards into an overlapping reading frame (~1 frame) and continues by translating a new sequence of amino acids1. Despite extensive biochemical and genetic studies, there is no clear mechanistic description for frameshifting. Here we apply single-molecule fluorescence to track the compositional and conformational dynamics of individual ribosomes at each codon during translation of a frameshift-inducing mRNA from the dnaX gene in *Escherichia coli*. Ribosomes that frameshift into the –1 frame are characterized by a tenfold longer pause in elongation compared to non-frameshifted ribosomes, which translate through unperturbed. During the pause, interactions of the ribosome with the mRNA stimulatory elements uncoouple EF-G catalysed translocation from normal ribosomal subunit reverse-rotation, leaving the ribosome in a non-canonical intersubunit rotated state with an exposed codon in the aminoacyl-tRNA site (A site), tRNA3Cyts sampling and accommodation to the empty A site and EF-G action either leads to the slippage of the tRNAs into the –1 frame or maintains the ribosome into the 0 frame. Our results provide a general mechanistic and conformational framework for –1 frameshifting, highlighting multiple kinetic branchpoints during elongation.

Despite detailed biochemical and genetic studies, the mechanism of –1 programmed ribosomal frameshifting (PRF) remains poorly understood, with at least three groups of models attempting to explain frameshifting9,10. The 3’ hairpin11 and 5’-internal Shine–Dalgarno sequence12, (in some prokaryotic cases) pause the ribosome over the slippery sequence, which is necessary but not sufficient to drive efficient –1 PRF. How these structural elements induce the pause and operate together to manipulate the ribosomal reading frame are not known. During a –1 frameshift, the anticodon of the tRNAs must detach from the mRNA and re-associate in the –1 frame. However, this slippage may occur at distinct points during the elongation cycle: (1) during accommodation of the A-site tRNA, (2) subsequent to accommodation, but before peptidyl transfer, (3) during EF-G catalysed translocation9,10, or (4) after translocation but before the next round of elongation. As the exact timing of frameshifting is unknown, the precise position of the ribosome over the slippery sequence during the slippage is unclear9,10. Finally, as –1 PRF has been shown to occur at approximately 1% to 80% efficiency depending on the sequence9, what determines whether one particular ribosome will frameshift or not remains elusive.

The dynamic and stochastic nature of frameshifting requires direct observation of single ribosomes translating multiple codons of an mRNA. We harness here single-molecule fluorescence and zero-mode waveguides (ZMWs) instrumentation14 to track ribosome progression on mRNAs and observe –1 translational frameshifting in real-time13. Conformational changes underlying elongation, involving rotational movements of the small (30S) ribosomal subunit body with respect to the large (50S) ribosomal subunit, were monitored during translation by site-specifically labeling the 30S with Cy3B and 50S with BHQ-2 (a non-fluorescent quencher), allowing for Förster resonance energy transfer (FRET) between the two dyes14,15. During normal translation elongation, aminoacyl-tRNA–EF-Tu–GTP ternary complex accommodation to the A site followed by peptide bond formation drives the non-rotated to rotated state transition (low to high Cy3B intensity, or high to low FRET), whereas EF-G catalysed translocation drives the rotated to non-rotated transition (high to low Cy3B intensity)9. Thus, one round of high-low-high FRET (low-high-low Cy3B intensity) corresponds to a single ribosome translating one codon (see Extended Data Fig. 1)9. Arrival and departure of the dye-labelled ligands such as Cy5–tRNAs and Cy5–EF-G can be simultaneously observed as a sequence of fluorescent pulses9,15. We applied this approach to the –1 frameshift sequence from the dnaX gene in the *E. coli*, which contains an internal Shine–Dalgarno sequence and a slippery –A AAA AAG– sequence followed by a 3’ RNA hairpin12,13.

We observed –1 frameshifting directly on a dnaX frameshift sequence, designed such that ribosomes that frameshift will translate 9 codons and stop at a stop codon in the –1 frame, whereas ribosomes that do not frameshift will translate 12 codons until a stop codon in the 0 frame (Fig. 1a). By delivering total tRNA (tRNA3Cyts) ternary complex, EF-G, and BHQ-50S to immobilized Cy3B-30S preinitiation complexes (30S subunit–mRNA–initiator tRNA), we observe ribosomes that translate either the full 12 codons or only 9 codons, as measured by the number of intersubunit FRET cycles (see Extended Data Fig. 2). By determining the fraction of ribosomes that translate > 9 codons, or translate up to 9 codons, we obtain an estimate of the frameshifting percentage (75%), consistent with previously observed frameshifting efficiency10 (confirmed independently as shown in Extended Data Fig. 2b, c). The Shine–Dalgarno sequence and hairpin act as barriers to translocation, so mutations of the potential Shine–Dalgarno sequence and removal of the hairpin all decrease frameshifting efficiency as expected (Extended Data Figs 3 and 4).

Elongation of the dnaX mRNA is drastically and abruptly perturbed at the seventh FRET cycle (codon Lys7). Analysis of rates at each codon revealed a tenfold increase in the rotated state (waiting for EF-G and translocation) lifetime (96 ± 18 s vs 5–10 s for the other codons) at Lys7, corresponding to tRNA3Cyts(GCA21)-codon pair in the ribosomal peptidyl-tRNA site (P site) and the newly incorporated tRNA3Cyts(AAG24) codon pair in the A site (GCA27, AAA24), poised for translocation; non-rotated state lifetimes (waiting for ternary complex and peptide bond formation) remain constant at each codon (Fig. 1b–d). Furthermore by partitioning frameshifted vs non-frameshifted ribosomes, an increased rotated-state lifetime at codon Lys7 is observed only for frameshifted ribosomes (138 ± 31 s); non-frameshifted ribosomes translate through the frameshift site seemingly unaffected (13 ± 4 s) (Fig. 1e, confirmed independently in Extended Data Fig. 2d, e). The seventh FRET cycle is the hallmark of frameshifting and requires the slippery-site sequence. Partitioning between frameshifted and non-frameshifted ribosomes was assumed to
Figure 1 | Framesshifting is characterized by a long rotated-state pause.

a. Schematic of the mRNA used in this study, modified from the dnaX gene. b. Schematic of the Cy3/BHQ ribosome FRET signal, with each low-high-low Cy3B intensity cycle representing a ribosome elongating one codon. c. Sample traces of Cy3B (green) fluorescent intensity for framesshifted and non-framesshifted ribosomes translating with 80 nM EF-G and 1 μM tRNA

5 ternary complex. Codon Lys7 of the framesshifting site is shaded yellow. d. The mean rotated-state lifetime and non-rotated state lifetime for each FRET cycle. The non-rotated state lifetime is constant. There is

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gate the arrival of the sampling tRNALys, confirming that tRNALys translocation occurs during the pause induced by framesshift signal. Instead, we demonstrate that the initial branch point occurs before the pause, but all framesshifted ribosomes exhibit a pause.

