N6-methyladenosine in mRNA disrupts tRNA selection and translation-elongation dynamics

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N6-methyladenosine (forming m6A) is the most abundant post-transcriptional modification within the coding region of mRNA, but its role during translation remains unknown. Here, we used bulk kinetic and single-molecule methods to probe the effect of m6A in mRNA decoding. Although m6A base-pairs with uridine during decoding, as shown by X-ray crystallographic analyses of Thermus thermophilus ribosomal complexes, our measurements in an Escherichia coli translation system revealed that m6A modification of mRNA acts as a barrier to tRNA accommodation and translation elongation. The interaction between an m6A-modified codon and cognate tRNA echoes the interaction between a near-cognate codon and tRNA, because delay in tRNA accommodation depends on the position and context of m6A within codons and on the accuracy level of translation. Overall, our results demonstrate that chemical modification of mRNA can change translational dynamics.

Expression of genetic information in biology is regulated at various levels, among which the control of gene expression at the post-transcriptional level offers distinct advantages. By this mechanism, prompt responses to stimuli are attained, without perturbation of overall cellular translational dynamics, through bypassing time-consuming mRNA transcription and executing localized control before or during protein synthesis1. Recently discovered evidence of N6-methyladenosine (m6A), the most abundant post-transcriptional modification occurring in bacterial2 and eukaryotic mRNA3–5, has suggested a potential role of m6A modification in modulating translation3–6. The modification sites for m6A within mammalian cells are often within the coding regions and are present during translation3–6. The modification sites for m6A within mammalian cells are conserved and are dynamically regulated by m6A methyltransferases and demethylases3–5. Further, perturbing m6A modification disrupts a wide variety of cellular functions such as translational efficiency6, cell viability7–9 and development10–17, although precise roles of m6A in these processes remain unknown. Compared to unmodified adenosine, m6A has less stable base-pairing with uridine and destabilizes local RNA structures18; these characteristics may have important roles in m6A-induced cellular functions19,20.

Central to translation is the selection of cognate and the discrimination against noncognate codon-anticodon RNA duplex structures, which define the information transfer from the gene sequence encoded in the mRNA to the protein sequence. The translation machinery has universally evolved to include a sophisticated two-step tRNA selection process involving irreversible chemical reactions: first, the initial selection of tRNA before GTP hydrolysis and second, the kinetic proofreading of selected tRNA before peptide-bond formation. This two-step process serves to amplify the accuracy impact of the free-energy differences among cognate, near-cognate and noncognate codon-anticodon interactions, thus ensuring precise information transfer during translation. However, the potential perturbation of these processes by m6A modifications within the mRNA coding sequence and the resulting effects on the accuracy and rate of translation remain unknown. Here, we set out to monitor the effects of m6A modifications on translation-elongation dynamics by using a combined structural, kinetic and single-molecule approach.

RESULTS
Codons with m6A have slower translation-elongation dynamics
To probe the effect of m6A in tRNA selection dynamics over multiple codons, we directly observed an Escherichia coli ribosome translating an mRNA molecule containing a single-base m6A modification, by using single-molecule fluorescence and zero-mode waveguide (ZMW) instrumentation21 (schematics in Fig. 1a). The E. coli translation system has been used as a model system to study translational decoding22,23 and has several advantages, including that established purification methods exist for site-specifically labeled factors, and there are fewer factors involved during translation in this system than in eukaryotic systems. To observe global conformation changes of a ribosome during

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translation, we monitored intersubunit Förster Resonance Energy Transfer (FRET) between Cy3B and BHQ-2 (a nonfluorescent energy-transfer quencher), which were site-specifically attached to E. coli 30S and 50S subunits, respectively. For this, we tracked lifetimes of: (i) a nonrotated state before an intersubunit rotation of the 30S subunit relative to the 50S subunit upon cognate-tRNA accommodation in the aminocyl (A) site and peptidyl transfer and (ii) a rotated state before a reverse rotation upon elongation factor G (EF-G)-catalyzed translation elongation21,24,25 (as shown in schematics in Fig. 1b and sample trace in Fig. 1c). Further, by using fluorescently labeled E. coli lysine tRNA, Lys-(Cy5)5RNA17, we simultaneously tracked the times between binding, ribosomal passage and dissociation of cognate Lys-(Cy5)5RNA17 from unmodified and m6A-modified lysine codons in the A site of a translating ribosome. We observed near-simultaneous Lys-(Cy5)5RNA17 binding and ribosomal intersubunit rotation, which indicated cognate decoding of a lysine codon by Lys-(Cy5)5RNA17 accommodation and peptidyl transfer, whereas rare short Cy5 fluorescence pulses uncorrelated with Cy3B-BHQ-2 FRET efficiency indicated a transient binding of Lys-(Cy5)5RNA17 to the decoding complex. Correlation between fluorescently labeled tRNA pulses and intersubunit FRET signals allowed us to identify true translational complexes within ZMWs with substantial confidence.

