Supporting Information

Salsi et al. 10.1073/pnas.1410873111

SI Materials and Methods

Materials and Sample Preparation. Maleimide derivatives of the Cy3 and Cy5 (Combinix) fluorescent dyes were used for labeling EF-G and S12 as previously described (1). A cysteine-free variant of EF-G (C113D, C265A, C397S) and a single-cysteine EF-G variant (K541C) that were cloned into the pET-24 expression vector were kindly provided by Harry Noller (University of California, Santa Cruz) (2). A single-cysteine EF-G variant (N538C) was created by site-directed mutagenesis of a cysteine-free variant of EF-G. The H92A substitution was introduced into wild type and K541C variants of EF-G. Different variants of 6-histidine-tagged EF-G were expressed, purified, and labeled as previously described (2, 3). A single-cysteine variant of ribosomal protein S12 (C26S/C33A/C52A/C103T/L48C) was conjugated with Cy3 and incorporated into 30S subunits by in vitro reconstitution from purified 16S rRNA and the other 19 individually purified ribosomal proteins according to published procedures (1, 4).

Preparation of Ribosomal Complexes for smFRET Experiments. All ribosomal complexes used for imaging were assembled in polyamine buffer (20 mM Hepes-KOH, pH 7.5, 6 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM β-mercaptoethanol, 2 mM spermidine, 0.1 mM spermine). Ribosomal complexes carrying a single deacylated tRNA bound to the P site were assembled by incubating 300 nM 70S S12–Cy3-labeled ribosomes with 600 nM mRNA m291, preannealed to 800 nM biotin-labeled DNA primer, and 600 nM $tRNA^{Met}$ at 37 °C for 20 min. The antibiotic viomycin or hygromycin B was eventually added (0.5 mM) with an additional incubation at 37 °C for 10 min. The pretranslocation complex was assembled by incubation of 0.5 μM 70S–Cy3-labeled ribosomes with 1 μM mRNA m291 preannealed to 1.3 μM biotin-labeled DNA primer, and 1 μM *N*-acetyl-Met-$tRNA^{Met}$, at 37 °C for 20 min followed by incubation with a 1 μM EF-Tu·GTP·Phe-$tRNA^{Phe}$ ternary complex, at 37 °C for 15 min. The ternary complex was preassembled by incubating 20 μM EF-Tu with 2 mM GTP in polyamine buffer at 37 °C for 10 min, followed by an additional incubation with 3 μM Phe-$tRNA^{Phe}$ at 37 °C for 5 min. The antibiotic viomycin or hygromycin B (0.5 mM when present) was added simultaneously with the ternary complex. Ribosomal complexes were subsequently diluted to a final concentration of 1 nM and immobilized in the sample chamber assembled from quartz microscope slides and glass coverslips coated with a mixture of m-PEG and biotin-PEG and pretreated with neutravidin (0.2 mg/mL). Then, Cy5-labeled EF-G (up to 10 nM) was added to S12–Cy3-labeled ribosomes in the presence of GTP (0.5 mM) or GDPNP (3 mM). Fusidic acid was 40 μM when present. Viomycin and hygromycin B were 0.5 mM when present. To prevent photobleaching, all samples were imaged in polyamine buffer (50 mM Hepes·KOH, pH 7.5, 6 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM β-mercaptoethanol, 2 mM spermidine, 0.1 mM spermine) containing an oxygen-scavenging system (0.8 mg/mL glucose oxidase, 0.625% glucose, 0.02 mg/mL catalase, and 1.5 mM Trolox). Trolox was used to eliminate Cy5 blinking. FRET values did not depend on illumination intensity, which only affected the signal-to-noise ratio.