We next determined what is occurring during the pause that is characteristic of framesshifting. Normally translocation is coupled to ribosome reverse-rotation with decacylated tRNA in the ribosomal exit site (E site) departing rapidly after the ribosome reverse-rotates14. Using Cy3-labelled tRNAVal, we observed E site tRNA departure directly at the framesshifted site on a GCA21 (Ala) to GUA21 (Val) mRNA mutant, without affecting the framesshifting behaviour (Extended Data Fig. 7). We measured the departure of Cy3–tRNAVal relative to the Cy5–tRNA d5 arrival to the AAA24 (Lys7) codon in the A site, which defines the start of the long rotated-state pause, correlated to peptide bond formation and transition to the rotated state: departure of decacylated Cy3–tRNAVal relative to the arrival of Cy5–tRNA d5 at codon Lys7 estimates when and if translocation occurs during the pause. During translation of the dnaX mRNA, Cy3–tRNAVal departs on average 45 ± 11 s after the arrival of Cy5–tRNA d5 to the Lys7 codon (within a photobleaching time of 196.7 ± 28.1 s). This time decreases with increasing concentration of EF-G, confirming that tRNA departure is linked to translocation (Fig. 2a).

However, as the Cy3–tRNAVal residence time is much shorter than the rotated state lifetime (138 s), translocation occurs within the rotated state pause and precedes eventual reverse rotation. Thus translocation at Lys7 during framesshifting is uncoupled from reverse rotation of the ribosomal subunits and moves the ribosome A and P sites over the slippery sequence (AAA24 AAG27). Translocation in this case is still inhibited through the interactions of the hairpin and internal Shine–Dalgarno sequence, with time to translocation longer than normal translation.

Uncoupling of tRNA–mRNA translocation from reverse-rotation and E site tRNA departure creates a non-canonical intermediate in translation: the ribosome has a peptidyl-tRNA in the P site, but remains in a rotated intersubunit conformation. To delve into the nature of this intermediate framesshifting state and whether the A site is available for tRNA binding, we correlated Cy5–tRNA d5 binding and departure events with the ribosome FRET signal (Fig. 2b). Although the dnaX mRNA sequence consists of 4 Lys codons, 71% of elongating ribosomes exhibit > 4 Cy5–tRNA d5 pulses (Fig. 2c) (equal to the framesshifting percentage). The first three tRNA d5 pulses (Lys1, Lys5 and Lys7) show arrival rates and lifetimes consistent with elongation dynamics from intersubunit FRET data (Extended Data Fig. 8a, b); the third Lys7 pulse corresponds to the ribosome decoding AAA24 at the slippery site (lifetime of 119.4 s, consistent with the rotated state lifetime at Lys7). The existence of the fourth and subsequent tRNA pulses directly indicate that translocation has occurred during the long rotated state and the A site is now available for aminoa-

cyl-tRNA binding. After uncoupled translocation of the tRNA d5 to the P site, which would expose the fourth Lys codon (Lys8), tRNA d5 samples the A site codon multiple times (on average 2.3 times), resulting in a buildup of Cy5 intensity (from two Cy5–tRNA d5 bound to the ribosome, Fig. 2b, c) even though the rotated state is not the natural substrate for tRNA binding to the A site. Mutation of the slippery sequence (A21G–A24G) greatly suppresses additional sampling by Cy5–tRNA d5 (only 9.9% of elongating ribosomes exhibit > 4 pulses), indicating that multiple sampling events on Lys8 are characteristic of framesshifting and the long pause. Post-synchronization of the arrival of the fourth sampling tRNA d5 to the time of uncoupled translocation shows that translocation gates the arrival of the sampling tRNA d5, confirming that tRNA d5 is indeed sampling the A site codon exposed by translocation (Fig. 2d and Extended Data Fig. 8). Delivery of tRNA–EF–Tu–GDPNP (a non-hydrolysable analogue of GTP) instead of GTP decreases the tRNA pulse lifetimes from 38 ± 2 s to 2.1 ± 0.1 s, demonstrating that GTP hydrolysis by EF-Tu and subsequent accommodation of the tRNA into the ribosomal A site occur for these long-lived sampling pulses.

We propose that uncoupled + 3 translation creates weakened codon–anticodon–ribosome interactions, and that tRNA d5–sampling and accommodation at the AAG27 codon presented by the non-canonical rotated ribosome drives the ribosome into the + 1 frame and helps re-establish codon–anticodon interactions (see Extended Data Fig. 9). Consistent with this model, AAA26 (−1 frame) and AAG27 (0 frame) both...
Figure 2 | rRNA samples the rotated state after uncoupled translocation and defines the reading frame. a, Sample trace and time to translocation at the frameshift site, which is estimated by Cy5-rRNA\textsuperscript{Val} (green) departure from the E site during frameshifting relative to the arrival of Cy5–rRNA\textsuperscript{Lys} (red) at codon Lys\textsubscript{7} (shaded yellow) on a GCA\textsubscript{21} (Ala) to GU\textsubscript{A21} (Val) mRNA tran. As time to translocation is shorter than the rotated state pause, translocation from Lys\textsubscript{7} to Lys\textsubscript{8} occurs during the pause and is uncoupled from reversion. From left to right, n = 337, n = 449 and n = 455. Error bars, s.e. b, Sample trace of correlation of the Cy3B/BHQ ribosome FRET signal (green) with Cy5-rRNA\textsuperscript{Lys} (red), confirming the long pause at codon Lys\textsubscript{7} (shaded yellow). Upon reaching codon Lys\textsubscript{7}, additional tRNA\textsuperscript{Lys} pulses sample codon Lys\textsubscript{8} in the A site (shaded red), which results in a buildup of Cy5 intensity from the two Cy5–rRNA\textsuperscript{Lys} in the A and P sites of the ribosome. c, Fraction of elongating ribosomes exhibiting >4 Lys pulses (additional sampling pulses) for the dnaX\textsuperscript{Val} wild-type mRNA and the A21G–A24G mutant. From left to right, n = 179 and n = 147. d, Two-dimensional density plot of Cy5–rRNA\textsuperscript{Lys} sampling to codon Lys\textsubscript{8} in the A site post-synchronized to the time of uncoupled translocation (indicated by the green line). The sampling of rRNA\textsuperscript{Lys} at Lys\textsubscript{8} only begins after translocation. e, Rotated and non-rotated state lifetimes for the slippery sequence mutant (A\textsubscript{23}A\textsubscript{25}G\textsubscript{27} codon to AAA). There is now an extra subpopulation of ribosomes with a long rotated-state pause that does not lead to frameshifting, n = 310, error bars, s.e. f, Mutation of the last A\textsubscript{23}A\textsubscript{25}G\textsubscript{27} codon to UUU (Phe). Similar to e, there are two subpopulations within the non-frameshifted ribosomes. n = 353, error bars, s.e. g, Pathways of frameshifting for the various slippery sequence mutants, indicating how rRNA sampling defines the final reading frame.
Peptidyl transfer is probably inefficient, as the rotated ribosome probably proposed, the tRNA hybrid states may be destabilized, favouring a classic frameshifting. As recently events as well as increased mean EF-G lifetime during the seventh FRET cycle times can be observed.

