SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Confirming pause position and FRET cycle counts

To confirm our FRET cycle counts, we pre-stalled the ribosome at an Arg 22 codon at codon 22 by withholding Arg amino acidarginine from the total tRNA mix (ΔArg total tRNA). Then, by resuming translation with the addition of total tRNA mix, we observed that 64% of the ribosomes now stall after 24 codons (instead of 45 codons if translation began from initiation). This confirms our location of the stall at Gly45 without counting so many FRET cycles (**Figure S2B**).

The long rotated pause at codon 45 is a hallmark of bypassing. We confirmed the location of the pause by mutating the Asp44 codon prior to the takeoff site to Phe. This allows replacement of the total tRNA mixture with a ΔPhe total tRNA mixture supplemented with Cy5-labeled Phe-tRNA^{Phe} (Johansson et al., 2014). The appearance of a Cy5 pulse indicates the arrival of (Cy5)Phe-tRNA^{Phe}, correlated with the conformational signal at the corresponding codons. The Cy5 pulse at codon 44 allows more accurate identification of the long rotated state pause at codon Gly45, confirming our results from counting FRET cycles (**Figure S3A**). Similarly, changing the Leu after the Gly landing codon (Leu46) to Phe, allows using (Cy5)Phe-tRNA^{Phe} pulses to score for successful bypassing. Using this measurement, 29% of the ribosomes exhibit a scoring Cy5 signal at Leu46, slightly lower than our estimate for bypassing efficiency, due to the end of movie censoring and the long pause at Gly45, suggesting that most pausing ribosomes do indeed eventually bypass. (**Figure S3B**). These results confirm that bypassing is preceded by the long pause in the rotated state at Gly45.

Comparison of bypass and SecM stall

To probe the specific interaction mechanism of the nascent peptide with the exit tunnel, we can compare the potential interactions with those during SecM stalling. The peptide exit tunnel of the ribosome can accommodate a fully extended peptide of approximately 28 amino acids in the absence of secondary structure and peptides up to 40 amino acids in the presence of secondary structure within the exit (Tsalkova et al., 1998). The gene *60* nascent peptide modulates translation while still within the ribosome since it is positioned 16–31 amino acids distant from the take-off site (the interaction begins 5 codons before). The KKYK, however, is located near the exterior end of the exit tunnel, so the presumed interaction point with the ribosome is different from that of SecM, where the peptide interacts with the constriction point, formed by the large subunit proteins L4 and L22 (Nakatogawa and Ito, 2002; Tsai et al., 2014). Replacing the entire upstream nascent peptide with the minimal SecM sequence placed adjacent to the take-off site eliminates bypassing *in vivo* (**Figure 2C**). Coincidentally, if we put SecM at the corresponding location as the nascent peptide signal in *gene 60* with the important Phe, which is preceded

by a single Lys, at the corresponding position to the Tyr of *gene 60* KKYK, we recover efficient bypassing (**Figure 2C**). Our data indicate that the interaction point required for bypassing is closer to the exterior of the ribosome than the constriction point where SecM interacts.

Reagents and buffers for translation experiments

Mutant *E. coli* ribosomes from strains bearing hairpin loop extensions in phylogenetically-variable, surface-accessible loops of 16S rRNA in helix 44 (30S body), helix 33a of 16S rRNA (30S beak) and 23S rRNA in helix 101 (50S) were used to purify the subunits (Dorywalska et al., 2005). 70S tight-coupled ribosomes were first purified, and 30S and 50S subunits were prepared from dissociated 70S particles using buffer exchange (7.5 mM to 1 mM Mg^{2+}) and purification by sucrose gradient ultracentrifugation (10–40%) as previously described protocols (Marshall et al., 2008). The desired mutant subunit was then collected and store at -80°C. As 30S subunits purified through sucrose gradients have sub-stoichiometric S1, it is purified separately and added at a stoichiometric amount to our 30S subunits before experiments. S1 were prepared as described previously (Blanchard et al., 2004; Dorywalska et al., 2005; Marshall et al., 2008).

The 3′-dye labeled DNA oligonucleotides (labeled with Cy3B or BHQ-2) complementary to the mutant ribosome hairpins that are used to label the ribosomes were ordered from Trilink. The DNA sequence to label the mutant 30S (for both helix 44 and helix 33a) is 5' GGG AGA TCA GGA TA (Cy3B) 3', and the sequence to label the mutant 50S (helix 101) is 5' GAG GCC GAG AAG TG (BHQ) 3' (Marshall et al., 2008).