Single-Molecule FRET Data Acquisition and Analysis. Single-molecule FRET measurements were essentially taken as previously described (5, 6). An Olympus IX71 inverted microscope, equipped with a UPlanApo 60×/1.20 w objective lens, a 532-nm laser (Spectra-Physics) for excitation of Cy3 dyes, and a 642-nm laser (Spectra-Physics) for excitation of Cy5 dyes was used. Total internal reflection (TIR) was obtained by a quartz prism (ESKMA Optics). Fluorescence emission was split into Cy3 and Cy5 fluorescence using a dual view imaging system DV2 (Photometrics) equipped with a 630-nm dichroic mirror and recorded via an Andor iXon+ EMCCD camera. Movies were recorded using the Single software (downloaded from Prof. Taekjip Ha's laboratory website at the University of Illinois, Urbana–Champaign, physics.illinois.edu/cplc/software) (5), with the exposure time set at 100 ms. Data acquisition was carried out at room temperature. In equilibrium experiments, we typically took up to seven 5-min-long movies while imaging different sections of the slide for each sample. When EF-G was flowed into the sample chamber during imaging, one 10-min-long movie was acquired. EF-G was delivered using a J-KEM 1250 syringe pump. Before each measurement, we checked for nonspecific binding by adding fluorescently labeled ribosomes and EF-G to the slide in the absence of neutravidin and imaging the slide. Nonspecific binding was virtually absent. To minimize background fluorescence, our smFRET measurements were typically carried out at a 10-nM concentration of EF-G, which is below the previously reported 30 nM K_D for EF-G binding to the ribosome (7).

Collected datasets were processed with IDL and Matlab software, using scripts downloaded from a freely available source: physics.illinois.edu/cplc/software. Apparent FRET efficiencies (E_{app}) were calculated from the emission intensities of donor (I_{Cy3}) and acceptor (I_{Cy5}) as follows: $E_{app} = I_{Cy5}/[I_{Cy5} + I_{Cy3}]$. The FRET distribution histograms were built from traces that showed single-step disappearance for both Cy3 and Cy5 fluorescence intensities using a Matlab script generously provided by Prof. Peter Cornish (University of Missouri, Columbia). Single-step disappearance of acceptor signal, which was due to either photobleaching or EF-G disassociation, resulting in a reciprocal increase in donor fluorescence indicated the presence of an energy transfer before acceptor signal disappearance. We had about 15–25 traces per movie that fit this selection criterion. The relatively low number of smFRET traces per movie is due to the scarcity of EF-G binding events that likely is a result of using a concentration of EF-G below the K_D for EF-G binding to the ribosome (see above). All histograms were smoothed with a five-point window and fitted to Gaussian distributions using Origin software (OriginLab). Fitting residuals were random.

Stopped-Flow Measurements of Pre–Steady-State Translocation Kinetics. Kinetics of mRNA translocation were measured as previously described with minor modifications (8–10). Pretranslocation complexes were constructed by the incubation of 70S ribosomes (1 μM) with fluorescein-labeled mRNA (5′-GGC AAG GAG GUA AAA AUG UUU AAA-3′ fluorescein, synthesized by Dharmacon RNAi Technologies, 0.85 μM) and deacylated $tRNA^{Met}$ (2 μM) in polyamine buffer (30 mM Hepes·KOH, pH 7.6, 150 mM NH_4Cl, 6 mM $MgCl_2$, 2 mM spermidine, 0.1 mM spermine, 6 mM β-mercaptoethanol) for 20 min at 37 °C, followed by an incubation with *N*-acetyl-Phe-$tRNA^{Phe}$ (1.5 μM) for 30 min at 37 °C. Pretranslocation ribosomes were mixed with wild type or mutant variants of EF-G and GTP using an Applied Photophysics stopped-flow fluorometer. Final concentrations after mixing were: 35 nM ribosomes, 0.5 μM EF-G, and 0.5 mM GTP. Fluorescein was excited at 480 nm and fluorescence emission was detected using a 515-nm long-pass filter. All stopped-flow experiments were done at 23 °C; monochromator slits were adjusted to 9.3 nm. Translocation of mRNA resulted in partial quenching of fluorescein coupled to the 3′ end of the mRNA (8). Time traces were analyzed using

Pro-Data Viewer software (Applied Photophysics). As reported previously (9, 11, 12), the kinetics of mRNA translocation are clearly biphasic and are best fitted to the sum of two exponentials, corresponding to the apparent rate constants k_1 and k_2. Although the biphasic character of fluorescence changes associated with mRNA translocation is well documented, the physical basis of this phenomenon remains unclear. The rate of translocation was defined as the weighted average rate constant k_{av} (Table S1), calculated as the sum of k_1 and k_2 normalized to their respective contributions to the total amplitude of fluorescence change [$k_{av} = (k_1 \times A_1 + k_2 \times A_2)/(A_1 + A_2)$] (9).