The competition between slow peptide-bond formation and slow translocation explains heterogeneous protein products in prior frameshifting studies. Thus, frameshifting involves both EF-G and tRNA, and occurs at an unconventional point during elongation after translocation of the ribosome on to the slippery sequence (uncoupled with reverse-rotation) but before peptidyl transfer (Fig. 4), though the binding energy of the codon–anticodon pairing to be used to allow slippage, whereas both P- and A-site tRNAs are on the ribosome and redefine the translational frame. EF-G may facilitate slippage. As recently proposed, the tRNA hybrid states may be destabilized, favouring a classical-like conformation of the two tRNAs that promotes −1 slippage. Peptidyl transfer is probably inefficient, as the rotated ribosome probably does not position the two tRNAs correctly for peptidyl transfer to occur efficiently. EF-G eventually resolves the state and continues translation. The competition between slow peptide-bond formation and slow translocation explains heterogeneous protein products in prior frameshifting studies. Thus, frameshifting involves both EF-G and tRNA, and occurs at an unconventional point during elongation after translocation of the ribosome on to the slippery sequence (uncoupled with reverse-rotation) but before peptidyl transfer (Fig. 4), though the binding energy of the codon–anticodon pairing to be used to allow slippage, whereas both P- and A-site tRNAs are on the ribosome and redefine the translational frame. EF-G may facilitate slippage. As recently proposed, the tRNA hybrid states may be destabilized, favouring a classical-like conformation of the two tRNAs that promotes −1 slippage. Peptidyl transfer is probably inefficient, as the rotated ribosome probably does not position the two tRNAs correctly for peptidyl transfer to occur efficiently. EF-G eventually resolves the state and continues translation. The competition between slow peptide-bond formation and slow translocation explains heterogeneous protein products in prior frameshifting studies. Thus, frameshifting involves both EF-G and tRNA, and occurs at an unconventional point during elongation after translocation of the ribosome on to the slippery sequence (uncoupled with reverse-rotation) but before peptidyl transfer (Fig. 4), though the binding energy of the codon–anticodon pairing to be used to allow slippage, whereas both P- and A-site tRNAs are on the ribosome and redefine the translational frame. EF-G may facilitate slippage. As recently proposed, the tRNA hybrid states may be destabilized, favouring a classical-like conformation of the two tRNAs that promotes −1 slippage. Peptidyl transfer is probably inefficient, as the rotated ribosome probably does not position the two tRNAs correctly for peptidyl transfer to occur efficiently. EF-G eventually resolves the state and continues translation. The competition between slow peptide-bond formation and slow translocation explains heterogeneous protein products in prior frameshifting studies. Thus, frameshifting involves both EF-G and tRNA, and occurs at an unconventional point during elongation after translocation of the ribosome on to the slippery sequence (uncoupled with reverse-rotation) but before peptidyl transfer (Fig. 4), though
multiple pathways probably exist. The interplay of mRNA sequence and structure with ribosomal dynamics leads to branchpoints during elongation, creating non-canonical paused states that allow unusual events in elongation. Such states may be a central feature of translational control.

METHODS SUMMARY

All labelled ribosomes, factors, and tRNAs were prepared and purified as described. Unless noted otherwise, all experiments were performed under buffer conditions described in Methods. Data collection from ZMW chips was conducted using instrumentation and techniques described previously. Fluorescence traces were recorded at 10 frames per second for 8 min, with delivery of ligands to start the experiment at t = 10 s. Statistical analysis on those traces was also conducted as described before, using a custom software written in MATLAB (MathWorks). All error bars presented on figures are standard errors (s.e.).

Online Content

Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions J.C. performed all the experiments and the data analysis. J.C., A.P., T.D. and J.D.P. designed the project and wrote the manuscript. M.I. and S.E.O’L. assisted with reagent preparation. All authors discussed the results and commented on the manuscript.

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E. coli ribosomal subunits and translation factors were prepared and purified as described before. EF-G, were prepared as described previously for our single-molecule experiments. A semi-purified mix of all aminoacyl-tRNA synthetases (aaRS) was prepared from E. coli S30 extract. Total or Δ(Lys, Val, Phe or some combination) aminoacyl-tRNAs were incubated by adding total E. coli tRNA (Sigma-Aldrich) with all amino acids (minus Lys, Val, Phe or some combination for Δ mixes) together with the aaRS mix for 30 min at 37°C in a buffer consisting of Tris-HCl (50 mM, pH 7.5), KCl (50 mM), MgCl₂ (10 mM), and β-ME (3 mM), complemented with ATP (2 mM), phosphoenol pyruvate (PEP, 10 mM), pyruvate kinase (PK, 50 μg ml⁻¹), myokinase (2 μg ml⁻¹) and inorganic pyrophosphatase (PPase, 10 μg ml⁻¹). The aa-tRNAs were then purified by phenol extraction, ethanol precipitation, and gel filtration (Micro Bio-Spin Columns With Bio-Gel P-6 in Tris Buffer, Bio-Rad), and finally stored in small aliquots at −80°C. E. coli tRNA**P**h, tRNA**r**, and tRNA**v** were produced from Sigma-Aldrich, Phe-(Cy5)tRNA**P**h and Lys-(Cy5)tRNA**r** were labeled with Cy5- NHS (GE Lifesciences) at acp'U at position 47, purified as described previously, and aminoacylated as above. Val-(Cy3)RNA**v** was labeled with Cy3-maleimide (GE Lifesciences) at σ'U at position 8, purified and aminoacylated as above. Synthetic biotinylated mRNAs were produced from Dharmacon. All mRNAs had 5'-biotin followed by a 5'-UTR and Shine–Dalgarno sequence derived from gene 32 of the T4 phage (sequence described previously), an AUG start codon, followed by the sequence of interest (as indicated in the figures). All experiments were conducted in a Tris-based polymix buffer consisting of 50 mM Tris-acetate (pH 7.5), 100 mM potassium chloride, 5 mM ammonium acetate, 0.5 mM calcium acetate, 0.5 mM magnesium acetate, 0.5 mM EDTA, 5 mM putrescine–HCl and 1 mM spermidine. All single-molecule experiments had 4 mM GTP and were performed at 22°C. Single-molecule experiments. The 3'-dye labeled DNA oligonucleotides (labeled with Cy3B or BHQ-2) complementary to the mutant ribosome hairpins were prepared by incubating total E. coli tRNA (Sigma-Aldrich) with all amino acids (minus Lys, Val, Phe or some combination for Δ mixes) together with the aaRS mix for 30 min at 37°C in a buffer consisting of Tris-HCl (50 mM, pH 7.5), KCl (50 mM), MgCl₂ (10 mM), and β-ME (3 mM), complemented with ATP (2 mM), phosphoenol pyruvate (PEP, 10 mM), pyruvate kinase (PK, 50 μg ml⁻¹), myokinase (2 μg ml⁻¹) and inorganic pyrophosphatase (PPase, 10 μg ml⁻¹). The aa-tRNAs were then purified by phenol extraction, ethanol precipitation, and gel filtration (Micro Bio-Spin Columns With Bio-Gel P-6 in Tris Buffer, Bio-Rad), and finally stored in small aliquots at −80°C.

