Supplementary Note 1. Quantification of the stall on the 2′-*O*-methylated codon.

Our smFRET assay is performed at the sub-saturated levels of tRNA TC concentrations, limiting our non-rotated state lifetimes by the tRNA TC association kinetics. At this regime, the large increase in the non-rotated state lifetimes are likely due to the increase in the number of rejection of the cognate tRNA TC per one successful peptide-bond formation. This is further supported by multiple short sampling events observed on the 2′-*O*-methylated codon, showing the increase in the non-rotated state lifetime is not largely due to a lengthening of tRNA TC bound state, but due to an increase in the number of rejections of tRNA TC. The number of average cognate tRNA TC rejection prior to peptide bond formation on the unmodified Lys codon (AAA) has not been measured in our assay. However, by comparing the fold-difference of the non-rotated state lifetimes between the modified and unmodified codons, we can calculate the relative effect of modification in the tRNA rejection. More precisely, the fold-increase in the non-rotated state lifetimes is equivalent to the fold-reduction of $(k_{cat}/K_m)_{pep}$, which is a partition factor that quantifies the inverse of probability that tRNA binding leads to the dipeptide formation. This partition factor is related to an inverse of the probability of tRNA binding leads to a successful peptide-bond formation. Our use of mRNA constructs with two unmodified Lys codon followed by a 2′-*O*-methylated Lys codon allows an internally-controlled way of measuring the fold-reduction of $(k_{cat}/K_m)_{pep}$. Since we do not observe any systematic difference between non-rotated state lifetimes between two unmodified Lys codon preceding the modified Lys codon, we use the average of two to calculate the stall duration on the modified codon, and use a propagation of error method to calculate the standard error of the stall duration.

This is then corrected for the photobleaching/movie-length induced truncation of stall on the modified codon, which may bias our measurements to under-estimate the actual stall duration. Assuming near 100% processivity of elongation at the modified codon, and assuming the distribution of stall lifetime and photobleaching rate is exponentially distributed, if we observe those (n_P) experiments which lead to peptidyl transfer to estimate the average stalling rate (k_P) and neglect the other (n_I, with its rate k_I, related to photobleaching or movie length) experiments in which peptidyl transfer is not reached, then the true rate constant, k_P, for stalling is estimated as an apparent rate constant k: $k=k_P+k_I$. Since k is an over estimation (or under-estimation of its inverse, stall duration), which becomes more severe the bigger k_I is in relation to k_P. we calibrated this estimation error by obtain k_P from: $k_P=k*n_P/(n_P+n_I)$, where $n_P/(n_P+n_I)$ is obtained from the ratio between the number of ribosomes that lead to peptidyl transfer over the number of ribosomes that reached the modified codon (the number of translating ribosomes found in the **Supplementary Data Set 1**). The standard error is corrected using the same method as well, considering the error from the lifetime measurements would dominate the error from the counted number of translating ribosomes. The corrected stall duration and the standard errors are reported on **Figure 1d.**

Supplementary Note 2. Measuring the effect of 2′-*O*-methylation in the bulk kinetics assay.

In the bulk kinetics assay, delay in the rate of GTP-hydrolysis was measured by comparing $(k_{cat}/K_m)_{GTP}$ and maximal rate $(k_{cat})_{GTP}$ by which the ribosome binds to EF-Tu-GTP-Lys-tRNA^{Lys} and induces hydrolysis of GTP. In polymix buffer at 5 mM total [Mg^{2+}], the 2′-*O*-methylation decreased the $(k_{cat}/K_m)_{GTP}$ 300-fold (**Figure 4**), meaning that the initial codon selection parameter, *I*, favoring the unmodified case is 300. The effect of the 2′-*O*-methylation during proofreading was measured by comparing $(k_{cat}/K_m)_{dip,}$ the k_{cat}/K_m-value for fMet-Lys dipeptide formation. The proofreading factor, F, corresponds to the number of GTPs hydrolyzed per successful peptidyl