Multiple-Turnover Translocation Assay. Translocation activity of single-labeled EF-G constructs was measured by puromycin reactivity of ribosome-bound tRNA (Fig. S2). Pretranslocation ribosome complexes were assembled using 70S ribosomes (0.2 μM) incubated with mRNA m32 (0.4 μM) and deacylated $tRNA^{fMet}$ (0.4 μM) in polyamine buffer (see above) for 20 min at 37 °C. Radiolabeled *N*-acetyl-[^{3}H]Phe-$tRNA^{Phe}$ (0.35 μM) was subsequently added to the mixture and incubated for another 20 min at 37 °C. Translocation was catalyzed by the addition of GTP (0.5 mM) and EF-G at concentrations varying between 1 and 200 nM, followed by incubation for 2 min at 37 °C, i.e., under initial velocity conditions for 1–10 nM EF-G. Puromycin (1 mM) was then added, followed by incubation for 3 min at 37 °C. The *N*-acetyl-[^{3}H]Phe·puromycin product was separated from the reaction mixture by the addition of 70 μL extraction buffer ($MgSO_4$-saturated 0.3 M NaOAc, pH 5.3) to a final reaction volume of 80 μL, and then 1 mL ethyl acetate was added. The ethyl acetate fraction was separated from the aqueous phase by centrifugation and incubated with scintillation fluid, followed by the measurement of radioactivity.

Toeprinting Analysis. Toeprinting experiments were performed as previously described (13, 14). The pretranslocation complexes in Figs. S6 and S8 were assembled by incubating 70S ribosomes (1 μM) with *N*-acetyl-Met-$tRNA^{Met}$ (2 μM) and mRNA m291 (13) (2 μM) preannealed to [^{32}P]-labeled primer (13) for 20 min at 37 °C. Then, 1 μM EF-Tu·GTP·Phe-$tRNA^{Phe}$ ternary complexes were added to P-site tRNA-bound complexes followed by incubation at 37 °C for 15 min. The ternary complex was preassembled by the incubation of 10 μM EF-Tu with 2 mM GTP at 37 °C for 10 min, followed by the incubation with 3 μM Phe-$tRNA^{Phe}$ at 37 °C for 5 min. A fraction of pretranslocation ribosomes was preincubated with viomycin (0.2 mM) for 5 min at room temperature. Translocation was carried out by the incubation of pretranslocation complexes (0.5 μM) with 3 μM EF-G in the presence of 0.5 mM GTP and 0.25 mM fusidic acid for 20 min at room temperature (Fig. S6) or 10 min at 37 °C (Fig. S8). The pretranslocation complex in Fig. S3 was assembled by incubating 70S ribosomes (1 μM) with $tRNA^{Tyr}$ (2 μM) and mRNA m293 (13) (2 μM) preannealed to [^{32}P]-labeled primer (13) for 20 min at 37 °C. Then, *N*-acetyl-Phe-$tRNA^{Phe}$ (2 μM) was added to P-site tRNA-bound complexes followed by incubation for 30 min at 37 °C. Translocation was carried out by incubation of pretranslocation complexes with 1.5 μM EF-G and 0.5 mM GTP.

GTPase Assay. The rate of GTP hydrolysis by EF-G (H92A) was assayed using γ–^{32}P-labeled GTP according to previous studies (15). Vacant ribosomes (0.2 μM) were incubated with or without EF-G (0.5 μM) in reaction buffer [90 mM Hepes·KOH (pH 7.5), 100 mM NH_4Cl, 20 mM $Mg(OAc)_2$] with [γ-^{32}P]-GTP (10 μM) for various time points at room temperature. Following GTP hydrolysis, the reaction was quenched by adding 10 μL of the reaction mixture to 390 μL of 5% (wt/vol) activated charcoal resuspended in 50 mM NaH_2PO_4. Inorganic phosphate was separated from the reaction mixture by centrifugation at 7,000 × *g* for 10 min, and 25 μL of the supernatant was added to scintillation fluid, followed by the measurement of radioactivity.