Using this approach, we measured the rotated and nonrotated lifetimes for each codon during translation of a 12-codon mRNA sequence with repeating phenylalanine and lysine codons and an m6A modification at the third base of the eighth codon (lysine with AA(m6A)A codon), which we called Lys3 mRNA (Fig. 2a). In the presence of elongation factor Tu (EF-Tu)–GTP–Lys-(Cy5)5RNA17 ternary complex (TC) and EF-Tu–GTP–Phe-tRNA5Phe TC, we observed a three-fold increase in the nonrotated-state lifetime for a modified lysine codon relative to the nonrotated-state lifetime for nonmodified lysine codons in the same mRNA (Fig. 2b,c). These dynamic effects were specific to A-site occupancy by the modified codon; we did not observe other effects on translational dynamics as m6A entered the ribosomal entry channel (corresponding to translational dynamics on codons 4–6), entered the ribosomal A site (codon 7) or left the ribosome (codons 9–12) (Supplementary Fig. 1). These results are consistent with a model that ascribes the observed perturbation to A-site codon-anticodon interaction. Furthermore, we did not observe any effect on rotated-state lifetimes, thus suggesting that m6A does not affect the rates of translocation (Supplementary Fig. 1).

Our X-ray crystal structures of translational decoding complexes containing m6A-modified short RNA oligonucleotides further support the observations above. We purified and crystallized m6A-modified mRNA constructs used in single-molecule assays.

(a) mRNA constructs used in single-molecule assays. All mRNA constructs have six codons in the coding region and an m6A-modified codon in the fourth codon, except Lys3, in which a 12-codon-long construct was used to test the effect of m6A entering and leaving ribosome completely. (b) The nonrotated-state lifetimes at the lysine, glutamine or proline codons present in the mRNA used. Error bars, s.e.m. (95% confidence interval) from fitting a single-exponential distribution to the number of molecules (n) specified. (c) Fold increases of the rotated-state lifetimes of m6A-modified codons, compared across experimental conditions and mRNAs used. Error bars are calculated according to the propagation-of-error method from data shown in b.

**Figure 1** Single-molecule assay for observing translational dynamics on m6A-modified mRNA. (a) Experimental setup for single-molecule assays21,24,25. Preinitiation complex (PIC) containing Cy3B-labeled 30S ribosomal subunit, initiation factor 2 (IF2), fMet-tRNAfMet and the 5′-biotinylated m6A-modified mRNA of interest is immobilized to partially neutravidin-biotinylated polyethylene glycol–covered zero-mode waveguides (ZMWs). Experiments are started by illuminating ZMWs with green and red lasers and delivering BHQ-2-labeled 50S, EF-G, Cy5-labeled and unlabeled tRNA ternary complexes to immobilized PICs. (b) Expected sequences of fluorescence signals with Cy3B-BHQ-2 FRET and Cy5 pulses. The Cy3B signal reports on the rotational state of ribosomal subunits for translating each codon, and long Cy5 pulses indicate stable binding of Cy5-tRNA to the translational complex. (c) Sample fluorescence signal observed during an experiment with the m6A-modified mRNA sequence shown. Each Cy5 pulse is correlated with two Cy3B low-high-low cycles, corresponding to two rounds of elongation during which tRNA is bound to the translational complex (A-to-P and P-to-E movement from the perspective of transiting tRNA).

**Figure 2** Single-base m6A modification of codons delays tRNA accommodation. (a) m6A-modified mRNA constructs used in single-molecule assays. All mRNA constructs have six codons in the coding region and an m6A-modified codon in the fourth codon, except Lys3, which is modified in codon 3. In this 12-codon-long construct was used to test the effect of m6A entering and leaving ribosome completely. (b) The nonrotated-state lifetimes at the lysine, glutamine or proline codons present in the mRNA used. Error bars, s.e.m. (95% confidence interval) from fitting a single-exponential distribution to the number of molecules (n) specified. (c) Fold increases of the rotated-state lifetimes of m6A-modified codons, compared across experimental conditions and mRNAs used. Error bars are calculated according to the propagation-of-error method from data shown in b.
**Table 1 Data collection and refinement statistics**