A semi-purified mix of all aminoacyl-tRNA synthetases (aaRS) was prepared from *E. coli* S30 extract (Pavlov and Ehrenberg, 1996). Total or Δ(Phe) aminoacyl-tRNAs were prepared by incubating total *E. coli* tRNA (Sigma-Aldrich) with all amino acids (minus Phe 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), $MgCl_2$ (10 mM), and β-ME (3 mM), complemented with ATP (2 mM), phosphoenol pyruvate (PEP, 10mM), pyruvate kinase (PK, 50 μg/ml), myokinase (MK, 2 μg/ml) and inorganic pyrophosphatase (PP_iase, 10 μg/ml). aa-tRNAs were thereafter 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 in -80°C.

E. coli $tRNA^{Phe}$ were purchased from Sigma-Aldrich. Phe-(Cy5)$tRNA^{Phe}$ and Phe-(Cy3.5)$tRNA^{Phe}$ were labeled with Cy5-NHS or Cy3.5-NEHS, respectively, (GE Lifesciences) at acp^3U at position 47, purified as previously described (Blanchard et al., 2004; Uemura et al., 2010), and aminoacylated as above.

The biotinylated mRNAs were transcribed following published protocols with modifications (McKenna et al., 2007; Tsai et al., 2014). The plasmid constructs were purchased from Genscript containing a T7 RNA polymerase promoter, a 5'-UTR and Shine-Dalgarno sequence derived from gene 32 of the T4 phage (sequence described previously (Aitken and Puglisi, 2010)), the sequence of interest from gene 60 of T4 phage, a BlpI site for cloning and transcription purposes, followed by a T7 terminator sequence. Restriction digestion with BlpI was followed by *in vitro* run-off transcription with T7 RNA polymerase and purification with a Superdex 200 gel filtration chromatography column as described (McKenna et al., 2007). Direct biotinylation of the 5' end of the mRNA was achieved by doping the transcription reaction with 1:10 ratio of 5'-Biotin-G-Monophosphate (Trilink) with respect to GTP. The biotinylation efficiency is roughly 10%. The final mRNA storage buffer is 10 mM Bis-Tris pH 7.0, 100 mM NaCl, and 0.5 mM EDTA.

An example plasmid DNA construct that can be cloned into a PUC57 plasmid vector for *in vitro* transcription. The various mutations used in the paper are schematized in the figures, and will not be reproduced here. The T7 terminator sequence is not necessary due to the upstream restriction site. It is added just in case of undigested plasmids during *in vitro* transcription.

Example plasmid DNA sequence (wild-type *gene60*)

5'*TAATACGACTCACTATAGGG*CAACCTAAAACTTACACACGCCCCGGTAAGGAAATAAAAATG
AAATTTGTAAAAATTGATTCTTCTAGCGTTGATATGAAAAAATATAAATTGCAGAACAATGT
TCGTCGTTCTATTAAATCCTCTTCAATGAACTATGCGAATGTCGCTATTATGACAGACGCAGA
TCACGATGGATAGCCTTCGGGCTATCTATAGAAATACCTCATAATTAAGAGATTATTGGATT
AGGTTCTATTTATCCTTCTCTGCTCGGATTTTTTAGTAATTGGCCAGAATTGTTTGAGCAAGG
ACGAATTCGCTTTGTCAAAACTCCTGTAATCATCGCTCAGGTCGGTAAAAAACAAGAATGGT
TTTATACAGTCGCTGAATATGAGAGTGCCAAAGATGCTCTACCTAAACATAGCATCCGTTAT
ATTAAGGGACTTGGCTCTTTGGAAAAATCTGAATATCGTGAGATGATTCAAAACCCAGTATA
TGATGTTGTTAAACTTCCTGAGAACTGGAAAGAGCTTTTTGAAATGCTCATGGGAGATAATG
CTGACCTTCGTAAAGAATGGATGAGCCAGTAG**GCTGAGC**AATAA*CTAGCATAACCCCTTGGG
GCCTCTAAACGGGTCTTGAGGGGTTTTTTGACGTC* 3'

Underlined: mRNA coding sequence

Bold: BlpI restriction site

Italicized: T7 promoter and T7 terminator

The corresponding mRNA coding sequence (for the wild-type gene60) is shown below, starting from the AUG start codon. The various mutations used in the paper are schematized in the figures, and will not be reproduced here.