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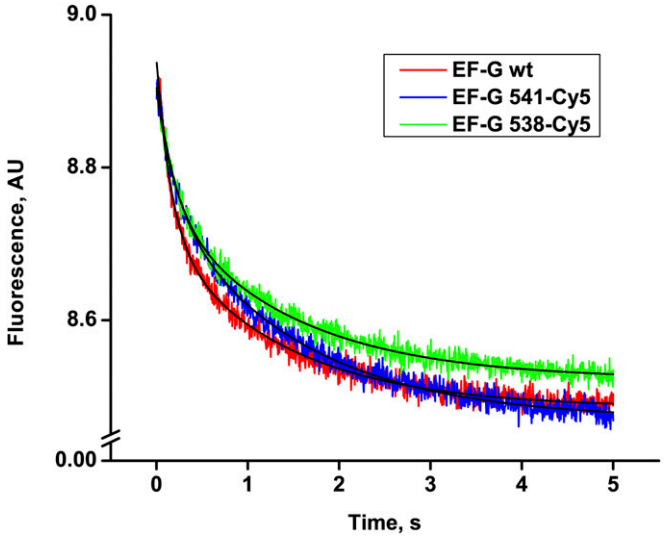

Fig. S1. Translocation activity of fluorescently labeled EF-G constructs under pre–steady-state conditions. Kinetics of mRNA translocation induced by wild-type EF-G (red), EF-G–541–Cy5 (blue), and EF-G–538–Cy5 (green). Kinetics of mRNA translocation were measured in pre–steady-state stopped-flow experiments by the quenching of fluorescein attached to the mRNA (10). Pretranslocation ribosomes containing $tRNA^{Met}$ in the P site and *N*-acetyl-Phe-$tRNA^{Phe}$ in the A site were mixed with GTP and EF-G. Double-exponential fits for fluorescein quenching (black curves) are reposted in Table S1.

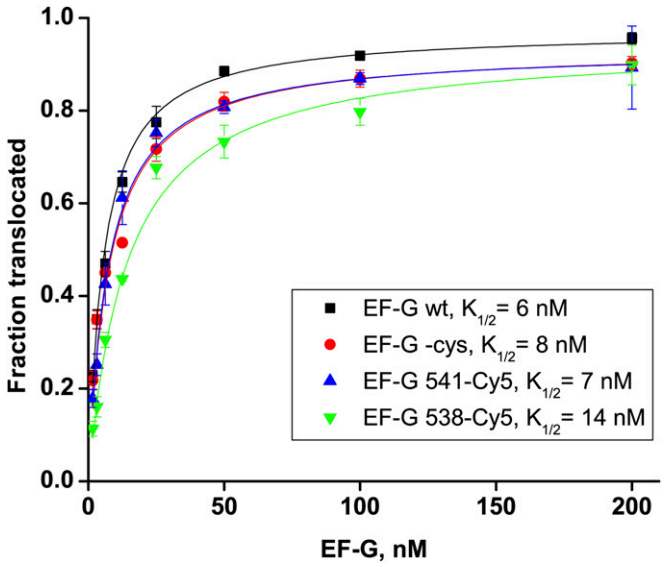

Fig. S2. Activity of single-labeled EF-G constructs in multiple turnover translocation. The fraction of translocated ribosomes as a function of EF-G concentration was measured by puromycin reactivity of ribosome-bound tRNA. Translocation in the presence of wild-type EF-G (black), cysteine-free EF-G (red), EF-G–541–Cy5 (blue), and EF-G–538–Cy5 (green) was carried out in the presence of GTP (0.5 mM) for 2 min at 37 °C . Ribosome concentration was 200 nM. $K_{1/2}$ values were determined by fitting the data to hyperbolas, which are shown as the respective colored lines. Error bars show SDs calculated from triplicate measurements.

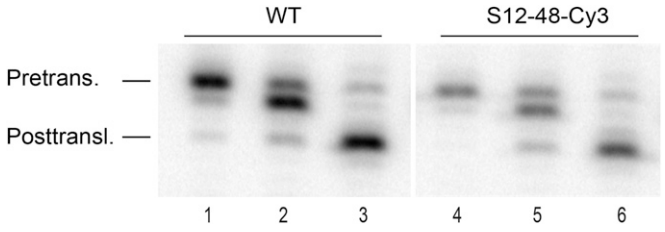

Fig. S3. Reconstituted ribosomes containing Cy3-labeled S12 are active in EF-G–catalyzed translocation. Toeprinting analysis of tRNA binding and translocation in wild-type ribosomes vs. S12-Cy3 reconstituted ribosomes. A pretranslocation complex was assembled by the binding of $tRNA^{Tyr}$ (P site, lanes 1 and 4), *N*-acetyl-Phe-$tRNA^{Phe}$ (A site, lanes 2 and 5), and mRNA m293 (13) and then incubated with EF-G and GTP (lanes 3 and 6) for 10 min at 37 °C.