| Data collection                  | Unmodified (PDB 4X62) | m6A +1 (PDB 4X64) | m6A +2 (PDB 4X65) | m6A +3 (PDB 4X66) |
|---------------------------------|-----------------------|-------------------|-------------------|-------------------|
| Space group                     | P4_1 2_2              | P4_1 2_2          | P4_1 2_2          | P4_1 2_2          |
| Cell dimensions                 |                       |                   |                   |                   |
| a, b, c (Å)                     | 400.8, 400.8, 175.1   | 400.5, 400.5, 175.5 | 401.4, 401.4, 175.9 | 401.3, 401.3, 175.6 |
| α, β, γ (°)                     | 90, 90, 90            | 90, 90, 90        | 90, 90, 90        | 90, 90, 90        |
| Resolution (Å)                  | 35.0-3.45             | 35.0-3.35         | 35.0-3.35         | 35.0-3.35         |
| Rmerge                          | 0.168 (0.984)         | 0.130 (0.993)     | 0.141 (0.929)     | 0.210 (0.961)     |
| t / σt                          | 15.10 (7.76)          | 12.29 (1.25)      | 19.08 (2.83)      | 9.04 (1.77)       |
| Completeness (%)                | 99.2 (100.0)          | 96.4 (91.4)       | 100.0 (100.0)     | 96.6 (98.7)       |
| Redundancy                      | 10.5 (10.7)           | 5.8 (2.7)         | 11.9 (9.2)        | 5.1 (4.9)         |
| Refinement                      |                       |                   |                   |                   |
| Resolution (Å)                  | 35.0-3.45             | 35.0-3.35         | 35.0-3.35         | 50.0-3.45         |
| No. reflections                 | 183,968 (5,548)       | 171,102 (1,033)   | 204,105 (5,914)   | 180,765 (5,622)   |
| Rwork / Rfree                   | 0.181 / 0.214         | 0.165 / 0.204     | 0.185 / 0.211     | 0.194 / 0.226     |
| (0.272 / 0.296)                 | (0.291 / 0.314)       | (0.261 / 0.303)   | (0.297 / 0.354)   |                   |
| No. atoms                       | Protein               | 19,121            | 19,121            | 19,121            |
| (PDB 4X62)                      | 32,874                | 32,875            | 32,875            | 32,875            |
| RNA                             | 782                   | 829               | 844               | 826               |
| Ligand/ion/water                |                       |                   |                   |                   |
| B factors                       | Protein               | 110.3             | 94.8              | 77.9              |
| (PDB 4X62)                      | RNA                   | 97.8              | 80.2              | 66.1              |
| r.m.s. deviations               | Ligand/ion/water      | 103.4             | 83.6              | 74.6              |
| Bond lengths (Å)                | 0.004                 | 0.004             | 0.005             | 0.005             |
| Bond angles (°)                 | 0.790                 | 0.859             | 0.819             | 0.801             |

One crystal was used for each data set. Values in parentheses are for highest-resolution shell.

**Thermus thermophilus** 30S ribosomal subunits and soaked them with an oligonucleotide corresponding to the modified anticodon stem loop (ASL) of human tRNA^Lys^3 and with four different short RNAs^{26-28} ((m^6A)AAUUU, A(m^6A)UUUU, AA(m^6A)UUU and AAAUUU, written from 5’ to 3’). From our four complete X-ray diffraction data sets with resolutions ranging from 3.35 Å to 3.45 Å for each crystallized sample (Table 1), we observed a well-defined difference electron density in the decoding site, showing canonical Watson-Crick base-pairing interaction for both A-U and m^6A-U in the ribosomal A site (Supplementary Fig. 2). At the current resolution, we could not obtain well-ordered electron density for the 6-methyl group of m^6A, perhaps because of rotational movement about the C-N bond. Our results were consistent with results from NMR experiments on m^6A base-pairing^{18}, which have shown exclusively canonical base-pairing between m^6A and U, despite the steric clash between the added methyl group and N^6 of adenosine. The structures obtained by X-ray crystallography indicated that the m^6A does not perturb canonical base-pairing in the final state of tRNA accommodation, whereas steric effects observed by NMR and manifested by decreased thermodynamic stability of modified A-U pairs may underlie the perturbed tRNA selection dynamics observed in our single-molecule assays.