transfer in the modified case divided by this number in the unmodified case. This means that F is given by the ratio between the ratio $[(k_{cat}/K_m)_{GTP}/(k_{cat}/K_m)_{dip}]^{AAmA}/[(k_{cat}/K_m)_{GTP}/(k_{cat}/K_m)_{dip}]^{AAA}$ (Hopfield, J. J. *Proc. Natl. Acad. Sci.* **71,** 4135–4139 (1974); Ninio, J. Kinetic amplification of enzyme discrimination. *Biochimie* **57,** 587–595 (1975)). We estimated F as 5 in polymix buffer at 5 mM total $[Mg^{2+}]$ (**Figure 5**), meaning in summary that TC discriminated against the modified codon by a factor of 300 in initial selection ($I = 300$; $I = 1/p_I$; p_I defined as a probability of bound TC hydrolyzes GTP during the initial selection, as in the main text) and a factor of 5 in proofreading ($F = 5$) giving an overall accuracy factor of 1500 ($A = I \times F = 1500$; $A = 1/p_A$; p_A defined as a probability of bound TC is accommodated for the successful peptidyl-transfer reaction, as in the main text) corresponding to a remarkably effective shut-down of modified codon reading. Additional information about the 2′-*O*-methylation was obtained by shifting the Mg^{2+} concentration from a near physiological (5 mM total) to a much higher value (15 mM total). In the latter case the rate constant for TC dissociation from the ribosome was greatly decreased (Vorstenbosch, E., Pape, T., Rodnina, M. V, Kraal, B. & Wintermeyer, W. *EMBO J.* **15,** 6766–6774 (1996); Johansson, M., Zhang, J. & Ehrenberg, M. *Proc. Natl. Acad. Sci.* **109,** 131–136 (2012)), the K_m-value for GTP hydrolysis in initial codon selection was unmeasurably small but the k_{cat}-value for this reaction, $(k_{cat})_{GTP}$, could be estimated as 26 s^{-1} in the absence and 0.1 s^{-1} in the presence of the 2′-*O*-methylation **(Figure 6b**). We have also found that the increase in $[Mg^{2+}]$ concentration from 5 to 15 mM reduces the 2′-*O*-methylation-induced proofreading of cognate ternary complex from $F = 5$ (80% rejection) to $F = 2$ (50% rejection) **(Figure 5)**. Although the small K_m-value for GTP hydrolysis at 15 mM total $[Mg^{2+}]$ did not allow for precise determination of $(k_{cat}/K_m)_{GTP}$, it is safe to say that when the $[Mg^{2+}]$ increases above its physiological range the accuracy of unmodified in relation to modified codon selection plummets to the point that the 2′-*O*-methylation is unable to shut down reading of modified codons.

Supplementary Note 3. Quantitative comparison between the bulk-kinetics and the single-molecule assays at two different Mg^{2+} concentrations.

At 5 mM $[Mg^{2+}]$ (1.3-1.9 mM free Mg^{2+} concentration), the reduction of $(k_{cat}/K_m)_{pep}$ (both the initial selection and the proofreading) measured in the bulk kinetics assay for AAmA codons predicted a 1500-fold reduction of $(k_{cat}/K_m)_{pep}$ ($A = 1/p_A$; **Supplementary Note 2**), which predicts nearly 7500s of the non-rotated state lifetime at the AAmA codon in the smFRET assay (average non-rotated state lifetime for unmodified Lys codons multiplied by $(k_{cat}/K_m)_{pep}$; 1500 * 5s = 7500s; average lifetimes in the **Supplementary Data Set 1**). This lifetime is far longer than the ~400s observation time defined by the dye photobleaching lifetime(Chen, J. *et al. Proc. Natl. Acad. Sci. U. S. A.* **111,** 664–9 (2014)). Assuming exponential distribution of lifetimes on the modified codon, the probability of observing translation event on the modified codon is 5 % (exponential function CDF = 1 – exp(-k*t) = 1 – exp(-400/7500) = 0.05), which matches the small number of translation events on the modified codon we observed in the smFRET assay (out of 158 ribosomes reaching the AAmA modified codon, 3 showed translation of the modified codon: 3/158 or 2 ± 1 %, considering Bernoulli distribution to estimate error). At higher $[Mg^{2+}]$ condition (7-7.5 mM free Mg^{2+} concentration, corresponding to 15 mM total $[Mg^{2+}]$ in the bulk kinetics assay and 10 mM total $[Mg^{2+}]$ in the smFRET assay), we used measurements made in both the smFRET assay and the bulk kinetics assay to calculate reduction of $(k_{cat}/K_m)_{GTP}$ in two independent ways. From the smFRET experiment, we observed 57-fold increase (s.e. estimated to be ±17-fold with the propagation of error) in the non-rotated state lifetimes at 10 mM Mg^{2+} condition (**Figure 1d; Supplementary Data Set 1**). This would be equivalent to 57-fold

reduction of $(k_{cat}/K_m)_{pep}$ in the bulk kinetics assay. Using the proofreading factor F = 2, and the definition of F from **Supplementary Note 2,** we calculated the fold-reduction of $(k_{cat}/K_m)_{GTP}$ as $[(k_{cat}/K_m)^{AAA}/(k_{cat}/K_m)^{AAmA}]_{pep}$ /F = 57/2 = 28.5 ± 9. Next, the bulk kinetics assay measured 0.1/second for the rate of GTP-hydrolysis, $(k_{cat})_{GTP}$, or lifetime of 10 seconds for GTP-hydrolysis (**Figure 6b**). Assuming tRNA is bound to the ribosome during these 10 seconds and given the average sampling lifetime of 0.5 seconds at this condition (**Figure 3c**), we would expect 20 ± 1 average number of sampling events in the smFRET experiments. These 20 sampling events will result in 20-fold increase in the non-rotated state lifetimes or 20-fold reduction in the $(k_{cat}/K_m)_{GTP}$, close to the independently calculated 28.5 ± 9-fold difference in the $(k_{cat}/K_m)_{GTP}$.