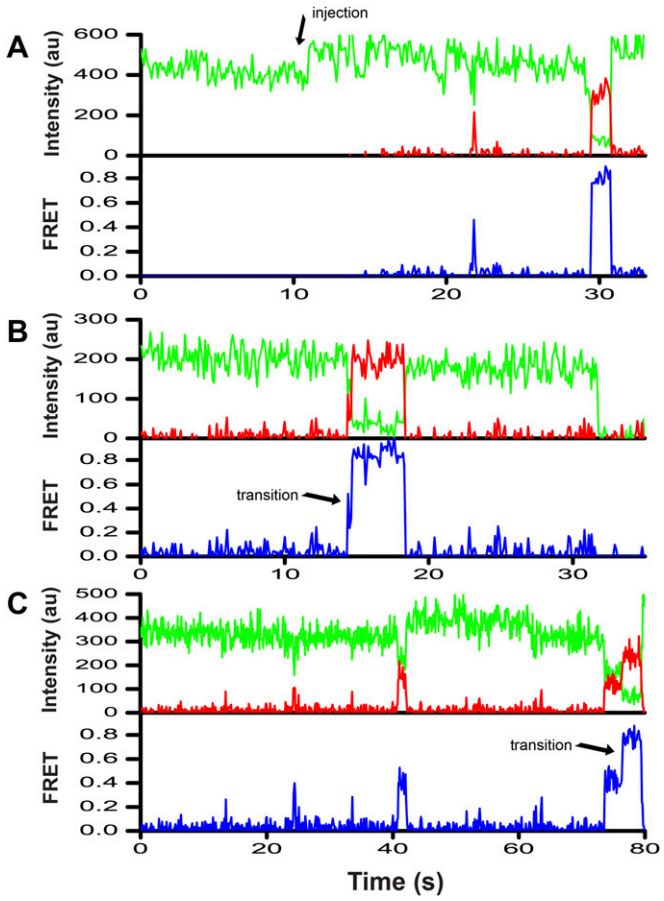

Fig. S4. Sampling of transient ~0.5 FRET state in smFRET traces in the absence of translocation inhibitors. EF-G–541–Cy5·GTP·Fus (*A* and *B*) or EF-G–538–Cy5·GTP·Fus (*C*) was added to S12–Cy3 labeled pretranslocation ribosomes. The traces show fluorescence intensities observed for Cy3 (green) and Cy5 (red) and the calculated apparent FRET efficiency (blue). All traces show transient sampling of a ~0.5 FRET state before the appearance of a 0.8 FRET state. The transition from the ~0.5 to the ~0.8 FRET state in *B* and *C* likely corresponds to a transition from the pre- to posttranslocation conformation of EF-G–ribosome complex.