**Codon context of m^6A affects decoding dynamics**

To determine the positional effects of m^6A modification within the codon on tRNA decoding, we monitored the translation of two six-codon-long mRNA constructs containing an m^6A modification at either the first or second base of the fourth codon, which we called Lys1 and Lys2 mRNA constructs, respectively (Fig. 2a). These short mRNAs with an m^6A-modified codon close to the start codon allowed the modified codon to enter the A site earlier in the experiment, thereby increasing the probability of observing its translation before reporter-dye photobleaching. Comparing the effect of m^6A in the different mRNAs, we found that the observed prolonged nonrotated-state lifetimes were greatest for m^6A at the first codon position (15-fold delay in accommodation compared to unmodified lysine codons), second longest for the second position (nine-fold) and shortest for m^6A in the third position (three-fold) (Fig. 2b,c). Prolonged nonrotated-state lifetimes on m^6A-modified codons indicated that the m^6A modification has an effect on the ternary-complex initial selection and/or the kinetic proofreading step during decoding before peptide-bond formation. In comparison, the greatest decreases in tRNA^Lys^3 selection rates are usually observed for single-base changes at position 2, and the smallest effects are observed at position 3 of the near-cognate codon^{22}. We next probed the role for the sequence context of m^6A modifications on decoding dynamics. For m^6A in *E. coli*, a consensus sequence of GGGC(m^6A)G has been reported^{2}. To test this more native codon context in which m^6A would most probably occur within *E. coli*, we used mRNA constructs with a (C(m^6A))G codon (glutamine) or a CC(m^6A)G codon (proline), which we named Gin2 and Pro3, respectively (Fig. 2a). We replaced tRNAs with Phe-(Cy5)tRNA^{Phe} and phenylalanine amino acid–depleted total aminoacylated tRNA mix to observe the correlation between the intersubunit FRET signal and Phe-(Cy5)tRNA^{Phe} transit during translation of these mRNAs, thus allowing us to score for actively translating complexes. Modifications in the glutamine or proline slowed translation elongation: we observed a five-fold increase in nonrotated-state lifetime for the C(m^6A)G glutamine codon compared with the other unmodified glutamine codons, and a three-fold increase in the nonrotated-state lifetime for the CC(m^6A)G proline codon compared to other proline codons. Our results showed that whereas the position of m^6A within a codon still delays tRNA decoding dynamics, the other bases within an m^6A-modified codon modulate the magnitude of the effect. Notably, replacing nearby A-U pairs with G-C pairs mitigated the m^6A-induced delay in tRNA incorporation.

m^6A delays distinct steps of tRNA incorporation differently

We next used bulk kinetic assays with quench-flow instrumentation to measure the effect of m^6A on different decoding steps. We used bulk kinetic assays to measure the ribosome-dependent rate of GTP hydrolysis by EF-Tu (k_{GTP}) and the rate of cognate-tRNA accommodation plus peptidyl transfer (k_{app}), which can reveal the effect of m^6A modification on these processes. We measured the rate of [3H]GTP hydrolysis by EF-Tu after mixing EF-Tu–GTP–Lys-tRNALys TC with 70S decoding complex in excess over TC and programmed with [M^3H]Met-tRNA^{Met} in the P site and an AAA or (m^6A)AA codon in the A site. The results showed slower GTP hydrolysis in decoding of the (m^6A)AA codon than decoding of the AAA codon (Fig. 3a). By measuring k_{GTP} at varying ribosome concentrations, we observed...
Figure 3  Single-base m6A modification slows down binding of ternary complexes to the A site of ribosomes during decoding and has a minor effect on the subsequent steps. (a) Kinetics of GTP hydrolysis after binding of Lys-tRNA\textsubscript{Lys} ternary complexes (0.3 \(\mu\)M) to 70S (1 \(\mu\)M) initiation complexes programmed with AAA or (m6A)AA in the A site. (b) Dependence of the rate of GTP hydrolysis, \(k_{\text{cat}}/K_m\), on ribosome concentration. (c) Estimates of the \(k_{\text{cat}}/K_m\) values for GTP hydrolysis from b. (d,e) Kinetics of GTP hydrolysis and formation of dipeptide fMet-Lys, measured simultaneously in the same experiment. The gray areas represent the total time for all kinetic steps after GTP hydrolysis on EF-Tu up to and including peptidyl transfer, from experiments shown in d and e. Supplementary Table 1 for data in c and f. Kinetic data in a, d and e are representative of three independent experiments. Error bars in b, c, and f represent s.d. (\(n = 3\) technical replicates), as calculated from the fitting procedure\(^{29}\).