mRNA coding sequence (wild-type)**:**

5'AUGAAAUUUGUAAAAAUUGAUUCUUCUAGCGUUGAUAUG*AAAAAAUAUAAAUUGCAGAAC*
*AAUGUUCGUCGUUCUAUUAAAUCCUCUUCA*AUGAACUAUGCGAAUGUCGCUAUUAUGACA
GACGCAGAUCA**CGAUGGA**<u>**UAGCCUUCGGGCUAUCUAUAG**</u>AAAUACCUCAUAAUUAAGA
<u>GAUUAUUGGA</u>UUAGGUUCUAUUUAUCCUUCUCUGCUCGGAUUUUUUAGUAAUUGGCCAG
AAUUGUUUGAGCAAGGACGAAUUCGCUUUGUCAAAACUCCUGUAAUCAUCGCUCAGGUC
GGUAAAAAACAAGAAUGGUUUUAUACAGUCGCUGAAUAUGAGAGUGCCAAAGAUGCUCU
ACCUAAACAUAGCAUCCGUUAUAUUAAGGGACUUGGCUCUUUGGAAAAAUCUGAAUAUC
GUGAGAUGAUUCAAAACCCAGUAUAUGAUGUUGUUAAACUUCCUGAGAACUGGAAAGAG
CUUUUUGAAAUGCUCAUGGGAGAUAAUGCUGACCUUCGUAAAGAAUGGAUGA 3'

<u>underlined</u>: coding gap

bold: hairpin

italicized: proposed nascent peptide signal K_{13}KYKLQNNVRRSIKSSS$_{29}$

Translation experiments with labeled tRNAs

Translation experiments using labeled tRNAs are performed in the same way as ribosome Cy3B/BHQ conformational FRET experiments described above with the following differences. Total tRNA mixture was charged with all amino acids except Phe (ΔPhe). The resulting mix ofΔPhe tRNAs was then used to form ternary complexes (Δ(Phe) aa-tRNA). Ternary complexes with the labeled tRNA are separately formed (Phe-(Cy5)tRNA^{Phe}). The delivery mix contains: 200 nM BHQ-50S, 1 μM IF2, 240 nM EF-G, 3 μM total tRNA ΔPhe ternary complexes, 200 nM Phe-(Cy5)tRNA^{Phe} ternary complexes, 4 mM GTP, 2.5 mM Trolox, and the PCA/PCD oxygen scavenging system in the Tris-based polymix buffer. During the experiment, the SMRT Cell is illuminated with both a green and a red laser.

Translation experiments for ribosome-mRNA FRET

The mRNA is labeled through a Cy5 labeled complimentary DNA oligonucleotide (Trilink) with a sequence 5' CTA AAA AAT CCG AGC AGA GAA GG (Cy5) 3' or 5' CCG AGC AGA GAA GGA TAA ATA GAA CC (Cy5) 3' downstream of the landing site, allowing for potential FRET between the mRNA and ribosome to monitor the bypass. To anneal, the Cy5 oligonucleotide is mixed with the mRNA at 2:1 molar ratio (2 μM oligonucleotide and 1 μM mRNA) in a buffer consisting of 20 mM cacodylate pH 7.0, 100 mM KCl, and 1 mM EDTA. The mixture is heated to 90°C for 3 minutes and slow cooled down to room temperature. At room temperature, $MgCl_2$ is added to a final concentration of 4 mM, and the labeled mRNA is stored at -80°C.

For the experiments involving ribosome-mRNA FRET, the procedure is similar to as described above. However, instead of using the helix 44 30S mutant, the helix 33a 30S mutant is used. This label is near the beak of the 30S subunit, which is close to the mRNA entrance channel and allows for FRET between the mRNA and 30S. The 30S subunit (final concentration = 1 μM) was mixed in 1:1 ratio with the same 3′ Cy3B-labeled oligonucleotide as the helix 44 30S mutant for 37°C for 10 min and then at 30°C for 20 min in a Tris-based polymix buffer system. The 50S subunit is not labeled.

30S PICs were formed by incubating the following at 37 °C for 10 minutes: 0.25 μM Cy3B-30S, pre-incubated with stoichiometric S1, 1 μM IF2, 1 μM fMet-tRNA^{fMet}, 50 nM mRNA, and 4 mM GTP to form 30S PICs in the polymix buffer. Before immobilization, we dilute the 30S PICs with our Tris-based polymix buffer containing 1 μM IF2 and 4 mM GTP down to 100 nM PIC concentration.

The delivery mix contains: 200 nM 50S (unlabeled), 1 μM IF2, 500 nM EF-G, 4.5 μM total tRNA ΔPhe ternary complexes, 240 nM Phe-(Cy3.5)tRNA^{Phe} ternary complexes, 4 mM GTP, 2.5 mM Trolox, and the PCA/PCD oxygen scavenging system in the Tris-based polymix buffer. During the experiment, the SMRT Cell is illuminated with only the green laser.