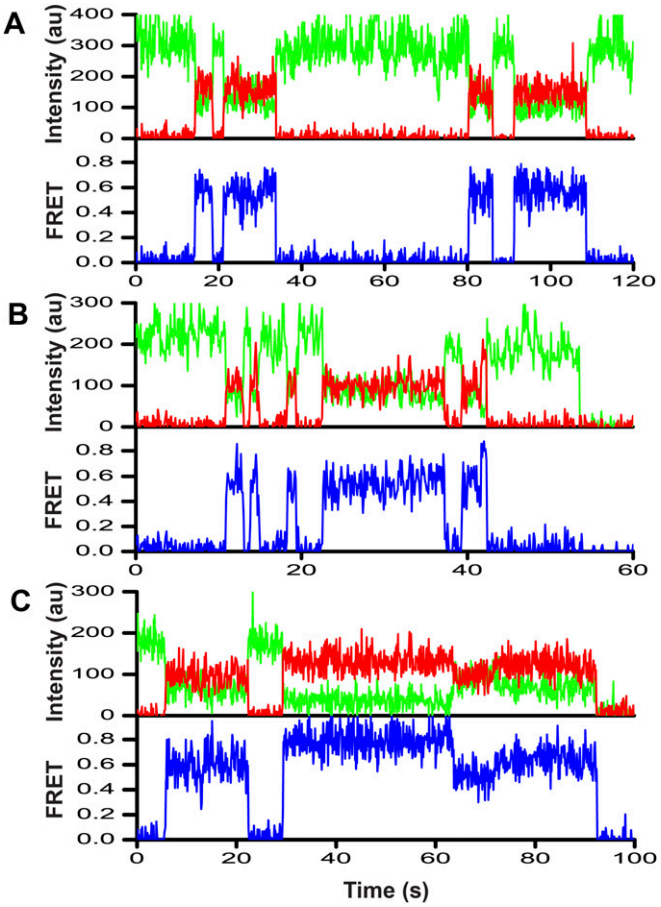

Fig. S5. Representative smFRET traces for EF-G–541–Cy5/S12–Cy3 pair showing EF-G binding to pretranslocation ribosomes preincubated with viomycin. Traces show fluorescence intensities observed for Cy3 (green) and Cy5 (red) and the calculated apparent FRET efficiency (blue). EF-G–541–Cy5·GTP·Fus was added to S12–Cy3–labeled pretranslocation ribosomes in the presence of viomycin. The trace in *A* shows a ∼0.55 FRET. Traces in *B* and *C* exhibit transitions between ∼0.55 and ∼0.8 FRET states.

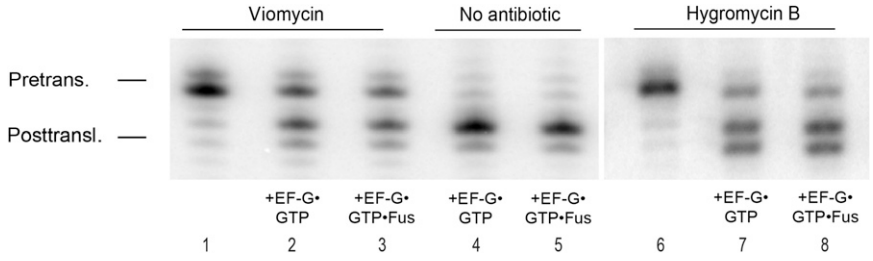

Fig. S6. Inhibition of translocation by viomycin and hygromycin B in a toeprinting translocation assay. Pretranslocation complexes were constructed by binding *N*-acetyl-Met-tRNA^{Met} to the P site of the 70S ribosome in the presence of mRNA m291 followed by binding Phe-tRNA^{Phe} to the A site in the presence of EF-Tu and GTP. Formation of the pretranslocation complex was evidenced by the appearance of a doublet toeprint (lanes 1 and 6). Pretranslocation complexes were then incubated with wild-type EF-G and GTP (lanes 2–5 and 7–8) for 20 min at room temperature in the presence of viomycin (lanes 2 and 3), hygromycin B (lanes 7 and 8) or in the absence of inhibitors of translocation (lanes 4 and 5). Fusidic acid was added in lanes 3, 5, and 8. Translocation shifted the toeprint stop by three nucleotides.