Figure 4  The effect of m6A in delaying tRNA-incorporation scales measured across methods. Comparison of fold difference in m6A\(^{\text{32}}\)-induced delay in tRNA shows agreement across different methods. Intersubunit FRET at 1.7 mM Mg corresponds to data from Figure 2, and quench-flow at 1.3 mM and 7.5 mM Mg corresponds to data from Figures 3 and 4, respectively. Intersubunit FRET at 11 mM magnesium concentration was measured as the ratio between two fittings of single-exponential distributions to the time between 50S joining and Lys-(Cy5)tRNA\textsubscript{Lys} arrival, which occurs almost simultaneously with the increase in intersubunit FRET efficiency, for both AAA and (m6A)AA (numbers of molecules analyzed (\(n = 113\) and 80 for AAA and (m6A)AA, respectively). The error for this measurement was calculated by the propagation of error method from the s.e.m. calculated in these two experiments. tRNA-tRNA FRET at 11 mM Mg was measured as indicated in the text.
formation. Assuming that dipeptide formation occurs concurrently with the rotation of ribosomal subunits, we were able to compare the measured lengthening of the nonrotated state due to m^6^A in single-molecule assays directly with the overall kinetic loss measured in bulk kinetic assays. By accounting for the difference in free-magnesium concentration, which affects the accuracy and efficiency of translation, we observed a linear relationship between magnesium concentration and the effect of m^6^A on tRNA selection (Fig. 4), analogously to relationships seen in near-cognate-tRNA selection22.

Probing the effect of m^6^A during early stages of decoding

To investigate in depth the effects of m^6^A at distinct stages during tRNA accommodation in the ribosome, we performed single-molecule experiments tracking FRET between fluorescently labeled tRNAs23. We have previously identified two different intermediate stages of translational decoding before accommodation, by using chemical agents known to inhibit translation at specific steps23,30. Without any perturbation to translation, we observed full accommodation of the tRNA in the A site, as indicated by the high FRET state (FRET efficiency near 0.85) between the P-site tRNA and the A-site tRNA. By substituting GTP with GDPNP, a nonhydrolyzable analog of GTP, we observed a mid-FRET state (FRET efficiency near 0.65) corresponding to the GTPase-active state before full accommodation. Further, in the presence of tetracycline, an antibiotic that blocks progression from the initial selection stage, FRET values were reduced to a low-FRET state (FRET efficiency near 0.4) (Fig. 5a). By comparing the evolution of tRNA-tRNA FRET signals from ribosomes programmed with either an unmodified or an m^6^A-modified lysine codon in the A site under different conditions, we tracked the effect of m^6^A on codon-anticodon interactions at distinct stages of translational decoding.

To validate our tRNA-tRNA FRET assay, we first measured the effects of m^6^A in the tRNA-accommodation stage and compared the results to those obtained above by bulk kinetics and single-molecule intersubunit FRET experiments. Using the same mRNAs as in the bulk kinetic experiments, we measured the Lys-(Cy5)tRNA^Lys^ selection time for either AAA or (m^6^A)AA in the A site after mixing EF-Tu–GTP–Lys-(Cy5)tRNA^Lys^ TC and the 70S decoding complex with fMet-(Cy3)tRNALys and the 70S decoding complex with tRNA-tRNA FRET signals from ribosomes programmed with either an unmodified or an m^6^A-modified lysine codon in the A site under different conditions, we tracked the effect of m^6^A on codon-anticodon interactions at distinct stages of translational decoding.

After observing the consistent effects of m^6^A among tRNA-tRNA FRET, bulk kinetic assays and intersubunit FRET assays, we perturbed tRNA selection by either substituting GTP with GDPNP or including the antibiotic tetracycline (Fig. 5a). In all three experimental conditions at high free-Mg^2^+ concentration (11 mM), the average FRET lifetimes decreased by 1.4- to 3.0-fold for (m^6^A)AA compared to the average FRET lifetimes for AAA, which indicated that the effect of m^6^A precedes the initial selection inhibited by tetracycline (Fig. 5b). Furthermore,
m^6A modification in the A-site codon slightly lowered the average FRET efficiency, especially in the presence of GDPNP (Supplementary Fig. 5), thus mirroring a previous result for selection of near-cognate tRNA in the A site^30. At low free-Mg^2+ concentrations (1 mM), the FRET lifetime decreased severely (Supplementary Fig. 6).