Before use, we pre-incubate a SMRT Cell V3 from Pacific Biosciences (Menlo Park, CA, USA), a zero-mode waveguide (ZMW) chip, with a 1 nm θ CLC Tn7 ligand solution in 50 mM Tris-acetate pH 7.5 and 50 mM KCl at room temperature for 5 min. The chip is then washed with Buffer 6 (50 mM Tris-acetate pH 7.5, 100 mM potassium chloride, 5 mM ammonium acetate, 0.5 mM calcium acetate, 0.5 mM magnesium acetate, and 0.5 mM EDTA). We then dilute the 30S PICs with our Tris-based polymix buffer containing 1 mM IF2 and 4 mM GTP down to 10 mM PIC concentration. The diluted PICs are then loaded into the SMRT cell at room temperature for 3 min to minimize the 30S PICs into the ZMW wells. We wash away excessive unbound material with our Tris-based polymix buffer containing 1 mM IF2, 4 mM GTP, 1 mM Trolox, and a PCA/PCD oxygen scavenging system (2.5 mM 3,4-dihydroxybenzoic acid and 250 nM protocatechuate deoxygenase). We formed ternary complexes between total charged E. coli tRNAs and EF-Tu(GTP) as described. Total or Δ(aa) aminoacyl-tRNA-EF-Tu-GTP ternary complexes were pre-formed by incubating (2 min at 37°C) the aa-tRNAs with fivefold excess of EF-Tu, GTP (1 mM), PEP (3 mM) and EF-Ts (40 mM) in polymix. The ternary complexes (1–6 μM) were added to BHQ-50S (200 nM), EF-G (80–480 nM), IF2 (1 μM), GTP (4 mM), 2.5 mM Trolox, and the oxygen scavenging system (PCA/PCD) to form a mixture in a polymix buffer. Many of the experiments are done at 1 μM ternary complexes and 80 nM EF-G (chosen to have well-defined, detectable FRET transition signals), unless indicated otherwise.

Before starting an experiment, the SMRT Cell is loaded into a modified PacBio RS sequencing 2. At the start of the elongation experiment, the instrument illuminates the SMRT cell with a green laser and then delivers 20 μl of a delivery mixture onto the cell surface at t = 10 s.

Translation experiments with labelled tRNAs. Translation experiments using labelled tRNAs are performed in the same way as the Cy3B/BHQ ribosome FRET experiments with the following differences. Total tRNA mixture was charged with all amino acids except the tRNA amino acid we wish to observe. The resulting mix of all charged tRNAs except tRNA of interest that remains uncharged was used to form ternary complexes. Ternary complexes with the labelled tRNA are separately formed. For example, we will form a ternary complex with Phe-(Cy5)tRNA**P**h and Lys-aa-tRNA (3 μM) mixture is used with 80–320 nM EF-G.

Correlation experiments with Cy5–EF–G. Cross-correlation experiments between our Cy3B/BHQ ribosome FRET signal and our EF-G binding signal are conducted exactly as above but with a different delivery mix and laser illumination. The delivery mix for the cross-correlation experiment contains: 200 nM BHQ2-50S, 1 mM IF2, 80–240 nM Cy5–EF-G, 1–3 μM total tRNA ternary complexes, 4 mM GTP, 1 mM Trolox, and the PCA/PCD oxygen scavenging system in the Tris-based polymix buffer. The data presented in Fig. 4 are performed at 80 nM Cy5–EF-G and 1 μM total ternary tRNA complexes. Dual illumination of SMRT Cell with a green and a red laser are used.

ZMW instrumentation and data analysis. All single-molecule fluorescence experiments were conducted using a commercial PacBio RS sequencer that we modified to allow the collection of single-molecule fluorescence intensities from individual ZMW wells about 130 nm in diameter in four different dye channels corresponding to Cy3, Cy3.5, Cy5, and Cy5.5 (ref. 14). The RS sequence has two lasers for dye excitation at 532 nm and 632 nm. In all experiments, data was collected at 10 frames per second (100 ms exposure time) for 8 min. The energy flux of the green laser is 0.48 μW per μm² and the red laser is at 0.22 μW per μm².

Data analyses for all experiments are conducted with MATLAB (MathWorks) scripts written in-house. Briefly, traces from the ZMW wells are initially selected based on fluorescence intensity, fluorescence lifetime and the changes in intensity. Filtered traces exhibiting intersubunit FRET or single-molecule binding signals are subsequently selected for further data analysis. The FRET states are assigned as previously described based on a hidden Markov model based approach and visually corrected. All lifetimes were fitted to a single-exponential distribution using maximum-likelihood parameter estimation in MATLAB. 31. Marshall, R. A., Dorywalska, M. & Puglisi, J. D. Irreversible chemical steps control intersubunit dynamics during translation. Proc. Natl Acad. Sci. USA 105, 15364–15369 (2008).