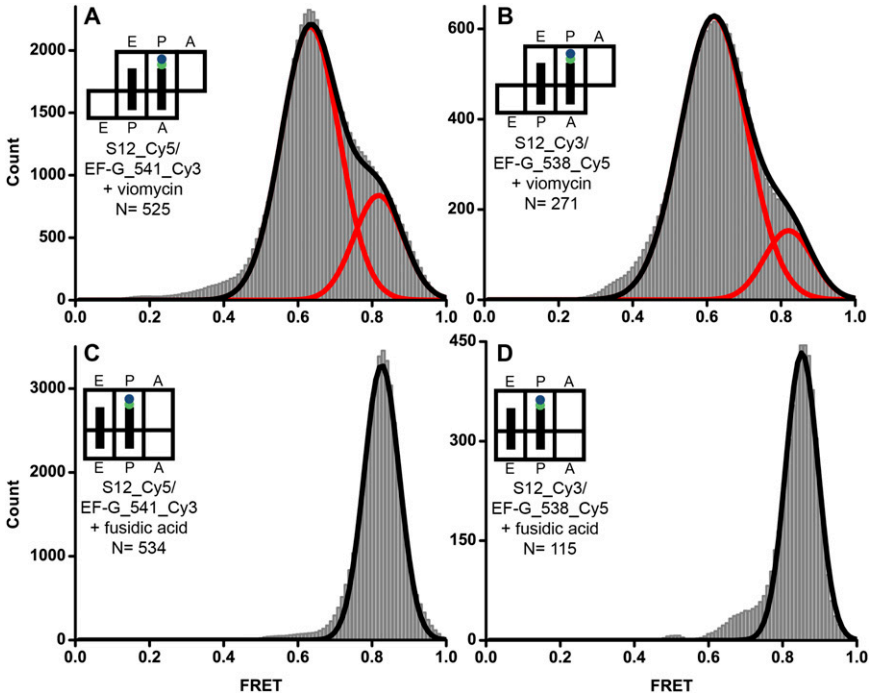

Fig. S7. Changes in FRET between fluorophores attached to protein S12 and EF-G are independent of orientation of donor and acceptor and labeling position on domain IV of EF-G. Histograms for FRET between Cy3 attached to position 541 of domain IV of EF-G and Cy5 coupled to protein S12 (*A* and *C*) or Cy3 coupled to protein S12 and Cy5 attached to position 538 of domain IV of EF-G (*B* and *D*). EF-G was incubated with pretranslocation ribosomes containing $tRNA^{Met}$ in the P site and *N*-acetyl-Met-Phe-$tRNA^{Phe}$ in the A site in the presence of GTP and viomycin and absence of fusidic acid (*A* and *B*) or in the presence of GTP and fusidic acid and absence of viomycin (*C* and *D*). *N* is the number of EF-G binding events in single-molecule traces compiled for each histogram. Red lines represent Gaussian fits; the black line represents the sum of two Gaussians.

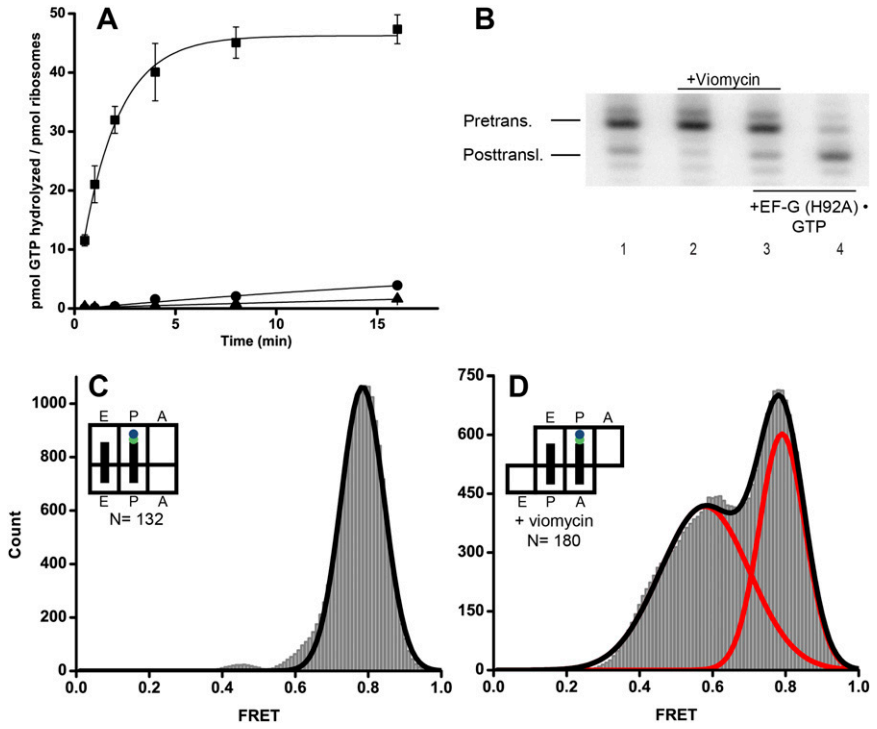

Fig. S8. The effects of substituting histidine 92 with alanine on structural rearrangements, GTPase, and translocation activity of EF-G. (*A*) Kinetics of ribosome-dependent multiple-turnover GTP hydrolysis by wild type (squares) and H92A (circles) variants of EF-G. Kinetics of GTP hydrolysis by wild-type EF-G in the absence of the ribosome are shown by triangles. Exponential fits are shown by black lines. (*B*) Toeprinting translocation assay. The pretranslocation complex (lanes 1 and 2) was constructed as in toeprinting experiments of Fig. S6 and then incubated with EF-G (H92A) and GTP (lanes 3 and 4) for 10 min at 37 °C. In lanes 2 and 3, the ribosome was preincubated with viomycin. (*C* and *D*) Histograms showing distribution of FRET values in EF-G–ribosomal complexes. EF-G (H92A)–541–Cy5·GTP was added to S12–Cy3-labeled pretranslocation ribosomes containing $tRNA^{Met}$ in the P site and *N*-acetyl-Met-Phe-$tRNA^{Phe}$ in the A site in the absence of translocation inhibitors (*C*) or the presence of viomcyin (*D*). *N* is the number of EF-G binding events in single-molecule traces compiled for each histogram. Red lines represent Gaussian fits; the black line represents the sum of two Gaussians.