**DISCUSSION**

Here, we investigated the effect of m^6A occurring within mRNA on translation, by using biochemical, structural and single-molecule methods. Our data show that the presence of an m^6A within a codon slows down cognate-tRNA decoding. Structural data from X-ray crystallography showed that a stable codon-anticodon interaction containing m^6A can form within the A site with little perturbation in local or global structure, but minor steric constraints due to the modification may lead to kinetic and thermodynamic perturbation in local or global structure, but minor steric constraints due to the modification may lead to kinetic and thermodynamic destabilization of the m^6A-U pairing, as shown previously^18. The greatest effect of m^6A on tRNA selection occurs at the most thermodynamically unstable steps of initial selection from codon-anticodon recognition through GTP hydrolysis by EF-Tu. The m^6A modification has a smaller but significant (1.5-fold) effect on proofreading after initial selection, as the ribosome evolves to the canonical accommodated codon-anticodon complex observed in our structures.

Our observations suggest that dynamic mRNA modifications within coding regions could be an important factor controlling translation-elongation dynamics locally and promptly at the post-transcriptional level. Regulating the local translation-elongation rate could in turn modulate coupled cotranslational process such as protein folding^31 and recognition of the nascent peptide chain by chaperones and/or signal recognition particles (Fig. 6), thus possibly resulting in different functional forms or localizations of protein products from a single gene.

**METHODS**

Methods and any associated references are available in the online version of the paper.

**Accession codes.** Coordinates and structure factors have been deposited in the Protein Data Base under accession codes PDB 4X62 (unmodified mRNA), PDB 4X64 (mRNA-1 ((m^6A)AAUUU)), PDB 4X65 (mRNA-2 (A(m^6A)AUUU)) and PDB 4X66 (mRNA-3 (AA(m^6A)UUUU)).

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

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**AUTHOR CONTRIBUTIONS**

J. Choi, K.-W.I. and H.D. performed all the experiments and the data analysis; J. Choi performed single-molecule experiments; K.-W.I. performed bulk kinetic experiments; H.D. performed X-ray crystallography, with the help of S.M.S. in material preparations. D.D. and G.R. provided reagents and conceived the project with J. Choi, K.-W.I., H.D., J. Chen, M.E. and J.D.P. I. Chen, A. Petrov and A. Prabhakar assisted in reagent preparation. J. Choi, K.-W.I., H.D., S.E.O’L., M.E. and J.D.P. wrote manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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Online Methods

Purification and crystallization of 30S ribosomal subunits. We purified and crystallized 30S ribosomal subunits from the *T. thermophilus* HB8 strain essentially as previously described. The m7A-modified mRNA fragments (with codon sequences underlined), 5′-m7A(UUUUUU)-3′, 5′-m7A(AUUUUU)-3′, and native mRNA 5′-AAUUU-3′ were purchased from Dharmacon. The presence of the m7A modification was confirmed by mass spectrometry. The ASL Lys3-tRNA (with anticodon sequence underlined, GCAGAC(U(AAG)(A))APCUUG) was a generous gift from P. Agris (University of Albany). 30S crystals were spatially transferred to the final buffer with 26% (v/v) 2-methyl-2,4-pentanediol (MPD) for cryoprotection, and soaking was performed in the final buffer supplemented with 200 μM of oligos of each mRNA, ASL Lys3-tRNA and 80 μM paromomycin for 48 h. Crystals were flash frozen for data collection by plunging directly into liquid nitrogen.

Data collection and refinement of structure obtained by X-ray crystallography. X-ray diffraction data were collected from a single crystal for all m7A-modified mRNA data sets, and two crystals were used for the unmodified mRNA data set. All data sets were collected with a Pilatus 6M detector at beamline BL12-2 at the Stanford Synchrotron Radiation Lightsource (SSRL). Diffraction data sets were processed with the HKL2000 package. Coordinates of the 30S-subunit structure excluding mRNA and ASL (PDB 3T11) with additional 30S rRNA and protein modifications were used for initial rigid-body refinement with Phenix. After simulated-annealing refinement, individual coordinates, three group B factors per residue, and TLS parameters were refined. Potential positions of magnesium or potassium ions were compared with those in a high-resolution (2.5 Å) 30S-subunit structure (PDB 2VRK) in COOT, and positions with strong difference density were retained. All magnesium atoms were replaced with magnesium hexahydrate. Water molecules located outside of significant electron density were manually removed. A similar refinement protocol was used for all data sets. Structure alignments were performed with the alignment algorithm PyMOL (http://www.pymol.org) with the default σ rejection criterion and five iterative alignment cycles. All X-ray crystal-structure figures were produced with PyMOL.