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Extended Data Figure 1 | Explanation and schematics of the experimental signals. a, Different stages of the elongation cycle during translation. Frameshifting has been proposed to occur at each of the steps: (1) during accommodation of the A-site tRNA\(^4\), (2) subsequent to accommodation, but before peptide bond formation\(^3\), (3) during RNA hybrid-state intermediates\(^10\), (4) during translocation\(^9\), and (5) at the start of the next round of elongation\(^11\). b, The ribosome starts each round of elongation in the non-rotated state. In this ‘locked’ state, the P-site tRNA is stably bound in the classical state, preserving the reading frame of the mRNA\(^18\). Upon A-site tRNA selection and peptide bond formation, the 30S subunit rotates 3–10\(^\mu\)anti-clockwise with respect to the 50S subunit to the rotated state (pre-translocation)\(^26,35,36\). This ‘unlocked’ state permits tRNA motions and the tRNA can fluctuate freely between the classical state and hybrid state\(^37\), thus facilitating translocation of tRNA and movement of ribosome by one codon over mRNA\(^38,39\). Peptide-bond formation also triggers spontaneous fluctuations of the L1 stalk between open and closed conformations as well as spontaneous rotations in ribosome conformations\(^36,40\). EF-G then catalyses translocation, and the ribosome returns to the non-rotated state (post-translocation). To monitor the rotational state of the ribosome in real time, we employed FRET between the small (30S) and large (50S) subunits. The 30S subunit was site-specifically labelled with Cy3B on helix 44, and a non-fluorescent quencher, BHQ-2, was placed on helix 101 of 50S subunit\(^18,31,32\). Reagent delivery of BHQ-50S, tRNA ternary complex, and EF-G to surface immobilized Cy3B-30S pre-initiation complexes in ZMW\’s results in IF2-guided 70S assembly during initiation and establishment of FRET between the two ribosomal subunits: upon subunit joining\(^11\), the green (Cy3B) intensity drops, which is followed by alternating low-high-low intensities. Each alternating cycle corresponds to the ribosome translating a single codon, with the two intensity states consistent with the two rotational states of the ribosome: the low intensity state (high FRET) defining the non-rotated (locked) ribosome conformation and the high intensity state (low FRET) the rotated (unlocked) conformation\(^11\). The rotated- and non-rotated-state lifetimes at each codon can be statistically analysed. c, During each cycle of elongation, the ribosome selects the aminoacyl-tRNA in a ternary complex with EF-Tu–GTP, and positions the tRNA in the A site. Upon A-site tRNA accommodation, the ribosome rapidly catalyses peptide bond formation with the P-site tRNA\(^33,38\). Translocation moves A- and P-site tRNA–mRNA complexes to the E and P site respectively, catalysed by the EF-G. The compositional dynamics of tRNA and EF-G on the ribosome, here defined as the relative timing of their arrival and departure during elongation, can be observed by labelling the tRNA or EF-G with Cy3 or Cy5. Cy5/Cy3–tRNA arrival to the surface immobilized ribosomes is marked by a red/green fluorescent pulse. Translation can be monitored by the arrival and departure of dye-labelled tRNA. Each productive tRNA binding event results in a fluorescence pulse that lasts as a ribosome translates 2 codons—beginning with arrival of tRNA in the A site, continuing through tRNA translocation to the P site, arrival of A-site tRNA to the next codon, a second round of translocation, and ending with spontaneous dissociation of tRNA from the E site\(^30\). To track tRNA and EF-G dynamics on translating ribosomes at near-physiological concentrations of fluorescent factors (0.1–1 \(\mu\)M), we used ZMW\’s to detect hundreds of individual ribosomes\(^30\). d, Substituting the traditional FRET acceptor, Cy5, with BHQ-2 allowed the use of Cy3 to label other translation components for correlation studies. The Cy3B intensity reports on the conformational state of the ribosome, whereas Cy5 pulses indicate arrival, occupancy and departure of ribosomal ligands.
Phe codon in the −1 frame confirms the characteristic long pause during frameshifting. 

**Extended Data Figure 2** | Phe codon in the −1 frame confirms the characteristic long pause during frameshifting. **a**, Histogram of the fraction of ribosomes translating to a particular codon for the dnaX wild-type mRNA, with a schematic. Many of the ribosomes translate up to 12 codons to a 0 frame stop codon, though a large percentage of ribosomes translate up to 9 codons to a −1 frame stop codon. There are also ribosomes that stall at codon 7, limited by Cy3B photobleaching or end of movie (8 min) from the long rotated state pause. Interestingly, there is also a number of ribosomes that stall at codon 8 (see Extended Data Fig. 10 for discussion). By parsing the number of ribosomes that translate beyond codon 9 and up to codon 9, the frameshifting percentage can be calculated (75%). However, as non-frameshifted ribosomes may terminate early, this would lead to a slight over-estimate of the frameshifting percentage (∼3–10%). The frameshifting efficiency has been independently confirmed using a Cy5–tRNA^Phe^ score, as described below. Number of molecules analysed, \( n = 256 \).

**b**, A UUC(Phe) codon is introduced in the −1 frame downstream of the slippery site. Frameshifting can be scored by an appearance of a Cy5 (red) pulse with Cy5–tRNA^Phe^ in addition to the Cy3B/BHQ ribosome FRET signal. This allows us to independently score for frameshifting. **c**, Using the Cy5–tRNA^Phe^ as a score to confirm frameshifting, we get the same dynamics and lifetimes: the non-rotated state lifetimes remain constant at each codon, and the rotated state lifetime increases tenfold at the seventh FRET cycle (codon Lys7 and Lys8 at the slippery sequence due to uncoupled translocation). This confirms and justifies our results in Fig. 1. Number of molecules analysed, \( n = 474 \). Error bars, s.e.

**d**, By using the Cy5–tRNA^Phe^ as a score, we can parse the rotated state lifetimes into ribosomes that frameshifted and ribosomes that did not frameshift. We also get the same results as Fig. 1: non-frameshifted ribosomes translate through the frameshift sequence seemingly unaffected; frameshifted ribosomes exhibit the characteristic long-rotated state pause at the seventh FRET cycle (codon Lys7 and Lys8 due to uncoupled translocation). Number of molecules analysed, \( n = 474 \). Error bars, s.e.
Extended Data Figure 3 | Hairpin and the internal Shine–Dalgarno sequence are important for frameshifting. 

**a** mRNA sequence of the no hairpin (no HP) mutant. The mRNA consists of the same sequence as the wild-type *dnaX* frameshift sequence, but with the sequence after the UGA stop codon in the 0 frame in the hairpin deleted. **b** Non-rotated and rotated state lifetimes in the presence of 80 nM EF-G and 1 μM tRNA<sub>tot</sub>. The non-rotated state lifetimes are constant at each codon. There is an increase in rotated state lifetime at codon Lys7. Number of molecules analysed, \( n = 124 \). Error bars, s.e.

**c** mRNA sequence of the no Shine–Dalgarno (no SD) mutant. The mRNA consists of the same sequence as the wild-type *dnaX* frameshift sequence, but with the original internal Shine–Dalgarno sequence GGGAGC mutated to AGGCGC, decreasing the rRNA–mRNA interaction energy from \(-4.70\) kcal mol\(^{-1}\) to 0.00 kcal mol\(^{-1}\). **d** Non-rotated and rotated state lifetimes in the presence of 80 nM EF-G and 1 μM tRNA<sub>tot</sub>. The non-rotated state lifetime is constant. There is an increase in rotated state lifetime at codon Lys7. Number of molecules analysed, \( n = 225 \). Error bars, s.e.