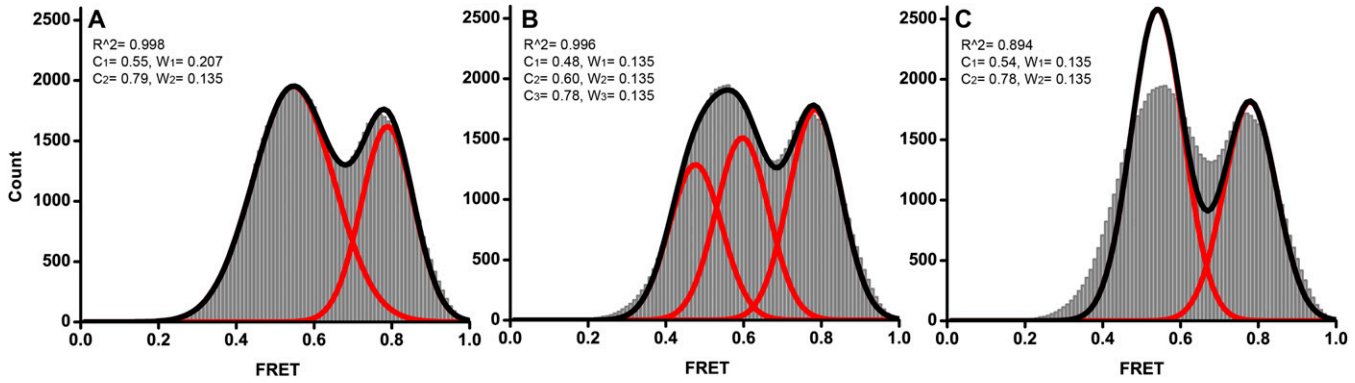

Fig. S9. Statistical analysis of the FRET distribution histogram obtained for EF-G–541–Cy5·GTP incubated with S12–Cy3-labeled pretranslocation ribosomes in the presence of fusidic acid and viomycin (Fig. 3*C* of the main text). The FRET distribution histogram built from 869 traces was fitted to two (*A* and *C*) or three (*B*) Gaussians with unconstrained (*A*) or self-consistent (*B* and *C*) widths. Red lines represent Gaussian fits; the black line represents the sum of Gaussians. The R^2 value, the center of the fitted peaks (c) and the width of the fitted peaks (w) are reported within each panel. Fitting the FRET distribution to three Gaussians (*B*) instead of to two Gaussians with self-consistent widths (*C*) satisfies the *F* test ($P < 0.01$).

Table S1. Translocation activity of fluorescently labeled EF-G constructs under pre–steady-state conditions

EF-G variant	k_1, s^{-1}	k_2, s^{-1}	$A_1/(A_1 + A_2)$	k_{av}, s^{-1}
WT	5.4 ± 0.9	0.7 ± 0.1	0.49 ± 0.03	3.0 ± 0.5
EF-G 538-Cy5	7.1 ± 3.0	0.7 ± 0.1	0.40 ± 0.02	3.3 ± 1.3
EF-G 541-Cy5	5.4 ± 1.6	0.7 ± 0.1	0.34 ± 0.04	2.3 ± 0.7
EF-G (H92A) 541-Cy5	2.0 ± 0.5	0.10 ± 0.02	0.13 ± 0.01	0.4 ± 0.1

Rates of translocation catalyzed by Cy5-labeled mutant variants of EF-G were measured in pre–steady-state stopped-flow kinetic experiments as described in *SI Materials and Methods*. EF-G and ribosome concentrations were 0.5 μM and 35 nM, respectively. k_1 and k_2 are the rate constants of double-exponential fits of the mRNA translocation data; $A_1/(A_1 + A_2)$ is the relative contribution of the faster phase to the total amplitude of fluorescein quenching. Weighted average values (k_{av}) for mRNA translocation rates were calculated by combining the rate constants derived from the two-exponential fits: $k_{av} = (k_1A_1 + k_2A_2)/(A_1 + A_2)$.