Reagents and buffers for single-molecule experiments. Reagents and buffers for intersubunit FRET single-molecule experiments, including modified *E. coli* ribosomal subunits capable of incorporation of fluorescent dye–labeled DNA oligonucleotides, translation factors (IF2, EF-Tu, EF-G and EF-Ts), S1, and aminoacyl-tRNA total RNA mix (purchased from Sigma-Aldrich), Cy3-labeled tRNA (purchased from Chemical Block, labeled at the elbow position with Cy5 NHS ester purchased from GE Healthcare), Cy5-labeled tRNA (purchased from Sigma-Aldrich, labeled at the elbow position with acp3U47), Cy5 NHS ester purchased from GE Healthcare) and tRNA (purchased from Chemical Block, labeled at the elbow position with acp3U47 with Cy5 NHS ester purchased from GE Healthcare)) were added to Cy5-labeled lysine and phenylalanine aminoacyl-tRNA–EF-Tu–GTP ternary complexes for experiments with Lys1, Lys2 and Lys3 mRNA constructs and Cy5-labeled phenylalanine and total tRNA-charged phenylalanine-depleted amino acids for experiments with Gln2 and Pro3 mRNA constructs were preformed by incubating (2 min at 37 °C) the aminoacyl-tRNA with a five-fold excess of EF-Tu, GTP (1 mM), PEF (3 mM) and EF-Ts (40 mM) in poly mix. The ternary complexes (final concentrations of 10.8 nM for Lys-(Cy5)tRNA, 17.5 nM for Phe-tRNA, 27 nM for Phe-(Cy5)tRNA and 750 nM for phenylalanine-depleted total aminoacylated tRNA) were added to BHQ-2–50S (200 nM), EF-G (120 nM), IF2 (1 mM), GTP (4 mM), 2.5 mM Trolox, and the oxygen-scavenging system (PCA/PCD) to form a delivery mix in poly mix buffer. Before the start of each experiment, the SMRT Cell was loaded into a modified PacBio RS sequencer. At the start of each elongation experiment, the instrument illuminated the SMRT cell with a green laser and then delivered 20 μl of a delivery mixture onto the cell surface at t = 0 s.

Single-molecule tRNA-tRNA FRET experiments on a TIFRM instrument. The preparation for tRNA-tRNA FRET experiments was the same as in ZMW experiments, with the following exception: the wash and delivery mixture contained an additional 10 mM Mg(OAc)2 (nearly 11 mM free-magnesium concentration, where applicable), 1 mM IF2, 10.8 nM Lys-(Cy5)tRNA and 10 nM Cy5-labeled lysine, and 100 μM of tetracycline (where applicable).

TIFR and ZMW instrumentation and data analysis. Single-molecule intersubunit FRET and Lys-(Cy5)tRNA-tRNA-transit experiments were conducted with a commercial PacBio RS sequencer that we modified to allow the collection of single-molecule fluorescence intensities from individual ZMW wells of approximately 130 nm in diameter in four different dye channels corresponding to Cy3, Cy3.5, Cy5, and Cy5.5. The RS sequencer had two lasers for dye excitation at 532 nm and 632 nm. In all experiments, data were collected at ten frames per second (100-ms exposure time) for 8 min. The energy flux of the green laser was 0.60 mW pm−2, and the red laser was at 0.10 mW pm−2.

The instrumental setup for TIFRM was as previously described. For excitation of Cy3 (or Cy3B), a diode-pumped solid-state 532-nm laser at 1 kW cm−2 was used. A Quad-view device (Photometrics) separated fluorescence emissions into four channels, two of which were filtered to correspond to the emission spectra of Cy3B and Cy5. The signal was then projected onto two 512 × 512 pixel quadrants of an EMCCD camera (Andor Technology). Videos were recorded (typically at ten frames per second with a total of 1,500 frames collected, but for experiments with tetracycline, we used 20 frames per second with a total 2,400 frames), with the MetaMorph software package (Molecular Devices).

Data analyses for all experiments are conducted with in-house–written MATLAB (MathWorks) scripts. Briefly, traces from either the ZMW wells or immobilized complexes on TIFRM slides were initially selected on the basis of fluorescence intensity, fluorescence lifetime and the changes in intensity. Exhaustively filtered traces exhibiting intersubunit FRET or single-molecule binding signals were then selected for further data analysis. The FRET states were assigned as previously described, with a hidden Markov model–based approach, and visually corrected. For tRNA-tRNA FRET data, FRET values less than 0.35 were assigned to no-FRET state, as previously described. For tetracycline experiments, FRET events with FRET values higher than 0.5 were rejected to account for incomplete binding of tetracycline to ribosomes. In single-molecule experiments was determined by the number of molecules analyzed. All lifetimes were

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fitted to a single-exponential distribution using maximum-likelihood parameter estimation in MATLAB.