**e** Frameshifting percentages of the no Shine–Dalgarno and no hairpin mutant. Without the Shine–Dalgarno sequence, frameshifting percentage drops by half. Without the hairpin, frameshifting percentage drops to a quarter of the wild-type sequence. This indicates that both the internal Shine–Dalgarno sequence and the hairpin are required for efficient frameshifting. These stimulatory elements may present a barrier and tension to translocation that is a prerequisite for efficient frameshifting.
Extended Data Figure 4 | Hairpin and internal Shine–Dalgarno sequences increases the energy barrier to translocation. a, Translation of a short linear mRNA, 6(FK), in the presence of 80 nM EF-G and 1 mM tRNAtot, with a sample trace. b, Histogram of fraction of ribosomes translating to a particular codon. Most of the ribosomes translate up to 12 codons. Ribosomes translate <12 codons are due to photobleaching of the Cy3B dye, or non-processive ribosomes. This gives us a background level of ~3–10% for our frameshifting efficiency analysis. The small number of ribosomes that translate beyond codon 12 are probably errors in our statistical analysis or read-through of the stop codon. Number of molecules analysed, n = 462. c, Rotated and non-rotated state lifetimes are fairly constant. Number of molecules analysed, n = 462. Error bars, s.e. d, Translation of a Phe-Lys sequence preceded by an internal Shine–Dalgarno sequence (same Shine–Dalgarno sequence used in the dnaX frameshift mRNA of this study) in the presence of 80 nM EF-G and 1 mM tRNAtot, with an example trace. e, Histogram of fraction of ribosomes translating to a particular codon. Number of molecules analysed, n = 462. f, There is an increase in rotated state lifetime at codon 5–7. There is an increase in the rotated state lifetimes ~3–4-fold over 3–5 codons downstream of the Shine–Dalgarno-like sequence, whereas the non-rotated state lifetime remains unaffected. The internal Shine–Delgano-like sequences may base pair with the 3’ end of the 16S rRNA and slow down ribosomes in the pre-translocation state, echoing several work done previously by tracking ribosome movement and ribosome profiling. g, Translation of a Phe-Lys sequence followed by a hairpin (same hairpin used in the dnaX frameshift mRNA of this study) in the presence of 80 nM EF-G and 1 μM tRNAtot, with an example trace. h, Histogram of fraction of ribosomes translating to a particular codon. Number of molecules analysed, n = 332. i, Non-rotated state lifetimes are fairly constant. There is an increase in rotated state lifetime at codon 5, exactly 3 codons before the start of the hairpin, placing the ribosome directly at the first遇到 of the hairpin. The relative position also matches where we see the long-rotated state pause during frameshift. This echoes the work which showed the ribosome is capable of translating through the secondary structure through two mechanisms: ribosome translocating when encountering an open-state junction, occurring naturally or induced by the ribosome, or mechanically unwinding by the ribosome when encountering a closed-state junction. When the ribosome encounters an open-state junction, translation proceeds at a constant rate; however, when a closed-state junction is encountered, the ribosome actively unwinds the secondary structure, resulting in a slight waiting time for translocation, after which the hairpin is biased by the ribosome into an open-state and translation occurs normally. The shunt to either pausing in the rotated state (which leads to uncoupled translocation) or normal translation during frameshifting is probably due to this mechanism. Number of molecules analysed, n = 332. Error bars, s.e.
Extended Data Figure 5 | Dynamics of frameshifting at different factor concentrations. a, Example trace and schematic of a ribosome translating the dnaX frameshift mRNA at much higher factor concentrations (6 μM tRNAtot and 480 nM EF-G). b, Frameshifting efficiency does not depend on EF-G and tRNAtot concentrations. c, Increasing the tRNAtot and EF-G concentrations twofold (from 1 μM tRNAtot and 80 nM EF-G to 2 μM tRNAtot and 160 nM EF-G) decreases both the rotated state lifetime and non-rotated state lifetimes. This confirms that our ribosome FRET signal depends correctly on factor concentration. Number of molecules analysed, n = 256 (1 μM tRNAtot and 80 nM EF-G), n = 234 (2 μM tRNAtot and 160 nM EF-G). Error bars, s.e. d, Increasing the tRNAtot concentration (from 1 μM to 3 μM) while keeping EF-G concentrations constant decrease the non-rotated state lifetimes threefold as expected. The rotated state lifetimes remain the same except for codon Lys7; this is expected because the rotated state lifetime depends only on concentration of EF-G. Unexpectedly, the rotated state lifetime at codon Lys7 (Lys8 after uncoupled translocation) is also slightly decreased, suggesting a linkage between tRNA and EF-G dynamics at that long rotated-state stall. This echoes our results in Fig. 2 that tRNA (tRNALys in this case) samples the uncoupled rotated-state after translocation, and the tRNA sampling and accommodation and EF-G sampling may help to re-establish the ribosome’s reading frame and reverse-rotate subsequently. Thus, increasing tRNA concentrations (especially tRNALys in this case) will decrease the long rotated-state lifetime. Number of molecules analysed, n = 526. Error bars, s.e. e, Increasing the EF-G concentration (from 80 nM to 240 nM) while keeping tRNAtot concentration constant decreases the rotated state lifetime threefold. The non-rotated state lifetime, which depends on the tRNA concentration, remains the same. However, the decrease in the rotated lifetime at codon Lys7 is only around twofold, rather than threefold as expected. This echoes our result in Fig. 2 as well as in b above, suggesting that tRNA sampling also plays a role at this codon. Number of molecules analysed, n = 314. Error bars, s.e. f, Increasing the EF-G and tRNAtot concentrations further to 6 μM tRNAtot and 480 nM EF-G further decreases the rotated and non-rotated state lifetimes. However, the rotated state lifetime at codon Lys7 remains the same when compared with 2 μM tRNAtot and 160 nM EF-G. The long tRNA sampling events observed in Fig. 2 may be contributing to the long-rotated state lifetime at codon Lys7.
Extended Data Figure 6 | Slippery sequence mutation (A21G–A24G) decreases frameshifting percentage. a, Sample trace of a ribosome translating the dnaX A21G–A24G mutant mRNA in the presence of 80 nM EF-G and 1 μM tRNA_{tot}. There seems to be a slightly longer pause at codon Lys7. b, Histogram of the fraction of ribosomes translating to a particular codon for the dnaX A21G–A24G mutant mRNA. Most of the ribosomes translate up to 12 codons to a 0 frame stop codon. The buildup of ribosomes stalled at codon 9 present during frameshifting disappears. By parsing the number of ribosomes that translate beyond codon 9 and up to codon 9, the frameshifting percentage can be calculated (12%). c, The rotated-state lifetime. The long stall at Lys7 is decreased with the slippery site mutant, suggesting that the extra-long pause is indeed a result of frameshifting. The slight increase in lifetime at Lys7 is due to the effects of the hairpin and internal Shine–Dalgarno sequence. Number of molecules analysed, n = 230. Error bars, s.e. d, A UUC(Phe) is introduced in the −1 frame downstream of the slippery site of the A21G–A24G mutant, similar to above. The A21G–A24G mutation is known to decrease frameshifting efficiency down to background levels19. e, The non-rotated state lifetime and rotated-state lifetime match with our results using codon counting (see above). In the absence of frameshifting, there is still an increase in rotated state lifetime at codon Lys7, due to the increased energy barrier to translocation by the hairpin and internal Shine–Dalgarno sequence, though this increased lifetime is still much less than the Lys7 rotated state lifetime during frameshifting. Number of molecules analysed, n = 538. Error bars, s.e. f, Using Cy5-tRNAPhe as a score for frameshifting, frameshifting percentage matches with our previous results. The slippery sequence A21G–A24G mutant decreases frameshifting percentage down to background levels. Number of molecules analysed, from left to right, n = 474, n = 538.
**Extended Data Figure 7 | tRNA dynamics during frameshifting with the dnaX GCA(Ala) to GUA(Val) mutant mRNA.**