Note that in **Figure 6**, we only see a single FRET transition and do not see further FRET transitions during scanning. This is probably due to the position of the beak labeling relative to the mRNA, which is not located directly at the mRNA entrance channel but ~50Å away, so the distance dependence may not be linear.

Data analysis

Briefly, the background intensity level of the ZMW chip is first determined by finding the average intensities of the lowest 30% mean intensity ZMWs. This is possible since the chip is usually loaded according to Poisson statistics, where the theoretical maximum for loading density to prevent doubly loaded ZMW wells is ~30%. The loading density is controlled by the loading concentration, which needs to be optimized for each type of immobilization molecule. For the pre-initiaion complexes in this study, we load at 100 nM, to give around 15% loading efficiency (~20000 molecules out of 150,000 ZMWs). Then, ZMWs showing stable fluorescence are selected based on a fluorescence cut-off of 2 standard deviations above the background lasting over 10 seconds. Furthermore, ZMWs at the center of the chip (center-most 40% of the ZMWs) are selected where mixing is immediate since the delivery occurs at the center of the chip. This gives ~6000 ZMW wells with some sort of stable high fluorescent signal. The traces are further filtered by selecting for traces showing an initial high green Cy3B intensity (representing Cy3B-30S-PICs initially immobilized) followed by a drop in intensity to 40~60% of the initial intensity (a low intensity state representing BHQ-50S joining to the Cy3B-30S-PICs) and then

multiple rounds (at least one round) of intersubunit FRET transitions (fluctuating between the low intensity state (40~60% of initial intensity) and a high intensity state that is lower than the initial intensity (60~80% of initial intensity)). This allows for selecting actively translating ribosomes, to distinguish from traces with poor photophysics or poor signal to noise ratio, and to distinguish doubly loaded ZMWs (since these traces will show additional photobleaching steps); these traces (~500 traces) are selected for further analysis. Thus, data that contain multiple dyes or only a single donor without subunit joining were not included in subsequent analysis. The FRET states are then assigned as previously described based on a hidden Markov model based approach with 2 states (Aitken and Puglisi, 2010; McKinney et al., 2006) and visually inspected (Aitken et al., 2010) and corrected if necessary. For example, if a trace contains multiple rounds of FRET cycles is interrupted by either Cy3B photobleaching or BHQ photobleaching and then resurrection or other photophysical events, then only the first portion of the trace is used and assigned; the remaining portion is not assigned since the FRET cycle count is disrupted. All lifetimes were fitted to a single-exponential distribution using maximum-likelihood parameter estimation in MATLAB. Clustering of bypassed ribosomes from non-bypassed ribosomes and identification of the rotated state pause at codon 45 is as our previous work on -1 frameshifting (Chen et al., 2014) and by a 2-class k-means clustering of the lifetime at codon 45. For correlation experiments of intersubunit FRET with Cy5-tRNA^{Phe} signal, the traces are selected not only for the FRET signals, but also presence of the Cy5-tRNA signal. The FRET signal and tRNA signal are assigned separately as describe above. For the ribosome-mRNA FRET experiments, the traces are selected for Cy3B/Cy5 FRET signals and the presence of Cy3.5-tRNA signal.

In vivo bypassing assays: plasmid construction and Western blot analysis

The *E. coli* strains DH5a and Bl21 DE3 Star were used for plasmid propagation and western blot analyses, respectively. Strains were grown in Luria–Bertani (LB) plus or minus isopropyl-β, D-thiogalactopyranoside (IPTG).

Mutation constructs were derived by two-step polymerase chain reaction with appropriately designed primers (Integrated DNA Technologies), and cloned with SpeI and BamHI restriction sites in the vector pJ307 (GST-MBP-His fusion vector) (Antonov et al., 2013)

For the Western blot analysis, overnight cultures of strains expressing the appropriate plasmid were diluted 1:100 in LB Broth, grown for 2 h at 37^{o}C, and then induced with 1mM IPTG for an additional 15mins at 37^{o}C. Crude extracts were obtained by culture centrifugation and resuspending the bacterial pellet in Laemmli sample buffer.

Proteins were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Protran), which were incubated at 4°C overnight with primary

antibodies. Immunoreactive bands were detected on membranes after incubation with appropriate fluorescently labelled secondary antibody using a LI-COR Odyssey® Infrared Imaging Scanner. The amounts of 'take-off' and bypassing product were quantified by ImageQuant. The bypassing efficiency was estimated as the ratio of the amount of bypass product to the total amount of non-productive take-off plus bypass products.

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