**Bulk kinetic experiments.** The low-Mg\(^{2+}\) buffer was polymix buffer\(^{40}\) containing 95 mM KCl, 5 mM NH\(_4\)Cl, 0.5 mM CaCl\(_2\), 8 mM putrescine, 1 mM spermidine, 5 mM potassium phosphate, 1 mM DTE and 5 mM Mg(OAc)\(_2\), supplemented with energy-regeneration components containing 1 mM ATP and 1 mM GTP for the ribosome mixture or 2 mM ATP for the ternary-complex mixture, 10 mM phosphoenolpyruvate (PEP), 50 μg/ml pyruvate kinase (PK), and 2 μg/ml myokinase (MK). The high-Mg\(^{2+}\) buffer contained 10 mM extra Mg(OAc)\(_2\) in addition to low-Mg\(^{2+}\) buffer. Under the assumption that PEP chelates Mg\(^{2+}\) with a K\(_d\) of 6 M, and one ATP or GTP molecule chelates one Mg\(^{2+}\) (ref. 27), the free-Mg\(^{2+}\) concentrations were calculated as 1.3 and 7.5 mM for low- and high-Mg\(^{2+}\) buffers, respectively.

Fast kinetic measurements were performed at 20 °C in a temperature-controlled quench-flow instrument (RQF-3; Kintek), in which ribosome and ternary-complex mixtures were rapidly mixed, and the reactions were stopped by quenching with 17% (final) formic acid at different incubation times. Ribosome and ternary-complex mixtures were prepared as previously described\(^{22,27}\), except here [\(^{3}H\)]GTP instead of [\(^{3}H\)]GDP was used for preparing ternary complexes. The ribosome mixture contained 705 ribosomes (variable concentrations as indicated in the experiments), [\(^{3}H\)]Met-tRNA\(^{f}_{\text{Met}}\) (1.2× ribosomes), mRNA (encoding AUG-AAA-stop or AUG-(m\(_6\)A)AA-stop; 1.5× ribosomes), IF1 (1.5× ribosomes), IF2 (0.5× ribosomes) and IF3 (1.5× ribosomes). The ternary-complex mixture contained E. coli tRNA\(^{f}_{\text{Met}}\) (4 μM), 0.5 μM EF-Tu, 0.5 μM [\(^{3}H\)]GTP, 0.2 mM lysine and 1.5 units/μL Lys-tRNA synthetase. The extent of [\(^{3}H\)]GDP and [\(^{3}H\)]Met-Lys formation was quantified by HPLC equipped with a β-ram model 4 radioactivity detector (IN/US Systems) as previously described\(^{22}\). Rates of GTP-hydrolysis (k\(_{\text{GTP}}\)) and dipeptide formation (k\(_{\text{dip}}\)) were estimated by fitting the data into a single-exponential model and a two-step exponential model, respectively, as previously described\(^{29}\). Ribosomes were always in excess over ternary complexes so that rates were limited by ribosome concentration.

To measure proofreading during tRNA selection, two ribosome mixtures (1 μM), one displaying AAA and the other displaying (m\(_6\)A)AA in the A site, were mixed with the same ternary-complex mixture (0.5 μM) in parallel. The lower plateau of dipeptide fMet-Lys formation for (m\(_6\)A)AA indicated the rejection of tRNA\(^{f}_{\text{Met}}\) after GTP hydrolysis. The proofreading factor f was given by the ratio of maximal yield of fMet-Lys formed between reactions with AAA and (m\(_6\)A)AA codons.

To estimate the compound rate constant, k\(_{\text{pep}}\), for all subsequent steps after GTP hydrolysis on EF-Tu up to and including peptidyl transfer, we measured GTP hydrolysis and dipeptide formation in the very same experiment. Under low-Mg\(^{2+}\) conditions, experiments were performed with 2 μM of ribosomes programmed with AAA or 4 μM of ribosomes programmed with (m\(_6\)A)AA, and 0.5 μM of ternary complexes. Under high-Mg\(^{2+}\) conditions, experiments were performed with 0.7 μM ribosomes programmed with AAA or (m\(_6\)A)AA, and 0.2 μM ternary complexes. Taking the proofreading factor f into account\(^{41}\), k\(_{\text{pep}}\) was calculated as 1/fk\(_{\text{GTP}}\) = f\(\cdot(1/k_{\text{dip}} - 1/k_{\text{GTP}})\).

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