**a** The 3 nucleotides upstream of the slippery sequence (GCA(Ala)) are mutated to GUA(Val) (named the C20U mutant) so that E-site tRNA dynamics can be observed during frameshifting since tRNAVal can be labelled with Cy3-maleimide (see Fig. 2). This allows us to estimate the time to translocation during the long rotated-state pause at codon Lys7, as translocation of the Cy3–tRNAVal from the P-site to the E-site leads to rapid departure of the tRNAVal and disappearance of the Cy3 signal. We want to make sure that the C20U mutation does not affect frameshifting dynamics. The non-rotated state and rotated state lifetimes, as well as frameshifting percentages, are consistent with what we have observed before for the wild-type sequence. Number of molecules analysed, n = 266. Error bars, s.e.

**b** tRNA-tRNA FRET between Cy3-tRNAVal and Cy5-tRNAlys

Extended Data Figure 7 | tRNA dynamics during frameshifting with the dnaX GCA(Ala) to GUA(Val) mutant mRNA. a, The 3 nucleotides upstream of the slippery sequence (GCA(Ala)) are mutated to GUA(Val) (named the C20U mutant) so that E-site tRNA dynamics can be observed during frameshifting since tRNAVal can be labelled with Cy3-maleimide (see Fig. 2). This allows us to estimate the time to translocation during the long rotated-state pause at codon Lys7, as translocation of the Cy3–tRNAVal from the P-site to the E-site leads to rapid departure of the tRNAVal and disappearance of the Cy3 signal. We want to make sure that the C20U mutation does not affect frameshifting dynamics. The non-rotated state and rotated state lifetimes, as well as frameshifting percentages, are consistent with what we have observed before for the wild-type sequence. Number of molecules analysed, n = 266. Error bars, s.e. b, Post-synchronization density plot of tRNA–tRNA FRET between the Cy3–tRNAVal in the P site and the incoming Cy5–tRNAlys at Lys7 in the A site at the slippery sequence. The tRNAs are in a hybrid state upon encountering of hairpin and engagement with the internal Shine–Dalgarno sequence. Thus, uncoupled translocation occurs with normal tRNA hybrid state formation. After translocation, the Cy3–tRNAVal departs from the ribosome, resulting in a disappearance of FRET. After translocation and uncoupling with ribosome reverse-rotation, the now P-site tRNAlys is probably in a ‘distorted’ conformation, according to the structure by Namy et al. Number of molecules analysed, left n = 227, right n = 337.
Extended Data Figure 8 | tRNA\textsubscript{Lys} transit and sampling dynamics.

a. Example trace of Cy5–tRNALys\textsuperscript{54} transit during translation of the dnaX wild-type mRNA, indicating the definition of pulse lifetime and time between pulse.

b. The time between tRNA\textsubscript{Lys} pulse and lifetime of each pulse for the first three Lys codons are consistent with what is expected, and decrease expectedly with the increase of EF-G concentration and tRNAtot concentration. Error bars, s.e.

c. Rotated state pause (codon Lys7). Number of molecules analysed, from left to right, \( n = 179 \), \( n = 212 \), \( n = 180 \) and \( n = 162 \). Error bars, s.e.

By translating the AAG(\textAAA) mutant in the presence of Cy5–tRNALys\textsuperscript{54}, we see similar dynamics as the dnaX wild-type sequence. Cy5–tRNALys\textsuperscript{54} samples the A site at codon Lys8 after uncoupled translocation at the frameshift site. The fraction of ribosomes exhibiting \( >4 \) tRNA\textsubscript{Lys} pulses are the same for the wild-type sequence and the AAG(\textAAA) mutant. Number of molecules analysed, \( n = 212 \) (AAG(\textAAA)) and \( n = 454 \) (AAG(\textAAA)). Error bars, s.e.

d. The mean number of tRNALys sampling pulses, the mean arrival time, and the mean lifetimes of the sampling pulses to the long rotated-state are the same for the AAG(\textAAA) mutant and the dnaX wild-type sequence. Number of molecules analysed, \( n = 212 \) (left, wild-type AAG), \( n = 454 \) (right, AAG(\textAAA)). Error bars, s.e.
Extended Data Figure 9 | tRNA sampling dynamics and slippage during frameshifting. a, Sample traces of Cy5–tRNA<sup>5′</sup> (red) sampling to the A-site Phe8 codon during the long rotated-state pause correlated with Cy3B/BHQ ribosome FRET signal (green) for the AAG(UUU) mutant. b, By translating the AAG(UUU) mutant in the presence of Cy5–tRNAPhe (red) and correlating with the Cy3B/BHQ ribosome FRET signal (green), we can observe the fraction of ribosomes exhibiting only 1 Cy5–tRNA<sup>Phe</sup> pulse or >1 Cy5–tRNA<sup>5′</sup> pulse sampling to the long rotated state pause after codon Lys7. There is a significant number of ribosomes exhibiting >1 Cy5–tRNA<sup>Phe</sup> pulse even when there is only one Phe codon, suggesting that even without frameshifting, many of the ribosomes still pause in an uncoupled rotated state after Lys7, where tRNAPhe samples the exposed UUU codon in the A site. Number of molecules analysed, n = 106. c, The arrival time of the first tRNA sampling to the long stalled codon for wild-type mRNA (with Cy5–tRNA<sup>3′</sup>) and AAG(UUU) with Cy5–tRNA<sup>Phe</sup> are the same. Although frameshifting in principle could occur through an incomplete +2 translocation<sup>x</sup> with weakened codon–anticodon–ribosome interactions and the final reading-frame determined through the Lys8 tRNALys transit through the hairpin and internal Shine–Dalgarno interaction. For +2 translocation, we would expect to see tRNA sampling to both −1 frame and the 0 frame of A-site codon. For the AAG(UUU) mutant, tRNAPhe will sample the 0 frame A<sup>1</sup> frame A<sup>2</sup>U<sub>25</sub>U<sub>26</sub>U<sub>27</sub> whereas tRNA<sup>3′</sup> will sample the −1 frame A<sup>2</sup>U<sub>25</sub>U<sub>26</sub>U<sub>27</sub>. As Cy5–tRNA<sup>Phe</sup> arrival times and lifetimes for sampling to the AAG(UUU) mutant match Cy5–tRNA<sup>3′</sup> arrival times and lifetimes for the wild-type sequence, there is probably no competition between tRNA<sup>3′</sup> and tRNA<sup>Phe</sup>, suggesting that the AUA codon is not initially exposed for tRNA<sup>3′</sup> sampling. Furthermore, for the +2 model, we would not expect a AAG(UUU) mutation to lead to a decrease in frameshifting efficiency. Thus, our results favour a +3 translocation followed by a −1 shift driven by sampling, accommodation and base-pairing stability. Unfortunately, our single-molecule assay is blind to the actual movement of the ribosome on the mRNA, so the details of this mechanism will require further exploration. See Extended Data Fig. 10 for possible implications for heterogeneous frameshift products observed previously<sup>d</sup>. Error bars, s.e. Number of molecules analysed, from left to right, n = 212, n = 106. d, Mean sampling lifetime and mean sampling arrival time for Cy5–tRNAPhe to the Phe8 codon for the AAG(UUU) mutant. The arrival time and lifetime are the same as Cy5–tRNA<sup>3′</sup> sampling to the Lys8 codon for the dnaX wild-type sequence. Number of molecules analysed n = 106. e, Example trace for Cy5–tRNA<sup>3′</sup> transit through the dnaX AAG(UUU) mutant mRNA. For the AAG(UUU) mutant, we see only three Lys pulses, as expected, since the fourth Lys codon (Lys8) is mutated to a Phe codon. Most of the ribosomes (~80%) exhibit only three Cy5–tRNA<sup>3′</sup> pulses, indicating that the additional Cy5–tRNA<sup>3′</sup> sampling pulses we saw characteristic of frameshifting are indeed sampling to the Lys8 codon. Sampling now is by tRNAPhe, which are dark and invisible to our observations. The time between pulses are consistent with both the wild-type sequence and AAG(AAA) mutant. The lifetime of the third pulse (at Lys7) is long, consistent with the long pause at that codon. Number of molecules analysed, n = 318. Error bars, s.e. f, Sample trace for Cy5–tRNA<sup>3′</sup> transit through the dnaX AAG(AAC) mutant mRNA. For the AAG(AAC) mutant, we see mostly only three Lys pulses (~75%) since the fourth Lys codon (Lys8) is mutated to an Asn codon. Number of molecules analysed n = 406. This further argues against the +2 translocation model (see e). For +2 translocation, we would expect to see long tRNA<sup>3′</sup> sampling to the −1 frame AAA codon, which is not observed significantly. The additional Cy5–tRNA<sup>3′</sup> pulses have a shorter lifetime and longer arrival time when compared with the translation of the wild-type mRNA, suggesting that these pulses are non-cognate sampling to the AAC codon in the 0 frame or sampling unstably to the AAA codon in the −1 frame. Even though our data support a +3 translocation followed by a −1 slippage, multiple frameshifting pathways probably occur. The details of this mechanism will require further exploration.
Extended Data Figure 10 | Heterogeneous frameshift products. a, Two different protein products are possible after −1 frameshifting, dependent on whether peptide bond formation occurs during sampling in the −1 or 0 frame. For the first scenario, tRNA sampling to the last three nucleotides of the slippery sequence (YYZ) redefines the ribosome in the −1 frame (YYY), after which the tRNA dissociates to leave an empty A-site codon. After the long rotated state is reverse-rotated by EF-G, tRNAYYY decodes that codon normally, creating a frameshift product denoted by XXY-YYY. For the second scenario, peptide bond formation occurs after slippage of tRNAYYZ into the −1 frame; peptide bond formation occurs slowly, as the P- and A-site tRNAs would probably not be positioned correctly in the rotated ribosomal conformation. EF-G would then normally and rapidly resolve the newly-created A/P hybrid state and the ribosome reverse-rotates. In this case, the frameshift product will be denoted by XXY-YYZ.

b, Histogram of the fraction of ribosomes translating to a particular codon for the dnaX AAG(UUU) mRNA, with a schematic. As the frameshifting percentage for the AAG(UUU) sequence is low, we see that most of the ribosomes translate up to 12 codons to a 0 frame stop codon. However, there is a significant number of ribosomes that translate to 11 codons (~5%), compared to ~5% for our previous experiments. There are two possible scenarios for tRNA^{Phe} sampling to the long rotated-state pause at codon Lys7 correlated with Cy3B/BHQ ribosome FRET signal (green), showing the two possible scenarios for tRNA sampling. Case 1 (as described in a) leads to correlation of tRNA arrival and ribosome rotation after the long rotated state pause whereas case 2 leads to overlap of a tRNA^{Phe} pulse with the reverse-rotation of the long pause. Both scenarios occur when translating the AAG(UUU) mutant, with ~58% of ribosomes for case 1 and 42% for case 2. For the second case, the time between the last Cy5–tRNA^{Phe} arrival and the ribosome reverse-rotation is 27.2 s, much longer than the 7.7 s during normal decoding and translocation, suggesting a slow peptidyltransfer reaction. Our results provide a possible explanation for why heterogeneous frameshifting products are observed in many frameshift systems. Number of molecules analysed, n = 533. c, Sample traces of Cy5–tRNA^{Phe} (red) sampling to the long rotated-state pause at codon Lys7 correlated with Cy3B/BHQ ribosome FRET signal (green), showing the two possible scenarios for tRNA sampling. Case 1 (as described in a) leads to correlation of tRNA arrival and ribosome rotation after the long rotated state pause whereas case 2 leads to overlap of a tRNA^{Phe} pulse with the reverse-rotation of the long pause. Both scenarios occur when translating the AAG(UUU) mutant, with ~58% of ribosomes for case 1 and 42% for case 2. For the second case, the time between the last Cy5–tRNA^{Phe} arrival and the ribosome reverse-rotation is 27.2 s, much longer than the 7.7 s during normal decoding and translocation, suggesting a slow peptidyltransfer reaction. Our results provide a possible explanation for why heterogeneous frameshifting products are observed in many frameshift systems. However, the details of this mechanism will require further exploration. Number of molecules analysed, n = 55.